

TOLEROGENIC DENDRITIC CELLS AND REGULATORY T CELLS AS THERAPEUTICS FOR IMMUNE-MEDIATED DISORDERS

EDITED BY: Djordje Miljkovic and Piotr Trzonkowski
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TOLEROGENIC DENDRITIC CELLS AND REGULATORY T CELLS AS THERAPEUTICS FOR IMMUNE-MEDIATED DISORDERS

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Advance in Targeted Immunotherapy for Graft-Versus-Host Disease

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Graft-versus-host disease (GVHD) is a serious and deadly complication of patients, who undergo hematopoietic stem cell transplantation (HSCT). Despite prophylactic treatment with immunosuppressive agents, 20–80% of recipients develop acute GVHD after HSCT. And the incidence rates of chronic GVHD range from 6 to 80%. Standard therapeutic strategies are still lacking, although considerable advances have been gained in knowing of the predisposing factors, pathology, and diagnosis of GVHD. Targeting immune cells, such as regulatory T cells, as well as tolerogenic dendritic cells or mesenchymal stromal cells (MSCs) display considerable benefit in the relief of GVHD through the deletion of alloactivated T cells. Monoclonal antibodies targeting cytokines or signaling molecules have been demonstrated to be beneficial for the prevention of GVHD. However, these remain to be verified in clinical therapy. It is also important and necessary to consider adopting individualized treatment based on GVHD subtypes, pathological mechanisms involved and stages. In the future, it is hoped that the identification of novel therapeutic targets and systematic research strategies may yield novel safe and effective approaches in clinic to improve outcomes of GVHD further. In this article, we reviewed the current advances in targeted immunotherapy for the prevention of GVHD.

Keywords: graft-versus-host disease, immunotherapy, immune inhibitors, immune cells, hematopoietic stem cell transplantation

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is used to treat hematologic malignancies. Conditional on surviving the first 2 years after BMT, 5 years survival generally exceeds 70% (1). Graft-versus-host disease (GVHD) is a serious life-threatening complication in patients undergoing HSCT resulting from donor T lymphocytes activated by host antigen-presenting cells (APCs), and resulting in an inflammatory response and immune system disorders (2–5). GVHD includes two phases of pathological progress, namely acute GVHD (aGVHD) and chronic GVHD (cGVHD). 20–80% of recipients would develop aGVHD after allogeneic hematopoietic cell transplantation. And the incidence rates of cGVHD range from 6 to 80%, depending on risk factors and diagnostic criteria used (1).

Despite considerable advances in knowing of the predisposing factors, pathophysiology, and diagnosis of aGVHD and cGVHD, a standard therapeutic strategy is still lacking (6–8). The American Society of Blood and Marrow Transplantation developed some recommendations for treatment of aGVHD. The standards of care treatments that are used now days include first-line treatment and second-line treatment. Steroid remains the mainstay of first-line treatment in grades II–IV GVHD. Standard treatment with prednisone showed an overall complete response (CR) rate of 48%, while

the percentage of steroid-refractory aGVHD (SR-aGVHD) is approximately 50%. Criteria and indications for secondary systemic therapy of aGVHD have not been systematically defined. Secondary systemic therapy may be indicated earlier in patients who cannot tolerate high-dose glucocorticoid treatment. Agents for second-line treatment of aGVHD include immunosuppressive agents (mycophenolate mofetil, pentostatin, cyclophosphamide) and monoclonal antibodies (mAbs), and so on. The use of cyclophosphamide at high doses to prevent GVHD would reduce the cumulative 1-year incidence of cGVHD to 15% or less (9).

Apart from systemic corticosteroids, the therapies targeting immune cells and immune molecular have been applied currently to inhibit GVHD (10–15). Regulatory T cells (Tregs), tolerogenic dendritic cells (TDCs), or mesenchymal stromal cells (MSCs) display considerable benefit in the relief of GVHD through the deletion of alloactivated T cells. mAbs targeting cytokines or signaling molecules have been demonstrated to be potent therapeutic candidates for the prevention of GVHD. In this paper, we reviewed current advances in immunotherapy for GVHD.

IMMUNE CELLS FOR THE TREATMENT OF GVHD

Donor T cells recognize alloantigens and are activated in GVHD process. The activation of dendritic cells (DCs) plays critical roles in the initiation of GVHD. Targeting immune cells, such as Tregs, as well as TDCs or mesenchymal stromal cells (MSCs) display considerable benefit in reducing GVHD through the deletion of alloactivated T cells. Coating allogeneic T cells showed significantly improved survival rate and relief GVHD. Moreover, the coating allogeneic T cells did keep still the effect of graft-versus-leukemia (GVL) (Table 1).

Therapeutic Potential of Tregs for GVHD

Host alloantigens are first recognized by donor T cells, and donor T cells become activated in GVHD process. An imbalance between Tregs and Th17 also involves in the pathological process. Tregs could suppress a wide range of cell types including B cells, T cells, APCs, and NK cells in MHC-unrestricted way. The roles for Tregs in inhibition of GVHD after HSCT have been demonstrated in both murine and human studies. As the negative regulators of immune responses to alloantigen, alloantigen-specific Tregs are critical for maintaining alloantigen-specific tolerance. Tregs strongly inhibited the division, expansion, and differentiation of donor T cells, suggesting a therapeutic potential of Tregs for GVHD. Tregs could protect from both acute and cGVHD (16). Tregs decrease the risk of allotransplant rejection through preventing autoimmune and allergic reactions (17, 18). Infusion of donor Tregs could prevent successfully aGVHD in mice and has shown promise in phase I clinical trials. Early Treg migration into lymphoid tissue and sustained donor Treg presence were important for GVHD prevention. Compared with control group, infusion of clinical-grade Tregs could also delay the onset of GVHD without causing any obvious toxicity or death in mice model (19) (Figure 1).

CCR8 could potentiate the survival of Tregs by promoting the interactions with DCs (20). Enforced IL-10 expression converts human CD4⁺ T cells into T regulatory type 1-like (CD4^{IL-10}) cells that suppress effector T cells *in vitro* and mouse models (21). Short-lived apoptotic protein Fas ligand (FasL) increases the suppressive activity of Tregs and ameliorates GVHD severity (22). Pharmacological blockade or genetic deficiency of C3aR/C5aR signaling could augment the generation of induced Treg (iTreg), stabilize Foxp3 expression, and resist iTreg conversion to effector T cells producing IFN- γ /TNF- α , resulting in limiting GVHD (23). Natural Tregs might induce tolerance in

TABLE 1 | Immune cells and monoclonal antibodies (mAbs) to cytokines for the treatment of graft-versus-host disease (GVHDs).

Different classes	Cells or agents	Mechanisms	Types of GVHD	Clinical translation
Immune cells	Regulatory T cells	Suppress the functions of T cells, natural killer (NK) cells, B cells, and APCs	aGVHD and cGVHD	Phase I clinical trials
	Tolerogenic dendritic cells	Modulate cytokines secretion, expand Foxp3 ⁺ Treg, and suppress allo-CD4 ⁺ T cell proliferation	aGVHD	Preclinical animal study
	Mesenchymal stromal cells	Regulate immunity by interacting with innate immune cells and adaptive immune cells	aGVHD	Being used in clinic
	MSC-ICOS-EGFP	Induce CD4 ⁺ T cell apoptosis, suppress Th1 and Th17 polarization, and promote Th2 polarization	aGVHD	Preclinical animal study
	Coating donor T cells	Block the direct contact between donor T cells and host APCs	aGVHD	Preclinical animal study
mAbs to cytokines	Daclizumab (humanized IL-2R α mAb)	Inhibit activated alloreactive T cells	Gastrointestinal and hepatic aGVHD, steroid-refractory aGVHD (SR-aGVHD)	Being used in clinic
	Basiliximab (IL-2R α mAb)	Inhibit activated alloreactive T cells	aGVHD and cGVHD	Being used in clinic
	Inolimomab (IL-2R α mAb)	Inhibit activated alloreactive T cells	SR-aGVHD	Being used in clinic
	Infliximab	Inhibit TNF- α signaling and functions of T cells, NK cells, B cells, and APCs	SR-aGVHD	Being used in clinic
	Etanercept	Inhibit TNF- α signaling and functions of T cells, NK cells, B cells and APCs	Skin and gut aGVHD	Being used in clinic

aGVHD, acute graft-versus-host disease; APC, antigen-presenting cell; cGVHD, chronic graft-versus-host disease; mAb, monoclonal antibody.

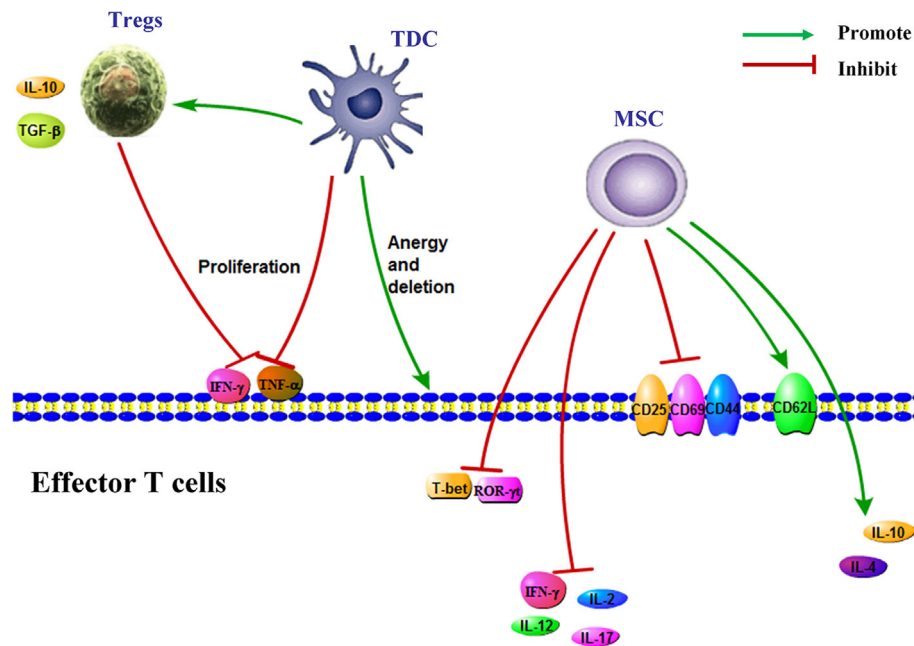


FIGURE 1 | Immune cells for the treatment of Graft-versus-host disease. Regulatory T cells (Tregs) secrete IL-10 and TGF-β and inhibit effector T cell proliferation and the production of IFN-γ and TNF-α. TDCs have low expression of MHC and co-stimulatory molecules, express high levels of immunosuppressive cytokines, expand Foxp3⁺ Treg and suppress allo-CD4⁺ T cell proliferation, and induce the anergy and deletion of effector T cell. MSC induce the expression of CD62L and the production of IL-4 and IL-10 in T cell and inhibit the expressions of ROR-γt, CD25, CD69, and CD44 and proinflammatory cytokines production. Abbreviations: MSC, mesenchymal stem cell; TDC, tolerogenic dendritic cells; Treg, regulatory T cell.

allogeneic cell and organ transplantations. It was more efficient that alloantigen-specific Tregs controlled mice GVHD than that of polyclonal Tregs (24).

Clinical trials have been showed that Tregs had potential effects in preventing GVHD in patients undergoing allo-HSCT. Six independent trials showed the feasibility and safety of Treg-based approaches. Either freshly isolated or *ex vivo* expanded FOXP3⁺ Tregs were infused in patients undergoing allo-HSCT for onco-hematological diseases. Treg-treated patients, the cumulative incidence of relapse was significantly lower than in historical controls. The group of M. G. Roncarolo has completed a phase-I clinical trial in which IL-10-anergized T cells containing Tregs were injected in patients undergoing haploidentical-HSCT. Donor-derived IL-10-anergized T cells specific for host allo-antigens were generated *in vitro* through activation of T cells by host-derived APCs in the presence of exogenous IL-10. M. G. Roncarolo demonstrated that no acute adverse events and only mild GVHD (grades II or III responsive to therapy) were observed after infusion of IL-10- anergized T cells (25, 26).

In addition to the role of CD4⁺ Tregs in suppressing excessive immune responses, CD8⁺ Tregs have also been reported to contribute in maintaining immune tolerance. Human alloantigen-specific CD8^{hi} Tregs have been generated in a large scale by Tu's research group from University of Hong Kong. Tu's research group demonstrated that *ex vivo*-induced CD8^{hi} Tregs controlled GVHD in an allospecific manner by reducing alloreactive T cell proliferation as well as decreasing inflammatory cytokine and chemokine secretion within target organs through a CTLA-4

dependent mechanism. These CD8^{hi} Tregs induced long-term tolerance effectively without compromising general immunity and graft-versus-tumor activity (27).

Martelli et al. reported that donor-derived Tregs, coinfused with conventional T cells (Tcons), could protect recipients against GVHD and prevent posttransplant leukemia relapse in phase II study (28). These findings demonstrate the immunosuppressive potential of Tregs in suppressing GVHD without loss of the benefits of graft-versus-leukemia (GVL) activity. It is very encouraging that the results of clinical trials applying Tregs in allo-HCT, which provides a basis for solid organ transplantation in future trials.

Treatment of GVHD With TDCs

The activation of DCs from donor and recipient plays an important role in the initiation of GVHD. Donor T cells could be activated by host DCs alone. TDCs are essential for both peripheral and central tolerance (29). In thymus, TDCs take part in the autoreactive immature T cells deletion through presenting self-antigens. In periphery, the interactions between TDCs and T cells induce tolerance and the subsequent induction of Tregs, T cell deletion, and T cell anergy (30). TDCs express high levels of immunosuppressive cytokines and express low levels of MHC and co-stimulatory molecules. TDCs could reduce the severity of aGVHD by expanding Foxp3⁺ Treg, suppressing allo-CD4⁺ T cell proliferation and decreasing cytokines secretion. TDCs potently induce and maintain tolerance to a greater extent compared with conventional DCs. Donor or host TDCs promote allograft

survival in mice. Furthermore, the levels of IL-10 and TGF- β in serum were significantly increased and the percentage of Foxp3⁺ cells continually elevated in the mice treated with TDCs (31) (**Figure 1**).

The use of TDC has shown great potential, and administration of TDC prolongs graft survival. Recipient DCs, donor DCs, or donor antigen-pulsed recipient DCs have been used in preclinical studies. Compared to immune inhibitor alone, autologous TDCs and suboptimal immune inhibitor combination are able to induce antigen specific graft tolerance and long-term allograft survival. Similar TDCs in different animal models (mice and non-human primates) were derived, and the protective abilities of these TDCs were confirmed *in vitro* and *in vivo*. In rats, mice, and nonhuman primates, bone marrow progenitors cultured with low doses of granulocyte macrophage colony-stimulating factor could generate TDCs. Autologous TDCs are more effective than allogeneic TDCs in prolonging allograft survival. The mechanisms involved in the tolerance induced by autologous TDC might be that autologous TDC could specifically induce INF-gamma production by TCR $\alpha\beta$ ⁺CD3⁺CD4⁻CD8⁻ cells (double-negative T cells). It indicates the practical advantages of autologous TDCs as a therapeutic tool in clinic. This strategy may also help reduce the immunosuppressive load in grafted patients and, therefore, limit the harmful effects of immune inhibitors (32–34). The studies of TDCs in aGVHD treatment have being in preclinical study phase.

Mesenchymal Stromal Cells for the Treatment of GVHDs

As a heterogeneous cell population, human multipotent mesenchymal stromal cells (MSCs) are present in many tissues and have immunomodulatory properties. MSCs downregulate immunity by interacting with innate immune cells [including macrophages, natural killer (NK) cells, and DCs], and adaptive immune cells (including B and T cells). MSCs have been clinically applied to treat autoimmune diseases and GVHD (35, 36). Since a 9-year-old boy with SR-aGVHD was first treated with haploidentical third-party derived MSCs, a number of clinical trial studies have suggested that MSC infusion might be effective and safe in aGVHD treatment (37). A phase II/III study using MSCs for grades II or III aGVHD was conducted by Muroi et al. 25 patients (grade IV, 3 patients and grade III, 22 patients) were enrolled and treated with MSC infusions. The rate of CR and partial response (PR) was 24% (6 patients) and 36% (9 patients), respectively, at 4 weeks after the first MSC infusions. And the adverse drug reaction commonly associated with MSC was not found. This result suggested that MSCs was effective for SR-aGVHD (38). The safety and feasibility of bone marrow-derived MSC was assessed in a phase I multicenter study with 40 patients. Overall response rate (ORR) was 67.5%, with 27.5% CR. The overall survival rate at 1 and 2 years was 50.0 and 38.6%, respectively, and the median survival time was 1.1 years from the first MSC administration (39). These findings show that MSC can be safely administered on top of conventional immunosuppression for SR-GVHD treatment.

Dotoli et al. reported that 46 patients received treatment with MSC infusion as salvage therapy for SR-aGVHD III/IV. The cumulative dose of MSCs was 6.81×10^6 /kg in a median of three

infusions (range, 1–7). Result showed that 50% (23/46) presented clinical improvement all of the patients, The CR rate and PR rate was 13 and 61%, respectively. And 26% patients presented transient PR. 4.3% patients had acute side effects, such as blurred vision, vomiting, nausea, and cell infusion. These results show that this kind of therapeutic way is safe for SR-aGVHD (40). Placenta-derived decidual stromal cells (DSCs) are more immunosuppressive than MSCs and are used for aGVHD after HSCT as a novel therapy. Baygan et al. assessed the safety and adverse events of DSCs in 44 patients, and 40 controls were given. The result showed that 1-year survival rate was 67% for DSC treatment in GVHD patients, which was significantly better than control group (41). Jurado et al. reported adipose tissue-derived MSCs may be considered safe and feasible for cGVHD in combination with immunosuppressive therapy, which would likely have an impact on the course of cGVHD (42).

MSC ameliorated the pathological changes of liver and gut, and increased significantly survival in mouse aGVHD model. MSC therapy could directly inhibit the proliferation of donor CD4⁺ T cell and reduce the production of TNF-alpha (43). The beneficial effect of human MSCs was also associated with the alternation multiple aspects of mouse T cell activation. Moreover, the effects are specific to MSCs, non-MSC control cell lines were incubated with T cells, which had no any effects on the proliferation and activation of T cell (44). Bone marrow MSCs inhibit CD8⁺ T cell-mediated activation by decreasing the secretion of indoleamine 2, PGE2, TGF- β , and 3-dioxygenase and reducing the expression of natural-killer group 2, member D (NKG2D, activating/co-stimulatory receptor) (45) (**Figure 1**).

However, the safety of MSC has been focused attention on the possible malignant transformation due to mutations acquired during the large-scale expansion *in vitro*. A few studies described spontaneous oncogenic transformation in murine MSC (46). In order to investigate the frequency of cytogenetic alterations in a broad “collection” of clinical-grade BM-MSC products, Capelli et al. performed cytogenetic analysis of preparations expanded under Good Manufacturing Practice conditions. Their conclusion was that the presence frequency of spontaneous, non-clone, and non-recurrent mutations was not low, but the clone mutations obtained were not associated with a malignant transformation and transformed phenotype *in vitro* (47). Nevertheless, for safety reasons, the lack of clone chromosome aberrations or the presence of non-clone chromosome anomalies on 10% or less of metaphases were set as release criteria before MSC distribution for exploitation in clinical trials (48).

MSC-^{ICOS-EGFP} is a potent strategy for the prevention and treatment of aGVHD. MSC-^{ICOS-EGFP} could induce more the apoptosis of CD4⁺ T cell and suppress the polarization of Th17 and Th1, and promote Th2 polarization. In the MSC-^{ICOS-EGFP} treatment group, the levels of IL-4, IL-10 in serum were high, and the low levels of IL-2, IFN- γ , IL-12, IL-17A were found. MSC-^{ICOS-EGFP} could also induce the expression of STAT6, GATA-3 and inhibit STAT4, T-bet, ROR- γ t expression (49). Despite substantial progress, how MSCs module immune responses during an aGVHD episode remains to be elucidated. The future studies of MSCs in aGVHD will lead to stepwise improvements in product selection, timing, dose, frequency, and method of administration. The optimization

of MSC infusion therapy in aGVHD may supports the best use of MSC in other diseases of immunity and inflammation.

Nanoencapsulation of Allogeneic T Cells Mitigates GVHD

The activation of recipient APCs and donor T cells play key roles in the initiation progress of aGVHD. Therefore, the blockade of donor T cell activation by systemic immunosuppression is a common approach to combat aGVHD (50, 51). Coating donor T cells with nanoscale biocompatible and biodegradable film without significantly changing the size and surface charge of T cells is desired to block the direct contact between host APCs and donor T cells to minimize GVHD in allogeneic transplantation. In our lab, we tested if the temporary immunoisolation achieved by coating donor T cells with a biodegradable and biocompatible porous film of alginate and chitosan could relieve GVHD without compromising GVL effect. The results showed that nanoencapsulation had no impact on the phenotype of T cells *in vitro* in terms of viability, proliferation, size, cytokine secretion, and cytotoxicity effect against tumor cells. Lethally irradiated mice were transplanted with the encapsulated allogeneic T cells and bone marrow cells, the results exhibited that compared to the transplantation of nonencapsulated allogeneic T cells and BMCs, the survival was significantly improved and GVHD was reduced together with minimal liver damage and enhanced engraftment of donor BMCs. Moreover, the nanoencapsulation did not alter the GVL effect of encapsulated donor T cells (52). This finding suggest that nanoencapsulation of T cells with

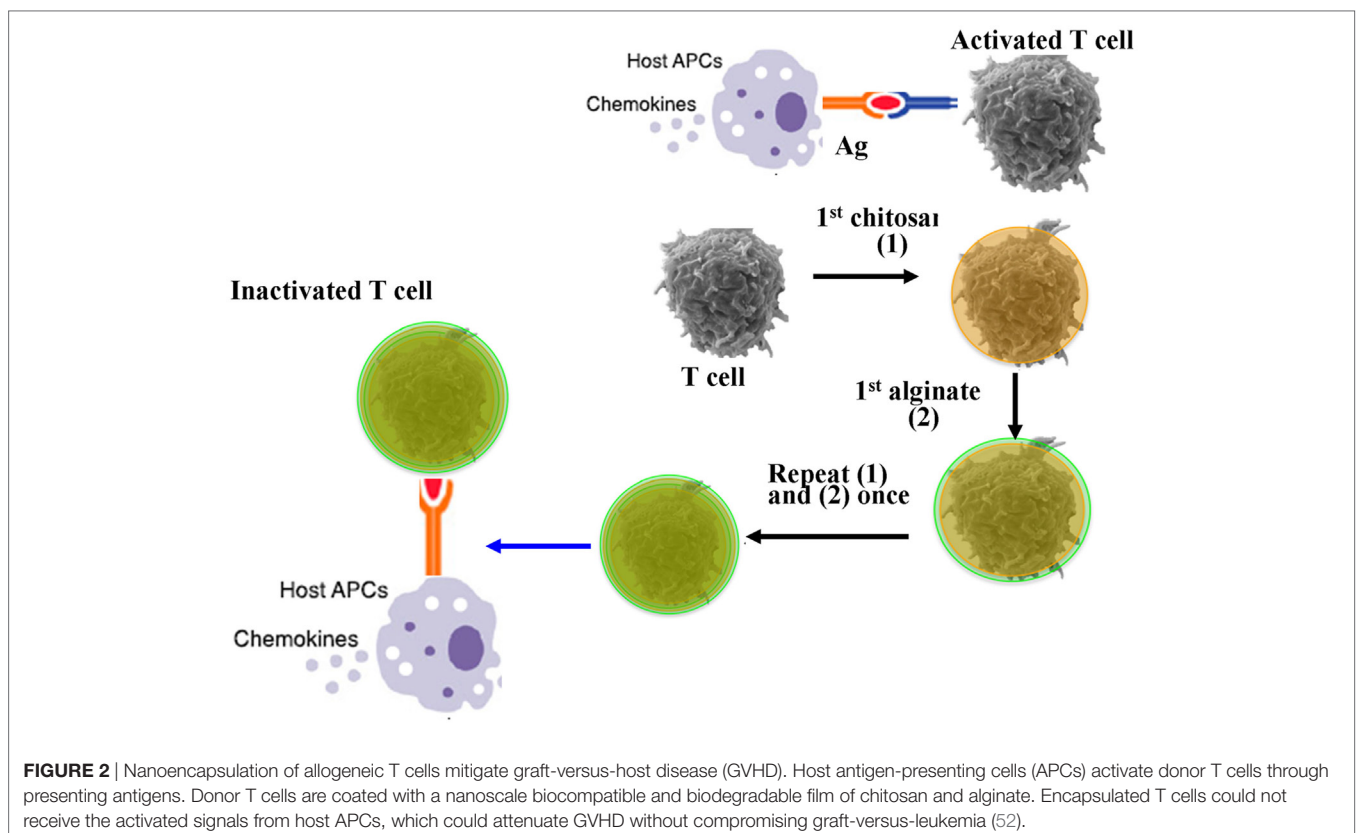
nanoscale, biodegradable, and biocompatible porous materials is a potentially effective and safe strategy to improve allogeneic HSC transplantation for hematological malignancies and other inflammatory and immune diseases (Figure 2).

TARGETING CYTOKINES FOR THE TREATMENT OF GVHD

Inflammatory cytokines secreted by activated T cells, macrophages, DCs, such as TNF- α and IL-2, and so on, are key inflammatory mediators of GVHD and may be important therapy targets. Various mAbs to cytokines secreted by effector cells in GVHD have been researched for aGVHD treatment. These mAbs include anti-TNF- α antibodies, IL-2 receptor antagonists, and so on (Table 1). Overall, the response rates are about greater than 60%, although the long-term survival still remains suboptimal (53).

Interleukin-2 Receptor Antagonists and Ultra Low-Dose IL-2

The mAbs of IL-2 receptor targeting activated T cells have been investigated in SR-aGVHD treatment. Daclizumab is a humanized mAb against IL-2 receptor alpha subunit (IL-2Ra) and has been demonstrated to be effective and safe for adults refractory GVHD. Hamidieh et al. reported that daclizumab was given intravenously, and then was given again on a 10- to 14-day interval for maximum five times if necessary. The results showed that the long-term evaluation of daclizumab might be relatively safe and



effective treatment in most of the severe SR pediatric patients with gastrointestinal aGVHD, although infection occurred frequently. 10 patients responded completely to daclizumab, only one patient responded partially to daclizumab, while there were remaining two patients failing to respond (54). 13 pediatric patients with refractory aGVHD were treated with daclizumab. After 30 days of daclizumab treatment, the CR rate and PR rate was 46 and 46%, respectively, in all patients, the CR rate and PR rate were both higher than that of control group. Moreover, the cutaneous aGVHD patients achieved CR. 50 and 30% had CR and PR, respectively, in gastrointestinal patients, whereas 11 and 55% of hepatic aGVHD patients reached CR and PR, respectively (55).

Combination treatment with infliximab and daclizumab is an effective therapy for SR-aGVHD patients and might be associated with decreased infection-related mortality compared to the monotherapy of infliximab or daclizumab (56). Seventeen GVHD received a combination anti-cytokine therapy of daclizumab and infliximab. This result suggested that the combination of anti-cytokine therapy of infliximab and daclizumab has significant activity in aGVHD (57). Basiliximab, another monoclonal antibody against IL-2R α , prevents graft failure in renal transplantation and is also capable to effectively treat SR-GVHD. The side effects of daclizumab and basiliximab were tolerable and moderate. The prophylactic effects of daclizumab or basiliximab against GVHD in 82 peripheral blood stem cell transplantation patients were evaluated. The incidence rate of grades II–IV and III–IV aGVHD were 35.4 and 15.9%, respectively. 38.7% of evaluable patients suffered from cGVHD. Daclizumab or basiliximab could contribute to favorable outcome by preventing GVHD efficiently. Compared to daclizumab, basiliximab has superior activity against cGVHD, although has a similar effect on aGVHD (58, 59). Inolimomab (anti-IL-2R α) is a mAb targeting IL-2 receptor subunit CD25 that predominantly inhibits activated alloreactive T cells. Several reports have showed encouraging results in SR-aGVHD treatment with inolimomab (60). But the combination of etanercept and inolimomab failed to improve the dismal prognosis of severe SR-aGVHD (61). Ultra low-dose (ULD) IL-2 could expand Tregs without diminishing antileukemic activity and antiviral activity in GVHD treatment. Tregs expressing high level of IL-2 receptor may selectively expand in response to low-dose IL-2 insufficient to stimulate effector T cell populations, thereby preventing GVHD (62).

Anti-TNF Alpha mAbs

Infliximab is an anti-TNF alpha mAb that is effective for treatment of patients with SR-aGVHD (15, 63). However, some studies show controversial results. Couriel's research group reported that 63 refractory aGVHD patients were included and randomized to receive either steroid therapy alone or steroid plus infliximab. Results showed that there were not significantly different between the two groups in GVHD-related mortality, non-relapse mortality, and overall survival (63). The possible explanation is that CD4⁺ Tregs express TNF receptor type 2 (TNFR2) and TNF-alpha could increase Treg activity through TNFR2. The sole defect of TNF production by donor T cells was sufficient to completely abolish the Treg suppressive effect in GVHD. Infliximab might reduce the inhibitory activity of Treg. The control of GVHD by

Tregs was fully abolished by blocking TNFR2 or TNFR2-deficient Tregs (64).

Anti-TNF-alpha treatment enables to reduce corticosteroid dose without aggravating GVHD. Reduction of methylprednisolone and administration of infliximab could get rapid improvement of depression induced by steroid without aggravating GVHD (65).

Etanercept is a fusion protein of recombinant human soluble TNF-alpha receptor and has a modest effect on SR-aGVHD with tolerable side effects (66). 13 SR-aGVHD patients received etanercept therapy, six patients responded to etanercept, and the best response was seen in the patients with gastrointestinal aGVHD (67). The patients with grade I aGVHD receiving etanercept and topical corticosteroids showed a more decrease in the progression of grade than that of control group with corticosteroids alone (68). Etanercept had a down-grading effect on aGVHD, although no patient experienced a complete remission. In addition, gut and skin GVHD were also well controlled by etanercept, whereas hepatic GVHD was not the case (69).

TARGETING CD MOLECULES FOR THE TREATMENT OF GVHD

Effector cells involving in GVHD express many CD molecules, these CD molecules promote the differentiation, activation, maturation, and survival of effector cells. mAbs to CD molecules on cell surface have been investigated for GVHD treatment. These mAbs include anti-CD83, anti-CD132, anti-CD20, and anti-CD28 mAbs, and so on (Table 2), while anti-CD20 mAb has been extensively studied in cGVHD (53).

Soluble CD83 Molecules

CD83 belongs to immunoglobulin (Ig) superfamily and is a highly glycosylated type I transmembrane glycoprotein. As a marker of mature DCs, CD83 is expressed on DCs and activated lymphocytes and is essential for longevity of CD4⁺ T cells and thymus maturation. In addition, CD83 is also involved in the maturation, homeostasis, and function of peripheral B cell (70). Soluble CD83 (sCD83) may be derived from proteolytic cleavage of membrane-bound CD83. It has been demonstrated that sCD83 has therapeutic effects against GVHD by blocking CD83-ligand interaction in animal models. sCD83 is capable of inducing donor-specific allograft tolerance and attenuating DC maturation, and also inhibits the proliferation of DC-dependent alloantigen-specific T cell to prevent the rejections of cardiac allograft. Furthermore, sCD83 could also attenuate innate and adaptive immune responses, which results in preventing chronic rejection in a rat model with renal transplant (71, 72).

Anti-CD83 Antibodies

It has been found that polyclonal or mAbs targeting CD83 can reduce GVHD symptoms through depleting activated CD4⁺ effector T cells and CD83⁺ DCs. In a human T cell-dependent peripheral blood mononuclear cell transplanted SCID (hu-SCID) model, CD83 antibody suppressed the alloproliferation of T lymphocytes but did not prevent engraftment of human T cells, including cytotoxic T lymphocytes (CTL) responsive to viruses and malignant cells. Polyclonal CD83 antibody for GVHD

TABLE 2 | Monoclonal antibodies (mAbs) to CDs and signaling molecules for the treatment of graft-versus-host disease (GVHD).

Different classes	Cells or agents	Mechanisms	Types of GVHD	Clinical translation
mAbs to CDs	sCD83 antibody	Inhibits DC-dependent cell proliferation and attenuate DC maturation	Prevent aGVHD in cardiac allograft	Preclinical animal study
	Rituximab (CD20 mAb)	Modulates cytokines secretion, expands Foxp3 + Treg, and suppresses allo-CD4+ T cell	Prevent cGVHD and preserve graft-versus-leukemia effect	Being used in clinic
	Anti-CD132 mAb	Inhibits granzyme B production in CD8+ T cells	Reverse liver and lung fibrosis in cGVHD	Phase II clinical trials
	Anti-CD45RC mAb	Induced rapid death of CD45RC high T cells through intrinsic cell signaling	Inhibited aGVHD in immune-humanized NSG mice	Preclinical animal study
	Anti-CD28 mAb	Suppress effector T cells, enhance regulatory T cells function and immune tolerance	Prevent aGVHD in mice	Preclinical animal study
	Anti-CD28 Fab antibody	Inhibits T cell expansion	Prevented aGVHD and cGVHD in mice	Preclinical animal study
Signaling molecules	DNMAML1	Blocks notch receptors and decreased Ras/MAPK and NF- κ B activity	Decreased mortality and severity of aGVHD	Preclinical animal study
	R788 (inhibitor of Syk)	Downregulate the expressions of CXCR4, MCP-1, MIP-1 α , IFN- γ , IL-13, IL-17A	Attenuated the severity and fibrosis of cGVHD	Preclinical animal study
	PIAS3 [signal transducer and activator of transcription 3 inhibitor (STAT3)]	Blocks the IL-2-induced proliferation and provides selected immunosuppression	Attenuates the clinical and histopathological severities of aGVHD	Preclinical animal study
	KD025 (Rho-associated coiled-coil containing protein kinase2 inhibitor)	Inhibits the secretion of IL-21, IL-17, and IFN- γ , decreases phosphorylated STAT3	Suppresses murine and human cGVHD	Preclinical animal study
	Ruxolitinib (Janus kinases 1/2 inhibitor)	Impairs the differentiation of CD4+ T cells and increases FoxP3+ regulatory T cells	aGVHD and cGVHD	Multiple centers clinical trials

aGVHD, acute graft-versus-host disease; APC, antigen-presenting cell; cGVHD, chronic graft-versus-host disease; DNMAML1, Dominant negative form of Mastermind-like 1; mAb, monoclonal antibody; UCBT, umbilical cord blood transplantation.

has been suggested to have the outcome of depletion of CD83⁺ DCs and mediates to suppress T cell proliferation. Anti-CD83 antibody treatment may leave tolerogenic and nonactivated CD83-tolerogenic DCs, which may induce Tregs with potential allo-suppressive benefits. Since activated CD4⁺ T cells also express CD83, anti-CD83 antibodies may also deplete the activated CD4⁺ effector T cells (73, 74). Therefore, administration of anti-CD83 antibodies may attenuate GVHD. Investigating the underlying mechanisms is likely to provide improved control of GVHD.

Anti-CD20 Monoclonal Antibody

B cells involve in the pathogenesis of cGVHD. Rituximab, which is a monoclonal antibody of anti-CD20, has been investigated in cGVHD treatment and demonstrated to have some benefit. Cutler et al. reported that the response rate was 70% in 21 steroid-refractory cGVHD patients treated by rituximab (75). Similarly, the response rate to rituximab therapy was 66% in seven studies involving 111 patients by meta-analysis (76). Before the signs of cGVHD, administration of anti-CD20 mAb can prevent the induction of autoimmune-like cGVHD and preserve GVL effect; however, there is little effect if rituximab is administered after cGVHD onset (77).

Anti-CD132 Monoclonal Antibody

CD132 is a subunit of the common gamma chain of the interleukin receptors for IL-2, IL-7, IL-9, IL-4, IL-21, and IL-15. The levels of these cytokines were shown to be high in aGVHD and cGVHD patients. Anti-CD132 monoclonal antibody could potentially reduce aGVHD with respect to survival, GVHD

histopathology, and the production of cytokines, such as TNF, IFN- γ , and IL-6. Anti-CD132 mAb afforded the protection from GVHD partly through inhibiting the production of granzyme B in CD8⁺ T cells. Also, T cells treated with anti-CD132 mAb displayed naive phenotype and showed decreased phosphorylation of JAK3. Additionally, anti-CD132 mAb reversed liver and lung fibrosis, and pulmonary dysfunction in the treatment of established cGVHD comparing with control group (78).

Anti-CD45RC Monoclonal Antibody

CD45RC, a different isoform of CD45, plays an important role in thymocyte maturation and T cell activation and function. CD45RC is expressed at high levels on B cells, NK, and CD8⁺ T cells. CD4⁺ and CD8⁺ Foxp3⁺ Tregs do not express CD45RC and have strong immunoregulatory properties. Anti-CD45RC is a potent therapeutic candidate to induce transplantation tolerance in human. Anti-human CD45RC treatment inhibited GVHD in immune-humanized NSG mice. Administration of anti-CD45RC antibody could induce transplant tolerance associated with inhibition of allogeneic humoral responses in a rat cardiac allotransplantation model. Compared to control group, anti-CD45RC mAb induced rapid death of CD45RC^{high} T cells through intrinsic cell signaling, and preserved CD4⁺ and CD8⁺ CD45RC^{low/-} Tregs, which are able to adoptively transfer donor-specific tolerance to grafted recipients (79).

Agonistic Anti-CD28 Monoclonal Antibody

Anti-CD28 monoclonal antibody targeting CD28 costimulatory molecule may be used as novel therapeutic agents to abrogate

pathogenic T cell responses by selective depletion of activated T cells. Anti-CD28 antibodies differentiate from CTLA4Ig and cannot block CTLA-4 and PDL-1 coinhibitory signals. Anti-CD28 antibodies have the efficacies in suppressing effector T cells while enhancing Tregs function and immune tolerance. Administration of anti-CD28 mAb could inhibit donor T cell expansion and T cell costimulation and prevent GVHD in mice. Anasetti team found that anti-CD28 treatment prevented GVHD by selectively depleting alloantigen-activated donor T cells. Depletion of activated T cells mediated through CD28 did not depend on the expression of death receptors Fas, TNFRI, and TNFRII (80). FR104 is a novel humanized pegylated anti-CD28 Fab antibody fragment presenting a long elimination half-life in monkeys. *In vitro*, compared to control group, FR104 failed to induce human T cell proliferation and cytokines secretion, even in the presence of anti-CD3 antibodies. Administration of FR104 inhibited T cell expansion and prevented GVHD in humanized NOD/SCID mice in a CTLA4-dependent manner (81).

TARGETING SIGNALING MOLECULES FOR THE TREATMENT OF GVHD

Inflammatory cytokines, such as IL-2, TNF- α , and IL-17, and so on, mediate some signaling pathways through binding to receptor. These signaling pathways, such as Notch signaling pathway, Janus kinase (JAK)/STAT signaling pathway, participate in gene expression, cells interaction, cell proliferation, and activation, and so on in the pathological process of GVHD. Various inhibitors targeting key molecules in signaling pathways in GVHD have been investigated for GVHD treatment (Table 2).

Notch Inhibitor for the Treatment of GVHD

Notch signaling is a cell-cell communication pathway, which plays an important role in the development and immunity of T cell. Notch ligands bind to four Notch receptors (Notch1-4), which leads to the activation of proteolytic receptor. Dominant negative form of Mastermind-like 1 (DNMAML1) blocks the transcriptional activation downstream of all Notch receptors. DNMAML1 markedly decreased mortality of aGVHD in mice. DNMAML1 in donor T cells led to decreasing aGVHD severity markedly, without causing global immune suppression. Alloreactive T cells expressing DNMAML1 displayed increased expansion of Tregs and decreased production of inflammatory cytokines, leading to attenuating target organ damage (82). In addition, alloreactive T cells expressing DNMAML1 could also decrease the activation of Ras/MAPK and NF- κ B signaling pathways (83) (Figure 3).

Inhibition of c-Rel

NF- κ B signaling plays important roles in immunity and oncogenesis and might be therapeutic targeting. c-Rel is one of NF- κ B family members and a subunit of NF- κ B. A novel strategy was developed that to ameliorate GVHD while preserving GVT activity by suppression of c-Rel (84). IT-901, which is a bioactive derivative of naphthalene thiobarbiturate, could potentially suppress GVHD and preserve GVL effect though inhibiting c-Rel in allogeneic transplantation. The major mechanisms of IT-901 were to reduce alloactivation and impair negative feedback on

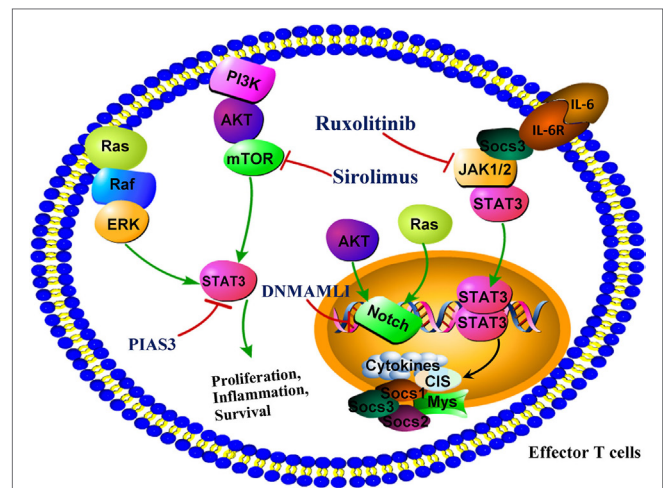


FIGURE 3 | Targeting signaling molecules for the prevention of graft-versus-host disease (GVHD). DNMAML1 blocks the transcriptional activation downstream of all Notch receptors and reduces markedly GVHD severity. PIAS3, a protein inhibitor of activated signal transducer and activator of transcription 3 (STAT3), attenuated the clinical and histopathological severities of aGVHD. Ruxolitinib, which is a specific Janus kinase 1/2 inhibitor, represents a novel targeted approach in GVHD by suppression of proinflammatory signaling that mediates tissue damage. Sirolimus, a mTOR inhibitor, is effective in reducing incidence of GVHD after allo-HSCT. Abbreviations: DNMAML1, dominant negative form of Mastermind-like 1; mTORC, mammalian target of rapamycin complex; NOTCH, notch promoter; PIAS3, protein inhibitor of activated STAT3.

IL-2 production, resulting in the expansion of Tregs. Further preclinical assessment revealed the antitumor properties of IT-901 in the treatment of human B-cell lymphoma. This finding suggests that IT-901 is a novel therapeutic agent to ameliorate GVHD and treat lymphoid tumors (85).

Syk Inhibitor

Syk, which is a protein tyrosine kinase, plays a key role in transmitting signals from receptors on cell surface. Syk phosphorylation increased in CD11b⁺ cells and lymphocytes during allogeneic transplantation. R788 is a potent inhibitor of Syk and attenuates the severity and fibrosis of cGVHD. The elevated expression of CXCR4 on T cells, B cells, and CD11b⁺ cells was significantly downregulated by R788. In addition, compared to control group, R788 inhibited the proliferation of CD11b⁺ cells and reduced mRNA expression levels of MCP-1, MIP-1 α , IFN- γ , IL-13, IL-17A, and TGF- β 1 in skin comparing with control group (86).

Inhibitor of Signal Transducer and Activator of Transcription 3 (STAT3)

Signal transducer and activator of transcription 3 is a pivotal transcription factor for Th17 differentiation. The roles of STAT3 in cGVHD were demonstrated in mice model. Mice transplanted with inducible STAT3-deficient T cells had the same pulmonary function as healthy negative controls (87). PIAS3, a protein inhibitor of STAT3, inhibits STAT3 activation and significantly ameliorates the histopathology and clinical severities of aGVHD

involving liver, lung, intestine, and skin comparing with control group. Inhibition of aGVHD by PIAS3 was largely associated to upregulating Th2 and Treg and downregulating Th17 and Th1 (88) (**Figure 3**).

KD025 is a selective inhibitor of Rho-associated coiled-coil containing protein kinase2 and effectively attenuates cGVHD in multiple animal models. Mice treated with KD025 resulted in normalization of pulmonary function, which resulted from a marked reduction of collagen deposition and antibody in lungs. Compared to control group, the frequency of T follicular helper cells decreased and T follicular regulatory cells increased in the spleens of mice treated with KD025, at the same time, STAT3 expression decreased. KD025 also inhibits the production of IL-17, IFN- γ , and IL-21, reduces protein expression of interferon regulatory factor 4 accompanied by decreasing phosphorylated STAT3 in peripheral blood mononuclear cells from the patients with active cGVHD (87).

JAK 1/2 Inhibitor Improve Survival of Mice With aGVHD

The important roles of host inflammatory response governed by JAK 1/2 have been also highlighted through some novel insights into the pathology of aGVHD. Activated JAK 1/2 are required for T effector cell responses. Ruxolitinib is a specific JAK 1/2 inhibitor. The potent anti-inflammatory properties of ruxolitinib have been demonstrated by preclinical study. Ruxolitinib might be a novel potential approach for GVHD by suppression of tissue damage mediated by proinflammatory signaling. Ruxolitinib could increase FoxP3⁺ Tregs and impair the differentiation of CD4⁺ T cells into the cells producing IL17A and IFN-gamma (89, 90) (**Figure 3**).

Ruxolitinib was recently employed to treat SR-GVHD and a promising ORRs was found. In a retrospective survey, 95 SR-GVHD patients from 19 stem cell transplant centers in the United States and Europe received ruxolitinib as salvage therapy. Results showed that the ORR in SR-aGVHD was 81.5%, and the ORR in SR-cGVHD was 85.4%. The 6-month survival was 79 and 97.4% for SR-aGVHD and SR-cGVHD, respectively. The ORR and survival rate were both higher than control group. However, the adverse effects were observed in both SR-aGVHD and

SR-cGVHD patients during ruxolitinib treatment, such as the reactivation of cytomegalovirus and cytopenia (91). Khandelwal et al. reported that ruxolitinib was less efficacy in children HSCT patients with a high rate of reversible adverse effects in children with SR-aGVHD (92).

CONCLUSION

The physiopathology of GVHD is complicated involving a variety of immune cells and molecules. The treatment and management of GVHD may be proposed, trialed, and ultimately validated. Although there is good evidence supporting treatment of both aGVHD and cGVHD with steroids, clearly there remains an unmet clinical need to develop novel safe therapeutic approaches. Some potential therapy strategies are being found, in particular, the roles of Tregs, TDCs, coating T cell, and MSC in GVHD treatment. IL-2Ra, anti-TNF alpha monoclonal antibody, targeting CD molecules and signaling molecules have been demonstrated to be effective for GVHD. The effects of Tregs against GVHD are very encouraging, but Tregs might also diminish the GVL effects mediated by NK or T cell. Despite substantial progress, how MSCs module immune responses when administered peripherally during an aGVHD episode remains to be elucidated. However, these therapy strategies remain to be validated in clinic. In addition, individualized therapy should be considered based on the characterization of GVHD pathophysiological mechanisms involved, stages, and subtypes. It is anticipated that the novel therapeutic targets and promising strategies may improve the outcomes of GVHD in the future.

AUTHOR CONTRIBUTIONS

LZ wrote the paper. JY collected data, and WW revised and wrote the paper.

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Expanded Regulatory T Cells Induce Alternatively Activated Monocytes With a Reduced Capacity to Expand T Helper-17 Cells

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Regulatory T cells (Tregs) are essential in maintaining peripheral immunological tolerance by modulating several subsets of the immune system including monocytes. Under inflammatory conditions, monocytes migrate into the tissues, where they differentiate into dendritic cells or tissue-resident macrophages. As a result of their context-dependent plasticity, monocytes have been implicated in the development/progression of graft-vs-host disease (GvHD), autoimmune diseases and allograft rejection. In the last decade, Tregs have been exploited for their use in cell therapy with the aim to induce tolerance after solid organ transplantation and for the treatment of autoimmune diseases and GvHD. To date, safety and feasibility of Treg infusion has been demonstrated; however, many questions of how these cells induce tolerance have been raised and need to be answered. As monocytes constitute the major cellular component in inflamed tissues, we have developed an *in vitro* model to test how Tregs modulate their phenotype and function. We demonstrated that expanded Tregs can drive monocytes toward an alternatively activated state more efficiently than freshly isolated Tregs. The effect of expanded Tregs on monocytes led to a reduced production of pro-inflammatory cytokines (IL-6 and tumor necrosis factor- α) and NF- κ B activation. Furthermore, monocytes co-cultured with expanded Tregs downregulated the expression of co-stimulatory and MHC-class II molecules with a concomitant upregulation of M2 macrophage specific markers, CD206, heme oxygenase-1, and increased interleukin-10 production. Importantly, monocytes co-cultured with expanded Tregs showed a reduced capacity to expand IL-17-producing T cells compared with monocyte cultured with freshly isolated Tregs and conventional T cells. The capacity to decrease the expansion of pro-inflammatory Th-17 was not cytokine mediated but the consequence of their lower expression of the co-stimulatory molecule CD86. Our data suggest that expanded Tregs have the capacity to induce phenotypical and functional changes in monocytes that might be crucial for tolerance induction in transplantation and the prevention/treatment of GvHD and autoimmune diseases.

Keywords: regulatory T cells, immunoregulation, monocytes, alternatively activated macrophages, cell therapy

INTRODUCTION

Regulatory T cells (Tregs) maintain peripheral immunological tolerance by controlling autoreactive T cells and dampening inflammation (1). These cells represent 5–10% of all the circulating CD4⁺ T lymphocytes and constitutively express high level of CD25 and FOXP3 (2, 3). Tregs can be broadly divided in two main subpopulations: thymic derived Tregs (4) and peripherally induced Tregs generated by the stimulation of conventional T cells (Tconv) under specific tolerogenic conditions (5, 6). Tregs use a plethora of mechanisms to suppress the activation and proliferation of different immune cell subsets (7–9). The release of immunosuppressive cytokines such as interleukin-10 (IL-10) (10) and transforming growth factor- β (TGF- β) is essential for Treg function as they can modulate both T lymphocytes and antigen-presenting cells (APCs) activation (11). IL-10 can downregulate MHC-class II and co-stimulatory molecules on dendritic cells (DCs) (12, 13) and, at the same time, reduce the production of IL-6, IL-1 β (14), and tumor necrosis factor- α (TNF- α) (15). However, in inflamed tissues, Tregs interact mainly with monocytes and macrophages. These cells are involved in both innate and adaptive immunity as they have the potential to phagocytose and kill bacteria, produce cytokines, and process/present antigen to lymphocytes (16–18). IL-10, together with TGF- β , can drive the differentiation of monocytes into M2 type c macrophages (19, 20). Compared with the classical M1 macrophages, these cells express high levels of the hemoglobin-haptoglobin scavenger receptor (CD163) and secrete less pro-inflammatory cytokines (21). Moreover, M2c can promote kidney repair *in vivo* by deactivating endogenous renal macrophages and by inhibiting CD4 T cells proliferation (20). Recently, it has been shown that IL-10 released by Tregs during the co-culture with monocytes, induced an upregulation of CD163 and CCL18 followed by reduced release of pro-inflammatory cytokines after LPS stimulation (22). In addition, IL-10 is involved in the control of genes implicated in the clearance of oxidative stress such as heme oxygenase-1 (HO-1) (23). This enzyme plays an essential role in suppressing immune responses during inflammation (24) autoimmune diseases (25) and allograft rejection (26).

Regulatory T cells can additionally exert their immunosuppressive function by contact-dependent mechanisms. They are the only T-cells that constitutively express cytotoxic T-lymphocyte antigen-4 (CTLA-4) (27). This molecule binds the same ligands as CD28, CD80, and CD86, thus limiting co-stimulatory signals during T cell activation. CTLA-4 can also downregulate DCs' activity *via* trans-endocytosis of CD80 and CD86 resulting in diminished co-stimulation and T cell anergy (28).

In addition, the interaction between monocytes and Tregs *in vitro* induces the upregulation of the mannose scavenger receptor (CD206), a specific marker for M2a macrophages (22).

Current strategies for clinical management of transplant recipients and for the treatment of graft-vs-host disease (GvHD) involve the use of immunosuppressive drugs (29, 30). However, they do not fully prevent chronic graft rejection or GvHD and they are linked to morbidity and mortality. For this reason, Tregs have been extensively studied as therapeutic tool for the generation of tolerance in solid organ transplantation and for the treatment of

autoimmune disorders and GvHD. Freshly isolated Tregs using Good Manufacturing Practice (GMP) protocols (31) have been infused in phase I clinical trials with no side effects (32–34). However, preclinical studies have also shown that expanded Tregs are more suitable in preventing graft rejection and GvHD than freshly isolated Tregs (35). We have recently developed a clinically applicable protocol for the expansion of human Tregs (36, 37) which involves the use of rapamycin and IL-2.

With the aim of better understanding the mechanisms adopted by expanded Tregs in the induction of tolerance, we have settled an *in vitro* model to study whether Tregs can induce an anti-inflammatory phenotype in monocytes. Monocytes display extreme plasticity in response to signals from the microenvironment and their presence in rejecting allograft tissue is associated with worse graft function and/or survival (38). We hypothesized that the modulation of monocytes by Tregs might be a key mechanism in the induction of tolerance. The data obtained here suggest that expanded human Tregs induce an alternative activation status in monocytes with the potential to support the long-term acceptance of an allograft or to reduce the high inflammatory status which is critical for the progression of GvHD and autoimmune diseases.

MATERIALS AND METHODS

Cell Isolation and Expansion

Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained from anonymized human leukocyte cones supplied by the National Blood Transfusion Service (NHS blood and transplantation, Tooting, London, UK). Human studies were conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of Guy's Hospital (Reference 09/H0707/86). Informed consent was obtained from all healthy donors prior to enrollment into the study. PBMCs were isolated by lympholyte (1.077 g/cm³) gradient stratification (Lymphoprep; Axis-Shield, Norway). Subsequently, highly purified CD4⁺CD25⁺, CD4⁺CD25[−], CD4 (from HLA-A2⁺ donors), and CD14⁺ cells (from HLA-A2[−] donors) were isolated using specific immunomagnetic cell isolation Kits (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Freshly isolated T cells were frozen and used when needed. Tregs expansion has been executed as already published by us (36, 39). Briefly, cells were cultured in X-Vivo (Lonza, UK) supplemented with 5% of Human Serum AB Male (BioWest, France) and 100 nM of rapamycin (LC-Laboratories, USA). Cells were then activated with anti-CD3/CD28 beads (ratio bead:cell of 1:1; Invitrogen, UK). IL-2 (1,000 IU/mL; Proleukin, Novartis, UK) was added at day 4 post activation and replenished every 2 days. Cells were re-stimulated every 10–12 days and used after 36 days from the first activation (three rounds of stimulation). Expanded cells were frozen and used when needed.

Flow Cytometry and Cytokine Evaluation

Freshly isolated Tregs, Tconv, and expanded Tregs have been phenotypically evaluated by flow cytometry using antibodies listed in Table S1 in Supplementary Material.

After detaching, monocytes were incubated with Human TruStain FcX™ (Fc Receptor Blocking Solution, BioLegend, USA) for 10 min and then stained with Live/Dead Yellow (Thermo Fisher Scientific, UK) and extracellular antibodies as listed in Table S1 in Supplementary Material for 30 min at 4°C. For intracellular staining, T cells were activated with PMA/ionomycin for 5 h at 37°C. Cells were then stained with, anti-HLA-A2 and CD45RO followed by cell permeabilization and intracellular staining using anti-IL-17 (BL168, BioLegend, USA), anti-IL-4 (8D4-8, Thermo Fisher Scientific, UK), and anti-IFN- γ (B27, Thermo Fisher Scientific, UK). Permeabilization was performed with the FOXP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, UK) 30 min/4°C according to the manufacturer's instructions. Samples were acquired on LSR-Fortessa flow cytometer and files analyzed using Flow Jo 9.7.5 (Tree Star Inc., USA). Supernatants from activated T cells and monocytes were used to detect cytokines production using LEGENDplex Human Th-Cytokine Assay and Human panel 2 Cytokine Assay (BioLegend, USA) according to the manufacturer's instructions. Cytokines were acquired on a FACSCanto II (BD Biosciences, USA). Data analysis was carried out on BioLegend's LEGENDplex Data Analysis Software. TGF- β evaluation from monocytes and T cells has been performed using Human TGF- β 1 Platinum ELISA kit (Thermo Fisher Scientific, UK).

Suppression Assay

1×10^5 CD4⁺CD25⁻ (T-effectors) were labeled with CFSE (2.5 μ M; Molecular Probe, USA) and cultured alone or at different ratios with freshly isolated Tregs, Tconv, and expanded Tregs. Effector T cells were stimulated with anti-CD3/CD28 beads (Thermo Fisher Scientific, UK) in U-bottom 96-well plates and incubated at 37°C, 5% CO₂ for 5 days. Data were acquired on LSR-Fortessa and analyzed with Flow Jo 9.7.5 software (Tree Star Inc., USA). Suppression of proliferation by Tregs was analyzed as previously described (40).

Monocytes-T Cells Co-Culture

A co-culture experiment was settled in 48-well plates pre-coated with anti-CD3 monoclonal antibody (50 ng/mL; clone UCHT1; BioLegend, USA). 0.5×10^6 monocytes (HLA-A2⁻) have been co-cultured in presence of 0.25×10^6 T cells (HLA-A2⁺) for 6 days as detailed in **Figure 1**. The resultant monocyte populations have been identified as M₂₅₊ (monocytes co-cultured with freshly isolated Tregs), M₂₅₋ (monocytes co-cultured with Tconv), and M_{exp} (monocytes co-cultured with expanded Tregs). Cells were then detached using Accutase (STEMCELL Technologies, UK) and labeled with Live and Dead dying (Thermo Fisher Scientific, UK) and HLA-A2 antibody (Miltenyi, Germany) for sorting, following the gate strategy reported in Figure S1 in Supplementary Material. After sorting, 50×10^3 cells per condition were placed in a new 96-well plate and left at 37°C overnight to ensure a complete adhesion. Adhering cells have been stimulated 24 h with LPS (50 ng/mL) and then co-cultured, for other 6 days, with allogeneic CD4 T cells (10^5 per condition, HLA-A2⁺) in the presence of soluble anti-CD3 (clone OKT3 50 ng/mL; eBioscience). To understand the mechanisms behind the monocytes/CD4 interaction, M₂₅₋ have been cultured for 6 days in presence/absence of tocilizumab (100 ng/mL, Roche UK) infliximab (100 ng/mL, Napp Pharmaceutical group, UK) and abatacept (Orencia® Bristol Mayer Squibb, USA). Specifically, to ensure a complete block of TNF- α and the co-stimulation during the co-culture, M₂₅₋ have been pre-incubated with Abatacept and Infliximab for at least 1 h; similar to selectively block the IL-6 receptor only on T cells, CD4 have been pre-incubated with Tocilizumab. Drugs have been replenished after 3 days.

To evaluate the effects of cytokines released by monocytes on T cell activation, 1×10^5 CD4⁺ T cells have been activated using anti-CD3/CD28 microbeads (beads to cells ratio 1:4) (Thermo Fisher Scientific, UK) in X-vivo and M₂₅₋ conditioned medium.

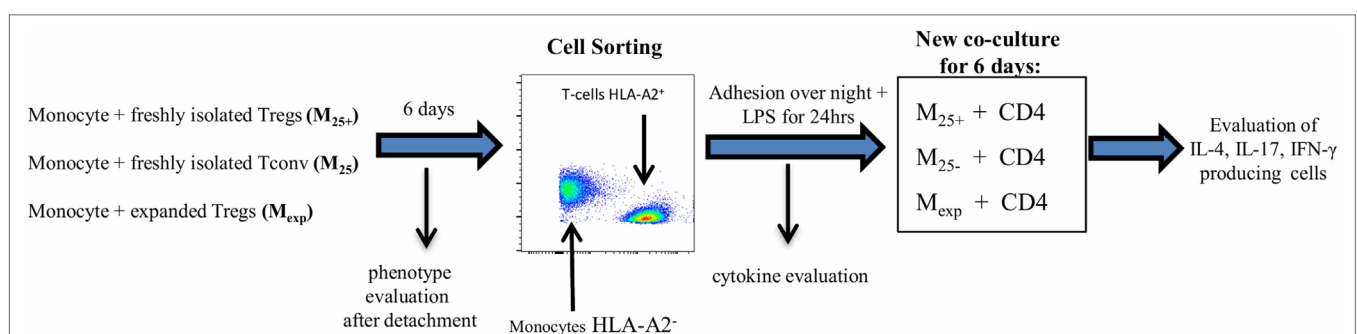


FIGURE 1 | Experimental protocol. Co-culture experiments have been settled in 48-well plates pre-coated with anti-CD3 monoclonal antibody (50 ng/mL). 0.5×10^6 monocytes (HLA-A2⁻) have been cultured in the presence of 0.25×10^6 T cells (HLA-A2⁺) for 6 days. The resultant monocyte populations have been identified as M₂₅₊ [monocytes co-cultured with freshly isolated CD4⁺CD25⁺ regulatory T cells (Tregs)], M₂₅₋ [monocytes co-cultured with freshly isolated CD4⁺CD25⁻ conventional T cell (Tconv)], and M_{exp} (monocytes co-cultured with expanded Tregs). Cells were then detached and labeled with Live/Dead dying and HLA-A2 antibody for cell sorting following the gate strategy reported in Figure S1 in Supplementary Material. At the same time, the expression of CD86, CD14, CD80 CD40, CD206, CD163, and HLA-DR has been evaluated by flow cytometry. After sorting, 150×10^5 cells have been lysed for WB analysis and 50×10^3 cells per condition were placed in a new 48-well plate and left at 37°C overnight to ensure complete adhesion. Adhering cells have been stimulated with LPS for 24 h and then co-cultured, for other 6 days, with allogeneic CD4 T cells (10^5 per condition, HLA-A2⁺ coming from the same donor of the Tconv/Treg) in presence of soluble anti-CD3 (50 ng/mL). Intracellular staining to evaluate IL-4, IL-17, and IFN- γ -producing CD4 T cells has been executed at the end of the co-culture. Supernatant after LPS stimulation (50 ng/mL) has been used for cytokine evaluation and for the experiment using the M₂₅₋ conditioned medium.

Western Blot Analysis

Sorted cells were pelleted and lysed with cold RIPA buffer (Thermo Fisher Scientific, UK) containing protease inhibitors 1× (Calbiochem, Germany). Protein lysates were denatured by adding 2× Laemmli buffer (Bio-Rad, USA) containing 5% β -mercaptoethanol (Sigma-Aldrich, Germany). Protein samples were separated on 10% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, USA). Membranes were blocked in 5% non-fat dry milk (Bio-Rad, USA) in PBS 0.1% Tween-20 for 1 h at room temperature and incubated with phospho- and total-p65 (Ser536) (both from Cell Signaling Technology, USA), HO-1 (Abcam, UK), and β -actin (Santa Cruz Biotechnology, USA) antibodies overnight at 4°C. Proteins were detected with chemiluminescence detection reagents (Bio-Rad, USA) after HRP conjugated secondary antibody (Cell Signaling Technology, USA) incubation using ImageQuant LASS4000 mini (GE Healthcare Life Science, UK) and quantified using Image Studio Lite version 5.2 (LI-COR Biosciences, USA).

Statistical Analysis

Statistical analyses were performed using Prism Version 7 software (Graph-Pad, USA). Data were expressed as mean \pm SEM where applicable using bar charts. Unpaired *t*-test has been used to evaluate different T cell subsets. One-way ANOVA followed by Tukey test was used for monocytes experiment and all *p* values were considered significant when ≤ 0.005 . Specifically for *p* values: **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

RESULTS

Expansion of Tregs Using Rapamycin Increases Their Tolerogenic Functions

To test the effects of expanded Tregs on monocytes, CD4⁺CD25⁺ were isolated and expanded as detailed in Section “Materials and Methods.” At the end of the culture, we evaluated the phenotype and function of expanded Tregs compared with freshly isolated Tconv and Tregs.

As shown in **Figures 2A,B**, the percentage of CD4⁺CD25⁺CD127^{low} and CD4⁺CD25⁺FOXP3⁺ was increased after expansion (74.7 ± 4.9 vs 97.2 ± 1.2 and 75.3 ± 5.1 vs 97.3 ± 0.5 , respectively). Noteworthy, in this study, we used a protocol mirroring the GMP Treg isolation showing the presence of a small fraction of cells negative for FOXP3 and positive for CD127. Similarly, we were able to detect FOXP3-positive cells in the Tconv fraction (**Figures 2A,B**).

The phenotypic analysis of freshly isolated Tregs, Tconv, and expanded Tregs showed an increased expression of functional markers such as CTLA-4, CD39, and GARP especially on expanded Tregs (**Figures 2C,D**). No statistically significant differences were found in the expression of HELIOS and TIGIT between freshly isolated and expanded Tregs. However, in comparison with Tconv, both freshly isolated and expanded Tregs expressed high level of these two markers (**Figures 2C,D**).

To further characterize the Tregs, we tested their suppressive ability. As expected, expanded Tregs showed the highest capacity to inhibit the proliferation of co-cultured CFSE-labeled

effector cells when compared with freshly isolated Tregs and Tconv (**Figure 2E**).

Finally, to establish the cytokine profile produced by the different cell preparations, we activated freshly isolated Tregs, expanded Tregs, and Tconv *in vitro* for 3 days using anti-CD3/CD28 beads. Compared with freshly isolated Tregs and Tconv, expanded Tregs produced less IL-6, IL-17, and TNF- α (**Figure 2F**). Furthermore, both freshly isolated and expanded Tregs produced more IL-10 and TGF- β compared with Tconv (**Figure 2F**). Although no statistically significant differences have been found, both freshly isolated and expanded Tregs tended to release more IL-13 than Tconv.

Overall, these results confirmed that expanded Tregs have an increased capacity to generate tolerance in comparison to freshly isolated Tregs and Tconv.

Expanded Tregs Differentially Activate Monocytes Inducing a Unique Population

To test the capacity of Tregs to activate/modulate CD14⁺ monocytes, we co-cultured HLA-A2⁺ monocytes with HLA-A2⁺ expanded Tregs, freshly isolated Tregs, and Tconv as detailed in **Figure 1**. We evaluated the impact of this interaction by measuring on monocytes the expression of CD80, CD86, CD14, CD206, CD163, CD40, and HLA-DR at day 0 and after 6 days of culture (Figure S2 in Supplementary Material; **Figure 3A**). Monocytes co-cultured with expanded Tregs (M_{exp}) expressed less HLA-DR both as percentage and MFI compared with monocytes co-cultured with Tconv (M_{25-}). Freshly isolated Tregs showed the capacity to reduce HLA-DR expression on monocytes (M_{25+}), but not to the same extent than expanded Tregs. Of note, no significant differences were found in the expression of HLA-DR between freshly isolated and cultured monocytes (Figure S2 in Supplementary Material; **Figure 3A**).

As Tregs have the potential to produce cytokines involved in the differentiation of M2 macrophages, we measured the expression of M2 specific markers such as CD206 and CD163. After 6 days of co-culture, the expression of these markers was increased in all the conditions when compared with the freshly isolated cells. However, monocytes co-cultured with either freshly isolated Tregs or expanded Tregs tended to upregulate the expression of CD163 more than M_{25-} , while M_{exp} showed the highest levels (MFI) of CD206.

The analysis of the co-stimulatory molecules revealed a reduction of CD86 expression in M_{25+} , M_{25-} , and M_{exp} compared with freshly isolated monocytes. In detail, the comparison between M_{25+} , M_{25-} , and M_{exp} showed that the highest expression of CD86 was on M_{25-} (**Figure 3A**). Conversely, CD40 was increased in all the conditions when compared with freshly isolated monocytes and M_{25-} showed the highest MFI (**Figure 3A**).

CD80 expression was not detected in all conditions before and after co-culture (data not shown). Of note, all monocytes from the co-cultures were positive for CD14, although in M_{25-} CD14 MFI was significantly reduced compared with M_{25+} (**Figure 3A**).

We then performed an automated clustering method including t-distributed stochastic neighbor embedding to use SNE visually (viSNE) as a mean of identifying different cell populations (41). Using the most representative markers commonly

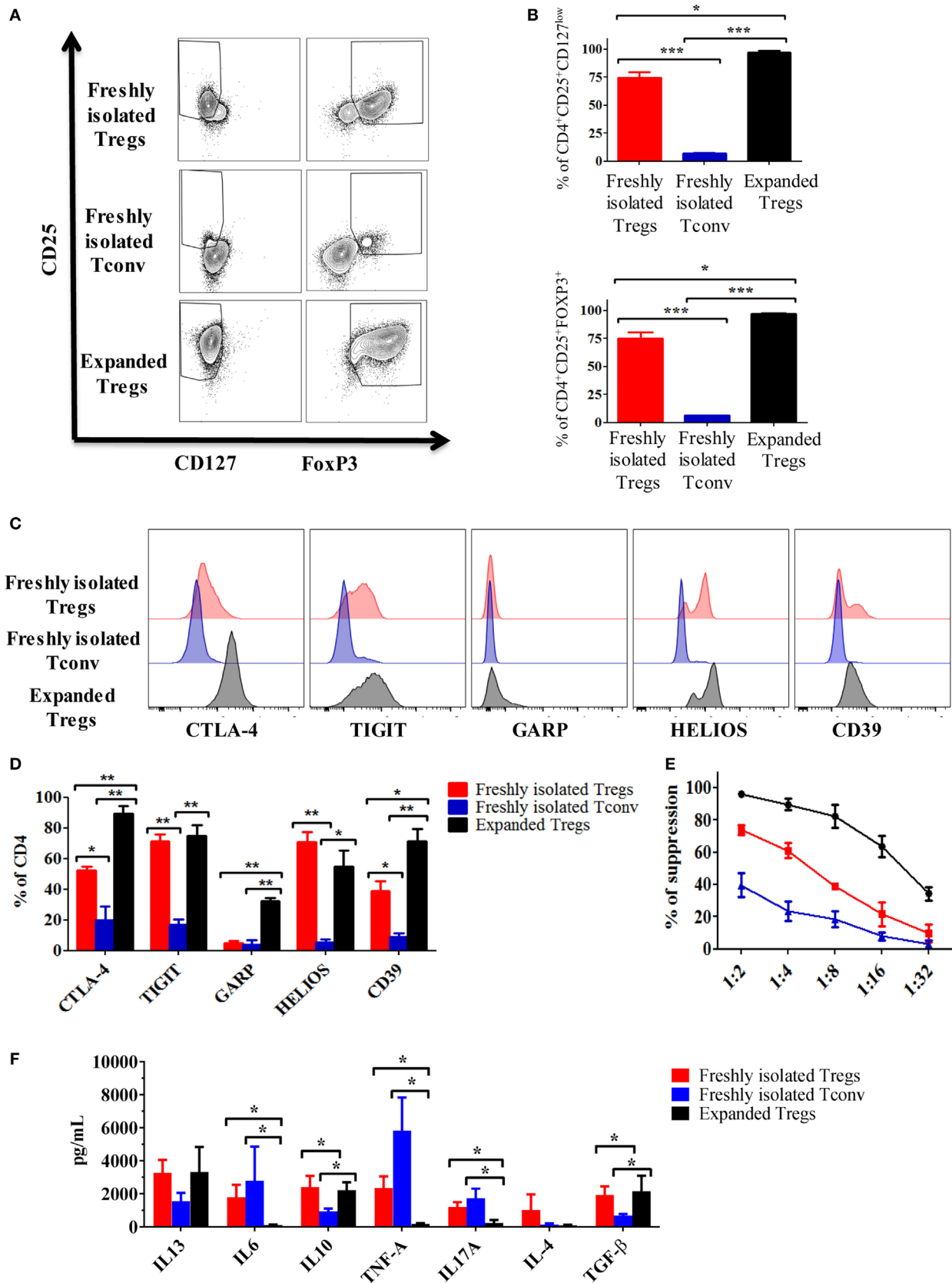


FIGURE 2 | Continued

FIGURE 2 | Expansion of regulatory T cells (Tregs) using rapamycin increases their immunosuppressive capacity. **(A)** Representative dot plots showing the expression of CD25 vs FOXP3 and CD127 in expanded Tregs, freshly isolated Tregs and conventional T cell (Tconv). **(B)** Cumulative data of six independent experiments showing the percentages of CD4⁺CD25⁺CD127^{low} and CD4⁺CD25⁺FOXP3⁺ in expanded Tregs, freshly isolated Tregs and Tconv. Representative histogram **(C)** and cumulative data **(D)** of six independent experiments showing the expression of cytotoxic T-lymphocyte antigen-4 (CTLA-4), TIGIT, GARP, HELIOS, and CD39 in expanded Tregs, freshly isolated Tregs and Tconv. Data are expressed as percentage of expression of CD4⁺ cells. **(E)** Suppressive ability at different ratios of expanded Tregs, freshly isolated Tregs and Tconv vs third party Teff. Means of six independent experiments are expressed as percentage of inhibition of the Teff proliferation. **(F)** Cytokine production by expanded Tregs, freshly isolated Tregs and Tconv after 3 days stimulation with anti-CD3/CD28 beads (4:1 cells to beads ratio). Data are expressed as mean of six independent experiments. In all the experiments, data are presented as mean \pm SEM and analyzed using unpaired *t*-test with **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

used to discriminate M2 macrophages (CD86, CD14, CD206, CD163, and HLA-DR), we showed that M₂₅₊, M₂₅₋, and M_{exp} clustered individually, confirming that expanded Tregs differentially activate monocytes compared with Tconv and freshly isolated Tregs (Figure 3B). In particular, we were able to detect a unique cluster (Figure 3B) which included CD14-positive cells expressing high levels of CD206, CD163 with low or no expression of CD86 and HLA-DR (Figure 3B). These data were confirmed by flow cytometry (Figures 3C,D) as the percentage of CD14⁺CD206⁺CD163⁺CD86⁻ cells was 5.15 ± 1.26 , 3.07 ± 0.7 , and 14 ± 3.4 in M₂₅₊, M₂₅₋, and M_{exp} respectively. In line with viSNE analysis, the MFI for HLA-DR on CD14⁺CD206⁺CD163⁺CD86⁻ was significantly reduced when monocytes were co-cultured with expanded Tregs (Figure 3D). Similar to what we have shown by analyzing the monocyte populations as a whole, expanded Tregs were more powerful in reducing HLA-DR than freshly isolated Tregs.

In conclusion, we showed that the interaction of expanded Tregs with monocytes is more powerful in reducing co-stimulatory and MHC-class II molecules and in the upregulation of M2 macrophages specific markers.

M_{exp} Showed a Reduced Activation Status Compared With M₂₅₊ and M₂₅₋

After activation, monocytes orchestrate both the initiation and resolution of inflammation mediating either pro-inflammatory or anti-inflammatory immune responses. For this reason, we investigated the activation status of M₂₅₊, M₂₅₋, and M_{exp} cells. In monocytes, NF- κ B is an important transcriptional factor linked to the production of pro-inflammatory cytokines and cell surface receptors (42). Therefore, we evaluated the phosphorylation of NF- κ B in sorted cells at the end of the co-culture as described in Figure 1. As shown in Figure 4A, we found a decreased activation of NF- κ B in monocytes co-cultured with Tregs, and this effect was more pronounced in those cells cultured with expanded Tregs. Indeed, NF- κ B (p65) phosphorylation was significantly reduced in M_{exp} compared with M₂₅₋, although no statistical difference was observed between M_{exp} and M₂₅₊ (Figure 4A).

Due to the different activation status of monocytes, we then evaluated their capacity to produce cytokines in response to LPS stimulation. As shown in Figure 4B, M_{exp} had a reduced capacity to secrete TNF- α compared with M₂₅₋, while IL-6 and IL-12 were reduced in both M₂₅₊ and M_{exp} compared with M₂₅₋. Conversely, M_{exp} produced more IL-10 in comparison to either M₂₅₋ or M₂₅₊. Other cytokines such as IL-1 α , IL-1 β , IL-18, and TLSP were not detected in any condition (data not shown). Similarly, no differences were found in the production of TGF- β (Figure 4B).

As IL-10 controls genes implicated in the clearance of oxidative stress, we evaluated the expression of HO-1. This enzyme catalyzes heme degradation into carbon monoxide (CO), ferrous iron, and biliverdin. These cyto-protective molecules have anti-oxidant and anti-inflammatory properties, and they have been linked to both GvHD prevention (43) and induction of tolerance (26). As expected, HO-1 expression was upregulated in M_{exp} compared with M₂₅₊ and M₂₅₋ (Figure 4C).

Here, we showed that expanded Tregs reduced monocytes activation and the release of pro-inflammatory cytokines. In addition, they increase the expression of molecules (IL-10 and HO-1) able to inhibit inflammation.

M_{exp} Reduced the Generation of IL-17-Producing Cells due to Their Lower CD86 Expression

Although DCs are considered the main APCs able to activate and drive the differentiation of T helper cells, monocytes/macrophages can play a similar role (44). To test the capacity of M₂₅₊, M₂₅₋, and M_{exp} monocytes to influence T helper differentiation/proliferation, sorted cells (Figure S3 in Supplementary Material) were co-cultured with CD4⁺ T cells as described in Figure 1. After 6 days of co-culture, the percentages of IL-4, IL-17, IFN- γ , and IL-17/IFN- γ -producing cells were evaluated. As shown in Figures 5A,B, both freshly isolated and expanded Tregs reduced the percentage of IL-17 and IL-17/IFN- γ -producing cells compared with Tconv. Importantly, M_{exp} were more powerful in reducing the percentages of IL-17-producing cells compared with M₂₅₊ (10.9 ± 1.7 vs $6.6 \pm 0.8\%$, respectively). No statistically significant differences were found in the percentages of IL-4 and IFN- γ -producing cells. Finally, in none of the conditions, we could detect a stable induction of Tregs (data not shown).

The differentiation of IL-17-producing cells is mediated by IL-23, IL-1 β , TNF- α , and IL-6 (45); however, this is not solely cytokine dependent (46). As M_{exp} produced low levels of IL-6 and TNF- α with a reduced expression of co-stimulatory molecules, we co-cultured M₂₅₋ and CD4⁺ T cells together with factors inhibiting either cytokines or co-stimulatory molecules expression. Three different blocking monoclonal antibodies were used in this experiment: infliximab (anti-TNF- α), tocilizumab (anti-IL-6 receptor), and abatacept (CTLA-4 fusion protein used to block CD80/CD86 co-stimulation). We found that the capacity of M₂₅₋ to increase IL-17-producing cells was drastically reduced only in the presence of abatacept (Figures 5C,D). Of note, this was not due to cell death as the numbers at the end of the culture were comparable between CD4⁺ T cells co-cultured with M₂₅₋ alone or

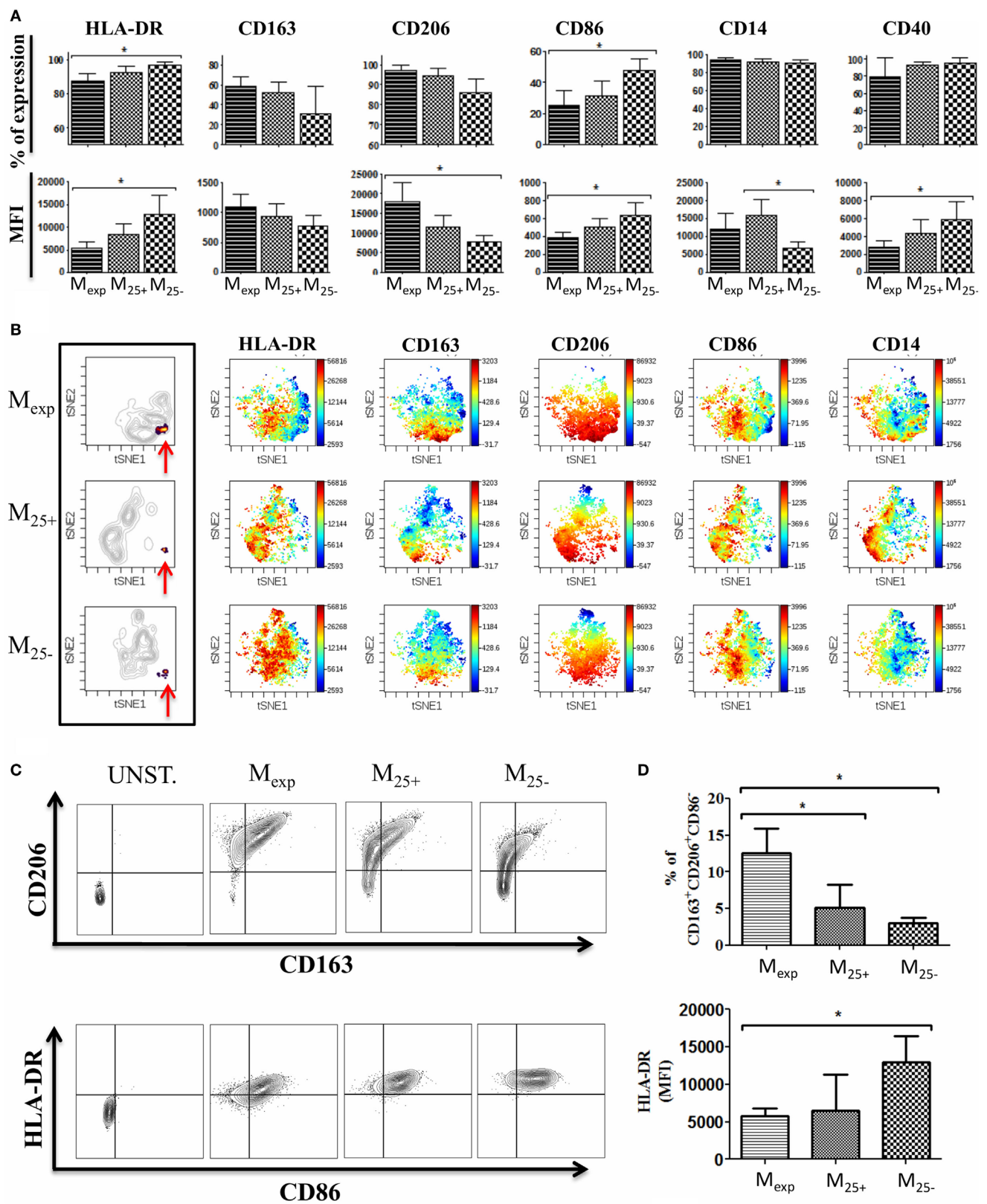


FIGURE 3 | Continued

FIGURE 3 | Phenotype of monocytes after co-culture with T cells. **(A)** Expression of HLA-DR, CD86, CD14, CD206, CD163, and CD40 evaluated by flow cytometry on monocytes co-culture with expanded regulatory T cells (Tregs) (M_{exp}), freshly isolated Tregs (M_{25+}), and freshly isolated conventional T cell (M_{25-}) for 6 days. Percentages of expression (upper panels) and MFI (lower panels) of 10 independent experiments. **(B)** Visualization of one representative automated clustering method including t-distributed stochastic neighbor embedding (t-SNE) highlighting the unique cluster (indicated by the red arrows) in M_{exp} , M_{25+} , and M_{25-} followed by the intensity of expression of HLA-DR, CD86, CD14, CD206, and CD163. **(C)** Representative plots showing CD206 and CD163 expression (upper panels) and HLA-DR plus CD86 on CD206⁺CD163⁺ cells (lower panel) in unstained control, M_{exp} , M_{25+} , and M_{25-} cells. **(D)** Cumulative data of six independent experiments showing the percentages of CD14⁺CD206⁺CD163⁺CD86⁺ in M_{exp} , M_{25+} , and M_{25-} (upper panel) and their HLA-DR expression (lower panel). In all the experiments, data, presented as mean \pm SEM, were analyzed using one-way ANOVA followed by Tukey with $*p < 0.05$.

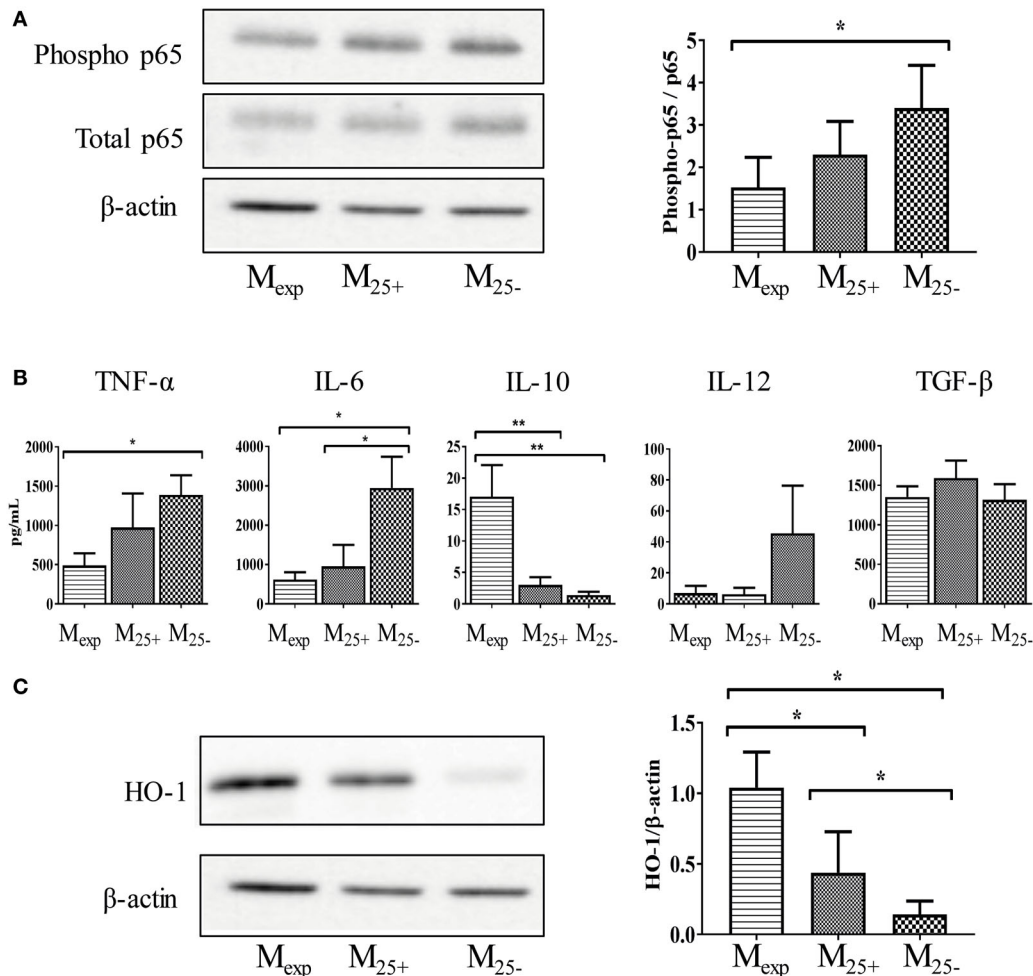
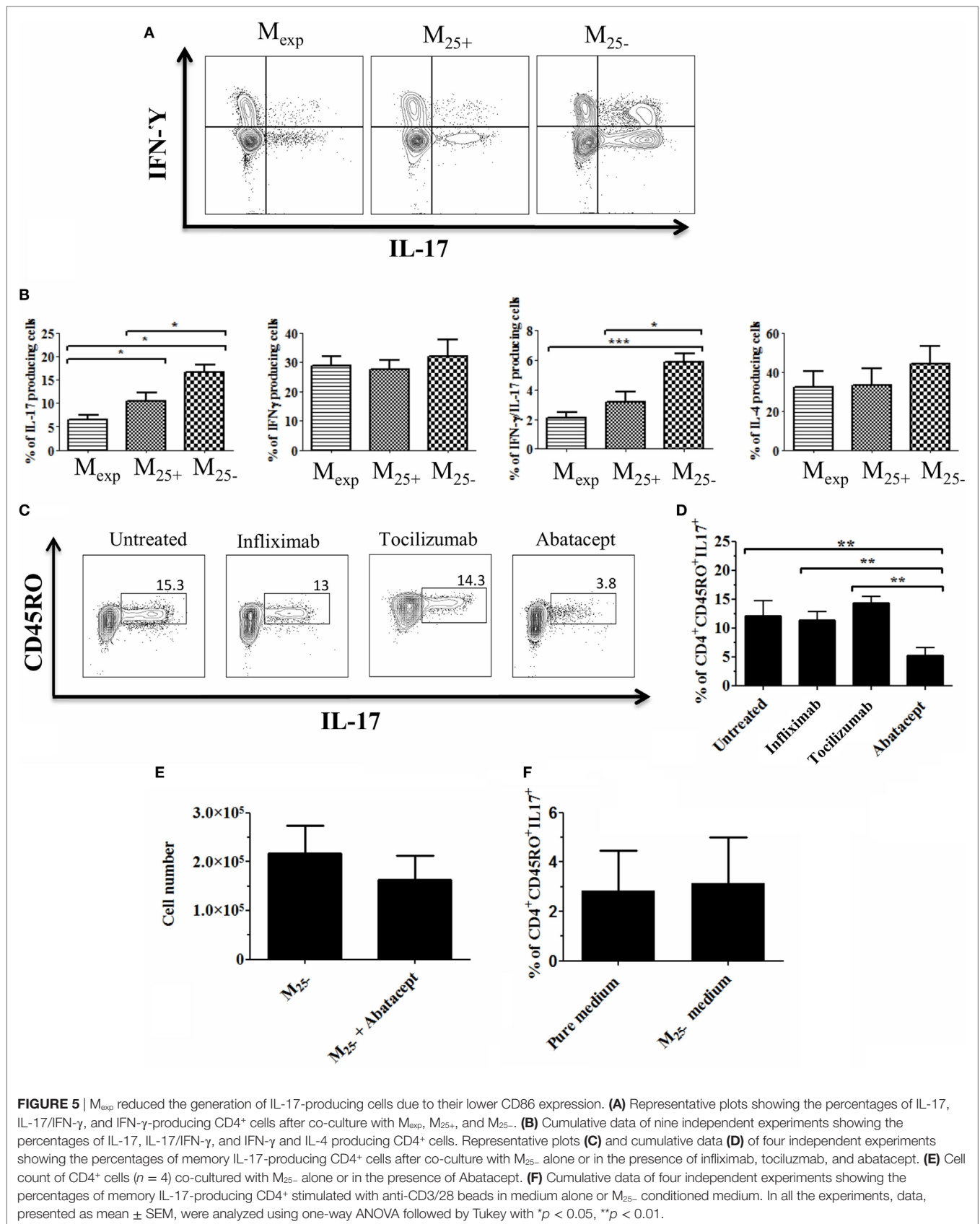


FIGURE 4 | M_{exp} showed a reduced activation status compared with M_{25+} and M_{25-} . **(A)** Representative WB showing the expression of phosphorylated and total p65 and β -actin in M_{exp} , M_{25+} , and M_{25-} (left panels); cumulative data of four independent experiments showing the ratio between phosphorylated and total p65 (right panel) in M_{exp} , M_{25+} , and M_{25-} cells. **(B)** Cytokine production of sorted M_{exp} , M_{25+} , and M_{25-} after LPS stimulation (50 ng/mL) for 24 h. **(C)** Representative WB showing the expression of β -actin and heme oxygenase-1 (HO-1) in M_{exp} , M_{25+} , and M_{25-} (left panels); cumulative data of four independent experiments showing the ratio between HO-1 and β -actin (right panel) in M_{exp} , M_{25+} , and M_{25-} cells. In all the experiments, data, presented as mean \pm SEM, were analyzed using one-way ANOVA followed by Tukey with $*p < 0.05$, $**p < 0.01$. Full length-blots are presented in Figure S4 in Supplementary Material. The same representative β -actin blot is shown in panels **(A,C)**.

in the presence of abatacept (**Figure 5E**). To further confirm the role of co-stimulation in the expansion of IL-17-producing T cells in our experimental condition, CD4⁺ T cells were stimulated with anti-CD3/CD28 beads in medium coming from M_{25-} culture (conditioned medium). No difference was observed between the

two culture conditions confirming the need for cell:cell contact in Th-17 proliferation (**Figure 5F**).

Overall, our data suggest that M_{exp} had a reduced capacity to generate/expand IL-17- and IL-17/IFN- γ -producing T cells due mainly to their lower CD86 expression.



DISCUSSION

Over the past years, Tregs moved from promising cell candidates for tolerance induction to a therapeutic tool for the treatment of GvHD (47), autoimmune disorders (48), or to induce transplantation tolerance (49). Results from the first clinical trials showed that Tregs could be purified (47), expanded in GMP facilities (50), and re-infused in patients (32, 51). To date, the GMP immunomagnetic Treg isolation (CliniMACS) does not allow the purification of a highly pure FOXP3⁺ Treg product (47) as the cell fraction is contaminated with activated Tconv. To avoid the infusion of activated cells, we and others have developed Treg expansion protocols that include rapamycin (36, 52). These protocols have been demonstrated to be a successful approach for large-scale generation of functionally potent and phenotypically stable Tregs. Up to this stage, our group has positively completed two clinical trials investigating the safety of infusing *ex vivo* expanded Tregs in solid organ transplantation (the ONE Study and Thrill in kidney and liver transplantation, respectively). The outcomes of these trials are very promising; however, many questions remain unanswered, including which cells are targeted by Tregs *in vivo*.

Our expansion protocol allowed the proliferation of FOXP3⁺ cells reducing contaminants from the freshly isolated fraction. Consistently, here we have shown that expanded Tregs do not produce TNF- α , IL-6, and IL-17 and, at the same time, release high level of IL-10 and TGF- β . Expanded Tregs also express high level of functional markers like CTLA-4, GARP, and CD39. Furthermore, rapamycin expanded Tregs are more powerful than freshly isolated Tregs in driving monocytes differentiation toward alternatively activated macrophages (AAMs). In 2007, Tiemessen et al. described the ability of freshly isolated Tregs to drive monocytes toward AAMs (22). The authors showed that the co-culture of Tregs with monocytes upregulated M2-specific markers, reduced their NF- κ B activation, and the release of pro-inflammatory cytokines. In line with these results, we found that expanded Tregs are more efficient than freshly isolated cells in doing so.

Tumor necrosis factor- α is crucial in all the phases of GvHD pathophysiology (53), and high concentrations of circulating TNF- α are considered as an immunological marker of graft rejection (54). CD14⁺ monocytes from healthy control cultured in the presence of TNF- α and GM-CSF differentiates into mature DCs or activated macrophages. Mature DCs expressed higher level of CD86 compared with those generated by GM-CSF and IL-4. As a consequence, these cells induced resting CD4 T cells to secrete IL-17. This evidence suggests that the priming of monocytes with TNF- α influences Th-17 responses induced by monocyte-derived mature DC (55). In our study, expanded Tregs decreased the expression of CD86 on monocytes during the co-culture. This effect might depend on the lack of TNF- α production, concomitant with the high level of IL-10 released by expanded Tregs. In addition, the presence of high level of CTLA-4 on these cells might also play a role in mediating the active removal of CD86 from cell surface. Importantly, the low levels of CD86 found in M_{exp} are essential in reducing their capacity to induce IL-17-producing T cells, as demonstrated in the co-culture of M₂₅₋ and CD4⁺ T cells where IL-6, TNF- α , and the

co-stimulatory molecules were blocked. Th-17 have a detrimental effect in solid organ transplantation and GvHD (56, 57). Recently, we published that spontaneously kidney-tolerant recipients exhibited reduced Th-17 responses compared with patients with chronic rejection and healthy individuals. These data suggested that defective pro-inflammatory Th-17 responses might contribute to the maintenance of a stable graft function in the absence of immunosuppressive agents (58). In addition, a recent study showed that monocytes isolated from patients with acute or chronic GvHD induced more Th-17 cells *in vitro* compared with monocytes from patients without GvHD and healthy donors (33). Altogether, these data confirmed that controlling IL-17-producing T cells might be crucial for the induction of tolerance in transplantation and for the treatment of GvHD. Finally, TNF- α is also responsible for the activation of NF- κ B (59). Therefore, the reduced production of TNF- α by expanded Tregs may explain the decreased phosphorylation of NF- κ B that, in turn, lead to a reduced capacity to produce pro-inflammatory cytokines in M_{exp} compared with M₂₅₋.

In 2007, Tiemessen et al. hypothesized that the upregulation of CD206 by Tregs is contact dependent (22). In fact, when monocytes and Tregs were cultured together in the same well, but kept separated by a permeable support, monocytes were not able to upregulate CD206. However, in 2014, Fernando et al. (60) showed that during the *in vitro* differentiation of monocytes in M2a macrophages using IL-4 and IL-13, IL-6 enhanced the expression of CD206 in monocytes. In our culture conditions, expanded Tregs did not produce IL-6 compared with freshly isolated Tregs and Tconv reinforcing the idea that CD206 induction Treg-mediated might be controlled by a contact dependent mechanism. However, further investigations are needed to fully understand how T cells control the expression of CD206.

Both freshly isolated and expanded Tregs produced high level of IL-10 and TGF- β compared with Tconv; these anti-inflammatory cytokines have been linked with monocyte conversion to M2 type c macrophages (19). Our results showed consistently that both M₂₅₊ and M_{exp} had a reduced expression of CD86, CD40, and HLA-DR compared with M₂₅₋, although statistically significant results were only obtained in the co-culture with expanded Tregs. This is probably due to the production of pro-inflammatory cytokines, such as TNF- α (released by the freshly isolated Treg fraction), which has been shown to induce CD40 and CD86 in monocytes/macrophages. Another protein upregulated in M_{exp} compared with M₂₅₊ and M₂₅₋ was HO-1. This enzyme has an essential role in suppressing immune responses linked to inflammation (24), autoimmune diseases (25), and allograft rejection (26). Specifically, the pre-treatment of pancreatic allografts prior to transplantation with an HO-1 inducer increased graft survival due to the reduction of pro-inflammatory and an increase in anti-inflammatory cytokines (61). HO-1 has a role in GvHD prevention as well; the high HO-1 expression in GvHD target organs may attenuate the acute phase of the disease through the regulation of the balance between Th-17 and Tregs (43). The role of HO-1 in macrophage polarization has been demonstrated (62), with M2 macrophages expressing high levels of this enzyme. HO-1 expression can be regulated by IL-10 signaling and *vice versa*. Specifically, IL-10-mediated induction

of HO-1 has been shown to require activation of STAT-3 and PI3K pathways in macrophage cell lines (62); subsequently, CO, the HO-1 bio-product, increases IL-10 production. In our model, monocytes treated with Tregs, upregulated HO-1 expression. In particular, M_{exp} were the cells with the highest HO-1 expression and the highest IL-10 production. This is in line with the positive feedback circuit between HO-1 and IL-10 reported in literature. These findings together with the reduced level of NF- κ B phosphorylation might explain the observed reduced capacity of M_{exp} to produce IL-6 and TNF- α .

Overall, we have shown the capacity of expanded Tregs to alternatively activate monocytes/macrophages by contact and no-contact dependent mechanisms. Importantly and relevant to the *in vivo* impact of Treg therapy, expanded Tregs induced a subset of monocytes/macrophages that are characterized by a unique signature. This might explain why we were able to detect the CD14⁺CD206⁺CD163⁺CD86⁻ cell population with low HLA-DR expression in the M_{exp} group.

In conclusion, the capacity of expanded Tregs to induce AAMs might shed light on the mechanisms adopted by expanded Tregs to favor tolerance *in vivo*. Our cell therapy protocol aims to modulate the ratio between activated effector cells and Tregs. Increasing the level of Tregs over the effector T cells might drive the monocytes differentiation toward a population producing low level of TNF- α and IL-6 and high level of IL-10. Furthermore, the Treg-induced monocytes might be able to clear, by HO-1, the inflammation-mediated oxidative stress and contribute to reduce the infiltrating IL-17-producing cells responsible of graft rejection and GvHD progression. In conclusion, our *in vitro* model showed how expanded Tregs may favor the organ acceptance or reduce chronic inflammation. This might be exerted directly, inducing AAMs and/or indirectly through the reduction of IL-17-producing cells.

ETHICS STATEMENT

Peripheral blood mononuclear cells were obtained from anonymized human leukocyte cones supplied by the National

Blood Transfusion Service (NHS blood and transplantation, Tooting, London, UK). Human studies were conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of Guy's Hospital (Reference 09/H0707/86). Informed consent was obtained from all healthy donors prior to enrolment into the study.

AUTHOR CONTRIBUTIONS

MR conception design, collection and assembly data, data analysis and interpretation, and manuscript writing. GF, NT, RM, and EN-L collection and assembly data, data analysis, and interpretation. RL intellectual input and critical revision of the article. GL and CS conception and design, critical revision of the article for important intellectual contents.

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Lactobacillus sakei WIKIM30 Ameliorates Atopic Dermatitis-Like Skin Lesions by Inducing Regulatory T Cells and Altering Gut Microbiota Structure in Mice

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Lactobacillus sakei WIKIM30 is a Gram-positive facultative anaerobic bacterium isolated from kimchi, a Korean fermented vegetable food. In this study, we found that WIKIM30 promoted regulatory T cell (Treg) differentiation by inducing dendritic cells with tolerogenic properties. The production of the T helper (Th) 2-associated cytokine interleukin (IL)-4 was decreased, but that of the Treg-associated cytokine IL-10 was increased in splenocytes from ovalbumin-sensitized mice treated with WIKIM30. We also investigated the inhibitory capacity of WIKIM30 on the development of 2,4-dinitrochlorobenzene-induced atopic dermatitis (AD), a Th2-dominant allergic disease in mice. Oral administration of *L. sakei* WIKIM30 significantly reduced AD-like skin lesions and serum immunoglobulin E and IL-4 levels while decreasing the number of CD4⁺ T cells and B cells and the levels of Th2 cytokines (IL-4, IL-5, and IL-13) in peripheral lymph nodes and enhancing Treg differentiation and IL-10 secretion in mesenteric lymph nodes. In addition, WIKIM30 modulated gut microbiome profiles that were altered in AD mice, which showed increases in *Arthromitus* and *Ralstonia* and a decrease in *Ruminococcus* abundance. These changes were reversed by WIKIM30 treatment. Notably, the increase in *Ruminococcus* was highly correlated with Treg-related responses and may contribute to the alleviation of AD responses. Together, these results suggest that oral administration of *L. sakei* WIKIM30 modulates allergic Th2 responses enhancing Treg generation and increases the relative abundance of intestinal bacteria that are positively related to Treg generation, and therefore has therapeutic potential for the treatment of AD.

Keywords: atopic dermatitis, lactic acid bacteria, *Lactobacillus sakei*, regulatory T cells, gut microbiota, kimchi

INTRODUCTION

Atopic dermatitis (AD) is an allergic skin disease accompanied by chronic inflammation that is characterized by severe itching, redness, dryness, and eczematous skin lesions and is associated with high serum level of immunoglobulin (Ig)E (1). Although the pathogenesis of AD is not fully understood, it is known to be caused by immune dysregulation resulting from the complex interaction

of environmental and genetic factors. T helper (Th) 2 responses are predominant in the acute stage of AD, whereas Th1 and Th17 responses contribute to disease pathogenesis at the chronic stage (2–4). Thus, AD lesions show accumulation of infiltrated inflammatory cells and destruction of the skin barrier due to an imbalance in Th responses (2).

Various therapeutic approaches have been used to alleviate chronic inflammation in AD, including the administration of probiotics in recent therapeutic trials (5, 6). Probiotics are live nonpathogenic bacteria that provide health benefits when consumed such as strengthening of the gastrointestinal (GI) epithelial barrier, inhibiting the growth of enteric pathogens, improving the balance of gut microbiota, and enhancing host intestinal immune function (7, 8). Immune homeostasis in the gut is achieved through interaction with epithelial cells, dendritic cells (DCs), macrophages, and lymphocytes. DCs are specialized antigen-presenting cells that stimulate immune responses by modulating T cell function (9, 10). Ingested probiotics induce DC maturation and migration to the mesenteric lymph node (MLN), where they increase immune tolerance by stimulating the generation of regulatory T cells (Tregs) (11). CD103⁺ DCs are critical for mediating immune tolerance in the GI system (12, 13). During the allergic response, Tregs migrate into peripheral tissues and inflammation draining lymph nodes, leading to Treg-mediated immune suppression of Th2 immune responses (14–17).

Microorganisms colonizing the gut play important roles in host health, including digestion of dietary components, production of metabolites (fatty acids, glycolipids, and vitamins), and regulation of immune system maturation (18). Maintaining proper gut microbiota is critical for host immune homeostasis; perturbation of the gut microbiota balance is correlated with the development and progression of various disorders including allergic diseases (19, 20). Modulating gut microbiota composition and function is a promising strategy for their treatment. Emerging clinical evidence indicates that probiotics can restore gut microbiota composition and promote the beneficial functions of gut microbiota, thereby improving disease symptoms (21). However, the underlying mechanisms on how this alleviates diseases remain to be elucidated.

In this study, we isolated *Lactobacillus sakei* strain WIKIM30 from kimchi, a Korean traditional fermented food, and investigated its immunomodulatory properties in a mouse model of AD induced by 2,4-dinitrochlorobenzene (DNCB). We found that WIKIM30 induced the transformation of DCs to a tolerogenic form that promoted Treg differentiation *in vitro* and improved AD symptoms *in vivo* through modulation of immune responses and gut microbiome composition.

MATERIALS AND METHODS

Isolation and Preparation of *L. sakei* WIKIM30

Lactobacillus sakei WIKIM30 was isolated from homemade kimchi in Korea. The kimchi was homogenized in a stomacher, and the homogenate was passed through the filter bag and

diluted before it was spread onto a de Man, Rogosa, and Sharpe (MRS; BD Biosciences, Franklin Lakes, NJ, USA) agar plate that was then incubated at 30°C for 2 days. The resultant lactic acid bacteria (LAB) colonies were isolated by sequential culturing and identified based on the 16S rRNA gene sequence. Sequence data were aligned and compared to those in the GenBank database. A phylogenetic analysis of the 16S rRNA gene sequence in the isolate revealed a 99.86% similarity to that of *L. sakei*; it was thus deposited in the Korean Federation of Culture Collection as *L. sakei* KFCC 11625P.

WIKIM30 was cultured overnight at 30°C in MRS broth. The culture was diluted 1:200 in fresh medium and cultured for a second night for maximal growth. The optical density at 600 nm (OD₆₀₀) was measured, and the number of colony-forming units (CFU) was determined from standard growth curves. For all cultured bacterial strains, an OD₆₀₀ value of 1 corresponded to 1×10^8 CFU/ml, which was confirmed by plating serial dilutions on MRS agar plates. After overnight culture, bacteria were washed in fresh, sterile phosphate-buffered saline (PBS; pH 7.4) and immediately administered to the mice, which received either sterile PBS or 2×10^9 CFU bacteria in 200 μ l PBS by intragastric gavage every day.

In Vitro Culture and Stimulation of Murine Bone Marrow-Derived DCs (BMDCs)

Bone marrow (BM) cells were isolated and cultured as previously described (22, 23). Femora and tibiae from 6-week-old male BALB/c mice were removed and stripped of muscles and tendons. The bones were rinsed in PBS and then crushed with a mortar to release BM cells. After washes with Roswell Park Memorial Institute (RPMI)-1640 medium, BM cells (2×10^6) were seeded in Petri dishes in 10 ml complete RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M β -mercaptoethanol in the presence of 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA). The cells were incubated for 10 days at 37°C. On day 3, the culture medium was supplemented with fresh complete RPMI-1640 containing 20 ng/ml murine GM-CSF, and on day 8, the medium was replaced with fresh complete RPMI-1640 containing 20 ng/ml murine GM-CSF. On day 10, immature DCs were collected and seeded in a 96-well plate at 5×10^5 cells/well. The cells were either left unstimulated or were stimulated with *L. sakei* WIKIM30 (1:5 cell to bacteria ratio) or LPS (100 ng/ml) for 24 h at 37°C. The culture supernatant was then collected and TNF- α , interleukin (IL)-12p70, and IL-10 levels were evaluated by flow cytometry using a Cytometric Bead Array kit (BD Biosciences, San Jose, CA, USA). For phenotypic analysis, cells were stained for the DC marker CD11c and CD11b; activated DC markers CD40, CD69, CD80, CD86, and MHCII; tolerogenic DC markers inducible T-cell costimulator ligand (ICOS-L), programmed death ligand (PD-L)1, and CD103; or appropriate isotype controls (BD Biosciences) and analyzed by flow cytometry. Data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

BMDC/T Cell Cocultures

The BMDC and T cell coculture system was adapted from a previous study (24). Briefly, immature BMDCs were stimulated with 100 ng/ml of LPS or WIKIM30 for 24 h at 37°C. Unstimulated or stimulated BMDCs (1×10^5 cells/well) were washed and cocultured with naïve CD4⁺ T cells (3×10^5 cells/well) purified from the spleen of BALB/c mice in the presence of soluble anti-CD3 monoclonal antibody (mAb) (2 µg/ml) for 4 days. Naïve CD4⁺ T cells were purified using the Naïve CD4⁺ T Cell Isolation kit (130-094-131; Miltenyi Biotec, San Diego, CA, USA).

Animal Studies

Wild-type male BALB/c mice were purchased from OrientBio Co. (Gwangju, Korea). Two to three mice were housed in each ventilated cage at a temperature of $22 \pm 2^\circ\text{C}$ and relative humidity of $55 \pm 5\%$ under a 12:12-h light/dark cycle in a pathogen-free animal facility at the World Institute of Kimchi. The mice were fed standard chow and had free access to water. To investigate the beneficial effects of LAB on AD, AD-like lesions were induced with DNCB (Sigma-Aldrich, St. Louis, MO, USA) as previously described (6). Mice were randomized into the following four groups ($n = 5$ each): naïve (treatment-naïve mice fed vehicle), negative control (mice treated with DNCB and fed vehicle), positive control (mice treated with DNCB and fed ketotifen at 1 mg/kg), and WIKIM30 (mice treated with DNCB and fed *L. sakei* WIKIM30). The dorsal skin was shaved, and 200 µl of 1% DNCB in acetone/olive oil (3:1) was applied twice a week. After 3 weeks, 0.2% DNCB was applied to the dorsal skin once a week. WIKIM30 or PBS (200 µl) was administered to the mice by intragastric gavage once daily. The vehicle and LAB were administered for 42 days. The severity of dermatitis in the dorsal skin of DNCB-induced AD mice was evaluated based on five symptoms (erythema/hemorrhage, edema/excoriation, erosion, scarring/dryness, and lichenification), which were scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). Dermatitis score was defined as the sum of individual scores. On day 43, the mice were sacrificed under CO₂, and blood samples, dorsal skin, spleen, MLNs, and peripheral (P) LNs (axillary and inguinal LNs) were collected for further analysis.

Measurement of Total Serum IgE and Cytokine Levels

Blood samples were collected from mice after sacrifice, and serum samples were obtained by centrifugation ($3,000 \times g$, 10 min). Serum IgE and IL-4 levels were measured using the OptEIA Mouse Sets (BD Biosciences). Single-cell suspensions from spleen, PLN, and MLN samples were obtained by mechanical disruption in 0.5 ml complete RPMI-1640. The cells were seeded in a 96-well plate at 5×10^5 cells/well and cocultured with anti-CD3/CD28 mAbs (1 µg) for 24 h at 37°C. IL-4, -5, -10, and -13 and IFN-γ concentrations in the supernatant were quantitated by flow cytometry using a Cytometric Bead Array kit.

Histological Analysis

The dorsal skin of mice was removed, fixed in 10% phosphate-buffered formalin, embedded in paraffin, and cut into

sections that were stained with hematoxylin and eosin (H&E) for evaluation of edema. Other sections were stained with Toluidine Blue for detection of mast cells.

Flow Cytometry Analysis

Mesenteric lymph nodes isolated from each group of mice and CD4⁺ T cells cocultured with LPS- or WIKIM30-treated BMDCs were labeled with fluorescein isothiocyanate-conjugated anti-mouse CD4, allophycocyanin (APC)-conjugated anti-mouse CD25, peridinin chlorophyll-Cy5.5-conjugated anti-mouse T cell receptor β (BD Biosciences), or phycoerythrin (PE)-conjugated anti-mouse Forkhead box (Fox)p3 (eBioscience, San Diego, CA, USA) mAb. Flow cytometry was performed on a FACSCanto II system (BD Biosciences), and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Intracellular Cytokine Staining

Isolated peripheral lymph node cells were cultured in the presence of 20 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1 mM ionomycin (Sigma-Aldrich), and 10 µg/ml brefeldin A for 4 h. Following surface antigen staining, cells were treated with fixation and permeabilization solutions (eBioscience) according to the manufacturer's instructions and then stained with APC-conjugated anti-IFN-γ (clone XMG1.2; BD Biosciences) and PE-conjugated anti-IL-4 (clone 11B11; BD Biosciences) mAbs, with isotype-matched antibodies used as controls.

Gut Microbiota Analysis

Fresh fecal samples were collected from mice and immediately stored at -150°C until processing. Fecal DNA was isolated using the FastDNA Spin kit (MP Biomedicals, Santa Ana, CA, USA). PCR amplification was performed using barcoded primers targeting the V3 to V4 region of the bacterial 16S rRNA gene. Amplicons were sequenced using the 250-bp paired-end read strategy on the MiSeq sequencing system (Illumina, San Diego, CA, USA). Fast length adjustment of short reads was used to merge reads based on the Greengenes database. Output data were analyzed using BIOPLUG software (Chunlab, Seoul, Korea).

Statistical Analysis

Statistical analysis was performed using Prism v.6.0 software (GraphPad Inc., La Jolla, CA, USA), and results are presented as mean \pm SE. Treatment effects were evaluated with the Student's *t*-test; $p < 0.05$, $p < 0.01$, and $p < 0.001$ was considered statistically significant. To examine the relationship between gut microbiota abundance and various parameters related to AD and WIKIM30 in mouse groups, a principal component analysis was performed using XLSTAT (Addinsoft, Paris, France).

RESULTS

WIKIM30 Modulates DC and T Cell Function *In Vitro*

Th1 and Th2 immunity and immune tolerance are regulated by DCs (10). The ability of DCs to prime the T cell response depends on the expression of co-stimulatory molecules and secretion

of cytokines (25). To evaluate the immunomodulatory role of WIKIM30, we examined changes in the phenotype and function of murine BMDCs following treatment with WIKIM30. We found that WIKIM30 treatment led to increased levels of inflammatory cytokines (TNF- α , IL-6, and IL-12p70) as well as immunoregulatory cytokine (IL-10) compared to unstimulated BMDCs. TNF- α , IL-6, and IL-12p70 production in WIKIM30- and LPS-treated BMDCs was comparable (Figure 1A), while IL-10 production in WIKIM30-treated BMDCs was markedly higher than that in LPS-treated BMDCs (Figure 1B). In addition, we compared the pattern of cytokines secretion by WIKIM30 treatment with that of different toll-like receptors (TLR) ligands (Figure S1 in Supplementary Material). Treatment of BMDCs with Pam3Cys-Ser-Ly4 (PAM, TLR1/2), LPS (TLR4), and Pam2CGDHPKHPKSF (FSL-1, TLR6/2) induced comparable levels of TNF- α , and IL-6, whereas only LPS induced that of IL-12p70 with WIKIM30. IL-10 production was observed in LPS- and FSL-1-treated BMDCs, which were lower than that in WIKIM30-treated BMDCs. Next, we analyzed the expression of surface molecules including markers of activated DCs (CD40, CD69, CD80, CD86, and MHCII) and tolerogenic DCs (ICOS-L, PD-L1, and CD103) in BMDCs treated with WIKIM30 and found that CD40, CD69, CD80, CD86, and MHCII levels were comparable to those in LPS-treated BMDCs (Figures 1C,D). However, tolerogenic DC markers such as PD-L1 and CD103 were more highly expressed in WIKIM30-treated than in LPS-treated BMDCs (Figures 1E,F).

Since tolerogenic DCs are known to have an immunoregulatory effect on CD4⁺ T cells (26, 27), we investigated whether WIKIM30-treated BMDCs promote the differentiation of naïve T cells into Tregs. The generation of CD4⁺CD25⁺Foxp3⁺ Tregs was significantly increased in CD4⁺ T cells cocultured with WIKIM30-treated BMDCs compared to those cultured with untreated BMDCs (Figure 1G). Indeed, WIKIM30 treatment increased the levels of IL-10 but not of IL-4, IL-17, or IFN- γ in the supernatant (Figure 1H). These results indicate that WIKIM30 can induce the differentiation of tolerogenic DCs, which in turn promote the generation of CD4⁺CD25⁺Foxp3⁺ T cells.

Oral Administration of WIKIM30 Suppresses DNCB-Induced AD

To clarify whether WIKIM30 modulates T cell immune responses, we measured the levels of cytokines produced by splenocytes from OVA-sensitized BALB/c mice, which were re-stimulated with OVA in the presence and absence of WIKIM30 (Figure S2 in Supplementary Material). WIKIM30 strongly inhibited the release of the Th2 cytokine IL-4, while enhancing that of IL-10 in an antigen-specific manner. Based on our observation that WIKIM30 modulates the Th1/Th2 response and induces an anti-inflammatory response *in vitro*, we investigated whether WIKIM30 can alleviate AD—which is characterized by Th2 polarization (28)—using a DNCB-induced AD mouse model. The mice were orally administered PBS (AD group), the anti-histamine agent ketotifen (Keto group), or WIKIM30 (WIKIM30 group) for 42 days (Figure 2A). WIKIM30 intake ameliorated AD-like lesions compared to the AD group. Indeed, dermatitis

scores—which integrate individual scores for four dermatitis symptoms (erythema, edema, erosion, and dryness)—were significantly reduced in the WIKIM30 as compared to the AD group (Figure 2B). We next examined the effect of WIKIM30 intake on the production of IgE and IL-4, which is a hallmark of AD caused by a strong Th2 immune response. As shown in Figures 2C,D, serum IgE and IL-4 levels were markedly lower in the WIKIM30 group (39 and 19%, respectively) than in the AD group. Histological analysis showed that dorsal skin thickening and inflammatory cell infiltration in AD mice were reversed, as determined by H&E staining (Figure 2E), whereas the accumulation and degranulation of mast cells, which mediate hypersensitivity by releasing inflammatory mediators, was suppressed by WIKIM30 treatment relative to the AD group, as determined by Toluidine Blue staining (Figure 2F). Collectively, these data demonstrate that oral administration of WIKIM30 effectively alleviates AD-like symptoms.

WIKIM30 Suppresses Th2 Immune Responses in PLNs

The inguinal and axillary LNs located near AD-like skin lesions are active sites of immune cell accumulation, proliferation, and differentiation. Compared to naïve mice, total numbers of lymphocytes including CD4⁺ T cells, CD8⁺ T cells, and CD19⁺ B cells were expanded in PLNs with AD, but WIKIM30 treatment significantly inhibited lymphocyte recruitment to PLNs (Figure S3 in Supplementary Material). To determine whether oral administration of WIKIM30 regulates the Th1/Th2 immune balance in lymphoid organs, we examined the frequencies of CD4⁺ cells expressing IL-4 and IFN- γ at a single-cell level by intracellular cytokine staining. There were more IL-4-producing CD4⁺ T cells in AD mice, while the frequency of IL-4-producing CD4⁺ T cells was lower in the WIKIM30 group than in AD group mice. Meanwhile, the frequency of CD4⁺ T cells expressing IFN- γ was comparable across groups (Figure 3A). We also measured the levels of Th1 and Th2 cytokines secreted in the supernatant following polyclonal stimulation of PLN cells. WIKIM30 treatment reduced the levels of IL-4, IL-5, and IL-13 relative to the AD group (Figure 3B). However, IFN- γ production was comparable across groups (Figure 3C). These results indicate that WIKIM30 exerts a protective effect against AD by suppressing the Th2 immune response.

WIKIM30 Promotes Treg Differentiation and Function in MLNs

The gut mucosal immune system contributes to immune tolerance by modulating the ratio of effector T cells to Tregs. We therefore investigated whether WIKIM30 intake influences immunomodulation in the gut-draining lymph nodes. WIKIM30 treatment significantly increased the MLN CD4⁺CD25⁺Foxp3⁺ Treg population compared to all other groups (Figure 4A). IL-10 production in polyclonally stimulated MLN cells showed a similar trend, while IL-4 and IFN- γ levels were comparable across groups (Figure 4B). The movement of Tregs into inflammatory draining lymph nodes is critical for the acquisition of immunosuppression (15, 16). The CD4⁺CD25⁺Foxp3⁺ Treg

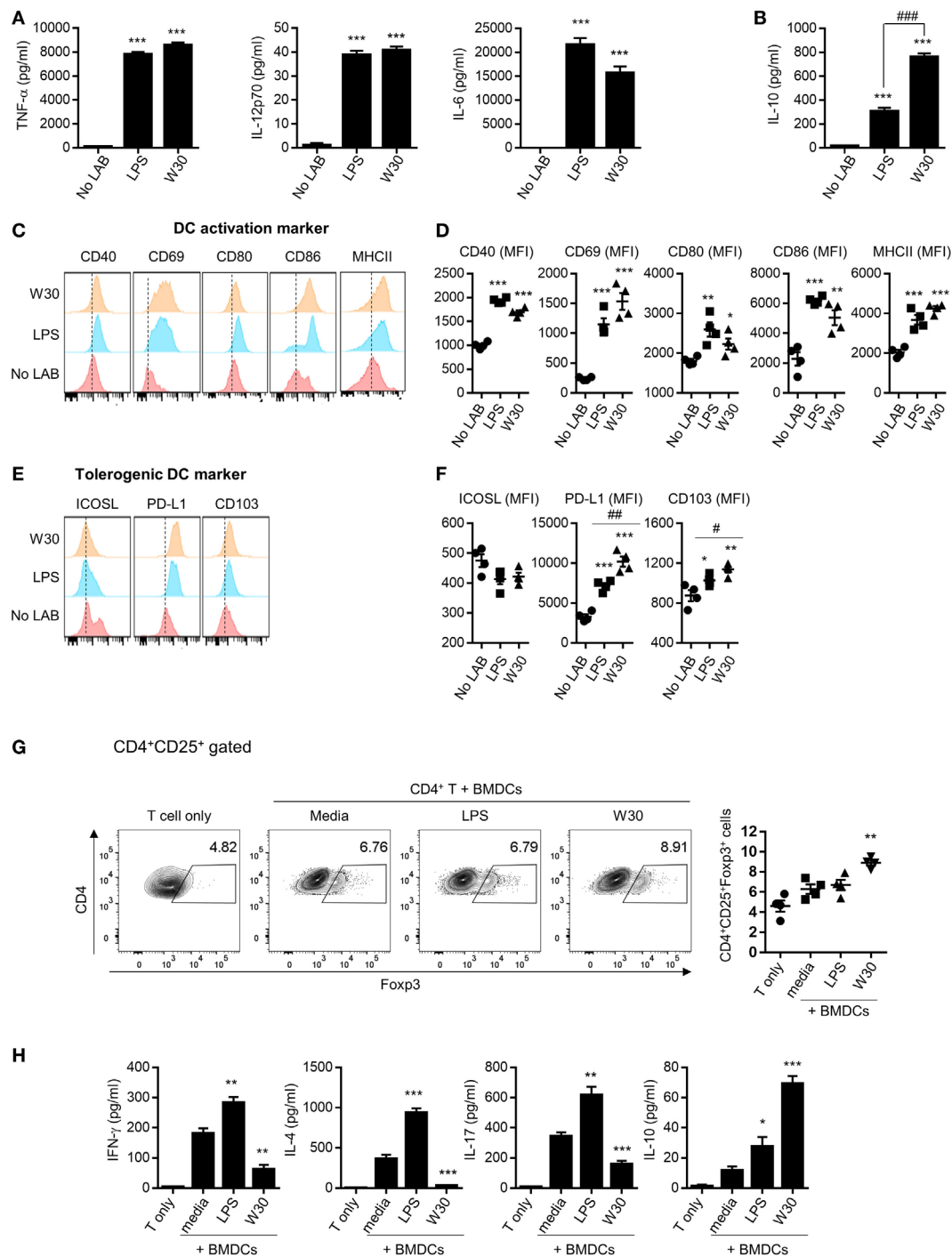


FIGURE 1 | WIKIM30 modulates dendritic cell (DC) and T cell function *in vitro*. Bone marrow-derived DCs (BMDCs) were generated from naive bone marrow cells of BALB/c mice in the presence of granulocyte-macrophage colony-stimulating factor. BMDCs were cultured with or without LPS and WIKIM30 for 24 h. **(A,B)** Expression levels of pro-inflammatory [TNF- α , interleukin (IL)-12p70, and IL-6] or anti-inflammatory (IL-10) cytokines in the culture supernatant measured with the cytometry bead array. **(C,D)** Surface expression of markers related to DC maturation (CD40, CD69, CD80, CD86, and MHCII) or tolerogenic DCs [inducible T-cell costimulator ligand (ICOS-L), programmed death ligand (PD-L)1, and CD103] was detected by flow cytometry. * $p < 0.05$ vs. no lactic acid bacteria (LAB) group; # $p < 0.05$ vs. LPS group. **(E,F)** BMDCs were stimulated with LPS or WIKIM30 for 24 h and cocultured with CD4⁺ T cells isolated from BALB/c mice in the presence of anti-CD3 monoclonal antibody for 4 days, and the proportion of CD4⁺CD25⁺Foxp3⁺ regulatory T cells analyzed by flow cytometry **(G)** while expression levels of cytokines (IFN- γ , IL-4, IL-17, and IL-10) in the culture supernatant were measured with the cytometry bead array **(H)**. Data are representative of three independent experiments. Student's *t*-test (unpaired); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. medium group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. LPS group.

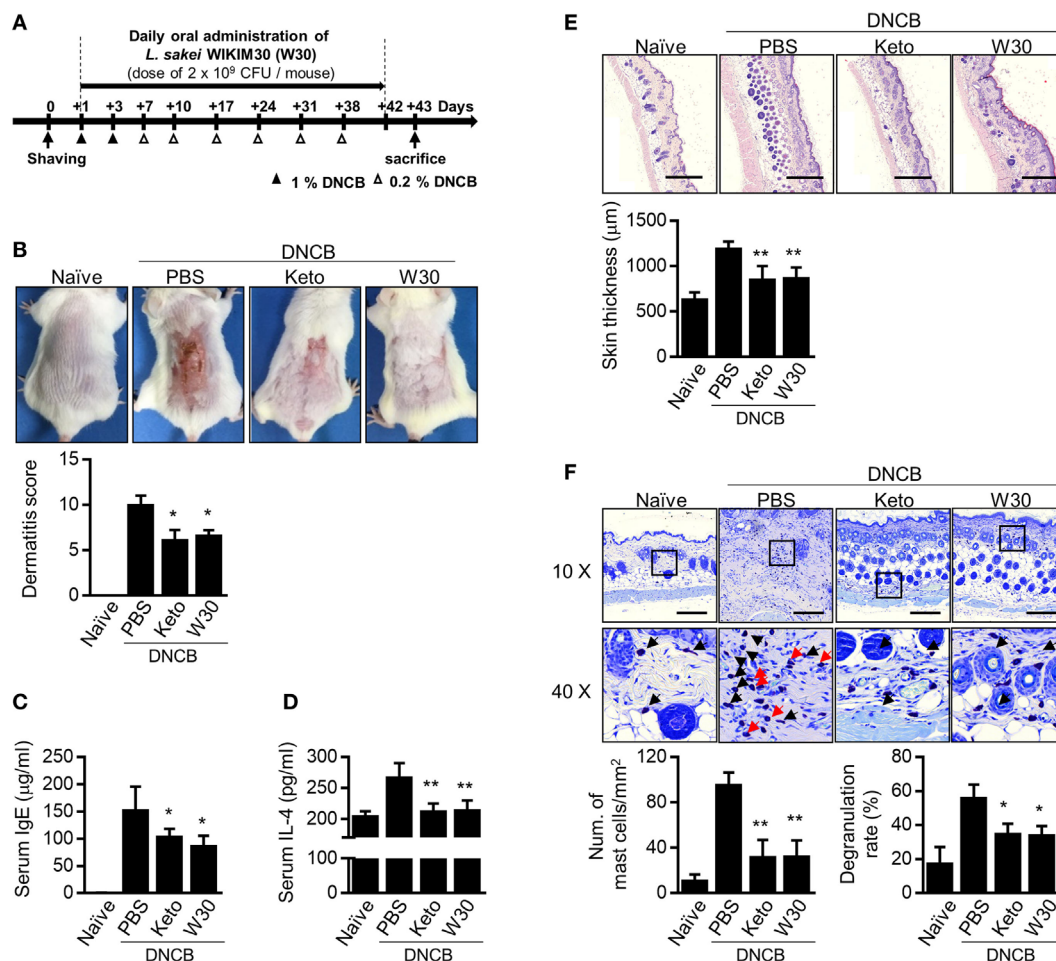


FIGURE 2 | Oral administration of *Lactobacillus sakei* WIKIM30 ameliorates atopic dermatitis (AD)-like symptoms. **(A)** Experimental design. To induce AD-like immunologic features and skin lesions in mice, DNCB was applied to the dorsal skin. Phosphate-buffered saline (PBS), ketotifen, or *L. sakei* WIKIM30 was orally administered once daily for 6 weeks. **(B)** AD-like skin lesions were evaluated by visual observation. **(C,D)** Serum immunoglobulin (Ig)E and interleukin (IL)-4 levels were detected by sandwich enzyme-linked immunosorbent assay. **(E,F)** Dorsal skin sections were stained with hematoxylin and eosin or Toluidine Blue. Skin thickness and cell infiltration **(E)** and mast cell number and degranulation **(F)** were evaluated by histological analysis. Data represent the mean \pm SE of $n = 5$ mice per group in three independent experiments. Student's *t*-test (unpaired); * $p < 0.05$, ** $p < 0.01$ vs. AD group.

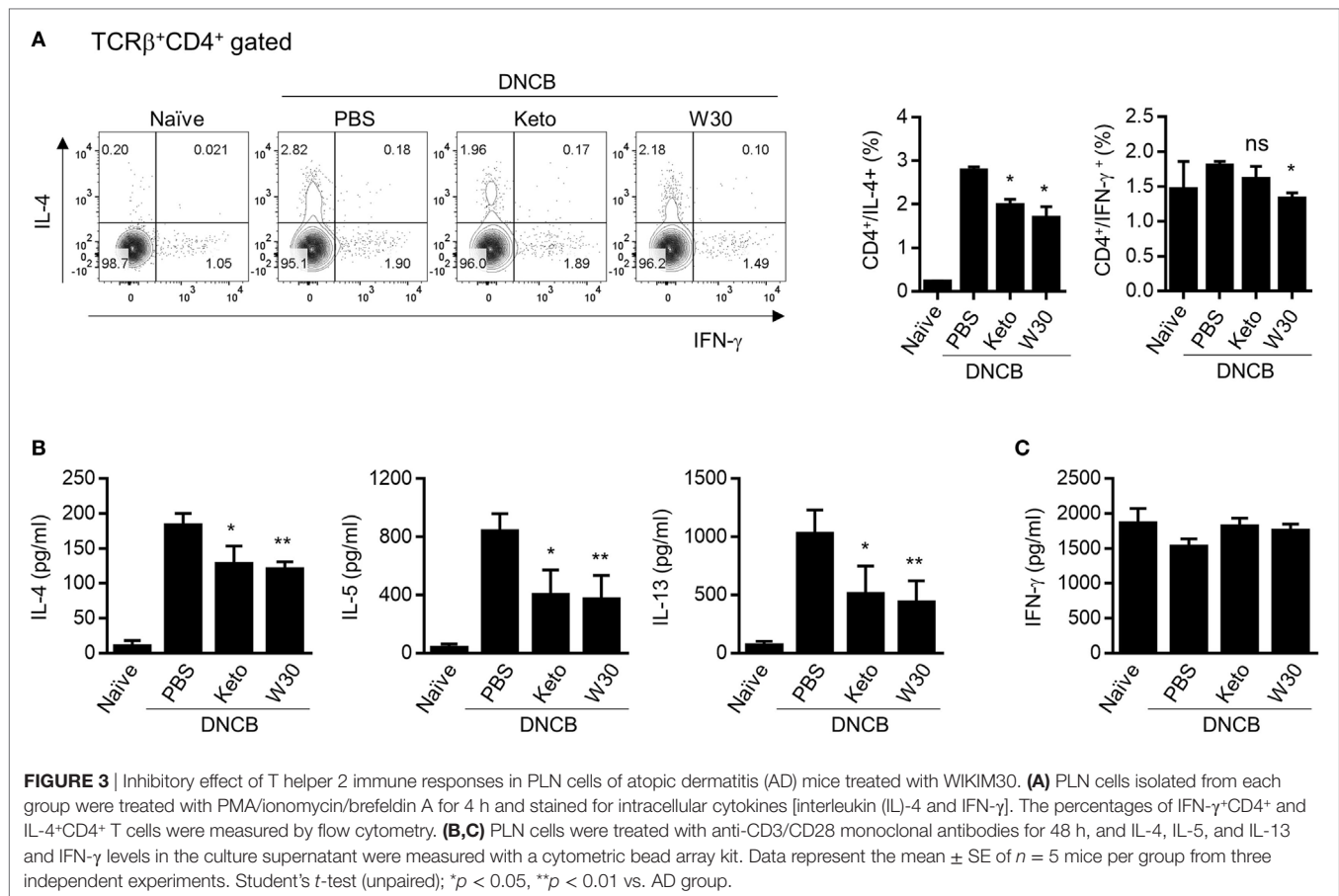
population in PLNs was also increased by WIKIM30 treatment (Figure S4 in Supplementary Material). These results suggest that oral administration of WIKIM30 induces Treg differentiation and IL-10 production, thereby suppressing the Th2-dominant immune response in AD.

WIKIM30 Modulates Gut Microbiota Composition in AD Mice

Emerging evidence has shown that dysbiosis of gut microbiota is associated with AD development. To determine whether WIKIM30 can counter this phenomenon, we analyzed fecal bacteria obtained from mice in each group by 16S rRNA high-throughput amplicon sequencing. After quality filtering, 1,491,125 high-quality sequences remained, with an average of 99,408 reads per sample (range: 53,518–144,901). The Wilcoxon rank-sum test was used to determine the richness and diversity

indices of each group (Figure 5A). The Chao1 index indicated that the richness of fecal microbiota was lower in the WIKIM30 group than in the naïve and AD groups. According to the Shannon and Simpson diversity index, fecal microbiota diversity was increased in AD as compared to the naïve group, but this was restored by WIKIM30 treatment (Figure 5A). A beta diversity analysis using the Bray–Curtis similarity index and principal coordinate analysis plots generated from the calculated beta diversity revealed differences in gut microbiota composition among the three groups (Figure 5B).

To determine whether the protective effects of WIKIM30 against AD are correlated with alterations in the gut microbiota, we analyzed differences in bacterial abundance among groups. To identify the specific bacterial taxa in each group, gut microbiota compositions were compared by the linear discriminant analysis effect size method (Figure S5 in Supplementary Material). At the genus level, the AD-associated decrease in the relative

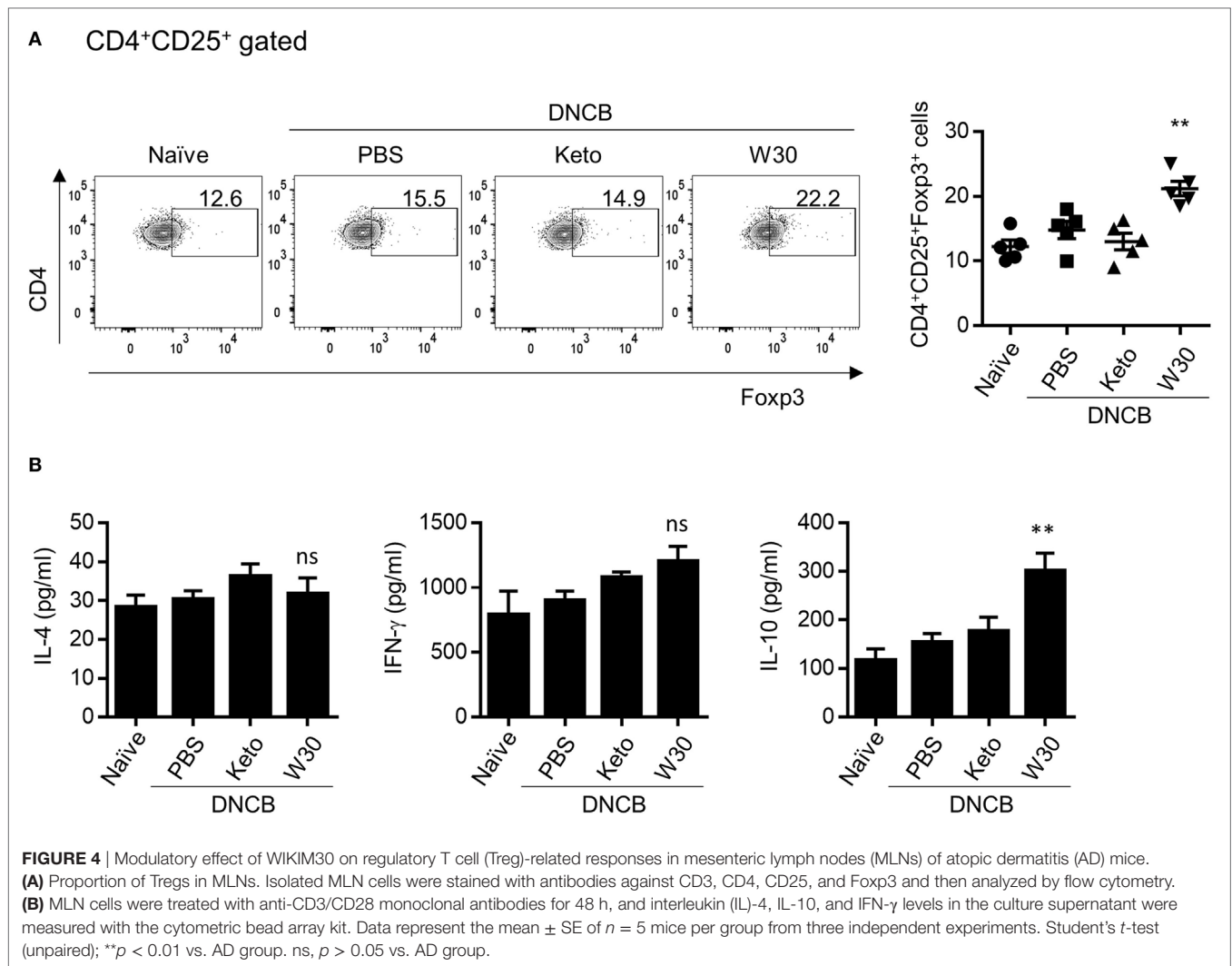


abundance of *Ruminococcus* was restored by WIKIM30 treatment (**Figure 6A**). Indeed, the relative abundance of *Arthromitus* and *Ralstonia* increased by AD sensitization was reversed by WIKIM30 treatment (**Figures 6B,C**). We also performed a correspondence analysis of fecal microbiota composition to determine the effect of WIKIM30 on immune response (**Figure 6D**; Figure S6 in Supplementary Material). Alterations in the abundance of genus *Arthromitus* and *Ralstonia* were positively correlated with Th2-related responses in AD, whereas the change in *Ruminococcus* abundance was positively correlated with Treg-related responses induced by WIKIM30 treatment. These data indicate that WIKIM30 may restore immune balance in AD through modulation of the gut microbiota.

DISCUSSION

In this study, we investigated whether *L. sakei* WIKIM30—a probiotic strain isolated from kimchi—can alleviate AD as well as the underlying mechanism through *in vitro* and *in vivo* studies. Initiation of an immune response by ingested probiotics depends on antigen-presenting cells in the GI tract, including DCs (21). DCs recognize bacterial components (e.g., pathogen-associated molecular patterns) through pattern recognition receptors (PRRs) such as TLRs (29). Thus, DCs stimulated by specific bacterial components on TLRs or pathogen-derived molecules can

promote different immune responses by inducing distinct T cell subtypes. For example, LPS from *Escherichia coli* (30), flagellin (31), viral double-stranded RNA (32), and bacterial CpG DNA (33) promote the Th1 response, whereas LPS from *Porphyromonas gingivalis* (34), helminth components (35), and cholera toxin (36) promote the Th2 response. Filamentous hemagglutinin from *Bordetella pertussis* stimulated DCs that enhanced Treg differentiation and showed increased IL-10 production (37). Likewise, different bacteria including WIKIM30 variably influence DC function, which can be detected based on the expression patterns of cytokines and surface molecules. Treatment of BMDCs with WIKIM30 stimulated the production of both pro- and anti-inflammatory cytokines and increased the activation of tolerogenic DCs, suggesting that WIKIM30 contains factors that modulate immune activation and tolerance (**Figures 1A,B**; Figure S1 in Supplementary Material). Collectively, these data indicate that WIKIM30 may contain immune modulatory components that engage PRRs like TLR1/2, TLR4, and TLR6/2; identifying these can provide insight into the mechanism of DC polarization toward a tolerogenic phenotype. Moreover, our coculture experiments using WIKIM30-treated BMDCs and naïve CD4⁺ T cells showed that WIKIM30-treated DCs increased the Treg-related response while decreasing responses related to Th1, Th2, and Th17 (**Figure 1F**), providing evidence that WIKIM30-treated BMDCs are polarized toward tolerogenic DCs.



To investigate the immune modulatory effects of WIKIM30 *in vivo*, we established a mouse model of DNCB-induced AD, which exhibits a polarized Th2 response. Oral administration of WIKIM30 inhibited the production of serum IgE and IL-4—a Th2-associated immune response—in these mice (Figure 2). WIKIM30 treatment restored the skin epidermis and decreased the number of CD4⁺ T cells and B cells as well as Th2 cytokine expression in the PLN (Figure 3) of AD model mice, whereas the proportion of Tregs in the MLN (Figure 4) as well as in the PLN (Figure S4) was increased in the WIKIM30-treated group. Tregs can rapidly migrate into inflamed draining lymph nodes and tissue by expressing chemokine receptors [C-C chemokine receptor type (CCR)4 and CCR6] (16). These data demonstrate that the movement of Tregs generated by WIKIM30 administration in MLN into inflammatory sites may inhibit the Th2-dominant immune response of AD; thus, WIKIM30 may be a tolerogenic DC inducer in the GI tract. However, additional studies are needed to confirm the direct action of WIKIM30 in promoting tolerogenic DC generation in the GI immune system.

Previous studies have reported that changes in gut microbiota profiles contribute to the development of AD by affecting the Th2-dominant immune response. In human studies, infants with AD showed increased populations of *Faecalibacterium prausnitzii* subspecies (38) and *Staphylococcus aureus* (39). Furthermore, the abundance of genus *Clostridium* was increased whereas that of *Bacteroides* was decreased in AD patients (40). Although we were unable to detect *F. prausnitzii* or *S. aureus* in AD mice, the abundance of *Clostridium* and *Bacteroides* showed similar patterns to those in humans. Among the mice with oxazolone-induced AD, mice with higher susceptibility to AD showed increased abundance of *Bacteroides uniformis* as well as species belonging to the family Lachnospiraceae and an unclassified genus of the family Rikenellaceae, whereas those with lower susceptibility showed greater numbers of unclassified *Bacteroides* species (41).

In this study, we found that oral administration of WIKIM30 modulates the structure of gut microbiota that may influence allergic immune responses in AD mice. The proportion of genus *Ruminococcus* was significantly decreased in AD, but this was restored by WIKIM30 treatment; thus, an increase in

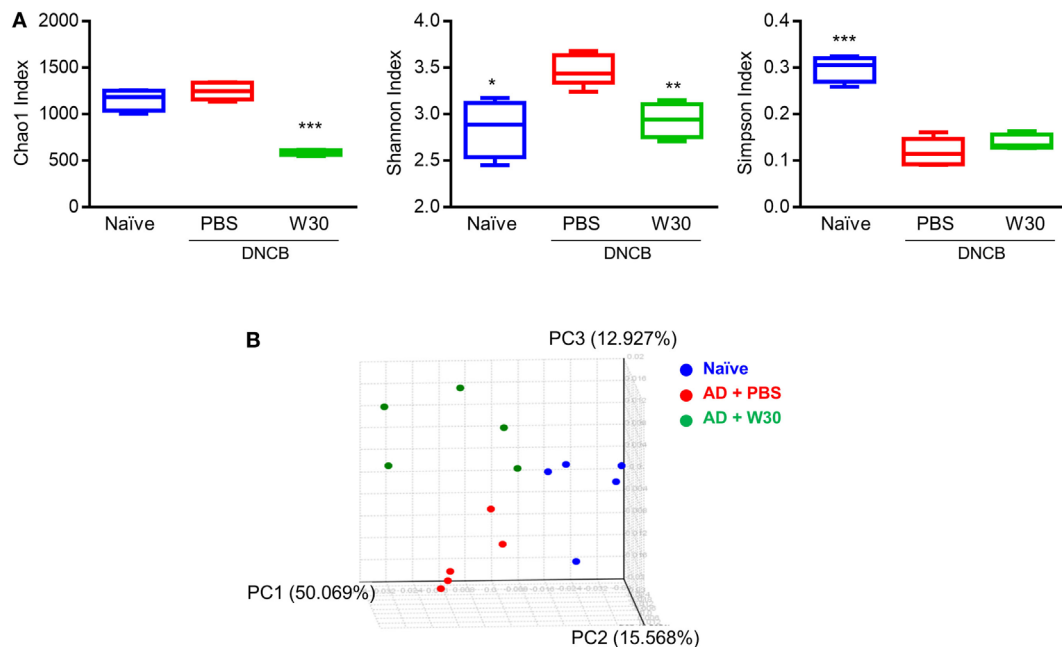


FIGURE 5 | Effect of WIKIM30 on alpha and beta diversity. Alpha and beta diversity of gut microbiota in normal mice and in atopic dermatitis (AD) mice without or with WIKIM30 treatment was evaluated. **(A)** Richness of gut microbiota is indicated by the Chao1 index; diversity is indicated by the Shannon and Simpson indices. Student's *t*-test (unpaired); **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. AD group. **(B)** Beta diversity pattern determined by principal coordinate analysis. Naïve, normal mice (blue); AD, DNFB-induced AD mice (red); W30, DNFB-induced AD mice with oral administration of WIKIM30 (green).

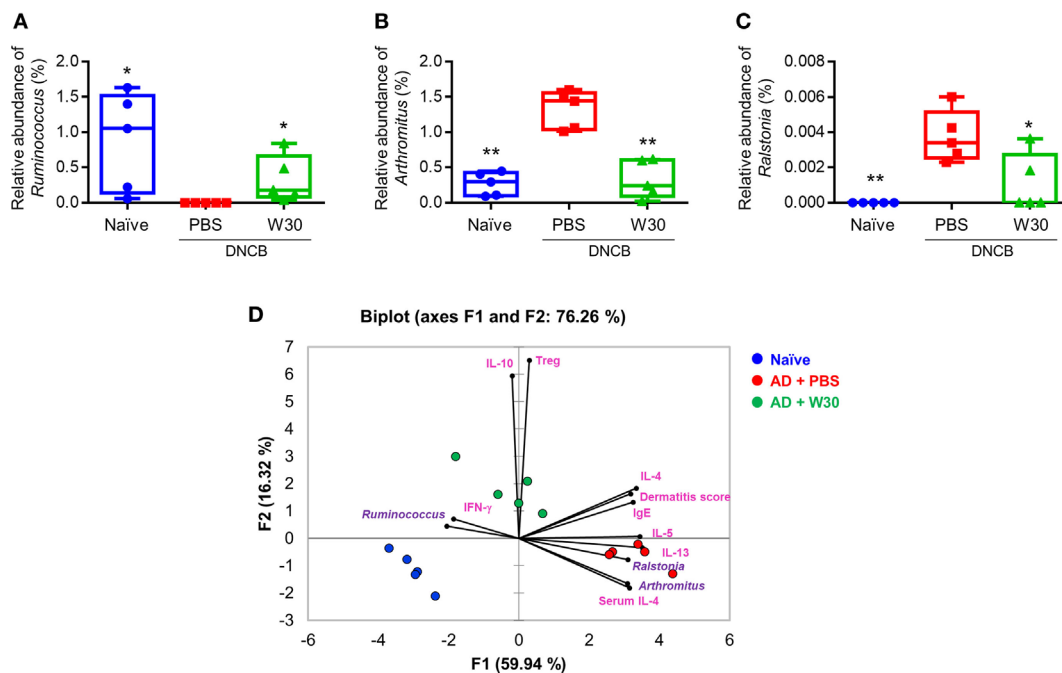


FIGURE 6 | Effect of WIKIM30 on gut microbiota composition. Composition of the gut microbiota in normal mice and in atopic dermatitis (AD) mice without or with WIKIM30 treatment. **(A–C)** Relative abundance of *Ruminococcus*, *Arthromitus*, and *Ralstonia* in each group. Student's *t*-test (unpaired); **p* < 0.05, ***p* < 0.01 vs. AD group. **(D)** Correspondence analysis of fecal microbiome composition and immune response. Naïve, normal mice (blue); AD, DNFB-induced AD mice (red); W30, DNFB-induced AD mice with oral administration of WIKIM30 (green).

the relative abundance of *Ruminococcus* may be positively correlated with Treg-related responses induced by WIKIM30. On the contrary, the relative abundance of *Arthromitus* and *Ralstonia* were elevated in AD and were reduced by WIKIM30 treatment, indicating that increases in the abundance of these two genera may be positively correlated with Th2-related responses in AD (Figure 6D). Previous studies have described the roles of *Ruminococcus*, *Arthromitus*, and *Ralstonia* in immune responses. For instance, *Ruminococcus albus*, *R. bromii*, and *R. callidus* are more highly represented in healthy individuals than in Crohn's disease patients (42), whereas *Ruminococcus* numbers are reduced in psoriatic arthritis patients (43). *Arthromitus* is a commensal segmented filamentous bacterial species that is known to induce the differentiation of CD4⁺ T cells into Th17 cells (44). In addition, *Ralstonia* is more abundant in patients with asthma than in those with non-asthmatic chronic rhinosinusitis (45) and is associated with the inflammatory response in Parkinson's disease (46) and the mucosal Th2 response in food allergy (47). These data demonstrate that WIKIM30 alleviates AD symptoms by increasing Treg-related or decreasing AD-related gut bacteria. However, mechanism on how probiotics directly alter gut microbiota structure remains to be determined.

In conclusion, our results showed that WIKIM30 stimulates the generation of Tregs directly by inducing tolerogenic DCs or indirectly by modulating gut microbiome profiles to suppress immune responses in AD. These findings suggest that administration of WIKIM30 modulates the gut microbiome, which can be an effective therapeutic strategy for alleviating AD.

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ETHICS STATEMENT

All animal procedures were performed according to the National Institutes of Health Guidelines for the Humane Treatment of Animals with approval from the Institutional Animal Care and Use Committee of the World Institutes of Kimchi (WIKIM IACUC 201509, 201601, and 201701).

AUTHOR CONTRIBUTIONS

H-JC, M-SK, and SKL conceived and designed the experiments; M-SK, SKL, J-YJ, JL, HKP, NK, MY, M-YS, and HEJ performed the experiments; M-SK, SKL, J-YJ, YJO, SWR, and H-JC analyzed the data; M-SK, SKL, and H-JC prepared the manuscript; and H-JC supervised the study.

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Searching for the Transcriptomic Signature of Immune Tolerance Induction—Biomarkers of Safety and Functionality for Tolerogenic Dendritic Cells and Regulatory Macrophages

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The last years have witnessed a breakthrough in the development of cell-based tolerance-inducing cell therapies for the treatment of autoimmune diseases and solid-organ transplantation. Indeed, the use of tolerogenic dendritic cells (tolDC) and regulatory macrophages (Mreg) is currently being tested in Phase I and Phase II clinical trials worldwide, with the aim of finding an effective therapy able to abrogate the inflammatory processes causing these pathologies without compromising the protective immunity of the patients. However, there exists a wide variety of different protocols to generate human tolDC and Mreg and, consequently, the characteristics of each product are heterogeneous. For this reason, the identification of biomarkers able to define their functionality (tolerogenicity) is of great relevance, on the one hand, to guarantee the safety of tolDC and Mreg before administration and, on the other hand, to compare the results between different cell products and laboratories. In this article, we perform an exhaustive review of protocols generating human tolDC and Mreg in the literature, aiming to elucidate if there are any common transcriptomic signature or potential biomarkers of tolerogenicity among the different approaches. However, and although several effectors seem to be induced in common in some of the most reported protocols to generate both tolDC or Mreg, the transcriptomic profile of these cellular products strongly varies depending on the approach used to generate them.

Keywords: biomarkers, tolerogenic dendritic cells, regulatory macrophages, tolerance mechanisms, genetic markers, immune tolerance, regulatory dendritic cells

INTRODUCTION

The immune system develops complex and sophisticated reactions, which are able to differentiate between what is dangerous and what is innocuous for the host (1), thus specifically attacking pathogens and other potentially dangerous antigens while remaining unresponsive against whether non-dangerous or self-molecules. This balance between immunogenicity and tolerance is orchestrated in the periphery by professional antigen-presenting cells (APC), such as dendritic

cells (DC) and, in a lesser extent, macrophages, which direct the immune response depending on the characteristics of the antigen and the cytokine milieu they encounter (2). Briefly, DC are in charge of both the initiation of the adaptive immune response and the control or abrogation of the inflammatory processes once the immunogenic antigen has been cleared. For this regulatory role, DC can deploy several mechanisms such as the induction of anergy or deletion of the activated immune cells, as well as, the activation of regulatory T cells (Treg) in an antigen-specific manner. Therefore, since DC have the potential to both stimulate or inhibit immune responses, the role of these cells in the immune system is complex and bidirectional (3–6). By their part, macrophages also play a minor role as APC, developing some of the regulatory processes mentioned above, although their main function consists in the clearance of cell debris, pathogens and other molecules after the immune response has concluded (7).

Eventually, the immune homeostasis can be disturbed due to a malfunction of the immune system, thus setting up immunogenic responses toward self-antigens from specific tissues and organs, which may lead to the development of autoimmune diseases. In the last years, there has been a significant progress in the knowledge of the mechanisms of immune regulation mediated by APC. Consequently, the development of novel autologous cell therapies capable of re-educating the immune system toward a tolerogenic profile has been postulated as a promising therapeutic alternative to conventional, unspecific immunomodulatory and immunosuppressive drugs, which often present severe side effects and a relatively poor efficacy (8).

So far, a wide variety of *in vitro* protocols has been established for the generation of immune tolerance-inducing DC—or tolerogenic DC (tolDC)—and regulatory macrophages (Mreg). Moreover, some of these cell products have been successfully translated from the bench to the bedside in the last few years, being tested in Phase I clinical trials in patients with autoimmune diseases—such as type 1 diabetes, rheumatoid arthritis or Crohn's disease—as well as, kidney transplantation, demonstrating in all cases that tolerogenic cell therapies are safe and well tolerated, without relevant side effects (9–13). In addition, many other studies are currently ongoing (14). These results, therefore, support the use of tolDC and Mreg as novel and safe approaches aiming to restore the immune tolerance. However, given the wide variety of protocols available for the generation of these cell products, finding objective and measurable biomarkers to characterize tolDC and Mreg and compare their characteristics between different approaches and laboratories remains one of the main obstacles to overcome.

In this context, the identification of differentially expressed (up- or down-modulated) genes in tolDC and/or Mreg constitutes one of the best tools for the definition of biomarkers of tolerogenicity, since they can provide more robust and reliable information compared to conventional methods such as phenotypical characterization by flow cytometry (with high variability) or functional studies (which require several days), as it will be further discussed below. In the case of tolDC and Mreg, these biomarkers would be able to guarantee the proper generation of the therapeutic cell product, ensuring that the cells are both safe and tolerogenic. Therefore, the ideal biomarker

would be one that is selectively overexpressed or repressed in the tolerance-inducing cell product compared to its respective mature immunogenic steady-state control condition.

With that purpose, here we review the main human tolDC- and Mreg-inducing protocols reported on the literature. We specifically focus on the different agents and drugs used to generate these cell products, in order to define a catalog of genes and/or proteins induced by these stimuli and thus try to find potential and universal biomarkers of tolDC and Mreg.

TOLEROGENTIC DENDRITIC CELLS AS KEY TOLERANCE-INDUCING PLAYERS AND THEIR TRANSCRIPTOMIC SIGNATURE

DC constitute a heterogeneous subset that includes classical, plasmacytoid, and monocyte-derived myeloid DC (15). In their immature state (iDC), DC are mainly antigen-capturing cells with tolerance-inducing functionality. However, once in the presence of a pro-inflammatory stimulus such as TNF- α , lipopolysaccharide (LPS) or IL-1 β , they can differentiate into immunogenic mature DC (mDC). By their part, mDC are capable of priming and activating T cells to initiate an immune response after providing the three required activation signals of the immune synapsis once a specific and immunogenic antigen has been recognized. During this maturation process, an upregulation of the expression of human leukocyte antigen (HLA) molecules, as well as, of other costimulatory molecules such as CD40, CD80, CD83, or CD86 takes place, along with an increase in the production of IL-12 and other proinflammatory cytokines (2, 3, 8).

However, a third type of DC has been defined in the last years, combining immune tolerance-inducing properties with a stability against maturation stimuli, called tolerogenic DC (tolDC). It is not clear whether tolDC constitute a different DC subset by themselves or if they are mere maturation-impaired iDC, although there seems to be a consensus about which features they have to possess in order to develop their regulatory function. Thus, tolDC usually present one or more of these characteristics: a semi-mature phenotype, with low expression of co-stimulatory (CD80, CD86, CD83) and HLA molecules, a maintained CCR7-dependant migratory ability toward the secondary lymphoid organs, an increased IL-10 production accompanied by low or null IL-12 and IFN- γ secretion, a lowered T cell-proliferation priming capability, potential to induce Treg and stability against maturation in front of a proinflammatory milieu. Specifically the latter, which has been described in the majority of these studies, probably constitutes the most important feature among all of them (16–18).

Importantly, tolDC can be differentiated *in vitro* from peripheral blood monocytes in the presence of a determined tolerogenic-inducing agent. Indeed, a wide variety of protocols have emerged in the last 20 years describing the induction of tolDC with several stimuli, such as anti-inflammatory cytokines—IL-10 (19, 20), TGF- β (20, 21)—, pharmacological agents and immunosuppressant compounds—rapamycin (20, 22, 23), different corticosteroids (24), dexamethasone (20, 23, 25, 26),

vitamin D3 (20, 23, 27) or a combination of both dexamethasone and vitamin D3 (28)—, several drugs and blocking molecules— aspirin (29), mitomycin C (30), the NF- κ B inhibitor BAY11-7082 (11)—and other strategies, such as genetic engineering for the selective repression or induction of key molecules and pathways (10, 31), among many others further discussed below. Generally, most of these protocols share several features in common, such as the differentiation of monocytes in the presence of GM-CSF and IL-4, as well as, the addition of a maturation stimulus (which usually includes different combinations of LPS, monophosphoryl lipid A, TNF- α , IL-1 β , prostaglandin E2, and/or IL-6), with few exceptions.

Either if we assume tolDC are a specific DC subset *per se* or just a modified state of iDC, there must be some footprint left by this condition. At the transcriptomic level, as already hinted, some obvious downregulated candidates would be the genes encoding co-stimulatory molecules or pro-inflammatory cytokines. However, those features would be shared with steady state iDC, thus making them useless in terms of differentially characterizing tolDC. In fact, ideally, a comparison against both immature and immunogenic control conditions should be taken into account in the search of specific genetic biomarkers, something that has not been considered in the majority of the reviewed studies. An ideal candidate should be, furthermore, clearly differentiated by a matter of full induction or repression, as a slight increase/decrease of its expression could be ambiguous and would always require the use of robust controls, which is not always possible.

Consequently, many research groups have been working on the identification of genetic markers for human tolDC, and deep transcriptomic studies are becoming more frequent each year. However, and although several studies have described a pool of markers for some specific tolerogenic cell products, common genetic biomarkers have not been found yet.

Glucocorticoids and Immunomodulatory Molecules in the Generation of Tolerogenic Dendritic Cells

Since mDC are immunogenic cells, or, in other words, promoters of inflammatory responses, the use of corticosteroids and other immunosuppressant drugs has been widely reported for the generation of tolDC. Rapamycin (20, 22, 23, 32, 33) and a combination of hydrocortisone and clobetasol-17-propionate (24), but especially dexamethasone (20, 23, 25, 26, 32–45), have all been used for the generation of tolDC. As a glucocorticoid-induced molecule, the expression of the gene encoding the anti-inflammatory mediator known as glucocorticoid-induced leucine zipper (GILZ) (46) has been reported strongly up-modulated in many of these studies, thus making it a good albeit predictable marker for tolDC generated with this kind of immunomodulatory agents. Furthermore, other molecules related with the complement and the immune system have been found commonly up- or down-modulated in several of these tolDC protocols, such as the anti-inflammatory cytokine IL-10 (up-regulated), the pro-inflammatory cytokine IL-12 or the fascin 1-encoding gene *FSCN1* (both down-modulated), which

are common features that define these cells (32). The full list of differentially expressed molecules reported for each of the abovementioned protocols and their respective references can be found on **Table 1**.

Dexamethasone-induced tolDC (dexa-tolDC) are one of the most widely implemented approaches worldwide for the generation of human tolDC, and are being or have been tested on clinical trials for the treatment of numerous autoimmune diseases, such as Crohn's disease (<http://www.clinicaltrials.gov>, NCT02622763) (12), rheumatoid arthritis (<http://www.clinicaltrials.gov>, NCT03337165; NCT03337165) and both multiple sclerosis or neuromyelitis optica (<http://www.clinicaltrials.gov>, NCT02283671). Several studies have reported the differential up-modulation of genes *CIQA* (encoding the C1q complement protein, chain A) (34, 35), *CD163* (34, 35), *GILZ* (32, 35, 36), *MERTK* (encoding the MER Proto-Oncogene Tyrosine Kinase, also used as a marker in the abovementioned clinical trial for Crohn's disease) (12, 26, 35) and *ZBTB16* (encoding zinc finger and BTB domain containing protein 16) (34, 35) in dexa-tolDC, thus making them the most relevant candidate biomarkers for this specific protocol. Additionally, the differential expression of *IDO1*, the gene encoding the indoleamine 2,3-dioxygenase—a molecule widely related to the induction of immune tolerance (65)—, has also been reported in dexa-tolDC. However, there is some controversy in this regard, as it has been found both up- (35) and down-modulated (32) in different studies. Besides, other induced genes described in studies using dexamethasone, relevant by their role in the modulation and mediation of different mechanisms of the immune system—with their respective encoded proteins in brackets—are *CD300LF* (CD300 molecule-like, family member F), *F13A* (coagulation factor XIII A), *FCGR2B* (Fc fragment of IgG receptor IIb), *FCGR3A* (Fc fragment of IgG receptor IIIa), *MRC1* (mannose receptor C-type 1), and *STAB1* (stabilin 1), as well as, other non-immune related genes like *FTL* (ferritin light chain), *IMDH2* (inosine monophosphate dehydrogenase 2), and *SOD2* (superoxide dismutase 2). Furthermore, the combination of dexamethasone with rosiglitazone has also been reported for the generation of tolDC, highlighting the induction of *FABP4* (fatty acid-binding protein 4) with this protocol, but specially also of *GILZ* gene (47).

The generation of human rapamycin-modulated tolDC (rapa-tolDC) is the second most reported protocol of this group of pharmacological and immunomodulatory agents. However, transcriptomic studies in tolDC generated with this strong immunosuppressant drug are not as predominant as those induced with dexamethasone. Yet, several genes have been postulated as candidate biomarkers for rapa-tolDC, both immune-related—*ANXA1* (annexin 1), *CIQC*, *CTSC* (cathepsin C) and *GILZ*—and non-immune-related —*GPX1* (Glutathione Peroxidase 1), *IMDH2*, *OSF1* (pleiotrophin) and *TPPI1* (tripeptidyl peptidase 1)—. Interestingly, all these genes have also been described in common with dexa-tolDC (32).

Additionally, the immunostimulant TLR3 ligand polyinosinic:polycytidylic acid (poly I:C) has also been reported to induce human tolDC, although in an inconsistent and poorly efficient manner. Nevertheless, the differential up-modulation

TABLE 1 | Differentially up- and down-modulated genes and proteins in the most reported human tolDC-inducing protocols.

	Protocol	Type	Up-modulated molecules	Down-modulated molecules	References
tolDC	Dexamethasone	Gene	ANXA1, C1QA, C1QC, C1QTNF1, C3AR1, CCL17, CD163, CD300LF, CD32, CFH, CLIC2, CSGALNACT1, CTSC, DCR3, EP2, EP3, F13A, FCGR2A, FCGR2B, FKBP5, FOXO3, FPR1, GILZ, GPX1, IDO1, IL10, IL12A, IL27B, IMDH2, JAG1, MERTK, MRC1, MT1, NCF1, OSF1, P2RY14, SLC39A8, SOD2, STAB1, TPP1, ZBTB16	CCL22, CD1C, FCER1A, IDO1, IL12B, LAMP3, MMP12, ZNF366	(26, 32, 34–36, 38, 39, 41)
		Protein	CYP1B1, DAB2, DPYD, FCER1G, FCGR3A, FTL, GCLC, IVNS1ABP, LRRC25, MCTP1, MERTK, NUDT16, PDCD4, PECAM1, RNASE6, RNASET2, SIGLEC5, SLC02B1	FSCN1	(12, 34, 37)
		miRNA	<i>miR-328-5p, miR-638, miR-663, miR-762, miR-1275, miR-1228, miR-1909</i>	<i>miR-142-5p</i>	(40)
	Dexamethasone + rosiglitazone	Gene	FABP4, GILZ		(47)
	Dexamethasone + vitamin D2	Protein	ERK1/2, IDO, JNK/SAPK, mTOR, p38 MAPK, STAT3		(48)
	Dexamethasone + vitamin D3	Gene	<i>ACADM, ACADVL, ACO1, ACO2, ACOX2, ACSS1, ALDH2, ATP5G3, ATP5J, ATP5O, BLVRB, C1orf162, C1QA, CCR5, CD14, CD209, CD274, CD52, CLIC1, COX11, COX6A1, COX7A2, CTSB, CTSD, CTSH, CYC1, DHRS9, EIF3B, EIF3C, EIF3CL, EIF4A3, FBP1, FCGR2B, FCGR3A, FN1, FTH1, FTL, G6PD, GAPDH, IDH3A, IDH3B, ILT3, LDHB, LILRB4, MATK, MCEMP1, MDH2, ME1, ME3, NDUFB9, NDUFS1, NDUFS8, NOS3, PCK2, PDHA1, PDXK, PIK3R1, PKM2, PNP, PRDX3, PTPN6, RAC2, RGCC, RPS12, RPS19, RPS21, RPS6KA1, RPS6KA2, SDHA, SLC11A1, SLC27A5, SLC2A1, SLC2A5, SNCA, SUCLG1, SUCLG2, TCEB1, TGFB1, TP53, TPI1, UQCR10, UQCR11, UQCRB, UQCRC1</i>	<i>ACTB, ADAM12, ADAM19, ANKRD33B, AOC1, CD25, CD40, CD80, CD83, CD86, DPYSL2, EHF, FSCN1, GPR157, ICOSLG, IKZF1, IKZF4, IL12B, IL2RA, ORMDL3, PIK3CG, PLEKHA5, PPP1R16B, PTPN2, SH2B3, TYK2, WDR1</i>	(49–51)
		Protein	<i>ADK, AKR1A1, ALDH2, ALDOA, ATP5H, ECHS1, FBP1, FTL, G6PD, GPD2, GALK, MPDH2, PGAM, PGM1, PKM2, PNP, PRDX6, TALDO1, TKT, TPI1</i>	<i>DPYSL2, ENO1, FSCN1, HSPD1, PDIA3</i>	(37)
	Hepatocyte growth factor	Gene	IL10		(52)
	IFN-γ	Gene		IRF4, RELB, IL12p40	(53, 54)
	IL-10	Gene	ANXA1, C1QC, CTSB, CTSC, CTSL, F13A, FTH1, GILZ, HLA-DOB, IL8, LILRB3, MRC1, STAB1, THBS1, TPP1	CD74, LAMP3	(32, 41, 55)
	IL-10 + IL-6	Gene	CTSB, CTSL, FTH1, HLA-DOB, IL-8, THBS1	CD74	(55)
	Poly I:C	Gene	IDO1, PDL1		(56, 57)
	Rapamycin	Gene	ANXA1, C1QC, CTSC, GILZ, GPX1, IMDH2, OSF1, TPP1	RALDH1	(32)
	Retinoic acid	Gene	ALDH1A1, ALDH1A2, CD141, GARP		(58, 59)
	TGF-β	Gene	ANXA1, CTSL, CXCL1, CXCR3, FTH1, HLA-DOB, IL8, LILRB3, THBS1	CD74, STAB1	(32, 55)
	TX527 (vitamin D3 analog)	Protein	<i>ACADVL, ACO2, ACOX1, ATP5A1, CTSD, CTSS, COPG, FBP1, G6PD, HADHA, IDH3A, MnSOD, OGDH, PCK2, PKM2, PRX3, PTM, UQCRCF51</i>	<i>ACAT1, ARCN1, DLD, PA28beta, PTM, RabGDI</i>	(60)
	Vitamin D3	Gene	ALOX5, ATP5A1, CAMP, CCL22, CD14, CD300LF, CMYC, CYP24, CYP24A1, CYP27B1, GILZ, GLUT3, HK3, ILT3, IRF8, LDHA, LGALS9, PDHA1, PFKFB4, PIK3CG, PRKAA1, THBD, VDR	CD1A, CD1C, CD1E, CD36, CD80, F13A, IER3, IRF4, LAMP3	(32, 36, 41, 61–63)
		Protein	AKT, FTL, GSK-3b, mTOR	FSCN1, SOD2	(37)
		miRNA	<i>miR-378</i>		(64)

Genes validated by qPCR or proteins validated by western blot are shown in bold.

of both *IDO1* and *PD-L1*, two genes involved in the induction and maintenance of immune tolerance, has been confirmed by quantitative PCR for these cells (56, 57). As for tolDC induced with hydrocortisone and clobetasol-17-propionate, no transcriptomic biomarkers have been reported.

Vitamins A and D Modulate the Transcriptomic Footprint of Tolerogenic Dendritic Cells

As reviewed by Mora et al. (66), vitamins A and D exert important immunomodulatory properties. While vitamin A and specifically its metabolite, retinoic acid, have been reported to have an influence in T cell differentiation and proliferation, as well as, Treg induction, vitamin D plays an important role as an immunoregulatory agent in the inhibition of T cell proliferation and the reduction of IL-2 and IFN- γ secretion. Furthermore, the absence or low levels of vitamin D in the organism has been widely linked to an increase in the incidence of autoimmune diseases.

The tolerogenic-inducing properties of 1,25-dihydroxycholecalciferol, the active form of vitamin D3, over DC (vitD3-tolDC) have been widely reported *in vitro* in many studies performed with murine (67–70) and even cattle cells (71), although we will only focus on biomarkers of human vitD3-tolDC (20, 23, 27, 32, 33, 36, 37, 41, 44, 61–64, 72–74). As a measurement of its relevance, such is the importance of vitD3-tolDC in the field of tolerogenic cell products that even two clinical trials are already ongoing for the treatment of multiple sclerosis using this cell product in Badalona, Spain (<http://www.clinicaltrials.gov>, NCT02903537) and in Antwerp, Belgium (<http://www.clinicaltrials.gov>, NCT02618902). Several transcriptomic and proteomic pre-clinical studies in human vitD3-tolDC have evidenced several genes and proteins strongly induced with this approach, including immune-related molecules—*CCL22* (62, 63), *ILT3* (immunoglobulin-like transcript 3) (36), *CD300LF* (62) or *GILZ* (32), these last two in common with dexa-tolDC—and oxidative metabolism enzymes and regulators—*GLUT3* (glucose transporter 3), *LDHA* (lactate dehydrogenase A), *mTOR* (mammalian target of rapamycin), *PDHA1* (pyruvate dehydrogenase E1, subunit alpha 1) or *PFKFB4* (fructose-2,6-bisphosphatase) (63)—, as well as direct targets of the response to vitamin D3 through the interaction with its receptor, like *CYP24A1* (cytochrome P450, family 24, subfamily A, member 1) (41, 61–63) and of course *VDR* (vitamin D receptor) (41). By their part, the repression of several co-stimulatory, pro-inflammatory, and antigen presenting genes and molecules like *CD1A*, *CD1C*, *CD80*, *FSCN1* or the transcription factor *IRF4* has been reported at the transcriptomic and proteomic levels (37, 62). Additionally, a synthetic structural analog of vitamin D3, TX527, has also been used for the induction of human tolDC (60). However, and although the up-modulation of the ATP synthase F1 subunit alpha-encoding gene (*ATP5A1*) was reported in common with vitD3-tolDC, the transcriptomic resemblance was more relevant with tolDC induced with a combination of dexamethasone and vitamin D3, a strategy that will be further discussed in the

next section. Nevertheless, some of these induced molecules consist of mostly metabolic-related genes—*ACADVL* (Acyl-CoA dehydrogenase very long chain), *ACO2* (aconitase 2), *FBP1* (fructose biphosphatase 1), *IDH3A* (isocitrate dehydrogenase 3, subunit alpha), *PCK2* (phosphoenolpyruvate carboxykinase 2) and *PKM2* (pyruvate kinase M2)—and *CTSD*, encoding the protease cathepsin D (37, 49, 50).

The use of vitamin A-derived molecules like retinoic acid, however, has not been so widely reported for the generation of human tolDC and only the selective up-regulation of *ALDH1A1* and *ALDH1A2* genes, encoding the aldehyde dehydrogenase 1 family members A1 and A2—involved in the metabolism of retinoic acid—has been reported, as well as, the induction of *CD141* and *GARP* genes (58, 59). Other differentially expressed genes induced by the protocols mentioned in this section are shown in Table 1.

The Synergic Effect of Dexamethasone and Vitamin D

Since dexamethasone and vitamin D treatments alone are able to generate tolDC, the combination of both of them is expected to induce synergic effects that would strengthen the tolerogenic functionality of these cells. Consequently, the simultaneous use of dexamethasone and vitamin D3, or vitamin D2 in a few cases (48, 75), has become one of the most widely reported human tolDC-generating protocols *in vitro*. Indeed, these cells have even reached the clinical phase for the treatment of rheumatoid arthritis, with successful results regarding the safety and tolerability of the product (<http://www.clinicaltrials.gov>, NCT01352858) (13).

As expected, the genetic signature of dexamethasone + vitamin D-induced tolDC (vtdx-tolDC) reported in pre-clinical studies partially overlaps with that reported for each or both of these treatments alone to generate human dexa- and vitD3-tolDC. In fact, the analysis of the reported data for these protocols showed that *C1QA*, *FCGR2B*, *FCGR3A* and *IDO1* genes were found induced in common with dexa-tolDC (34, 35, 38, 48, 50) and *CD14*, *ILT3*, *mTOR* and *PDHA1* were shared with vitD3-tolDC (36, 48–50, 62, 63). Nevertheless, our analysis evidenced that the up-regulation of *FTL* and the suppression of *FCSN1* genes were the only genetic modulations in common between these three protocols (34, 37, 50, 63). Interestingly, the function of the proteins encoded by all these genes is strongly related to the modulation of the immune system. Surprisingly, however, there was a pool of genes that were only described for vtdx-tolDC but not for either dexa-tolDC nor vitD3-tolDC, such as *CTSB*, *DHRS9* (dehydrogenase/reductase 9), *FTH1* (ferritin heavy chain 1), *RGCC* (regulator of cell cycle), *SLC11A1* (solute carrier family 11 member 1), *TBET* or *TGFB1* (49–51). Indeed, after our study, it is worth noting that out of 64 up-modulated genes and/or proteins reported for dexa-tolDC, 29 genes for vitD3-tolDC and 102 genes for vtdx-tolDC, only 4 genes could be found in common between vtdx-tolDC and each treatment separately, as shown in the Venn diagram in Figure 1. The chances are, however, that many of these genes could simply not be detected or were overlooked in the validation process of the separated

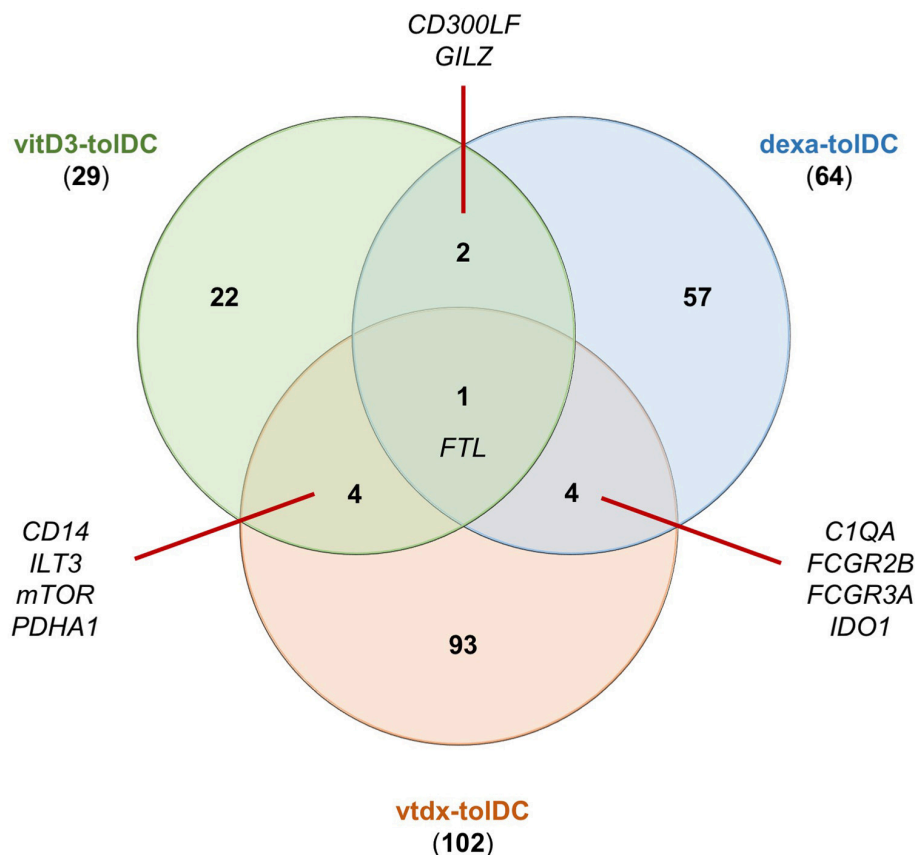


FIGURE 1 | Common up-regulated genes in tolDC induced with either vitamin D3, dexamethasone or the combination of both. The numbers in the Venn diagram indicate the number of reportedly induced genes for each condition alone or in combination with one or both of the others. Dexa-tolDC: dexamethasone-induced tolDC; vitD3-tolDC: vitamin D3-induced tolDC; vtdx-tolDC: dexamethasone + vitamin D3-induced tolDC.

protocols due to intrinsic limitations of the methodologies used, as it is known that biases frequently appear in high throughput transcriptomic and proteomic techniques. For this same reason, for instance, some already mentioned immune-related and metabolic genes were detected simultaneously induced in vtdx-tolDC and tolDC generated in the presence of the vitamin D3 analog TX527—*ACADVL*, *ACO2*, *CTSD*, *FBP1*, *G6PD* (glucose-6-phosphate dehydrogenase), *IDH3A*, *PCK2*, *PKM2*)—(37, 49, 50, 60). Although the down-modulation of genes is not as relevant toward the identification of transcriptomic biomarkers, it is nonetheless worth noting that the *FSCN1* gene has been found repressed in vtdx-tolDC, dexa-tolDC, and vitD3-tolDC at the same time (37, 50). **Table 1** shows a complete list of the differentially expressed genes and proteins reported in protocols using a combination of dexamethasone and vitamin D derivatives.

The Effect of Cytokines and Growth Factors in the Induction of Tolerogenic Dendritic Cells

Many different kinds of cytokines have been used for the induction of human tolDC, ranging from anti-inflammatory—IL-10 (19, 20, 32, 33, 41, 55, 76–78), TGF- β (21, 32, 33, 55, 79) or

both (80)—to even immunostimulatory molecules—IFN- γ (53, 54) or a combination of IL-6 with IL-10 (55)—, but also several growth factors—hepatocyte growth factor (52) and low-doses of GM-CSF alone (81).

As previously mentioned, the secretion of IL-10 is one of the most sought features of tolDC due to its anti-inflammatory and regulatory properties. Consequently, the generation of tolDC in the presence of exogenous IL-10 (IL10-tolDC) constitutes one of the most implemented protocols for the generation of this type of regulatory cell products. In fact, many of the genes and molecules already cited for other protocols, with immune or metabolic involvement, have also been found induced in IL10-tolDC, such as, *ANXA1*, *C1QC*, *CTSB*, *CTSC*, *CTSL* (cathepsin L), *F13A*, *FTH1*, *HLA-DOB*, *IL-8*, *LILRB3* (leukocyte immunoglobulin-like receptor B3), *MRC1*, *STAB1*, *THBS1*, *TPP1* and, especially for its repeated prevalence, *GILZ* (32, 55). Also, and in line with the traditional concept of tolDC, the down-modulation of the antigen presenting molecule *CD74* (also known as HLA-DR) (55) and *LAMP3* (lysosomal-associated membrane protein 3), typically found on iDC (41), has been reported. Interestingly, the combined exposure in front of both of IL-10 and IL-6 for the generation of tolDC performed in one of the previously cited articles did not seem to change the transcriptomic profile

of these cells, as many of the above mentioned genes were also found accordingly induced or repressed like they were in IL10-tolDC (55).

The use of TGF- β for the *in vitro* differentiation of tolDC is not as widely established as IL-10, but still some potential biomarkers have been described, both exclusively for this product (the immune related-genes *CXCL1* and *CXCR3*) and in common with other regulatory cells (*ANXA1*, *CTSL*, *FTH1*, *HLA-DOB*, *IL-8*, *LILRB3*, *THBS1*) (32, 55). Just like in IL10-tolDC, *CD74* appears differentially repressed in TGF- β -induced tolDC but, controversially, also does *STAB1*, reportedly up-modulated in the former protocol (55). As far as we are concerned, no potential transcriptomic or proteomic markers have been reported in cells induced with the combination of IL-10 and TGF- β for the generation of human tolDC.

Surprisingly, IFN- γ has also been described in a couple of publications for the generation of tolDC, even though it does not constitute the most obvious strategy due to its proinflammatory properties. Nevertheless, these studies have reported the selective reduction in the expression of the pro-inflammatory genes *IRF4*, *RELB*, and *IL12p40* in this cell product (53, 54). Consequently, the down-modulation of these genes is in line with the expected anti-inflammatory profile for tolDC, and even *IRF4* has also been reported as differentially repressed in vitD3-tolDC, as mentioned above (62). All the biomarkers described within the protocols mentioned in this section are shown in **Table 1**.

Finally, the differentiation of stable tolDC from monocytes in the presence of low doses of GM-CSF, and in the absence of IL-4 in the culture, has also been reported in humans (81), but also in animal models (82–84). In fact, their clinical use is being tested under the context of a multicentre trial named *The ONE Study ATDC* in living-donor renal transplantation (<http://www.clinicaltrials.gov>, NCT02252055) (85). However, any potential biomarker in human low-dose GM-CSF-induced tolDC has been reported yet.

Genetic Changes Induced in Tolerogenic Dendritic Cells Generated With Other Strategies

The pharmacological agents and factors mentioned so far comprise the most predominant strategies in the literature for the induction of tolDC, but there is still a wide variety of drugs, proteins and several treatments with the potential of generating this type of regulatory DC products. However, provided that the aim of this review is to look for universal biomarkers of immune tolerance, we have also considered these approaches. In fact, a significant amount of studies have reported the differential expression of several genes and molecules that could become potential biomarkers for their respective and specific protocols, generating tolDC in the presence of different organic compounds—such as the *Aspergillus* cell wall (32), curcumin (86), mitomycin C (87), paeoniflorin (88), phosphatidylserine liposomes imitating apoptotic bodies (89)—, other cell types—mast cells (90) and trophoblasts (91)—and a variety of agents, conditions and/or molecules—for instance a combination of the complement protein C5a and LPS (92), seminal plasma (93), the Wnt-5a protein (94) or even the deprivation of tryptophan in

the culture (95)—. However, there are still many other different strategies without transcriptomic or proteomic studies reported in the literature that are, therefore, outside of the objective of this review. The full list of differentially expressed genes and molecules in the protocols mentioned in this section is presented in **Table 2**.

A totally different approach to generate tolDC consists in using targeted genetic engineering in order to achieve cells with specific functional features either silenced or induced. There are several strategies reported in this regard, ranging from the impairment of immunogenic properties—such as silencing the expression of *CD40*, *CD80*, and *CD86*, already tested in type 1 diabetes patients, which was the first clinical trial using a tolerogenic cell therapy (10, 98) (<http://www.clinicaltrials.gov>, NCT00445913)—to selectively inducing the production of several anti-inflammatory cytokines like IL-10 and TGF- β (31, 99), overexpressing the IL-12 and IL-23-suppressor factor SOCS-3 (100) or transfecting the cells with a modified *CTLA4* construct that inhibits the expression of the co-stimulatory molecules CD80 and CD86 (101). Surprisingly, some approaches using genetic manipulation achieved to generate human IL-10-producing DC through the induction of, *a priori*, immunogenic functions such as the CD40-CD40L signaling pathway (102). However, the definition of transcriptomic biomarkers for tolDC induced by genetic engineering would not be of much utility, provided that the differentially expressed genes or proteins to check would be precisely those that have been specifically induced or repressed by the procedure itself.

GENERATION OF REGULATORY MACROPHAGES: DIFFERENCES AND SIMILARITIES WITH TOLEROGENIC DENDRITIC CELLS

Mreg constitute one of the three main macrophage subtypes, being the other two the classical macrophages and the so-called alternatively activated macrophages, or M2 macrophages. However, and as reviewed by Fleming and Mosser (103), Mreg present unique features: they are characterized by their ability to modulate the immune system toward a regulatory T_H2 response through the production of IL-10 and a limited or absent secretion of IL-12 mediated by the activation of the ERK cascade. In addition, these cells present an increased antigen-presenting functionality with an elevated expression of HLA class II and B7 co-stimulatory molecules. And this is, probably, the crucial feature in which Mreg and M2 macrophages differ the most, because although both subsets exhibit immunoregulatory properties, the ability to induce antigen-specific responses is limited in M2 macrophages due to their low HLA expression. However, Mreg are considered to deploy their potent T cell suppressor functions mainly through three non-antigen-specific mechanisms: via IFN- γ -induced IDO activity, by a contact-dependent deletion of activated T lymphocytes or mediating the induction of TIGIT⁺ FoxP3⁺ Treg (9, 96, 104, 105).

Just like tolDC, human macrophages can be generated *in vitro* by differentiating them from monocytes. Briefly, classical

TABLE 2 | Differentially up- and down-modulated genes and proteins in other human tolDC- and Mreg-inducing protocols.

	Protocol	Type	Up-modulated molecules	Down-modulated molecules	References	
tolDC	<i>Aspergillus</i> cell wall	Gene	<i>ANXA1, STAB1, GILZ, IDO, RALDH1, RALDH2</i>	<i>F13A, MRC1</i>	(32)	
	C5a and LPS	Gene	<i>RGCC, FERMT2, SLC39A14, TNFSF14, TGFB1</i>	<i>IL12B, FOXO1</i>	(92)	
	Curcumin	Gene		<i>RELB</i>	(86)	
	Mast cells	Gene	<i>IDO1, NFKB1, NFKB2, RELB, SOCS5</i>	<i>SOCS3</i>	(90)	
	Mitomycin C	Gene	<i>ADM, CSF2RA, DDIT3, FDXR, GAB2, LILRB4, LRDD, MAFB, MAP4K4, PERP, TNFRSF10B, TRAF4, TSC22D3</i>	<i>CFLAR (FLAME-1, I-FLICE, Usurpin), NRG2</i>	(87)	
	Paeoniflorin	Gene	<i>IDO1</i>		(88)	
	Phosphatidyl-serine liposomes	Gene	<i>CLCN6, CYTH4, IFNLR1, LAIR1, LDLR, MFSD2A, NFKBIA, PLAUR, PPME1, SHB, SLC43A3, TNFAIP3, TNFSF14, VEGFA</i>	<i>ALKBH1, ATP10D, AURKA, BCL2L1, BLCAP, BST1, BTBD3, BTK, BUB1, C9orf64, CASP3, CBX4, CD1D, CDC23, CDC42SE1, CDK13, CDYL2, CKAP2, CLCN3, CSRP2BP, CUL3, DAPP1, DCAF12, DCAF7, DCLRE1A, DCTD, DDO, DYRK2, EHBP1, ERLIN1, FBXO25, FBXO36, FRAT2, FZD5, GIMAP4, GLRX, GOLPH3L, GTF2B, HHEX, HPGD, ICK, KBTBD6, KIF11, KIF20B, LMNB1, LNX2, MAPRE2, MCM4, MCPH1, MDM1, MEF2C, MEGF9, MIER3, MLH1, MNDA, MSH2, MYB, N4BP1, NCAPG2, NET1, NFIA, NSMCE4A, NUP160, PAQR8, PARG, PAXIP1, PCNA, PMP22, PROS1, RAB32, RAD51C, RCSD1, RMDN1, RMND5A, SCYL3, SEC22C, SKI, SLAMF6, SLC10A7, SLC40A1, SMC2, SNN, SNX18, SOCS2, STIM2, STX3, TIMMDC1, TNFRSF11A, TPK1, TRIM5, UBE2E3, UBFD1, UNC50, VWA5A, WRNIP1, ZBED3, ZBTB39, ZBTB5, ZFP36L2, ZNF436</i>		(89)
	Seminal plasma	Gene	<i>COX2, TGFB1</i>	<i>CD1A</i>	(93)	
	Trophoblasts	Gene	<i>IDO1</i>		(91)	
	Tryptophan-deprived	Gene	<i>CHOP, ILT3</i>		(95)	
Mreg	Wnt5a	Gene	<i>ID3, IRF1, IRF2, SOCS3, TLR1</i>	<i>ID2, IRF8, TLR3, TLR4, TLR5</i>	(94)	
	M-CSF + IFN- γ	Gene	<i>ALDH1A1, ALDH1A2, CD1D, DHRS9</i>		(96)	
	M-CSF + LPS	Protein		<i>IL-12p35, IL-12p40, IL23-p19</i>	(97)	

Genes validated by qPCR or proteins validated by western blot are shown in bold.

macrophages are obtained in the presence of GM-CSF, and M2 and Mreg macrophages are generated in the presence of M-CSF, but with different supplementary treatments. While M2 macrophages are normally achieved using M-CSF + IL-4 and/or other T_H2 cytokines, Mreg are treated with M-CSF + LPS or IFN- γ for a brief period of time (106, 107). This combination of M-CSF and a short and complementary pro-inflammatory treatment is precisely the responsible for the strong induction of IL-10 production, something that both stimuli alone fail to achieve in macrophages (108, 109).

The generation of human Mreg is not as widely extended as tolDC, and consequently the number of protocols describing the differentiation of these cells is much more reduced. However, several molecules have already been postulated as potential biomarkers for these cell products. So far, transcriptomic studies have only been performed over LPS (LPS-Mreg) and IFN- γ -activated Mreg (IFN-Mreg). The former, LPS-Mreg, were initially described as IL-10-producing M2 macrophages, and their impaired IL-12 and IL-23 production was confirmed by qPCR (97, 110). However, IFN-Mreg are more widely reported

and studied, especially considering their translation into the clinic, where they have already been used for the treatment of living-donor renal transplant-recipient patients (<http://www.clinicaltrials.gov>, NCT00223067 and NCT02085629) (9, 105). This product is obtained by the stimulation of M-CSF-differentiated macrophages with IFN- γ , after 7 days of culture (9, 105, 111), and a strong up-modulation of *ALDH1A1*, *ALDH1A2* and *CD1D* genes has been reported, as well as the induction of *DHRS9* as a specific IFN-Mreg biomarker (96). The detailed list of markers reported in pre-clinical human Mreg protocols is shown in Table 2.

Nevertheless, although the list of genetic biomarkers described in Mreg is short, the identification of *DHRS9* in IFN-Mreg achieves a high relevance in the context of immune tolerance biomarkers, provided that the enzyme encoded by these gene seems to be involved in the biosynthesis of retinoic acid (112). As commented above, this compound is a vitamin A-derived molecule that can be used to differentiate human monocytes into tolDC. Interestingly, both *ALDH1A1* and *ALDH1A2* genes have been identified as differentially induced

TABLE 3 | Differentially expressed genes reported in at least two different protocols for the generation of human tolDC and/or Mreg.

Gene	Name	Modulation	Repeats	Protocols	References
<i>ACADVL</i>	Acyl-CoA Dehydrogenase Very Long Chain	Up	2	Dexa+vitD3, TX527	(49, 60)
<i>ACO2</i>	Aconitase 2	Up	2	Dexa+vitD3, TX527	(49, 60)
<i>ALDH1A1</i>	Retinaldehyde Dehydrogenase 1	Up	3	Asp, IFNg Mreg, RA	(58, 96)
<i>ALDH1A2</i>	Retinaldehyde Dehydrogenase 2	Up	2	IFNg Mreg, RA	(58, 96)
<i>ANXA1</i>	Annexin A1	Up	5	Asp, dexa, IL10, rapa, TGFb	(32)
<i>ATP5A1</i>	ATP Synthase 5 Alpha Subunit 1	Up	2	TX527, vitD3	(60, 63)
<i>C1QA</i>	Complement C1q A Chain	Up	2	Dexa, dexa+vitD3	(34, 35, 50)
<i>C1QC</i>	Complement C1q C Chain	Up	3	Dexa, IL10, rapa	(32)
<i>CD14</i>	Cluster of Differentiation 14	Up	2	dexa+vitD3, vitD3	(50, 62)
<i>CD1A</i>	CD1a Receptor	Down	2	Sem, vitD3	(50, 93)
<i>CD1C</i>	CD1c Receptor	Down	2	Dexa, vitD3	(35, 62)
<i>CD300LF</i>	CD300 Molecule Like Family Member F	Up	2	Dexa, vitD3	(34, 62)
<i>CD80</i>	Cluster of Differentiation 80	Down	2	Dexa+vitD3, vitD3	(50, 62)
<i>CTSB</i>	Cathepsin B	Up	3	Dexa+vitD3, IL10, IL10+6	(50, 55)
<i>CTSC</i>	Cathepsin C	Up	3	Dexa, IL10, rapa	(32)
<i>CTSD</i>	Cathepsin D	Up	2	Dexa+vitD3, TX527	(49, 60)
<i>CTSL</i>	Cathepsin L	Up	3	IL10, IL10+6, TGFb	(55)
<i>DHRS9</i>	Dehydrogenase/Reductase 9	Up	2	Dexa+vitD3, IFNg Mreg	(49, 96)
<i>F13A</i>	Coagulation Factor XIII A	Up	2	Dexa, IL10	(32)
		Down	2	Asp, vitD3	(32)
<i>FBP1</i>	Fructose-Bisphosphatase 1	Up	2	Dexa+vitD3, TX527	(37, 49, 60)
<i>FCGR2B</i>	Fc Fragment Of IgG Receptor IIb	Up	2	Dexa, dexa+vitD3	(34, 49)
<i>FCGR3A</i>	Fc Fragment Of IgG Receptor IIIa	Up	2	Dexa, dexa+vitD3	(34, 49)
<i>FSCN1</i>	Fascin Actin-Bundling Protein 1	Down	3	Dexa, dexa+vitD3, vitD3	(37, 50)
<i>FTH1</i>	Ferritin Heavy Chain	Up	4	Dexa+vitD3, IL10, IL10+6, TGFb	(50, 55)
<i>FTL</i>	Ferritin Light Chain	Up	3	Dexa, dexa+vitD3, vitD3	(34, 37, 50)
<i>G6PD</i>	Glucose-6-Phosphate Dehydrogenase	Up	2	Dexa+vitD3, TX527	(37, 50, 60)
<i>GILZ</i>	Glucocorticoid-Induced Leucine Zipper	Up	6	Asp, dexa, RGZ, IL10, rapa, vitD3	(32, 35, 36, 47)
<i>GPX1</i>	Glutathione Peroxidase 1	Up	2	Dexa, rapa	(32)
<i>HLA-DOB</i>	Human Leukocyte Antigen Class II, DO Beta Chain	Up	3	IL10, IL10+6, TGFb	(55)
<i>IDH3A</i>	Isocitrate Dehydrogenase 3 Alpha	Up	2	Dexa+vitD3, TX527	(49, 60)
<i>IDO1</i>	Indoleamine 2,3-Dioxygenase	Up	7	Asp, dexa, dexa+vitD2, mast, pae, pIC, tropho	(32, 35, 48, 57, 88, 90, 91)
<i>IL-10</i>	Interleukin 10	Up	2	Dexa, hepa	(35, 52)
<i>IL-12</i>	Interleukin 12	Down	5	C5a, dexa, dexa+vitD3, IFNg, LPS Mreg	(35, 38, 50, 53, 54, 92, 97)
<i>IL-8</i>	Interleukin 8	Up	2	IL10, IL10+6	(55)
<i>ILT3</i>	Immunoglobulin-Like Transcript 3	Up	4	Dexa+vitD3, mitC, tryp, vitD3	(36, 50, 87, 95)
<i>IMDH2</i>	Inosine Monophosphate Dehydrogenase 2	Up	2	Dexa, rapa	(32)
<i>IRF4</i>	Interferon Regulatory Factor 4	Down	2	IFNg, vitD3	(54, 62)
<i>LAMP3</i>	Lysosome-Associated Membrane Protein 3	Down	3	Dexa, IL10, vitD3	(41)
<i>LILRB3</i>	Leukocyte Immunoglobulin Like Receptor B3	Up	2	IL10, TGFb	(55)
<i>MRC1</i>	Mannose Receptor C-Type 1	Up	2	Dexa, IL10	(32)
<i>mTOR</i>	Mammalian Target Of Rapamycin	Up	2	Dexa+vitD2, vitD3	(48, 63)
<i>OSF1</i>	Pleiotrophin	Up	2	Dexa, rapa	(32)
<i>PCK2</i>	Phosphoenolpyruvate Carboxykinase 2	Up	2	Dexa+vitD3, TX527	(49, 60)
<i>PDHA1</i>	Pyruvate Dehydrogenase E1 Alpha 1 Subunit	Up	2	Dexa+vitD3, vitD3	(49, 60)

(Continued)

TABLE 3 | Continued

Gene	Name	Modulation	Repeats	Protocols	References
<i>PIK3CG</i>	Phosphatidylinositol-3-Kinase Subunit Gamma	Up	2	Dexa+vitD3, vitD3	(49, 50, 63)
<i>PKM2</i>	Pyruvate Kinase Muscle Isozyme M2	Up	2	Dexa+vitD3, TX527	(37, 49, 60)
<i>RELB</i>	RelB Transcription Factor, NF- κ B Subunit	Down	2	Cur, IFN γ	(53, 54, 86)
<i>RGCC</i>	Regulator Of Cell Cycle	Up	2	C5a, dexa+vitD3	(50, 92)
<i>STAB1</i>	Stabilin 1	Up	3	Asp, dexa, IL10	(32)
<i>TGFB</i>	Transforming Growth Factor Beta	Up	3	C5a, dexa+vitD3, sem	(51, 92, 93)
<i>THBS1</i>	Thrombospondin 1	Up	3	IL10, IL10+6, TGF β	(55)
<i>TNFSF14</i>	TNF Superfamily Member 14	Up	2	C5a, lipo	(88, 92)
<i>TPP1</i>	Tripeptidyl Peptidase 1	Up	3	Dexa, IL10, rapa	(32)

The column "Modulation" indicates if a determined gene has been found up- or down-modulated, and the field "Repeats" indicates the amount of different protocols in which each gene or protein has been described. The abbreviations stand for either tolDC induced with asp, *Aspergillus* cell wall; C5a, C5a, and LPS; cur, curcumin; dexa, dexamethasone; dexa+vitD2, dexamethasone + vitamin D2; dexa+vitD3, dexamethasone + vitamin D3; hepa, hepatocyte growth factor; IFN γ , IFN- γ ; IL10, IL-10; IL10-6, IL-6 + IL-10; mast, mast cells; mitC, mitomycin C; pae, paeoniflorin; pIC, Polyinosinic:polycytidylic acid; RA, retinoic acid; rapa, rapamycin; RGZ, rosiglitazone; sem, seminal plasma; TGF β , TGF- β ; tropho, trophoblasts; tryp, tryptophan deprivation; vitD3, vitamin D3; or regulatory macrophages induced with IFN γ Mreg, IFN- γ ; LPS Mreg, lipopolysaccharide.

in retinoic acid-generated tolDC (58) as well as IFN-Mreg, making them two interesting candidates for the characterization of at least this couple of different tolerance-inducing cell products. Furthermore, the differential up-modulation of *DHRS9* has also been reported in vtdx-tolDC, also discussed above (49). Consequently, since these cells are generated with both dexamethasone and vitamin D3, a clear relation between the transcriptomic profile of both IFN-Mreg and tolDC induced with either vitamin A or D is likely to exist. For this reason, further studies and validations in this direction could be of great interest, as potential common biomarkers of two different immune-regulatory myeloid cell-derived products could be identified.

SUMMARY AND CONCLUDING REMARKS

The identification of robust biomarkers for the characterization of tolerogenic and immunoregulatory cell products constitutes one of the last steps needed to take the final leap toward the broad application of these novel autologous antigen-specific therapies in the clinic. Specifically, their key importance resides in their capability to provide a fast and reliable quality control of the proper generation, functionality and safety of tolDC and Mreg.

In this article we have performed an exhaustive review of the currently published human tolDC- and Mreg-generating protocols that have reported potential biomarkers for these cells, with the aim of elucidating if a common transcriptomic or proteomic pattern relating all of them could be drawn. However, as it has been discussed, albeit many genes and molecules have been found separately induced using different strategies to generate these immunoregulatory cell products, so far, there is not a biomarker or a pool of biomarkers that can functionally characterize or at least identify the entirety of the studied protocols. Nonetheless, this is not necessarily bad news, as the chances of identifying a common biomarker were slim given the overwhelming variety of approaches and cell types reported in this review. As already mentioned above, the immune

system can deploy several strategies for the induction of tolerance that modulate many different immune and non-immune related pathways and transcriptomic cascades, thus making this goal even more unlikely. However, it is also worth noting that finding biomarkers provided only by the tolerance-inducing mechanisms could also be misleading; for instance, DC subtypes like iDC are capable of developing some tolerogenic functions, but still they could not be applied as a therapeutic approach in autoimmune diseases provided their lack of stability against pro-inflammatory stimuli, as discussed above.

Still, despite the consideration of such a wide variety and heterogeneity of protocols for the induction of regulatory cells, a significant amount of differentially expressed genes encoding several anti-inflammatory and immunomodulatory molecules has been reported in very different protocols, for instance *IDO1* (in 7 approaches) *GILZ* (in 6 approaches) or *ANXA1* (in 5 approaches). Similarly, the down-modulation of the pro-inflammatory cytokine IL-12 has been reported in 5 different tolDC-inducing strategies. In other words, in this review we have gathered all the genes and proteins that have been described separately with each of the approaches for the generation of tolDC and Mreg in the literature, and we have subsequently compared and put them all together in order to evidence potential common biomarkers between them. The complete list of the genes that have been reported in studies with at least two different approaches for the generation of human tolDC and/or Mreg are shown in **Table 3**. Therefore, the general idea that lies behind these reported molecules is that all the considered tolerogenic-inducing agents are modulating the cells toward a regulatory profile that might be partially shared between some approaches, but that is often achieved through different mechanisms and biological pathways that are strongly dependent on the stimuli used to generate them.

Consequently, this review evidences that the definition of strong biomarkers for tolDC and Mreg is still needed, but also that, although a universal transcriptomic profile of immune

tolerance induction might not be achievable, the elaboration of useful panels of biomarkers can still be feasible for determined pools of tolerogenic products. Bearing that in mind, our work could therefore serve as a starting point for developing and guiding further research in this field. For instance, one of the next steps that could be taken in this regard could be to specifically try to validate some of the above discussed genes in different protocols in which they have not been explicitly reported, either because they have been already identified in several approaches—like *IDO1* or *GILZ*—or because the stimuli used to induce the tolerogenic status share some functional or structural resemblance that might translate into the induction of common pathways and metabolic processes. In other words, with this review we intend to provide a useful reference of currently described biomarkers from which direct the investigation of new genes and proteins, most likely protocol-specific.

Thus, the combination of both stimulus-specific and some other partially-common differentially expressed genes could potentially lead to the development of transcriptomic panels of tolerogenic functionality. After all, provided that the relevance of tolerance-inducing cell therapies in the treatment of autoimmune diseases and solid organ transplantation rejection is becoming hugely relevant in the last years, the need for adequate and objective biomarkers is increasing accordingly. And in this context, the definition of panels of tolerogenic functionality for

at least a limited pool of protocols would consequently provide a robust tool for the establishment of reliable quality and safety controls for trials using tolDC- and/or Mreg-based therapies in the near future, which would also allow to properly compare them and therefore to dramatically accelerate their translation into the clinic.

AUTHOR CONTRIBUTIONS

EM-C, JN-B, and MM conceived the manuscript. JN-B wrote the manuscript. EM-C and MM reviewed the manuscript.

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GDF15 Regulates Malat-1 Circular RNA and Inactivates NF κ B Signaling Leading to Immune Tolerogenic DCs for Preventing Alloimmune Rejection in Heart Transplantation

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Recombinant human growth differentiation factor 15 (rhGDF15) affects dendritic cell (DC) maturation. However, whether GDF15 is expressed in DCs and its roles and signaling in DCs remain largely unknown. It is unclear whether GDF15-DCs can induce immune tolerance in heart transplantation (HT). This study aims to understand the impact of endogenous GDF15 on DC's development, function, underlying molecular mechanism including circular RNA (circRNA). This study will also explore GDF15-DC-mediated immune modulation in HT. Bone marrow (BM) derived DCs were cultured and treated to up- or down regulate GDF15 expression. Phenotype and function of DCs were detected. Expression of genes and circRNAs was determined by qRT-PCR. The signaling pathways activated by GDF15 were examined. The impact of GDF15 treated DCs on preventing allograft immune rejection was assessed in a MHC full mismatch mouse HT model. Our results showed that GDF15 was expressed in DCs. Knockout of GDF15 promoted DC maturation, enhanced immune responsive functions, up-regulated malat-1 circular RNA (circ_Malat 1), and activated the nuclear factor kappa B (NF κ B) pathway. Overexpression of GDF15 in DCs increased immunosuppressive/inhibitory molecules, enhanced DCs to induce T cell exhaustion, and promoted Treg generation through IDO signaling. GDF15 utilized transforming growth factor (TGF) β receptors I and II, not GALT. Administration of GDF15 treated DCs prevented allograft rejection and induced immune tolerance in transplantation. In conclusion, GDF15 induces tolerogenic DCs (Tol-DCs) through inhibition of circ_Malat-1 and the NF κ B signaling pathway and up-regulation of IDO. GDF15-DCs can prevent alloimmune rejection in HT.

Keywords: GDF15, circular RNA, circ_Malat-1, tolerogenic DCs, heart transplantation, immune suppression, tolerance

INTRODUCTION

Heart transplantation (HT) is the gold standard for the treatment of patients with end stage heart failure or severe coronary artery disease. Despite improvements in short-term outcomes, long-term patient and graft survival remain suboptimal due to the toxic side effects associated with long-term use of immunosuppressive drugs. An ideal treatment able to induce allograft-specific tolerance in transplant recipients, which enables long-term graft survival while avoiding the need for immunosuppression and its associated adverse effects, is urgently needed. More detailed elucidation of new immune suppressive molecules and signaling pathways will help develop innovative new immune rejection treatment strategies for HT.

Dendritic cells (DCs) are highly specialized and functionally diverse antigen-presenting cells (APCs) and are responsible for the control of the innate and adaptive immune responses. Originating from bone marrow (BM) progenitor cells, DCs can differentiate into immune responsive or immune suppressive/tolerogenic, depending on stimulus. In organ transplantation, DCs play a crucial role in determining the fate of a transplanted organs: rejection or acceptance by a host, depending on their immune suppressive or immune responsive function (1). Specifically, immune tolerogenic DCs (Tol-DCs) specifically are positively related to allograft survival and in nonhuman primates, have been shown to prolong allograft survival in a variety of organ transplantation models (2). In contrast, immunogenic DCs initiate graft rejection. Tol-DCs could be induced by many strategies including treatment with maturation-inhibiting agents, (3) blockade of costimulatory molecules with antibodies (4) and siRNA (5) as well as pre-treatment with immunosuppressant (6). We previously demonstrated that *in vitro* generated Tol-DCs could prevent allograft rejection in animal HT models (6–11), implying that Tol-DCs might be an ideal treatment for transplantation. Nevertheless, immune tolerance induction still needs to be further explored and improved.

GDF15, also named as macrophage inhibitor cytokine (MIC-1), is a divergent member of the TGF- β superfamily, and is associated with many diseases including cardiovascular disease and cancer (10–12). It displays immune suppressive function (12–14). Zhou et al. reported that treatment with recombinant GDF15 (rhGDF15) suppresses expression of surface molecules CD83, CD86, and HLA-DR in DC, thereby preventing the recruitment of T cells leading to acceleration of tumor growth in a cancer model (15). It suggests that GDF15 might be a good candidate for preventing immune rejection in HT. However, it is unknown yet whether DCs express GDF15 and what roles it plays in DC development and by what molecular mechanism(s) they function. Whether GDF15-modulated DCs can prevent transplant hearts (allografts) from immune rejection in HT remains to be addressed.

Moreover, recently emerging evidence is showing that a new class of endogenously expressed non-coding RNAs named as circular RNAs (circRNAs) regulate gene expression and function (16, 17). circRNAs are produced from back splicing and form a covalently closed loop without free terminals (18). CircRNAs play

critical roles in physiological and pathological processes due to their special structure, abundance, conservation across species, and functions. However, there are no reports about circRNAs in DC development and any associations between GDF15 and circRNA are unknown.

In this study, we aimed to investigate the role of endogenous GDF15 in DC development and DC-mediated immune tolerance induction, and signaling pathways activated by GDF15. We also aimed to reveal the involvement of circRNA in DC development and association with GDF15 in DCs and to determine the impact of GDF15-regulated DCs in preventing allograft rejection and immune tolerance induction in HT.

MATERIALS AND METHODS

Animals

C57B/6 wild type mice and BALB/c mice were purchased from Charles River Laboratories (Charles River Canada, Saint-Constant, Canada). Whole genome GDF15 knock-out (KO) mice, generated on a C57BL/6 using standard gene-targeting techniques, were kindly provided by Professor Se-Jin Lee at John Hopkins University (Baltimore, MD). GDF15 Transgenic (TG) mice ubiquitously expressing high levels of human GDF15 (hNAG1) under the control of the chicken β -actin promoter (CAG) were kindly provided by Dr. Seung J. Baek at the University of Tennessee (Knoxville, TN, USA) (19). Male C57BL/6 (H-2b) and BALB/c (H-2d) mice at the age of 9–10 weeks were used as donors and recipients, respectively. Animals were housed at the Conventional Animal Care Facility, University of Western Ontario, and were cared for in accordance with the guidelines established by the Canadian Council on Animal Care. All animal experiments in this study were approved by the Committee of Animal Use of the University of Western Ontario.

DC Culture

Bone marrow (BM) derived DCs were cultured from BM progenitor cells as previously described (9). Briefly, BM cells were flushed from the femurs and tibias of C57BL/6, GDF15 KO, or GDF15 TG mice, washed and cultured in 6-well plates in the presence of 10 ng/ml of recombinant GM-CSF and recombinant mouse IL-4 (Peprotech, Rocky Hill, NJ) at 37°C in 5% humidified CO₂. Non-adherent cells were removed (Day 2) and fresh medium was added. Half of the medium was replaced every 2 days.

DC Transfection and Infection

Day 5 cultured BM derived DCs (1 million/well) were plated in a 12 well plate and transfected with 1 μ g GDF15 siRNA using lipofectamine 2000 (Life technologies, Burlington, Canada) at the ratio of 1:2 for 4 h. Twenty-four hours after transfection, LPS (50 ng/ml) was added to the medium for 3 h and then the cells were re-collected for further experiments.

Infection of DCs with GDF15 expression adenovirus was conducted on day 2. Day 2 cultured BM-derived DCs (2 million/well) were plated in a six well plate and infected with

human GDF15 expression adenovirus (GDF15-Ad) or Null-Ad (SignaGen Laboratories, Gaithersburg, MD 20884-0661) with multiplicity of infection (MOI) of 100 in 1 ml medium for 6 h. Six hours after infection, fresh additional 3 ml of DC medium was added to the DCs and allowed to continue culture. Half of the culture medium was replaced every other day, until the time for the next experiments.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using Trizol (Invitrogen). cDNA was synthesized from RNA using oligo-(dT) primer and reverse transcriptase (Invitrogen). Primers used for the amplification of murine GDF15, IDO 1, IDO2, IL-2, IL-1 β , RelA, Rel B, PD-1, TIM-3, PD-L1, BTLA, GFRAL, and GAPDH genes are listed in **Supplemental Table 1**. qPCR was conducted in the CFX connectTM Real Time System (BioRad, Mississauga, Ontario) or Stratagene Mx3000P QPCR System (Agilent Technologies, Lexington, MA) using SYBR green PCR Master Mix (Froggabo Inc., ON, Canada) and 100 nM of forward and reverse primers. The PCR condition was 95°C for 2 min, 95°C for 10 s, 58°C for 10 s, and 72°C for 20 s (40 cycles).

Circular RNA Malat-1 Expression and Sequencing

cDNA was synthesized from total RNA using hexamer random primers and reverse transcriptase according to instruction of the manufacturer (Invitrogen). Divergent primers spanning the junction of circ-Malat-1 (Forward: gccttgccctaatacacaga; and Reverse: ttgtggggagacctgaaac) were designed and used for qPCR. qPCR was conducted in the CFX connectTM Real Time System (BioRad) using SYBR green PCR Master Mix (Froggabo Inc.) and 100 nM of forward and reverse primers. The PCR condition was 95°C for 2 min, 95°C for 5 s, 60°C for 10 s, and 72°C for 10 s (40 cycles).

Regular PCR using Taq DNA polymerase was conducted to amplify the circ-Malat-1 fragment. The amplified fragment was subjected to DNA sequencing at The Robarts Research Institute in London Ontario, Canada.

Flow Cytometry

Flow cytometry was performed to characterize the phenotype of DCs and T cells using a Calibur flow cytometer (Becton Dickinson, San Jose, CA) or Cyto Flex S (Beckman). Antibodies were purchased from eBioscience, San Diego, CA.

DC and T cell subsets were analyzed by means of two- or three-color staining with various combinations of mAbs. DCs were stained with FITC- or PE-CD86, FITC- or PE-CD11c, PE-CD80, PE or APC CD-83, FITC-MHCII, PE-PD-L1, and PE-Cy5-CD40 monoclonal antibodies. For T cells, PE-Cy5-CD4, PE-CD25 and FITC-FoxP3, Percp-eFluor710-PD-1, APC-BTLA, and PE-TIM 3 conjugated anti-mouse monoclonal antibodies were used for staining. Foxp3 expression was assessed by intracellular staining, using a cell permeabilization kit (eBioscience). Appropriate isotype controls were included.

Western Blotting

DCs were collected and washed with PBS. Total proteins were extracted with RIPA buffer containing protease inhibitor MSCF followed by three cycles of 5 s sonication. For heart tissue, 10 mg of heart tissues were homogenized with PRIPA buffer containing protease inhibitor MSCF using manual homogenizer on ice prior to sonication. Cell lysate and tissue lysate was centrifuged for 20 min at 15,000 rpm and supernatant was collected. The concentration of protein was measured using the Bradford method with Bradford 1 \times Dye reagent (BioRad, Mississauga, Ontario, Canada). Twenty micrograms total protein was loaded on 12% polyacrylamide gel and run for 60–90 min at 100 V. Separated proteins were transferred to PCM membrane. Transferred membranes were blocked with 5% fat-free milk powder in TBST for 30 min at room temperature and then blotted with the primary antibodies against mouse or human GDF15, TGF β RI, and TGF β RII (1:1,000 dilution, Sigma), phosphorylated Rel A p65, (1:1,000 dilution, Cell Signaling Technology, Danvers, MA), total Rel A p65 (1:1,000 dilution, Cell Signaling Technology), and β -actin (1:4,000 dilution, Santa Cruz Biotechnologies, San Diego, CA) at 4°C for overnight. The blotted membranes were washed with TBST containing 0.25% Tween-20 for 10 min at room temperature and repeatedly washed for three times. Washed membranes were blotted with appropriated second antibodies (Santa Cruz Biotechnologies) for 30 min at room temperature. Proteins were developed with ECL kits (Bio-Rad, Hercules, CA 94547) and visualized by FluorChem M system (ProteinSimple, San Jose, CA).

Immunoprecipitation Assays

DCs were collected and washed with cold PBS and lysed with RIPA buffer containing protease inhibitor MSCF for 5 min on ice. Cell lysate was centrifuged at 10,000 rpm for 5 min at 4°C. Supernatant was collected and protein concentration was measured using the Bradford method with Bradford 1X Dye reagent (Bio-Rad). Two hundred micrograms total proteins were used and 2 μ g GDF15 Abs (K-13, Santa Cruz) were added to the protein and incubated at 4°C overnight. 18 μ l of Protein A/G plus Agarose IP reagent beads (Santa Cruz) were added to each sample and incubated at 4°C for 3 h. The mixture was centrifuged at 2,500 \times g for 5 min at 4°C. The pellets were washed with 1 ml PBS for three times. After the final cycle of washing, 20 μ l of PBS and 10 μ l 6X loading buffer was added to each sample. The suspended sample was boiled at 95°C for 5 min to denature protein and spanned down at 2,500 \times g at 40°C for 1 min. Denatured samples were subjected to Western blotting.

Mixed Lymphocyte Reaction (MLR)

A mixed lymphocyte reaction was conducted to measure T cell proliferation. T cell proliferation was measured using a carboxyfluorescein succinimidyl ester (CFSE) dilution assay *in vitro* (20).

For a CFSE dilution assay, T cells (2 \times 10⁵/well) from naïve BALB/c mice were labeled with CFSE and then co-cultured with allogeneic BM-derived DCs cultured from C57BL/6 mice at a ratio of DC:T = 1:5 cells in 200 μ l of complete RPMI 1640 medium (Life Technologies). Cells were cultured at 37°C in

a humidified atmosphere of 5% CO₂ for 3 days. The dilution of CFSE was measured by flow cytometry with a Calibur flow cytometer (Becton Dickinson, San Jose, CA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of IFN- γ , TGF β , and IL-10 in DC the culture medium were determined using mouse IFN γ , TGF β , and IL-10 Quantikine ELISA kit (R&D Systems) according to the manufacturer's protocol. Optical density values were measured at 450 nm on an ELISA plate reader.

Heterotopic Cardiac Transplantation

Recipient mice (BALB/c) were intravenously treated with 1 million of DCs infected with human GDF15 expressing adenovirus through the tail vein of mice seven days prior to HT. Recipient BALB/c mice were anesthetized with Ketamine/Xylene and subjected to intra-abdominal allogeneic cardiac transplantation using the hearts from fully MHC-mismatched C57BL/6 mice according to our well-established procedure (10). After transplantation, recipients daily received sub dose rapamycin (1 mg/kg) through intra-peritoneal (*i.p.*) injection from days 0 to 7. Pulsation of implanted heart grafts was monitored daily by direct abdominal palpation in a double-blind manner to determine graft rejection/survival.

Statistical Analysis

In this study, data were reported as the mean \pm SEM. Quantitative real-time PCR data were analyzed using one-way ANOVA or student's *t*-test. Allograft survival among experimental groups was compared using the log-rank test. Differences with *P*-values < 0.05 were considered significant.

RESULTS

GDF15 Is Expressed in DCs and Up-regulated by Exogenous rhGDF15

It has been reported that exogenous rhGDF15 affects DC maturation and function (15). However, it remains unknown whether DCs themselves express GDF15 and whether exogenous rhGDF15 affects endogenous GDF15 expression in DCs. To address these questions, BM-derived DCs from wild type C57BL/6 mice were cultured *in vitro* with RPIM1640 in the presence of GM-CSF and IL4. RNA were extracted from DCs on different culture days. GDF15 expression in DCs was measured by qRT-PCR. As shown in **Figure 1A**, GDF15 was detectable on day 2 of the culture and its expression levels gradually increased over time.

Next, rhGDF15 was added to DC culture at various concentrations on different days of culture to determine the effect of exogenous rhGDF15 on GDF15 expression in DCs. Addition of exogenous rhGDF15 up-regulated the expression of GDF15 in DCs, and the increase of expression levels was both dose (**Figure 1B**) and treatment duration dependent (**Figure 1C**). GDF15 expression in DCs was also confirmed at the protein level by Western blotting (**Figure 1D**).

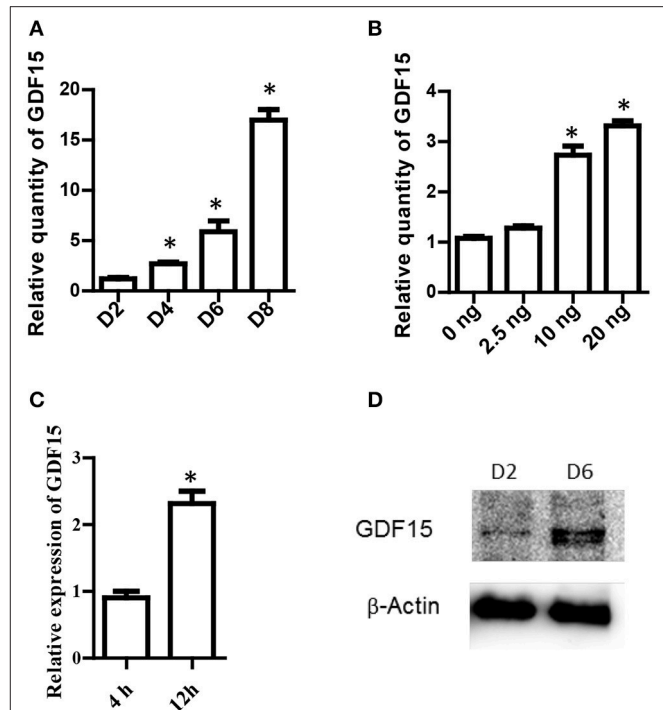


FIGURE 1 | GDF15 expression in DCs. **(A)** A time course of GDF15 expression in DCs. DCs (10^6 cell/well) were cultured from bone marrow progenitors in the presence of IL-4 and granulocyte/macrophage colony stimulating factor (GM-CSF). Cultured DCs were collected on day 2, 4, 6, and 8 for RNA extraction. The level of GDF15 mRNA was detected by qRT-PCR and GAPDH was used as loading control. Relative quantity of GDF15 mRNA was the expression of GDF15 in Day 2 DCs. $n = 4$, One way ANOVA was conducted for statistical analysis. **(B)** rhGDF15 increased GDF15 expression in DCs. BM-derived DCs were cultured and a variety of concentrations of rhGDF15 were added on day 4. Twenty-four hours after treatment, GDF15 expression in DCs was detected using qRT-PCR. GDF15 expression was normalized with control DCs without rhGDF15 treatment. $n = 4$, One way ANOVA was conducted for statistical analysis. **(C)** The up-regulation of GDF15 in DCs by rhGDF15 was time related. BM-derived DCs were treated with 2.5 ng/ml rhGDF15 on day 5. $n = 3$, the student *T*-test was conducted for statistical analysis. GDF15 expression in DCs was detected 4 h and 12 h after addition of rhGDF15 by qRT-PCR. The expression of GDF15 in DCs treated for 4 h was used as a normalizer. **(D)** GDF15 protein expressed in DCs. Total protein was extracted from day 2 and 6 *in vitro* cultured DCs. GDF15 expression at the protein levels was determined by western blotting using GDF15 Abs. β -Actin was used as a loading control. Images were representatives of $n = 3$, * $P < 0.05$.

Deficiency of GDF15 Promotes DC Maturation

To study the impact of endogenous GDF15 on DC development, DCs were cultured from GDF15 KO and WT mice, respectively. The expression of MHCII and co-stimulatory molecules (CD40, CD83, and CD86), which are commonly used as DC maturation markers, was measured by flow cytometry and compared between groups. As shown in **Figure 2A**, GDF15 KO-DCs expressed significantly higher levels of MHCII, CD40, CD83, and CD86 than WT DCs, indicating that GDF15 deficiency promotes DC maturation.

To confirm the above effect of endogenous GDF15 on DCs development, DCs from WT C57BL/6 mice were transfected with

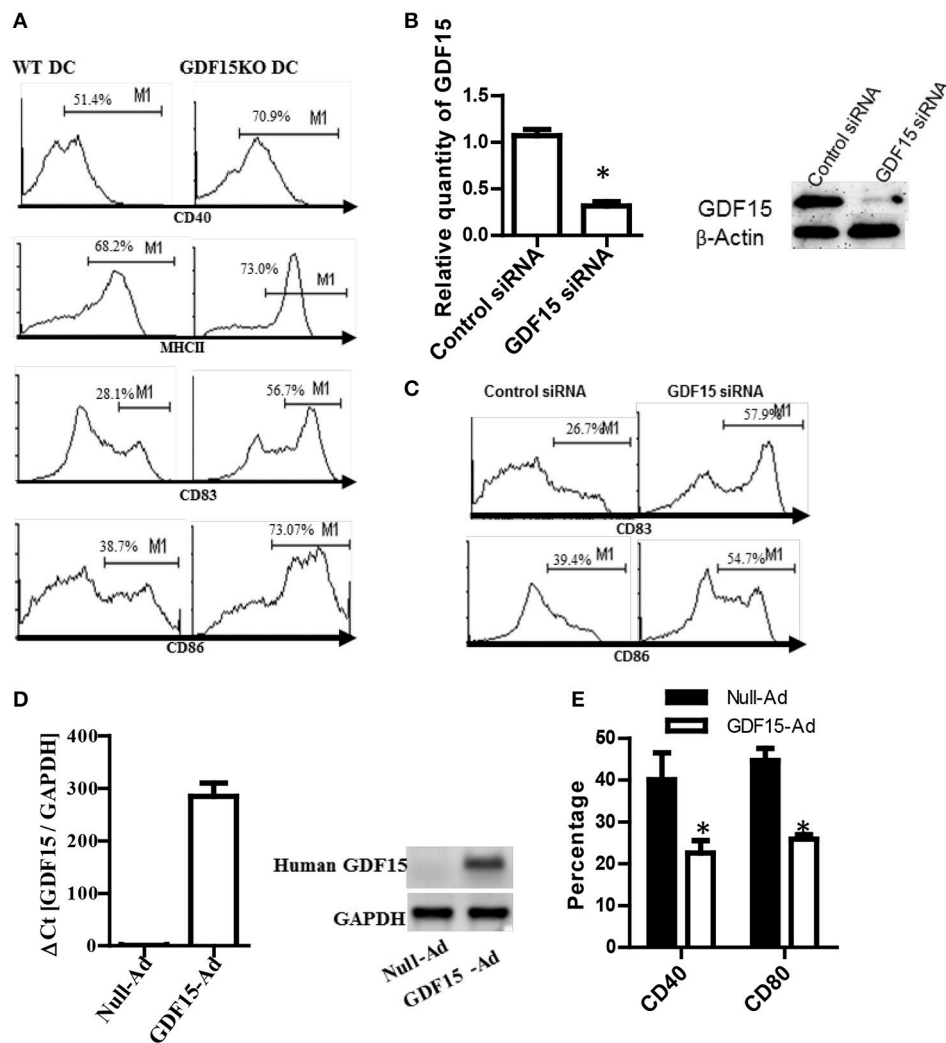


FIGURE 2 | Knockout of GDF15 promoted DC maturation. **(A)** GDF15 KO DCs promoted DC maturation. BM-derived DCs were cultured from GDF15 KO and WT mice. On day 6, DCs were stimulated with 50 ng/ml LPS for 3 h. DC maturation was detected by measuring the expression of MHCII, CD40, CD83, and CD86 using flow cytometry. Images were representatives of $n = 4$. **(B)** GDF15 silenced GDF15 expression in DCs. Day 5 DCs cultured from WT mice were transfected with GDF15 siRNA for 24 h. GDF15 expression was determined by qRT-PCR (left) and Western blotting (right). $n = 3$, the student T -test was conducted for statistical analysis of qRT-PCR results. **(C)** GDF15 siRNA increased the expression of CD83 and CD86 in DCs. DCs were transfected with GDF15 siRNA. 24 h after transfection, CD83 and CD86 expression was detected by flow cytometry. Data were representatives of $n = 3$. **(D)** GDF15-Ad increased GDF15 expression in DCs. Day 4 DCs were infected with 100MOI of adenovirus expressing human GDF15 qRT-PCR (left, $n = 3$) and Western blotting (right). Data were representatives of $n = 3$. **(E)** GDF15-Ad decreased CD40 expression in DCs measured by flow cytometry. $n = 3$, the student T -test was conducted for statistical analysis. * $P < 0.05$.

siRNA specifically targeting GDF15 on day 5 to knock down GDF15 expression in DCs. 48 h after siRNA transfection, the expression of GDF15, CD86, and CD83 was detected by qRT-PCR, Western blotting and by flow cytometry, respectively. The results show that GDF15 siRNA effectively knocked down the expression of GDF15 expression at the mRNA levels (Figure 2B, left) and protein levels (Figure 2B, right). The expression of CD86 and CD83 was up-regulated in GDF15-siRNA transfected DCs as compared with control GL2 siRNA transfected DCs (Figure 2C), which is consistent with the result from KO DCs.

Furthermore, BM-DCs were cultured from WT C57BL/6 mice and then treated with adenovirus expressing the

human GDF15 gene (GDF15-Ad) on day 2 *in vitro* to over-express GDF15. As expected, DCs infected with GDF15-Ad significantly over-expressed human GDF15 (Figure 2D). Over-expression of GDF15 resulted in a reduction of CD40 and CD80 (Figure 2E). Addition of rhGDF15 or GDF15-Ad to KO DCs reduced CD40 and CD80 compared to untreated KO DCs (Supplemental Figure 1). We also observed that DCs cultured from GDF15 TG mice presented the similar phenotype of DCS treated with rhGDF15 (data not shown). These data suggest that GDF15 expression level negatively correlates DC development and maturation.

Lack of GDF15 Enhances the Capacity of DCs to Activate T Cells

DCs are professional antigen presenting cells that are the unique cells able to activate naïve T cell response. To determine the effect of GDF15 on the capacity of DCs to activate naïve allogeneic T cells, an MLR was performed to measure T cell proliferation using a CFSE-dilution assay. As shown in **Figure 3A**, there was quicker dilution of CFSE labeled on T cells co-cultured with GDF15 KO DCs than those co-cultured with WT DCs, indicating that GDF15 deficiency enhances the capacity of DCs to activate naïve allogeneic T cells. In contrast, the T cells co-cultured with GDF15 expressing adenovirus treated DCs proliferated less than those co-cultured with control DCs (**Figure 3B**). This data indicates that GDF15 attenuated DC function to activate naïve T cells, which is consistent with the result from Zhou et al. (15).

In addition, the expression of cytokines which are required for DCs to activate and polarize T cells was measured. Over-expression of GDF15 by infecting DCs with GDF15 expressing adenovirus decreased the expression of IL-2, IFN- γ , and IL12p40 (**Figure 3C**), and increased the expression of TGF- β and IL10 (**Figure 3D**). Addition of rhGDF15 had similar results to GDF15 adenovirus (data not shown). The results indicated that GDF15 inhibited Th1-producing DCs that are characterized by high secretion of IL-12 and IFN- γ and low production of IL-10 (21).

GDF15 Promotes Treg Generation Depending on IDO and Increases Immune Checkpoint Molecules

Generation of regulatory T cells (Treg) is one of the critical characteristics of Tol-DCs. To determine whether GDF15 induces Tol-DCs, the ability of GDF15 treated DCs to generate Tregs was studied. DCs with different levels of GDF15 were co-cultured with allogeneic naïve T cells from a BABL/c mouse at a ratio of DC: T = 1:10 *in vitro* for 5 days. CD4⁺CD25⁺FoxP3⁺ Tregs were measured by triple staining with CD4, CD25, and FoxP3 Abs followed by flow cytometry. 14% of CD4⁺CD25⁺FoxP3⁺ Tregs were detected in the T cells cultured with untreated WT DCs, 24% in rhGDF15 treated DCs, 13% in control siRNA transfected DCs, and 9% in GDF15 silenced DCs (**Figure 4A**), indicating that GDF15 promotes Tol-DCs to generate Tregs. We also found that DCs from GDF15 KO mice significantly reduced Treg generation while DCs from GDF15 TG mice significantly increased Treg, as compared to wild type DCs (**Supplemental Figure 2**).

It has been reported that IDO, an immunosuppressive molecule expressed in immunosuppressive DCs, plays a vital role in immune tolerance induction (22, 23) and is critical for DCs to generate Tregs (24). We proposed that GDF15 might enhance DCs to induce Treg through IDO. Therefore, IDO expression was first detected by qRT-PCR. As shown in **Figure 4B**, both rhGDF15 and GDF15-Ad treatment up-regulated the expression of IDO 1 (**Figure 4B**, left) and IDO 2 (**Figure 4B**, right) in DCs. Moreover, the expression levels of IDO in DCs positively correlated with the concentration of rhGDF15 (data now shown).

To verify whether IDO is involved in Treg induction by GDF15-DCs, BM-derived DCs was cultured from IDO KO

mice and then treated with rhGDF15 or GDF15 siRNA 24h after treatment. DCs were then subjected to a co-culture with allogeneic T cells for 5 days to produce Tregs. As shown in **Figure 4C**, the percentage of Tregs did not significantly change among the control, rhGDF15 treated DCs and GDF15 siRNA silenced DCs when IDO was knocked out as compared their controls, indicating GDF15 enhances Treg generation through up-regulation of IDO.

PD1 and PD-L1 signaling and TIM 3-galactin 9 signaling have been recently recognized as one of the important immune suppression mechanisms of DC-mediated immunosuppression (25). Overexpression of PD-L1 by DCs inhibited CD4⁺T cell activation (26). Accordingly, the expression of immune checkpoint genes PD-1L, TIM3, and galectin 9 was detected. The results showed that over-expression of GDF15 increased PD-L1, TIM3, and galectin 9 expression (**Figure 4D**), suggesting GDF15 induced exhausted DCs.

GDF15 Reduces Circular Malat-1 (Circ_Malat-1) Expression and Inhibited the NF κ B Pathway

Circular RNA (circRNA) is a newly discovered non-coding RNA with 5' and 3' end covalent closed structure. A growing number of studies have shown that circRNA is actively involved in various physiological and pathological processes. However, it has not been reported whether circRNAs are also involved in development of DCs and in Tol-DC induction by GDF15. circ_Malat-1 was the third highly expressed circRNA in DCs and that mature DCs expressed circRNA_Malat-1 3-fold times greater than immature DCs shown in our circRNA microarray data (data not shown). Literature has reported that long non-coding RNA Malat-1, which is the parental RNA of circRNA Malat-1, regulates macrophages and DCs (27, 28). Therefore, we proposed that GDF15 may affect the expression of circ_Malat-1 expression since GDF15 inhibits DC maturation. qRT-PCR was conducted using divergent primers that can only amplify circRNA, not linear RNA, to detect circ_Malat-1. As shown in **Figure 5A**, DCs expressed circ_Malat-1 and both LPS and CD40L that are stimulators for DC maturation significantly up-regulated circ-Malat-1 expression in DCs as compared with unstimulated DCs. The expression of malat-1 circular RNA (circ_Malat 1) was increased in GDF15 KO DCs as compared with WT DCs (**Figure 5B**). In contrast, treatment with GDF15 expressing adenovirus (GDF15-Ad) reduced circ_Malat 1 expression (**Figure 5B**) as compared with control adenovirus (Null-Ad). To confirm PCR specificity to circRNA, DNA sequencing was also performed on PCR products of circ_Malat-1. As shown in **Figure 5C**, the amplified PCR fragment contained the junction sequence ataccggttt caaggt(3') c(5')tcc ccacaa, confirming that the divergent primers amplified circ_Malat-1, not linear Malat-1 transcript.

Nuclear factor-kappa B (NF κ B) is essential for DC maturation (29) and interruption of the pathway will impair the maturation of DCs and their function (30). There are some contradictory reports on whether GDF15 inhibits vs. activates the NF κ B pathway (31–33). Nevertheless, the relationship between GDF15

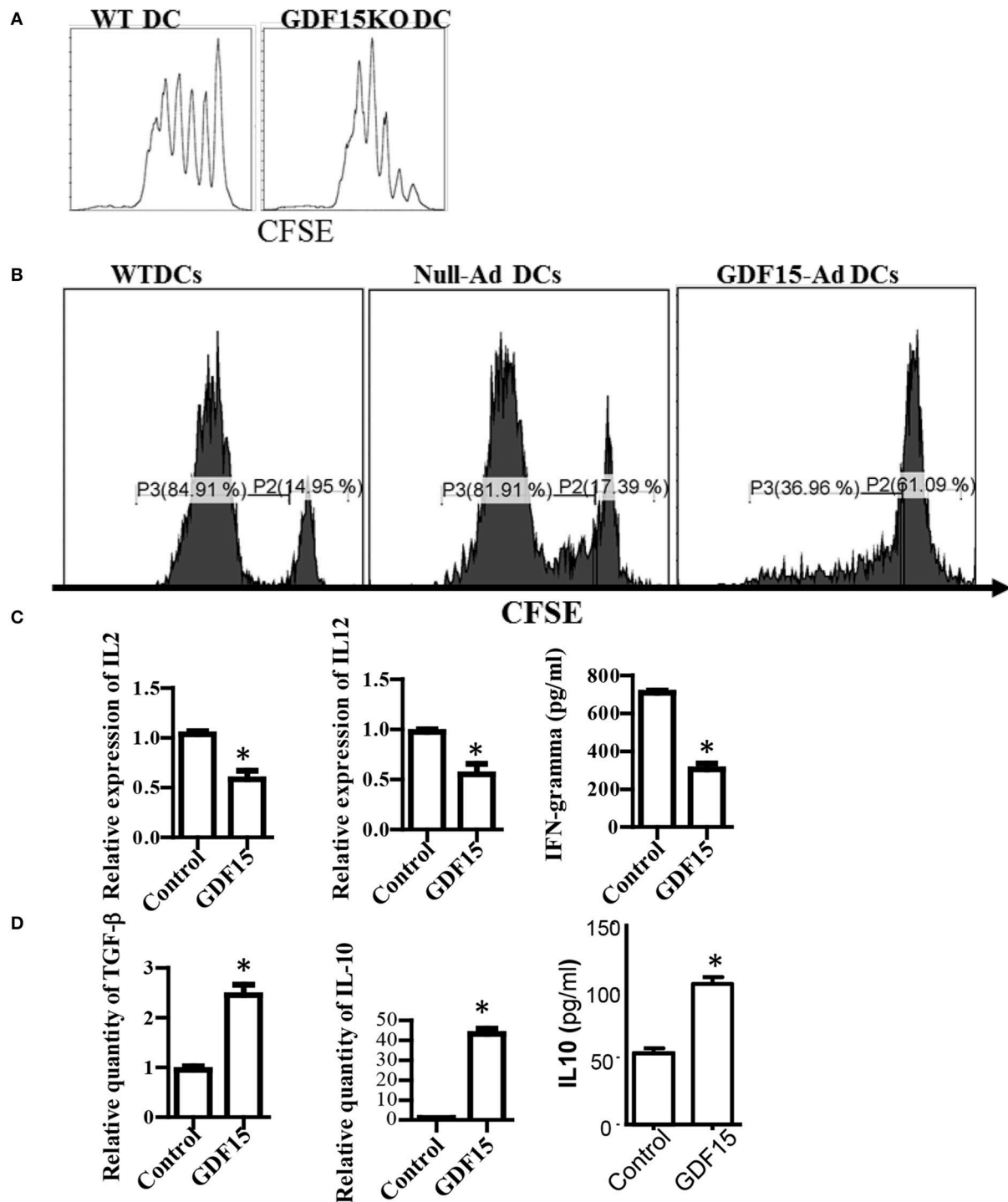


FIGURE 3 | GDF15 impaired DC's capacity to activate T cells. **(A)** GDF15 KO DCs increased T cell proliferation. BM-derived DCs were cultured from WT or GDF15 KO mice. On day 6, an MLR was performed in which different cultured DCs were used as stimulators and allogeneic (BALB/c) CFSE-labeled T cells were used as responders. Proliferation was measured by CFSE dilution. Data were representatives of $n = 3$. **(B)** GDF15-Ad DCs inhibited T cell proliferation. BM-derived DCs were infected with GDF15-Ad on day 2 and an MLR was conducted to measure T cell proliferation. Data were representatives of $n = 4$. **(C)** GDF15 decreased the expression of proinflammatory genes. RNA was extracted from DCs treated with GDF15-Ad or Null-Ad. Expression of IL2 and IL-12 was detected by qRT-PCR and IFN γ in the culture medium was detected by ELISA. $n = 3$, the student T -test was conducted for statistical analysis. **(D)** GDF15 increased immunosuppressive cytokines IL10 and TGF- β . The expression of TGF- β and IL10 in the cells was detected by qRT-PCR and expression of IL10 in culture medium was detected by ELISA. $n = 3$, the student T -test was conducted for statistical analysis. * $p < 0.05$.

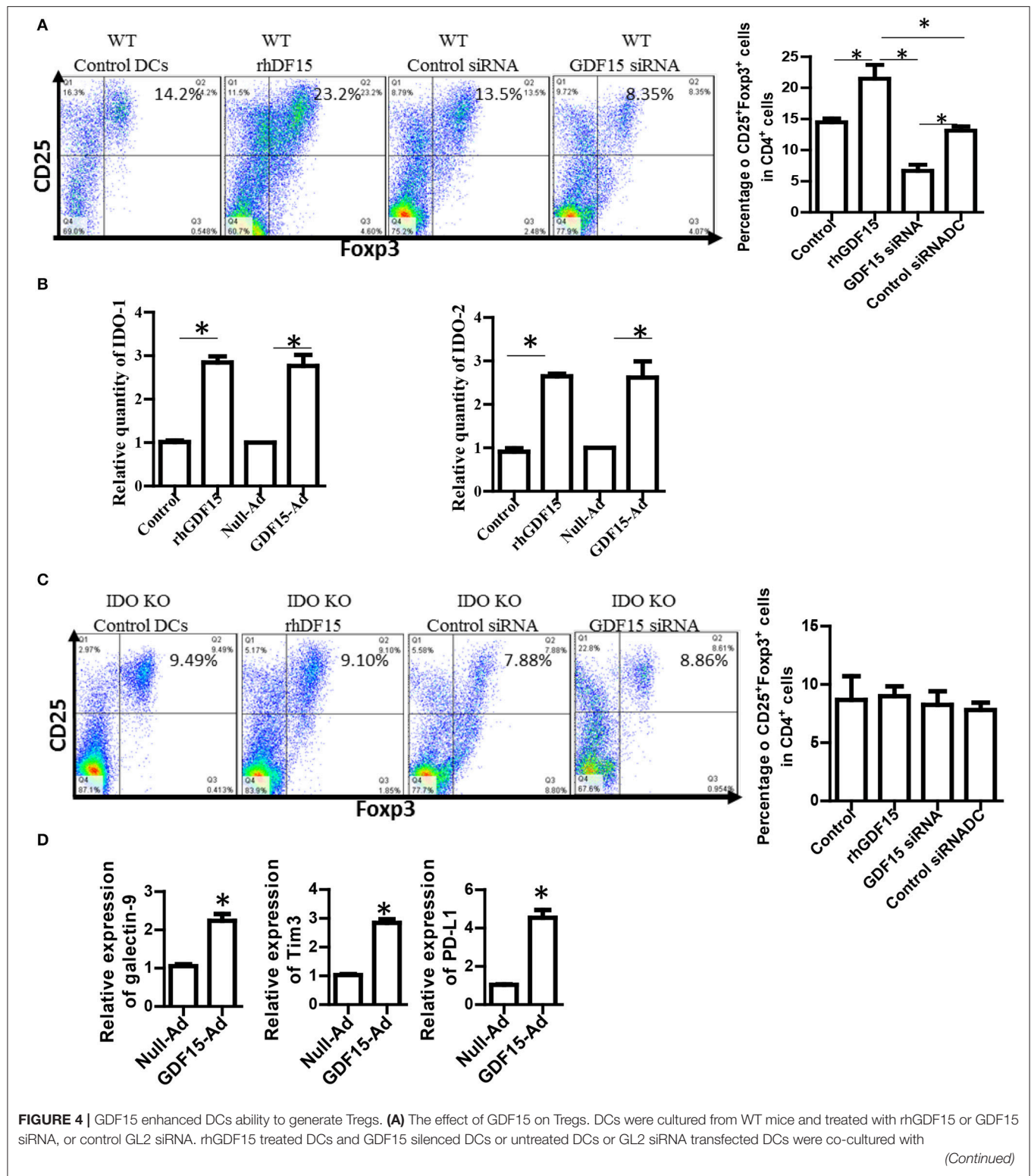


FIGURE 4 | allogeneic naïve T cells from BABL/c mice at the ratio of 1:10 for 5 days. CD4⁺CD25⁺FoxP3⁺ cells were detected by flow cytometry. Representative images (upper) and summarized data (lower) from $n = 5$ experiments. The student *t*-test was conducted to compare data between rhGDF15 treated DCs and untreated DCs and between GDF15 siRNA and GL2 siRNA. * $P < 0.05$. **(B)** GDF15 increased the expression of IDO 1 and IDO2. DCs were cultured and treated with rhGDF15, GDF15 expression adenovirus (GDF15-Ad), or control null adenovirus (Null-Ad). Expression of IDO 1 and IDO2 in DCs was detected by qRT-PCR, $n = 3$, the student *t*-test was conducted to compare data between rhGDF15 treated DCs and untreated DCs and between GDF15-Ad and Null-Ad. * $P < 0.05$. **(C)** Deficiency of IDO in DCs eliminated the effect of GDF15 on Treg generation. DCs were cultured from IDO KO mice and treated with rhGDF15 or GDF15 siRNA, then subjected to co-cultured with allogeneic T cells for 5 days. CD4⁺CD25⁺FoxP3⁺ cells were detected by flow cytometry. * $P < 0.05$. **(D)** GDF15 up-regulated immune inhibitory ligands. DCs were cultured and treated with GDF15-Ad or Null- Adenovirus. Expression of GDF15, Galectin 9, PD-L1 and TIM-3 was detected by qRT-PCR. Control adenovirus infected DCs were used as a normalizer. $n = 3$, the student *t*-test was conducted to compare data between Null-Ad and GDF15-Ad. * $P < 0.05$.

and NFκB signal in DCs remains unknown. We first detected the expression of Rel A and Rel B, which are members of the NFκB family using qRT-PCR. Over-expression of GDF15 significantly reduced the expression of Rel A (**Figure 5D**, left) and Rel B (**Figure 5D**, right). We next detected phosphorylation of Rel A p65 in DCs by Western blotting. We found that the expression levels of phosphorylated p65 were decreased both in DCs treated with GDF15-Ad (**Figure 5E**) and in GDF15 TG DCs (**Supplemental Figure 3**), as compared with null-Ad treated DCs or WT DC. By contrast, phosphorylated p65 was increased in DCs from GDF15 KO mice (**Supplemental Figure 3**). We also found that GDF15-Ad decreased total p65 expression in DCs (**Figure 5E**). Taken together, the data suggested that GDF15 inhibited DC maturation through inhibition of the NFκB signaling pathway.

GDF15 Utilizes TGF-β Receptors

GDF15 is a growth differentiation factor and functions through interactions with its receptor(s). It has previously been reported that GDF15 may use TGF-β receptors (TGF-β R) in non-brain tissues but this finding is contested. More recently, GDNF family receptor α-like (GFRAL), that is exclusively expressed in the brain, (17, 19) has been reported as a receptor of GDF15 used by neuron cells (34, 35). We performed RT-PCR to detect the expression of GFRAL and TGF β receptor I and II in DCs and splenocytes. Our data showed that there was no amplification of GFRAL using three pairs of primers located at three different positions in the GFRAL gene (**Figure 6A**), indicating that GFRAL is not expressed in both DCs and splenocytes. In contrast, DCs expressed TGF β receptor I and II (**Figure 6A**).

To test whether GDF15 uses TGF-β Rs, Ly364947, an inhibitor specific to TGF-β RI, and Ly2109761, a TGFβ RI and II dual inhibitor (36) were added to rhGDF15-treated DCs. The expression of MHC II co-stimulatory molecules CD40, CD80, CD83, CD86, and CCR7 was detected. As shown in **Figure 6B**, both Ly 364947 and Ly 2109761 resumed the expression of the aforementioned genes inhibited by rhGDF15. Data indicates that GDF15 might utilize TGF-β RI and TGF-β RII to modulate DCs.

To further confirm which receptor GDF15 utilizes, an IP assay was performed. DCs were treated with GDF15-Ad or Null-Ad on day 2 *in vitro*. On day 6, proteins were extracted from cultured DCs and GDF15 Abs were used to pull down proteins bound to GDF15, followed by Western blotting with primary Abs targeting TGF-β receptor I and II independently. We found that both TGF-β receptor I and receptor II were pulled

down by GDF15 Ab (**Figure 6C**), although the bands for TGF receptor I were thicker than receptor II. The Data indicates that GDF15 utilizes TGF-β receptor I and receptor II for its signaling.

GDF15-Induced Tol-DC Prevents Alloimmune Rejection and Prolonged Allograft Survival in Heart Transplantation

Tol-DCs have been demonstrated to be able prevent alloimmune rejection in organ transplantation (25). We investigated whether GDF15 treated DCs can prevent alloimmune rejection and immune tolerance in allogeneic HT using a murine model. BM-derived DCs were cultured from donor mice and infected with GDF15-Ad on day 2. 1 million of GDF15-Ad treated DCs or Null-Ad treated DCs were intravenously injected into recipient Babl/C mice 7days prior to transplantation. Recipient mice daily received sub-dose rapamycin (1ug/kg, s.c.) from days 0 to 7 after transplantation. Heartbeat was monitored by palpation and graft rejection was defined as the cessation of heartbeat. As shown in **Figure 7A**, the average survival of allografts in control mice was 14 and 77.5 days for grafts in GDF15-DC treated mice. 4 allografts (two-third) in recipients treated GDF15-Ad DC still had very strong heartbeat 100 days post transplantation, indicating that GDF15-Ad DC significantly prolonged allograft survival.

At the endpoint of the experiment, CD4⁺CD25⁺FoxP3⁺ Tregs that are able to induce immune tolerance were detected. Our results show that Treatment with GDF15-DCs increased CD4⁺CD25⁺FoxP3⁺ Tregs (**Figure 7B**), suggesting more Treg in mice with GDF15-DCs.

T cell exhaustion has been discussed as one of mechanisms of immune tolerance (37). Our data described in **Figure 4D** show that GDF15 up-regulated immune inhibitory ligands and suggested that GDF15 induced exhaustive DCs that could induce T cell exhaustion. Accordingly, the expression of inhibitory receptors PD-1, Tim3, and BTLA that are highly expressed by exhausted T cells was detected. We found PD-1, Tim3, and BTLA were expressed at higher levels in splenic T cells from the immune tolerant mice treated with GDF15-Ad DCs than those from control-DCs treated rejected animals (**Figure 7C**), implying that GDF15-DCs induced T cell exhaustion. Induction of T cell exhaustion is another mechanism contributing to prevention of allograft immune rejection.

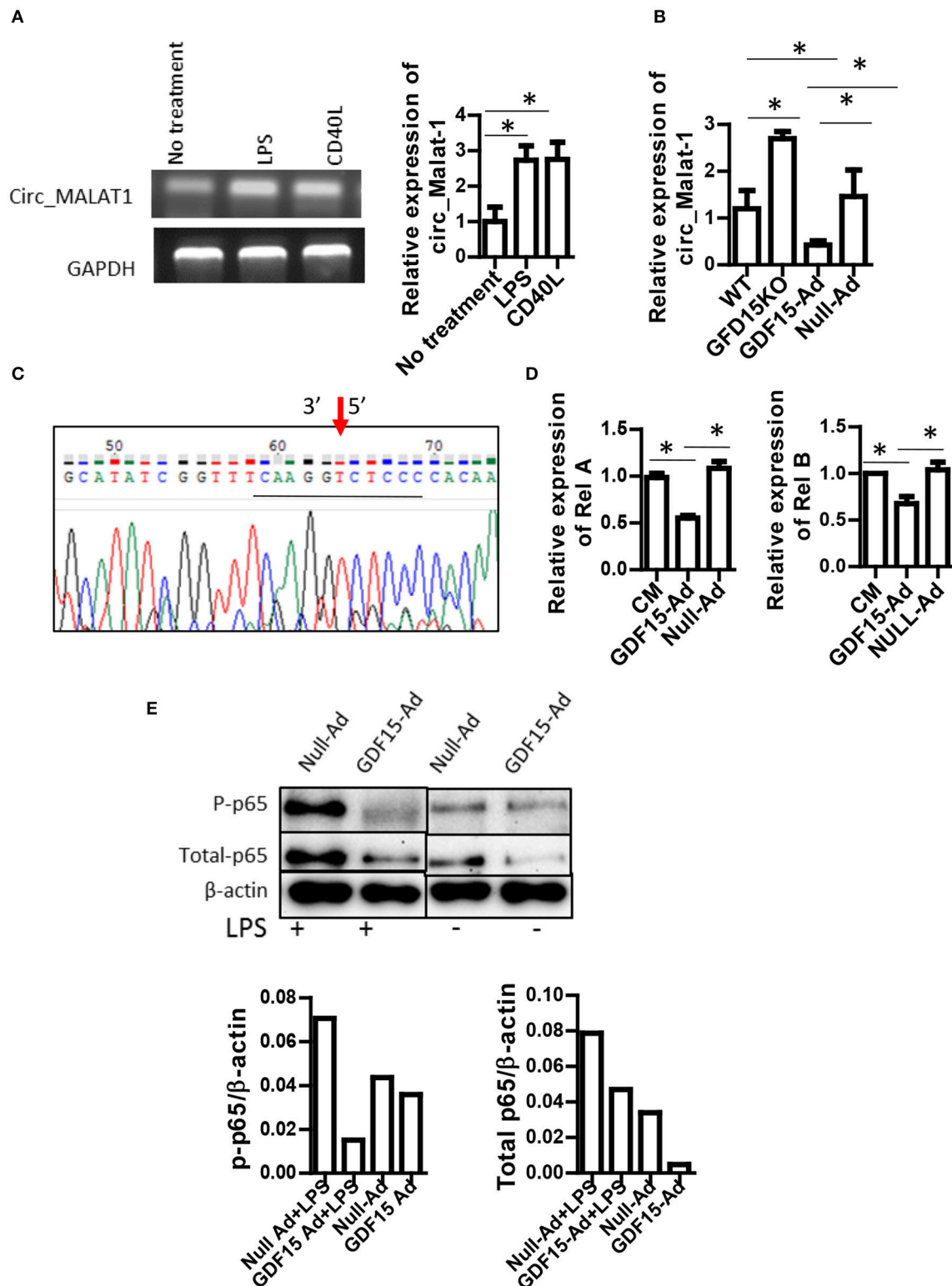


FIGURE 5 | GDF15 reduced circ_Malat-1 expression and inhibited the NF κ B pathway. **(A)** circ_Malat-1 expression in DCs. BM derived DCs were cultured from WT mice and treated with 50 ng/ml CD40L or 10 ng/mL LPS for 24 h. Untreated DCs were used as control. RNA was extracted and circ_Malat-1 expression was detected by RT-PCR. Left: regular PCR, representative images for $n = 3$; Right: qPCR, $n = 3$. One way ANOVA was conducted for statistical analysis $^*P < 0.05$. **(B)** GDF15 (Continued)

FIGURE 5 | negatively regulated circ_Malat-1 expression. The expression of circ_Malat 1 was detected in GDF15 KO DCs, WT DCs or GDF15-Ad or control Null-Ad treated DCs by qRT-PCR. $n = 3$, One way ANOVA was conducted for statistical analysis $*P < 0.05$. **(C)** Representative DNA sequence from DNA sequencing of RT-PCR products. circ_Malat-1 was amplified using divergent primers and the PCR products was then subjected to DNA sequencing using forward primer of circ_Malat-1. Arrow: pointing to the junction of circ_Malat-1. The sequencing containing the circ_Malat-1 conjunction sequence gcataatcggttt caaggt ctcc ccacaa was presented. The central conjunction sequence was underlined. **(D)** The expression of Rel A and Rel B by qRT-PCR. DCs were treated with GDF5-Ad, or Null-Ad. The expression of Rel A and Rel B were measured by qRT-PCR 48 h after infection. One way ANOVA was conducted for statistical analysis $n = 3$ $*P < 0.05$. **(E)** *GDF15 inhibited the NFκB signaling pathway*. Phosphorylated p65 and total p-65 protein was detected by western blotting using phosphorylated p65A Abs and p65 Abs. Representative image of western blotting (upper) and relative quantity of protein (low) from $n = 4$ experiments. Samples treated with LPS and samples without LPS treatment were loaded separately by other samples for PAGE.

DISCUSSION

In this study, we, for the first time, reported that BM-derived DCs express GDF15 and deficiency of GDF15 accelerates DC maturation and enhances DC's immune response. We first discovered that circ-Malat-1 is differentially expressed in mature DC vs. immature DCs and that GDF15 negatively regulates circ-Malat-1 expression in DCs. We demonstrated that overexpression of GDF15 promoted exhaustive DCs to induce T cell exhaustion and enhanced Treg generation that was IDO-dependent. We also demonstrated that administration of GDF15 treated DCs prevented allograft injection and promote immune tolerance induction in HT.

GDF15 is a divergent member of the TGF-β superfamily (38). Its immunosuppressive function was first demonstrated in pregnancy, as GDF15 is highly expressed in the placenta during pregnancy and is essential to prevent rejection of the fetus (39). Zhou group reported that treatment with rhGDF15 *in vitro* prevents DC maturation and impairs the anti-tumor immune response of DCs (15). However, the authors did not address whether GDF15 is expressed by DCs, what roles endogenous GDF15 plays and how GDF15 impacts DCs at molecular level. In this study, we conducted a series of investigations on GDF15 expression in DCs. We showed that GDF15 is expressed in DCs and that its expression level is increased as DCs develop and differentiate. This increase in GDF15 expression might result from the stimulation of GM-CSF which was added to the culture medium during entire DC culture since GM-CSF has been reported to affect GDF15 expression (40). Exogenous rhGDF5 as well as GDF15-Ad treatment increased endogenous GDF15 expression in DCs and the increasing levels are dose dependent.

We further demonstrated the role of endogenous GDF15 expressed in DCs using GDF15 KO DCs or siRNA. Deficiency or knockdown of GDF15 in DCs increased the expression of MHCII, CD40, CD80, CD83, and CD86, while over expression of GDF15 either by using GDF15 TG DCs or infection with GDF15-Ad reduced the expression of these molecules, in comparison with WT untreated DCs. Our study demonstrated that exogenous and endogenous GDF15 modulated DCs toward immature DCs characterized by low expression of MHC class II and co-stimulatory molecules, and resulted DCs failed on recognition, priming and activation of T cells and presented Tol-DCs phenotype. We also found that over expression of GDF-15 led to a decrease in NFκB family members Rel A and Rel B, IL-2, IFN-γ, and IL-12p40, and an increased expression of TGF-β, and IL-10. These findings imply that GDF15 inhibited Th1-producing DCs.

We reported for the first time that over expression of GDF15 in DCs significantly up-regulated immunosuppressive genes IDO1, IDO2, and inhibitory molecules PD-L1 and galectin-9, while reducing Rel A and Rel B, and inactivating the NFκB pathway. Subsequently, these GDF15 over-expressing DCs failed in the recognition, priming and activation of T cells, thus presenting an immunosuppressive function. Moreover, over-expression of GDF15 enhanced DCs to generate CD4⁺CD25⁺Foxp3⁺Tregs which is one of the key features of Tol-DCs and IDO is necessary for Treg generation by GDF15. These findings suggest that GDF15 is able to induce Tol-DCs. Administration of these GDF15-over-expressed Tol-DCs *in vivo* successfully prevented alloimmune rejection and prolonged graft survival in HT. The current study presented a new method to induce Tol-DCs by over-expression of GDF15 and a new potential treatment for the protection of transplanted hearts from immune rejection.

The receptor GDF15 utilizes remains unclear. More recent studies have shown that GFRAL, not TGF beta receptor is the receptor for GDF15 in control of body weight loss (34, 35). Interestingly, GFRAL is exclusively expressed in brain tissue; and not in other tissues (34, 41). We detected GFRAL expression by RT-PCR and demonstrated that GFRAL was not highly expressed in DCs. This dilemma implies that there must be some other receptors GDF15 uses since GDF15 signaling was demonstrated in many other organs and non-neuron cells including DCs (15). Several early studies have suggested that GDF15 might utilize TGF-β RII and signal through canonical TGF-β receptors in heart cells (42, 43). In this study, TGF-β R inhibitors and pull-down assays were used to investigate whether GDF15 used TGF-β RII in DCs. Addition of Ly 364947, an inhibitor specific to TGF-β RI, and Ly2109761, a dual inhibitor of TGF-β RI and RII, abolished the effect of rhGDF15 on DCs. Our IP assay showed that GDF15 binds to both TGF-β R I, and RII. Taken together, our data identifies TGF-β RI and II as receptors for GDF15. TGF-β receptors could lead to the activation of multiple pathways including the NFκB, ERK, PI3K, and SMAD signaling pathways in cancer cells and heart cells (44).

The NFκB pathway is essential for DC maturation (29) and interruption of the pathway will impair the maturation of DCs and their function (30). In this study, we found that Rel A and Rel B and phosphorylation of p65 was increased in GDF15 KO DCs, but is decreased in GDF15 TG DCs and GDF15-treated DCs. The expression of AKT was increased in GDF15 over-expressing DCs and both the p38MAPK and ERK pathways seemed unaffected (data not shown). Taken together, our study demonstrates that GDF15 inhibits the NFκB pathway, resulting in the arrest of DC

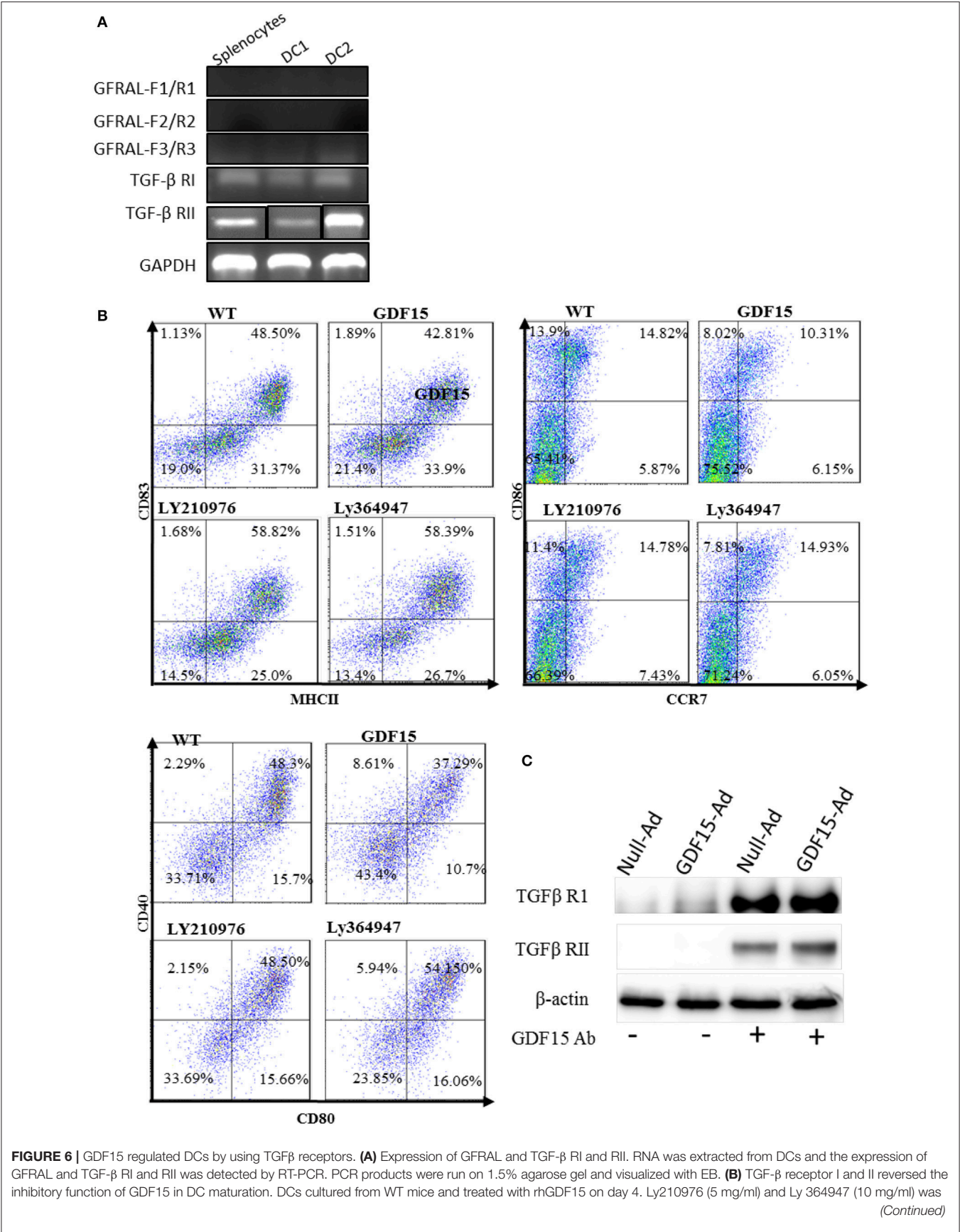


FIGURE 6 | added to rhGDF15 treated DCs on day 5, respectively. 24 h later, DCs maturation was determined by measuring expression of MHCII, CD40, CD80, CD83, CD86, and CCR7 flow cytometry. Data were representatives of three independent experiments. **(C)** IP assays. Total protein was extracted from the above DCs treated with GDF15-Ad or control Adenovirus and an IP assay was employed to pull down GDF15 bound protein using GDF15 Abs and control Ig Abs. IP protein was subjected to western blotting with TGFβ RI and RII Abs respectively. Representative of image of $n = 4$ experiments. PCR products for TGF-β RII from Splenocytes (SP) and two DC samples (DC1 and DC 2) were loaded separately by other genes products on an agarose gel and the image shown was modified by deletion of other genes' bands between TGF-β RII's bands.

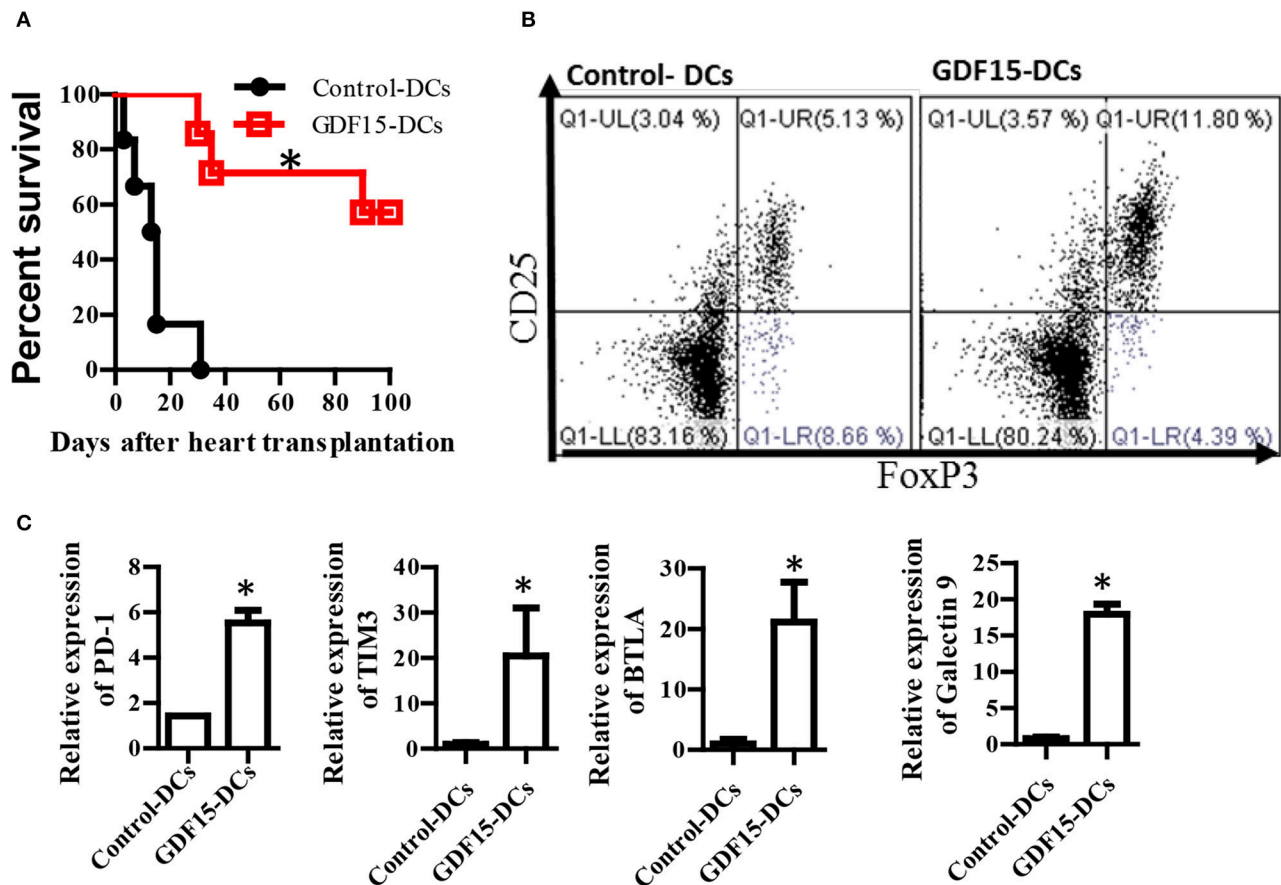


FIGURE 7 | GDF15 regulated DCs prevent allograft rejection in heart transplantation and augment tolerance induction and T cell exhaustion. **(A)** Allograft survival. Donor derived (C57BL/6) DCs were cultured and treated with GDF15-Ad or Null-Ad on day 2. 1 million day 7 DCs were i.v. injected into recipient BABL/c mice 7 days prior to heart transplantation. Allogeneic heart transplantation was conducted between C57BL/6 and BABL/c mice. Recipient mice daily received 1 g/kg rapamycin for 7 days from day 0 post transplantation. The cessation of heartbeat was defined as rejection. $n = 6$. **(B)** CD4⁺CD25⁺Foxp3⁺ Tregs in splenic cells. At the end of experiments, mice splenic cells were isolated from recipient mice on day 100 to detect CD4⁺CD25⁺Foxp3⁺ Tregs by flow cytometry. Representative images (upper) and relative quantity of FoxP3 protein from $n = 3$ * $P < 0.05$. **(C)** Inhibitory receptor expression. RNA was extracted from the above rejected and tolerant recipient mice. Gene expression of inhibitory receptors PD-1, TIM3, BTLA and galectin 9 was detected by qRT-PCR. $n = 3$, the student t -test was conducted for statistical analysis * $P < 0.05$.

maturation, reduction in pro-inflammatory cytokine production, and suppression of DC immune function.

More importantly, this study, for the first time, discovered the involvement of circRNA in DC development and the link between GDF15, circ_Malat-1 and immunosuppressive function. circRNA is single stranded non-coding RNA which forms a covalently closed continuous loop (45). It is abundant, stable with a longer half- life and conserved across species. circRNAs regulate gene expression and function by acting as sponges of microRNA (miRNA) (46) and interacting with proteins (16, 17).

Most circRNAs identified are generated from exons by back-splicing events and are called exonic circRNAs. circ_Malat-1 we reported here is intronic circRNA from a long non-coding RNA Malat-1, existing in the nucleus. There are no reports about circ_Malat-1. Our study shows that circ_Malat-1 was expressed in DCs at certain abundance and that circ_Malat-1 was expressed in mature DCs about 2-fold greater than in immature DCs. GDF15 significantly reduced the expression of circ_Malat-1. Taken together, circRNA is involved in DC development and circ_Malat-1 in mature DCs was significantly expressed at

2-fold higher than in immature DCs. GDF15 negatively regulates circ_Malat 1. However, the function of circ_Malat-1 needs to be further studied in the future.

In summary, we for the first time demonstrated that DCs express GDF15 and discovered a new impact of GDF15 in DC development and immune modulation such as increasing immunosuppressive molecule and immune inhibitory molecule expression, T cell exhaustion and Treg generation. We also demonstrated that administration of GDF15 regulated DCs can prevent allograft rejection and promote immune tolerance in HT. We dissected the mechanisms by which GDF 15 regulates the immune response and prevents immune rejection. On top of these findings, we first demonstrated the involvement of circRNA in DC development and GDF15 regulation of circ_Malat-1. This study also enhances the understanding of the development of mechanism-based therapies for preventing allograft rejection in transplantation. It provides a potential therapy for preventing immune rejection.

AUTHOR CONTRIBUTIONS

YZ, GZ, YL, RC, and DZ: data collection and analysis; VM, TM, and KL: data discussion and manuscript writing; XZ: project design, data collection, analysis, and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02407/full#supplementary-material>

Supplemental Table 1 | Primer sequences for qPCR.

Supplemental Figure 1 | rhGDF15 increased DC maturation. DCs (10^6 cell/well) were cultured from bone marrow progenitors in the presence of IL-4 and granulocyte/macrophage colony stimulating factor (GM-CSF). rhGDF15 were added on day 4. DC maturation was detected by flow cytometry.

Supplemental Figure 2 | Treg generation. DCs were cultured from WT, GDF15 KO, and GDF15 Tg mice. These Cs cultured for 7 days were collected and co-cultured with allogeneic naïve T cells from BABL/c mice at the ratio of 1:10 for 5 days. CD4⁺CD25⁺FoxP3⁺ cells were detected by flow cytometry. Representative images were from $n = 3$ experiments.

Supplemental Figure 3 | GDF15 TG DCs expressed lower levels of Phosphorylated p65. Phosphorylated p65 and beta-actin in DCs was detected by western blotting using phosphorylated p65A Abs and beta-actin Abs. Representative of image of western blotting (upper) and relative quantity of protein (low) from $n = 3$ experiments.

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Distinct Role of IL-27 in Immature and LPS-Induced Mature Dendritic Cell-Mediated Development of CD4⁺ CD127⁺3G11⁺ Regulatory T Cell Subset

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Interleukin-27 (IL-27) plays an important role in regulation of anti-inflammatory responses and autoimmunity; however, the molecular mechanisms of IL-27 in modulation of immune tolerance and autoimmunity have not been fully elucidated. Dendritic cells (DCs) play a central role in regulating immune responses mediated by innate and adaptive immune systems, but regulatory mechanisms of DCs in CD4⁺ T cell-mediated immune responses have not yet been elucidated. Here we show that IL-27 treated mature DCs induced by LPS inhibit immune tolerance mediated by LPS-stimulated DCs. IL-27 treatment facilitates development of the CD4⁺ CD127⁺3G11⁺ regulatory T cell subset *in vitro* and *in vivo*. By contrast, IL-27 treated immature DCs fail to modulate development of the CD4⁺CD127⁺3G11⁺ regulatory T cell sub-population *in vitro* and *in vivo*. Our results suggest that IL-27 may break immune tolerance induced by LPS-stimulated mature DCs through modulating development of a specific CD4⁺ regulatory T cell subset mediated by 3G11 and CD127. Our data reveal a new cellular regulatory mechanism of IL-27 that targets DC-mediated immune responses in autoimmune diseases such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE).

Keywords: dendritic cell, immune tolerance, immunotherapy, regulatory T cell, IL-27

INTRODUCTION

Interleukin-27 (IL-27) is an important cytokine that plays a critical role in regulation of immune responses *in vivo* (1). Previous research has shown that IL-27 is an anti-inflammatory cytokine. For example, it blocks Th17-mediated immune responses (2). However, recent data also indicate that IL-27 plays the role of an inflammatory cytokine that facilitates T cell-mediated immune responses (3–5). These contradictory results suggest the complex regulatory mechanisms of IL-27 *in vivo* (1).

CD127 is a subunit of interleukin-7 (IL-7) receptor. It is composed of 459 amino acids and is expressed in mature T cells, monocytes and macrophages. In particular, CD127 is a biomarker of regulatory T cells (T_{regs}) (6, 7). Several sub-populations of T_{regs} have been defined according to the importance of CD127 expression on CD4⁺ T cells (8).

3G11 is a membrane antigen expressed on murine CD4⁺ T cells. 3G11 is a ganglioside with mobility between GD1a and GD1b complexes in the human brain. 3G11 antigen is identified as the disialoganglioside IV3(NeuAc)2-GgOse4Cer. The immune function of 3G11 on CD4⁺ T cells has not been fully elucidated. Recent research demonstrated that 3G11 may be a biomarker of T_{reg} subsets (8–13).

Dendritic cells (DCs) are important immune regulatory cells that play a central role in development of T cells such as CD4⁺ T helper cells. DCs modulate development and differentiation of T cells through production of multiple cytokines such as IL-27 (5). It is not clear whether IL-27 can affect DC-mediated CD4⁺ T cell immune responses. The effect of IL-27 on development of CD4⁺ regulatory T cells was examined in this project. Our data show that IL-27 modulates development of mature DC-mediated differentiation of T_{reg} subsets.

Regulatory T cells are important immune cells *in vivo* and they play a central role in induction of immune tolerance and anti-inflammatory responses (14, 15). Multiple subsets of T_{regs} have been reported. For example, our previous data show that there are two subpopulations of T_{regs} including CD4⁺CD25⁺FoxP3⁺GITR⁺CD127⁺3G11⁺ and CD4⁺CD25⁺FoxP3⁺GITR⁺CD127⁺3G11[−] T_{reg} subsets *in vivo*. Although the immune function of these two new subsets of T_{regs} is unclear, the number of T_{reg} sub-populations mediated by 3G11 and CD127 is different in mice with experimental autoimmune encephalomyelitis (EAE) development and those with immune tolerance. However, the regulatory mechanisms of CD4⁺CD127⁺3G11⁺ T_{regs} are still unclear (8). Our project is focused on whether or not IL-27 plays an important role in the development of CD4⁺CD127⁺3G11⁺ T_{regs} mediated by immature or mature DCs induced by LPS. Our results will show that IL-27 modulates development of CD4⁺CD127⁺3G11⁺ T_{regs} mediated by mature DCs, and they may help to reveal a new mechanism of IL-27 in mature DC-mediated immune responses.

MATERIALS AND METHODS

Mice

Wild type C57 BL/6J female mice (8–12 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred in Thomas Jefferson Animal Care facilities and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Immunogen and Peptide

Mouse MOG_{35–55} peptide (MEVGWYRSPFSRVVHLYRNGK), an ingredient of myelin oligodendrocyte glycoprotein (MOG), was obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA).

Isolation of Bone Marrow

As described previously, femurs and tibiae were isolated from muscle tissue of mice. The intact bones were then sterilized with 70% ethanol for 5 min and washed with phosphate-buffered saline (PBS). Bone ends were cut and the bone marrow was

flushed with PBS. Cellular clusters within the bone marrow suspension were disintegrated and washed with PBS (8, 16–20).

Bone Marrow-Derived DC Culture

As described previously, leucocytes from bone marrow were fed in bacteriological 100 mm Petri dishes (Falcon, Becton Dickinson, Heidelberg, Germany) at 2×10^6 cells per dish. Cells were cultured in RPMI1640 complete medium (Gibco-BRL, Eggenstein, Germany) including penicillin (100 U/ml, Sigma, St. Louis, MO, USA), streptomycin (100 U/ml, Sigma), L-glutamine (2 mM, Sigma), 2-mercaptoethanol (2-ME, 50 μ M, Sigma), 10% heated, inactivated and filtered (0.22 μ m, Milipore, Inc., Bedford, MA, USA) Fetal Calf Serum (FCS, Sigma) and granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech, Rocky Hill, NJ, USA) at 20 ng/ml at day 0 (10 ml medium per dish) (8, 16–20).

At day 3, 10 ml fresh medium with GM-CSF (20 ng/ml) was added to each dish and, at day 6, half of the medium (about 10 ml supernatant) was collected and centrifuged at 300 g for 5 min. Subsequently, cells were re-suspended in 10 ml fresh medium with GM-CSF (20 ng/ml) and were then re-fed in the original dish. Only non-adherent cells (DCs) were harvested and seeded in a fresh dish; 10 ml fresh medium including GM-CSF (20 ng/ml) was added at day 8 (8, 16–20).

Cells were also treated with lipopolysaccharide (LPS, Sigma) for 24 h at 1 μ g/ml. LPS was isolated from *K. Pneumoniae*. DCs or LPS-treated DCs were pulsed with 0.1 μ M MOG peptide for 30 min and then washed twice with PBS at 300 g \times 5 min before i.v. transfer to EAE mice. DCs were treated with IL-27 at 100 ng/ml for 72 h before conducting flow cytometry assay or i.v. transfer experiments. Fresh non-adherent DCs were then collected and washed with PBS at 300 g for 5 min and characterized by flow cytometry or i.v. transferred to EAE mice. More than 90% of cells expressed DC marker CD11c (8, 16–20).

Flow Cytometry

MOG-primed T lymphocytes were isolated from EAE mice and incubated with anti-mouse CD4 (Pacific blue), CD25 (APC), CD127 (PerCP-Cy5.5), 3G11 (PE-Cy7) and GITR (APC-Cy7) antibodies (Biolegend). Cells were washed twice with 5% FCS in PBS at 300 g for 5 min, fixed with 5% formalin in PBS at 4°C for 2 h and then permeated for intracellular staining (8, 16–20).

For intracellular staining, spleen cells were stimulated by leukocyte activator (BD) for 6 h. Splenocytes were then washed twice with 5% FCS in PBS at 300 g for 5 min and fixed with 5% formalin (Sigma) in PBS at 4°C for 2 h. After cells were washed with permeabilization buffer (Biolegend) twice at 300 g \times 10 min, anti-mouse FoxP₃ (PE) antibody (Biolegend) was incubated with cells at 4°C for 24 h. Cells were then washed with permeabilization buffer twice at 300 g for 5 min, re-suspended in 0.5 ml cell staining buffer (Biolegend), and tested in a FACSaria (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software (Treestar, Ashland, OR, USA) (8, 16–20).

Generation of Effector T Cells *in vitro*

C57 BL/6J mice were immunized with MOG_{35–55} peptide (Invitrogen) 200 μ g, QuilA (Sigma) 20 μ g, and keyhole limpet

Abbreviations: APC, Allophycocyanin; CD, Cluster of differentiation; CFA, Complete Freund's adjuvant; DC, Dendritic cell; EAE, Experimental autoimmune encephalomyelitis; FCS, Fetal Calf Serum; Fig, Figure; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL, Interleukin; i. p., Intraperitoneal; i.v., Intravenous; KLH, Keyhole limpet hemocyanin; LPS, Lipopolysaccharide; MOG, Myelin oligodendrocyte glycoprotein; 2-ME, 2-mercaptoethanol; MS, Multiple sclerosis; PBS, Phosphate-buffered saline; pDCs, Plasmacytoid dendritic cells; PI, Propidium Iodide; PT, Pertussis toxin; s.c., Subcutaneous; SD, Standard deviation; SEM, Standard error of arithmetic mean; Th, T helper cells.

hemocyanin (KLH, Sigma) 20 μ g per mouse at day 0. Spleen cells were then isolated at day 10 after immunization. CD4⁺ T lymphocytes were purified with mouse CD4⁺ T cell subset column kit (R&D Systems). CD4⁺ T cells (1×10^6 cells/per well) were co-cultured with DCs at 10:1 (T cells: DCs) and pulsed with MOG_{35–55} peptide at 0.1 μ M in complete medium with mouse IL-2 at 1 ng/ml for 3 days. Cells were harvested and MOG-primed CD4⁺ T cells were gated and analyzed by flow cytometry (8, 16–20).

EAE Induction and Treatment

C57BL/6J mice (female, 8–12 week) were immunized with MOG_{35–55} peptide/complete Freund's adjuvant (CFA, Sigma) at 200 μ g/200 μ l/per mouse (subcutaneous injection, s.c.). Pertussis toxin (PT, Sigma) was simultaneously injected at 200 ng/per mouse (intraperitoneal injection) and the second PT injection was conducted after 48 h. EAE was assessed following standard clinical scores: 0.5: paralysis of half the tail, 1: paralysis of whole tail, 2: paralysis of tail and one leg, 3: paralysis of tail and two legs, 4: moribund, 5: death (8, 16–20).

Mice were divided into five groups. DCs were washed with PBS twice and were immediately injected via tail vein (3×10^5 cells/per mouse/per time) on days 11, 14, and 17 post-immunization (p.i): (1) injected with PBS only (EAE control); (2) injected with DCs pulsed with MOG peptide; (3) injected with IL-27-treated DCs pulsed with MOG peptide; (4) injected with LPS-DCs pulsed with MOG peptide; (5) injected with LPS and IL-27-treated DCs pulsed with MOG peptide (8, 16–20).

At day 25 p.i., splenocytes were isolated and stimulated with MOG peptide (0.1 μ M) and mouse IL-2 (1 ng/ml) for 3 days. Cells were then harvested for flow cytometry assay (8, 16–20).

Statistical Analysis

Experimental data were analyzed using Prism software (GraphPad, La Jolla, CA, USA). A two-way ANOVA test was performed for analysis of clinical score of EAE; *t* tests were conducted for analysis of flow cytometry data. Error bars represent the mean and standard deviation (SD) or standard error of arithmetic mean (SEM). Results are considered to show a significant difference if the *P* value is less than 0.05 (8, 16–20).

RESULTS

IL-27-Treated Immature DCs do not Affect Expression of T_{reg}-Associated Molecules on CD4⁺ T Cells

Since CD25, CD127, FoxP3, GITR, and 3G11 are T_{reg}-associated molecules and expressed on CD4⁺ T cells, we supposed that IL-27-treated DCs may affect expression of CD25, CD127, FoxP3, GITR, and 3G11 on CD4⁺ T cells and then regulate development of T_{regs} via modulating expression of T_{reg}-associated molecules. To test whether IL-27-treated immature bone marrow-derived DCs can affect protein expression of T_{reg}-associated molecules on MOG-primed CD4⁺ T cells, DCs (Thin line) or IL-27-treated DCs (Thick line) were pulsed with MOG peptide and co-cultured

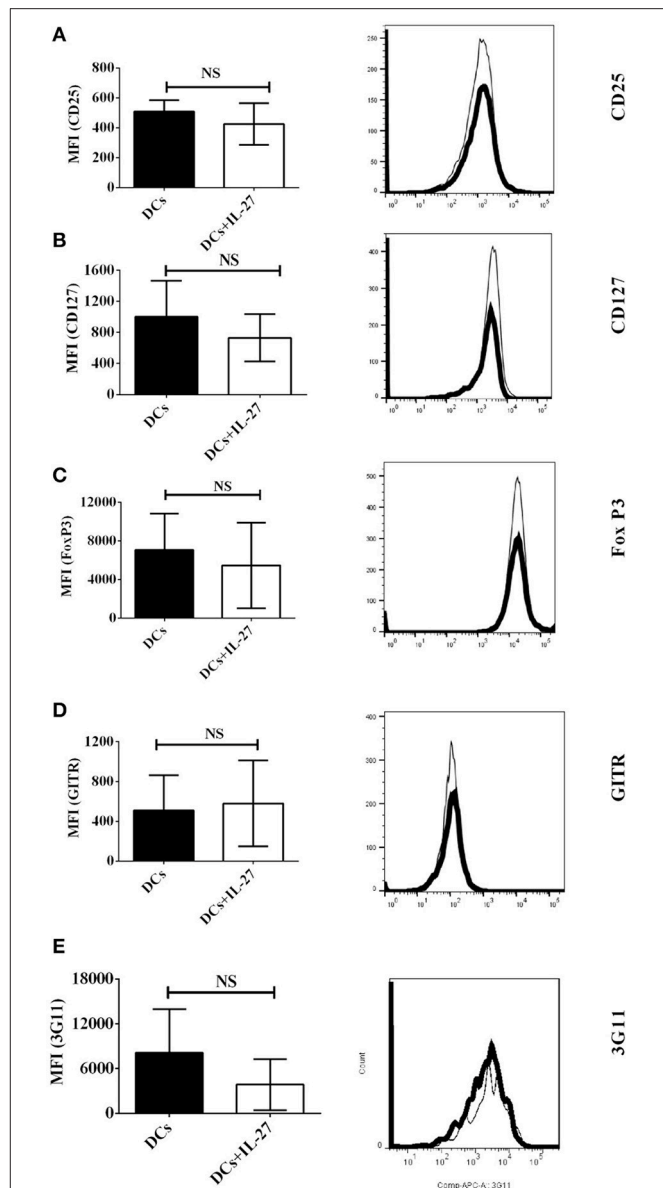


FIGURE 1 | Expression of T_{reg}-associated molecules on MOG-primed CD4⁺ T cells co-culture with immature DCs (Thin line) or IL-27-treated immature DCs (Thick line) pulsed with MOG peptide *in vitro*. C57 BL/6J mice were immunized with MOG (200 μ g)/Quil A (20 μ g) / KLH (20 μ g)/per mouse at day 0. Splenocytes were harvested at day 10. CD4⁺ T cells were then isolated using mouse CD4⁺ T cell subset column kit (R and D Systems). CD4⁺ T Lymphocytes were re-stimulated with MOG peptide (0.1 μ M) and IL-2 (1 ng/ml) for 72 h. Cells were then stained by anti-mouse CD25 (A), CD127 (B), FoxP3 (C), GITR (D), and 3G11 (E) antibodies. Protein expression of T_{reg}-associated molecules on CD4⁺ T cells is shown. Error bars indicated in this figure represent mean and SD of triplicate determinations of mean fluorescence intensity (MFI) of T_{reg}-associated molecule expression on CD4⁺ T cells (*n* = 3, *t* test, *P*_A = 0.4198; *P*_B = 0.4450; *P*_C = 0.6047; *P*_D = 0.8372; *P*_E = 0.2523; NS, no significant difference).

with MOG-primed CD4⁺ T cells. The expression of CD25, CD127, FoxP3, GITR, and 3G11 on CD4⁺ T cells co-cultured with MOG-loaded DC or MOG-pulsed DC (MOG-DCs) treated

with IL-27 is shown (Figures 1A–E). The experimental data indicate that there is no significant difference in expression of T_{reg} -associated molecules on $CD4^+$ T cells incubated with MOG-DCs or MOG-DCs-treated with IL-27.

Nor do IL-27-Treated Mature DCs Induced by LPS Affect Expression of T_{reg} -Associated Molecules on MOG-Primed $CD4^+$ T Cells

Although IL-27-treated immature DCs do not affect protein expression of T_{reg} -associated molecules on $CD4^+$ T cells (Figure 1), it is unclear whether or not mature DCs induced by LPS can do that. To detect whether or not IL-27 treatment can modulate mature DC-mediated expression of T_{reg} -associated molecules on $CD4^+$ T cells, MOG-pulsed mature bone marrow-derived DCs induced by LPS were treated with IL-27 (Thick line) or without IL-27 treatment (Dot line) and co-cultured with MOG-primed $CD4^+$ T cells. The expression of CD25, CD127, FoxP3, GITR, and 3G11 on $CD4^+$ T cells is demonstrated (Figures 2A–E). Our data indicate that expression of T_{reg} -associated molecules on $CD4^+$ T cells co-cultured with IL-27-treated mature DCs is similar to that on $CD4^+$ T cells co-cultured with mature DCs without IL-27 treatment. It can be concluded that IL-27 treatment does not modulate either immature or mature DC-mediated expression of CD25, CD127, FoxP3, GITR, and 3G11 on MOG-primed $CD4^+$ T cells.

IL-27 Treatment Facilitates Development of $CD4^+CD127^+3G11^+ T_{regs}$ Mediated by LPS-Induced Mature DCs

Although immature and mature DCs treated with IL-27 do not affect expression of T_{reg} -associated molecules on $CD4^+$ T cells (Figures 1, 2), we assumed that IL-27-treated immature or mature DCs may still modulate development of T_{reg} sub-populations. To test whether IL-27 can affect immature and mature DC-mediated development of $CD4^+$ T_{reg} subsets, immature, and mature DCs were incubated with or without IL-27 treatment. Immature and mature DCs were then pulsed with MOG peptide and co-cultured with MOG-primed $CD4^+$ T cells. Phenotypes of $CD4^+$ T_{regs} -mediated by CD127 and 3G11 are shown (Figure 3). The experimental results indicate that immature DCs treated with IL-27 cannot modulate development of $CD4^+CD127^+3G11^+ T_{reg}$ subset; however, LPS-induced mature DCs treated with IL-27 can enhance development of the $CD4^+CD127^+3G11^+ T_{reg}$ sub-population (Figure 3). This suggests that LPS may modulate mature DC-mediated development of $CD4^+$ T_{reg} subsets *in vitro*.

IL-27-Treated Mature DCs Block Immune Tolerance Induced by LPS-Stimulated DCs *in vivo*

We have investigated the effect of immature and mature DCs treated with IL-27 on development of T_{regs} *in vitro* (Figures 1–3). It is necessary for establishment of *in vivo* model to detect whether or not immature and mature DCs treated with IL-27

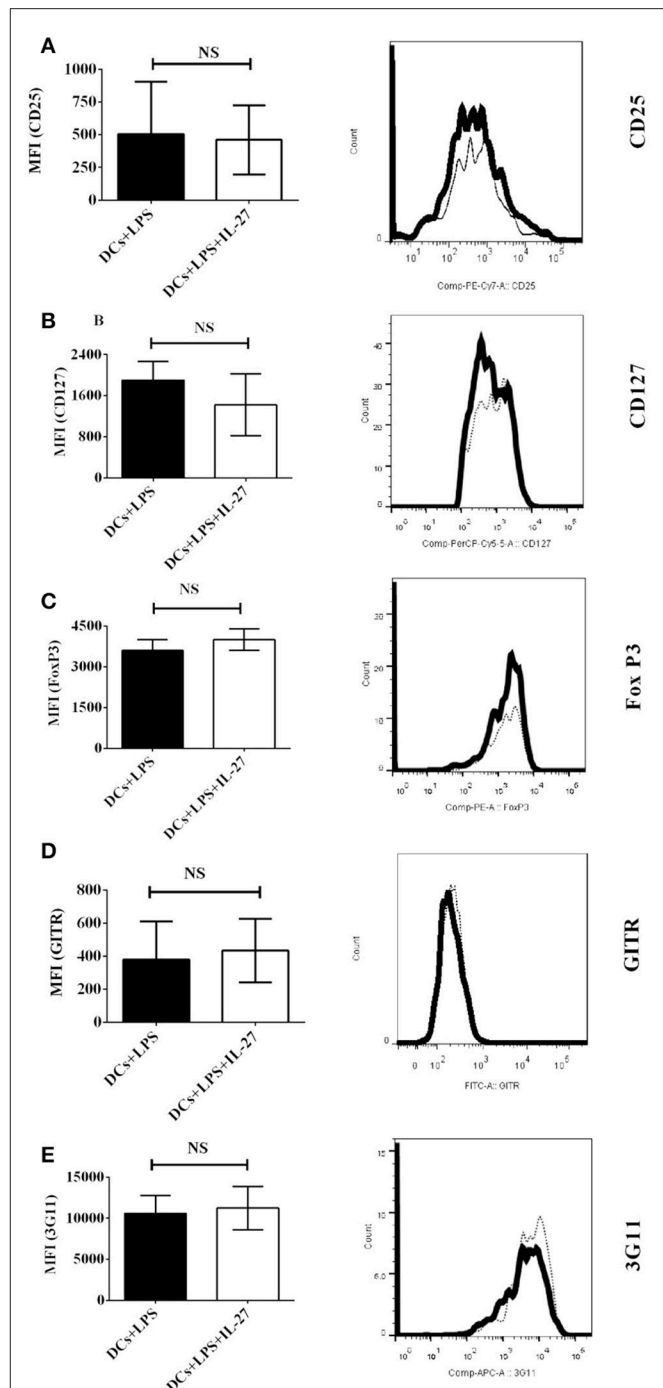


FIGURE 2 | Protein expression of T_{reg} -associated molecules on MOG-primed $CD4^+$ T cells incubated with LPS-induced mature DCs or IL-27-treated mature DCs pulsed with MOG peptide *in vitro*. Bone marrow-derived dendritic cells were stimulated with LPS (1 μ g/ml) for 24 hrs. LPS-stimulated DCs were also simultaneously incubated with IL-27 (20 ng/ml) (Thick line) for 72 hrs or had no IL-27 treatment (Dot line). DCs were then co-cultured with MOG-primed $CD4^+$ T cells as shown in Figure 1. Protein expression of CD25 (A), CD127 (B), FoxP3 (C), GITR (D), and 3G11 (E) on $CD4^+$ T cells is shown. Error bars indicated in this figure represent mean and SD of MFI of T_{reg} -associated molecules expressing on $CD4^+$ T cells in three independent experiments ($n = 3$, t test, $P_A = 0.8809$; $P_B = 0.3012$; $P_C = 0.2879$; $P_D = 0.7744$; $P_E = 0.7549$; NS, no significant difference).

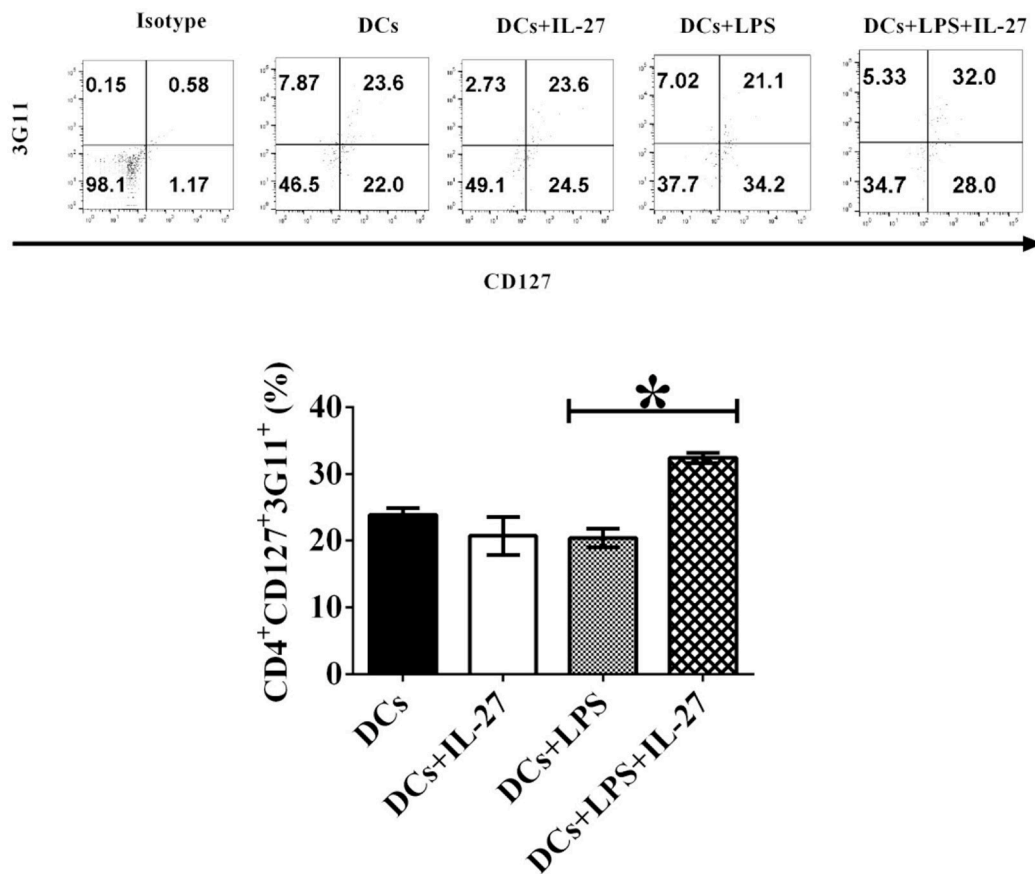


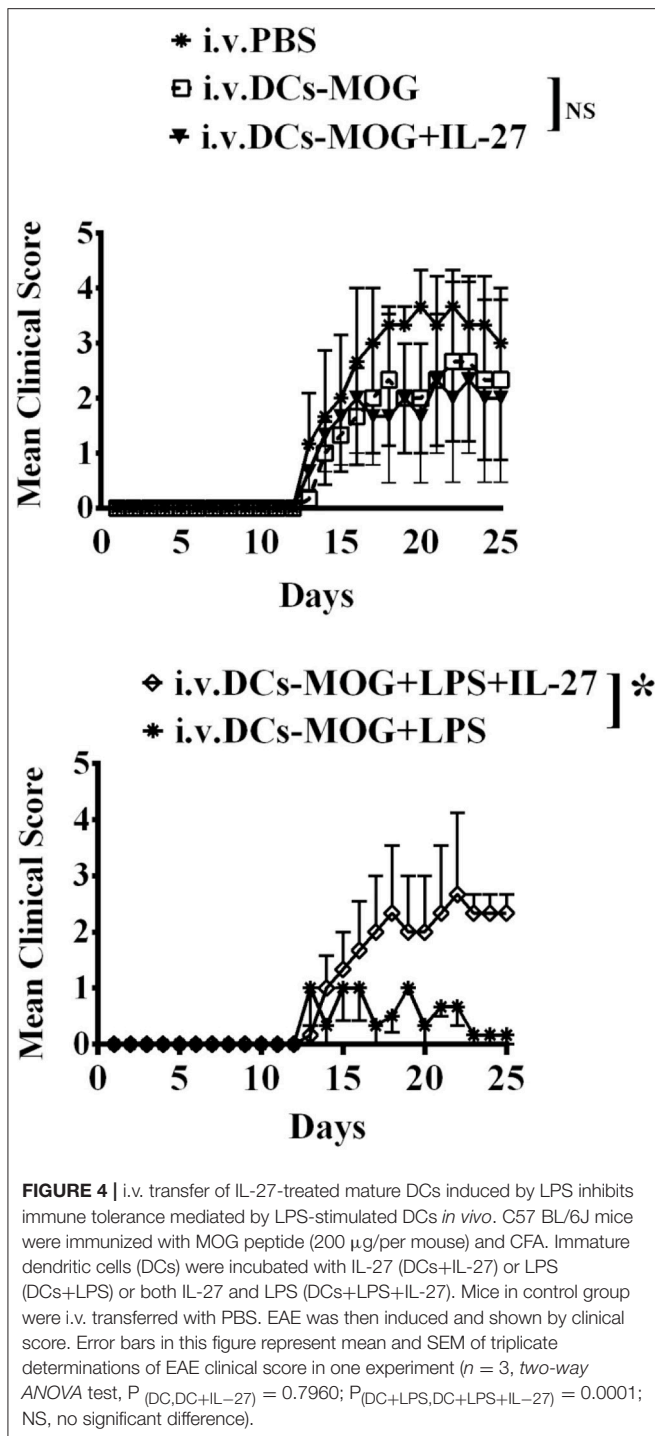
FIGURE 3 | IL-27 treatment facilitates LPS-induced mature DCs-mediated development of CD4⁺CD127⁺3G11⁺ T_{reg} subset *in vitro*. Bone marrow-derived DCs shown in **Figures 1, 2** were treated with IL-27 (20 ng/ml, 72 h) or/and LPS (1 μg/ml, 24 h) or without LPS or IL-27 incubation. DCs were then co-cultured with MOG-primed CD4⁺ T cells indicated in **Figures 1, 2** for 72 h. CD4⁺CD25⁺FoxP3⁺GITR⁺ cells were gated. The frequency of CD127⁺3G11⁺ T_{regs} is shown. Isotype control is CD4⁺ T cells incubated with isotype control antibodies. Error bars shown in this figure represent mean and SD of frequency of CD4⁺CD127⁺3G11⁺ T_{regs} in three independent experiments ($n = 3$, t test, $P_{(DC,DC+IL-27)} = 0.1357$; $P_{(DC+LPS,DC+LPS+IL-27)} = 0.0002$).

can regulate development of T_{regs}. To test whether or not IL-27 treated immature and mature DCs can affect MOG-primed CD4⁺ T cell-induced autoimmunity *in vivo*, immature and LPS-induced mature DCs were pulsed with MOG peptide and incubated with or without IL-27 treatment. Immature and mature DCs were then i.v transferred into C57BL/6J mice immunized with MOG peptide to induce EAE. Our data indicate that IL-27 treatment blocks immune tolerance mediated by LPS-induced mature DCs. By contrast, IL-27 treatment does not affect the development of EAE in mice that are i. v. transferred with immature DCs (**Figure 4**). The experimental results suggest that IL-27 may affect mature DC-mediated immune responses but that it does not affect immature DC-mediated immune responses.

IL-27 Treated Immature DCs do Not Affect Expression of T_{reg}-Associated Molecules on CD4⁺ T Cells *ex vivo*

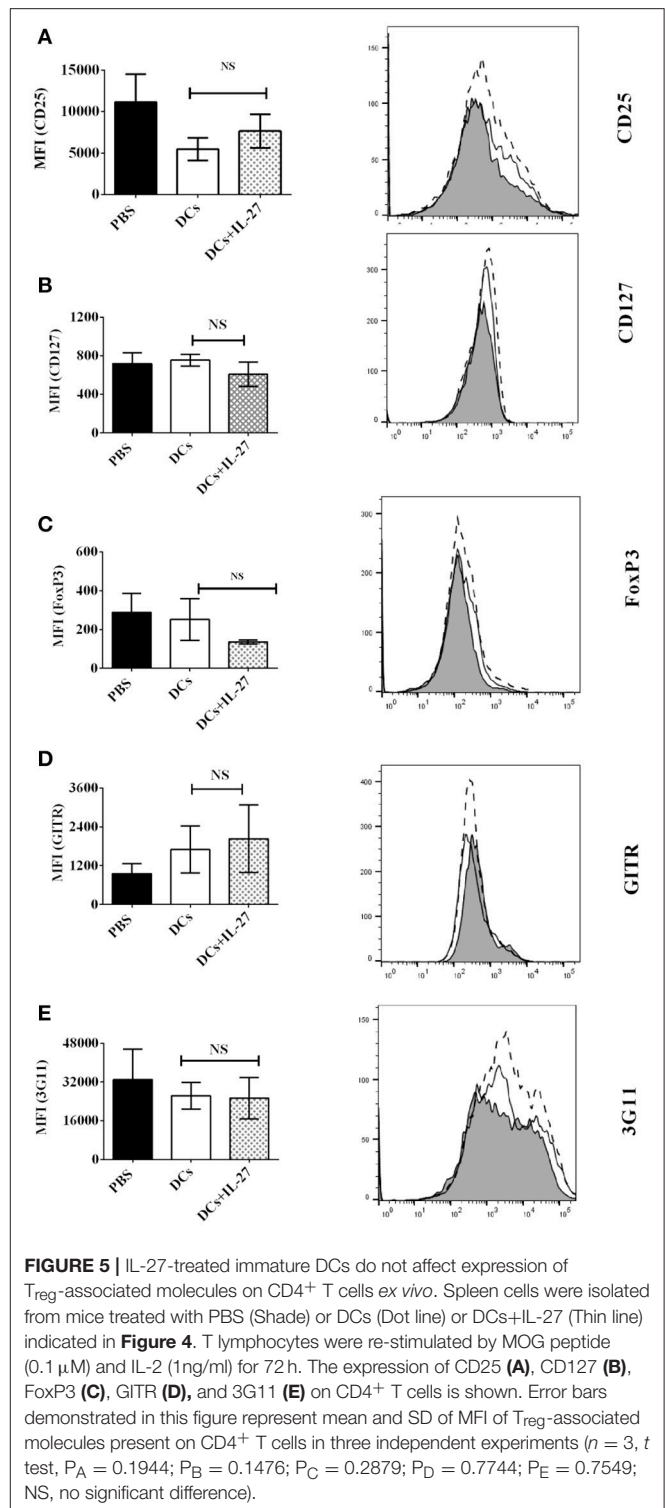
Our data of *in vitro* assay have shown that IL-27-treated immature DCs do not affect expression of T_{reg}-associated

molecules on CD4⁺ T cells (**Figure 1**), however, it is still unknown whether or not IL-27-treated immature DCs can affect expression of T_{reg}-associated molecules on CD4⁺ T cells *in vivo*. To test whether or not IL-27-treated immature DCs can modulate expression of T_{reg}-associated molecules on CD4⁺ T cells *in vivo*, MOG peptide-pulsed immature DCs incubated with IL-27 or without IL-27 treatment were i.v. transferred into EAE mice shown in **Figure 4** at day 11, 14, and 17 after immunization. Lymphocytes were isolated from mice which are i.v transferred with IL-27-treated DCs or immature DCs without IL-27 treatment shown in **Figure 4** at day 25. Expression of T_{reg}-associated molecules on CD4⁺ T cells was detected using flow cytometry. Our results demonstrated that there is no difference in expression of T_{reg}-associated molecules, including CD25, CD127, FoxP3, GITR, and 3G11, on CD4⁺ T cells isolated from mice that are i.v. transferred with IL-27-treated immature DCs or DCs without IL-27 incubation (**Figures 5A–E**). Our data suggest that IL-27-treated immature DCs do not affect development of T_{regs} *in vivo*.

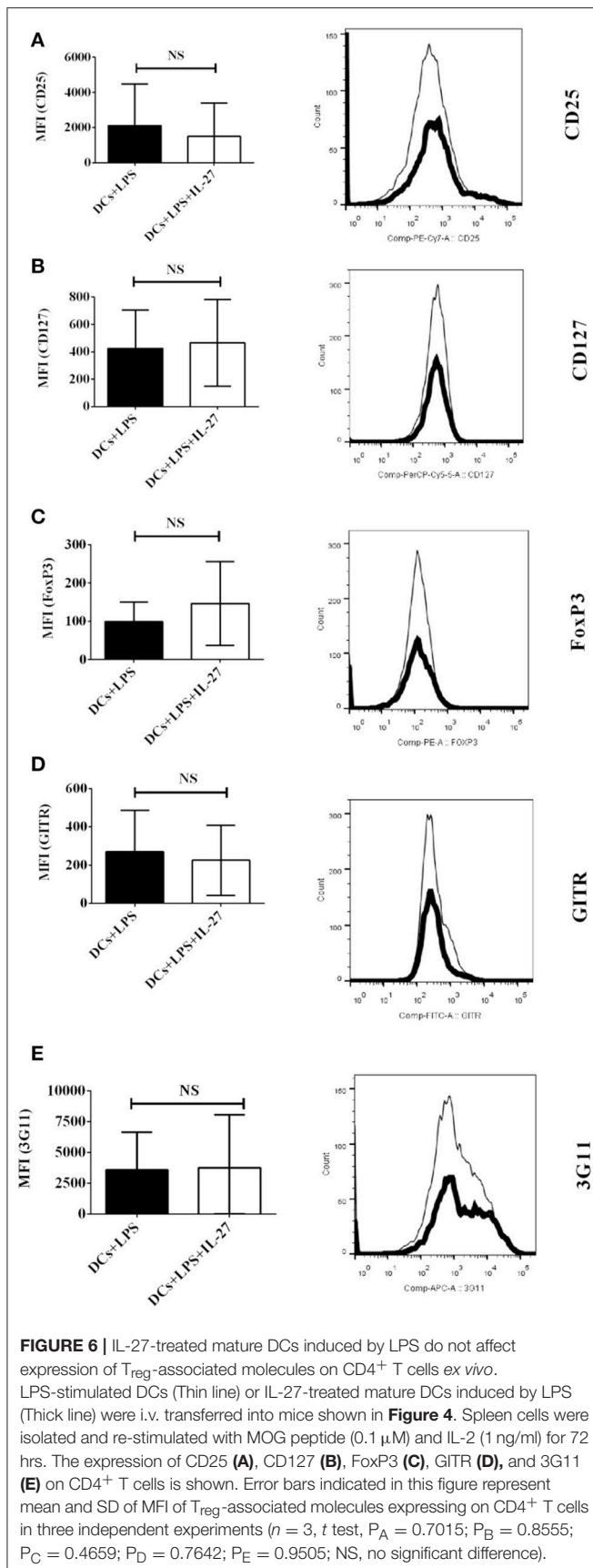


IL-27-Treated Mature DCs do Not Affect Expression of T_{reg}-Associated Molecules on CD4⁺ T Cells *ex vivo*

We have testified that IL-27-treated mature DCs do not affect expression of T_{reg}-associated molecules on CD4⁺ T cells *in vitro* (Figure 2), however, it is still unclear that whether or not IL-27-treated mature DCs can modulate expression of T_{reg}-associated



molecules on CD4⁺ T cells *in vivo*. To determine whether IL-27-treated mature DCs can modulate expression of T_{reg}-associated molecules on CD4⁺ T cells *in vivo*, IL-27-treated mature DCs induced by LPS or mature DCs without IL-27 treatment were i.v. transferred into EAE mice shown in Figure 4 at day 11, 14



and 17 after immunization. Protein expression of CD25, CD127, FoxP3, GITR, and 3G11 on CD4⁺ T cells was tested using flow cytometry. The experimental data show that i.v. transfer of IL-27-treated mature DCs or mature DCs without IL-27 treatment failed to modulate expression of T_{reg}-associated molecules on CD4⁺ T cells (**Figures 6A–E**). Our results suggest that IL-27-treated mature DCs do not affect development of T_{regs} through regulating expression of T_{reg}-associated molecules on CD4⁺ T cells *in vivo*.

IL-27-Treated Mature DCs Facilitate Development of CD4⁺ CD127⁺3G11⁺ T_{reg} Subset *ex vivo*

Our results of *in vitro* assay demonstrated that IL-27-treated mature DCs elicit development of CD4⁺ CD127⁺3G11⁺ T_{reg} subset (**Figure 3**), however, it is unknown whether or not IL-27-treated mature DCs also can facilitate development of CD4⁺ CD127⁺3G11⁺ T_{reg} subset *in vivo*. To detect whether or not IL-27-treated mature or immature DCs can regulate development of T_{reg} sub-populations *in vivo*, LPS-induced mature DCs and immature DCs without LPS stimulation were pulsed with MOG peptide and incubated with or without IL-27 treatment. IL-27-treated immature/mature DCs or immature/mature DCs without incubation with IL-27 were i.v. transferred into EAE mice shown in **Figure 4** at day 11, 14 and 17 after immunization. Lymphocytes were isolated from mice that had been i.v. transferred with IL-27-treated immature/mature DCs or immature/mature DCs without IL-27 treatment shown in **Figure 4** at day 25. Our results indicate that immature DCs incubated with or without IL-27 treatment did not modulate development of CD4⁺3G11⁺CD127⁺ T_{reg} subset but that mature DCs treated with IL-27 facilitated development of CD4⁺ CD127⁺3G11⁺ T_{reg} sub-populations *ex vivo* (**Figure 7**). Our data suggest that IL-27-treated mature DCs may block autoimmunity (**Figure 4**) through upregulation of CD4⁺CD127⁺3G11⁺ T_{regs} development *in vivo*.

DISCUSSION

IL-27 is a novel cytokine whose immune function has not yet been fully elucidated. Chiyo et al. reported that tumor cells expressing IL-27 activate CD4⁺ T helper cells, CD8⁺ cytotoxic T lymphocytes and natural killer cells (21, 22). IL-27 shows an effect of anti-tumor immunity as a possible therapeutic target for cancer (21). IL-27 plays an important role in T cell differentiation and regulation of T cell-mediated immune responses. For example, IL-27 produced by dendritic cells facilitates the polarization of T helper 1 cells in Lewis rats (23). Harker et al. recently found that IL-27-mediated signaling is necessary for anti-viral immunity (24). Moreover, IL-27 promotes differentiation of T helper 17 cells *in vivo* (25). Our data also indicate that IL-27 inhibits LPS-induced mature DC-mediated immune tolerance. These data suggest that IL-27

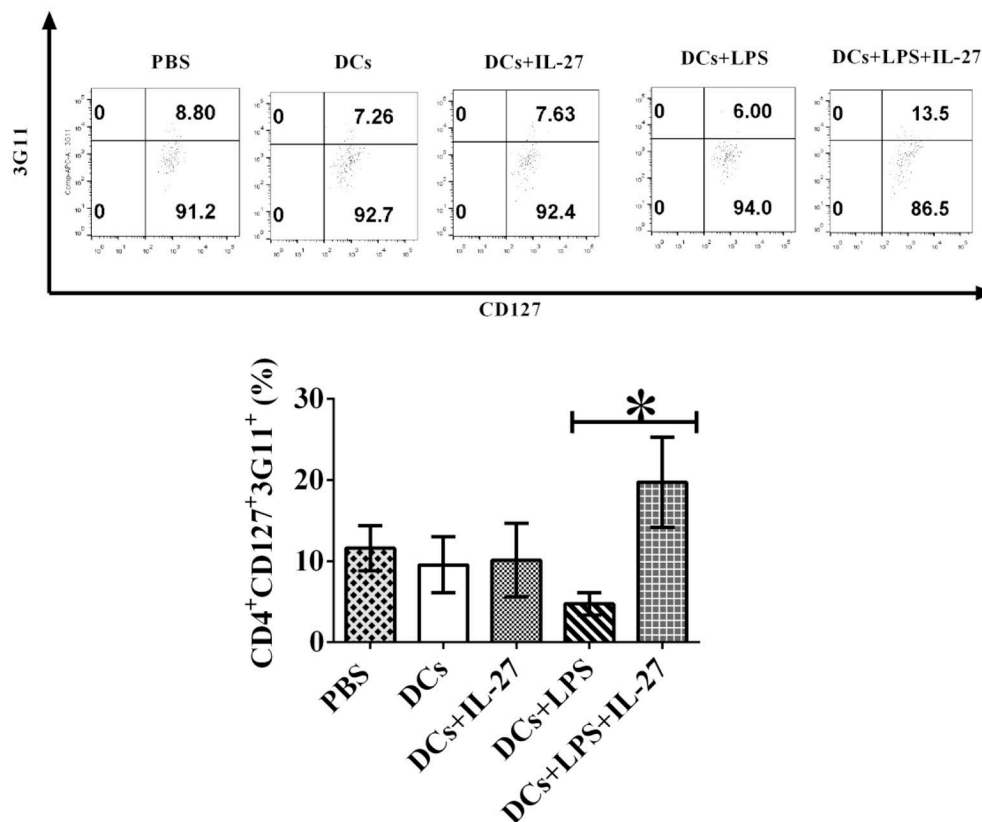


FIGURE 7 | IL-27 facilitates LPS-stimulated mature DC-mediated development of $CD4^+CD127^+3G11^+$ T_{reg} subset *ex vivo*. Bone marrow-derived DCs were pulsed with MOG peptide and treated with IL-27 (20 ng/ml, 72 h) (DCs+IL-27) or LPS (DCs+LPS) (1 μ g/ml, 24 h) or both LPS and IL-27 (DCs+LPS+IL-27). These DCs were then i.v. transferred into mice with EAE shown in **Figure 4**. Mice treated with PBS are control. Spleen cells were isolated and re-stimulated with MOG peptide (0.1 μ M) and IL-2 (1 ng/ml) for 72 h. Cells were collected and $CD4^+CD25^+FoxP3^+GITR^+$ T_{regs} were gated. The frequency of $CD127^+3G11^+$ cells is demonstrated. T lymphocytes incubated with isotype control antibodies are isotype control. Error bars indicated in this figure represent mean and SD of frequency of $CD4^+CD127^+3G11^+$ cells in three independent experiments [$n = 3$, t test, $P_{(DC,DC+IL-27)} = 0.8682$; $P_{(DC+LPS,DC+LPS+IL-27)} = 0.0105$].

is a pro-inflammatory cytokine and a positive regulator in T cell-mediated immune responses.

Interestingly, the experimental data also indicate that IL-27 is an anti-inflammatory cytokine and inhibits development of autoimmunity *in vivo*. For instance, Mascanfroni et al. reported that IL-27 induces expression of CD39 on DCs and blocks development of T helper 1 and 17 cells to inhibit EAE induction (26). Tsumarkidou et al. also found that tolerogenic $CD1c^+$ DCs regulate development of T_{regs} via IL-27/IL-10 inducible co-stimulatory ligands (27). Rostami et al. have published data showing that the induction of peripheral tolerance is dependent on IL-27-mediated signal transduction pathway in DCs (28). These data suggest that regulatory mechanisms of IL-27 in the immune system are extremely complex and that IL-27 may play a dual role in equilibrium between autoimmunity and immune tolerance. Our results demonstrate that IL-27 does not affect immature DC-mediated immune responses but that it facilitates mature DC-mediated $CD4^+CD127^+3G11^+$ T_{reg} development. This suggests that the immune function of IL-27 on DCs is dependent on their maturation.

The cellular and molecular regulatory mechanisms of IL-27 have been recently investigated. For example, it is known that IL-27 can modulate development and biological function of T helper 17 cells, dendritic cells, NK cells and neutrophils (22, 25, 29, 30). IL-27 produced by DCs is necessary for trafficking of T_{regs} to locate in tumor (31), and pulmonary $CD1c^+$ DC-mediated development of T_{regs} is dependent on IL-27/IL-10/inducible costimulator ligand (27). IL-27 also facilitates development of T_{reg} and induces immune tolerance *in vivo* (32). By contrast, our data indicate that IL-27-treated mature DCs elicit development of $CD4^+CD127^+3G11^+$ T_{reg} subset and inhibits mature DC-mediated immune tolerance *in vivo* (**Figure 7**).

The immunological significance of this study is that we find a new subset of $CD4^+$ T_{regs} mediated by 3G11 and CD127. Biological function of $CD4^+CD127^+3G11^+$ T_{regs} may be different from that of conventional $CD4^+$ T_{regs} . Previous studies showed that $CD4^+$ T_{regs} is a negative regulator of autoimmunity. By contrast, the frequency of $CD4^+CD127^+3G11^+$ T_{regs} increases in mice in which LPS-stimulated DC-mediated immune tolerance is inhibited. This new sub-population of T_{regs}

may be a positive regulator which facilitates T cell-mediated immune responses *in vivo*. This has never been reported.

IL-27 can act as both pro-and anti-inflammatory cytokine, however, molecular mechanisms of IL-27 to modulate autoimmunity and immune tolerance have not yet been fully elucidated. Our data indicated that IL-27 does not affect immature DC-mediated immune responses, however, IL-27 can block immune tolerance induced by LPS-stimulated mature DCs. IL-27 acts as a pro-inflammatory cytokine to inhibit immune function of LPS-treated mature DCs. Interestingly, IL-27 only elicits development of CD4⁺CD127⁺3G11⁺ T_{regs} mediated by mature DCs induced by LPS. IL-27 does not affect that of T_{reg} subset mediated by immature DCs. It may be dependent on maturation of DCs whether IL-27 plays a role of pro- or anti-inflammatory cytokine *in vivo*.

The interesting question is how IL-27 regulates LPS-stimulated mature DC-mediated immune tolerance *in vivo*. There is little amount of data to reveal it. The molecular mechanisms of IL-27 to block immune tolerance mediated by LPS-treated mature DCs should be investigated in the future so that a new immune therapy using CD4⁺CD127⁺3G11⁺ T_{reg} sub-population can be designed to treat human diseases.

In summary, Our results imply that CD4⁺CD127⁺3G11⁺ cells may be a type of positive T_{regs} which are different from

conventional CD4⁺ T_{regs} which inhibit autoimmunity *in vivo*. This new subset of T_{regs} are CD127 positive cells and conventional CD4⁺ T_{regs} express CD127 with low level, although both of them are CD4⁺CD25⁺FoxP3⁺GITR⁺ cells. Biological function of CD4⁺CD127⁺3G11⁺ T_{regs} may be different from that of conventional CD4⁺CD25⁺CD127^{low}FoxP3⁺GITR⁺ T_{regs}. Immune functions of this new CD4⁺CD127⁺3G11⁺ T_{reg} subset should be investigated in future studies.

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

AUTHOR CONTRIBUTIONS

FZ designed and conducted experiments for this research project. G-XZ and AR supervised the research and reviewed the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Third-Party Allogeneic Mesenchymal Stromal Cells Prevent Rejection in a Pre-sensitized High-Risk Model of Corneal Transplantation

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High-risk cornea transplant recipients represent a patient population with significant un-met medical need for more effective therapies to prevent immunological graft rejection due to heightened anti-donor immune response. In this study, a rat model of pre-existing anti-donor immunity was developed in which corneal allografts were rejected earlier than in non-pre-sensitized recipients. In this model, third-party (non-donor, non-recipient strain) allogeneic mesenchymal stromal cells (allo-MSC) were administered intravenously 7 and 1 days prior to transplantation. Rejection-free graft survival to 30 days post-transplant improved from 0 to 63.6% in MSC-treated compared to vehicle-treated control animals ($p = <0.0001$). Pre-sensitized animals that received third-party allo-MSC prior to transplantation had significantly higher proportions of CD45⁺CD11b⁺ B220⁺ monocytes in the lungs 24 h after the second MSC injection and significantly higher proportions of CD4⁺ FoxP3⁺ regulatory T cells in the graft-draining lymph nodes at the average day of rejection of control animals. In *in vitro* experiments, third-party allo-MSC polarized primary lung-derived CD11b/c⁺ myeloid cells to a more anti-inflammatory phenotype, as determined by cytokine profile and conferred them with the capacity to suppress T cell activation via prostaglandin E₂ and TGFβ1. In experiments designed to further validate the clinical potential of the protocol, thawed cryopreserved, third-party allo-MSC were shown to be similarly potent at prolonging rejection-free corneal allograft survival as their freshly-cultured counterparts in the pre-sensitized high-risk model. Furthermore, thawed cryopreserved third-party allo-MSC could be co-administered with mycophenolate mofetil without adversely affecting their immunomodulatory function. In conclusion, a clinically-relevant protocol consisting of two intravenous infusions of third-party allo-MSC during the week prior to transplantation, exerts a potent anti-rejection

effect in a pre-sensitized rat model of high-risk corneal allo-transplantation. This immune regulatory effect is likely to be mediated in the immediate post-transplant period through the promotion, by allo-MSCs, of alternatively-activated macrophages in the lung and, later, by enhanced regulatory T-cell numbers.

Keywords: mesenchymal stromal cells, cornea transplantation, high-risk, pre-sensitization, regulatory T cells, immunomodulation, third-party

INTRODUCTION

High immunological risk cornea transplant recipients have a much higher rate of rejection and lower long-term graft survival than conventional cornea transplant recipients (1). Factors that place patients at high-risk of rejection include corneal ulcers, bullous keratopathy or a failed previous graft (1, 2). These conditions frequently lead to increased blood and lymphatic vessel infiltration to the recipient graft bed and/or some pre-existing donor-specific cellular immune response (3). As outcomes for lower-risk cornea transplant recipients, such as those receiving a first graft for keratoconus and other non-inflammatory conditions, have been optimized through technical developments, the immunological focus for cornea transplant-related research is shifting toward improving the therapeutic options and outcomes for high-risk patients (3). Pre-clinical high-risk cornea transplantation models have mainly involved the formation of a pre-vascularised graft bed, either by placing sutures into the recipient cornea 7–14 days prior to transplantation (4–6) or causing a chemical injury to the cornea prior to transplantation (7). These models result in ingress of blood and lymphatic vessels to the recipient graft bed which removes the immune privileged nature of the anterior chamber of the eye. This permits free access and egress of immune cells to the cornea, leading to accelerated graft rejection (4–7). Another high-risk transplant scenario arises when the allograft recipient has pre-existing anti-donor immunity—a situation that is likely to arise in the setting of re-transplantation following immune-mediated failure of one or more prior grafts. Indeed, a pre-existing immunity to a single antigen mismatch has been shown to significantly accelerate graft rejection in a mouse model (8).

Cornea transplantation has been recognized as a target for mesenchymal stromal cell (MSC) therapy for several years. A number of research groups, including our own, have demonstrated the therapeutic potential and mechanism of action of systemically-administered MSC in models of corneal allo-transplantation (9–14). It is widely accepted that MSC possess potent anti-inflammatory capabilities and modulate the activity of a range of immune effector cells including T cells, B cells, dendritic cells and macrophages (11, 14–16). This modulation occurs both through cell-cell contact and through the paracrine release of soluble factors such as nitric oxide (NO), prostaglandin E₂ (PGE₂), tumor necrosis factor-inducible gene 6 protein (TSG-6), indoleamine 2,3-dioxygenase (IDO) and several others (17–19). However, a number of critical questions remain to be answered in regard to the use of MSC in cornea transplantation; most notably whether autologous or allogeneic

MSC represent the optimal cell source. In a conventional risk rat model, we have previously shown that autologous MSC are ineffective while allogeneic, either donor-derived or third-party, are highly effective at prolonging corneal allograft survival when administered prior to transplantation (11). In contrast, Omoto et al. showed, in a fully MHC-mismatched mouse model, that autologous MSC administered post transplantation significantly prolonging corneal transplant survival (12). MSC can be sourced from different tissues including bone marrow and adipose tissue. In the setting of corneal transplantation, tissue source of MSC may also have an impact on efficacy. A number of studies have demonstrated reduced rejection with administration of bone marrow-derived MSC (11, 12). In contrast, in a high-risk (pre-vascularized) rabbit model, Fuentes-Julien et al. reported lack of a beneficial effect of adipose-derived MSC injected intravenously at 4 separate time-points (20). Despite this result, the impact of dosing strategy, cell source, and allogenicity could all play significant roles, and further investigation of MSC efficacy in high-risk cornea transplantation is needed.

Another relevant concern with regard to the clinical translation of MSC immunomodulatory therapy for high-risk corneal transplantation is the co-administration of cells with immunosuppressive drugs. This issue has been studied to some extent in animal models of solid organ transplantation. For example, in a heart transplant model, Eggenhofer et al. showed that MSC and mycophenolate mofetil (MMF) had a synergistic effect when administered in combination which resulted in increased graft survival (21). Cell therapy approaches such as MSC also have other significant logistical challenges when being administered in a clinical setting. These include how the cells will be transported and stored and whether they require reconstitution at the bedside. For this reason, an “off the shelf” allogeneic cell product manufactured from healthy donors then delivered frozen and administered directly after thawing with no requirement for cell culture facilities at the clinical site is often considered to be optimal for the widespread clinical application of MSC and other cell therapies (22).

In this study, we aimed to determine whether intravenously delivered third-party MSC (fully allogeneic to both donor and recipient) were capable of prolonging rejection-free survival of corneal allografts in the setting of pre-existing anti-donor immunity. We also sought to investigate the potential *in vivo* immunomodulatory mechanisms of third-party allo-MSCs in high-risk corneal transplant recipients and the feasibility of using a cryopreserved cell preparation in combination with the commonly prescribed immunosuppressant drug MMF.

MATERIALS AND METHODS

Cornea Transplantation

Male Lewis (RT-1^l) and Dark Agouti (DA; RT-1^{av1}) rats aged 8–14 weeks were purchased from Envigo (Huntingdon, UK) and housed in a fully-accredited bio-resource. All procedures were approved by the NUI Galway Animal Care Research Ethics Committee and authorized by the Health Product Regulatory Authority (HPRA) of Ireland. Orthotopic corneal transplantation was performed on Lewis rats using DA donor corneas as reported previously (23). Corneal opacity was the primary indicator of graft rejection and was evaluated three times per week based on the following scale: 0-completely transparent cornea; 0.5-slight corneal opacity, iris structure easily visible; 1.0-low corneal opacity with visible iris details; 1.5-moderate corneal opacity, iris vessels still visible; 2.0-moderate opacity, only some iris details visible; 2.5-high corneal opacity, only pupil margin visible; 3.0-complete corneal opacity, anterior chamber not visible. Grafts were considered rejected if they reached an opacity score of ≥ 2.5 on two consecutive observations or a score of 3.0 on one occasion. Neo-vascularisation was assessed based on the number of quadrants of the donor cornea in which vessels were present. Corneal edema was quantified as central corneal thickness using a pachymeter (Micro Medical Devices, Calabasas, CA, USA) based on the following scale: 0-0-200 μm ; 1-200-300 μm ; 2-300-400 μm ; 3-400 μm +. Animals with surgical complications were excluded.

Pre-sensitisation

For donor-specific sensitization, splenocytes were isolated from healthy 6–12 weeks old male DA rats. Briefly, the spleen was isolated using aseptic technique post-mortem and stored in sterile phosphate buffered saline (PBS). Under a laminar flow hood, a single cell suspension was obtained by mashing the spleen through a 40 μm cell strainer (Fisher-Scientific, Wexford, Ireland). Red blood cells were lysed using ACK buffer for 5 min at room temperature. Splenocytes were washed and counted then re-suspended at a concentration of 20×10^6 cells/ml in sterile PBS. Lewis rats were injected subcutaneously with 10×10^6 DA splenocytes in 0.5 ml of sterile PBS 14 days prior to cornea transplantation.

MSC Culture, Characterization, and Administration

Wistar Furth (WF) rat MSC were isolated from the bone marrow of the femurs and tibiae of 6–10 week old male WF rats. Briefly, the rats were euthanised humanely and the bone of the legs dissected away under sterile conditions. The legs were transferred to a Biological Safety Cabinet and the bone marrow was flushed from the bones, red blood cells were lysed and the mononuclear cells were counted. Cells were seeded in tissue culture flasks at a density of 9×10^5 cells per cm^2 and cultured under standard culture conditions (24). MSC characterization was performed for standard surface markers by flow cytometry. (Supplementary Figure 1). For *in vivo* administration, MSC were trypsinised and counted then suspended at 1×10^6 cells/ml in sterile PBS. For preparation of cryopreserved MSC, the cells

were cultured to passage 2 (P2) then were lifted by trypsinization, washed, re-suspended at 1×10^6 cells/ml in human serum albumin with 10% DMSO and cooled slowly to -80°C for 24 h before being transferred to liquid nitrogen. Aliquots of cryopreserved MSC were thawed rapidly in a 37°C water bath immediately prior to administration. Based on our previous data (11), freshly cultured or thawed, cryopreserved MSC were injected intra-venously through a tail vein at 7 days and 1 day prior to cornea transplantation. In the case of cultured MSC, cells were trypsinised and resuspended in PBS at a concentration of 1×10^6 cells per ml. In the case of cryopreserved MSC, controlled animals were administered an equal volume of 10% DMSO in human serum albumin, while for cultured MSC treated animals, control animals received an equal volume of PBS. 1×10^6 MSC were administered through the lateral tail vein using a 25 gauge needle slowly over 1 min.

Mycophenolate Mofetil Preparation and Administration

Mycophenolate mofetil (MMF; CellCept[®], Roche Pharmaceuticals, Basel, Switzerland) was administered at a dose of 20 mg/kg daily from 1 day prior to 6 days post-transplant (Figure 7A) (21). MMF was resuspended in 5% glucose solution at a concentration of 20 mg/ml. Animals were weighed prior to injection and an appropriate volume of MMF was administered intra-peritoneally using an insulin syringe. Vehicle-treated animals were administered the corresponding volume of 5% glucose solution only.

Allo-Antibody Assay

A total of 600 μl of blood was drawn from a tail vein at day 0 from pre-sensitized and naïve animals. The blood was layered onto Ficoll Paque (GE Healthcare Life Sciences, Buckinghamshire, UK) and centrifuged at 400 g for 20 min. Plasma was isolated by carefully removing the upper layer from the tube. Aliquots of 50 μl of plasma were incubated with 1×10^6 donor derived (DA) splenocytes for 2 h at 4°C . The splenocytes were washed and incubated with FITC-conjugated anti-rat IgG2 antibodies for 40 min at 4°C . Mean fluorescence intensity was analyzed on a FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer (25).

Corneal Histology and Infiltrating Cell Quantification

Whole eyes were removed by dissection immediately after euthanasia. Eyes were fixed in 10% formalin and embedded in paraffin wax using an automatic tissue processor (Leica ASP300, Leica, Wetzlar, Germany). Sections of 5 μm thickness were cut on a rotary microtome (Leica) and fixed to glass slides. Hematoxylin and eosin staining was performed as previously described (11). Sections were imaged at 10X magnification and images taken using an Olympus BX61 microscope (Olympus, Tokyo, Japan). Infiltrating cells were quantified by counting the number of distinct cells visible in the corneal stroma of 3 separate 10X fields of view per animal.

Immune Cell Profiling

Animals were euthanized at day 0 and day 10 for immune cell profiling experiments. Spleen, lungs and draining lymph nodes were dissected immediately after euthanasia. Single cell suspensions were prepared from spleens and lymph nodes by mashing the tissue through a 40 μ m cell strainer with PBS. Single cell suspensions were obtained from lungs by first cutting the lungs into small (~ 2 mm²) pieces and digesting for 2 h at 37°C in 100 U/ μ l Collagenase IV (Sigma-Aldrich) and 200 U/ μ l DNase I (Bioline, London, UK) followed by mashing through a 40 μ m cell strainer. Single cell suspensions were stained with fluorochrome-conjugated antibodies and data were acquired on a FACSCanto II (BD Biosciences) flow cytometer. Data were analyzed using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR, USA).

Co-culture Experiments

CD11b/c⁺ myeloid cells were enriched from lung-derived cell suspensions by magnetic column separation using anti-rat CD11b/c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommended protocol. Isolated CD11b/c⁺ cells were co-cultured at a ratio of 1:1 with MSC in a 96-well round-bottomed plate for 48 h in a humidified incubator at 37°C and 5% CO₂. Supernatants were saved and CD11b/c⁺ cells were re-enriched from the mixture by the same magnetic column separation procedure.

Whole lymph nodes cells were prepared from the subcutaneous lymph nodes of healthy Lewis rats between 5 and 12 weeks of age. The lymph nodes cells were washed with PBS and stained with CellTrace Violet (Fisher Scientific Ltd., Dublin, Ireland) according to the manufacturer's recommended protocol. Aliquots of 1×10^5 CellTrace Violet-labeled lymph node cells were added to the wells of 96-well round-bottomed tissue culture plates with or without anti-rat CD3/CD28 beads at 1:1 ratio in T cell medium (RPMI 1,640 supplemented with 10% fetal calf serum, 50 μ M β -mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mmol/l sodium pyruvate and 2 mmol/l L-Glutamine). In various experiments, MSC were added at T cell:MSC ratio of 50:1 or CD11b/c⁺ cells were added at 2:1 T cell:myeloid cell ratio for 4 days prior to analysis by flow cytometry.

For flow cytometry analysis, T cells were stained with fluorochrome conjugated antibodies for surface expression of CD4, CD8, and CD25. Intracellular staining for FoxP3 was performed according to the recommended protocol using the FoxP3 staining buffer kit (Fisher Scientific Ltd.). Briefly, cells were first stained for surface expression of CD4, CD8, and CD25 and washed in FACS buffer (PBS, 2% FBS and 0.05% Sodium Azide). Cells were then resuspended in 200 μ l Fixation/Permeabilisation buffer and incubated at 4°C overnight. The next morning, the cells were washed twice with permeabilisation buffer and stained with anti-FoxP3-PE-Cy7 (eBioscience) in permeabilisation buffer for 45 min at 4°C. Finally, cells were washed once more in permeabilisation buffer, re-suspended in FACS buffer and analyzed for surface marker expression, proliferation and FoxP3 expression on a FACSCanto II flow cytometer. Proliferation was analyzed using FlowJo

software by identifying each generation on the histogram and comparing the proliferation of MSC or myeloid-containing wells with stimulated or unstimulated lymph node cells.

Assays for Nitric Oxide (NO), Prostaglandin E₂ (PGE₂), and Transforming Growth Factor β (TGF β)

PGE₂ and TGF β 1 ELISA kits were purchased from R&D Systems, Abingdon, UK. Analyte levels were detected using these kits according to manufacturer's instructions. The presence of nitric oxide (NO) in culture supernatants was analyzed by Griess assay. Aliquots of 100 μ l of supernatant were added to 96-well flat-bottom optical plates and an equal volume of Griess reagent (1% sulphanilamide and 0.1% N-1-(naphthyl)-ethylenediamine-diHCl in 2.5% H₃PO₄) was added to each well. Absorbance was measured immediately at 540 nm on a plate reader (Perkin Elmer, Dublin, Ireland). NO concentration was calculated using a sodium nitrite standard curve (24).

Statistical Analysis

All data were analyzed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA, USA) and were expressed as mean \pm SEM. Log-rank (Mantel-Cox) or one-way ANOVAs with Tukey's post-test were used to determine statistical differences among groups as appropriate. For all experiments, statistical significance was assigned at $p < 0.05$.

RESULTS

Generation of Pre-sensitized Cornea Transplant Model

Pre-sensitization of Lewis rats to DA allo-antigens was confirmed by detection of anti-DA IgG2 antibodies in serum samples from rats that had received subcutaneous inocula of DA splenocytes 14 days previously but not in sera from naïve Lewis rats (Figures 1A,B) (11, 24). Subsequently, in DA-to-Lewis corneal allotransplants, pre-sensitized graft recipients were shown to have accelerated rate of rejection compared to naïve recipients (Figure 1C). In this experiment, average day of rejection (ADR) for pre-sensitized recipients was 11.46 compared to 19.25 for naïve recipients. It was concluded that the inoculation of recipients with corneal donor strain splenocytes 14 days prior to transplantation provided a robust, high immunological risk model in which to investigate the effects of third-party allo-MSC administration.

Intravenous Administration of Third-Party allo-MSC Increases Rejection-Free Survival of High Immunological Risk Corneal Transplants

We have previously shown that a protocol consisting of two pre-transplant intravenous injections of third-party allo-MSC is similarly efficient to corneal donor-specific allo-MSC at modulating graft rejection in a conventional risk rat corneal transplant model (11). Using the same injection strategy (Figure 2A), it was observed that the rejection rate of corneal

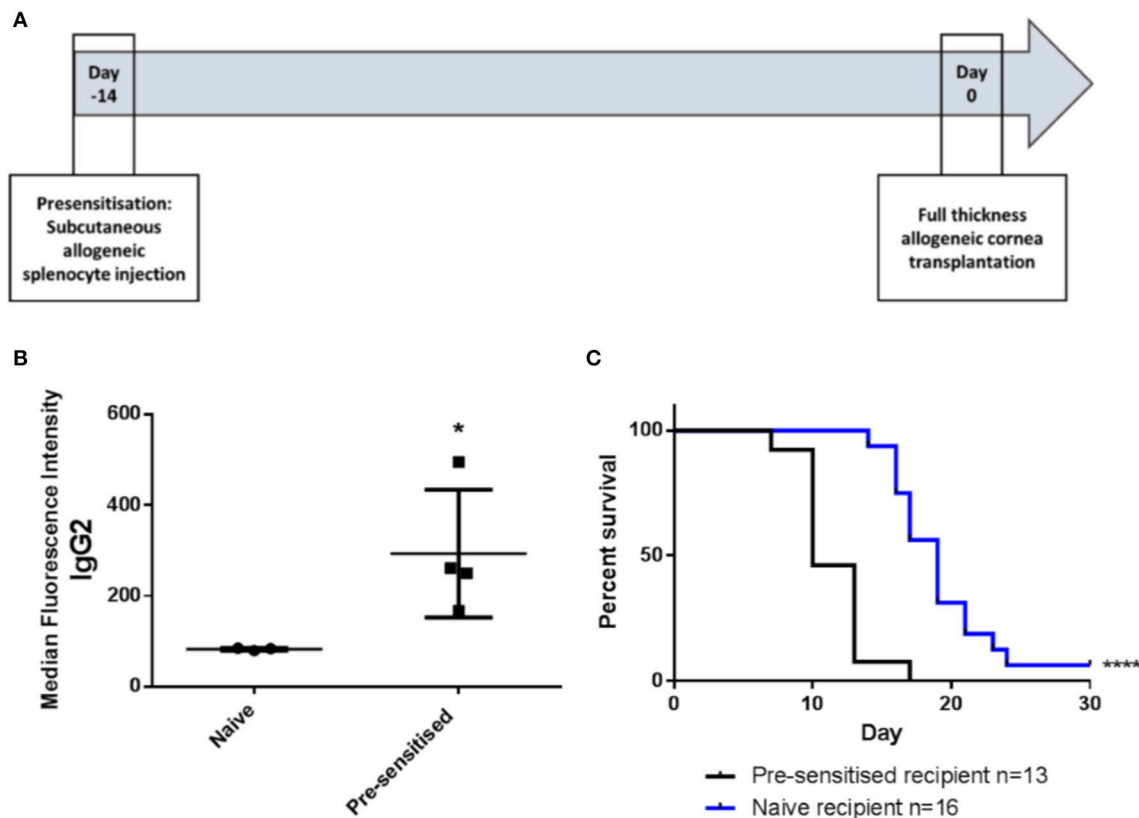


FIGURE 1 | DA-splenocyte pre-sensitized Lewis rats develop anti-DA IgG antibodies and reject corneal allografts earlier than non-pre-sensitized Lewis rats. **(A)** Lewis rats were pre-sensitized with 10×10^6 DA rat (donor) splenocytes 14 days prior to cornea transplantation. **(B)** Graph showing results of flow cytometry analysis of DA rat splenocytes incubated with serum samples taken at the time of transplantation from non-pre-sensitized (naïve; $n = 3$) and pre-sensitized ($n = 4$) Lewis rats followed by staining with anti-rat-IgG2-FITC. Individual results (symbols) and mean \pm SEM for the two groups are shown and expressed as median fluorescence intensity on the FL1 channel. **(C)** Kaplan-Meier plots depicting DA to Lewis rat corneal allograft rejection-free survival in naïve ($n = 16$; blue line) and pre-sensitized ($n = 13$; black line) animals. Statistical significance was determined using a one tailed Student's *t*-test **(B)** or Log-rank (Mantel-Cox) test **(C)** as appropriate * $p < 0.05$, **** $p < 0.0001$.

transplants in pre-sensitized recipients was strongly reduced by intravenous injections of third-party allo-MSC at day-7 and day-1 (**Figure 2B**). In this experiment, the 30-day rejection-free survival of MSC-treated animals was 63.6% compared to 0% in the untreated group. In keeping with a potent immunomodulatory effect, the trends for mean graft opacity and neovascularisation scores were lower for MSC-treated animals throughout the 30-day post-transplant observation period (**Figures 2C,D**). At day 10, which approximates the ADR for untreated animals in this model, the mean opacity score of MSC-treated animals was 2.05 ± 0.11 compared to 2.40 ± 0.15 for the controls (**Figure 2E**). At this same, key time-point, mean neovascularisation scores for the MSC- and control-treated animals were 0.09 ± 0.09 and 1.53 ± 0.39 (**Figure 2F**) and the mean numbers of infiltrating cells, as determined by analysis of H&E stained tissue sections, were 130.33 ± 61.72 and 382.33 ± 39.7 respectively (**Figures 2G,H**). Thus, in the pre-sensitized, high immunological risk rat corneal allograft model, administration of third-party allo-MSC exerted a potent protective effect against acute rejection that was comparable to the previously-reported effect in a conventional risk model.

MSC Administration Results in Localized, Proportionate Increases in Regulatory Immune Cells

In order to study the effects of third-party allo-MSC therapy on the dynamics of potential regulatory immune cell populations, samples of various organs from three groups of Lewis rats (naïve, pre-sensitized untreated and pre-sensitized allo-MSC-treated) were analyzed at two time-points by multi-color flow cytometry. On the prospective day of transplantation (day 0), cell suspensions from lungs, spleens and draining (submandibular) lymph nodes were assessed for immune cell subtypes by multi-color flow cytometry (**Figures 3A,B**). In pre-sensitized, allo-MSC-treated animals, higher mean proportions of lung $CD45^+$ cells were double-positive for CD11b and B220 (13.5%) in comparison to naïve (7.2%) and untreated pre-sensitized (7.6%) animals (**Figure 3C**). No differences in the proportions of $CD11b^+ B220^+$ cells were observed among the total $CD45^+$ populations of the spleens and draining lymph nodes from the 3 groups at this time-point (**Figures 3D,E**). Of note, $CD45^+ CD11b^+ B220^+$ cells have been shown to be increased in the

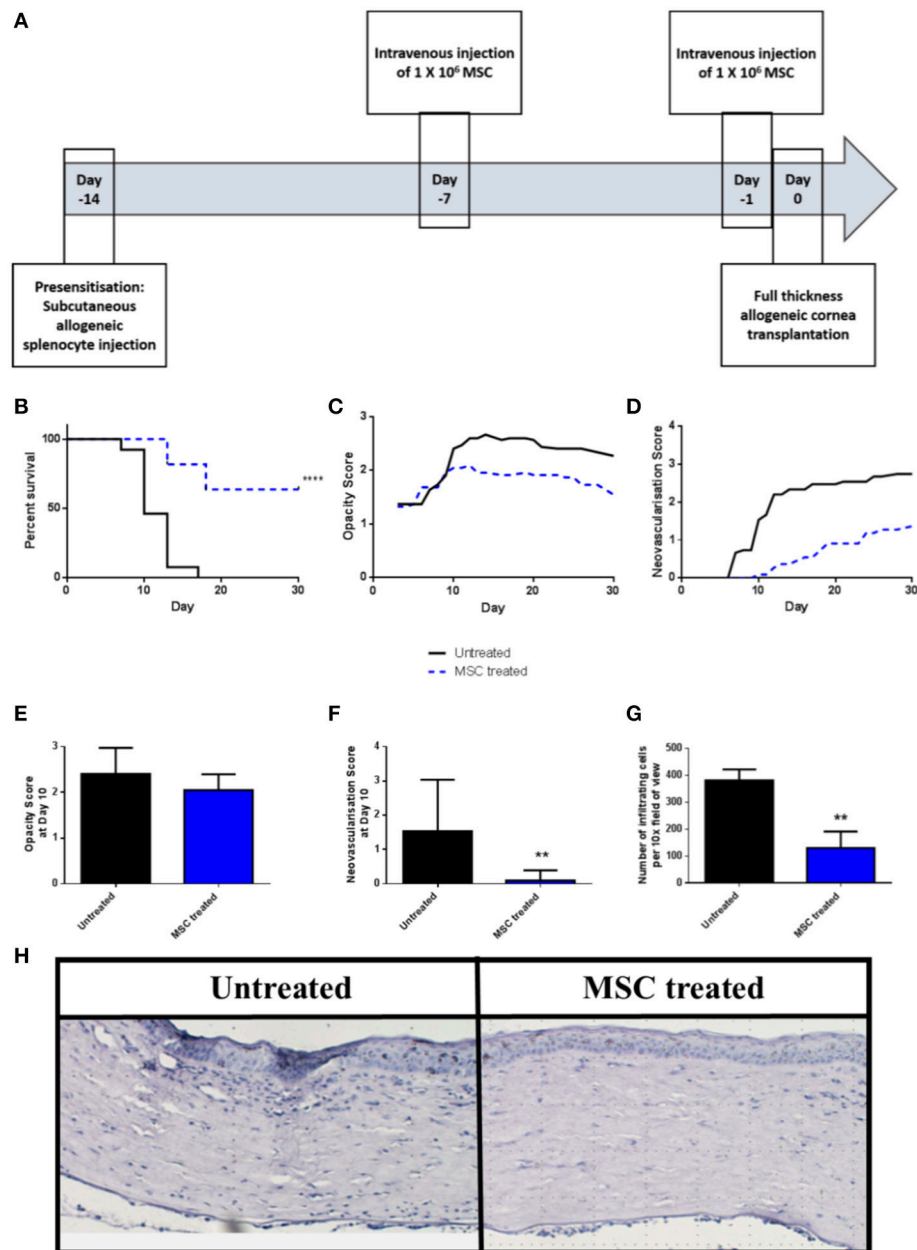


FIGURE 2 | Third-party allo-MSC are capable of prolonging rejection-free corneal allograft survival in pre-sensitized recipients. **(A)** Schematic representation of administration of third-party allo-MSC in the pre-sensitized high-risk rat corneal transplant model. **(B)** Kaplan-Meier curves demonstrating rejection-free survival of pre-sensitized Lewis recipients of DA corneas that received third-party allo-MSC injections (MSC-treated, $n = 13$; blue dashed line) or vehicle injections (Untreated, $n = 11$; black line) up to 30 days post-transplant. **(C,D)** Trend-lines for corneal allograft opacity scores **(C)** and neovascularisation scores of the two groups of pre-sensitized corneal transplant recipients up to day 30 post-transplant. **(E–G)** Corneal allograft opacity scores **(E)**, neovascularisation scores **(F)** and number of corneal infiltrating cells per 10X field **(G)** on day 10 post-transplant for untreated (black bar) and MSC treated (blue bar) groups. All data are presented as mean \pm SEM. **(H)** Representative examples of H&E stained sections of corneal allografts of untreated and MSC-treated pre-sensitized Lewis rats at day 10 post-transplant. Statistical significance was determined using a Log-rank (Mantel-Cox) test or one-tailed Student's t -test or as appropriate $**p < 0.01$, $****p < 0.0001$.

lungs of mice following administration of human MSC and to be sufficient for prolongation corneal allograft survival (13). No significant changes in proportions of T cell populations were observed in any of the organs examined at this time-point (data not shown). It should be noted that MSC have also previously been reported to migrate to other tissues, including spleen, lymph

nodes, thymus and others and that these cells could exert effects in cells in these organs or in the circulation which were not detected here.

The same immunological profiling was then carried out on tissues from groups of pre-sensitized animals that had been transplanted 10 days previously and treated either with

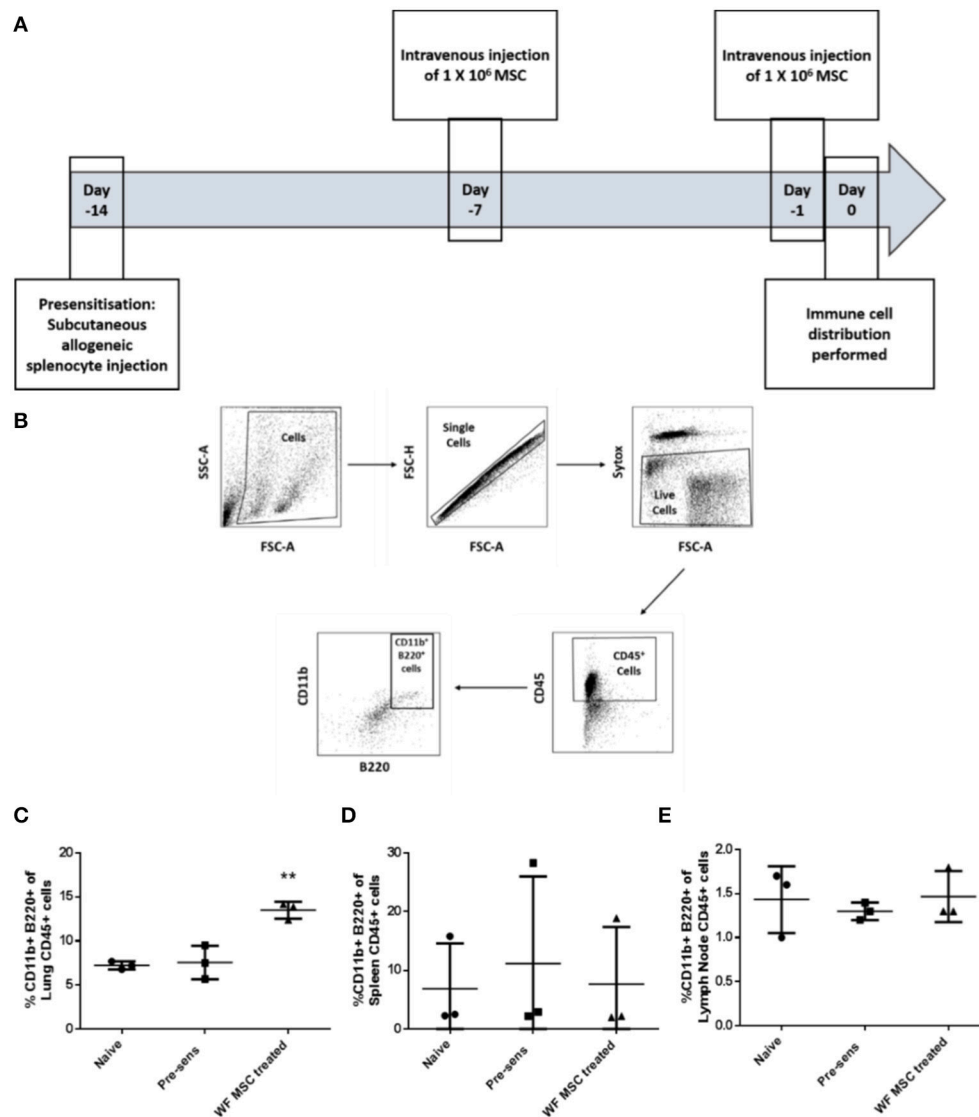


FIGURE 3 | Third-party allo-MSC administration to pre-sensitized Lewis rats results in a proportionate increase in lung CD11b⁺/B220⁺ cells on the day of transplantation. **(A)** Schematic representation of experiment to evaluate immune cell profiles in pre-sensitized Lewis rats treated with third-party allo-MSC 1 and 7 days earlier. **(B)** Gating strategy used to gate CD11b⁺ B220⁺ cells from tissues of transplanted rats **(C–E)** Graphs showing results of flow cytometry analysis of the proportion of CD45⁺ cells co-expressing CD11b and B220 in the lungs **(C)**, spleens **(D)** and submandibular lymph nodes **(E)** of non-pre-sensitized/untreated rats (naive, $n = 3$); pre-sensitized/untreated rats (Pre-sens, $n = 3$) and pre-sensitized/third-party allo-MSC treated rats (WF MSC treated, $n = 3$). Results for individual animals (symbols) and mean \pm SEM for each group (horizontal lines with error bars) are shown. Statistical analysis was performed using one-way ANOVA with Tukey's post-test, ** $p < 0.01$.

third-party allo-MSC or untreated (Figure 4A). At this time-point, a trend toward a reduction in the proportion of CD3⁺ CD4⁺ CXCR3⁺ Th1 cells in the draining lymph node was observed (Figure 4C) as well as an increase in the proportion of CD3⁺ CD4⁺ FoxP3⁺ regulatory T cells (mean 4.4% in untreated compared to 7.9% in MSC-treated, pre-sensitized animals; Figures 4B,D). Importantly, increased regulatory T cells has been previously shown to prolong corneal allograft survival (5). These changes in proportions of T cell populations were only observed in the draining lymph nodes, indicating a localized corneal transplant-specific effect.

MSC-Educated Lung Myeloid Cells Exhibit an Immunosuppressive Phenotype

To determine if third party allo-MSC directly increase the proportion of potentially regulatory CD45⁺CD11b⁺ B220⁺ cells among native lung myeloid cells *ex vivo*, CD11b/c⁺ cells were isolated from the lungs of naïve Lewis rats by magnetic column separation. The isolated cells were then cultured for 48 h under 3 conditions: Unstimulated, stimulated with IFN γ /LPS alone and cultured in the presence of WF allo-MSC for 48 h (Figure 5A). CD11b/c⁺ cells were then re-separated from MSC by magnetic column separation and cultured alone for 24 h.

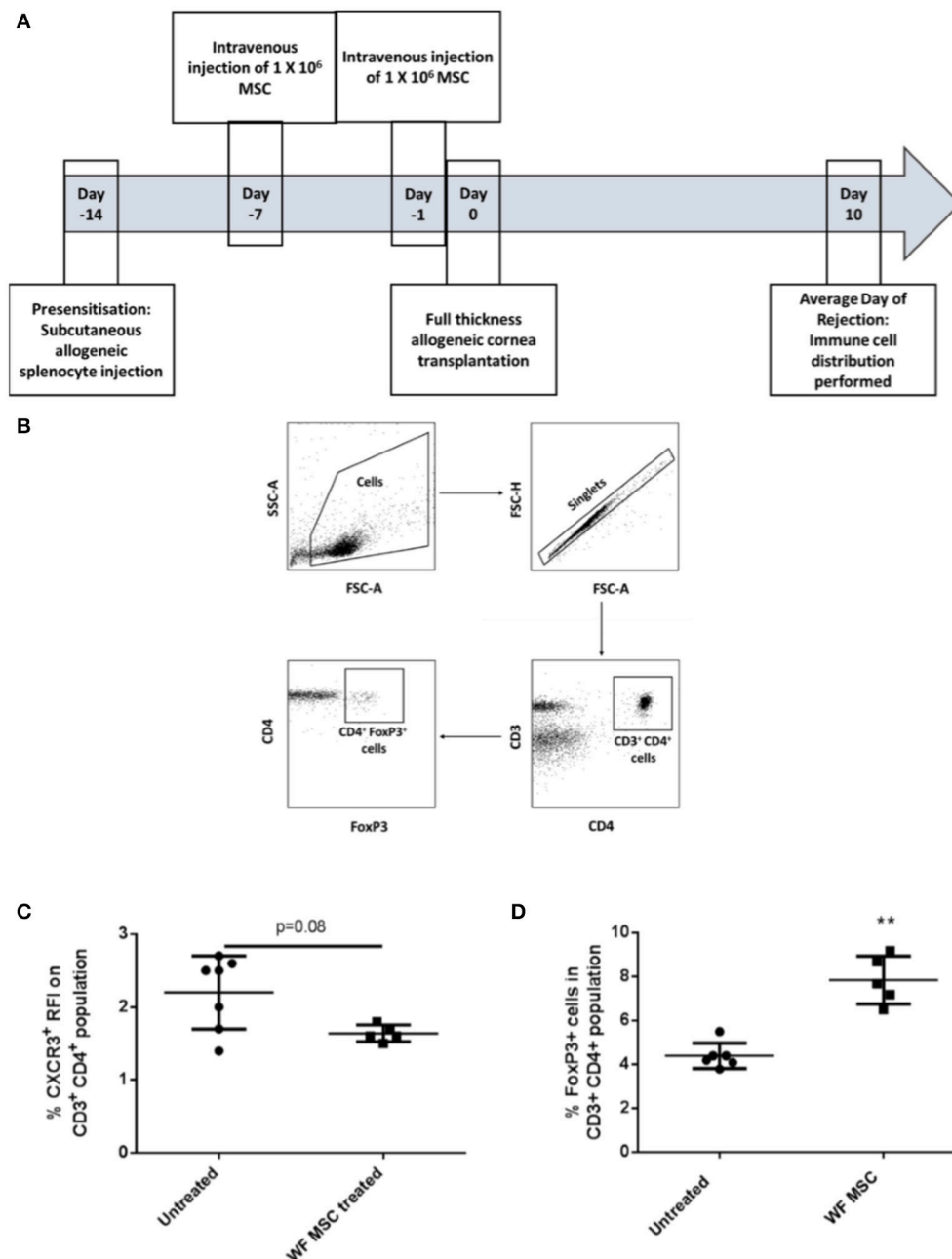


FIGURE 4 | Third-party allo-MSC administration to pre-sensitized Lewis rat corneal transplant recipients results in a proportionate increase in draining lymph node FoxP3⁺ (regulatory) CD4⁺ T-cells cells 10 days after transplantation. **(A)** Schematic representation of experiment to evaluate immune cell profiles at day 10 post-transplant in pre-sensitized Lewis rat recipients of DA corneal allografts following treatment with third-party allo-MSC. **(B)** Gating strategy used to identify FoxP3⁺ cells by flow cytometry (C and D). Graphs showing results of flow cytometry analysis of the proportions of CD3⁺CD4⁺ T-cells co-expressing CXCR3 **(C)** and FoxP3 **(D)** in the graft-draining lymph nodes of pre-sensitized/untreated rats (Pre-sens, $n = 6$) and pre-sensitized/third-party allo-MSC treated rats (WF MSC treated, $n = 5$). Results for individual animals (symbols) and mean \pm SEM for each group (horizontal lines with error bars) are shown. Statistical analysis was performed using one-way ANOVA with Tukey's post-test, ** $p < 0.01$.

In this experiment, however, no increase in the proportion of CD45⁺CD11b⁺B220⁺ cells was observed following allo-MSC co-culture, suggesting that the higher proportion of B220⁺ myeloid cells in lungs of allo-MSC-treated animals

was not explained by direct induction of this marker on resident, CD11b⁺B220⁺ myeloid cells (**Figure 5B**). Nonetheless, significantly higher concentrations of PGE₂ and TGF β 1 were generated by myeloid cells that had been re-purified from

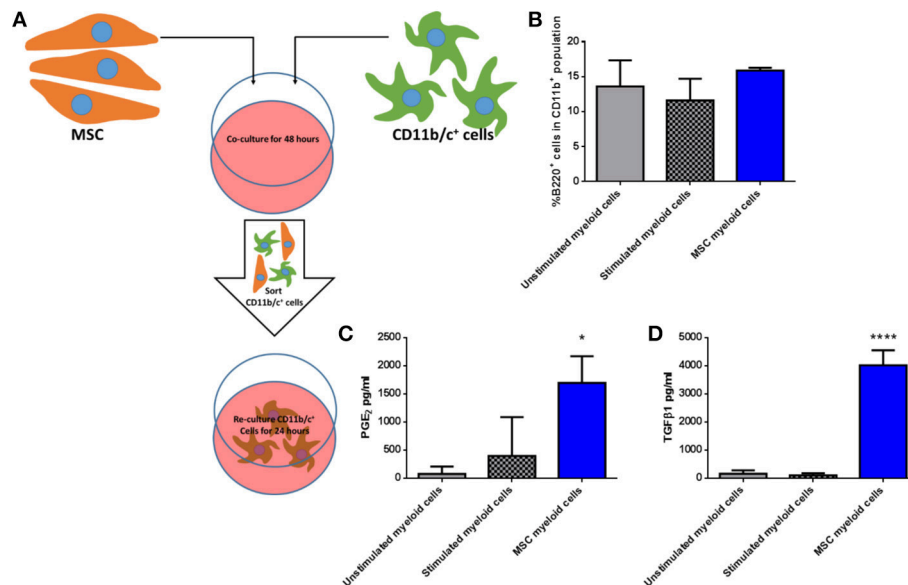


FIGURE 5 | Allogeneic MSC polarize macrophages to a more immunomodulatory phenotype *in vitro* (A) Schematic representation of the co-culture of CD11b/c⁺ (myeloid) cells isolated from Lewis rat lungs with Wistar Furth MSC or control conditions for 48 h followed by re-purification and culture for 24 h. (B) Graph of the proportions of B220⁺ cells among CD11b⁺ cells following 48 h culture Lewis rat lung myeloid without further stimulus (Unstimulated myeloid cells), with IFN γ /LPS stimulation (Stimulated myeloid cells) and with Wistar Furth MSC (MSC myeloid cells). (C,D) Graphs of the concentrations of PGE₂ (C) and TGF β 1 (D) in 24 h culture supernatants of CD11b/c⁺ Lewis rat lung myeloid cells re-purified from the same three 48-h culture conditions. All results are presented as mean \pm SEM with $n = 3$ in each group. Statistical analyses were performed using one way ANOVA with Tukey's post-test, * $p < 0.05$, **** $p < 0.0001$.

cultures containing allo-MSC compared to unstimulated or IFN γ /LPS stimulated myeloid cells (Figures 5C,D).

Next, CD11b/c⁺ cells re-isolated from the three culture conditions were co-cultured with syngeneic fluorescently-labeled lymphocytes in the presence of anti-CD3/anti-CD28 stimulation for 4 days (Figure 6A), following which proliferation was quantified by flow cytometry based on dilution of the fluorescent dye. As shown in Figures 6B,C, MSC-educated lung myeloid cells suppressed the proliferation of CD4⁺ and CD8⁺ T cells to a greater degree than unstimulated lung myeloid cells, while IFN γ /LPS stimulated lung myeloid cells had no suppressive effect. This suppression was accompanied by an increase in the PGE₂ concentration (Figure 6D) as well as a trend toward an increase in TGF β 1 (Figure 6E) in the culture supernatants of the T cell stimulation cultures containing MSC-educated lung myeloid cells. It was concluded that direct contact between allo-MSC and primary lung myeloid cells results in a deviation of the myeloid cell activation response toward an anti-inflammatory, immunosuppressive phenotype.

Cryopreserved MSC Are Capable of Prolonging Corneal Allograft Survival

For translation of an allogeneic cell therapy to the clinic, logistical concerns like transport of cells from the production site to the clinical site, formulation of the sample and manipulations required at the bedside become major concerns (26). For instance, the ability to thaw and directly administer cells at the bedside may be critical for clinical cost-effectiveness and

ease of use. To address this issue in the context of high-risk corneal transplantation, *in vitro* and *in vivo* experiments were carried out using freshly-thawed cryopreserved third-party allo-MSC. As shown in Figure 7A, thawed, cryopreserved allo-MSC mediated equivalent suppression of CD4⁺ T cell proliferation as freshly cultured cells. Furthermore, when administered to pre-sensitized animals at the same dose and time-points as for experiments using freshly cultured cells (Figure 7B), thawed, cryopreserved, third-party allo-MSC proved similarly capable of modulating corneal transplant rejection (Figures 7C–E). Cryopreserved allo-MSC-treated animals had an ADR of 21.5 days compared to 10.3 days for vehicle-treated animals.

Finally, to determine whether the allo-MSC-associated immunomodulation is compatible with peri-transplant administration of a clinically-relevant immunosuppressant, a group of pre-sensitized DA-to-Lewis corneal transplant recipients was treated with a combination of thawed, cryopreserved third allo-MSC and daily injections of MMF between day–1 and day 6 post-transplant (Figure 7B). As shown in Figure 7C, the combined treatment also resulted in a significant prolongation of corneal allograft rejection-free survival with ADR of 27. It was concluded that, in addition to their potent and consistent immunomodulatory effects, the translational potential of allo-MSC for amelioration of rejection in high immunological risk cornea transplant recipients is further enhanced by their compatibility with cryopreservation and with co-administration with MMF.

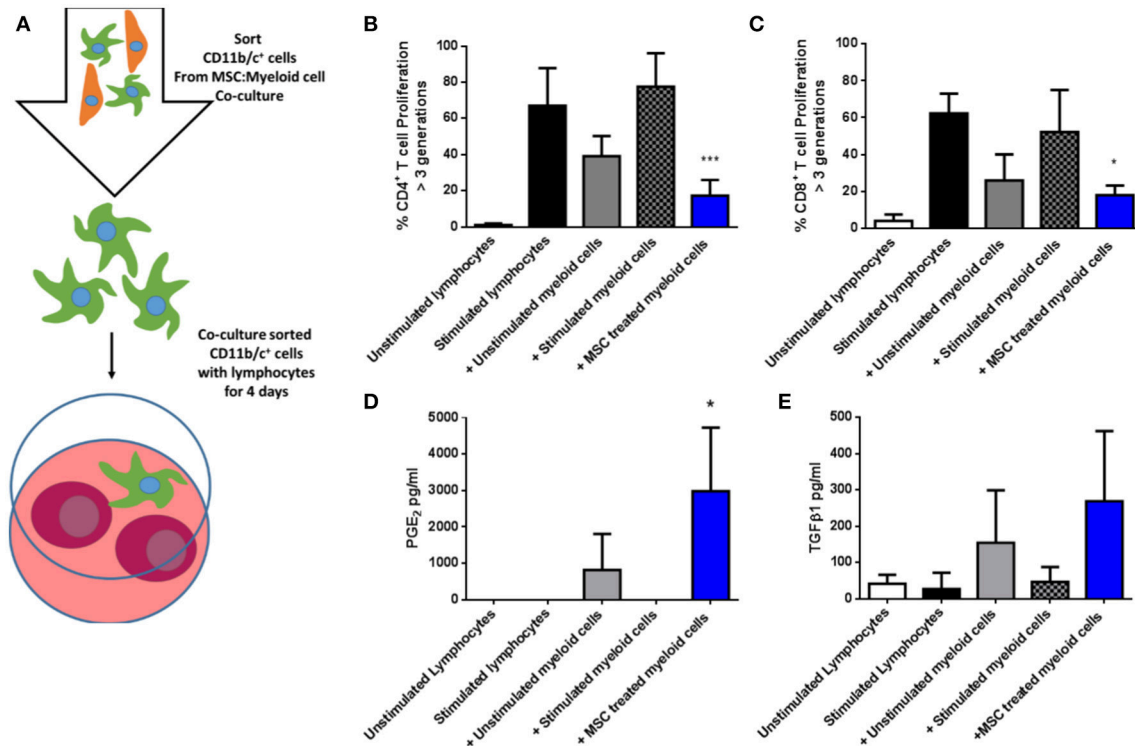


FIGURE 6 | Allogeneic MSC-educated lung myeloid cells more potently suppress T-cell proliferation. **(A)** Schematic representation of the addition of Lewis rat lung CD11b/c⁺ (myeloid) cells re-sorted from co-cultures with Wistar Furth MSC or control conditions and then added to syngeneic lymphocytes, with, or without anti-CD3/anti-CD28 stimulation for 4 days. **(B,C)** Graphs of CD4⁺ **(B)** and CD8⁺ **(C)** T-cell proliferation, quantified by flow cytometry-based fluorescent dye dilution as the proportion of cells that had proliferated through >3 generations, in 4-day cultures of Lewis rat splenocytes under five conditions: No stimulation (Unstimulated lymphocytes); Anti-CD3/CD28 stimulation alone (Stimulated lymphocytes); Anti-CD3/CD28 stimulation + re-purified unstimulated Lewis rat lung myeloid cells + Unstimulated myeloid cells; Anti-CD3/CD28 stimulation + re-purified, IFN γ /LPS-stimulated Lewis rat lung myeloid cells (+ Stimulated myeloid cells) and Anti-CD3/CD28 stimulation + re-purified Wistar Furth MSC co-cultured Lewis rat lung myeloid cells (+ MSC treated myeloid cells). **(D,E)** Graphs of the concentrations of PGE₂ **(D)** and TGF β 1 **(E)** in 4-day Lewis rat splenocyte cultures under the same five conditions. All data are presented as mean \pm SEM with $n = 3$ in each condition. Statistical analysis was performed using one way ANOVA with Tukey's post-test, * $p < 0.05$, *** $p < 0.001$.

DISCUSSION

Patients at higher risk of corneal allograft rejection due to pre-existing inflammation or anti-donor immunity suffer from immune-mediated corneal rejection at an earlier time and higher frequency than patients classified as non-high-risk (1–3). A major issue in the clinical management of high-risk corneal allograft recipients is the reluctance to administer systemic immunosuppressive drug treatments due to the risk of side effects and the fact that, unlike other organ transplants, a corneal transplant failure is not a life threatening event (27, 28). This means that high-risk patients present a potential unmet clinical need which could be ameliorated with MSC therapy.

Pre-sensitization with donor-derived splenocytes resulted in an earlier and very consistent rejection profile in the DA-to-Lewis rat cornea transplantation model. This rejection was associated with rapid opacification and neovascularisation of the graft and a demonstrable pre-existing allo-immunity in the form of anti-DA IgG2 antibodies. Similar to our previous results in a conventional risk rat corneal transplant model, we show here that third-party allo-MSC are capable of significantly prolonging

corneal allograft survival to day 30 in this high-risk model (11). Third-party MSC are the most relevant cells to use as, in the clinical setting for MSC therapies, it is likely that cells from a source allogeneic to both donor and recipient will have to be used for reasons of logistics and cost (11). Additionally, reports in recent years have shown the importance of activation of MSC with pro-inflammatory cytokines such as interferon- γ , tumor necrosis factor- α and interleukin-1 β for their ability to manifest immunomodulatory effects (29–31). *In vivo*, this stimulus can come from introducing the MSC to an already-inflamed environment or through allo-recognition of the cells by the host immune system with subsequent production of pro-inflammatory factors (25, 32, 33). Thus, infusing an allogeneic cell, such as the Wistar Furth MSC employed in this study, may result in allo-recognition upon infusion and subsequent activation of the MSC by the host's immune system (18, 19).

The first organ which intravenously-infused MSC encounter is the lung. It is well established in animal models that MSC become lodged in the lungs soon after infusion and that the vast majority do not migrate to other tissues (10, 13, 34). In addition to residing in the lung after infusion, the half-life of infused MSC

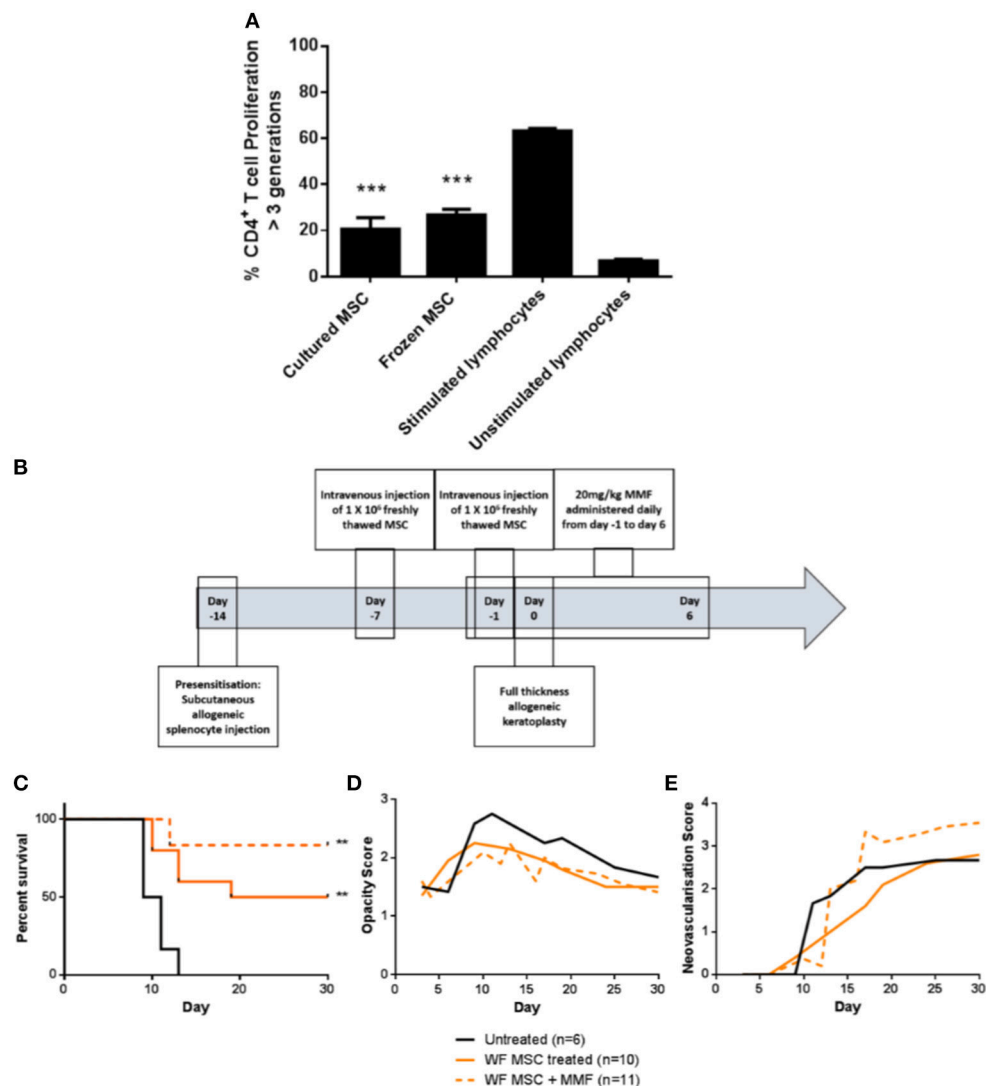


FIGURE 7 | Cryopreserved third party allo-MSC are immunosuppressive and capable of prolonging corneal allograft survival without and with mycophenolate mofetil co-administration in pre-sensitized recipients. **(A)** Graph of CD4⁺ T-cell proliferation, quantified by flow cytometry-based fluorescent dye dilution as the proportion of cells that had proliferated through >3 generations, in 4-day cultures of Lewis rat splenocytes under four conditions: Anti-CD3/CD28 stimulation + freshly cultured Wistar Furth MSC (Cultured MSC); Anti-CD3/CD28 stimulation + thawed, cryopreserved Wistar Furth MSC (Frozen MSC); Anti-CD3/CD28 stimulation alone (Stimulated lymphocytes) and No stimulation (Unstimulated lymphocytes). **(B)** Schematic representation of administration of thawed, cryopreserved (freshly thawed) third-party allo-MSC and MMF in the pre-sensitized high-risk rat corneal transplant model. **(C)** Kaplan-Meier curves demonstrating rejection-free survival of pre-sensitized Lewis recipients of DA corneas that received no treatment (Untreated, $n = 6$); thawed, cryopreserved third-party allo-MSC (WF MSC, $n = 10$) and thawed, cryopreserved third-party allo-MSC with MMF (WF MSC + MMF, $n = 11$). **(D,E)** Trend-lines for corneal allograft opacity scores **(D)** and neovascularisation scores **(E)** of the same three groups of pre-sensitized corneal transplant recipients up to day 30 post-transplant. Statistical significance was determined using a Log-rank (Mantel-Cox) test, ** $p < 0.01$ compared to Untreated group. *** $p < 0.001$.

is remarkably short considering the long-term benefits they can exert (10, 13, 34). Ko et al. in an important recent study, showed that intravenously administered MSC primed CD11b⁺ B220⁺ lung myeloid cells in the mouse to prevent corneal allograft rejection (13). Here we show that these cells are also increased in the lungs of pre-sensitized high-risk rat cornea transplant recipients 24 h after administration of the second of two third-party allo-MSC infusions. The data presented here add to previous studies showing that MSC are transient mediators which

may imprint their immunomodulatory phenotype onto relevant recipient cells to indirectly exert their long-term effect. In the case of this study, evidence of imprinting of MSC immunomodulatory phenotype included an almost 2-fold increase in the proportion of regulatory T cells in the draining lymph node during the time-period in which rejection typically manifests in this high-risk model. Regulatory T cells in the draining lymph node have been shown to be critically important for corneal allograft survival (35, 36) and depletion of these cells negatively impacts the

survival of the graft (37). Indeed, several groups have shown that adoptive transfer of regulatory T cells and other *in vivo* strategies to increase the numbers of regulatory T cells can have beneficial effects on transplant survival (5, 38–40).

In *in vitro* experiments, we examined whether third-party allo-MSC directly induced an increase in the proportion of CD11b⁺ B220⁺ within a purified population of CD11b/c⁺ lung cells. However, we observed that there was no increase in the proportion of these cells in a co-culture with CD11b/c⁺ lung cells and MSC suggesting that, once localized to the lung, MSC may recruit CD11b⁺ B220⁺ and polarize these cells to a more immunomodulatory phenotype rather than switching the expression of previously lung resident CD11b⁺ B220[−] cells. The production of immunomodulatory proteins such as TGF- β 1 and PGE₂ could polarize these CD11b/c⁺ cells to a more immunomodulatory phenotype. Third-party allo-MSC-educated CD11b/c⁺ cells were furthermore demonstrated to have enhanced anti-proliferative potency against activated T cells and to release immunomodulatory mediators such as PGE₂ which, in addition to the above mentioned reduction in pro-inflammatory mediators, could all combine to result in an increase in the proportion of regulatory T cells and subsequent promotion of allograft survival.

Translation of a cell therapy such as MSC will likely involve one site producing cells for transport to and administration at several clinical sites. A therapy which can be stored, easily transported and administered in the clinic without a requirement for cell culture facilities is, therefore, ideal to benefit the maximum number of patients. A frozen cell product which can be thawed and directly administered to the patient fulfills the requirements for an easily adoptable cell therapy. Therefore, we determined whether MSC that had been cryopreserved in HSA/DMSO could prolong corneal allograft survival in the pre-sensitized model. Despite existing evidence that cryopreserved MSC may have impaired immunomodulatory capacities, including reduced expression of immunosuppressive molecules and impaired responses to pro-inflammatory stimuli (41), we found that cryopreserved MSC were equally capable of prolonging allograft survival as cultured cells. This important finding further demonstrates the translatability of an MSC therapy for high-risk cornea transplant patients who may have pre-existing allo-immunity.

Additionally, in a high-risk cornea transplant setting, it is likely that clinicians will co-administer MSC with an already established immunosuppressive drug regimen. We sought, therefore, to determine whether a peri-transplant course of MMF would augment or interfere with the efficacy of MSC in the high-risk pre-sensitized CT model. MMF was chosen for several reasons. Firstly, unlike other immunosuppressive drugs such as corticosteroids, it does not alter the expression of MSC immunomodulatory factors (42) and has been shown to exert a synergistic effect with MSC in a pre-clinical heart transplant model (21). Secondly, MMF is currently prescribed to patients receiving high-risk cornea transplants including re-grafts (43, 44). Our observation that co-administration of MMF did not diminish the anti-rejection of third-party allo-MSC therapy (and trended toward

further increase in rejection-free survival) in this high-risk model provides additional evidence of clinical translational potential.

In conclusion, we have shown for the first time that pre-transplant intravenous administration of third-party allo-MSC results in distinct immune modulatory effects that overcome pre-existing anti-donor immunity to prolong rejection-free survival of corneal allografts in a rat model. Furthermore, the experimental cell therapy regimen described here appears to be compatible with prior cryo-preservation and with co-administration of a relevant immunosuppressive drug. These findings open the door to clinical translation of off-the-shelf allo-MSC products for recipients of high-risk corneal transplants who continue to suffer from very poor long-term graft survival rates. The results of this study, particularly those related to the co-administration of cryo-preserved allo-MSC and MMF have formed the basis of a regulatory submission for a Phase Ib clinical trial in corneal re-graft patients. This trial will determine the safety and feasibility of co-administration of allo-MSC and MMF in the setting of high risk cornea transplantation.

AUTHOR CONTRIBUTIONS

When determining authorship, the following criteria should be observed: Substantial contributions to the conception or design of the work PL, MG, and TR or the acquisition PL, NM, OT, KL, MM, and BC analysis PL, NM, OT, KL, MM, and BC or interpretation of data for the work PL, AR, MG, and TR. Drafting the work or revising it critically for important intellectual content PL, AR, MG, and TR. Final approval of the version to be published PL, NM, OT, KL, MM, BC, AR, MG, and TR. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved PL, NM, OT, KL, MM, BC, AR, MG, and TR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02666/full#supplementary-material>

Supplementary Figure 1 | MSC were characterized by flow cytometry using standard markers (A) CD90, (B) CD73, (C) MHC-I, (D) MHC-II, (E) CD45, and (F) CD86.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A GMCSF-Neuroantigen Tolerogenic Vaccine Elicits Systemic Lymphocytosis of CD4⁺ CD25^{high} FOXP3⁺ Regulatory T Cells in Myelin-Specific TCR Transgenic Mice Contingent Upon Low-Efficiency T Cell Antigen Receptor Recognition

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Previous studies showed that single-chain fusion proteins comprised of GM-CSF and major encephalitogenic peptides of myelin, when injected subcutaneously in saline, were potent tolerogenic vaccines that suppressed experimental autoimmune encephalomyelitis (EAE) in rats and mice. These tolerogenic vaccines exhibited dominant suppressive activity in inflammatory environments even when emulsified in Complete Freund's Adjuvant (CFA). The current study provides evidence that the mechanism of tolerance was dependent upon vaccine-induced regulatory CD25⁺ T cells (Tregs), because treatment of mice with the Treg-depleting anti-CD25 mAb PC61 reversed tolerance. To assess tolerogenic mechanisms, we focused on 2D2-FIG mice, which have a transgenic T cell repertoire that recognizes myelin oligodendrocyte glycoprotein peptide MOG35-55 as a low-affinity ligand and the neurofilament medium peptide NFM13-37 as a high-affinity ligand. Notably, a single subcutaneous vaccination of GMCSF-MOG in saline elicited a major population of FOXP3⁺ Tregs that appeared within 3 days, was sustained over several weeks, expressed canonical Treg markers, and was present systemically at high frequencies in the blood, spleen, and lymph nodes. Subcutaneous and intravenous injections of GMCSF-MOG were equally effective for induction of FOXP3⁺ Tregs. Repeated booster vaccinations with GMCSF-MOG elicited FOXP3 expression in over 40% of all circulating T cells. Covalent linkage of GM-CSF with MOG35-55 was required for Treg induction whereas vaccination with GM-CSF and MOG35-55 as separate molecules lacked Treg-inductive activity. GMCSF-MOG elicited high levels of Tregs even when administered in immunogenic adjuvants such as CFA or Alum. Conversely, incorporation of GM-CSF and MOG35-55 as separate molecules in CFA did not support Treg induction. The ability of the vaccine to induce Tregs

was dependent upon the efficiency of T cell antigen recognition, because vaccination of 2D2-FIG or OTII-FIG mice with the high-affinity ligands GMCSF-NFM or GMCSF-OVA (Ovalbumin323-339), respectively, did not elicit Tregs. Comparison of 2D2-FIG and 2D2-FIG-*Rag1*^{-/-} strains revealed that GMCSF-MOG may predominantly drive Treg expansion because the kinetics of vaccine-induced Treg emergence was a function of pre-existing Treg levels. In conclusion, these findings indicate that the antigenic domain of the GMCSF-NAg tolerogenic vaccine is critical in setting the balance between regulatory and conventional T cell responses in both quiescent and inflammatory environments.

Keywords: FOXP3, Tregs, GM-CSF, neuroantigen, EAE, tolerance, multiple sclerosis

INTRODUCTION

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) marked by periodic focal attacks on white and gray matter myelin accompanied by significant diffuse myelin and axonal injury and atrophy (1–7). MS often begins as an overt relapsing-remitting disease accompanied by an insidious, clinically-subvert progression of disability. After 10–25 years, the disease often transitions from a succession of punctate attacks to a secondary progressive phase marked by incessant white and gray matter degeneration and increasing levels of physical and cognitive impairment (8, 9). MS is widely considered to be an autoimmune disease of the CNS driven by molecular mimicry against environmental antigens complemented by genetic influences that drive autoimmunity and impair the homeostatic regulatory responses needed to maintain tolerance in CNS tissues (10–12). Several studies have provided evidence that MS is associated with deficient function of regulatory T cells, including the canonical CD25^{high} FOXP3⁺ regulatory T cells (Tregs) (13–15). Current therapies for MS however are not designed to restore a homeostatic balance needed for adaptive tolerance and disease resolution. Instead, first-line immunomodulatory drugs lack disease-specific activity and thereby exhibit modest efficacy and do not alter the long-term disease trajectory in most MS patients (16). Second-line broad-spectrum, immunosuppressive drugs block both pathogenic and adaptive immunity and may result in an immunocompromised state, opportunistic infection, and/or cancer (17–19). Tolerogenic vaccines are qualitatively distinct and are designed to restore myelin-specific Treg responses and specifically repair a major defect underlying MS susceptibility. Tolerogenic vaccines for MS that have been tested in pre-clinical models of EAE are based on diverse technical platforms

that include naked synthetic peptides, DNA vectors, antigen-expressing dendritic cell APC/leukocytes, engineered viral vectors, antigen-bearing vesicles/nanoparticles, among several others (20–26). Many of these vaccine platforms however lack robust therapeutic efficacy (27). Ideally, tolerogenic vaccines should exhibit prophylactic and therapeutic activity, attenuate the pathogenic T conventional cell (Tcon) repertoire, and enhance the Treg repertoire even within the confines of proinflammatory environments (28).

Fusion proteins containing GM-CSF as the N-terminal domain and a dominant encephalitogenic neuroantigen (NAg) as the C-terminal domain represent an emerging vaccine platform that may fulfill these criteria. GMCSF-NAg tolerogenic vaccines were effective in three different EAE models including monophasic EAE in Lewis rats, relapsing-remitting EAE in SJL mice, and chronic EAE in C57BL/6 mice (28–32). Recombinant proteins comprised of rat GM-CSF fused to the Myelin Basic Protein (MBP) 69–87 peptide, murine GM-CSF fused to the Proteolipid Protein (PLP)139–151 peptide, and murine GM-CSF fused to the MOG35–55 peptide were used in the respective EAE models. In each system, GMCSF-NAg was an effective prophylactic that prevented the subsequent induction of EAE. GMCSF-NAg was also a therapeutic intervention that elicited remission when administered after the onset of severe paralytic EAE (29–32). The tolerogenic mechanism of GMCSF-MOG was unique because the fusion protein did not require a quiescent environment to inhibit EAE (29). Rather, GMCSF-MOG was effective when administered adjacent to or within a MOG35–55/CFA encephalitogenic emulsion even when MOG35–55 was at a 75:1 molar excess compared to GMCSF-MOG. This observation negates the dogma that tolerogenic vaccines require a quiescent non-inflammatory environment for the efficient induction of tolerance. The relevant target of GMCSF-NAg *in vivo* is thought to be myeloid APC, because *in vitro* analyses revealed that GMCSF-NAg fusion proteins targeted NAg for enhanced antigen presentation by myeloid APC *in vitro*. For example, a GMCSF-NAg fusion protein that included the 69–87 epitope of MBP engendered an approximate 1,000-fold enhancement of antigenic potency by a mechanism that was blocked by free GM-CSF and that depended on physical linkage of the GM-CSF and NAg domains (32). Interleukin-4 (IL4)-NAg and macrophage colony stimulating factor (M-CSF)-NAg fusion proteins targeted NAg for enhanced presentation by B cells and macrophages,

Abbreviations: APC, antigen presenting cell(s); CFA, Complete Freund's Adjuvant; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FIG, Foxp3-IRES-GFP knock-in allele; GM-CSF, granulocyte-macrophage colony stimulating factor; IV, intravenous; M-CSF, macrophage colony stimulating factor; MFI, mean fluorescence intensity; mAb, monoclonal antibody; MS, multiple sclerosis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; NAg, neuroantigen(s); NFM, neurofilament medium protein; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; PLP, proteolipid protein; SC, subcutaneous; SE, Standard error of the mean; Tcon, conventional T cell(s); TCR, T cell antigen receptor; Treg, regulatory T cell(s).

respectively. But IL4-NAg lacked tolerogenic activity (33), and MCSF-NAg did not approximate the tolerogenic activity of GMCSF-NAg (32). These findings suggested that GMCSF-NAg fusion proteins have the core activities needed for tolerogenic vaccination.

The use of GM-CSF as a tolerogenic fusion partner is intriguing because GM-CSF has classically been considered a proinflammatory cytokine, and GM-CSF has been successfully used as an immunogenic domain in GMCSF-antigen fusion vaccines in models of cancer and infectious disease (34–42). Nonetheless, GM-CSF is also known to elicit differentiation of regulatory DC and myeloid-derived suppressor cells that in turn elicit regulatory T cells to inhibit autoimmune disease (43–45). Administration of GM-CSF inhibits autoimmune disease in several pre-clinical disease models, including experimental myasthenia gravis, autoimmune diabetes, and experimental autoimmune thyroiditis (46–55). Conversely, deficiency of GM-CSF is associated with susceptibility to autoimmune diabetes and systemic lupus erythematosus (56, 57). The activity spectrum of GM-CSF is therefore complex and contradicts simple perspectives as a pro-inflammatory or anti-inflammatory cytokine. One possibility is that GM-CSF amplifies immunogenic or tolerogenic activity of associated antigens based on the intrinsic T cell antigen receptor recognition events that are dominant in a particular locale. If so, then GMCSF-NAg fusion proteins would amplify the intrinsic immunogenic or tolerogenic activity of the covalently-tethered NAg domain.

To address this hypothesis, the current study focused on 2D2-FIG strain of MOG-specific mice that have a transgenic T cell repertoire specific for the low affinity ligand MOG35-55. This study revealed that subcutaneous (SC) administration of GMCSF-MOG in saline elicited a major population of FOXP3⁺ Tregs comprising ~20–40% of all circulating T cells within ~3–4 days. The GMCSF-MOG vaccine also imposed a desensitized phenotype upon the 2D2 T cell repertoire, as shown by reduced circulating T cell numbers, down-regulated CD3/TCR expression on a per cell basis, and expanded percentages of CD4⁽⁻⁾ 2D2 T cells. In contrast, GMCSF-based fusion proteins incorporating highly agonistic antigens NFM13-37 or OVA323-337 lacked robust Treg-induction activity in 2D2-FIG and OTII-FIG models, respectively. Thus, the antigen-targeting and adjuvant activities of the GM-CSF domain of GMCSF-antigen fusion proteins may simply amplify the intrinsic antigenic activity and efficiency of TCR-antigen recognition events to set the balance of Treg/Tcon responses. These data indicate that the vaccine-targeted presentation of the low-affinity MOG35-55/I-A^b by myeloid APC is a key parameter for induction of a predominant Treg response and establishment of CNS-specific tolerance. Because CNS myelin peptides are uniformly subject to self-tolerance and exhibit weak, inefficient interactions with the T cell repertoire, GMCSF-NAg fusion proteins may have wide applicability as tolerogenic vaccines in CNS-targeted autoimmune disease. The extrapolation is that most self-proteins, when incorporated as GMCSF-antigen fusion proteins, would drive dominant Treg responses to elicit immunological tolerance.

MATERIALS AND METHODS

Mice

C57BL/6J (000664), B6.Cg-Foxp3^{tm2Tch}/J (FIG Foxp3-IRES-GFP 006772), B6.129S7-Rag1^{tm1Mom}/J (Rag1^{-/-} 002216), C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J (2D2 MOG35-55-specific TCR transgenic 006912), and B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII OVA323-339-specific TCR transgenic 004194) mouse strains were obtained from the Jackson Laboratory (Bar Harbor, ME) and were housed and bred in the Department of Comparative Medicine at East Carolina University Brody School of Medicine. 2D2-FIG, 2D2-FIG-Rag1^{-/-}, and OTII-FIG mice were obtained through intercross breeding. Animal care and use was performed in accordance with approved animal use protocols and guidelines of the East Carolina University Institutional Animal Care and Use Committee.

Reagents and Recombinant Proteins

Synthetic peptides MOG35-55 (MEVGWYRSPFSRVVHLYRNGK), NFM13-37 (RRVTETRSSFSRVSGSPSSGFRSQS), and OVA323-339 (ISQAVHAAHAEINEAGR) were obtained from Genscript (Piscataway, NJ). Derivation, expression, purification, and bioassay of the murine GM-CSF and GMCSF-MOG fusion proteins were described in previous studies (30, 31). These fusion proteins as well as GMCSF-OVA and GMCSF-NFM were comprised of the murine GM-CSF cytokine as the N-terminal domain, the amino acid sequence comprising the relevant antigenic peptide domain, and an 8-histidine C-terminus. GM-CSF contained the 8-histidine tag C-terminus but did not contain an antigenic peptide domain. GMCSF-MOG, GMCSF-NFM, and GMCSF-OVA contained MOG35-55, NFM13-37, and OVA323-339 peptides, respectively. These fusion proteins did not contain linkers in the GM-CSF/antigenic peptide/8-histidine-tag junctions. These recombinant proteins were isolated from stably-transfected human embryonic kidney (HEK) cells or from Chinese hamster ovary (CHO) cells. Expression supernatants were concentrated on YM10 ultrafiltration membranes and were directly applied to Ni-NTA Agarose columns (Qiagen, Chatsworth, CA) followed by extensive washing of the resin bed (50 mM NaH₂PO₄, 500 mM NaCl, with 10, 20, or 60 mM imidazole, pH 8.0). Recombinant proteins were eluted with 250 mM imidazole (pH 8.0) and were concentrated and diafiltered in Amicon Ultra-15 centrifugal filter devices (EMD Millipore, Billerica, MA). Protein quantity was assessed by absorbance at 280 nm, and purity was assessed by SDS-PAGE.

Generation, Purification, and Administration of Monoclonal Antibodies (mAbs)

Hybridomas secreting the PC61-5.3 mAb or a rat IgG1 isotype control were described previously (58). Hybridoma cells were cultured in supplemented DMEM in C2011 hollow fiber cartridges (FiberCell Systems, Inc., Frederick, MD). Hybridoma supernatants were clarified at 7,200 x g, precipitated with 50% ammonium sulfate, and dissolved in PBS. MAb preparations were purified on protein G agarose columns. Antibody was eluted with

200 mM glycine at pH 3.0 and immediately neutralized by 1M Tris buffer of pH 9.0. The purity of the antibody was verified by SDS-PAGE.

Flow Cytometric Analyses of Lymphocytes, Splenocytes, and Peripheral Blood Mononuclear Cells (PBMC)

Blood was collected from the submandibular vein into 200 μ l of sodium citrate (130 mM). Inguinal lymph nodes and spleen were dissected from mice and placed into 10 ml of HBSS. Dissected lymph nodes and spleen were pressed through a wire mesh screen and a 70 μ m cell strainer (Corning, NY) to obtain single-cell suspensions. Cells were washed in 3 ml HBSS with 2% heat-inactivated FBS and stained with designated cocktails of fluorochrome-conjugated antibodies for 1 h at 4°C in the dark. After staining whole blood, erythrocytes (RBC) were lysed with 1:10 HBSS for 20 s at 4°C followed by addition of 2X PBS. Alternatively, RBCs were lysed by incubating samples for 10 min on ice with 3 ml of ammonium chloride lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1.2 mM EDTA- pH 7.2). Lysis was repeated when necessary. Samples were then washed 1 time with HBSS/2% FBS and were analyzed on a Becton-Dickson LSRII flow cytometer (San Jose, CA) with FlowJo software (Ashland, OR). In designated experiments, reference “counting” beads were added to samples immediately before flow cytometric analysis (AccuCount blank particles or FITC-, PE-, or APC-conjugated EasyComp fluorescent particles 3.0–3.4 μ m, Spherotech, Lake Forest, IL). The use of reference beads enabled comparisons of absolute cell numbers among different samples. For intercellular staining of FOXP3 and Ki67, blood was collected, and RBCs were lysed as previously described. PBMC were fixed for 10 min using 2% paraformaldehyde (PFA) and were fixed/permeabilized with 1 mL of ice cold 100% methanol for 30 min. Cells were then stained with antibody cocktails against both surface markers and intercellular targets for 30 min at room temp. Cells were extensively washed between PFA, methanol, and staining treatments using PBS + 2% FBS. Fluorochrome-conjugated mAbs were obtained from BioLegend and included CD3-BV421, PE/Dazzle 594, or PE (17A2 or 145-2C11), CD4-BV785, PE, or APC (GK1.5), CD25-BV421 (PC61), CD44-BV421 (IM7), CD45-BV785 (30-F11), CD62L-APC (MEL-14), TCR-V α 3.2-PE (RR3-16), TCR-V β 11-PE or AF647 (KT11), TCR-V β 5.1,5.2-PE (MR9-4), TCR-V α 2-APC (B20.1), Neuropilin-PE (3E12), and LAP-PE (TW7-16B4). Comparisons among three or more groups were analyzed by use of ANOVA, which was interpreted with a Holm-Sidak multiple comparisons test. Pairwise comparisons were analyzed by two-tailed *t*-tests for data that passed Normality (Shapiro-Wilk) and Equal Variance (Brown-Forsythe) tests. A *P*-value < 0.05 was considered significant. Error bars represent standard error of the mean (SE) unless designated otherwise.

Measurement of GM-CSF Activity and Antigen-Specific T Cell Responses

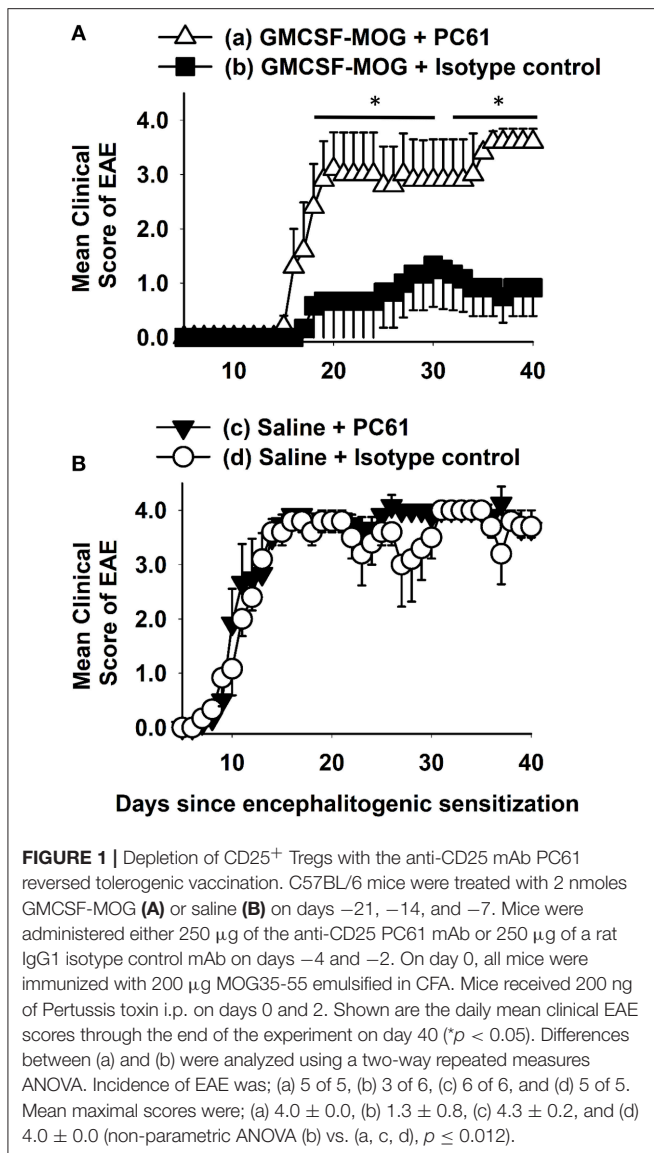
To measure antigen-specific proliferation, 2D2 or OTII T cells (2.5×10^4 /well) were cultured with irradiated splenocytes (3,000 rads, 2.0×10^5 cells/well) in the presence of designated antigen

concentrations. To measure GM-CSF activity, C57BL/6 bone marrow cells were cultured with designated concentrations of GM-CSF or GM-CSF fusion proteins. Cultures were pulsed with 1 μ Ci [³H]thymidine (6.7 Ci/mmol, New England Nuclear, Perkin Elmer, Waltham, MA, USA) during the last 24 h of a 72-h culture. Cultures were harvested onto filters by use of a Tomtec Mach III harvester (Hamden, CT, USA). [³H]thymidine incorporation into DNA was measured by use of a Perkin Elmer MicroBeta2 liquid scintillation counter. Error bars represented standard deviation of triplicate sets of wells.

Induction and Assessment of EAE

CFA (Incomplete Freund's Adjuvant with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra, BD Biosciences, Franklin Lakes, NJ) was mixed 1:1 with MOG35-55 in phosphate-buffered saline. The CFA/antigen mixture was emulsified by sonication. EAE was elicited by injection of 200 μ g MOG35-55 in a total volume of 100 μ l emulsion via three SC injections of 33 μ l across the lower back. Each mouse received separate intraperitoneal injections (200 nanograms i.p.) of *Pertussis toxin* in PBS on days 0 and 2. All immunizations were performed under isoflurane anesthesia (Abbott Laboratories, Chicago, IL). Mice were assessed daily for clinical score and body weight. The following scale was used to score the clinical signs of EAE: 0, no disease; 0.5, partial paralysis of tail without ataxia; 1.0, flaccid paralysis of tail or ataxia but not both; 2.0, flaccid paralysis of tail with ataxia or impaired righting reflex; 3.0, partial hind limb paralysis marked by inability to walk upright but with ambulatory rhythm in both legs; 3.5, same as above but with full paralysis of one leg; 4.0, full hindlimb paralysis; 5.0, total hindlimb paralysis with forelimb involvement or moribund. A score of 5.0 was a humane endpoint for euthanasia.

EAE incidence was the number of EAE-afflicted mice compared to the total group size. Maximal scores were calculated as the most severe EAE score for each mouse. Mice that did not exhibit EAE had a score of zero, and these scores were included in the group average. Mice that exhibited humane endpoints as assessed by body weight loss, body score, or clinical score of 5.0 were subjected to humane euthanasia and were omitted from scoring thereafter. Time-course graphs portrayed daily mean maximal scores. Cumulative and maximal EAE scores were converted to ranked scores and analyzed by non-parametric ANOVA. To calculate percent maximal weight loss, 100% body weight was assigned as the maximal body weight obtained from day 1 through day 10, and daily body weights were calculated for each day after normalization to this 100% value. The minimum body weight was defined as the lowest body weight after normalization to the 100% value during the span of day 11 until the end of the experiment. Maximal weight loss was calculated by subtraction of the normalized minimum value from the 100% value. Negative weight loss values represented weight gain. Weight loss was analyzed by parametric ANOVA. Non-parametric and parametric ANOVA were assessed with a Bonferroni *Post Hoc* test unless noted otherwise. Incidence of EAE was analyzed pair-wise by Fisher's Exact Test. Mean EAE and weight loss data were shown with the standard error of the mean (SE).



Preparation of GMCSF-MOG in Saline, Alum, and CFA

Vaccines containing GMCSF-MOG, GMCSF-OVA, GMCSF-NFM, GM-CSF, MOG35-55, or GM-CSF + MOG35-55 were administered at a dosage of either 2 or 4 nmoles as designated in the figure legends. CFA-based vaccines were prepared with equal parts of CFA and vaccine proteins/peptides (in PBS) for a total injection volume of 100 μ l. The CFA/vaccine mixture was emulsified by sonication and injected via two SC injections of 50 μ l across the back hindquarters. Conversely, vaccines in saline (no extrinsic adjuvant) were prepared in 200 μ l of PBS and were administered SC by two injections of 100 μ l each in the back hindquarters. Vaccines administered intravenously (IV) were given in 100 μ l of PBS and injected retro-orbitally. Alum-based vaccines were prepared by mixing equal volumes of Alhydrogel adjuvant (InvivoGen) and vaccine proteins/peptides (in PBS) for a total injection volume of 150 μ l per mouse. The Alum/vaccine

mixture was incubated for 1 h on ice with continuous agitation to allow the protein/peptide to attach to the Alum gel. The vaccine was administered SC by two injections of 75 μ l each in the back hindquarters.

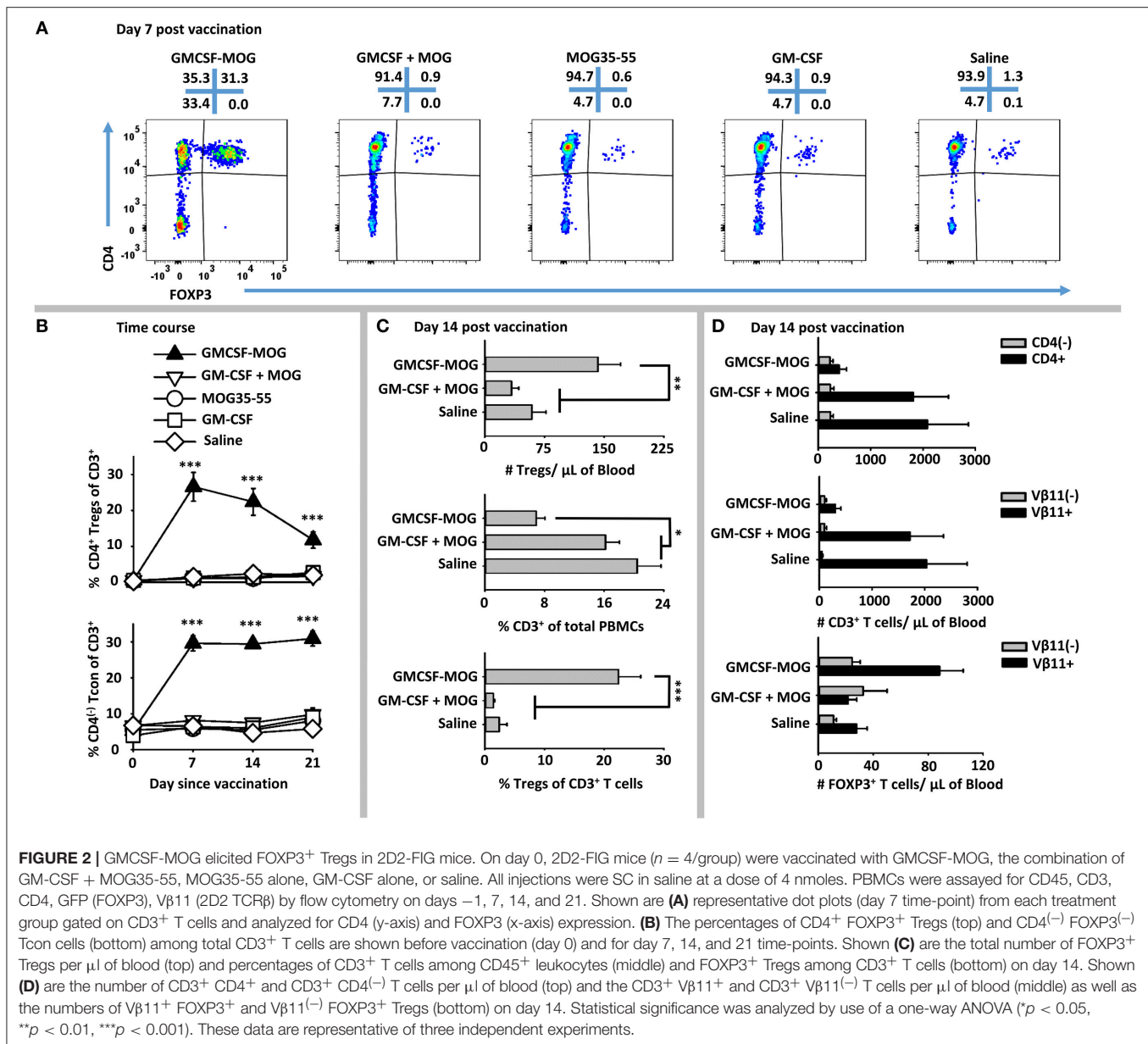
RESULTS

Depletion of FOXP3⁺ CD25⁺ Tregs With the Anti-CD25 mAb PC61 Reversed Tolerogenic Vaccination

We hypothesized that GMCSF-based tolerogenic vaccines mediated tolerance via induction of CD25^{high} FOXP3⁺ Tregs (Figure 1). To address this question, we pretreated C57BL/6 with 2 nmoles GMCSF-MOG (A) or saline (B) on days -21, -14, and -7 and then administered the anti-CD25 PC61 mAb or a an IgG1 isotype control mAb on days -4 and -2 to deplete CD25⁺ FOXP3⁺ Tregs *in vivo* (59). Mice were then subjected to active induction of EAE on day 0. Pretreatment with the anti-CD25 PC61 mAb but not the isotype control antibody eliminated circulating CD25⁺ Tregs (data not shown). Pretreatment with the anti-CD25 PC61 mAb reversed the suppressive action of the tolerogenic vaccine such that the PC61-treated mice acquired full susceptibility to EAE and showed a chronic course of paralytic EAE equivalent to mice in the control groups (Figures 1A,B). PC61 had no effect on mice vaccinated with saline, presumably because Tregs played a minimal role in mice that were fully susceptible to EAE (Figure 1B). These results were mirrored by maximal weight loss: (a) $20.3\% \pm 4.6\%$, (b) $6.7\% \pm 4.2\%$, (c) $31.3\% \pm 1.0\%$, and (d) $26.4\% \pm 4.2\%$; b vs. c, d, $p < 0.004$. In conclusion, the GMCSF-MOG vaccine elicited CD25⁺ Tregs that were required for the inhibitory action of the tolerogenic vaccine. Thus, these data revealed a causal link between GMCSF-MOG vaccination, CD25⁺ Tregs, and tolerance induction in EAE.

GMCSF-MOG Elicited a Robust FOXP3⁺ Treg Response in 2D2-FIG Mice

To address whether GMCSF-MOG expanded MOG-specific Tregs, we used 2D2-FIG mice that had a transgenic MOG-specific T cell repertoire and a GFP reporter of FOXP3 expression. 2D2-FIG mice were vaccinated SC with 4 nmoles of GMCSF-MOG, GM-CSF + MOG35-55, MOG35-55, GM-CSF in saline or with saline alone (Figure 2). A “day 0” baseline revealed that Tregs comprised <1.5% of all circulating T cells in naïve 2D2-FIG mice. By day 7 after vaccination with GMCSF-MOG, FOXP3⁺ Tregs comprised ~30% of all circulating T cells whereas mice vaccinated with control vaccines “GM-CSF + MOG35-55,” MOG35-55 alone, GM-CSF alone, or saline had baseline levels of Tregs (<1.5%) (Figure 2A). Previous research showed that covalent linkage of GM-CSF and NAg was required for the tolerogenic activity of GMCSF-MOG (30–32). Covalent linkage of GM-CSF to MOG35-55 was also required for induction of FOXP3⁺ Tregs (Figure 2A). Time course studies revealed that GMCSF-MOG vaccination increased Treg percentages to 27% of the circulating CD4⁺ T cell pool by day 7 and that significant percentages of these Tregs were maintained through days 14 (22%) and 21 (12%) (Figure 2B, top). Thus, after the

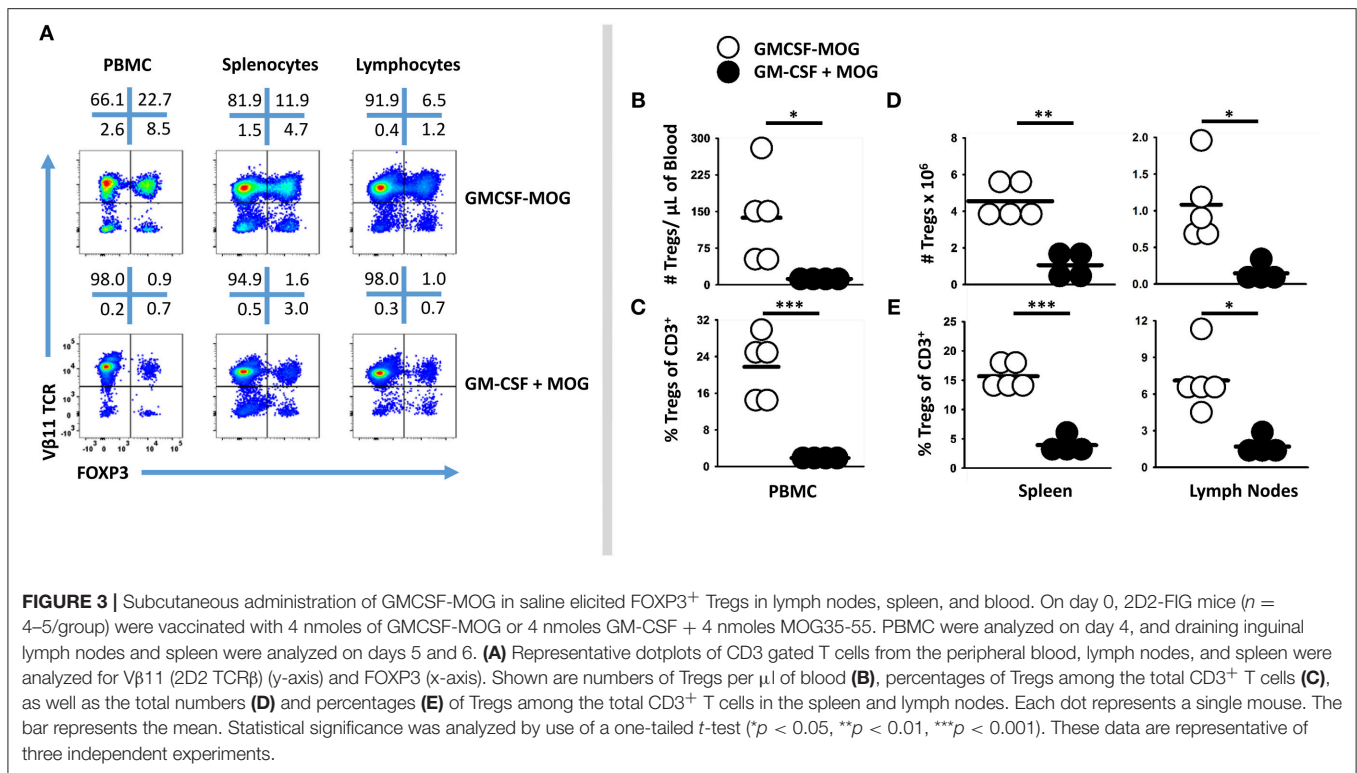


initial vaccine-mediated inductive event, Treg percentages gradually attenuated throughout the remainder of the experiment.

GMCSF-MOG vaccination resulted in an increased percentage of CD4⁻ CD3⁺ Tcon cells, such that 30% of the circulating Tcon cells lacked CD4 expression as compared to 5% of T cells in control groups on days 7, 14, and 21 (Figure 2B, bottom). The absolute number of CD4⁻ T cells/ μL of blood however was unchanged among all vaccine groups but the absolute number of CD4⁺ T cells was significantly diminished in mice that received GMCSF-MOG ($\sim 400/\mu\text{L}$ blood) as compared to control groups “GM-CSF + MOG35-55” or saline ($\sim 2,000/\mu\text{L}$ of blood) (Figure 2D, top). These data indicate the GMCSF-MOG acted indirectly to increase

percentages of CD4⁻ T cells by depleting CD4⁺ T cells rather than expanding the CD4⁻ T cell subset. Thus, GMCSF-MOG primarily affected the most reactive T cells (i.e., the CD4⁺ subset rather than the relatively non-reactive CD4⁻ subset).

GMCSF-MOG vaccination also reduced the percentages of circulating CD3⁺ T cells per total PBMCs by ~ 2.5 fold ($\sim 7\%$ CD3⁺ T cells) compared to a baseline of 16–20% T cells in control groups that received “GM-CSF + MOG35-55” or saline (Figure 2C, middle). GMCSF-MOG selectively eliminated MOG-specific V β 11⁺ (2D2 TCR β) CD3⁺ Tcon cells ($\sim 300/\mu\text{L}$ of blood) as compared to control groups ($\sim 2,000/\mu\text{L}$ of blood), whereas V β 11⁻ CD3⁺ T cells numbers remained unchanged (Figure 2D, middle). Thus,



GMCSF-MOG exhibited antigen specificity by depleting NAg-reactive Vβ11⁺ T cells while sparing non-specific Vβ11⁽⁻⁾ T cells.

GMCSF-MOG vaccination resulted in ~140 Tregs/μl of blood compared to 50–60 Tregs/μl of blood in control groups (Figure 2C, top). GMCSF-MOG also selectively expanded the number of Vβ11⁺ Tregs compared to control groups whereas Vβ11⁽⁻⁾ Tregs numbers were unchanged (Figure 2D, bottom). These data indicated that at least two factors accounted for the elevated percentages of Tregs (Figure 2C, bottom), including an increase in the absolute numbers of circulating Tregs and a decrement in the absolute numbers of Tcon cells. Overall, these data indicate that GMCSF-MOG effectively targeted MOG35-55 to myeloid APC to expand MOG-specific CD4⁺ Tregs and deplete CD4⁺ MOG-specific Tcon cells while preserving CD4⁽⁻⁾ Tcon cells.

GMCSF-MOG Elicited a System-Wide FOXP3⁺ Treg Lymphocytosis in Lymph Nodes, Spleen, and Blood

GMCSF-MOG primed a system-wide Treg response in that the vaccine elicited high frequencies of FOXP3⁺ Tregs in the spleen, draining inguinal lymph nodes, and blood (Figure 3A). 2D2-FIG mice were vaccinated with GMCSF-MOG or “GMCSF + MOG35-55” on day 0, PBMC were analyzed on day 4, and lymphoid organs were analyzed on days 5 and 6. GMCSF-MOG vaccination induced high percentages of FOXP3⁺ Tregs in all three compartments including ~22, 15, and 7% of all T cells in PBMC, spleen, and lymph nodes, respectively (Figures 3A,C,E).

Mice that received the control vaccine “GM-CSF + MOG35-55” had relatively low frequencies of FOXP3⁺ Tregs (~1, 4, and 2% Tregs in PBMC, spleen, and lymph nodes, respectively). GMCSF-MOG increased the total number of Tregs as compared to the “GM-CSF + MOG35-55” control vaccine (Figures 3B,D). GMCSF-MOG vaccination resulted in $\sim 4.5 \times 10^6$ Tregs as compared to 1.0×10^6 Tregs per spleen in “GM-CSF + MOG” vaccinated mice. Similarly, GMCSF-MOG induced $\sim 1.2 \times 10^6$ Tregs in the inguinal lymph nodes as compared to 0.2×10^6 Tregs in control nodes. These results indicate that SC vaccination with GMCSF-MOG in saline elicited Treg responses throughout the secondary lymphoid organs and the circulation.

Booster Vaccination of GMCSF-MOG Maintained Circulating Levels of FOXP3⁺ Tregs

Booster immunizations were used to assess whether repeated immunization of GMCSF-MOG elicited sustained Treg responses. 2D2-FIG mice were given three injections (days 0, 7, 14), two injections (days 7, 14), or one injection (day 14) of GMCSF-MOG (Figure 4). On day -1, baseline FOXP3⁺ Tregs as a percentage of total CD3⁺ T cells in PBMC were <1.5% for all 16 mice (Figure 4A). Vaccination with GMCSF-MOG elicited circulating FOXP3⁺ Tregs by day 4 with a range of 5–29%. By day 11, mice receiving 2 immunizations exhibited percentages of Tregs ranging from 27 to 49% of all circulating T cells whereas mice receiving 1 immunization exhibited Treg percentages ranging from 6 to 32%. Percentages of circulating Tregs on day 18 ranged from 26 to 42% (3 injections, 3x),

33–50% (2 injections, 2x), 12–44% (single injection, 1x), and ~1% for saline treated mice. Major FOXP3⁺ subpopulations were noted in both transgenic Vβ11⁺ T cells and non-transgenic Vβ11⁽⁻⁾ populations whereas FOXP3⁺ T cells were exclusively CD4⁺ (Figure 4B). Major populations of Vβ11⁽⁻⁾ Treg were attributed to the overall loss of CD3⁺ Tcon cells, resulting in elevated percentages but not numbers of Vβ11⁽⁻⁾ Tregs. Elevated percentages of FOXP3⁺ T cells in vaccinated mice mirrored increased numbers of FOXP3⁺ T cells per μl of blood (Figure 4C). As measured by FOXP3⁺ Treg frequencies or absolute numbers, the disappearance of circulating Tregs occurred at similar rates in the 3x, 2x, and 1x vaccine groups. Thus, multiple boosters maintained Tregs in circulation during repeated immunizations. For example, the 3x vaccination group showed a longer duration of Treg presence in the circulation compared to the 1x vaccination group. However, after the last vaccination on day 14, Tregs disappeared from the blood at similar rates during the next 2–3 weeks.

GMCSF-MOG (1x, 2x, and 3x) resulted in the down-regulation of TCRα/β on a per cell basis (Figure 4D). Diminished expression of TCR Vβ11 expression however did not rebound to baseline levels during this time span. Rather, lower levels of TCR-Vβ11 expression instead appeared to represent a new set-point for the 2D2 repertoire. These findings indicated that GMCSF-MOG not only elicited a major FOXP3⁺ Treg population but also desensitized T cell antigen recognition among the Tcon repertoire.

GMCSF-MOG Induced a FOXP3⁺ Population With a Canonical Treg Phenotype

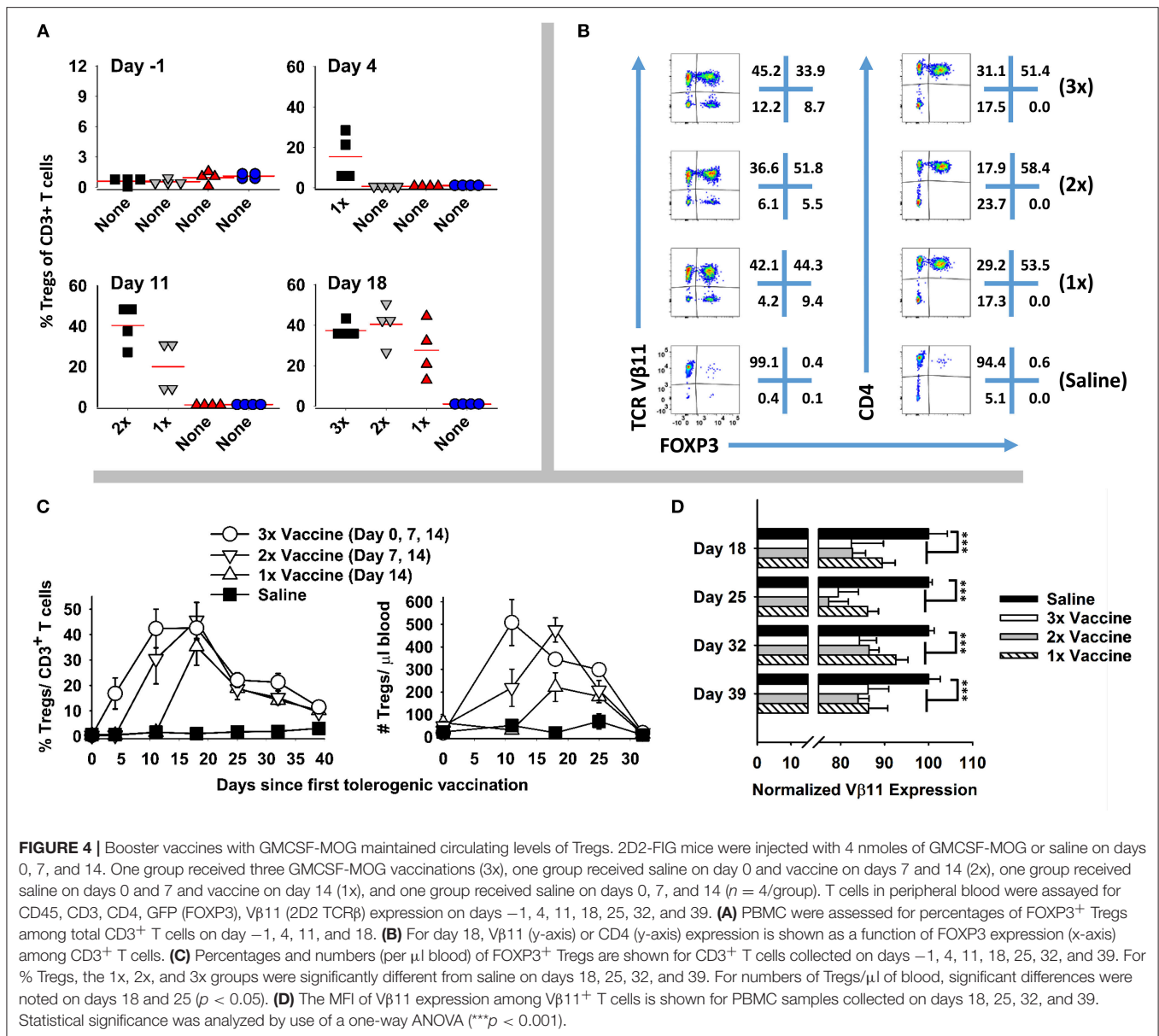
To determine the phenotype of GMCSF-MOG-induced Tregs, 2D2-FIG mice were vaccinated SC with GMCSF-MOG or “GM-CSF + MOG35-55” in saline. PBMC were analyzed on day 4 for CD44, CD62L, CD25, LAP, Neuropilin, and Ki67 expression (Figures 5A,B). GMCSF-MOG vaccinated mice exhibited significantly increased numbers and percentages of CD44^{high} CD62L^{low}, LAP^{high}, CD25^{high}, and Neuropilin^{high} Tregs in blood compared to control mice (Figures 5C,D). For example, in GMCSF-MOG vaccinated mice, ~8% of total T cells expressed a FOXP3⁺ CD44^{high}-CD26L^{low} phenotype compared to ~1% in control mice (Figure 5D). GMCSF-MOG vaccination also increased the percentages of T cells that expressed a FOXP3⁺, LAP^{high} (14%), CD25^{high} (18%), or Neuropilin^{high} (22%) phenotype compared to control mice in which <2% of the T cells were Tregs positive for any of the respective markers (Figure 5D). Interestingly, the two treatment groups did not differ in the percentages of Tregs that expressed these markers within the Treg pool, aside from a modest decrease (~13 %) in Neuropilin^{high} Tregs in GMCSF-MOG treated mice (Figure 5E). GMCSF-MOG vaccination also increased the number and percentages of T cells that were CD44^{high} Tregs compared to control mice (Figures 5F–G), whereas GMCSF-MOG vaccination increased percentages but not numbers of T cells that were CD44^{high} Tcon cells, a discrepancy that reflected the generalized loss of Tcon cells. GMCSF-MOG vaccination

increased both Treg and Tcon intracellular staining for Ki67, a marker of cell division, compared to control mice although Ki67 expression was upregulated 5-fold in Tregs vs. 2-fold in Tcon cells (Figure 5H). These data showed that GMCSF-MOG-induced Tregs have a phenotype similar to preexisting Tregs and that GMCSF-MOG favors the expansion of MOG-specific Tregs over MOG-specific Tcon cells.

The Antigenic Domain of GMCSF-Antigen Fusion Proteins Was a Major Parameter for Treg Induction

A major question was whether the GM-CSF domain or the antigenic domain of GMCSF-antigen fusion proteins represented the predominant variable polarizing T cells into the Treg lineage. To assess this issue, GMCSF-MOG, and GMCSF-OVA were compared for Treg induction in MOG-specific (2D2-FIG) vs. OVA-specific (OTII-FIG) mice. GMCSF-MOG and GMCSF-OVA were exquisitely specific in stimulating proliferation by MOG-specific T cells and OVA-specific T cells, respectively (Figures 6A,B). As expected, given that MOG represented a self-antigen and OVA represented a foreign antigen in mice, the antigenic activity of GMCSF-MOG and MOG35-55 in 2D2-FIG T cell cultures was substantially less potent than the respective GMCSF-OVA and OVA323-339 responses of OTII-FIG T cells. That is, 2D2 T cell responses to GMCSF-MOG and MOG35-55 were at least 100-fold less potent than those of OTII T cells to GMCSF-OVA and OVA323-339, respectively. Thus, MOG35-55 represented a low-affinity or inefficient T cell epitope whereas OVA323-339 represented a relatively high-affinity, high-efficiency T cell epitope in the respective systems. Both GMCSF-MOG and GMCSF-OVA displayed enhanced antigen potency as compared to their respective peptide counterparts, MOG35-55 and OVA323-339, which most likely reflected antigenic targeting to myeloid APC via GM-CSF and the GM-CSF receptor (CD116, CD131).

To assess induction of Tregs, GMCSF-MOG, GMCSF-OVA, or control vaccines GM-CSF (2D2-FIG) or saline (OTII-FIG) were used to vaccinate 2D2-FIG or OTII-FIG mice (Figures 6C–E). As expected, GMCSF-MOG vaccination caused a significant induction of absolute numbers and percentages of MOG-specific Tregs in 2D2-FIG mice (Figure 6C), including an average of 55% Tregs per the total CD3⁺ T pool by day 4 (Figure 6E, right panel). GMCSF-MOG however did not significantly elicit Tregs in OTII-FIG mice, which verified that specific T cell antigen recognition was a requirement for induction of Tregs (Figure 6D). In contrast, GMCSF-OVA vaccination of OTII-FIG or 2D2-FIG mice did not reliably elicit significant increases in the absolute numbers or percentage of Tregs compared to control mice (Figures 6C,D). These data indicated that specific T cell antigen recognition, although required in the GMCSF-MOG/2D2-FIG system, was not sufficient for induction of Tregs in the GMCSF-OVA/OTII-FIG system. These findings indicated that T cell antigen recognition was necessary but not sufficient for induction of Tregs. Rather, the quality of T cell antigen recognition was the critical parameter polarizing the Treg/Tcon balance, in that



the low efficiency ligand MOG35-55 in GMCSF-MOG was best adapted to support induction of Tregs. These experiments revealed that intrinsic qualities of the antigen covalently attached to the GM-CSF fusion partner played a key role in Treg induction.

The GM-CSF domain of GMCSF-MOG was also critical for induction of Tregs because the synthetic MOG35-55 peptide did not independently elicit Tregs (Figures 2, 3, 5). Rather, the covalently-linked GM-CSF and MOG35-55 domains were both required for efficient induction of Tregs. Interestingly, GM-CSF alone (i.e., GM-CSF, GMCSF-MOG in OTII mice, GMCSF-OVA in 2D2 mice) resulted in increased numbers and percentages of Tregs (2–10%) as compared to saline (<2% Tregs). However, these increases were transient (day 4) and were modest when compared to the effect of GMCSF-MOG vaccine in 2D2-FIG

mice (Figure 6E). These findings are consistent with previous studies showing that GM-CSF alone increased Treg proliferation in rodent models of autoimmune disease (48, 50, 51, 53, 54).

Induction of Tregs by GMCSF-MOG Was Associated With Inefficient TCR Ligation

To test whether low-efficiency TCR ligands are optimal for induction of Tregs, we devised an alternative experimental system based on the observation that the 2D2 TCR recognizes two distinct Nag, including MOG35-55 as a low affinity antigen and NFM13-37 as a high affinity antigen (60, 61). We derived an expression system for GMCSF-NFM, which exhibited GM-CSF activity equivalent to that of GMCSF-MOG, GMCSF-OVA and GM-CSF in bone marrow proliferation assays (Figure 7A). Each recombinant protein induced equivalent proliferation responses

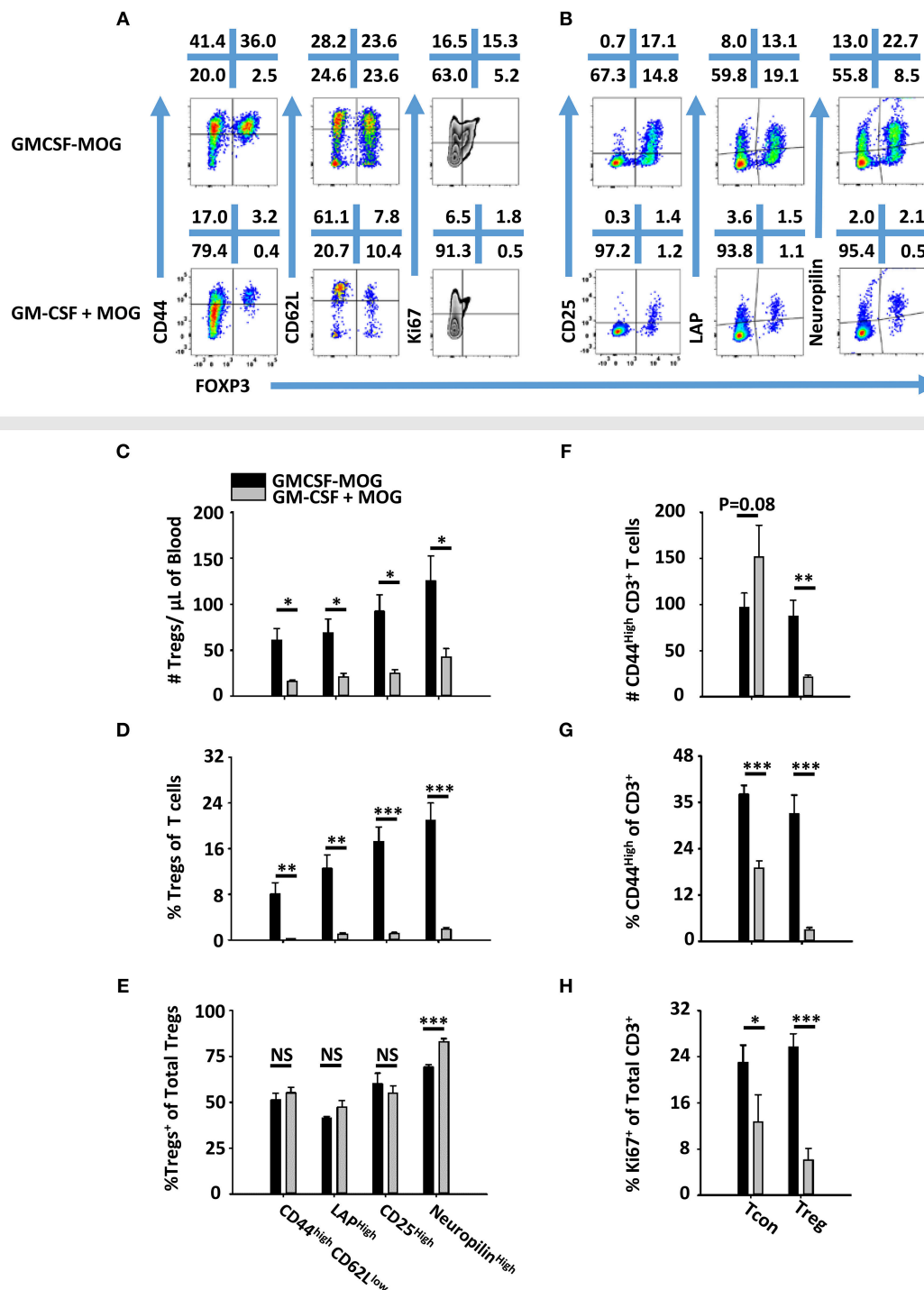


FIGURE 5 | GMCSF-MOG induced a FOXP3⁺ T cell population with a canonical Treg phenotype. On day 0, 2D2-FIG ($n = 4-5/\text{group}$) mice were vaccinated subcutaneously with 4 nmoles of GMCSF-MOG or 4 nmoles GM-CSF + 4 nmoles MOG35-55. PBMCs were analyzed on day 4. Shown (A) are representational dotplots of CD3⁺ T cells analyzed for CD44, CD62L, and Ki67 expression (y-axis) and (B) CD4⁺ T cells analyzed for CD25, LAP, and Neuropilin vs. FOXP3 expression (x-axis). Shown are numbers of Tregs per μL of blood (C) or percentages of Tregs (D) that express CD44^{high} CD62L^{low}, LAP, CD25, or Neuropilin among total T cells (D) or among Tregs (E). Shown are the Treg and Tcon cell numbers per μL of blood (F) and percentages of CD44^{high} Tcons or Tregs among CD3⁺ T cells (G). Shown are (H) percentages of Ki67⁺ Tregs or Tcons among CD3⁺ T cells. Statistical significance was analyzed by use of a one-tailed t -test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). These data are representative of two independent experiments.

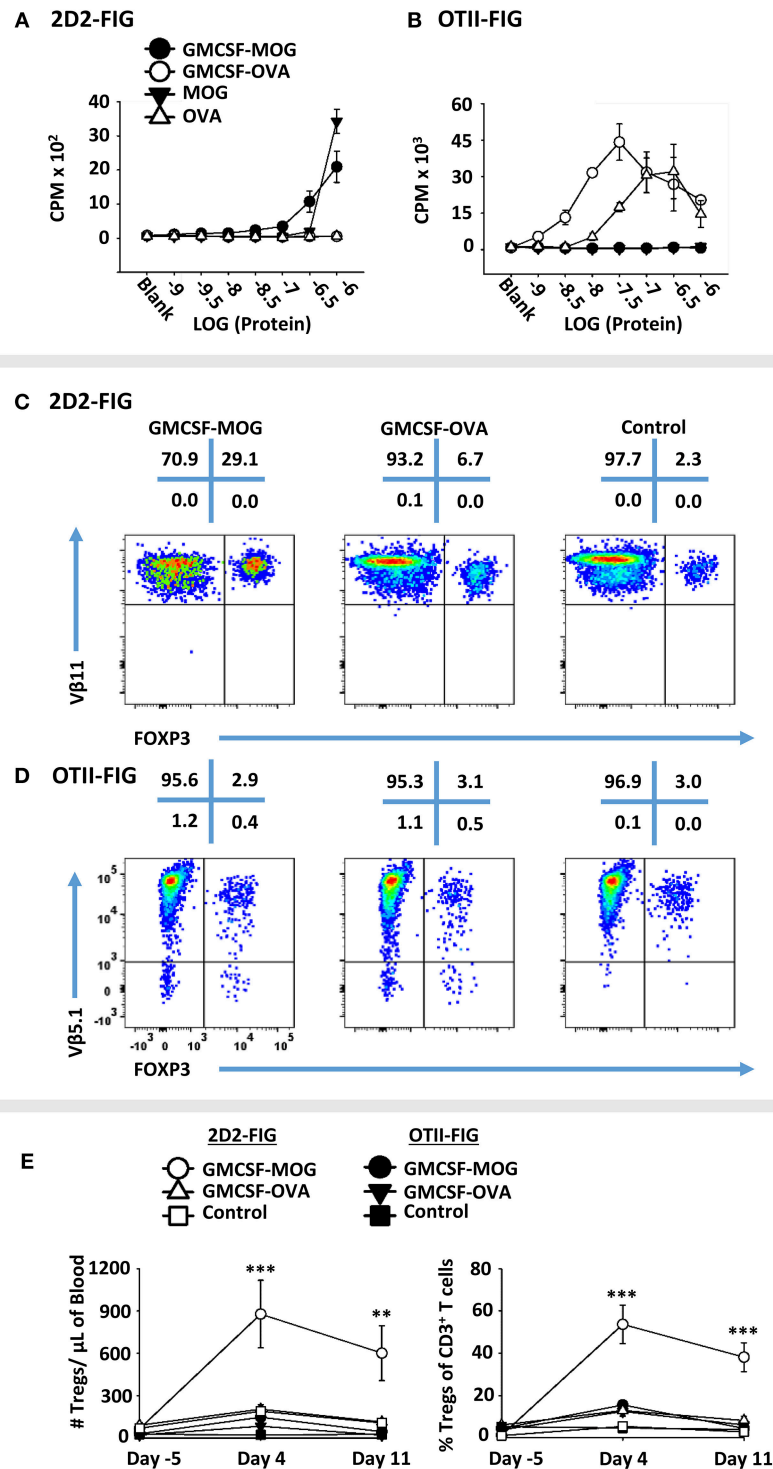


FIGURE 6 | GMCSF-MOG Treg induction was dependent upon the antigenic domain. Twenty-five thousand 2D2 T cells (**A**) or OTII T cells (**B**) from continuous T cell lines were cultured with 100,000 irradiated splenocytes and designated concentrations (x-axis) of GMCSF-MOG, GMCSF-OVA, MOG35-55, or OVA323-339. Cultures were pulsed with 1 μ Ci of [3 H]thymidine during the last 24 h of a 3-day culture, and counts per minute (CPM, y-axis) were measured on day 3. (**C–E**) On day 0, 2D2-FIG or OTII-FIG ($n = 3–4$ /group) were subcutaneously vaccinated with either 4 nmoles of GMCSF-MOG, 4 nmoles GMCSF-OVA, or with saline (OTII-FIG) or GM-CSF + MOG35-55 (2D2-FIG). PBMCs were assayed on days –5, 4, and 11 for CD3, CD4, Vβ11 (2D2 TCRβ), Vβ5.1 (OTII TCRβ) and FOXP3 expression. Representative dotplots of 2D2-FIG (**C**) and OTII-FIG (**D**) PBMCs were analyzed for Vβ11 or Vβ5.1 (y-axis) respectively and FOXP3 (x-axis) among CD3⁺ T cells. Shown (**E**) are Treg numbers per μ l of blood and Treg percentages of total CD3⁺ T cells on days –5, 4, and 11. Statistical significance was analyzed by use of a one-way ANOVA (** $p < 0.01$, *** $p < 0.001$). These data are representative of three independent experiments.

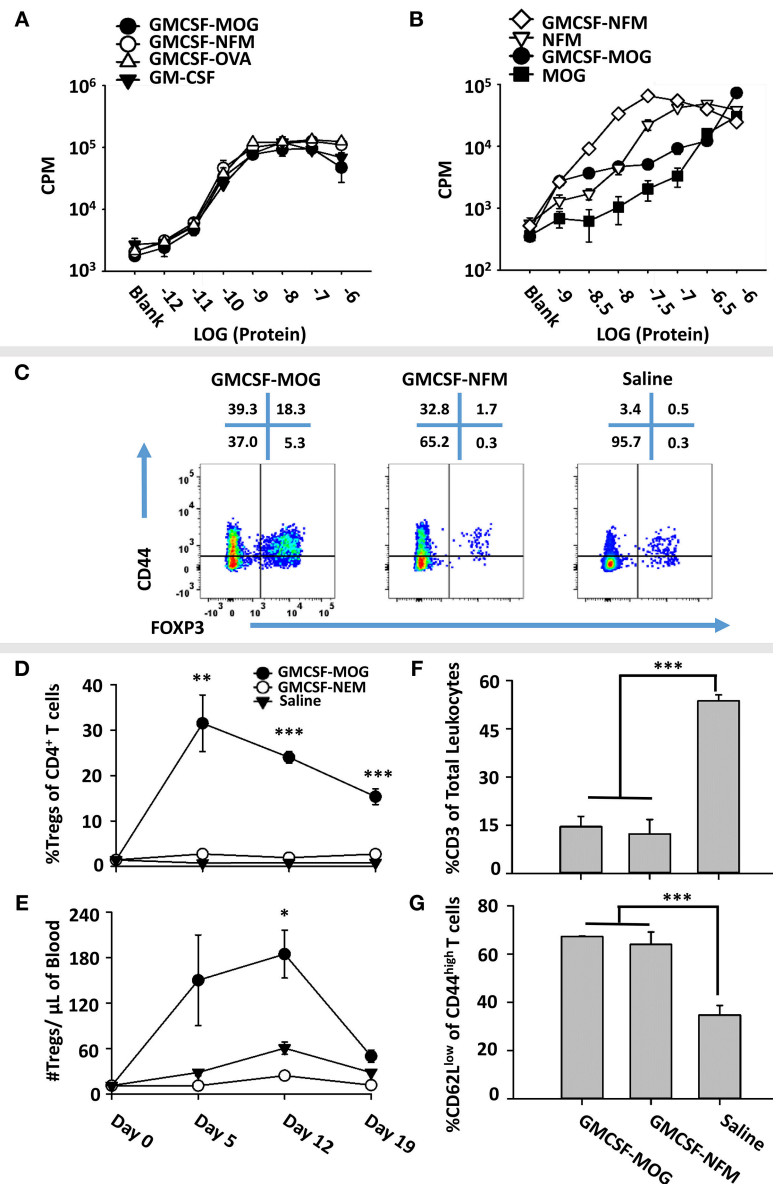
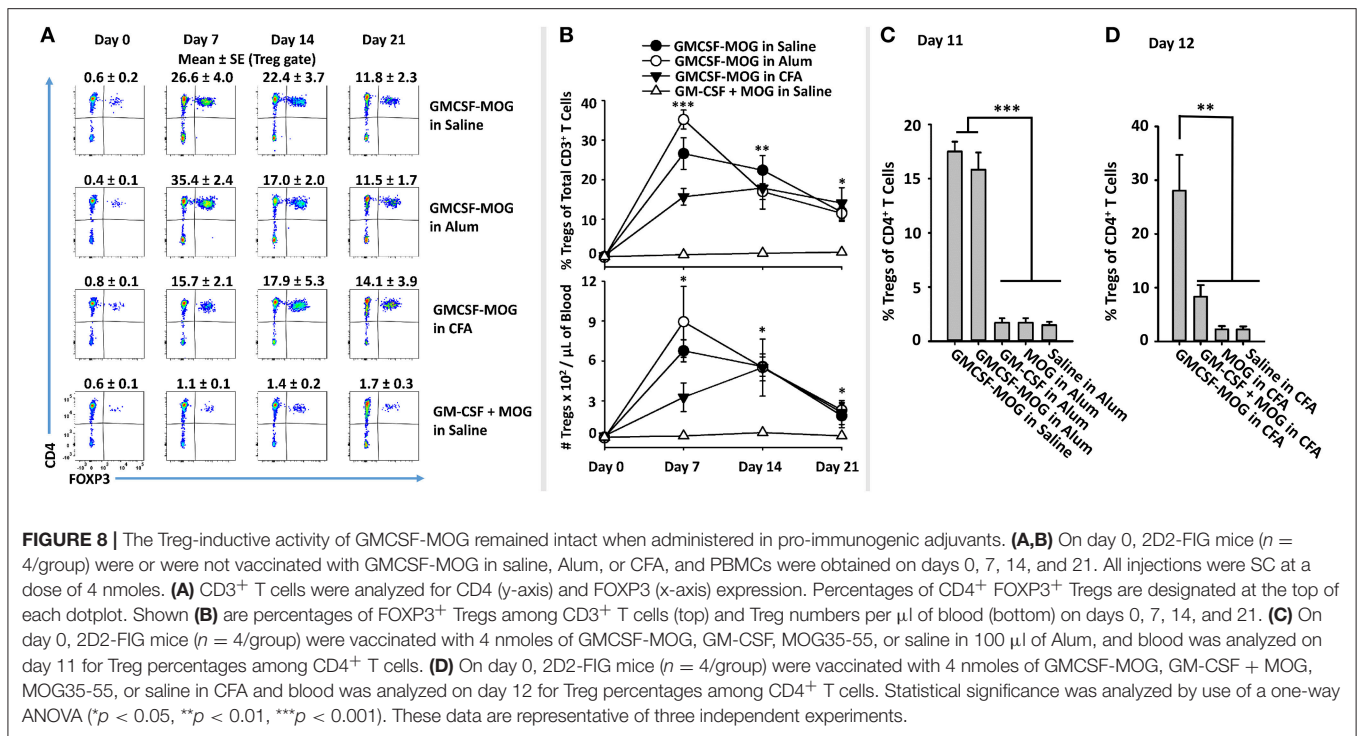


FIGURE 7 | Induction of Tregs by GMCSF-MOG was associated with inefficient TCR ligation. **(A)** Designated concentrations (x-axis) of GM-CSF, GMCSF-MOG, GMCSF-NFM, or GMCSF-OVA were incubated with 100,000 C57BL/6 bone marrow cells for 3 days. **(B)** 25,000 2D2 T cells were cultured with 100,000 irradiated splenocytes with designated concentrations (x-axis) of GMCSF-MOG, GMCSF-NFM, MOG35-55, or NFM13-37. **(A,B)** Cultures were pulsed with 1 μ Ci of [3 H]thymidine during the last 24 h of a 3-day culture. **(C–G)** On day 0, 2D2-FIG mice were subcutaneously vaccinated with either 4 nmoles of GMCSF-MOG ($n = 5$), 4 nmoles GMCSF-NFM ($n = 3$), or saline ($n = 3$). PBMCs were assayed on 5, 12, and 19 for CD3, CD4, V β 11 (2D2 TCR β), CD44, CD62L, and GFP (FOXP3) expression. The pre-day 0 bleed was derived from the average Treg percentages among CD4⁺ T cells and the average number of Tregs per μ L of blood ($N = 50$ mice from 3 independent experiments). **(C)** On day 12, PBMCs from GMCSF-MOG, GMCSF-NFM, or saline treated mice were analyzed for CD44 (y-axis) and FOXP3 (x-axis) among CD3⁺ CD4⁺ T cells. Shown **(D,E)** are Treg numbers per μ L of blood and Treg percentages among CD4⁺ T cells on days 5, 12, and 19. Shown are percentages of CD3⁺ T cells among total CD45⁺ leukocytes **(F)** and percentages of CD62L^{low} cells among CD44^{high} CD3⁺ CD4⁺ T cells **(G)** on day 12. Statistical significance was analyzed by use of a one-way ANOVA ($p < 0.05$, $**p < 0.01$, $***p < 0.001$). These data are representative of three independent experiments.

with a half-maximal stimulation in the 10–100 picomolar range. These assays confirmed that C-terminal antigenic domains did not affect potency of the GM-CSF cytokine. To measure activity of the antigenic domain, GMCSF-MOG, GMCSF-NFM, MOG35-55, and NFM13-37 were compared in 2D2 T cell proliferative assays (**Figure 7B**). GMCSF-NFM exhibited the

highest potency (half-maximal stimulation at $\sim 3.2 - 10$ nM). GMCSF-NFM was ~ 10 -fold more potent than NFM13-37 and was several orders of magnitude more active than either GMCSF-MOG or MOG35-55.

To assess induction of Tregs, GMCSF-MOG, GMCSF-NFM, or saline were used to vaccinate 2D2-FIG mice, and PBMCs



were analyzed on day 4 for Treg induction. GMCSF-MOG induced high percentages of FOXP3⁺ Tregs in the CD4⁺ T cell pool. Importantly, GMCSF-NFM lacked activities necessary for induction of Tregs (~1%) (Figure 7C). GMCSF-MOG induced a sustained Treg response as shown by high percentages of Tregs on day 5 (30%), day 12 (25%), and day 19 (20%) and increased numbers of Tregs/ μl of blood on day 5 (130/ μl) and day 12 (180/ μl). In contrast, GMCSF-NFM did not affect Treg numbers or percentages during the 19 days of the experiment (Figures 7D,E). These data support the hypothesis that GMCSF-antigen fusion proteins containing high-efficiency TCR ligands lack activities required for the robust induction of FOXP3⁺ Tregs. Although GMCSF-MOG and GMCSF-NFM differed qualitatively in activities needed for the induction of Tregs, both vaccines caused activation of the 2D2-FIG T cell repertoire as shown by the increased percentages of CD62L^{low} CD44^{high} T cells (Figure 7G). Both vaccines also caused the diminution of the 2D2 Tcon repertoire (Figure 7F), which may represent a separate mechanism of tolerance.

The Treg-Inductive Activity of GMCSF-MOG Remained Intact When Administered in Pro-immunogenic Adjuvants

Previous studies showed that GMCSF-MOG and GMCSF-PLP(139–151) imposed tolerogenic outcomes at relatively low doses even when emulsified with the respective encephalitogenic peptide in CFA in the C57BL/6 and SJL models of EAE (29). These data indicated that GMCSF-NAg exerted operational tolerance even in strong pro-inflammatory environments. For

example, inclusion of 1 n mole GMCSF-MOG with 77.5 nmoles of MOG35-55 in CFA strongly inhibited incidence, severity, and duration of EAE compared to mice immunized with the CFA/MOG35-55 emulsion without GMCSF-MOG. These data raised the important question of whether GMCSF-MOG would retain Treg inductive capacity in adjuvant primed proinflammatory environments.

To assess this question, GMCSF-MOG was prepared in saline, Alum, or CFA and injected into 2D2-FIG mice (Figures 8A,B). Alum vaccine formulations incorporated GMCSF-MOG, GM-CSF, MOG35-55, or saline into the Alum adjuvant. A single vaccination of GMCSF-MOG/CFA, GMCSF-MOG/Alum, and GMCSF-MOG in saline elicited high percentages of circulating Tregs that persisted through the 21-day assessment (Figure 8B). On day 7, the GMCSF-MOG/Alum and GMCSF-MOG/saline vaccines elicited higher Treg percentages than GMCSF-MOG/CFA although these differences disappeared by days 14 and 21 (Figure 8B). On day 11, GMCSF-MOG in saline and GMCSF-MOG/Alum both elicited high percentages of Tregs while GMCSF/Alum, MOG35-55/Alum, and saline/Alum did not elicit significant Treg responses (Figure 8C). These findings are consistent with the concept that low-efficiency T cell antigen recognition events are integrated within the confines of an immunological synapse and can support Treg induction without regard to local inflammatory environmental cues.

A related question was whether incorporation of GMCSF-MOG into CFA would relieve the strict requirement for covalent coupling of GM-CSF and NAg, because the two domains would be physically sequestered in the same antigenic depot. Vaccine formulations in CFA included GMCSF-MOG, “GMCSF + MOG35-55,” MOG35-55, or saline in CFA. GMCSF-MOG/CFA

induced a significant Treg response whereas the other CFA-based vaccine formulations including “GMCSF + MOG35-55” in CFA lacked robust Treg inductive capability (**Figure 8D**). These data indicate that GMCSF-MOG exerted a dominant Treg response even within the context of a CFA adjuvant-primed lymphatic drainage. However, co-localization of independent GM-CSF and NAg molecules in the same adjuvant-based antigenic depot did not relieve the requirement for covalent cytokine-NAg linkage. These data suggest that a continued requirement of GMCSF-NAg linkage is needed for Treg induction during and/or after the immunological processing of the CFA antigenic depot.

Subcutaneous and Intravenous Routes of GMCSF-MOG Administration Drove Robust Treg Responses

The observation that SC GMCSF-MOG vaccination elicited the highest Treg frequencies in the blood (30–40%) rather than the spleen (13–15%) or lymph nodes (6%) raised questions whether the Treg inductive response required a classical lymphatic drainage (**Figure 3**). The expectation was that SC injection of GMCSF-MOG would target MOG35-55 to myeloid APC in the draining lymphatics at the site of inoculation and that sensitization of the Treg response would occur in the draining lymphatics. Conversely, IV administration of GMCSF-MOG would predictably bypass the lymphatic drainage and instead introduce NAg directly into the blood, spleen, lung, and liver. To assess this question, the optimal route of GMCSF-MOG administration was tested by comparing SC or IV injections in 2D2-FIG mice. Notably, either route was equally effective. Administration of GMCSF-MOG via either SC and IV route caused robust Treg responses marked by ~25% Tregs (of the total CD4⁺ T cell population) as compared to mock (saline) vaccination (3% Tregs) when assessed on day 12 (**Figures 9A,B**). Both SC and IV vaccination also resulted in increased percentages of activated 2D2 T cells marked by high expression levels of CD44 and low levels of CD62L (**Figures 9A,E**). Although both SC and IV administration of GMCSF-MOG caused the activation of both Treg and Tcon subsets, the vaccine preferentially drove the expansion Tregs in both cases. Mice vaccinated by either route had significantly increased levels of Treg per μ l of blood (~180) as compared to saline (~60) (**Figure 9C**) and reduced percentages and numbers of circulating CD3⁺ T cells (~900) as compared to saline (~8,000) (**Figure 9D**). These results indicate that neither cutaneous APC nor draining lymphatics are required for GMCSF-MOG-mediated Treg induction.

Vaccine-Induced Kinetics Controlling Treg Emergence Was a Function of Pre-existing Treg Levels

A central question was whether tolerogenic GMCSF-MOG vaccination required pre-existing Tregs to stage the rapid and predominant FOXP3⁺ Treg response that occurred in 3–4 days among the circulating MOG-specific repertoire. That is, did GMCSF-MOG drive expansion of pre-existing Tregs or did GMCSF-MOG induce *de novo* differentiation of Tregs from naïve T cell precursors? To gain insight into this question,

2D2-FIG-*Rag1*^{−/−} mice were derived because these mice largely lack FOXP3⁺ Tregs of either thymic or peripheral origin. TCR transgenic *Rag1*^{−/−} naïve T cells however have the capacity to differentiate into iTregs/pTregs (i.e., inducible Tregs, peripheral Tregs) upon TGF- β signaling during cellular activation.

As expected, naïve 2D2-FIG-*Rag1*^{−/−} mice exhibited a substantial 120-fold reduction in Treg percentages in that 2D2-FIG-*Rag1*^{−/−} mice averaged 0.007% Tregs compared to 0.845% 2D2-FIG Tregs in the circulating CD4⁺ pool (**Figure 10A**). These data indicated that 2D2-FIG-*Rag1*^{−/−} mice had profound reductions in Tregs but were not devoid of Tregs. Notably, 2D2-FIG-*Rag1*^{−/−} mice exhibited substantially delayed kinetics in response to tolerogenic GMCSF-MOG, which elicited 1% and 30% Tregs in 2D2-FIG-*Rag1*^{−/−} and 2D2-FIG mice, respectively, by day 5 (**Figure 10C**, Top). In accordance, 2D2-FIG-*Rag1*^{−/−} mice and 2D2-FIG mice averaged 4 and 150 Tregs per μ l of blood respectively (**Figure 10C**, bottom) on day 5. By day 12 however, GMCSF-MOG vaccinated 2D2-FIG-*Rag1*^{−/−} mice had high numbers and percentages of circulating Tregs that closely approximated the circulating Treg population in 2D2-FIG mice (~20% Tregs, **Figure 10C**). Mice injected with saline or “GM-CSF + MOG” control vaccines did not exhibit significant increases in Tregs (**Figure 10B**). The delayed Treg induction in 2D2-FIG-*Rag1*^{−/−} mice was not due to deficient exposure to MOG35-55 because GMCSF-MOG caused equivalent decrements in circulating Tcon numbers and equivalent elevations in CD44^{high} CD62L^{low} T cell numbers (per μ l of blood) in both mouse strains by day 12 (**Figure 10D**).

Collectively, these data are consistent with the hypothesis that GMCSF-MOG vaccination selectively amplifies pre-existing Tregs to stage the rapid accumulation of Tregs. However, these data did not exclude the possibility that GMCSF-MOG may also drive *de novo* differentiation of naïve T cells into Tregs, perhaps abetted by pre-existing Tregs. Notably, when given sufficient time, GMCSF-MOG has sufficient tolerogenic efficacy to elicit large Treg populations in both 2D2-FIG-*Rag1*^{−/−} and 2D2-FIG mice. These data reveal that the tolerogenic activity of GMCSF-MOG does not require clonotypic diversity in the T cell repertoire or an intact B cell repertoire because 2D2-FIG-*Rag1*^{−/−} mice lack endogenous TCR-alpha chain rearrangements and are largely devoid of B cells.

DISCUSSION

The GM-CSF Domain of GMCSF-NAg Mediates Antigen-Targeting and APC-Conditioning to Elicit Tregs

Fusion proteins comprised of GM-CSF and major encephalitogenic peptides of myelin are potent NAg-specific tolerogenic vaccines. When administered before disease onset, these vaccines prevent the subsequent induction of EAE. When administration is initiated after onset of severe paralytic disease, these vaccines are therapeutic interventions that reverse EAE (28–32). This study provides two lines of evidence that GMCSF-NAg tolerogenic vaccines mediate NAg-specific tolerogenic activity, at least in part, by induction of CD25^{high} FOXP3⁺

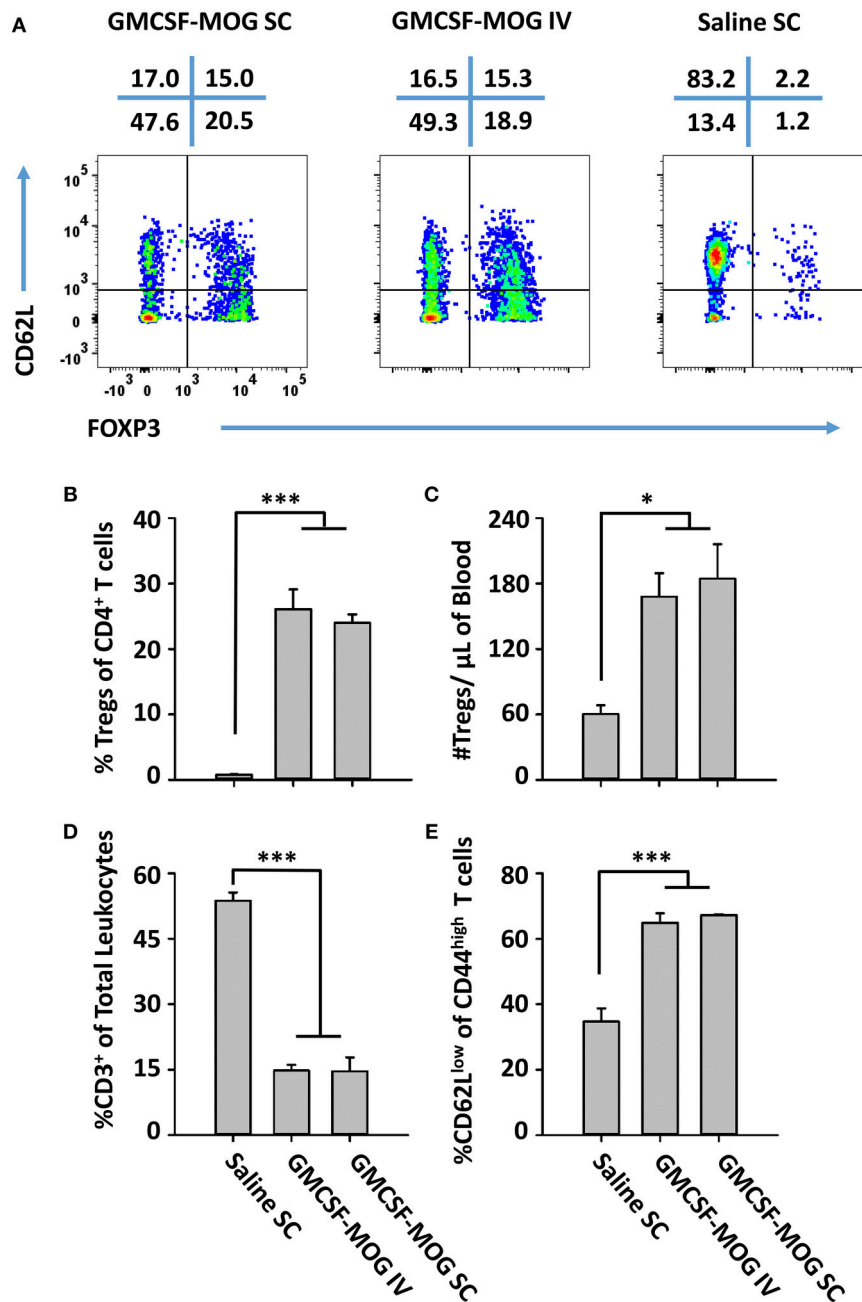


FIGURE 9 | GMCSF-MOG induced Tregs when administered intravenously. On day 0, 2D2-FIG mice were vaccinated with 4 nmoles of GMCSF-MOG intravenously via the retro-orbital route ($n = 5$) or by SC ($n = 5$) injection or were vaccinated SC with saline alone ($n = 3$). Blood was analyzed on day 12. **(A)** CD3⁺ CD4⁺ CD44^{high} T cells were analyzed for CD62L expression (y-axis) and FOXP3 (x-axis). The percentages of each quadrant are designated at the top of each dotplot. Shown **(B)** are the percentages of FOXP3⁺ Tregs among total CD3⁺ CD4⁺ T cells and **(C)** total numbers of FOXP3⁺ Tregs per μ L of blood. Shown **(D)** are percentages of CD3⁺ T cells among total leukocytes in the blood. Shown **(E)** are the percentages of CD62L^{low} T cells among CD44^{high} CD3⁺ CD4⁺ T cells. Mean percentages of CD44⁺ T cells in the CD3⁺ CD4⁺ T cell pool for the “GMCSF-MOG IV” and “GMCSF-MOG SC” groups ($61\% \pm 4\%$, $54\% \pm 4\%$, respectively) were significantly different from those for the saline group ($6\% \pm 1\%$) ($p \leq 0.001$). Statistical significance was analyzed by use of a One-way ANOVA ($*p < 0.05$, $***p < 0.001$). These data are representative of two independent experiments.

regulatory T cells (Tregs). First, the prophylactic activity of GMCSF-NAg was reversed by the subsequent treatment with an anti-CD25 mAb that depleted the CD25^{high} Treg subset *in*

vivo. GMCSF-MOG vaccinated mice treated with the anti-CD25 PC61 mAb exhibited severe EAE comparable to non-vaccinated littermates (**Figure 1**). Second, SC GMCSF-NAg administration

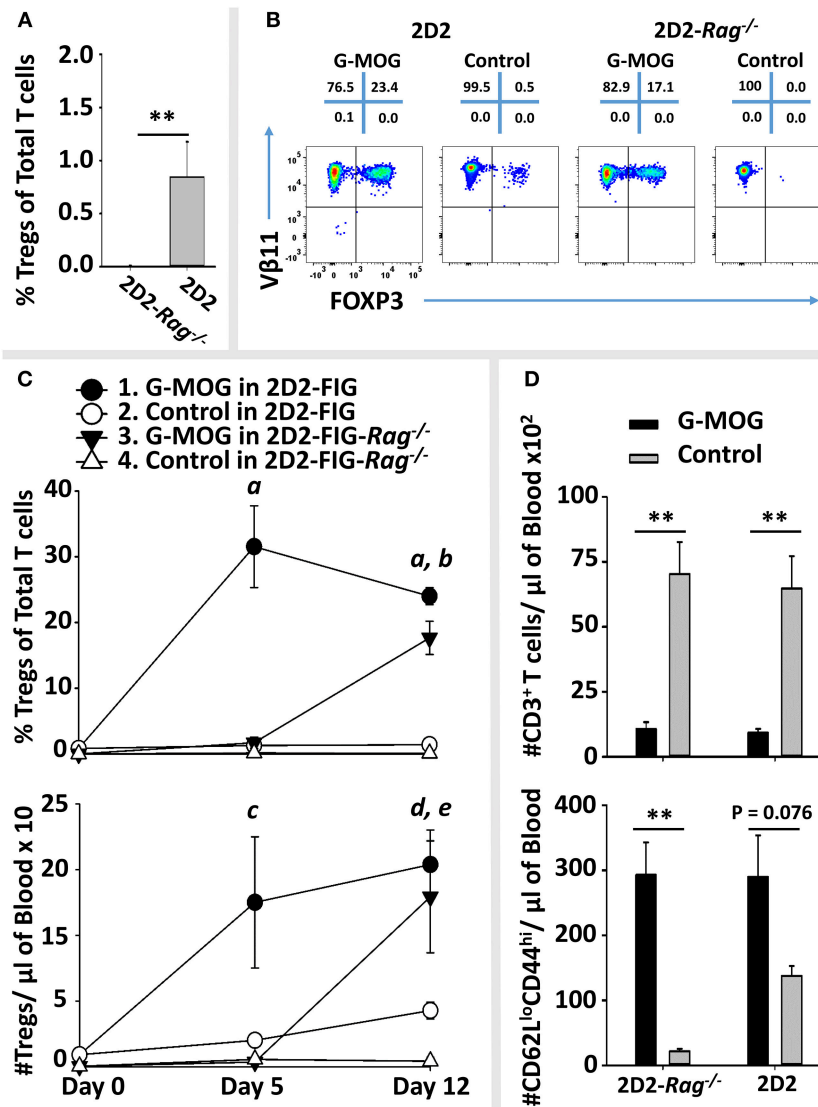


FIGURE 10 | Pre-existing FOXP3⁺ MOG-specific Tregs are associated with rapid expansion of Tregs following GMCSF-MOG (G-MOG) vaccination. **(A)** Shown are percentages of circulating FOXP3⁺ Tregs in the CD3⁺ CD4⁺ T cell pool of naïve untreated 2D2-FIG ($n = 13$) and 2D2-FIG-Rag1^{-/-} ($n = 19$) mice. **(B–D)** On day 0, 2D2-FIG ($n = 5$) and 2D2-FIG-Rag1^{-/-} ($n = 4$) mice were vaccinated subcutaneously with 4 nmoles of GMCSF-MOG or with control vaccines (saline alone in 2D2-FIG mice or “4 nmoles GM-CSF + 4 nmoles MOG35-55” in 2D2-FIG-Rag1^{-/-} mice). PBMCs were analyzed on day 5 and 12. **(B)** Shown are representative dotplots of CD3⁺ CD4⁺ T cells analyzed for FOXP3 expression (x-axis) and Vβ11 (y-axis) on day 12 post vaccination. **(C)** Shown are percentages (top) and numbers (bottom) of FOXP3⁺ Tregs in the CD4⁺ T cell pool on days 5 and 12. Group sizes for day 0 were supplemented with historical data (2D2-FIG mice, total $n = 50$ and $n = 13$; and 2D2-FIG-Rag1^{-/-} mice, $n = 19$ and $n = 10$) for calculation of average Treg percentages and Tregs per μl of blood, respectively. **(D)** Shown are the number of CD3⁺ T cells (top) and the number of CD62L^{low} CD44^{high} T cells (bottom) per μl of blood on day 12. Statistical significance was analyzed by use of a one-way ANOVA. Pairwise comparisons were performed by use of the Holm-Sidak method (** $p < 0.01$). Statistically significant ($p < 0.05$) pairwise comparisons for **(C)**: a, 1 vs. 2, 3, and 4; b, 3 vs. 2 and 4; c, 1 vs. 4; d, 1 vs. 2 and 4; e, 3 vs. 4. These data are representative of three independent experiments.

in saline elicited a rapid Treg response in 2D2-FIG mice that developed in 3 days and persisted as circulating Tregs for several weeks (Figures 2, 4, 5). The vaccine-induced Treg response was robust in that ~20–50% of circulating T cells were FOXP3^{high} and expressed the Vβ11⁺, Vα3.2⁺ 2D2 TCR. This MOG-specific Treg population was also expanded in the spleen and lymph nodes (Figure 3). These data indicated that GMCSF-NAg targeted NAg to a myeloid APC compartment specialized

for induction, expansion, and/or maintenance of FOXP3⁺ Tregs.

Several previous studies showed that GMCSF-NAg fusion proteins targeted NAg to myeloid APC for enhanced presentation *in vitro* (32). In the presence of enriched myeloid APC, GMCSF-NAg exhibited antigenic activity that was substantially more potent than NAg alone. For example, the antigenic activity of a rat GMCSF-NAg was 1,000-fold more potent than NAg alone, and

this potency enhancement was reversed by free soluble GM-CSF but not by free soluble M-CSF. Likewise, the antigenic potency of a MCSF-NAg fusion protein was blocked by free soluble M-CSF but not by GM-CSF. The enhanced potency of GMCSF-NAg was observed in cultures with purified myeloid APC but was absent in cultures of B cell or T cell APC (32). Murine fusion proteins, including GMCSF-PLP139-151 and GMCSF-MOG35-55 also exhibited an enhancement of antigenic potency due to antigenic targeting of the NAg domain to myeloid APC (30, 31). Antigenic targeting by GMCSF-NAg was also evident in antigen pulse experiments in which GMCSF-MOG was qualitatively more active than “GM-CSF + MOG35-55” in targeting NAg to myeloid APC for the subsequent NAg presentation (29). The GM-CSF domain of GMCSF-NAg not only facilitated antigen targeting for MHCII-mediated NAg presentation, the GM-CSF domain also elicited immunoregulatory activities including an IFN- γ -dependent competence for NAg-stimulated nitric oxide production that abrogated T cell expansion (29). These data indicated that the antigen-targeting and myeloid APC-conditioning activities of GMCSF-NAg may facilitate a mechanism of negative antigen presentation that favors regulatory T cell responses over immunogenic responses.

The physical linkage between the GM-CSF domain and the NAg domain was required not only for targeted antigen presentation *in vitro*, physical linkage was also required for tolerogenic activity *in vivo* as shown in rat and mouse models of EAE. GMCSF-NAg elicited tolerance that suppressed EAE whereas control vaccine formulations including a mixture of “GMCSF + NAg,” GM-CSF alone, NAg alone had no effect (30–32). Covalent GMCSF-NAg linkage was required in both prophylactic and interventional models of EAE. Covalent GMCSF-NAg linkage was also required for induction of a robust Treg response and depletion of the MOG-specific T cell repertoire (Figures 2, 3, 5). The requirement for GMCSF-NAg linkage may reflect a common mechanism of targeted antigen presentation as the basis for different dimensions of the vaccine-mediated response, including tolerance induction, therapeutic intervention, inhibitory antigen presentation, Treg induction, Tcon depletion, and TCR desensitization.

FOXP3⁺ Tregs have been studied extensively over the past several decades, but the “modus operandi” that governs Treg specification and function remains a mystery of contemporary immunology (62). Tregs are maintained in specialized environments characterized by cytokines and antigens that maintain viability, phenotypic stability, and functional activity. The cytokine environment that sustains Tregs is comprised of low-intensity, chronic IL-2 stimulation that favors competitive Treg dominance due to superlative CD25 expression (63). A specialized antigenic niche may also be a necessary foundation for the induction and maintenance of the FOXP3⁺ Treg repertoire (64–70). Although the existence of specialized antigenic compartments for Tregs is commonly assumed by many scientists, the physical and functional basis for such an antigen compartment remains hypothetical. This study supports the concept of an antigenic Treg niche, because a reasonable interpretation is that GMCSF-NAg selectively loads NAg into specialized antigen-processing domain of myeloid APC to confer

an antigenic niche that drives dominant NAg-specific FOXP3⁺ Treg responses.

The NAg Domain of GMCSF-NAg Vaccines Couples Inefficient TCR Recognition With Treg Induction in Both Quiescent and Inflammatory Environments

GMCSF-MOG imposed tolerance in quiescent non-stimulated environments as well as in strongly immunogenic environments, including a CFA-primed lymphatic drainage (29). Thus, GMCSF-NAg and the associated mechanism of inhibitory antigen presentation ameliorated EAE even when GMCSF-MOG or GMCSF-PLP was mixed in the CFA emulsion with the relevant encephalitogenic peptide. This observation contradicts the dogma that APC in quiescent “steady-state” environments are tolerogenic whereas APC in an activated inflammatory environment are immunogenic. Clearly, mechanisms of tolerance must also function in inflamed tissues during adaptive immune responses. Otherwise, immunity to foreign antigens would routinely lead to pathogenic autoimmunity, particularly in response to persistent environmental or infectious agents. Similar difficulties lie in ascribing the Treg niche to either immature vs. mature DC because both types of DC have suppressive properties in select experimental models (71–74). This “DC maturity” paradigm explains the adaptive exposure of peripherally-acquired antigens within secondary lymphoid organs but does not support a conceptually cohesive argument as to why immature vs. mature DC would, respectively, favor regulatory or immunogenic responses (or vice versa). GMCSF-MOG, when emulsified with CFA with a vast excess of encephalitogenic peptide, nonetheless inhibited EAE even though the draining lymphatics are replete with activated, mature DCs. These considerations indicate that the tolerogenic activity of GMCSF-NAg may involve parameters apart from quiescence or immaturity of the myeloid APC subset.

Although the GM-CSF domain was critical for antigen targeting, APC-conditioning, and inhibitory antigen presentation, a central finding of the current study was that the NAg domain was also critical for tolerogenic responses based on the quality of TCR-dependent signaling (Figures 6, 7). Thus, GMCSF-MOG and GMCSF-NFM interact with the same 2D2 transgenic TCR but did or did not exhibit Treg inductive activity in association with low vs. high efficiency TCR interactions, respectively. Likewise, GMCSF-OVA, which contained an epitope recognized as a high-affinity ligand in OTII-FIG mice, lacked Treg inductive activity. These findings indicate that GMCSF-targeting of low-efficiency vs. high efficiency NAg/TCR interactions favors differentiation of regulatory vs. conventional T cell subsets, respectively. One possibility is that inefficient TCR recognition resulted in low levels of CD40L induction on CD4⁺ T cells and consequently inefficient CD40 engagement and low levels of APC-mediated costimulation. Other related non-exclusive possibilities could be also considered. For example, targeted low-affinity TCR interactions may result in limiting levels of IL-2 sufficient to support CD25^{high} Treg responses. Low levels of IL-2 may be insufficient to sustain Tcon cells that express

intrinsically lower levels of CD25. Notably, low-affinity NAG may be the common rule because several GMCSF-NAG fusion proteins that incorporated diverse encephalitogenic myelin peptides, including MBP72-86, PLP139-151, and MOG35-55, were potent tolerogens in EAE. The low affinity nature of myelin peptides may reflect generalized mechanisms of self-tolerance, whereby high-affinity self-reactive clones are deleted during maturation and only low-affinity self-reactive clones persist in the periphery. Although high-affinity NFM13-37 peptide may represent an exception in regard to the specific 2D2 clonotype, the overall NFM-reactive repertoire may primarily comprise low-affinity clones because NFM13-37 lacks encephalitogenic activity in C57BL/6 mice (60).

As noted for tolerance induction, the ability of GMCSF-MOG to elicit Treg responses was replete in either quiescent or adjuvant-primed immunogenic environments. That is, GMCSF-MOG emulsified in the Th2 adjuvant Alum or the Th1-adjuvant CFA elicited Treg responses that were equal to that of GMCSF-MOG (in saline) during 14–21 days post-vaccination (**Figure 8**). Importantly, GMCSF-MOG/Alum or GMCSF-MOG/CFA both exhibited robust Treg-inductive activity, but a mixture of GM-CSF and NAG in CFA lacked Treg inductive capability. Thus, GMCSF-MOG exhibited a requirement for covalent linkage of cytokine and NAG domains when emulsified in CFA adjuvant or when administered in saline. One might assume that the incorporation of unlinked GM-CSF and NAG in an adjuvant would provide a non-covalent physical association in that both entities were physically sequestered in the same antigen depot, but this type of physical association was not sufficient to support the induction of Tregs. These data provide evidence that GMCSF-MOG must retain physically-connected cytokine and NAG domains after immunological extraction from the emulsion during subsequent and perhaps indirect antigen processing steps that lead to tolerogenic antigen presentation.

GM-CSF Has Complex Proinflammatory and Anti-inflammatory Activities

A substantial literature supports the concept that GM-CSF has a pro-inflammatory role in immunity, including autoimmunity and EAE. For example, GM-CSF-deficient mice exhibited profound resistance to EAE, and anti-GM-CSF mAb inhibited EAE (75–81). However, treatment of GM-CSF-deficient mice with an anti-CD25 mAb that depleted CD25^{high} FOXP3⁺ Tregs restored full disease susceptibility in both active and passive models of EAE (58). These data indicate that the Csf2^{-/-} mice lack the responsive capability to overcome the natural resistance of the Treg repertoire in this model of EAE. A substantial literature also supports the concept that GM-CSF has a profound tolerogenic role in immunological disease. Administration of exogenous GM-CSF is known to inhibit several models of autoimmunity, including experimental autoimmune myasthenia gravis, experimental autoimmune thyroiditis, and type I diabetes via the induction of “tolerogenic DC” and regulatory T cell subsets (43, 46–56, 82–91). This study may provide insight into immunogenic vs. tolerogenic roles of GM-CSF in that

GM-CSF-conditioned myeloid DC may integrate the intrinsic efficacy of TCR signaling within a local environment or across an immunological synapse to establish dominance of conventional or regulatory T cell responses.

The GMCSF-NAG Vaccines Have Unique Tolerogenic Activities

The observation that GMCSF-NAG, when emulsified in CFA, retains both Treg-inductive and tolerogenic activities reveals unique attributes of this tolerogenic vaccine platform. Many other types of tolerogenic vaccine, if emulsified in a pro-inflammatory adjuvant like CFA, would likely cause rather than prevent autoimmune pathogenesis. Another unique attribute of the GMCSF-NAG vaccine platform is that both SC and IV routes were equally effective for induction of robust Treg responses (**Figure 9**). Other vaccine platforms are contingent upon intravenous administration whereas SC administration is countermanded and potentially immunogenic. One assumes that SC administration of GMCSF-MOG introduces the vaccine in the lymphatic drainage to stage the tolerogenic response. However, the strong tolerogenic efficacy of intravenous GMCSF-MOG indicates that blood-borne immunological organs (spleen, liver, etc.) are sufficient to drive Treg responses without need for draining lymphatics.

The Expansive vs. Inductive Roles of GMCSF-MOG in Driving Emergence of MOG-Specific Tregs

At least two mechanisms may account for the differential kinetics of the vaccine-induced Treg response in 2D2-FIG-*Rag1*^{-/-} vs. 2D2-FIG mice. First, GMCSF-MOG may expand pre-existing Tregs and may lack efficacy for *de novo* induction of Tregs from naïve precursors. In this model, GMCSF-MOG vaccination of 2D2-FIG-*Rag1*^{-/-} mice may drive Treg expansion from the minute population of pre-existing Tregs, which would require a prolonged 10–12 day duration to reach peak accumulation. Second, GMCSF-MOG may induce the *de novo* differentiation of iTregs by mechanisms facilitated by pre-existing Tregs. In this case, Treg induction would be slower in 2D2-FIG-*Rag1*^{-/-} mice while pre-existing Tregs slowly facilitated expansion of peripherally-induced Treg populations by a mechanism of infectious tolerance. These models are not exclusive, and aspects of both models may drive the tolerogenic Treg response. Alternatively, the differential kinetics of the vaccine-induced Treg response in 2D2-FIG-*Rag1*^{-/-} vs. 2D2-FIG mice may reflect other unappreciated differences between the two strains apart from the pre-existing Treg levels. Future research will be needed to resolve these possibilities.

CONCLUSION

GMCSF-NAG fusion proteins comprise a unique tolerogenic vaccine platform. The emerging picture is that the GM-CSF domain interacts with the GM-CSF receptor (CD116, CD131)

on myeloid APC to target the NAg domain to myeloid APC contingent upon the covalent GMCSF-antigen linkage. The subsequent GM-CSF/receptor interaction conditions myeloid APC to express co-inhibitory activities that facilitate an overall process of inhibitory antigen presentation. Presentation of NAg domain as an inefficient TCR ligand to myelin-reactive CD4⁺ T cells drives desensitization of the respective myelin-reactive T cell repertoire and promotes the induction and outgrowth of myelin-specific CD25^{high} FOXP3⁺ Tregs. The overall vaccine response mediates active dominant tolerance in the context of inflammatory environments. This tolerogenic vaccine platform can provide mechanistic insight into basic Treg physiology while advancing a unique vaccine class to fulfill an unmet clinical need in MS.

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AUTHOR CONTRIBUTIONS

CM, AC, AB, and MM designed the project, provided intellectual input, analyzed the data, and wrote the manuscript. CM performed most of the experiments. AC was instrumental in the initiation of the project. AB and SE contributed to important aspects of the experimentation.

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Animal care and use was performed in accordance with approved animal use protocols and guidelines of the East Carolina University Institutional Animal Care and Use Committee.

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Ethyl Pyruvate Stimulates Regulatory T Cells and Ameliorates Type 1 Diabetes Development in Mice

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Type 1 diabetes (T1D) is an autoimmune disease in which a strong inflammatory response causes the death of insulin-producing pancreatic β -cells, while inefficient regulatory mechanisms allow that response to become chronic. Ethyl pyruvate (EP), a stable pyruvate derivate and certified inhibitor of an alarmin–high mobility group box 1 (HMGB1), exerts anti-oxidant and anti-inflammatory properties in animal models of rheumatoid arthritis and encephalomyelitis. To test its therapeutic potential in T1D, EP was administered intraperitoneally to C57BL/6 mice with multiple low-dose streptozotocin (MLDS)-induced T1D. EP treatment decreased T1D incidence, reduced the infiltration of cells into the pancreatic islets and preserved β -cell function. Apart from reducing HMGB1 expression, EP treatment successfully interfered with the inflammatory response within the local pancreatic lymph nodes and in the pancreas. Its effect was restricted to boosting the regulatory arm of the immune response through up-regulation of tolerogenic dendritic cells ($CD11c^+CD11b^-CD103^+$) within the pancreatic infiltrates and through the enhancement of regulatory T cell (Treg) levels ($CD4^+CD25^{high}FoxP3^+$). These EP-stimulated Treg displayed enhanced suppressive capacity reflected in increased levels of CTLA-4, secreted TGF- β , and IL-10 and in the more efficient inhibition of effector T cell proliferation compared to Treg from diabetic animals. Higher levels of Treg were a result of increased differentiation and proliferation ($Ki67^+$ cells), but also of the heightened potency for migration due to increased expression of adhesion molecules (CD11a and CD62L) and CXCR3 chemokine receptor. Treg isolated from EP-treated mice had the activated phenotype and T-bet expression more frequently, suggesting that they readily suppressed IFN- γ -producing cells. The effect of EP on Treg was also reproduced *in vitro*. Overall, our results show that EP treatment reduced T1D incidence in C57BL/6 mice predominantly by enhancing Treg differentiation, proliferation, their suppressive capacity, and recruitment into the pancreas.

Keywords: regulatory T cells (Treg), type 1 diabetes (T1D), ethyl pyruvate, inflammation, immunoregulation

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease that develops as a consequence of β -cell death and subsequent lack of insulin. The cells that infiltrate the pancreatic islets and destroy β -cells are usually M1 pro-inflammatory macrophages and dendritic cells (DC) that present autoantigens to $CD8^+$ cytotoxic lymphocytes, IFN- γ -producing Th1 and IL-17-producing Th17 lymphocytes. The opposing components of the immune response are M2 anti-inflammatory

macrophages, as well as tolerogenic DC that enable activation of regulatory T lymphocytes (Treg) (1). The balance between pro-inflammatory and regulatory cells is a common target for new experimental T1D therapeutics, for example, ethyl pyruvate (EP). This is a stable pyruvate derivative that possesses anti-oxidant and anti-inflammatory properties. It is a genuine inhibitor of high mobility group box 1 (HMGB1), a protein that, like histones, binds DNA and regulates transcription (2). More importantly, HMGB1 can be secreted by monocytes, macrophages and dendritic cells and can act as an alarmin to promote inflammation and generation of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) (3, 4). Either through inhibition of HMGB1 or interaction with other still unknown molecules, EP has been found beneficial in the treatment of various inflammatory disorders, including myocardial ischemia-reperfusion injury (5), sepsis (6), pancreatitis (7), colitis (8), inflammatory arthritis (9), and experimental autoimmune encephalomyelitis (10). Thorough analysis of EP's mechanism of action in the autoimmune process that targets CNS suggests that EP ameliorated the symptoms of experimental autoimmune encephalomyelitis through inhibition of HMGB1 secretion from microglia/macrophages (10, 11). The activity of phagocytic cells (ROS production and antigen presentation properties) and Th cells (IFN- γ and IL-17 production) was significantly impaired by the action of EP (10, 11). The effects of EP include more than only the inhibition of the pro-inflammatory response; EP also boosts the regulatory component of immunity, as recent findings indicate that EP promotes the differentiation of tolerogenic DC (tolDC) *in vitro* (unpublished data). However, there are no data on the possible effect of EP on Treg.

So far, EP has been mostly used to treat the secondary effects that diabetes and the resulting hyperglycemia have on the retina (12), kidneys (13), or liver (14). Having in mind that HMGB1 enhances the progression of T1D in NOD mice (15), the application of EP might prove beneficial for the treatment of T1D.

MATERIAL AND METHODS

Animals

C57BL/6 mice were kept at the animal facility at the Institute for Biological Research "Sinisa Stankovic," under standard conditions with free access to food and tap water. All experimental procedures were approved by the Ethic Committee at the Institute for Biological Research "Sinisa Stankovic" (App. No 01-11/17 - 01-2475) in accordance with the Directive 2010/63/EU.

T1D Induction and EP Treatment

T1D was induced in 2 months old male C57BL/6 mice using multiple low doses of streptozotocin (MLDS) that were given intraperitoneally for 5 consecutive days. Streptozotocin (STZ) (40 mg/kg bw, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in cold 0.1 M citrate buffer (pH 6) just prior to administration. Ethyl pyruvate (EP) (100 mg/kg bw, Sigma-Aldrich) was dissolved in Hartmann's solution (Hemofarm A.D., Vršac, Serbia) and administered intraperitoneally, starting from the first dose of STZ

for 9 days in total. STZ and EP injections were given 3 h apart. MLDS-treated group also received the diluent in equal volume. Mice were monitored for the development of hyperglycemia by weekly measurements of blood glucose levels, using a glucometer (Sensimac, IMACO GmbH, Lüdersdorf, Germany). Animals were considered hyperglycemic if their blood glucose level was higher than 11 mmol/l in non-fasted animals. The presence of ketones in urine (as an indirect measure of high glucose levels) was detected by URISCAN test strips (YD Diagnostics, Kyunggi-Do, South Korea). *Ex vivo* analyses were performed between the 11th and the 15th day after the first STZ injection.

Histological Analysis

To assess the incidence of inflammatory changes and the degree of islet cell destruction, pancreata were collected on the 35th day after the first STZ injection, embedded in paraffin, sectioned (5 μ m thick sections, at least 200 μ m between sections), stained with Mayer's hematoxylin (Bio-Optica, Milan, Italy) and examined by light microscopy (Zeiss Imager Z1, AXIO, Carl Zeiss Meditec AG, Oberkochen, Germany). Insulitis scoring was performed by examining at least 20 islets per pancreas and graded in a blinded fashion as follows: grade 0, intact islet; grade 1, peri-islet infiltrate; grade 2, heavy intra-islet infiltrate. Results are expressed as a percentage of graded islets out of the total number of islets.

Immunohistochemical staining was performed on pancreatic sections collected on the 15th day after the first STZ injection in the following manner. To assess the presence of insulin, PE conjugated rabbit anti-mouse insulin antibody (1:400, Cell Signaling Technology, Danvers, MA, USA) was used, while counterstaining was carried out with Hoechst 33342 dye (2 μ l/ml, ChemoMetec, Allerød, Denmark). For the negative control, rabbit anti-goat IgG-biotin (Vector Laboratories, Burlingame, CA, USA) coupled with streptavidin-PE (ThermoFisher Scientific, Waltham, MA, USA) was used. Image acquisition (20 \times) was performed using an AxioVision microscope (Carl Zeiss Meditec AG, Germany). The presence of insulin in the pancreatic islets was determined with Fiji, an open-source distribution of ImageJ software for biological image analysis (16). The acquired images were converted to gray scale, and fluorescence intensity was quantified by measuring the mean gray value, which represents the sum of gray values of all pixels in the selection divided by the number of pixels. For HMGB1 assessment, anti-mouse HMGB1 antibody (1:500, Invitrogen, Carlsbad, CA, USA) was paired with a secondary HRP conjugated anti-mouse IgG (1:1,000, Invitrogen), incubated with ExtrAvidin peroxidase (1:10, Sigma-Aldrich) and finally stained with DAB chromogen solution (1:50, DakoCytomation, Glostrup, Denmark), while counterstaining was carried out with hematoxylin. For the negative control, HRP conjugated anti-mouse IgG-treated sections were used. The presence of HMGB1 in pancreatic islets was measured in the following way. Pancreatic islets were photographed at the magnification of 20 \times , and HMGB1 positive regions were determined using the Color Picker Threshold Plugin within Icy, open-source bioimage processing software (17). Using a representative pancreatic section, at least 15 positive colors (shades of darker brown) and 15 negative

colors (blue, white and light brown) were selected as standard recognition patterns. Those were then applied to all pancreatic sections to differentiate between stained and unstained tissue regions. The presence of HMGB1 in pancreatic islets was expressed as a relative percentage of the HMGB1-positive islet area divided by the area of the whole pancreatic islet.

Cell Isolation

Spleen and pancreatic lymph node (PLN) cells were obtained by passing the tissue through the cell strainer (40 μ m) and after removal of the erythrocytes by RBC Lysis Buffer (eBioscience, San Diego, CA, USA). Pancreatic infiltrates were obtained by collagenase type V (Sigma-Aldrich) digestion of the pancreatic tissue (from which lymph nodes were previously removed). Tissue was cut into small pieces and shaken for 10 min at 37°C. Digests were passed through a 20 μ m cell strainer and washed several times in Hank's balanced salt solution. The cells were finally resuspended in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 1% penicillin and streptomycin (all from PAA Laboratories, Pasching, Austria), 2 mM L-glutamine and 25 mM HEPES. CD4⁺CD25⁻, CD25⁺ or CD25⁻ cells were marked for separation from the spleen, cervical and mesenteric lymph node cell suspension by incubation with biotin conjugated anti-mouse CD4 (1:60) or biotin conjugated anti-mouse CD25 (1:100) antibodies (both from eBioscience). They were then resuspended in cold magnetic bead buffer (PBS+0.5% BSA+2 mM EDTA) containing BD IMagTM Streptavidin Particles Plus-DM (1:20, BD Biosciences, Bedford, MA, USA). The appropriate cells were purified by placement in a BD IMagTM Cell Separation Magnet (BD Biosciences), 3 \times for 8 min, and finally resuspended in a T lymphocyte medium-RPMI 1640 supplemented with 10% FCS, 1% penicillin and streptomycin, 0.02 mM Na-pyruvate, 5 μ M β -mercaptoethanol, 2 mM L-glutamine, and 25 mM HEPES.

Detection of Extracellular and Intracellular Markers by Flow Cytometry

Surface molecules were detected on viable cells dispersed in PBS+1% BSA. The following antibodies were used: anti-mouse CD4 PerCP-Cyanine5.5 (rat IgG2a, κ), anti-mouse CD4-FITC (rat IgG2b, κ), CD4-APC (rat IgG2b, κ), anti-mouse CD8-PE (rat IgG2a, κ), anti-mouse B220-FITC (rat IgG2a, κ), anti-mouse CD19-PE-Cy5 (rat IgG2a, κ), CD11c-PE-Cy5 (Armenian hamster IgG), F4/80-FITC (rat IgG2a, κ), CD25-Alexa Fluor[®] 488 (rat IgG1, λ), CD25-PE (rat IgG1, λ), CD101-PE (rat IgG2a, κ), CD62L-PE-Cy7 (rat IgG2a, κ), CD206-PE (rat IgG2b, κ), CD127 APC-eFluor 780 (rat IgG2a, κ), MHC II-FITC (rat IgG2b, κ), CD357 (GITR)-FITC (rat IgG2b, κ), CD80-PE-Cy5 (Armenian hamster IgG), CD86-PE-Cy5 (rat IgG2a, κ), CD103-FITC (Armenian hamster IgG), CD5-FITC (rat IgG2a, κ), CD11a-FITC (rat IgG2a, κ), CD40-PE (Armenian hamster IgM, κ), anti-rat IgG-PE (all from ThermoFisher Scientific), CD11b (rat IgG2b, κ) (BD Biosciences), PD-1 (rat IgG2a, κ) (Abcam, Cambridge, MA, USA). The staining was performed for 30 min at 4°C. Regulatory T cells (Treg) were detected by Mouse Regulatory T cell Staining Kit (FoxP3) according to the manufacturer's instructions (eBioscience). For intracellular cytokine staining, cells were stimulated with Cell Stimulation

Cocktail (plus protein transport inhibitors) (1:500, eBioscience) for 4 h. Cells were fixed in 2% paraformaldehyde, permeabilized and stained with the following antibodies: anti-mouse IFN- γ -PE (rat IgG1, κ), IL-4-PE (rat IgG1, κ) (both from eBioscience) or IL-17-PE (rat IgG1, κ) (BD Biosciences). Isotype-matched controls were included in all experiments (eBioscience). For Ki67-FITC (goat polyclonal antibody) (SantaCruz Biotechnology, San Diego, USA), T-bet-PE (mouse IgG1, κ) and ROR γ T-PE (rat IgG1, κ) (eBioscience) antibodies, cells were permeabilized using the same protocol as for FoxP3 (Treg) detection. Cells were acquired on Partec CyFlow Space by FlowMax software (Partec, Görlitz, Germany) or by FACSCalibur and BD FACSaria III (BD Biosciences) and analyzed by FlowMax (Partec) or FlowJo software (Treestar, Ashland, OR, USA). Cells were first gated on live cells (empirically determined) and then further gated appropriately to the required analysis.

Measurement of Intracellular Nitric Oxide and Reactive Oxygen Species

4-Amino-5-methylamino-2',7'-difluoro-fluorescein diacetate (DAF-FM diacetate; Molecular Probes, Leiden, The Netherlands) was used as an indicator of intracellular nitric oxide (NO). Briefly, pancreatic infiltrates were incubated with 2 μ M DAF-FM diacetate for 1 h at 37°C, washed, and then incubated for 15 min at 37°C in phenol red- and serum-free RPMI-1640 for the completion of de-esterification of intracellular diacetates. The cells were then washed and resuspended in PBS. Green (FL1) fluorescence emission was measured with Partec CyFlow Space and analyzed by FlowMax software. Dihydrorhodamine 123 (DHR) was used to reactive oxygen species (ROS). After isolation, cells were immediately exposed to DHR (5 μ M) for 20 min at 37°C. After being washed, the cells were analyzed with the flow cytometer. The mean fluorescence intensity (MFI) as a measure of intracellular production of NO and ROS was measured in the macrophage gate (higher SSC level compared to the lymphocyte gate).

Immunoblot

Ex vivo isolated cells (5×10^6) from PLN or pancreatic infiltrates, purified CD25⁺ cells (5×10^5) or *in vitro* cultured CD4⁺CD25⁻ (5×10^6) were lysed in the buffer containing 62.5 mmol/l Tris-HCl (pH 6.8 at RT), 2% SDS, 10% glycerol, 50 mmol/l DTT, 0.01% bromophenol blue (all from Sigma-Aldrich). All samples were subjected to electrophoresis on 12% SDS-polyacrylamide gel (SDS-PAGE). After electro-transferring the samples to polyvinylidene difluoride membranes at 5 mA/cm², using a semi-dry blotting system (Semi-Dry Transfer Unit, GE Healthcare, Buckinghamshire, England), the membranes were blocked with PBST (PBS+0.1% Tween-20, Sigma-Aldrich)+5% BSA and probed with specific antibodies dissolved in PBST+1% BSA. Secondary antibodies for anti-mouse CTLA-4 (1:1,000, Invitrogen) were FITC conjugated anti-armanian and syrian hamster IgG cocktail (1:1,000, BD Biosciences) and HRP conjugated anti-mouse IgG (1:5,000, Invitrogen), for anti-mouse IL-10 (1:600, eBioscience) or anti-mouse IL-2 (1:500, eBioscience) it was HRP conjugated anti-rat IgG (1:5,000, eBioscience), and for anti-mouse HMGB1 (1:1,500, Invitrogen) or anti-mouse

β -actin (1:1,000, Sigma-Aldrich) it was HRP conjugated anti-mouse IgG (1:5,000, Invitrogen). Detection was performed by using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA), while photographs were made by X-ray films (Kodak, Rochester, NY, USA). Densitometry was performed with Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA), and the production of specific proteins was expressed relative to the production of β -actin.

In vitro Suppression Assay and Transmigration Assay

For the suppression assay, $CD4^+CD25^-$ cells isolated from either EP-treated or diabetic animals were first incubated with $2\ \mu\text{M}$ CFSE (Invitrogen) for 20 min at RT and 5 min at 37°C , washed and resuspended in the T lymphocyte medium. A U-bottom 96-well plate (Sardstedt, Numbrecht, Germany) was coated with anti-mouse CD3 ($1\ \mu\text{g}/\text{ml}$, eBioscience), and an equal number of purified $CD4^+CD25^-$ cells (25×10^3) was placed in each well. $CD25^+$ cells were then added in a series of dilutions, starting from 25×10^3 cells per well, and continuing to a $2\times$, $4\times$, $8\times$, and $16\times$ lesser number of cells. Certain wells contained only $CD4^+CD25^-$ cells, as control. The cells were also stimulated by the addition of anti-mouse CD28 ($1\ \mu\text{g}/\text{ml}$, eBioscience) to the T lymphocyte medium. After 3 days of cultivation, the cells were washed, resuspended in PBS and analyzed by flow cytometry.

For the transmigration assay, a special chemotaxis system was used (ChemoTX System, Neuro Probe Inc., MD, USA). $CD25^+$ or $CD25^-$ cells isolated from either EP-treated or diabetic animals (10^5 cells in $50\ \mu\text{l}$) were placed above a membrane and their migratory abilities were tested, either toward isolated pancreatic islets [isolation described in (18)], medium containing CXCL12 ($10\ \text{ng}/\text{ml}$, Gibco, ThermoFisher Scientific), or plain medium as control. After 4 h, the number of cells that migrated through the membrane and into the wells was determined by LUNA-IITM Automated Cell Counter (Logos Biosystems, Gyeonggi-do, South Korea).

In vitro Th Differentiation Assay

$CD4^+CD25^-$ cells (5×10^6) isolated from untreated healthy animals were stimulated in an adequate manner for the purpose of Th cell differentiation. All cells received stimuli from plate-bound anti-CD3 ($1\ \mu\text{g}/\text{ml}$) and soluble anti-CD28 antibodies ($1\ \mu\text{g}/\text{ml}$) (both from eBioscience). For Th1 differentiation the cells were additionally stimulated with IL-12 ($20\ \text{ng}/\text{ml}$, R&D Systems, Minneapolis, MN, USA) and anti-IL-4 antibody ($10\ \text{ng}/\text{ml}$, eBioscience), for Th17 differentiation the cells were stimulated with TGF- β ($10\ \text{ng}/\text{ml}$, R&D Systems) and IL-6 ($10\ \text{ng}/\text{ml}$, R&D Systems), while for Treg differentiation the cells were stimulated with either TGF- β ($2\ \text{ng}/\text{ml}$) and IL-2 ($10\ \text{ng}/\text{ml}$) (both from R&D Systems) (complete stimulation), or only with IL-2 (incomplete stimulation). EP ($125\ \mu\text{M}$) was administered 24 h after the beginning of the culture, and after 4 more days of cultivation the cells were analyzed for the presence of certain Th subsets by flow cytometry as described above.

ELISA

Splenocytes were cultured in the presence of LPS ($100\ \text{ng}/\text{ml}$) for 48 h and cytokine concentration in cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunc, Roskilde, Denmark) and anti-mouse paired antibodies according to the manufacturer's instructions. Supernatants from previously described *in vitro* differentiation experiments were also collected after 5 days of cultivation. Samples were analyzed in duplicate for murine TNF, IL-1 β , and TGF- β (eBioscience) and absorbance was measured by LKB microplate reader (LKB Instruments, Vienna, Austria) at 450 and 570 nm. A standard curve created from the known concentrations of appropriate recombinant cytokines was used to calculate concentration values of measured cytokines.

Statistical Analysis

Data are presented as mean \pm SD. The presented results are representative of four repeated experiments with comparable results. The significance of differences between groups was determined by two-tailed Student's *t*-test. In addition, for the analysis of the results with considerable deviations a Mann-Whitney non-parametric test was used since it produced more stringent *p*. The usage of this test is specified in the appropriate figure legends. Differences are regarded as statistically significant if *p* < 0.05. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

The Effect of EP on T1D Development

In order to estimate the effect of EP on T1D development in C57BL/6 mice, EP was applied prophylactically for 9 days starting from the first dose of STZ (Figure 1A). In contrast to the control mice that developed hyperglycemia on the 14th day, mice treated with EP had a significantly lower incidence of diabetes (Figure 1B). Also, the number of islets with mononuclear infiltrates (counted on the 35th day from the beginning of the experiment) was lower in EP-treated mice compared to diabetic mice (Figures 1C–E), while the presence of functional insulin⁺ β -cells was higher and similar to that in healthy islets (Figures 1F–I). Isotype staining for insulin is presented in Figure S1A. Since there is evidence of higher expression of HMGB1 in β -cells of NOD mice that develop spontaneous T1D (15), we measured the expression of HMGB1 in the β -cells of mice treated with MLDS. Compared to healthy, untreated mice, HMGB1 expression in diabetic mice was significantly higher, and EP-treatment successfully suppressed the observed change (Figures 1J–M). Background staining is presented in Figure S1B. In addition, throughout the examination period mice treated with EP gained weight normally (data not shown) and only 14% of EP-treated mice had ketones in urine compared to 71% of diabetic ones (Figure 1N).

The Influence of EP Treatment on Innate Antigen-Presenting Cells

Ex vivo analysis of cells from spleen, PLN and pancreatic infiltrates showed that EP treatment did not change the number

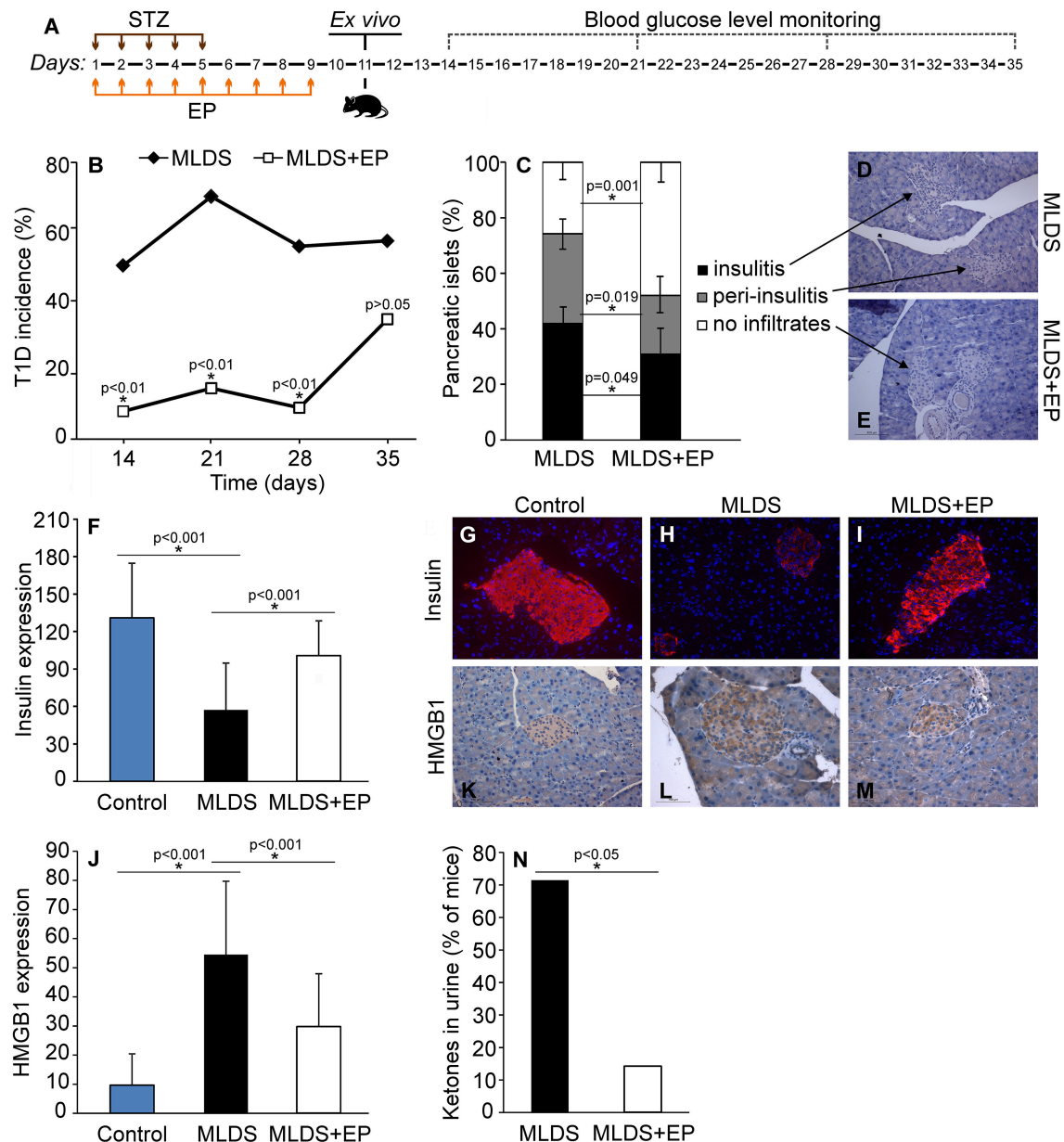


FIGURE 1 | The effect of EP on T1D development. **(A)** Diagram of diabetes induction, EP treatment and diabetes monitoring by weekly measurements of blood glucose levels. **(B)** T1D incidence presented as a proportion of C57BL/6 mice with glycemia higher than 11 mmol/L. **(C)** The proportion of pancreatic islets without immune cell infiltrates, with infiltrates surrounding the islets (peri-insulinitis) and with infiltrates within the islet (insulinitis). Representative images of pancreatic islets from MLDS-treated mice **(D)** or EP-treated mice **(E)**, stained with hematoxylin. **(F)** Insulin expression was determined by analyzing fluorescence intensity with Fiji software. Representative images of pancreatic islets from a control healthy animal **(G)**, MLDS-treated mice **(H)** or EP-treated mice **(I)** stained for insulin visualization (red) and with Hoechst 33342 (nucleus – blue). **(J)** HMGB1 expression within pancreatic β -cells was determined using the Color Picker Threshold Plugin within Icy software. Representative images of pancreatic islets from a control healthy animal **(K)**, MLDS-treated mice **(L)** or EP-treated mice **(M)** stained for HMGB1. **(N)** The proportion of mice with ketones in urine. All groups consisted of 7–10 animals. * $p < 0.05$ represents a statistically significant difference between MLDS+EP-treated compared to MLDS-treated mice.

of F4/80⁺ macrophages (Figure S2A), while it significantly affected the proportion of dendritic cells (Figure 2A). More precisely, the proportion of CD11c⁺ DC was significantly down-regulated in PLN, while their number was simultaneously increased within the pancreatic infiltrates (Figure 2A).

To explore whether EP changed the ratio between pro-inflammatory M1 and anti-inflammatory M2 macrophage subsets, we detected F4/80⁺CD40⁺ M1 and F4/80⁺CD206⁺ M2 macrophages in the pancreatic infiltrates. The results indicate that EP had no impact on either of the examined macrophage

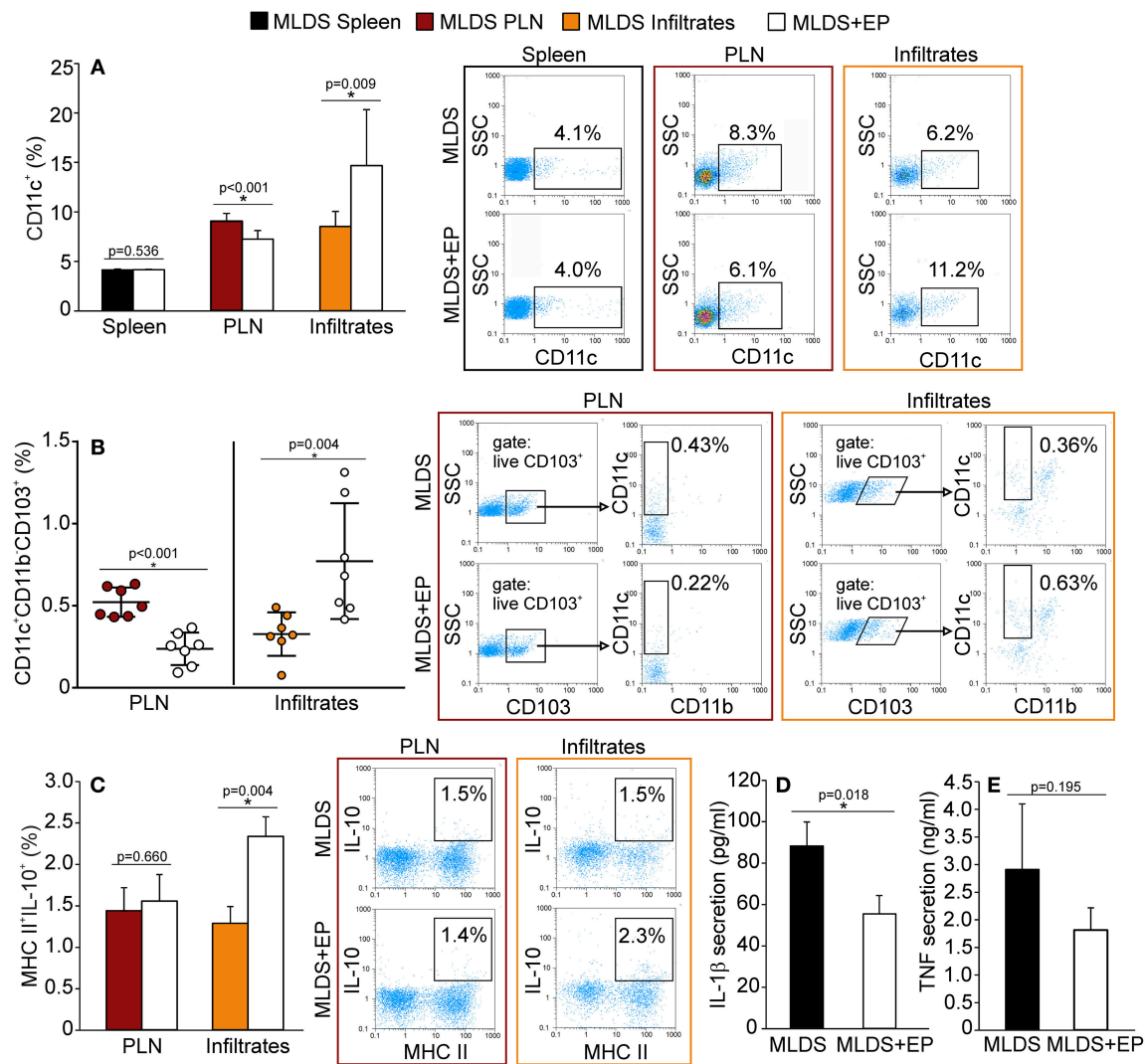


FIGURE 2 | The influence of EP on innate antigen-presenting cells. The proportion of all cells, isolated from the spleen, pancreatic lymph nodes (PLN) or pancreatic infiltrates, was measured by flow cytometry. **(A)** The proportion of CD11c⁺ dendritic cells, along with representative dot plots. **(B)** The proportion of tolerogenic DC (CD11c⁺CD11b⁻CD103⁺), along with representative dot plots (first gated on live CD103⁺ cells, followed by the gate on CD11c⁺CD11b⁻). Statistical analysis for CD11c⁺ and tolerogenic DC was performed by Mann-Whitney non-parametric test. **(C)** Proportion of IL-10⁺ cells within MHC II⁺ population, along with representative dot plots. Secretion of IL-1β **(D)** and TNF **(E)** from LPS-treated splenocytes cultured *ex vivo* for 48 h and measured by ELISA. All measurements were performed on samples from at least 7 animals per group. **p* < 0.05 represents a statistically significant difference between cells of MLDS+EP compared to those of MLDS-treated mice.

subsets (Figure S2B). However, it did increase the proportion of CD11c⁺CD11b⁻CD103⁺ tolerogenic DC in the pancreatic infiltrates (Figure 2B). These cells seemed to migrate from PLN to the pancreas, since their number was reduced in the nodes after EP treatment (Figure 2B).

To examine the function of antigen-presenting cells, we measured their ability to produce NO and ROS. Despite being an anti-oxidant, EP did not affect the production of NO or ROS in phagocytic cells within the pancreas (Figure S2C). Also, EP did not influence cells' antigen presentation capacity since the expression of co-stimulatory molecules CD80 and CD86 remained the same on MHC class II⁺ cells after EP

treatment (Figure S2D). However, EP significantly stimulated the suppressive function of MHC class II⁺ cells since the production of IL-10 in these cells was up-regulated (Figure 2C). Accordingly, when analyzing LPS-stimulated splenocytes, we found that cells isolated from EP-treated mice produced lower amounts of IL-1β (Figure 2D) compared to cells isolated from diabetic mice, while the secretion of TNF was similar (Figure 2E).

The Effect of EP on Adaptive Immunity in Diabetic Mice

Treatment with EP did not significantly change the proportion of CD4⁺, CD8⁺ or B lymphocytes in the spleen, PLN or pancreatic

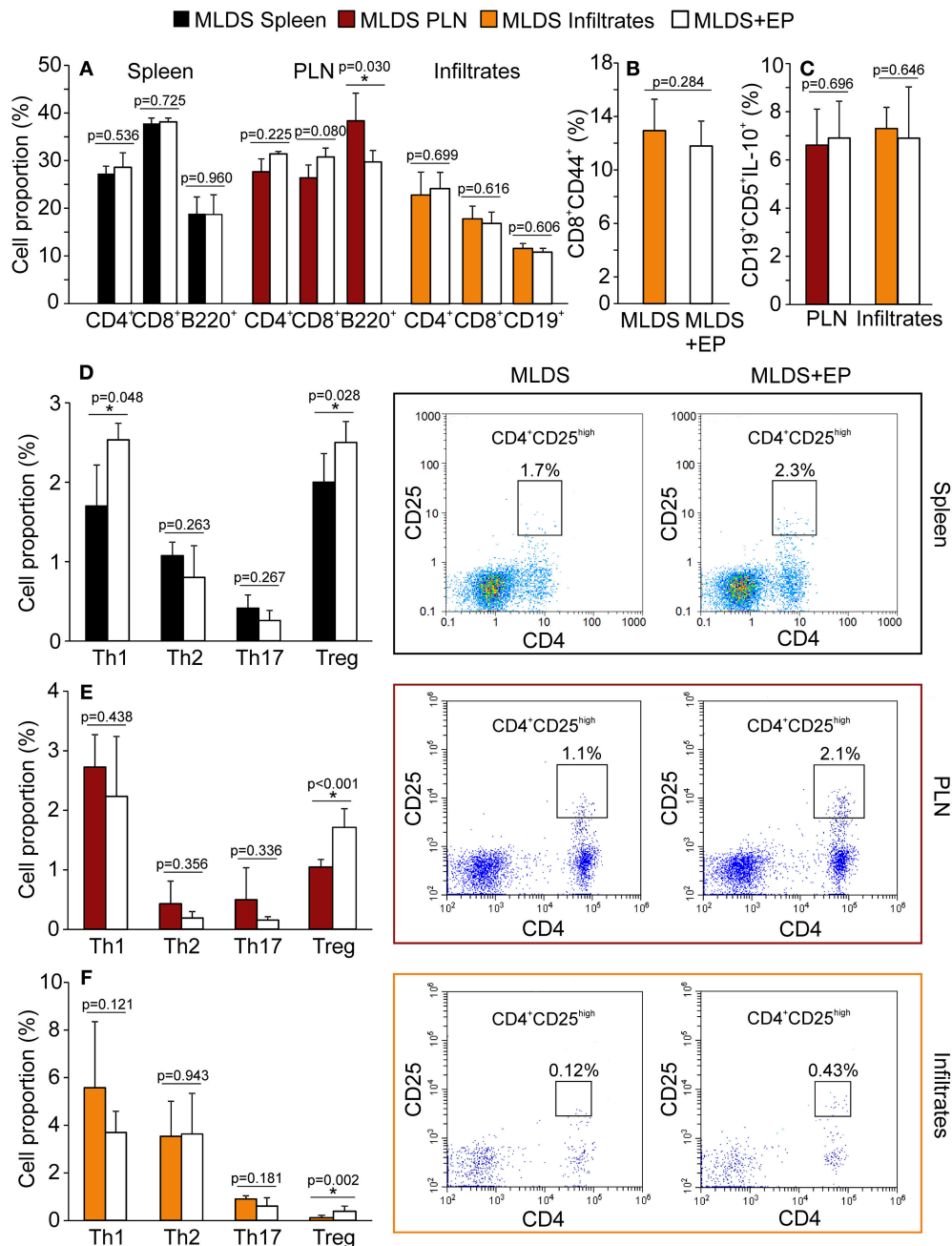


FIGURE 3 | Phenotypic analysis of adaptive immune cells after EP treatment. All cell proportions were measured by flow cytometry. **(A)** The proportion of Th (CD4⁺), cytotoxic lymphocytes (CD8⁺) or B lymphocytes (B220⁺ or CD19⁺) in spleen, PLN or pancreatic infiltrates. **(B)** The proportion of activated cytotoxic lymphocytes (CD8⁺CD44⁺) in the pancreatic infiltrates. **(C)** The proportion of regulatory B cells (CD19⁺CD5⁺IL-10⁺) within PLN and pancreatic infiltrates. The proportion of Th subsets: Th1 (CD4⁺IFN- γ ⁺), Th2 (CD4⁺IL-4⁺), Th17 (CD4⁺IL-17⁺) and Treg (CD4⁺CD25^{high}) within the spleen **(D)**, PLN **(E)**, and pancreatic infiltrates **(F)** of MLDS or MLDS+EP-treated mice. Representative dot plots for CD4⁺CD25^{high} on the right hand side. All measurements were performed on samples from 7 animals per group. * $p < 0.05$ represents a statistically significant difference between cells of MLDS+EP compared to those of MLDS-treated mice.

infiltrates, except for the reduction in B lymphocytes in PLN (**Figure 3A**). Further insight into CD8⁺ activity revealed that these cells were similarly activated in both diabetic and EP-treated mice (**Figure 3B**). As for B lymphocytes, the proportion of their regulatory subset (Breg, CD19⁺CD5⁺IL-10⁺) was the

same between the groups both in PLN and in the pancreatic infiltrates (**Figure 3C**). All gating strategies and representative dot plots are shown in **Figures S3A–F**. After examining the Th subsets (**Figures 3D–F**), we found that the proportion of CD4⁺CD25^{high} cells (Treg) was up-regulated after EP treatment

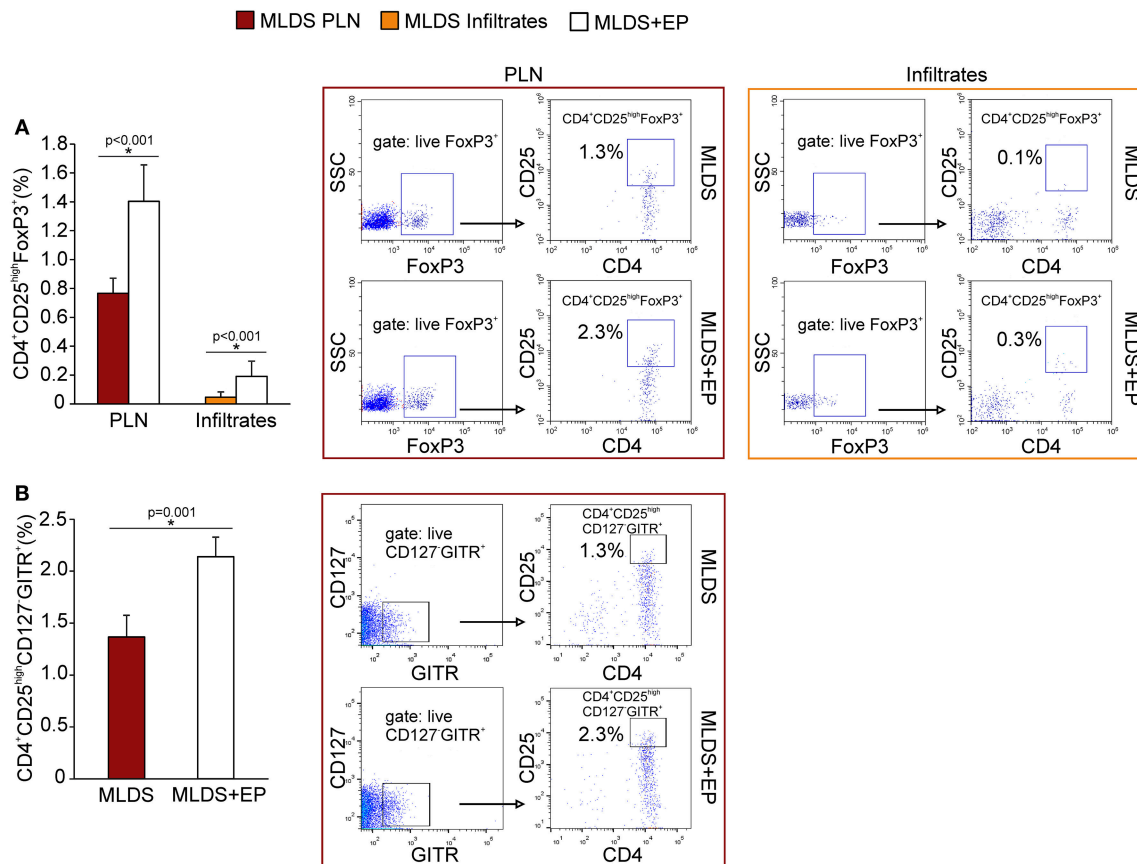


FIGURE 4 | Phenotypal characterization of Treg after EP treatment. **(A)** Proportion of Treg (CD4⁺CD25^{high}FoxP3⁺) within PLN and pancreatic infiltrates, along with representative dot plots (first gated on live FoxP3⁺ cells, followed by the gate on CD4⁺CD25^{high}). **(B)** The proportion of Treg within PLN that express Gitr and are negative for CD127, along with the representative dot plots (first gated on live CD127⁻Gitr⁺ cells, followed by the gate on CD4⁺CD25^{high}). All measurements were performed on samples from at least 7 animals per group. Statistical analysis was performed by Mann-Whitney non-parametric test. **p* < 0.05 represents a statistically significant difference between cells of MLDS+EP compared to those of MLDS-treated mice.

in all tested compartments. Since the most convincing effect of EP was exerted on Treg, further analysis was focused on Treg function within the local pancreatic environment. Gating strategies for Th1, Th2, and Th17 and representative dot plots are shown in Figures S4A–C.

In vivo EP Effect on Treg Function

To describe the phenotype of Treg in more detail, the expression of FoxP3 (signature transcription factor of Treg), Gitr (glucocorticoid-induced tumor necrosis factor receptor, a marker of naturally occurring Treg) and CD127 (α subunit of IL-7 receptor that is absent on Treg) was determined within CD4⁺CD25^{high} cells. The results indicate that EP increased the number of CD4⁺CD25^{high}FoxP3⁺ cells in PLN and pancreatic infiltrates (Figure 4A). Also, the proportion of CD4⁺CD25^{high}CD127⁻Gitr⁺ cells was increased after EP treatment (Figure 4B). The expression of Treg markers FoxP3, Gitr, PD-1 per cell did not differ between the groups (Figure S5A), while the expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4) in CD25⁺ cells from EP-treated mice

was significantly increased (Figure 5A). EP's effect on their suppressive function was also manifested through a higher proportion of TGF- β ⁺ Treg in EP-treated animals (Figure 5B), as well as through higher production of IL-10 in purified CD25⁺ cells (Figure 5C) and pancreatic infiltrates (Figure 5D). These data suggest that EP enhances Treg suppressive activity, which was confirmed in an *ex vivo* suppression assay (Figures 5E,F). Compared to CD25⁺ cells isolated from diabetic mice, cells from EP-treated mice were more potent in inhibition of CFSE-measured proliferation of CD4⁺CD25⁻ T effector cells. Overall, these results suggest that EP promoted Treg suppressive function through enhancing their capacity for effector T cell inhibition, both through cell-to-cell contact and secreted anti-inflammatory cytokines.

In vivo EP Effect on Treg Differentiation and Proliferation

The increased proportion of Treg found in both PLN and pancreatic infiltrates after EP treatment could be attributed either to increased *de novo* differentiation from naïve CD4⁺

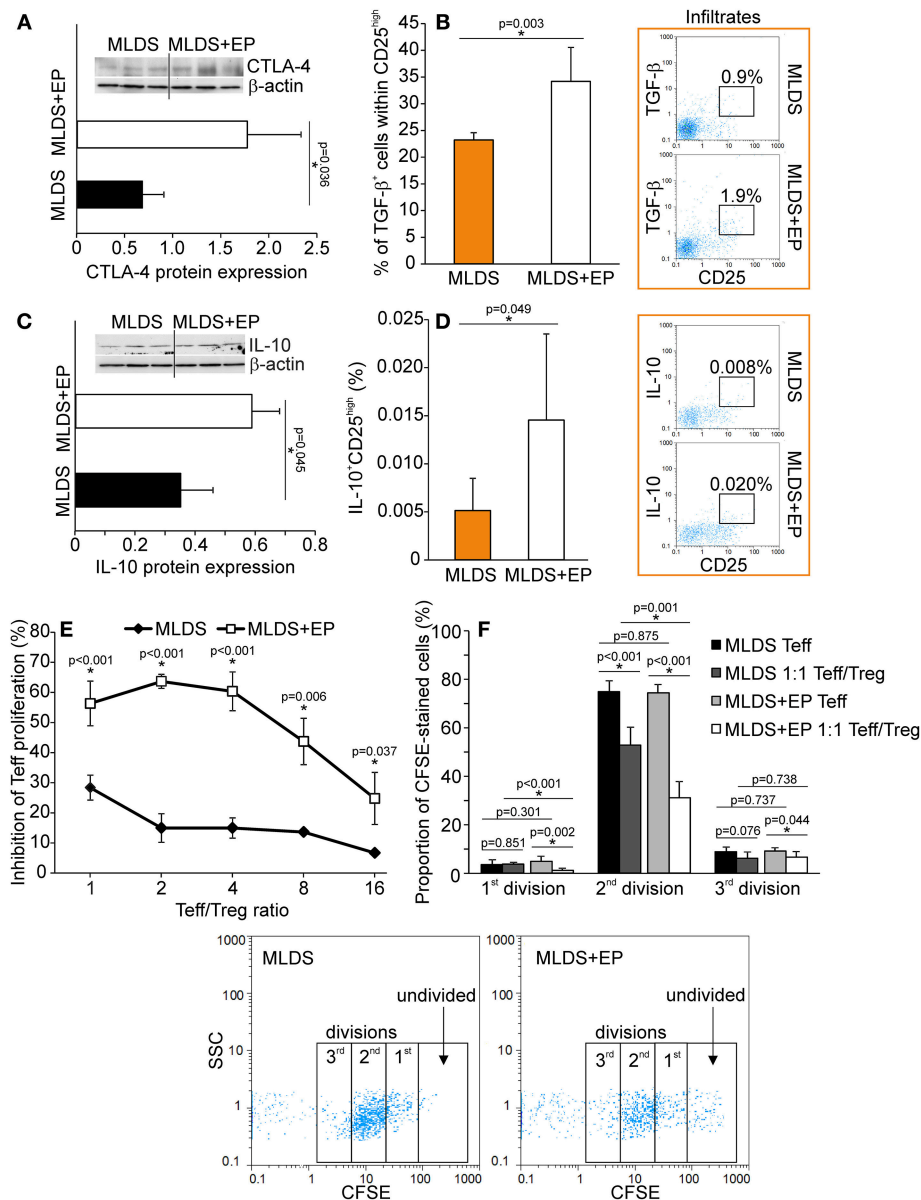


FIGURE 5 | Functional characterization of Treg after EP treatment. **(A)** CTLA-4 protein expression in CD25⁺ cells purified from the pool of lymphoid tissues, normalized to the expression of β-actin, along with the representative blot. **(B)** The proportion of TGF-β-expressing cells within Treg, along with representative dot plots. **(C)** IL-10 protein expression in CD25⁺ cells purified from a pool of lymphoid tissues, normalized to the expression of β-actin, along with the representative blot. **(D)** The proportion of IL-10-expressing Treg within pancreatic infiltrates, along with representative dot plots. **(E)** The level of inhibition of effector T cell (Teff-CD4⁺CD25⁻) proliferation after co-culture with Treg (CD4⁺CD25⁺) cells purified from a pool of lymphoid tissues of MLDS or MLDS+EP-treated mice. Proliferation was measured after 72 h of incubation by CFSE dilution (in Teff). **(F)** Proportion of divided Teff, or Teff cultured in the presence of Treg (1:1 ratio). Representative dot plots are given below. All measurements were performed on samples from 7 animals per group. **p* < 0.05 represents a statistically significant difference between cells of MLDS+EP compared to those of MLDS-treated mice.

cells or to the proliferation of existing ones. For peripheral differentiation of Treg, CD4⁺ cells require IL-2 and TGF-β in their surroundings (19). Although the presence of IL-2 in PLN stayed the same (Figure 6A), the proportion of TGF-β⁺ non-Treg cells was significantly elevated after EP administration (Figure 6B), suggesting that EP enhanced the TGF-β-mediated signaling pathway for enhanced Treg differentiation. However, it seemed that apart from differentiation, EP influenced Treg

proliferation as well, judging by the increased number of Ki67⁺ Treg in the pancreatic infiltrates of EP-treated mice compared to those isolated from diabetic mice (Figure 6C).

In vivo EP Effect on Treg Migration

Since the proportion of Treg was increased after EP treatment, the next step was to investigate their migratory capacities. In addition to the observed increased proliferation, the higher

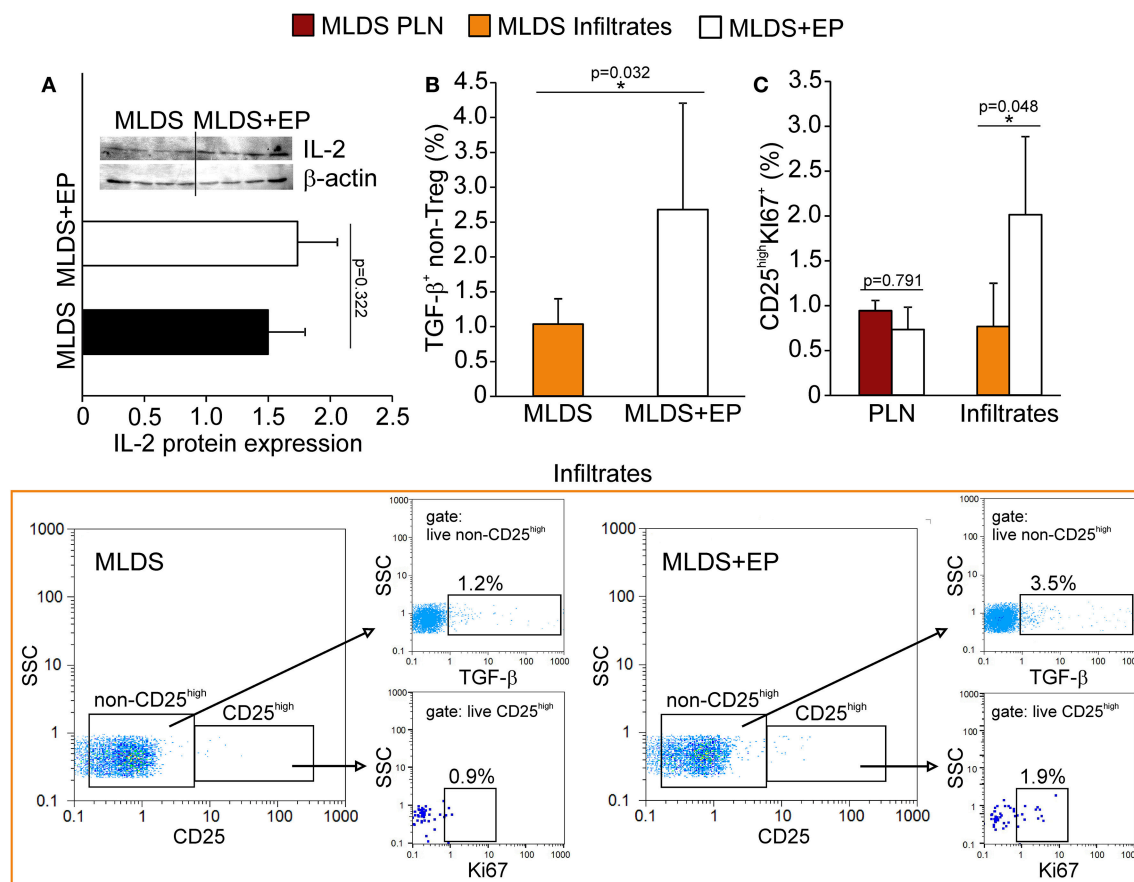


FIGURE 6 | The effect of EP on Treg differentiation and proliferation *in vivo*. **(A)** IL-2 protein expression in PLN, along with the representative blot. **(B)** The proportion of non-Treg producers of TGF- β in the pancreatic infiltrates, along with representative dot plots (first gated on live non-CD25^{high} cells, followed by the gate on TGF- β ⁺). **(C)** The proportion of proliferating Treg (CD25^{high}Ki67⁺) in PLN and pancreatic infiltrates. Representative dot plots (first gated on live CD25^{high} cells, followed by the gate on Ki67⁺) for pancreatic infiltrates are shown. All measurements were performed on samples from 7 animals per group. * $p < 0.05$ represents statistically significant difference between cells of MLDS+EP compared to those of MLDS-treated mice.

proportion of Treg might also result from their increased migration to the site of inflammation. We detected a higher expression of CD11a (subunit of LFA-1—integrin that drives cells from blood to tissues) and L-selectin (CD62L—adhesion molecule that enables transmigration) (Figure 7A) on Treg isolated from EP-treated mice, as well as an increased number of chemokine receptor CXCR3⁺ Treg (Figure 7B). The proportion of CXCR5⁺ Treg was the same between the groups (Figure S6A). These observations suggest that these cells migrated to the pancreas more readily in EP-treated mice than Treg from diabetic mice. This was confirmed in an *ex vivo* transmigration assay, in which CD25⁺ cells isolated from EP-treated mice migrated more efficiently toward pancreatic islets or toward a CXCL12 concentration gradient, compared to Treg isolated from diabetic mice (Figure 7C). Finally, EP increased the proportion of Treg that carried the CD103⁺ receptor in the pancreatic infiltrates (Figure 7D), while their proportion was similar in PLN of both diabetic and EP-treated mice (Figure 7D and Figure S6B) suggesting increased retention of Treg at the site of inflammation (20).

The Effect of EP on Treg Activation and Affinity for Th1 or Th17 Suppression

A higher proportion of Treg from EP-treated mice was CD44⁺, suggesting that they became activated (Figure 8A) and were able to inhibit the activation of effector Th cells (CD4⁺CD25^{med}) (Figure 8B). Induced peripheral Treg might express transcription factors such as T-bet or ROR γ T and these cells are thought to specifically inhibit effector T cell population, Th1 and Th17, respectively. Seemingly, in the pancreatic infiltrates EP significantly up-regulated the T-bet⁺ Treg population (Figure 8C), while the proportion of effector T-bet⁺CD25^{med} cells was reduced (Figure 8D). However, the proportion of ROR γ T⁺ Treg and effector ROR γ T⁺CD25^{med} was similar in both groups (Figures 8E,F). The expression of T-bet or ROR γ T per cell did not differ upon EP treatment (data not shown).

In vitro EP Effect on Treg Differentiation

Naïve CD4⁺CD25[−] cells were stimulated *in vitro* through their TCR in the presence of adequate cytokines or antibodies

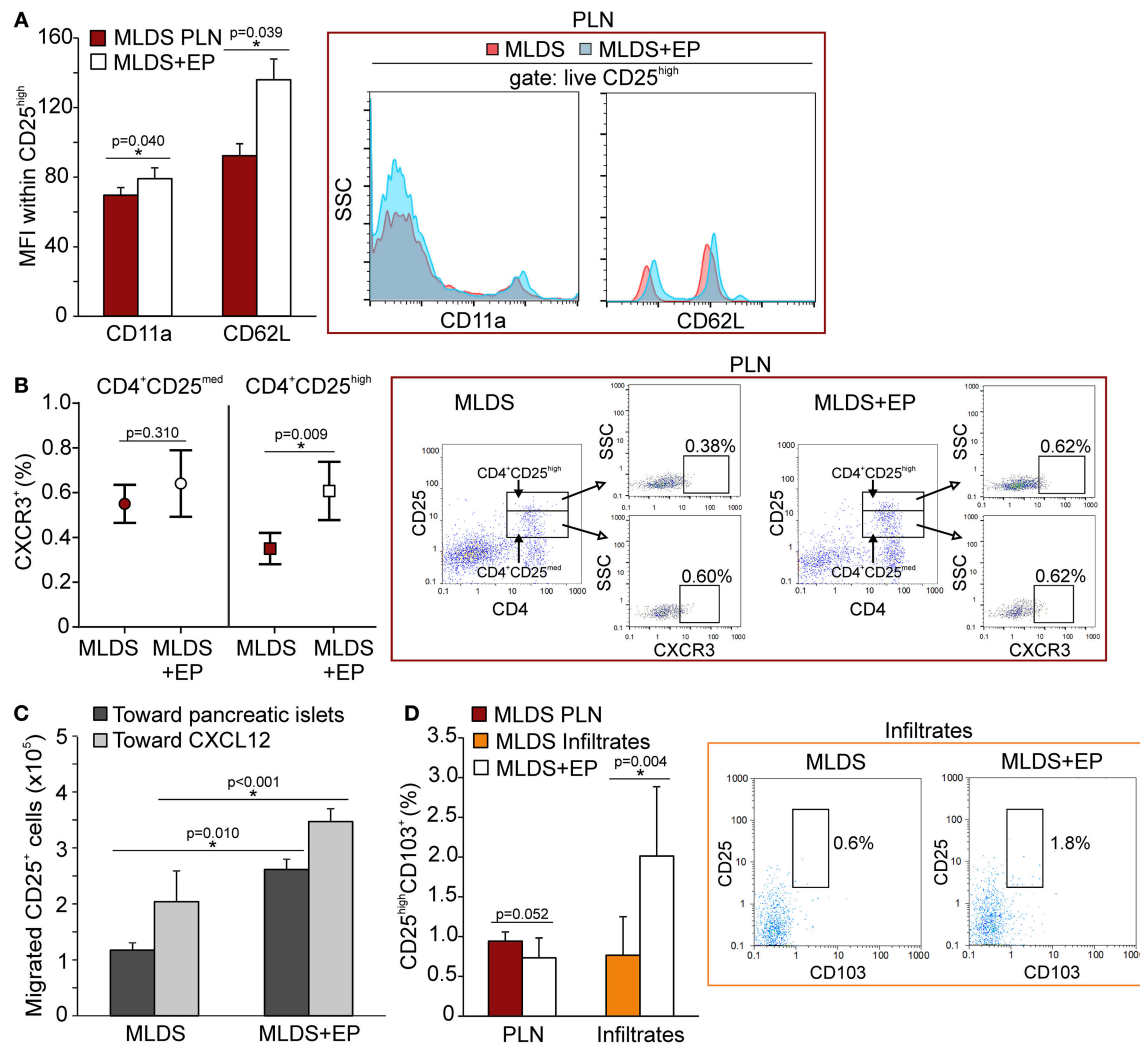


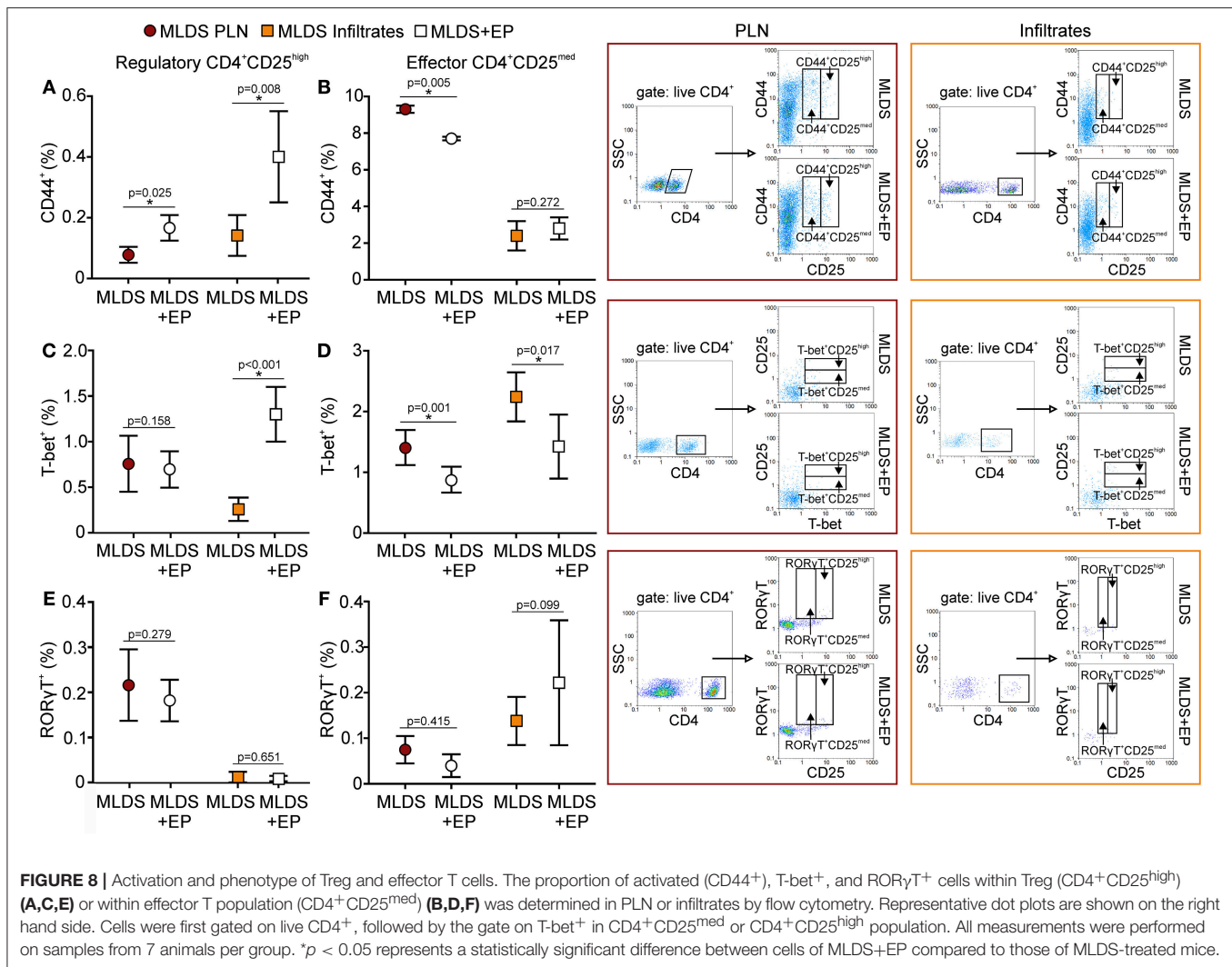
FIGURE 7 | The effect of EP on Treg migratory abilities. **(A)** CD11a and CD62L expression on Treg within PLN, measured by mean fluorescence intensity (MFI). Representative histograms are shown. **(B)** The proportion of CXCR3⁺ cells within activated Th cells (CD4⁺CD25^{med}) or within Treg (CD4⁺CD25^{high}) from PLN. Representative dot plots show the first gate on either live CD4⁺CD25^{med} or live CD4⁺CD25^{high} cells, followed by the gate on CXCR3⁺. **(C)** Migration of CD25⁺ cells purified from a pool of lymphoid tissues of MLDS or MLDS+EP-treated mice in a chemotaxis assay toward pancreatic islets or CXCL12 (10 ng/ml). **(D)** CD25^{high}CD103⁺ proportion within PLN and pancreatic infiltrates. Representative dot plots for pancreatic infiltrates are shown. All measurements were performed on samples from 7 animals per group. * $p < 0.05$ represents a statistically significant difference between cells of MLDS+EP compared to those of MLDS-treated mice.

that drive the differentiation of Treg, Th1 or Th17 cells. The addition of EP 24 h after the initial stimulation significantly promoted the differentiation of Treg, while the proportion of *de novo* differentiated Th1 and Th17 cells did not change compared to the cells cultured without EP (Figure 9A). The expanded Treg expressed PD-1, GITR and CTLA-4 surface markers (Figure 9B), but the levels of expression per cell did not differ compared to Treg developed without EP (data not shown). EP also stimulated Treg *in vitro* differentiation in the absence of TGF- β (incomplete stimulation) (Figure 9C), suggesting that EP might have stimulated, at least partly, the intracellular signaling events that mimicked the TGF- β trigger. Finally, EP stimulated IL-10 production in the CD4⁺ cultures exposed to the complete Treg stimulation (Figure 9D). These

data suggest that EP stimulates differentiation of Treg in culture.

DISCUSSION

EP exerted specific effect on the regulatory arm of the immune response and thus prevented the development of T1D in C57BL/6 mice. It promoted tolDC within the pancreas and influenced Treg biology at multiple levels: through the stimulation of Treg proliferation and *de novo* differentiation, through the increase of CTLA-4, TGF- β , and IL-10 expression, making them more suppressive, and through favoring their migration to and retention at the site of inflammation.



The β -cell death in the investigated mouse model of T1D occurs through the induction of oxidative stress by the action of streptozotocin (21). Since EP possesses anti-oxidant properties, to avoid possible false results due to interference of EP with streptozotocin, the application of these two chemicals was at least 3 h apart. β -cells are extremely sensitive to oxidative damage since they have low levels of protective anti-oxidant enzymes (22). When endangered, β -cells express alarmins. One of those is HMGB1, whose level in β -cells of NOD mice correlates with the initiation of T1D (15). Higher HMGB1 expression was also found in β -cells of C57BL/6 mice treated with streptozotocin. EP is usually characterized as an HMGB1 inhibitor and it undoubtedly did reduce HMGB1 presence in pancreatic β -cells. Aside from acting as an alarmin, HMGB1 might be detrimental to β -cells because it can induce ROS generation through the stimulation of NADPH oxidase (4). Therefore, its reduction in β -cells may have helped protect β -cells from ROS-induced apoptosis. Surprisingly, EP did not prevent ROS and RNI generation in phagocytic immune cells that entered the pancreas, suggesting that this function of antigen-presenting cells is resistant to EP's influence.

After the initial ROS and RNI-mediated β -cell destruction after streptozotocin treatment, immune cells infiltrate the pancreatic islets. Subsequent β -cell apoptosis then becomes a result of a fulminant inflammatory process. In addition to anti-oxidant properties, EP can block inflammation as well. EP was found effective in several animal models of diseases with a strong inflammatory component, for example experimental colitis and encephalomyelitis (8, 10, 11). Although little is known about EP's molecular targets in immune cells, its anti-inflammatory properties are again related to the down-regulation of HMGB1 in non-immune cells. When secreted, HMGB1 can bind cell-surface receptors for advanced glycation end products (RAGE) and in that way intensify inflammation through generation or propagation of cells of innate and adaptive immunity. Another way of a pro-inflammatory HMGB1 effect could be the activation of TLR4. For example, elevated HMGB1 expression in β -cells of NOD mice is detected by the surface TLR4 (15). This activation of TLR4 can trigger NF- κ B signal transduction, leading to the expression of pro-inflammatory cytokines and the up-regulation of leukocyte adhesion molecules, thereby promoting injury and

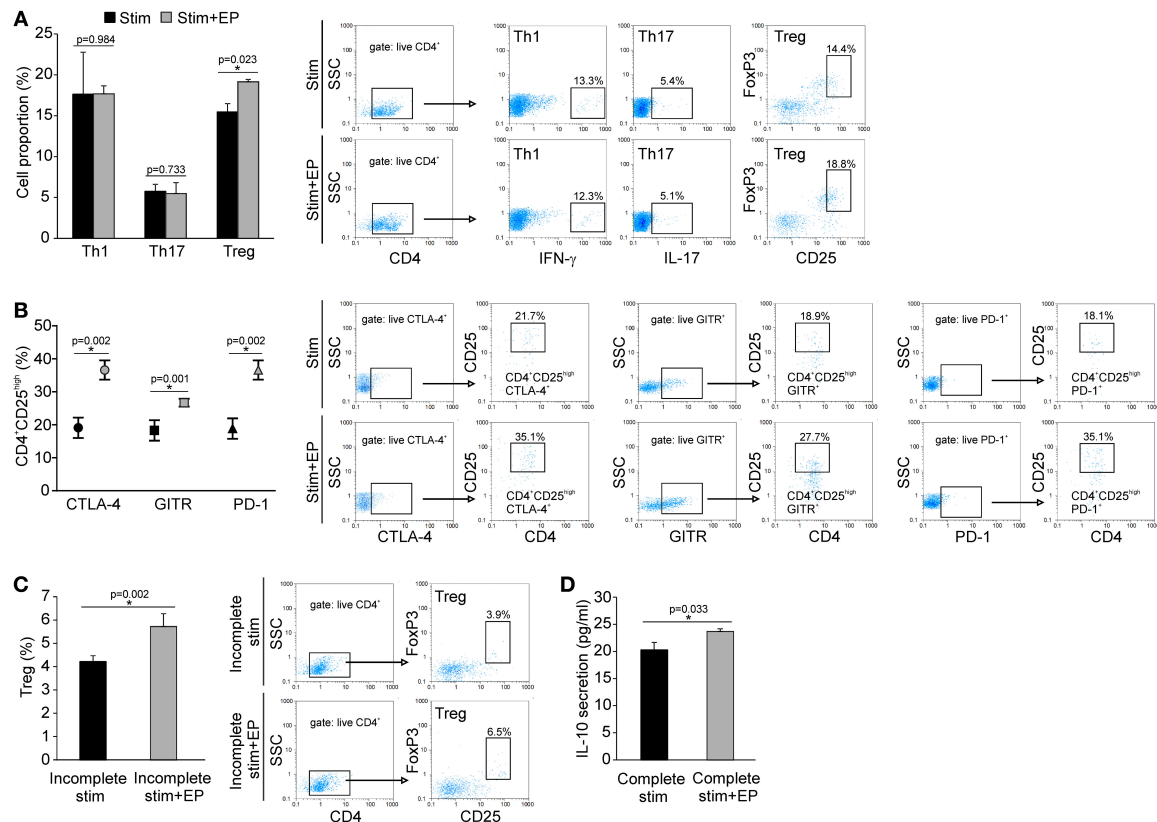
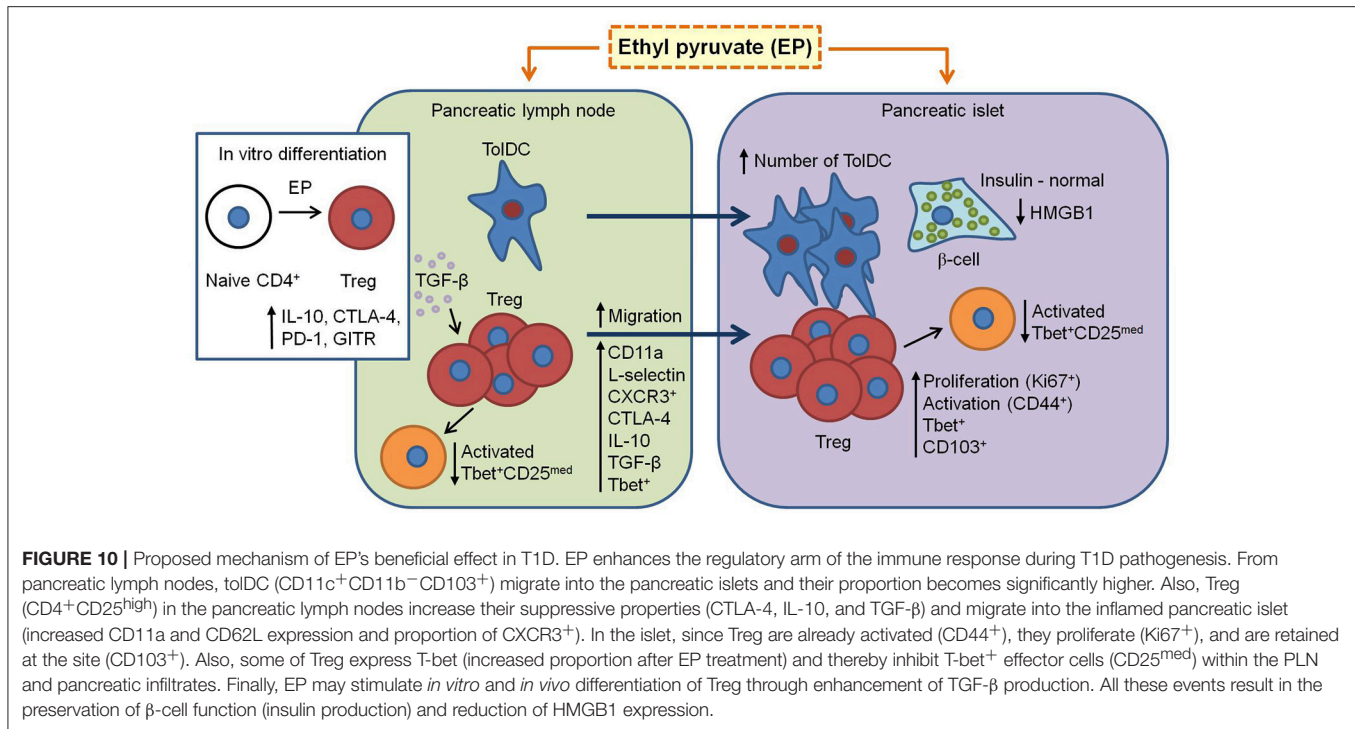


FIGURE 9 | *In vitro* effect of EP on Th cell differentiation. **(A)** The proportion of Th1 (CD4⁺IFN-γ⁺), Th17 (CD4⁺IL-17⁺), and Treg (CD4⁺CD25^{high}FoxP3⁺) cells after 96 h of incubation of CD4⁺CD25[−] cells with adequate stimulation (described in Material and methods) and in the presence of EP (125 μM), added 24 h after the start of cell cultivation. Representative dot plots are shown. Cells were first gated on live CD4⁺, followed by the gate on IFN-γ⁺, IL-17⁺ or CD25^{high}FoxP3⁺. **(B)** The proportion of Treg that expressed GITR, CTLA-4, and PD-1, determined by flow cytometry. Representative dot plots show the first gate on live CTLA-4⁺, GITR⁺ or PD-1⁺ population, followed by the gate on CD4⁺CD25^{high}. **(C)** The proportion of Treg after incomplete stimulation (anti-CD3+anti-CD28+IL-2) after 5 days of incubation of CD4⁺ cells in the presence or absence of EP. Representative dot plots show the first gate on live CD4⁺ cells, followed by the gate on CD25^{high}FoxP3⁺. **(D)** IL-10 concentration in supernatants of EP-treated CD4⁺ cells in the presence of complete Treg stimulation (anti-CD3/anti-CD28+IL-2+TGF-β), after 5 days of incubation, measured by ELISA. **p* < 0.05 represents a statistically significant difference between cells of MLDS+EP compared to those of MLDS-treated mice.

inflammation (23, 24). Consistent with these literature data, the other reason for the preservation of β-cell function after EP treatment was a lower degree of immune cell infiltration into the pancreatic islets (as determined by the insulitis scoring). Although the composition of these infiltrates was more or less the same regarding both cells of the innate and adaptive immunity, two major differences between EP-treated and diabetic mice have been observed. One is the higher presence of tolDC, and the other is the increased proportion of Treg. It was already shown that secreted HMGB1 stimulates dendritic cell maturation and *in vivo* homing to lymph nodes (25). So, the observed stimulating effect of EP on the differentiation of tolDC might be conducted through the inhibition of HMGB1 secretion. This was also confirmed *in vitro* where EP contributed immensely to the differentiation of tolDC from bone marrow precursors (personal communication—unpublished data). The higher presence of tolDC in the pancreatic infiltrates may not be due to *de novo* differentiation of immature DC, as these cells may come from pancreatic lymph nodes where we detected

a lower number of tolDC. Additionally, despite the fact that HMGB1 stimulates the differentiation of M1 macrophages, its inhibition by EP did not affect the ratio between M1 and M2 macrophages.

In the inflammatory setting during T1D progression, EP increased the number and the quality of Treg. The higher proportion of Treg could be a result of either enhanced proliferation or *de novo* differentiation. Proliferation of Treg was confirmed in the pancreatic islets, while for their differentiation we have only circumstantial evidence. For example, the level of TGF-β⁺ (non-Treg) cells was increased after EP treatment, suggesting that this TGF-β could drive new differentiation. Also, EP stimulated Treg differentiation *in vitro* in the circumstances of suboptimal stimulation (in the absence of TGF-β), suggesting that EP might trigger the same signaling pathways as TGF-β in naïve CD4⁺ lymphocytes. EP also enhanced the suppressive properties of Treg *in vivo*. It is already known that HMGB1 inhibits Treg function by down-regulating the costimulatory molecule CTLA-4, the Treg cell transcription factor Foxp3, and



the Treg suppressor cytokine IL-10 (26). Therefore, our results with *in vitro* and *in vivo* EP application strongly correlate with the observed inhibition of HMGB1 *in vivo* after EP treatment. More precisely, EP increased the expression of CTLA-4, which through binding to CD80 and/or CD86 probably suppressed the activation of antigen-presenting cells (27). Also, EP increased the proportion of TGF-β⁺ and IL-10⁺ Treg and thereby significantly up-regulated their immunosuppressive properties, as shown by their potency in the inhibition of T effector cell proliferation. These Treg that proliferated in response to EP treatment were probably the so-called induced or peripheral Treg, due to their strict necessity for exogenous lipids and pyruvate which they utilize in the Krebs cycle to acquire energy [reviewed in (28)]. Therefore, the addition of the stable form of pyruvate may have aided the differentiation and proliferation of peripheral Treg through a metabolic pathway as well.

Although the proportion of Treg was higher in both pancreatic lymph nodes and infiltrates examined after EP treatment, their migration to the pancreatic islets might be significantly increased if we take into account their *ex vivo* behavior in the transmigration assay. *Ex vivo* isolated Treg from EP-treated mice were attracted more efficiently to the pancreatic islets or to a CXCL12 chemokine gradient compared to the Treg from diabetic mice. Furthermore, they expressed higher levels of CXCR3, a chemokine receptor that responds to CXCL9, CXCL10, and CXCL11 and guides the cells to the site of inflammation (29), in this case the pancreatic islet where they are successfully retained (judged by CD103 expression). The expression of CXCR5 on CD25^{high} cells

(presumably regulatory follicular T cells) was the same in both diabetic and EP-treated mice, suggesting that EP did not specifically direct Treg to the germinal centers of pancreatic lymph nodes where they can help the differentiation of Breg (30). This result was corroborated by equal levels of Breg found in lymph nodes and infiltrates in mice of both groups.

It is a general opinion that Treg express signature transcription factors of pro-inflammatory cell subsets, thereby increasing their specificity for suppressing those particular Th subsets. Treatment with EP increased the proportion of T-bet⁺ Treg (T-bet⁺CD25^{high}) in the pancreatic infiltrates and simultaneously reduced the proportion of T-bet activated T cells (T-bet⁺CD25^{med}). However, there is a discrepancy between this result and the equal levels of Th1 cells observed in diabetic and EP-treated mice. This might stem from the fact that CD8⁺ cells may express CD25 and T-bet as well (31). Although the inhibition of HMGB1 can theoretically interfere with Th17 polarization (32, 33), our results indicate that HMGB1 reduction by the action of EP did not result in the down-regulation of Th17 response, which was corroborated by the equal levels of both activated effector RORγT⁺CD25^{med} cells and RORγT⁺ Treg after EP treatment.

In conclusion, this study proves the beneficial role of EP in the treatment of mouse T1D. It seems that EP favors the activation of the regulatory arm of the immune response and enhances its immunosuppressive properties (Figure 10). However, the question about EP's influence on the Treg metabolism still remains and should be explored as well.

AUTHOR CONTRIBUTIONS

All authors performed experimental work. IK and IS statistically analyzed the results. IS wrote the manuscript. All authors discussed data and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.03130/full#supplementary-material>

Figure S1 | Negative staining for insulin (A) and HMGB1 (B).

Figure S2 | The effect of EP on innate cells proportion and function. (A) F4/80⁺ cell proportion within the spleen and the pancreatic infiltrates. Representative dot plots for the spleen are shown on the right hand side. (B) The proportion of M1 (F4/80⁺CD40⁺) and M2 (F4/80⁺CD206⁺) macrophages within the pancreatic infiltrates, with representative dot plots below. (C) The intracellular production of

nitric oxide (NO) measured by DAF-FM mean fluorescence intensity (MFI) and reactive oxygen species (ROS) measured by DHR MFI within the pancreatic infiltrates, along with representative histograms. (D) The proportion of MHC class II⁺ antigen-presenting cells with co-stimulatory molecules CD80⁺ or CD86⁺ within pancreatic infiltrates, along with representative dot plots.

Figure S3 | Phenotypic analysis of adaptive immune cells after EP treatment. Representative dot plots of the proportion of cytotoxic lymphocytes (CD8⁺) or B lymphocytes (B220⁺ or CD19⁺) in spleen (A), PLN (B) or pancreatic infiltrates (C). Representative dot plots of the proportion of regulatory B cells (CD19⁺CD5⁺IL-10⁺) within PLN (D) and pancreatic infiltrates (E) (first gated on live IL-10⁺ cells, followed by the gate on CD19⁺CD5⁺). (F) Representative dot plots of the proportion of activated cytotoxic lymphocytes (CD8⁺CD44⁺) in the pancreatic infiltrates.

Figure S4 | Phenotypic analysis of adaptive immune cells after EP treatment. Representative dot plots of the proportion of Th (CD4⁺) and Th1 (CD4⁺IFN- γ ⁺), Th2 (CD4⁺IL-4⁺) and Th17 (CD4⁺IL-17⁺) within the spleen (A), PLN (B) and pancreatic infiltrates (C) of MLDS or MLDS+EP-treated mice (first gated on live CD4⁺ cells, followed by the gate on IFN- γ ⁺, IL-4⁺, or IL-17⁺).

Figure S5 | Characterization of Treg after EP treatment. (A) The expression of FoxP3, GITR, PD-1, and CD101 within CD4⁺CD25^{high} measured by mean fluorescence intensity (MFI), along with representative histograms.

Figure S6 | The effect of EP on Treg migratory abilities. (A) The proportion of CXCR5⁺ cells within activated Th cells (CD4⁺CD25^{med}) or within Treg (CD4⁺CD25^{high}) from PLN. Representative dot plots show the first gate on either live CD4⁺CD25^{med} or live CD4⁺CD25^{high} cells, followed by the gate on CXCR5⁺. (B) Representative dot plots for CD25^{high}CD103⁺ proportion within PLN.

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Past, Present, and Future of Regulatory T Cell Therapy in Transplantation and Autoimmunity

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Regulatory T cells (Tregs) are important for the induction and maintenance of peripheral tolerance therefore, they are key in preventing excessive immune responses and autoimmunity. In the last decades, several reports have been focussed on understanding the biology of Tregs and their mechanisms of action. Preclinical studies have demonstrated the ability of Tregs to delay/prevent graft rejection and to control autoimmune responses following adoptive transfer *in vivo*. Due to these promising results, Tregs have been extensively studied as a potential new tool for the prevention of graft rejection and/or the treatment of autoimmune diseases. Currently, solid organ transplantation remains the treatment of choice for end-stage organ failure. However, chronic rejection and the ensuing side effects of immunosuppressants represent the main limiting factors for organ acceptance and patient survival. Autoimmune disorders are chronic diseases caused by the breakdown of tolerance against self-antigens. This is triggered either by a numerical or functional Treg defect, or by the resistance of effector T cells to suppression. In this scenario, patients receiving high doses of immunosuppressant are left susceptible to life-threatening opportunistic infections and have increased risk of malignancies. In the last 10 years, a few phase I clinical trials aiming to investigate safety and feasibility of Treg-based therapy have been completed and published, whilst an increasing numbers of trials are still ongoing. The first results showed safety and feasibility of Treg therapy and phase II clinical trials are already enrolling. In this review, we describe our understanding of Tregs focussing primarily on their ontogenesis, mechanisms of action and methods used in the clinic for isolation and expansion. Furthermore, we will describe the ongoing studies and the results from the first clinical trials with Tregs in the setting of solid organ transplantation and autoimmune disorders. Finally, we will discuss strategies to further improve the success of Treg therapy.

Keywords: Tregs (regulatory T cells), transplantation, autoimmunity, cell therapy, clinical trial

INTRODUCTION

Since the discovery in 1969 of the suppressor T cells (1), the regulatory T cells (Tregs) research field has undergone an incredible boom over the years. Studies on the biology of Tregs have attracted a lot of attention and our knowledge about their development and differentiation has increased enormously. The breakthrough in this field dates back to the discovery, in 1995, of a subset of thymus derived CD4⁺ T cells expressing high levels of IL-2R α (CD25) able to protect thymectomized mice from autoimmunity (2). Since then, well-cited papers have shown the crucial role of Tregs in maintaining immune homeostasis (3) and preventing autoimmunity (4). Despite the improvement in Treg biology, nowadays there are no specific markers to characterize human Tregs. Differently from the mouse counterpart, the sole expression of CD25 and the transcriptional regulator forkhead box P3 (FOXP3) (5) is not sufficient for characterizing human Tregs, since effector T cells can upregulate these markers after activation. However, the methylation status of the Treg-specific demethylated region (TSDR) (6), a conserved non-coding element within the *FoxP3* gene locus, can be used for the identification of the “real” human Tregs (7). The analysis of TSDR methylation does not represent a suitable tool for their isolation; currently the expression of CD4, CD25, and lack of the α -chain of IL-7R (CD127) (8) are used for Tregs purification. In preclinical studies, human CD4⁺CD25⁺CD127⁻ Tregs have been shown to be effective in preventing Graft vs. Host Disease (GvHD) (9, 10), autoimmune diseases (11, 12) and delaying graft rejection (13, 14). The positive outcomes gave the rationale to apply Tregs for the treatment of human diseases and results from the first clinical trials with adoptively transferred Tregs were published in 2009 (15).

Solid organ transplantation represents the only treatment for end-stage organ diseases. Over the years, several strategies have been applied in order to improve transplantation outcomes and short-term graft survival (16). A better selection of donors and recipients associated with improved immunosuppressive schemes and patients’ management has been crucial for ameliorating the graft survival in early stages. Long-term organ acceptance is a different story, remaining constant over the past decades (17). The immunosuppressive regimen, consisting of a combination of different drugs, aims to dampen the response of the immune system to the graft. Although successful in controlling the immune response early post-transplant, it is linked with detrimental side effects. Cardiovascular diseases, cancer, kidney failure and infections represent the main side effects that can cause graft loss and death (18). Long-term outcomes and finally operational tolerance are key for a successful organ transplantation. Different strategies are under investigation with the aim to reduce the use of immunosuppressive drugs. In this scenario, Tregs might represent a valid solution for controlling the immune response and inducing transplantation tolerance.

Autoimmune disorders are chronic diseases caused by the breakdown of tolerance against self-antigens. Usually they

involve a specific region of the body such as the joints in rheumatoid arthritis (RA) or the pancreatic cells in type 1 diabetes mellitus (T1D). In other autoimmune diseases such as systemic lupus erythematosus (SLE) multiple areas are affected. The origin of autoimmune diseases is still a matter of debate; one hypothesis involves a failure in central and peripheral tolerance with the latter being associated with reduced Treg number or failure in their function (19). Furthermore, the combination of genetic and environmental risk factors has been implicated in the ontogenesis of autoimmunity as well (20). Similar to transplantation, immunosuppressive regimens aim to inhibit the activation of the immune system and reduce chronic inflammation. Different monoclonal antibodies targeting co-stimulatory molecules (21), cytokines (22), and lineage specific molecules (23) have been tested however, they all aim to target the immune and autoimmune responses leaving patients immunocompromised. For this reason, Tregs have been suggested as an effective tool for the treatment of autoimmune diseases.

TREGS ONTOGENESIS

The summation of the research over the past years has demonstrated that the thymus is the crucial organ for the generation of Tregs (24). Animal models have shown that the differentiation of thymus-derived Tregs (tTregs) depends on T cell receptor (TCR) signaling, particularly the strength and duration of the signal (25). Despite technical limitations, this has been confirmed in humans as well (24). In thymus, immature CD4 single positive (SP) cells receive a TCR signal of varied strength, which will drive their fate. Following a TCR signal of high strength, most CD4 SP cells undergo negative selection, whereas those receiving TCR signals of intermediate strength are able to escape deletion and are committed to differentiate into Tregs (26). Nevertheless, whether there are differences between TCR signals for conventional T cells (Tconv) and Tregs is still an open question. Some pieces of evidence so far support the idea of “quantitative” difference in signaling, but it is also plausible that TCR signals might be “qualitatively” different. Beyond TCR signaling, CD28 is also crucial in the generation of tTregs. In fact, both CD28-deficient and CD80-CD86-deficient mice have decreased number of Tregs (27). Several other factors, including NFAT/AP1, ICOS/ICOSL and thymic stromal lymphopoietin (TSLP) are involved in the transcriptional control of human Treg differentiation (28–30). FOXP3 expression requires the presence of γ chain cytokines (IL-2, IL-15, and IL-7) and the reduction of PI3K-mTOR signaling pathway. Mice deficient in IL-2 or IL-2R α have decreased number of FoxP3⁺ thymocytes, while ablation of IL-15 and IL-7 alone does not have such effect (5). The essential role of IL-2 in the generation of Tregs has been confirmed in humans as well (29). Phosphatidylinositol 3-kinase (PI3K) is induced by TCR and CD28 signaling and through the activation of Akt-mTOR pathway, antagonizes FOXP3 expression, thereby inhibiting the development and suppressive function of tTregs (31). Conversely, the hyper-activation of this pathway in Tconv cells has been suggested as a possible

mechanism to overcome Treg suppression (32). In recent years, it has been demonstrated that the demethylation status of a TSDR is essential for human Treg lineage maintenance (6). Therefore, both FOXP3 expression and TSDR demethylation are essential for tTreg lineage commitment. Together, these findings demonstrate that TCR signaling in combination with other cell-intrinsic and extrinsic signals orchestrates human tTreg cell differentiation.

In addition to tTreg, naïve FOXP3⁺CD4⁺ T cells can differentiate in the periphery to become FOXP3⁺ cells, which are known as induced Tregs (iTregs) or peripheral Tregs (pTregs). Differently from tTregs, the generation of iTregs is likely promoted by non-self-antigens (allergens, food, microbiota) (33). It has been also shown that a distinct TCR repertoire and ligand specificity support the generation of iTregs. These TCRs are of high affinity and their sequences only partially overlap with the TCRs used by tTregs. Additionally, an efficient induction of FOXP3 and iTreg generation occurs *in vivo* upon TCR stimulation together with suboptimal co-stimulation (decreased CD28 signaling) (34). Therefore, TCRs that recognize antigens to which an organism is chronically exposed promote the generation of iTregs. Low levels of costimulatory molecules in the presence of anti-inflammatory molecules secreted by tolerogenic DC cells (tDCs) promote the differentiation of iTreg cells as well (35). Beyond TCR signaling and suboptimal co-stimulation, the polarization of naïve CD4⁺ T cells into iTregs requires the combination of TGF- β and IL-2 (36). For example, several animal studies have shown that TGF- β /TCR-mediated iTreg cell generation is strictly dependent on IL-2 signaling. IL-2 promotes the activation of *Foxp3* locus through STAT5 and constrains the differentiation of activated CD4⁺ T cells into Th17 cells (37, 38). So far, *in vitro* TGF- β -induced iTreg cells have been considered a valid approach to study the development of iTreg cells *in vivo*. However, this experimental method fails in recapitulating the epigenetic and transcriptional characteristics of *in vivo* induced iTreg cells, namely transient suppressive ability and unstable FOXP3 expression, precluding therefore their use in the clinic (39). Inducible T regulatory type 1 (Tr1) cells are a subset of iTregs characterized by the ability to produce the immunosuppressive cytokine IL-10 (40). Tr1 can only transiently up-regulate FoxP3 following stimulation. These cells have been shown to maintain peripheral tolerance, modulate effector T cell responses in several autoimmune diseases and prevent allograft rejection (40). The possibility of generating *in vitro* expanded Ag-specific Tr1 cells has encouraged their clinical use in autoimmunity and Graft vs. Host Disease (GvHD).

Current evidence indicates that tTregs and iTregs are designated to play different roles in different tissues. Owing to the nature of iTreg differentiation induced by non-self-antigens and a particular TCR signaling combined with other signals, such as TGF- β and IL-2, these cells are assumed to be more functional for maintaining mucosal tolerance. iTregs may therefore control immune responses to commensal antigens and prevent allergic-type reactions.

“HETEROGENEITY OF TREGS”

Tregs in circulation are considered heterogeneous, this is mainly due to their plasticity and the capacity to acquire features specific to the type of immune response they control. In the literature, Tregs are divided in subpopulations according to the sites of differentiation and the expression of well-known functional markers. However, this does not allow a full distinction due to the overlap and redundancy between many of these parameters. For the first time in 2009, Miyara et al. demonstrated that human Tregs consists of three subpopulations based on their expression levels of FOXP3 and CD45RA (41). Tregs were classified as naïve/resting (CD45RA⁺FoxP3^{low}), effectors (CD45RA⁺FoxP3^{high}), and cytokine-producing (CD45RA⁺FoxP3^{low}). Naïve Tregs are considered the “real Tregs” arising from thymus with a fully demethylated *Foxp3* locus. Effectors Tregs are the active population *in vivo* while the cytokine-producing Tregs include those cells able to produce pro-inflammatory cytokines like IL-17 and IFN- γ but still able to suppress. More recently, human Tregs named T helper-(Th-) like Tregs have been described (42). These memory Tregs mirror the classical CD4⁺ Th population expressing the same chemokine receptors CXCR3, CCR6 and CCR4, typically expressed by T-bet⁺-Th1, ROR γ t⁺-Th17, and GATA3⁺-Th2, respectively. We have fully characterized these subsets showing their cytokine production, suppressive and migratory ability (43). *In vitro*, all Th-like Tregs are functional with no preferential suppressive ability toward the cognate Th counterpart. This highlights the importance of Th/Tregs co-localization for the control of the immune system activation. The ontogenesis of these subsets is still under debate due to the high Treg plasticity, which can be detrimental in the setting of autoimmune diseases (44). In this scenario, Tregs acquire Th phenotypes associated with a reduced function despite maintaining Foxp3 expression and demethylation. The frequency of Th1-like Tregs is increased in patients with T1D (45), multiple sclerosis (46) and autoimmune hepatitis (47) and it is associated with a reduced suppressive ability. Similarly, Th2-like Tregs are increased in the skin but not in the peripheral blood of patients with systemic sclerosis (48). Whereas, Th17-like Tregs are increased in psoriasis (49) patients and inflammatory bowel diseases (50, 51) with no loss of function. However, the origin and the fate of Th17-like Tregs is matter of debate as some authors suggested that they might represent a transient stage in the differentiation of Tregs into Th17 cells (52). Under inflammatory conditions and autoimmune diseases, FOXP3⁺ Tregs can convert into Th17 thus impairing immune homeostasis and contributing to the progression and pathogenesis of the disease (44). As already mentioned, the demethylation of the TSDR region is the key determinant for Treg stability and function. FOXP3 is known to neutralize ROR γ t transcription, the master transcription factor of IL-17 producing Th17 cells (53). Therefore, a highly stable Foxp3 expression *in vitro* is associated with a small risk for IL-17 production *in vivo* under inflammatory conditions.

MECHANISMS BY WHICH TREGS SUPPRESS

The mechanisms used by Tregs to suppress different immune cells can either be considered direct whereby Tregs themselves elicit a direct response on a target cell, or indirect, in which a third-party cell or molecule is affected and in turn suppresses the target cell (54). Examples of direct mechanisms include the secretion of cytokines such as IL-10, TGF β and IL-35 and the production of granzyme and perforin, enzymes leading to apoptosis in target cells (54). Indirect mechanisms include the expression of CD39/CD73, which deplete the microenvironment of extracellular ATP via the generation of adenosine and AMP, molecules with immunosuppressive effects (54). Alternatively, Tregs can influence changes in the microenvironment due to their high expression of CD25. The high expression of this receptor enables Tregs to uptake more IL-2 and “starve” the surrounding cells of this cytokine (55). When considering whether there is a “dominant” mechanism of suppression utilized by Tregs, it is important to understand that different mechanisms are utilized preferentially for the vast variety of target cells and microenvironments in which Tregs act. Below, we explore, more specifically the methods used on several “key” cell types from both the innate and adaptive arms of the immune system (Figure 1).

T-Lymphocytes

This includes of CD4⁺ and CD8⁺ cells. Tregs suppress CD4 T cell activation and proliferation by contact-dependent and contact-independent mechanisms [extensively reviewed in (56)]. An important factor for Treg suppressive ability on other T cells is their localization. This is in line with the mutual distribution of the Th-like Tregs and classical Th cells observed by us in human thymus, spleen, liver, and colon (43). Additionally, Tregs can also influence proliferation, activation and apoptosis of CD8⁺ T cells (57, 58). As a result, the induction of high affinity effector and memory CD8⁺ T cells is reduced.

B-Lymphocytes

B-Lymphocytes are important components of the adaptive immune system acting largely by the production of antibodies; however, they can act as APCs as well. B-cells require activation by T-cells following antigen recognition. Upon activation, B-cells differentiate into “effector” plasma cells which can produce antibodies. Tregs have the potential to suppress autoreactive B-cells in an antigen-specific manner and prevent the production of harmful autoantibodies. This suppression requires PD-1 expression on autoreactive B cells and expression of the two PD-1 ligands (PDL-1 and 2) on Treg (Figure 1) (59). In addition, Tregs are able to kill B-cell by releasing granzyme B and perforin (60).

Dendritic Cells

Dendritic cells (DCs) play a critical role in the regulation of the adaptive immune response by activating resting naïve T cells. In the setting of transplantation, they are key in mediating graft rejection through direct, indirect and semi-direct presentation [extensively reviewed by us in (61)]. Similarly, DCs have been

involved in the pathogenesis of many autoimmune diseases (62). Animal studies have shown that Tregs can interact with DCs in a leukocyte function-associated antigen-1 (LFA-1) dependent manner (63) and down-regulate the expression of CD80/CD86 on target cells by CTLA-4 (Figure 1). However, in CTLA-4 KO-mice Tregs could still suppress via compensatory mechanisms involving TGF- β and IL-10 (64). Furthermore, Tregs have the ability to increase the expression of Indoleamine 2,3-dioxygenase (IDO) in DCs (Figure 1) (56). This enzyme catalyzes the degradation of tryptophan to kynurenine leading to starvation of effector cells. Unlike effector T cells, Tregs can express LAG3 a homolog of the CD4 receptor. It binds to MHC-II with a significantly higher affinity than CD4 mediating the activation of PI3K/AKT, p42/44ERK, and p38MAPK pathways (65). As a result, DCs exhibit an increased expression of co-stimulatory molecules but reduced capacity to capture the antigens. In addition to these cell-contact depended mechanisms, Tregs can disrupt the microenvironment in the immunological synapse provided by DCs and essential for T cells proliferation. In detail, Tregs act either reducing the limiting enzyme for glutathione (GSH) synthesis or consuming extracellular cysteine (66, 67).

Monocytes

Under inflammatory conditions, monocytes migrate into the tissue, where they differentiate into dendritic cells or tissue-resident macrophages. Monocytes constitute the major cellular component in inflamed tissues and their regulation might be key in reducing chronic inflammation. Tregs have been demonstrated to directly act on monocytes inhibiting their cytokine secretion, differentiation and antigen presenting function. Following co-culture with Tregs, monocytes exhibited classical features of M2 macrophages such as increased expression of CD206 (mannose scavenger receptor) and CD163 (hemoglobin scavenger receptor), simultaneously these cells showed a reduced capacity to respond to pro-inflammatory stimuli as demonstrated by decreased production of IL-6 and TNF- α and decreased NF- κ B activation (68) (Figure 1). Recently we have shown that *ex vivo* expanded Tregs are more efficient in skewing monocytes toward a tolerogenic phenotype. Of note, monocytes co-cultured with expanded Tregs showed a reduced capacity to increase detrimental IL-17 producing T-cells when compared to freshly isolated Tregs (69). This mechanism was due to a decreased expression of CD86 by Tregs-conditioned monocytes.

Granulocytes

Granulocytes are a group of cells belonging to the innate immunity. Among them, neutrophils are the first to respond to sites of inflammation where they phagocytose pathogens, release proteolytic enzymes, and produce antimicrobial peptides. Dysfunction of these cells results in sustained inflammation which can cause a number of pathological conditions including sepsis (70) and autoimmune diseases (71). Tregs directly affect neutrophils (Figure 1) limiting their accumulation. They do this by decreasing the expression of chemoattractants, CXCL1 and CXCL2, thus preventing aberrant skin infiltration

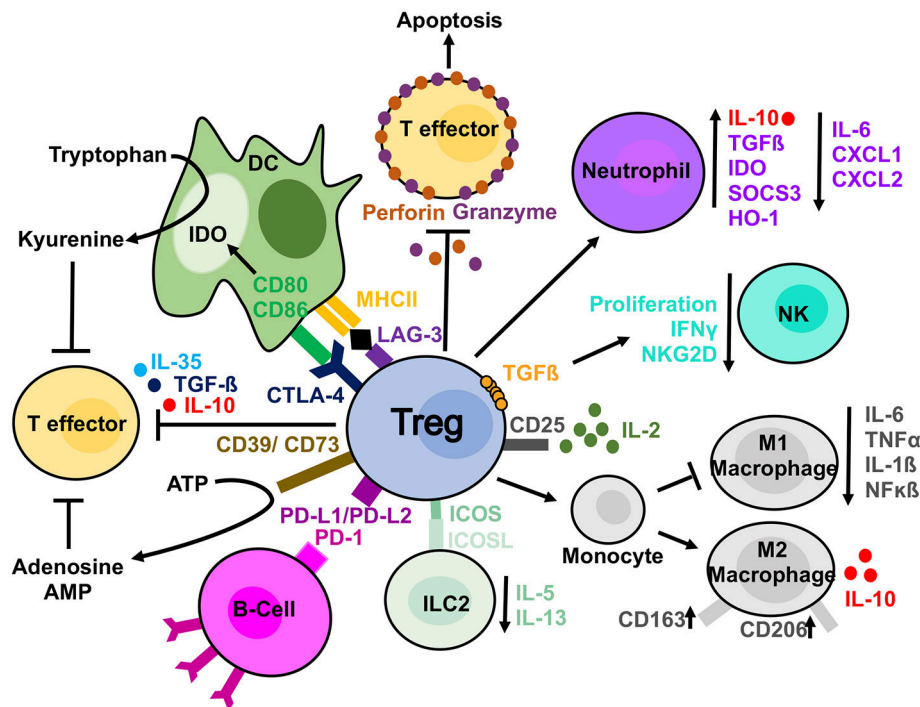


FIGURE 1 | Treg suppressive mechanisms. Tregs are able to suppress different cell types by direct and indirect mechanisms. Tregs can produce anti-inflammatory cytokines (IL-10, IL-35, and TGFβ) affecting T cells. In addition, they release perforin and granzyme, which damage target cell membrane leading to apoptosis. They can sequester, by the high expression of CD25, IL-2 from the microenvironment reducing effector T cells proliferation and exhibiting effector functions as well. Furthermore, NKs can be directly affected by Tregs in a membrane bound TGF-β dependent manner. Tregs have been observed to have a direct effect on B-cells via PDL1/PD-1 interaction and DCs via both CTLA-4 and LAG-3. CTLA-4 blocks co-stimulation reducing CD80/CD86 expression and it induces upregulation of IDO. The expression of CD39 on Tregs mediate the conversion of ATP to adenosine and AMP and reduce T effector proliferation. Tregs can also skew monocyte toward M2 macrophages and prevent their differentiation in pro-inflammatory M1 macrophages. They can similarly induce a suppressive phenotype in neutrophils and reduce ILC2 cytokine secretion.

(72). Furthermore, Tregs can also induce a more “immuno-suppressive” phenotype in neutrophils, thus skewing the microenvironment toward a less inflammatory one. Neutrophils co-cultured with Tregs produced more IL-10 and TGF-β together with a decreased IL-6 production (73). In addition, Tregs induced the expression of heme oxygenase-1, IDO and the suppressor of cytokine signaling 3 molecule (SOCS3) (73).

Basophils are another subgroup of granulocytes which are critical in mediating allergic and inflammatory responses. A recent publication showed that Tregs were able to activate resting basophils inducing their expression of CD69, CD203c, and CD13. Further to these, activated basophils were able to release IL-4, IL-8, and IL-13 (74).

Innate Lymphoid Cells (ILCs)

Recently described, ILCs are a subset of the innate immunity arising from a lymphoid precursor. They are divided into three groups depending on the expression of specific transcription factors and cytokines [extensively reviewed in (75)] Natural Killer cells (NKs) release cytokines, recruit other immune cells via IFN-γ and TNF-α production and have direct cytotoxic activity. Interactions between NKs and Tregs have been reported (76). During pregnancy, Tregs suppress NKs to create a tolerant

environment favoring the implant (77) while in tumors Tregs have the potential to block NKs generating an immune-suppressive environment, which favors cancer cell survival (76). Mechanisms of control exerted on NK cells by Tregs have been investigated (Figure 1). Following activation, Tregs suppress NKs via membrane bound TGF-β (78). This suppression results in the inhibition of NK cells’ effector functions and a down regulation of NKG2D receptors on cell surface. Further to this, by restricting the availability of IL-2 in the microenvironment, Tregs prevent the NKs from proliferating, secreting IFN-γ and enhancing missing self-recognition (76). Tregs can also affect ILCs2. In particular, iTregs but not tTregs have the ability to suppress ILC2 function (Figure 1) preventing their secretion of both IL-5 and IL-13 in an ICOS/ICOSL dependent-manner (79).

TREGS ISOLATION AND EXPANSION

Tregs can be isolated in large scale from either peripheral blood (PB) (80), umbilical cord-blood (UCB) (81) or thymus (82). To date, only Tregs isolated from UCB and PB have been tested in the clinic. The isolation under GMP condition was carried out by “CliniMACS” system (CliniMACS TM Instruments, Miltenyi Biotec) a clinical-scale magnetic enrichment of cells in a closed

and sterile system. The protocol consisted in either the depletion of CD8⁺ and CD19⁺ cells or CD8⁺ only followed by an enrichment of the CD25⁺ fraction. Although the CliniMACS has been used by several groups for isolating Tregs, cell purity represents an important limitation. As reported by Di Ianni et al., only 80% of the cells were FOXP3⁺ due to the presence of cell contaminants (83). The presence of non-FOXP3 cells might be deleterious especially if the generation of antigen specific cells has been planned. Another method used to isolate Tregs in the clinic is the flow cytometry-based purification. Similar to the cell sorter routinely used in the research field, cells can be isolated according to the expression of selected cell markers. To date, flow-sorted Tregs have been used in clinical trials outside the EU (84, 85) where cells have been selected according to the expression of CD4, CD25, and CD127 with high purity (>99%). However, in the last few years, different companies have started to develop GMP compatible cell sorter and fluorescent antibodies. This will allow the isolation of CD4⁺CD25⁺CD127^{low}CD45RA⁺ Tregs, a subset more suitable for long-term expansion due to an epigenetically stable FOXP3 expression and an increased resistance to Th17 conversion (86). Moreover, this method does not allow the isolation of activated effector cells that usually express intermediated levels of CD25 and are increased in autoimmune diseases. However, due to the high number of processed PBMCs at the beginning, a pre-enrichment for CD4⁺ cells might be necessary. As a consequence, the cost for a single preparation will increase considerably.

Another challenge for the research groups aiming to start/develop new clinical trials with Tregs has been their low number. In PB, Tregs are 5–10% of all the circulating CD4⁺ T cells. Although in CB and thymus the number of Tregs is higher, the infusion of a large cell number has been difficult to achieve. The first clinical trial in GvHD used freshly isolated Tregs (87). However, during the same period our group and others developed a clinically scalable protocol for their expansions (88, 89). Tregs are now routinely expanded *ex-vivo* in 36 days using anti-CD3/CD28-coated beads in the presence of high dose of IL-2 (polyclonal expansion) (90). In detail, our protocol involves the use of rapamycin, an immunosuppressant that inhibits the mechanistic target of rapamycin (mTOR) protein kinase. Rapamycin added during the course of the culture, inhibits exclusively the proliferation of effector T-cells. In addition, by blocking the signaling through AKT–mTOR–SMAD3, rapamycin favors FOXP3 upregulation (91). Rapamycin confers to the expanded Tregs higher stability and suppressive capacity; of note, Tregs from TD1 patients and patients with cirrhosis on a waiting list for a transplant expanded in the presence of rapamycin recover their suppressive ability (80, 92). Together with rapamycin and IL-2, Leventhal's group has developed a protocol to expand Tregs in the presence of TGF- β (93). At the end of the expansion, their product was more suppressive compared to the use of rapamycin alone and TSDR more demethylated compared with the freshly isolated counterpart. All-trans retinoic acid (ATRA) is another molecule that can be used for Tregs expansion. Similarly to TGF- β , ATRA can induce the generation of Tregs (94). During Treg expansion this molecule is essential for the upregulation of chemokine receptors

responsible for gut homing like CCR9 and integrin- α 4 β 7 (89). Due to its peculiarity, ATRA will be used for the expansion of Tregs in one of our clinical trials, “TRIBUTE” aiming to evaluate the infusion of *ex-vivo* polyclonally expanded naïve Tregs in patients with Crohn's Disease. Umbilical cord blood-derived Tregs have been isolated and expanded for the treatment of GvHD (95). Cells were stimulated with anti-CD3 mAb and artificial APC consisting of K562 cell lines (KT) engineered to express CD86 and the high affinity Fc Receptor (CD64) (KT64/86). During the 19 days culture, cells were supplemented with 300 IU/mL of IL-2. Finally, the generation and expansion of alloantigen specific Treg is a promising strategy that can be tested soon in the EU with the advent of the GMP-cell sorter. Our preclinical protocol for the generation and expansion of antigen-specific Tregs involves the co-culture of Tregs with CD40-activated allogeneic B-cells or donor-derived DCs in the presence of IL-2. Antigen-specific Tregs have been shown to be more powerful in suppressing alloimmune responses *in vitro* and *in vivo* compared to the polyclonally expanded Tregs (96).

CLINICAL TRIALS ENROLLING REGULATORY T CELLS

After 10 years from the first Treg infusion in patients with GvHD, several phase I or phase I/II clinical trials have been completed or started. They aim to test the safety, feasibility and efficacy of Treg infusion in the setting of solid organ transplantation (97) GvHD (98) and autoimmunity (84). In the next sections, we describe results from published studies in the setting of autoimmune diseases and transplantation giving an overview of the main clinical trials that are ongoing.

Tregs in Autoimmunity

The first-in-man clinical trial adopting Tregs in autoimmune diseases was the “CATS1” study, the results of which were published in 2012 by Desreumaux et al. (99) In this phase I/IIa, open-label, multicentric trial, 20 patients with active and symptomatic refractory Crohn's Disease were divided in 4 dose cohorts receiving a single infusion of 10⁶–10⁹ Tr1. Firstly, PBMCs were cultured in the presence of ovalbumin in medium supplemented with supernatant derived from *Drosophila* Schneider 2 (S2). S2 cells were previously transfected to produce IL-2 and IL-4 and express trans-membrane mouse anti-human CD3, CD80, and CD58. After 7 days of culture growing clones were harvested and tested for antigen specificity and Tr1 cell identity before being expanded on *Drosophila* feeder cells. Cell infusion has been considered safe and, the reported unexpected severe adverse events were correlated to the natural history of the disease rather than the treatment. In 2014 Marek-Trzonkowska et al., published the results from a prospective, non-randomized phase I trial with the purpose to evaluate safety and feasibility of the infusion of autologous *ex-vivo* expanded polyclonal Tregs in patients with recently diagnosed T1D (85). Twelve patients aged 7–18 were enrolled and compared with ten patients who met the eligibility criteria, whose blood could not be collected due to inappropriate venous access. Fresh blood

(250 mL) was collected and Tregs ($CD4^+CD25^+CD127^{low}$) were sorted and expanded using antiCD3/CD28 coated-beads, IL-2 and autologous serum without using rapamycin. Three patients received a single dose of 10×10^6 cells/kg, other three patients received 20×10^6 cells/kg, while six patients were offered a double dose up to a total 30×10^6 cells/kg. The last group was composed of those patients who showed good laboratory and metabolic response, but symptoms of disease progression after 6 months from the first infusion. After 1 year of follow-up, safety was proved by the absence of serious adverse events and eight patients showed signs of clinical remission, among those, two remained insulin-independent. Conversely, the untreated patients remained insulin-dependent with lower C-peptide levels.

In 2015, Bluestone et al. published results from an open-label, interventional phase I clinical trial conducted at University of California (San Francisco) and Yale University (84). They aimed to determine safety and feasibility of intravenous infusion of *ex-vivo* expanded autologous polyclonal Tregs in patients with T1D. Fourteen recently diagnosed T1D patients, six females and eight males, were divided into four dose cohorts, ranging from 0.05×10^8 cells for cohort one to 26×10^8 cells for cohort four, with 8-fold dose increase in each cohort. Two weeks before the planned single infusion, 400 ml of fresh peripheral blood was collected; Tregs were isolated via Cell Sorting and cultured for 14 days in the presence of antiCD3/CD28 coated-beads and IL-2. Out of the sixteen eligible patients, two did not receive treatment, due to failure in expanding Tregs. After a mean follow-up of 31 months, only three severe adverse events were reported (two hypoglycaemias, one ketoacidosis), while no opportunistic infections were observed. None of the total reported adverse events (mild to severe) was related to cell infusion. Seven patients (cohort 3 and 4) received deuterium-labeled Tregs in order to track the infused cells. After 1 day post infusion, Tregs could be detected in circulation with a peak observed by 7 to 14 days. The percentage of deuterium labeled Tregs dropped to 25% after 3 months remaining stable up to 9 months. After 1 year, deuterium was still detectable in four patients with no evidence of differentiation of Tregs into T effectors. Although this study showed a feasible and safe therapeutic approach to T1D, with stably suppressive Tregs, the small number of treated patients as well as the early phase of the trial could not help to shed light on the optimal dose and the impact of Tregs on the function of islet cells. To address these points, a multicentre phase II randomized, placebo-controlled double blind clinical trial (NCT02691247) is underway, with the purpose to evaluate, in young patients, safety and effect on beta cell function of a single dose (low 2.5×10^6 /kg vs. high 20×10^6 /kg compared to placebo) of autologous *ex-vivo* expanded polyclonal Tregs. Another ongoing phase I clinical trial (NCT02772679) is evaluating safety and optimal dosing of a single infusion of autologous *ex-vivo* expanded polyclonal Tregs ($CD4^+CD25^+CD127^{low}$) followed by injection of IL-2 in patients with T1D. The enrolled patients will be allocated into two dose-cohorts receiving 3×10^6 cells/kg and 20×10^6 cells/kg, respectively. IL-2 (1×10^6 IU) will be administered subcutaneously, daily, for the 5 consecutive days post infusion and repeated after 1 month. The primary outcome will be

the occurrence of any adverse events and the evaluation of distribution of deuterium-labeled Tregs.

In 2018, Dell'Era et al. published results from NCT02428309, a phase I non-randomized, open-label study originally set to evaluate escalating single dose of autologous, FACS-sorted and *ex-vivo* polyclonally expanded Tregs (1×10^8 , 4×10^8 , and 16×10^8 cells, respectively) in adult patients with active cutaneous Lupus (100). Due to screening failures and comorbidity burden, a single patient was recruited, who received 1×10^8 deuterium-labeled Tregs. The labeled cells in circulation were reduced after 4 weeks, while skin biopsies showed a marked increase in tissue Tregs and IL-17 production by both CD4 and CD8 cells. Along with the aforementioned, to date (end of November 2018) more studies underway (Table 1). NCT03239470 is a phase I open-label trial evaluating the infusion of a single dose (2.5×10^8 vs. 10×10^8 cells) of sorted autologous polyclonally expanded Tregs in adult patients with active cutaneous pemphigus. NCT03011021 is a phase I/II randomized open-label study, led by Central South University Changsha, evaluating the infusion of 2×10^6 /kg of umbilical cord blood Tregs along with liraglutide therapy in adult and elder patients with autoimmune diabetes. Patients will be allocated in four groups: Tregs+liraglutide+insulin, Tregs+insulin, liraglutide+insulin, insulin alone. The same center is leading another randomized, open-label phase I/II trial (NCT02932826) with the aim to compare, in T1D, the infusion of umbilical cord blood Tregs combined to insulin and insulin therapy alone. NCT02704338 is a phase I/II open-label study on the infusion of a single dose of 10 – 20×10^6 /kg autologous *ex-vivo* polyclonally expanded Tregs in patients aged 10–70 with autoimmune hepatitis (Nanjing Medical University). NCT03185000 (TRIBUTE) is a double-blind, placebo-controlled trial (King's College London), evaluating the infusion of sorted and polyclonally expanded $CD4^+CD25^+CD127^{low}CD45RA^+$ Tregs in adult patients diagnosed with moderate to severe Crohn's Disease not tolerating or responding to at least 2 standard treatments. Patients are divided in two groups, one receiving Tregs at week 0 and placebo at week 8, the other receiving placebo at week 0 and Tregs at week 8. Doses range from 0.5 – 1×10^6 /kg, up to 8 – 10×10^6 /kg.

Tregs in Solid Organ Transplantation

Few reports regarding the infusion of Tregs in solid organ transplantation have been published. In 2016, Todo et al. treated 10 consecutive patients with end-stage liver failure who underwent transplantation from a living donor with a cell product enriched in anergic and/or regulatory T lymphocytes (101). In detail, recipient lymphocytes and splenocytes (collected during the transplant) were cultured with irradiated donor cells in the presence of anti-CD80/CD86 antibodies for 2 weeks. Patients received a single infusion on day 13 post-transplantation. Although the cell product was contaminated by monocytes, DCs NK and B cells the numbers of Tregs infused ranged from 0.43×10^6 /kg to 6.37×10^6 /kg. From the 40 patients originally planned, the trial ended due to the acute cellular rejection during weaning in two patients with primary biliary cirrhosis and one with primary sclerosing cholangitis. Patients with no immunological related disease successfully

TABLE 1 | Ongoing clinical trials adopting tregs in autoimmunity.

Study ID	Phase	Indication	Enrollment/ Age	Product	Dose	Status
ISRCTN06128462	I	Type 1 Diabetes	12/ range 5–18	Polyclonally expanded tTregs (A)	10 and $30 \times 10^6/\text{kg}$	Completed
NCT02691247	II	Type 1 Diabetes	113/ range 8–17	Polyclonally expanded tTregs (A)	2.5 and $20 \times 10^6/\text{kg}$	Active, not recruiting
NCT02772679	I	Type 1 Diabetes	16/ range 18–45	Polyclonally expanded tTregs (A)	3 and $20 \times 10^6/\text{kg}$	Recruiting
NCT02428309	I	Cutaneous Lupus	NA/ range 18–60	Polyclonally expanded tTregs (A)	1, 4 and 16×10^8	Active, not recruiting
NCT03239470	I	Pemphigus	12/ range 18–75	Polyclonally expanded tTregs (A)	2.5×10^8 and 10×10^8	Recruiting
NCT03011021	I/II	Type 1 Diabetes	40/ >18	Polyclonally expanded tTregs (UCB)	$2 \times 10^6/\text{kg}$	Recruiting
NCT02932826	I/II	Type 1 Diabetes	40/ range 6–60	Polyclonally expanded tTregs (UCB)	$2 \times 10^6/\text{kg}$	Recruiting
NCT02704338	I/II	Autoimmune hepatitis	30/ range 10–70	Polyclonally expanded tTregs (A)	$10\text{--}20 \times 10^6/\text{kg}$	Unknown
NCT03185000	I/II	Crohn's Disease	20/ range 18–80	Polyclonally expanded naive tTregs (A)	0.5–1, 3–5 and $8\text{--}10 \times 10^6/\text{kg}$	Not yet recruiting

(A), autologous; **(UCB)**, umbilical cord blood.

tolerated the immunosuppression weaning started after 6 months post-transplantation and followed by a complete weaning at 18 months. Patients with acute rejection underwent on low dose of tacrolimus and mycophenolate mofetil. Although the infused cell product was contaminated with antigen specific effector cells, the authors presented this pilot study as a novel strategy for tolerance induction in patients undergoing liver transplantation for non-immunological diseases. To confirm this hypothesis, investigations are currently underway in a large group of patients excluding those ones with autoimmune disorders. In 2017, results from phase I, open-label pilot study conducted at the University of California (San Francisco) were published (102). They aim to test the feasibility of Treg isolation, expansion and infusion in kidney transplant recipients on immunosuppression with subclinical graft inflammation. Three kidney transplant recipients were enrolled according to their Kidney inflammation status detected during the 6-month post-transplant surveillance biopsy. Sorted Tregs ($\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{low}}$) were expanded as described above using medium containing deuterated glucose for further *in vivo* tracking. Patients received a single infusion of around 320×10^6 and were maintained under tacrolimus, mycophenolate mofetil and prednisone. Follow-up biopsies were performed at 2 weeks and 6 months post-infusion. None of the enrolled patients had infusion reactions and no infections or malignancies were observed during the 1 year follow-up period. The authors showed that infused Tregs peaked in circulation the first week with deuterium signals detectable during the first month after infusion in all subjects dropping near the detection limit at 3 months after infusion. Due to the low number of the enrolled patients, it is not possible to draw any conclusion of either safety or efficacy of Treg infusion isolated from kidney transplant recipients on immunosuppression with subclinical graft inflammation. However, following the results

of this pilot study new trials have been planned to test this strategy in a larger casuistic (**NCT02088931** and **NCT02711826**). In 2018, the results from the clinical trial conducted at the Northwestern University (Chicago) called TRACT have been published (93). This was a phase I dose escalation study infusing *ex vivo* expanded autologous polyclonal Tregs into living donor kidney recipients. Nine patients divided in 3 cohorts have been infused 60 days post transplantation with 0.5, 1, and 5×10^9 cells, respectively. Tregs were isolated from leukapheresis collected 1 month prior to the transplant and expanded *ex-vivo* for 21 days. Patients received alemtuzumab together with the transplant for a complete lymphodepletion and 2 days before the transplant, they were placed on tacrolimus and mycophenolate. At 2 months post-transplant, prior to Treg infusion, tacrolimus was stopped and switched to sirolimus. During the follow up, no serious adverse events attributable to Treg infusion were detected and the opportunistic infection seen were linked with the immunosuppressive regimen. The authors found an increased Treg number after the infusion compared to historical control patients under the same immunosuppressive regimen. The presence of donor specific antibodies was observed in two patients but, the authors stated that this was due to the suboptimal immunosuppression. Overall, the product was safe and the authors are planning a phase II trial.

Most of the clinical trials using Tregs to prevent rejection in solid organ transplantation are still ongoing (Table 2). We are part of The ONe Study consortium where eight academic institutions along Europe and US are testing safety and feasibility of different regulatory cell populations (Tregs, tolerogenic DCs and regulatory macrophages) in kidney transplant patients. The ONe Study UK (**NCT02129881**) involved our institute (King's College London) and Oxford University; autologous Tregs have been isolated from PB, magnetically enriched, polyclonally

TABLE 2 | Ongoing clinical trials adopting Tregs in transplantation.

Study ID	Phase	Indication	Enrollment/ Age	Product	Dose	Status
NCT02145325	I	Living donor kidney transplant	10/ range 18–65	Polyclonally expanded tTregs (A)	0.5, 1, 5 × 10 ⁹	Active but not recruiting
NCT02129881	I/II	Living donor kidney transplant	12/>18	Polyclonally expanded tTregs (A)	1, 3, 6 × 10 ⁶ /kg	Completed
NCT02371434	I/II	Living donor kidney transplant	9/ range 18–65	Polyclonally expanded tTregs (A)	0.5, 1, 3 × 10 ⁶ /kg	Unknown
NCT02244801	I/II	Living donor kidney transplant	16/ range 18–70	Donor-alloantigen-reactive tTregs (A)	300 and 900 × 10 ⁶	Completed
NCT02091232	I/II	Living donor kidney transplant	8/>18	Belatacept-conditioned tTregs (A)	300 and 900 × 10 ⁶	Active, not recruiting
NCT02166177	I	Liver transplant	9/ range 18–70	Polyclonally expanded tTregs (A)	0.5–1 and 3–4.5 × 10 ⁶ /kg	Completed
NCT02188719	I	Liver transplant	24/ range 21–70	Donor-alloantigen-Reactive Tregs (A)	50, 200, 800 × 10 ⁶	Recruiting
NCT02088931	I	Living donor kidney transplant	3/ range 18–50	Polyclonally expanded tTregs (A)	320 × 10 ⁶	Unknown
NCT02474199	I	CNI reduction in liver transplant	18/ range 18–70	Donor-alloantigen-Reactive Tregs (A)	400 × 10 ⁶	Recruiting
NCT02711826	I	Subclinical Inflammation in Kidney Transplantation	40/>18	Donor-alloantigen-Reactive Tregs (A)	1 × 10 ⁶ /kg	Recruiting
NCT01624077	I	Liver transplant	1/ range 10–65	Induced Tregs (A)	1 × 10 ⁶ /kg	Unknown
ISRCTN11038572	IIb	Living donor kidney transplant	136/>18	Polyclonally expanded tTregs (A)	5–10 × 10 ⁶ /kg	Not yet recruiting
NCT01446484	I	Kidney transplant (children)	30/ range 1–18	Polyclonally expanded tTregs (A)	200 × 10 ⁶	Unknown
NCT03577431	I/II	Liver transplant	9/ range 17–70	Belatacept-conditioned tTregs (A)	from 2.5 to 500 × 10 ⁶	Not yet recruiting
NCT03284242	NA	Kidney transplant	12/ range 18–65	Polyclonally expanded tTregs (A)	NA	Not yet recruiting

(A), autologous; NA, not available.

expanded and then infused with no adverse effects in 12 patients. Following the positive experience of this trial, a phase IIb trial (The TWO study **ISRCTN11038572**) will start at the end of 2018. In this new study, 34 renal transplant recipients will be enrolled and infused with expanded Tregs 6 months after transplantation. The primary outcome will be the incidence of acute rejection episodes at 12 months post-transplantation. The ONe Study-Charité in Berlin (**NCT02371434**) is also evaluating polyclonally expanded Tregs while, the group in Milan is testing the effects of Antigen-specific Tr1 (T10 cells). Tregs specific for the donor alloantigens (DarTregs) have been tested by the US-partner of the ONe study (University of California, **NCT02244801** and Massachusetts General Hospital, **NCT02091232**). In California, sorted Tregs have been co-cultured firstly with donor B cells activated using CD40L and then re-stimulated using antiCD3/CD28 coated-beads (96). In Boston, PBMCs were co-cultured for 72hrs with an equal number of irradiated kidney donor PBMCs (first-party stimulators) in the presence of belatacept (CTLA4 blocking Ig) and then re-stimulated with new first-party stimulator without co-stimulatory blockage (103). In both trials, patients have been divided in 2 cohorts receiving

300 × 10⁶ and 900 × 10⁶ of darTregs respectively 10 days after the transplant. The Russian State Medical University in Moscow is leading a phase I clinical trials where two doses of Tregs will be infused in pediatric patients after kidney transplantation (**NCT01446484**). Patients will be treated at day–21,–14 and the day of the transplant with alemtuzumab (monoclonal antibody specific for CD52). On day 0, patients will receive either tacrolimus or cyclosporine followed by mycophenolate mofetil at day 3. Sirolimus will start 1 month after the transplant together with the first infusion of Tregs. The second dose will be administrated after 3 months post-transplant. Although this clinical trial was supposed to end in 2014, no results are available. Other two clinical studies in the US (**NCT03284242** and **NCT02145325**) are testing safety and feasibility of Tregs after kidney transplantation. The ThRIL (**NCT02166177**) is a Phase I/IIa clinical trial conducted at King's College London. We aimed to test polyclonally expanded Tregs in liver transplant patients. The last patient was infused in 2017 and early data are already available. Two different multicentric studies running at the University of California (San Francisco), Northwestern University, (Chicago), and Mayo

Clinic (Rochester) are testing DarTregs in liver transplant recipient. The first one (NCT02474199) aims to test safety and feasibility of DarTregs infusion only, while in the second (NCT02188719) they aim to infuse Tregs and reducing the use of calcineurin inhibitors. In NCT02474199, patients will receive a target dose of 400×10^6 darTregs infused intravenously while in NCT02188719 four cohorts of patients will receive none, 50×10^6 , 200×10^6 , 800×10^6 darTregs, respectively. Both studies are still recruiting and results will not be available soon. In August 2018, at the Massachusetts General Hospital a new single-center, open-label, non-randomized clinical trial started (NCT03577431). In this phase I/II study they aim to use Tregs to facilitate immunosuppression withdrawal in liver transplant recipients. Similarly to what has been used for the kidney recipients in the ONE study, a cell product containing donor-specific hyporesponsive cells in association with allospecific Tregs will be used. This cell product is generated in mixed leukocyte reaction where donor and recipient cells are co-cultured in the presence of belatacept. They have planned the infusion of 2.5×10^6 up to 500×10^6 cells in nine patients.

FUTURE DIRECTIONS

Although preclinical studies have shown the capacity of Tregs to treat autoimmune diseases and prevent graft rejection, in the clinic we are still far away from these ultimate goals. The first clinical studies have shown the safety and feasibility of Tregs infusion and new phase II trials are now starting or being planned. The next steps will be crucial to define a standardized strategy for treating autoimmune disease and graft rejection. One important aspect is represented by the immunosuppressive regimen used to dampen the immune response. We have recently shown that immunosuppressive drugs like tacrolimus, mycophenolate and methylprednisolone reduced Tregs' viability and proliferation in a dose dependent manner (104). Therefore, the immunosuppressive regimen adopted might have an essential role for the efficacy of the Tregs therapy. This is the reason why different strategies are now under investigation with the aim to tailor the immunosuppressive regimen to the Tregs or find the best timing for their infusion. As described in the previous section, the infusion of Tregs can be executed when patients are under rapamycin treatment. Differently to the other immunosuppressive drugs, rapamycin favors the expansion of Tregs both *in vivo* (105) and *in vitro* (90, 92) supporting their action. We believe that Tregs need to be injected in combination with other therapies tailored to the type of disease that is to be targeted. In other words, combined therapy protocols might represent a winning strategy for the future. To date, low doses of IL-2 have been used for expanding endogenous circulating Tregs in autoimmunity (106) and GvHD (107) directly *in vivo*. The main issues of this approach are represented by both the half-life of the IL-2 in circulation together with the possible activation of other detrimental cells like NK or eosinophils. New clinical trials (NCT03556007, NCT03221179, and NCT03451422) are testing molecularly engineered IL-2 with an increased half-life. This will allow the minimization of the dose of IL-2

administrated and the development of a more specific therapy for Tregs. Another promising strategy of combined therapy in autoimmunity is represented by the engagement of the TNF receptor 2 (TNFR2). TNF-blocking strategies are effective for the treatment of rheumatoid arthritis (22, 108) however the inflammatory effect of the TNF-alpha is mediated by the receptor 1 (TNFR1) while the TNFR2 has been shown to induce immune modulation and tissue regeneration. Tregs express higher levels of TNFR2 compared to other T cells and its expression has been linked with Treg suppressive ability in both mice (109) and human (110, 111). Due to the role of this receptor, TNFR2 antagonism has been suggested as a new promising strategy in cancer immunotherapy specifically in ovarian, lung, and cutaneous T cell lymphoma (112, 113). In addition, a defective TNF/TNFR2 interaction is critical for Treg functionality in autoimmunity. For this reason, the combined infusion of antigen specific Tregs together with TNFR2 agonists might be a winning strategy. In the last years, several reports have shown how chronic inflammation changed the microbiome composition which is essential for developing regulatory pathways involved in the maintenance of the immune homeostasis (114). To further reduce the systemic inflammation, the infusion of Tregs can be applied in combination with Treg-inducing microbial as fecal microbiome transplantation is not yet approved in clinic due to safety reasons. Recent studies have highlighted the importance of short chain fatty acids derived from bacteria as main factors mediating Treg induction. Butyrate has been implied in the up regulation of anti-inflammatory genes in DCs. Furthermore, it enhances histone acetylation of the *Foxp3* locus and the stability of FOXP3 protein (115). Finally, polysaccharide A and cell surface β -glucan/galactan from *Bifidobacterium bifidum* were able to induce $\text{Foxp3}^+\text{IL-10}^{\text{high}}\text{IFN-}\gamma^{\text{low}}$ and $\text{Foxp3}^+\text{IL-10}^{\text{high}}\text{IFN-}\gamma^{\text{high}}$ Tregs, respectively (116). For this reason, Treg-inducing microbial components can ameliorate the outcome of cell therapy protocol adopting Tregs.

Treg localization and migration represent the main challenges in the field. Cells delivered specifically to inflamed area will increase enormously the positive outcome of the cell therapy protocol based on Tregs. As already discussed above in this review, the use of ATRA during Tregs expansion has been shown to induce chemokine receptor specific for gut homing. The discovery of the Th-like Tregs has opened another important avenue in selecting a population tailored to the type of disease. In autoimmunity, therapies targeting Th17-dependent pathways are associated with clinical benefits (117); in this scenario, Th17-like Tregs might be the ideal candidates for cell therapy protocols. Cardiac allograft vasculopathy after heart transplantation is linked to Th-1 and the use of Th1-like Tregs might be the ideal strategy. However, due to the low number of the Th-like Treg subpopulation, *ex-vivo* expansion is necessary. This might modify their phenotype especially if rapamycin or other drugs are added into the culture. Overall, new studies on these cells need to be conducted before including them as possible candidate for cell therapy. An important aspect that needs to be considered in developing Phase II/III trials will be the tracking of the infused cells. To date, only Tregs infused in patients with T1D have been monitored using deuterium labeling (84). However, this

strategy is limited to the circulating cells and it is not possible so far to gain information on the localization of Tregs in the tissues. For this reason, future trials need to test new approaches for a more specific cell detection. One possibility is represented by the sodium-iodide symporter (NIS) a molecule expressed on thyroid follicular cells and essential for the uptake of plasma iodide (118). The NIS gene was firstly cloned in 1996 by Dai et al. (119) and it is considered safe, non-immunogenic and non-invasive. Although it mediates mainly the transport of iodide into the cells, NIS can translocate several other substrates detected using different system like PET or SPECT/CT. Transducing Tregs with NIS will be key for understanding their localization and whether they successfully reach the target organ or tissue. In addition, compared to the iron oxide nano-particles that persist after death of the labeled cell, NIS works only on living cells allowing at the same time the evaluation of cells viability *in vivo*. We have already developed a protocol for transducing Tregs with NIS (120). NIS expressing self-specific Tregs were radiolabelled *in vitro* with Technetium-99m pertechnetate with no effects on cell viability, phenotype, and function. Moreover, we were able to detect these cells *in vivo* in the spleen of C57BL/6 mice 24 h after infusion by SPECT/CT. Lastly, enthusiasm is growing in the generation of antigen-specific Tregs by genetic engineering with chimeric antigen receptors (CARs). This new strategy [extensively reviewed in (121)] has been firstly developed in the tumor field and in 2017, the FDA approved the use of CAR T-cell for the treatment of acute lymphoblastic leukemia in children and advanced lymphomas in adults (122). In autoimmune diseases, CAR-T cells might be developed for targeting the pathological cells responsible of the autoimmune reaction (B cells). On the other hand, CAR can be transduced into Tregs to generate a population that is specific for a selected antigen. Due to the lack of self-protein exclusively expressed on the inflamed tissue, the generation of CAR specific Tregs in autoimmune diseases is challenging (123). Conversely, in transplantation, CAR-Tregs specific for MHC-I molecules have been generated. In the context of HLA-mismatched transplant, HLA class I specific-CAR-Tregs will target the transplanted organ without interfering with the recipient-immune system. We have shown how HLA-A2 (MHC class I protein) specific CAR Treg have the capacity

to prevent skin-graft rejection in a mouse model compared with polyclonally expanded Tregs (124). However, before being tested in the clinic, CAR-Treg stability is another issue to be solved together with cell homing capacity. In fact, as CAR-Tregs are antigen specific, they do not need to migrate into the lymph node but can be specifically directed into the target organ/tissue.

CONCLUSIONS

So far, many of the original questions for the use of Tregs in transplantation and autoimmunity remain unanswered. Results from the ongoing clinical trials will be crucial to better understand the tolerated Treg dose, timing of infusion and the immunosuppressive regimen to preserve/favor them. However, few data will be available on Tregs efficacy and whether or not we should engineer them for being antigen-specific or expressing molecules linked with the migration into the target tissue/organ. A big step forward to understand the real potential of Treg-based cell therapy will be there *in vivo* tracking. However, due to the high costs of cellular engineering and ethical approval this will not be revealed soon. For this reason, in the near future, the best strategy is represented by the combined therapy whereby antigen-specific Treg will be infused together with either low dose of IL2, rapamycin or in the future TNFR2 agonists.

AUTHOR CONTRIBUTIONS

MR and GF participated in manuscript writing and editing. CA and GG contributed to manuscript writing and figure development. GL contributed to manuscript editing.

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Automated Clinical Grade Expansion of Regulatory T Cells in a Fully Closed System

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Adoptive transfer of T regulatory cells (Treg) has been successfully exploited in the context of graft-versus-host disease, transplantation, and autoimmune disease. For the majority of applications, clinical administration of Treg requires laborious *ex vivo* expansion and typically involves open handling for culture feeds and repetitive sampling. Here we show results from our approach to translate manual Treg manufacturing to the fully closed automated CliniMACS Prodigy[®] system reducing contamination risk, hands-on time, and quality variation from human intervention. Polyclonal Treg were isolated from total nucleated cells obtained through leukapheresis of healthy donors by CD8⁺ cell depletion and subsequent CD25^{high} enrichment. Treg were expanded with the CliniMACS Prodigy[®] device using clinical-grade cell culture medium, rapamycin, IL-2, and α CD3/ α CD28 beads for 13–14 days. We successfully integrated expansion bead removal and final formulation into the automated procedure, finalizing the process with a ready to use product for bedside transfusion. Automated Treg expansion was conducted in parallel to an established manual manufacturing process using G-Rex cell culture flasks. We could prove similar expansion kinetics leading to a cell yield of up to 2.12×10^9 cells with the CliniMACS Prodigy[®] and comparable product phenotype of >90% CD4⁺CD25^{high}CD127^{low}FOXP3⁺ cells that had similar *in vitro* immunosuppressive function. Efficiency of expansion bead depletion was comparable to the CliniMACS[®] Plus system and the final ready-to-infuse product had phenotype stability and high vitality after overnight storage. We anticipate this newly developed closed system expansion approach to be a starting point for the development of enhanced throughput clinical scale Treg manufacture, and for safe automated generation of antigen-specific Treg grafted with a chimeric antigen receptor (CAR Treg).

Keywords: regulatory T cells, treg cell therapy, Treg expansion, closed system, automation, advanced therapy medicinal product, CliniMACS Prodigy

INTRODUCTION

Nine years after the first in-man report, there are currently close to 30 recruiting or ongoing clinical trials administering Treg in autoimmune diseases, solid organ transplantation, pro-inflammatory diseases and graft-versus-host disease (GvHD) (1, 2). Most clinical applications require *ex vivo* expansion of Treg, classifying the cell product as advanced therapy medicinal product (ATMP). Treg expansion requires activation through the T cell receptor (TCR) in the presence of high doses of IL-2 (3–5). Efficient good manufacturing practice (GMP) compliant protocols for Treg expansion have been developed by us and others (6–18) and in the case of CliniMACS isolated Treg, typically include rapamycin as cell culture medium supplement to prevent T effector cell outgrowth (11, 15, 17, 19–22). We reported manual Treg expansion for cGvHD treatment using cell differentiation bags (Miltenyi Biotec) (18, 23) and since then have changed to G-Rex100 cell culture devices (Wilson Wolf manufacturing) due to enhanced growth rates, likely related to optimized gas exchange through the permeable membrane bottom, and convenient handling. *Ex-vivo* Treg expansion for cellular therapy typically requires 2–5 weeks depending on the starting material and desired final dose. The long culture requires multiple feeding and stimulation steps realized by open handling in the majority of manufacturing processes. In our opinion, three challenges have to be overcome to make expanded Treg an attractive seminal product for prospective controlled trials and potential market launch. First, other than the vast majority of current expansion protocols, media and cytokine feeds, cell activation, optional transduction, and quality control (QC) steps should avoid open handling to ensure product and personnel safety. Second, hands-on labor should be minimized to standardize manufacturing and reduce manufacturing costs. Third, realization of individualized cellular therapy for large patient cohorts will be feasible if we can use automated closed manufacturing systems with small footprint. Here we present the first proof-of-principle study exploiting *ex-vivo* Treg expansion in the fully closed CliniMACS Prodigy® system (Miltenyi Biotec).

MATERIALS AND METHODS

The recently published minimum information about Treg cells (MITREG) checklist was followed for the preparation of this paper (24). See <http://w3id.org/ontolink/mitreg> for MITREG document and checklist.

Cell Source

Unstimulated leukapheresis containing ACD-A and heparin as anticoagulants were collected from healthy donors after informed consent at the Department of Transfusion Medicine, Medical Clinic I, Carl Gustav Carus University Hospital at TU Dresden with the use of a continuous-flow cell separator (Spectra Optia®; Terumo BCT). Peripheral blood mononuclear cells (PBMCs) used for functional assays were isolated from buffy coats by standard Ficoll (Lymphoprep™, Axis-Shield) density centrifugation as described earlier (25). Buffy coats were obtained from the Deutsches Rotes Kreuz-Blutspendedienst Nord-Ost

GmbH Sachsen as a side product of red blood cell isolation for clinical use. The study included sample drawing from healthy donors with informed consent approved by the local institutional review board (EK 206082008).

Treg Isolation

Apheresis products were stored overnight at 4°C before cell isolation on the following morning (day 0 of culture protocol). Treg cell isolation was performed as previously described (18). Briefly, Treg were isolated with clinical-grade reagents in a two-step procedure under GMP conditions with the use of the CliniMACS® Plus separation system (Miltenyi Biotec). Total leukocytes containing a maximum number of 4.0×10^9 CD8⁺ cells were used as starting material, allowing the usage of a single vial of CliniMACS CD8 Reagent (Miltenyi Biotec, 275-01). After depletion of CD8⁺ cells, the intermediate product was enriched for the CD25^{high} fraction (CliniMACS CD25 Reagent, Miltenyi Biotec, 274-01). As a modification of the previously published protocol (18), two washing steps were performed after CD25 labeling.

CD4⁺CD25[−] T Responder Cell Isolation

CD4⁺CD25[−] T cells were isolated from PBMCs, cryopreserved and later used as responder cells (Tresp) to test the *in vitro* function of the manufactured Treg in a proliferation-based suppression assay. CD4⁺CD25[−] cells were enriched by research scale magnetic activated cell sorting (MACS) in a two-step process using the CD4⁺ T Cell Isolation Kit human (Miltenyi Biotec) to enrich CD4⁺ T cells by negative isolation and the CD25 MicroBeads II human (Miltenyi Biotec) to deplete Treg following the manufacturer's recommendations. The enriched CD4⁺CD25[−] population was aliquoted into 2 ml cryotubes (Greiner Bio-One) containing 1.5×10^7 cells resuspended in 1 ml of CryoStor® CS10 solution (BioLife Solutions). Vials were readily placed into a CoolCell™ LX cell freezing container (Corning) and directly transferred to a −80°C freezer. Within 3 days, cryotubes were transferred to a liquid nitrogen tank for long term storage.

Phenotyping

Treg purity and viability was determined by flow cytometry (Canto II, BD Biosciences) from aliquots taken during isolation, during the *ex vivo* expansion protocol (day 8, 12), on the day of Treg harvest (day 13 or 14) and from the final ready-to-infuse product. Extracellular staining of $0.5\text{--}1 \times 10^6$ cells was performed in 100–200 µl of PBS or CliniMACS buffer. The following antibodies from BD Biosciences were used: CD45/CD3/CD4/CD8/CD16/56/CD19 (Multitest-6 color, 337166), CD25-V450 (clone M-A251), CD14-FITC (clone M5E2), CD127 PerCP5.5 (clone HIL-7R-M21), CD3 APC-H7 (clone SK7), CD45-V500 (clone HI30), CD45RO-PE (UCHL1). eBiosciences™/Invitrogen (ThermoFisher): CD45RA-V450 (clone HI100), CD62L-PE (clone DREG56). After incubation for 15 min at room temperature in the dark, cells were washed with 2 ml PBS (500 g, 5 min) and resuspended in 500 µl for flow cytometric analysis. Intracellular staining of FOXP3 was performed using the FOXP3/Transcription Factor

Staining Buffer Set (eBioscience™/Invitrogen, ThermoFisher) according to the manufacturer's recommendations and the FOXP3-PE-Cy7 conjugated antibody (clone PCH101) from the same manufacturer. Cell viability was determined by 7-AAD/AnnexinV staining using the AnnexinV Apoptosis Detection Kit eFluor™450 (eBioscience™/Invitrogen, ThermoFisher) as recommended in the datasheet.

Manual Treg Expansion

Freshly isolated cells were seeded at $0.3\text{--}0.5 \times 10^6$ cells/ml in G-Rex® 100 flasks (Wilson Wolf Manufacturing) containing TexMACS GMP Medium (Miltenyi Biotec) supplemented with 100 ng/ml MACS GMP Rapamycin (Miltenyi Biotec), 1,000 U/ml IL-2 (Proleukin S, Novartis Pharma), 5% cell therapy-grade pooled human AB serum (Blutspendedienst Tübingen, Germany) and MACS GMP ExpAct Treg beads (Miltenyi Biotec) at a cell to bead ratio of 1:4. Cells were cultured at 37°C and 5%CO₂. Cells were counted on days 5, 8, 10, and 12 and adjusted to $0.3\text{--}0.5 \times 10^6$ cells/ml (day 5, 8), $0.3\text{--}1.0 \times 10^6$ cells/ml (day 10) or $0.5\text{--}1.0 \times 10^6$ cells/ml (day 12) by partial (50%) media replacement. Fresh medium contained all additives except ExpAct Treg beads. Cells were re-stimulated with fresh beads at a 1:1 ratio on day 8. For direct comparison to the automated culture, manual G-Rex® 10 flask cultures containing the same isolated Treg pool at 10-fold lower cell number and volume as compared to the automated culture were performed.

Automated Treg Expansion

Automated cell culture was performed using the CliniMACS Prodigy® System (Miltenyi Biotec) equipped with a disposable CliniMACS Prodigy tubing set TS 510 (Miltenyi Biotec), which contains the single-use CentriCult cell culture and centrifugation chamber. $31\text{--}120 \times 10^6$ (mean, 56.9×10^6) cells were seeded on day 0 of the culture in 80–105 ml medium. Cells were cultured under defined conditions of CO₂ (5%) and temperature (37°C). Medium composition, partial media exchange timepoints and regimens were based on our manual SOP with the difference that cell concentrations were higher in the Prodigy® system in the second week of expansion due to volume restriction of 260 ml in the CentriCult unit. Different to the manual culture, the automated culture was agitated from day 5 to ensure optimal gas supply and for cultures showing a high expansion rate an additional media exchange was conducted on day 11. For the optimized automated protocol, re-stimulation with beads on day 8 was integrated as an automated 10 ml feed after volume reduction <100 ml. Agitation was paused for 1 h to facilitate early bead to cell contact before a fresh media feed was carried out and agitation was resumed. Automated QC sampling was realized through sampling pouches as part of the tubing set or through an additionally connected triple sampling adapter (Miltenyi Biotec).

Removal of ExpAct Treg Beads and Final Formulation

A customized process for fully automated expansion ExpAct Treg bead removal and final formulation in 0.9% NaCl/1%HA (Human Albumin 200 g/l, Baxter) infusion solution using the CliniMACS Prodigy® system was developed together with

Miltenyi Biotec and successively optimized. Treg culture in the CentriCult unit, bead removal over the column and final formulation were performed with a single CliniMACS Prodigy TS 510 tubing set. The final optimized process includes multiple washing steps to exchange the media to infusion solution, after which the cells pass the primed magnetic column in a two-stage process. The final product is harvested to a sealable cell bag (Cell differentiation bag, Miltenyi Biotec). ExpAct bead removal using the CliniMACS® Plus system was performed following the manufacturer's recommendations to compare the newly developed CliniMACS Prodigy® bead removal process with the standard procedure. Briefly, CliniMACS® Plus bead removal was performed using the CliniMACS® PBS/EDTA buffer (Miltenyi Biotec) supplemented with 0.5%HA (200 g/L, Baxter), the CliniMACS® Tubing Set LS (Miltenyi Biotec) and the software sequence Depletion 2.1.

Quantification of Residual ExpAct Treg Beads

3×10^7 cells of the final Treg product were transferred to a 15 ml tube and centrifuged (300 g, 10 min, RT). The supernatant was discarded and the cell pellet was lysed in 2 ml of distilled water, vortexed and incubated for 5 min at 37°C. Subsequently, 200 µl of a 2,000 U/ml DNase I stock solution (Roche) were added and the sample was incubated for 2 min at RT. After incubation, 6 ml of MACSQuant Washing Solution (Miltenyi Biotec) were added and the vial was incubated for 10 min at RT. After centrifugation at 1,400 g for 10 min at RT, the supernatant was carefully removed and the residual beads were resuspended in the remaining volume (<30 µl). Residual beads were quantified using a C-Chip™ Neubauer improved disposable counting chamber (Biochrom AG) and Türk's solution (Merck Millipore) at a 1:1 dilution ratio.

Treg Suppression Assay

Functionality of the final Treg product was assessed on the basis of the ability to suppress proliferation of allogeneic CD4⁺CD25[−] T effector cells (responder cells, Tresp). Cryostored Tresp and aliquots of manufactured Treg (analogously frozen), were thawed, washed with 10 ml X-Vivo15 media (Lonza, 04-418Q) containing 5% serum followed by a wash with 10 ml PBS. Tresp cells were stained with eFluor670 cell proliferation dye (Invitrogen) at a final concentration of 5 µM in PBS following the manufacturer's recommendations. Manufactured Treg were stained in parallel with eFluor450 cell proliferation dye (Invitrogen) at a final concentration of 10 µM to facilitate discrimination of both subsets at the time of assay readout. After incubation for 10 min at 37°C, cells were washed twice in 10 ml of cold X-Vivo15 containing 10% AB serum and resuspended in the same, but prewarmed medium for plating. 1×10^5 stained Tresp cells were seeded in a round bottom 96 well cell culture plate (ThermoFisher) and Treg were added to the Tresp cells to reach Treg:Tresp ratios of 1:32, 1:16, 1:8, 1:4, 1:2, and 1:1. αCD3/αCD28 coated beads (Dynabeads™ Human T activator, ThermoFisher) were added at a bead to total cell ratio of 1:75. In order to determine maximum proliferation of Tresp, Tresp were cultured with beads in the absence of Treg. All conditions

were seeded as triplicates. The final volume was adjusted to 250 μ l for all conditions. Plates were incubated at 37°C for 5 days. On day 5, the individual wells were harvested and stained for CD4-FITC (clone RPA-T4, BD Biosciences) and CD25-PE-Cy7 (clone MA251, BD Biosciences). Proliferating Tresp were defined as the percentage of eFluor670^{dim}CD25⁺ cells from the total Tresp population. The mean inhibition of proliferation (% suppression) found at the different Treg:Tresp ratios was calculated as $(\text{Proliferation}_{\text{Tresp only}} - \text{Proliferation}_{\text{Tresp}}) / \text{Proliferation}_{\text{Tresp only}} \times 100$. A nonlinear fit of the percentage of suppression for each condition vs. the number of added Treg for each condition was calculated using a four parameter dose-response curve algorithm in GraphPad V6.0c (GraphPad Software Inc., California, USA). The Treg:Tresp ratio responsible for 75% of inhibition of Tresp proliferation was determined as the ratio between the EC₇₅ value extrapolated from the non-linear fit and the total number of Tresp cells present in the assay (1×10^5 cells).

Statistical Analysis

Statistical analyses were conducted using Prism 7.03 GraphPad Software. Unless otherwise stated, results are reported as mean values with standard deviation (SD) and *P*-values determined by two-tailed paired or unpaired Student's *t*-test.

RESULTS

High Yield Isolation of Treg From Apheresis Products

CliniMACS[®] Treg isolation from leukapheresis through CD8 depletion and CD25 enrichment resulted in preparations with a mean purity of 72% CD4⁺CD25^{high}CD127^{low}FOXP3⁺ (range 56–87%, *n* = 12) and a mean yield of 61×10^6 total cells (range 27×10^6 – 155×10^6 , *n* = 11, data not shown). Representative flow cytometry plots before isolation, after CD8⁺ depletion and after final CD25^{high} enrichment are shown in **Figure 1A**. CD8⁺ depletion was highly efficient, with only mean 0.14% CD8⁺ T cells in the Treg-enriched isolated product (range 0–1.3, *n* = 16; **Figure 1B**). The main contaminating cells were CD25 expressing CD19⁺ B cells (mean 16.1%, range 5.1–31.8, *n* = 8), followed by CD4⁺CD25^{–/low} cells (CD4 resting) with a mean percentage of 8.07% (range 2.4–18.1, *n* = 8). Effector memory CD4⁺CD25⁺CD127⁺CD45RO⁺ (CD4 Teff) cells were present with a mean concentration of 1.47% (range 0.1–2.8, *n* = 8). NK cells expressing CD16/56 were infrequent (mean 0.62%, range 0.1–1.3, *n* = 8) and monocytes were present at a percentage of mean 1.14% (range 0.1–2.4, *n* = 4).

Treg Manufactured by Automated Closed-System Culture Are of High Purity and Vitality

Our standard large scale clinical-grade manual Treg expansion using G-Rex100 vessels resulted in cellular products with a Treg mean purity of 94.7% (range 89.5–98.1, *n* = 11) after 12–14 days of expansion (**Figure 2C**, upper panel). As published by us and other investigators, Treg expansion capacity is largely variable between donors (6, 13, 14, 17, 18, 25, 26). For

direct comparison, isolated Treg enriched populations were split 1:10 after QC sampling to allow for a manual medium scale expansion culture using G-Rex10 devices (10 cm²) in parallel to each CliniMACS Prodigy[®] culture (100 cm²). We successfully optimized feeding intervals, agitation modes, media exchanges, and bead restimulation on the CliniMACS Prodigy[®], reaching between 0.33 and 2.12×10^9 cells on day 13 of culture with maximal cell concentrations of up to 8.5×10^6 cells/ml at the time of harvest. **Figure 2A** shows the expansion curves for four CliniMACS Prodigy[®] manufacturing processes using starting material from four different donors. A lag phase seen in run A after bead restimulation was successfully diminished in later cultures through optimization of the restimulation process. **Figure 2B** illustrates the expansion kinetics for three optimized expansion cultures (cultures B, C, D) comparing the manual expansion in G-Rex10 flasks (orange symbols) to the automated expansion in the CliniMACS Prodigy[®] system (blue symbols). Our optimized Prodigy protocol allowed for similar kinetics as shown by the parallel expansion curves.

Treg phenotype has proven to be a critical factor for Treg stability over time. As shown by a number of investigators, loss of FOXP3 transcription factor expression occurs after prolonged *ex-vivo* culture and may correlate with reduced suppressor function (6–10, 27). The percentage of FOXP3 expressing cells within the final product is one of the release criteria in the majority of recent Treg therapy studies (13, 15, 28). We, therefore, compared Treg phenotype (CD4⁺CD25^{high}CD127^{low}FOXP3⁺) after manual and automated expansion cultures (**Figure 2C**, lower panel). At the end of culture, manually expanded Treg showed a mean purity of 95.6% (range 93.7–97.0; *n* = 4). CliniMACS Prodigy[®] expanded Treg of the same isolated batches were of 92.8% purity (range 89.8–94.5%, *n* = 4; *p* = 0.08). Taken together, our results indicate that the CliniMACS Prodigy[®] system is suitable for clinical Treg manufacturing in terms of cell yield and phenotype.

Successful Development of Automated Expansion Bead Removal and Final Formulation

Beads coated with antibodies against CD3 and CD28 are widely used as standardized T cell stimulus mimicking both TCR engagement and costimulation by antigen presenting cells. Their use in cell manufacturing for cellular therapy requires the removal from the product prior to infusion to avoid activation of endogenous T cells. Three protocols were developed along the optimization process. As outlined in **Table 1**, cell recovery was improved from initially only 47% to 62% with the third developed process. Lost cells were partially detected in the CentriCult unit with initially high numbers (66×10^6 cells in process II), but could be reduced by protocol optimization resulting in only 1.4 – 6×10^6 cells in the CentriCult unit after final formulation with process III (data not shown). Residual beads in the final product were microscopically quantified from a 30×10^6 cell QC sample after cell lysis and maximal volume reduction. Extrapolated residual bead content in the final product decreased to <400 beads per 100×10^6 cells for

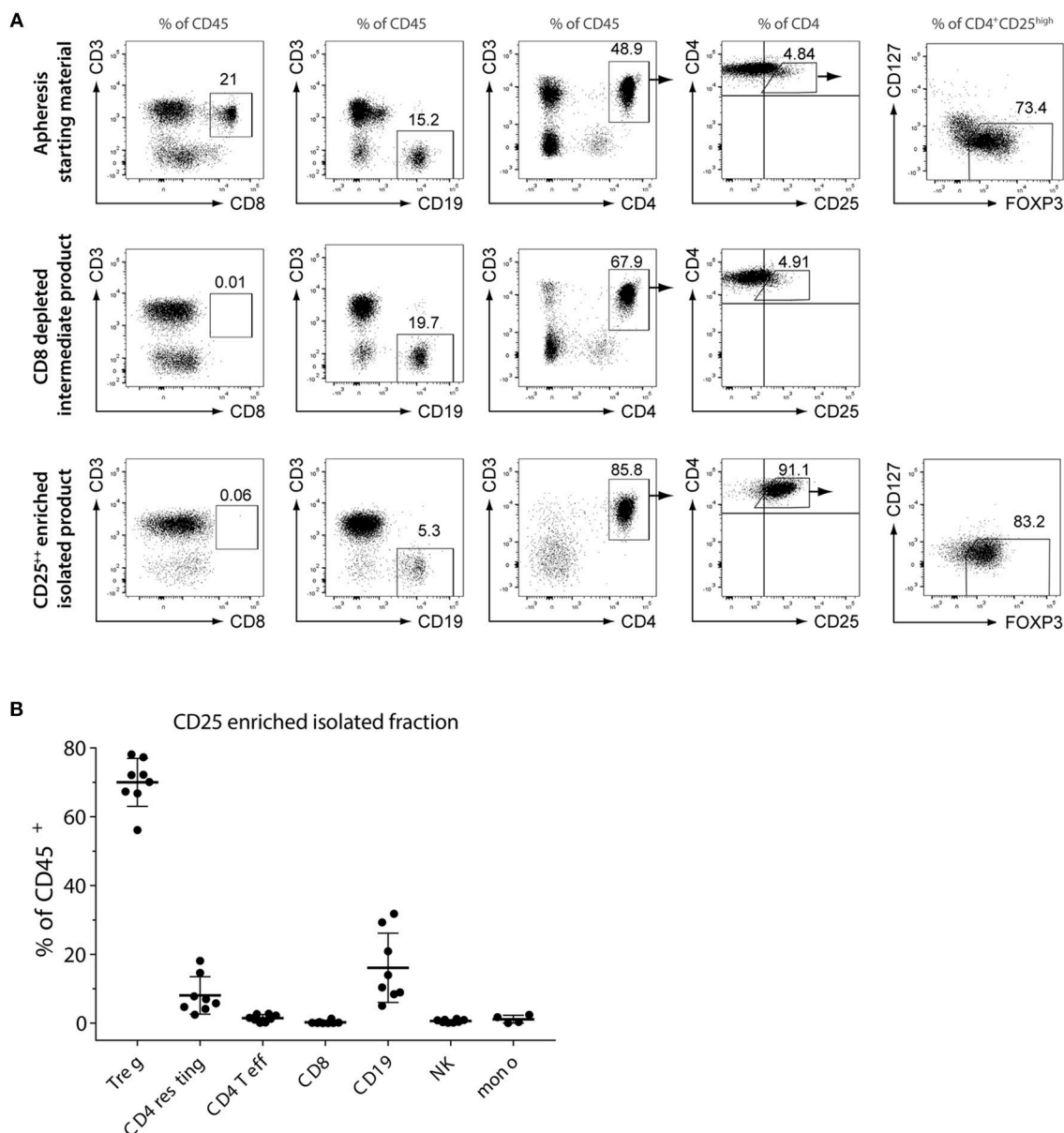


FIGURE 1 | Quantification of CD4⁺CD25^{high}CD127^{low}FOXP3⁺ Treg and other lymphocyte subsets before and after CD8⁺CD25^{high} enrichment. **(A)** Phenotyping of initial cell source (leukapheresis), CD8⁺ depleted intermediate product and final CD25^{high} Treg enriched fraction by flow cytometry. Shown are representative flow cytometry plots for one of the donors. **(B)** Relative percentage of Treg and contaminating CD4⁺CD25^{low} (CD4 resting), effector memory CD4⁺CD25⁺CD127⁺CD45RO⁺ (CD4 T eff), CD8 cytotoxic T cells, B cells, NK cells and monocytes. Shown are individual and mean percentages (solid line) of CD45⁺ leukocytes of each subset for $n = 8$ Treg isolations. Error bars = SD.

the optimized process (Table 1), marking the detection limit of our bead quantification method. To directly compare the optimized CliniMACS Prodigy[®] bead depletion process to the widely used CliniMACS[®] Plus bead removal process using the Depletion 2.1 program, the two systems were directly compared. To this end, on the day of harvest, 50% of the CliniMACS Prodigy[®] culture volume was drawn into a cell bag as part of the tubing set by a custom partial harvest process. The collected cell fraction was bead depleted on the CliniMACS[®] Plus device before manual final formulation. The remainder

of the culture was bead depleted using the optimized process on the CliniMACS Prodigy[®] system. Equal distribution of the culture was examined by cell counting. As depicted in Table 1 (frame), similar cell recovery and proportion of residual beads was observed for both methods. In conclusion, we were able to develop a custom process for the CliniMACS Prodigy[®] device that allows for fully automated efficient removal of ExpAct Treg expansion beads together with final formulation of the cell product, facilitating immediate bedside infusion after QC.

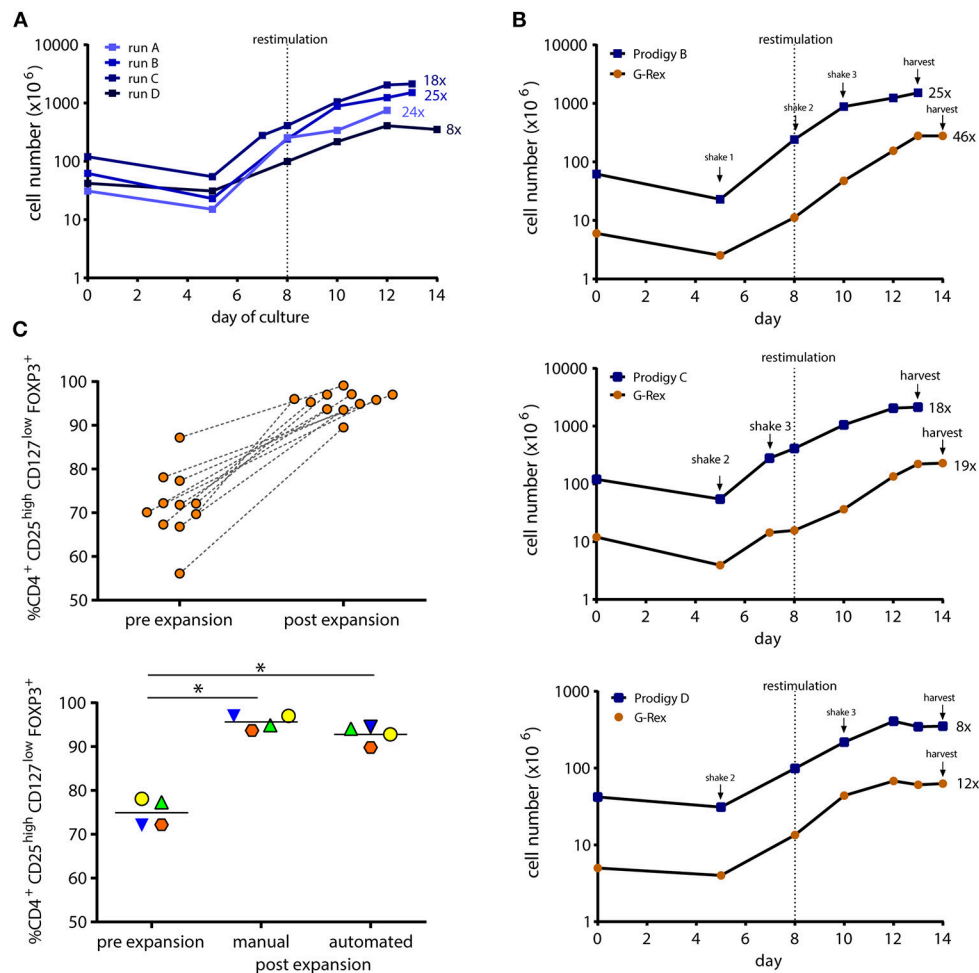


FIGURE 2 | Growth curves and purity of automated closed-system vs. manually manufactured Treg. Clinical grade isolated Treg were cultured for 12–14 days in the ClinMACS Prodigy® or G-Rex10 culture devices with high dose IL-2, rapamycin, and ExpAct Treg expansion beads. **(A)** Expansion curves of four automated ClinMACS Prodigy® cultures during process development and optimization. **(B)** The optimized automated expansion process was compared to parallel manual G-Rex®10 culture starting with 1/10 of ClinMACS Prodigy® seeding cell number of the same isolated Treg pool. Bead restimulation and agitation modes are depicted. Results from 3 donors are shown. Numbers followed by x indicate fold expansion values. **(C)** Treg purity before and after manual or automated expansion culture. Treg phenotype was analyzed by flow cytometry as CD4⁺CD25^{high}CD127^{low}FOXP3⁺. Shown is the percentage of Treg of all CD45⁺ for manually manufactured cultures (upper panel, $n = 11$, $p < 0.05$) and manual vs. ClinMACS Prodigy® expanded cultures (lower panel, $n = 4$, $p < 0.05$). Matching symbols indicate same starting material.

CliniMACS Prodigy® Manufactured Treg Are Highly Functional

Efficacy of Treg cellular therapy requires functionality of the final cell product. We compared the suppressive capacity of Treg manufactured by our manual vs. automated method. Functionality was measured as the ability of Treg to suppress α CD3/ α CD28 bead stimulated proliferation of Treg-depleted allogeneic CD4⁺ T responder cells (Tresp) in a 5-day assay. **Figure 3** shows the suppression assay results for Treg derived from three donors and expanded both by the manual and automated protocol. % of Tresp proliferation in absence and presence of increasing numbers of Treg is depicted in **Figure 3A**. For donor A, both ClinMACS Prodigy® and manually manufactured Treg suppressed 75% of Tresp proliferation at a Treg to Tresp ratio of 1:7 (**Figure 3B**). For donor B, the necessary

Treg to Tresp ratio was determined as 1:4 for the ClinMACS Prodigy® manufactured Treg, and 1:5 for Treg expanded by standard manual culture. Treg from Donor C showed the highest suppressive capacity. ClinMACS Prodigy® manufactured Treg suppressed 75% of Tresp proliferation at a Tresp ratio of 1:19, whereas the respective calculated ratio for manually expanded Treg was 1:22. Suppressive capacity thus clearly varied between donors but appeared to be independent of the manufacturing regimen.

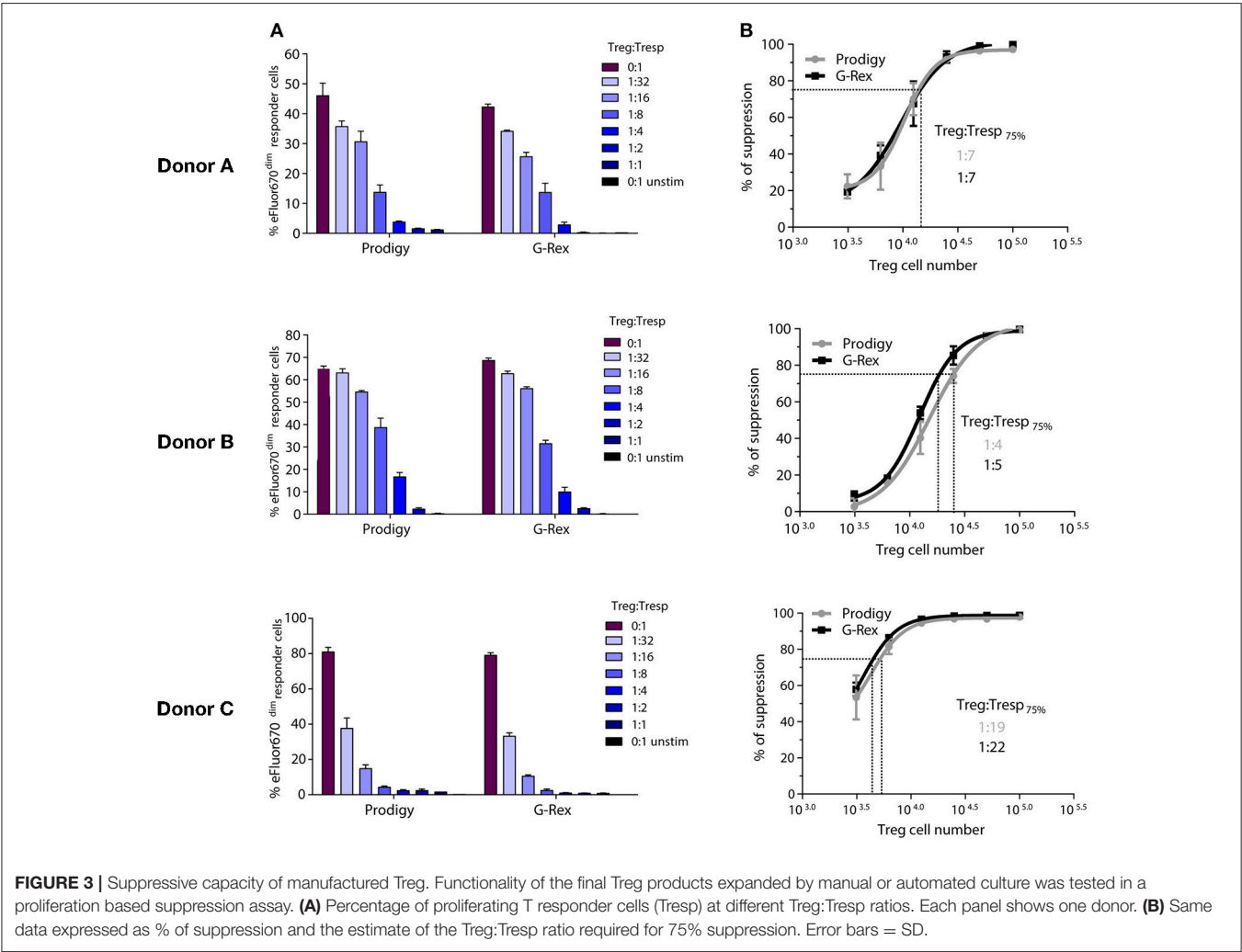
High Stability of Final Product for Cellular Therapy

Release relevant QC measurements for ATMP require stability of the final product over several hours. We thus determined

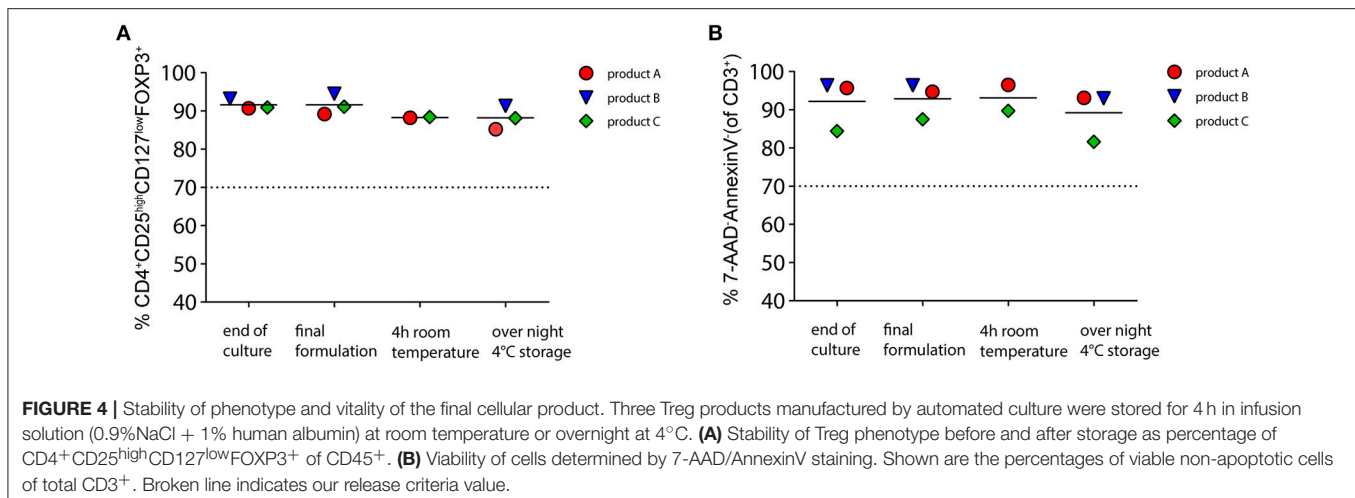
TABLE 1 | Development of expansion bead removal within the CliniMACS Prodigy® device.

Treg expansion		CliniMACS Prodigy®			
		Culture A	Culture B	Culture C	
		CliniMACS Prodigy®	CliniMACS Prodigy®	CliniMACS Prodigy®	CliniMACS® Plus
ExpAct Treg bead removal		Process I	Process II	Process III	Depletion 2.1
Input	Cell number	125 × 10 ⁶	759 × 10 ⁶	920 × 10 ⁶	1194 × 10 ⁶
	Beads to deplete	126 × 10 ⁶	245 × 10 ⁶	342 × 10 ⁶	444 × 10 ⁶
Final product	Cell recovery	47%	59%	62%	66%
	Residual beads	79,500	8,444	326	633
	(per 100 × 10 ⁶ cells)				

Specifications and results of three distinct customized processes for ExpAct Treg bead removal on the CliniMACS Prodigy® system. Results are shown for three individual cultures. Culture C was split by partial harvest and bead depletion was performed on the CliniMACS® Plus device in parallel to bead depletion on the CliniMACS Prodigy® system (process III). Shown are cell yield and bead removal efficiency for both devices (bold frame).



the viability and phenotype of the CliniMACS Prodigy®-manufactured final product in infusion solution over time. As depicted in **Figure 4**, all three tested products were phenotypically stable with only marginal changes after overnight storage ($p = 0.09$). All products had Treg purities well above our release criteria of 70% CD4⁺CD25^{high}CD127^{low}FOXP3⁺ at all tested timepoints (mean at the end of culture, 91.6%; mean after overnight storage, 88.2%). The viabilities of the three products



were above our release criterion of 70% 7-AAD⁻/AnnexinV⁻ live cells of total CD3⁺ at all time points. We observed a slight drop in viability after overnight storage (mean, 89.2%) as compared to the end of culture timepoint (mean, 92.2%; $p = 0.007$). Taken together, CliniMACS Prodigy[®] manufactured Treg can meet the release criteria for phenotype and vitality of our center also after overnight storage.

DISCUSSION

The development of closed cell manufacturing systems is important for the strongly growing ATMP field. Advantages over the still widely used open cell culture handling include enhanced safety for the personnel, reduced risk of product contamination, and consequently streamlined requirements by the regulatory authorities that may lower ATMP manufacturing cost in the near future. Several disposable cell culturing systems have been developed that allow closed system handling by sterile welding. Closed system Treg manufacturing in cell culture bags has successfully been developed in the context of the ONE study (17). The majority of closed systems, however, require hands-on user interaction at a similar degree to standard open cell culture systems. The CliniMACS Prodigy[®] system has the advantage of facilitating fully automated feeds, media exchanges, volume reduction and washing steps, reducing hands-on time and inter-operator product variability to a minimum. The device was successfully implemented for ATMP manufacturing for monocyte derived dendritic cells (29), CAR T cells (30–33) and natural killer (NK) cells (34, 35). Our proof-of-principle study is to our knowledge the first report of translating Treg manufacture to the CliniMACS Prodigy[®] system.

We demonstrated the feasibility of CliniMACS[®] Plus enrichment of Treg followed by CliniMACS Prodigy[®] automated Treg expansion yielding cell therapy relevant Treg numbers. Comparable to other investigators (17), Treg purity after CD8⁻CD25⁺ isolation is typically below 80% and in our hands largely dependent of the proportion of B cells as main contaminants. Purity could be enhanced by

B-cell depletion as published for expansion free Treg infusion (36). However, the increase in purity to typically >90% after rapamycin supplemented expansion, indicates sufficient Treg purity after isolation by the robust CD8⁻CD25⁺ CliniMACS process. Expansion kinetics and post expansion purity of Treg manufactured with the CliniMACS Prodigy[®] system were comparable to the manual expansion culture, and neither phenotype nor *in vitro* function significantly differed between both tested manufacturing methods. α CD3/ α CD8 coated beads are available from multiple manufacturers varying in bead material and size but with the common characteristic of being magnetic, facilitating their GMP compliant removal by the closed-system device CliniMACS[®] Plus. We achieved an automated CliniMACS Prodigy[®] based expansion bead removal process that was equally effective as the widely used bead depletion process on the CliniMACS[®] Plus instrument. Conveniently, the automated process could be designed to end with a readily formulated product that can be sealed off for bedside infusion. Functionality of the manufactured products was proven based on a proliferation based suppression assay. As seen by others, suppressive capacity varied between donors (17), which we hypothesize to be influenced by varying degrees of histoincompatibility of the Treg in respect to the allogeneic responder cells that were of the same batch for all assays. Importantly, suppressive capacities of Treg manufactured manually vs. automated showed comparable results for all donors. Quality control measurements are indispensable for ATMP product release. Release relevant measurements such as intracellular FOXP3 staining or the determination of residual expansion beads typically require multiple test laboratories and are time-consuming. The phenotypic and vitality stability over several hours and overnight, were, therefore, important findings which will be complemented by functional analysis data of the stored product in further manufacturing runs before regulatory approval.

A limitation of the procedure was that enrichment of Treg could not be integrated to the CliniMACS Prodigy[®] platform due to the unavailability of a certified CD8⁺ depletion process for

the device. Using a co-localized CliniMACS® Plus to isolate Treg is, however, more cost-effective than hypothetical Treg isolation on the CliniMACS Prodigy®. The reason is that the column of the Prodigy tubing set used for culture has to be reserved for expansion bead removal, and additionally required tubing sets for isolation would be more expensive than for the CliniMACS® Plus. Our findings are based on observations and data from five consecutive runs. We acknowledge this limitation of our study and see our development of automated Treg culture as a starting point for further optimization. A substantial fraction of the product is lost during expansion bead removal and for the QC sample for quantification of residual expansion beads. Bead-free stimuli would thus be of high interest to further enhance cell product yield. Another limitation is the restriction to the 260 ml CentriCult expansion vessel, which currently remains a disadvantage over cell culture bag based processes such as the process developed by Fraser et al. (17) even though cell densities exceed those of standard vessels due to the inclusion of culture agitation. Nevertheless, market launch of tubing sets with enhanced volume CentriCult units is expected to further increase achievable cell product sizes.

Taken together, we see a clear potential for the CliniMACS Prodigy® system in standardizing polyclonal clinical Treg expansion in the future that would facilitate decentralized manufacture in large multi-center studies. We furthermore anticipate our study to be the starting point of automated antigen specific Treg manufacture by TCR or CAR transduction using the CliniMACS Prodigy® device.

AUTHOR CONTRIBUTIONS

JMMM contributed to Treg manufacturing, performed experiments, contributed to data analysis and manuscript

writing. NM and KP manufactured Treg. DF headed the GMP facility and critically read the manuscript. TB and A-CF contributed to manufacturing optimization and critically read the manuscript. JK developed custom application processes for the CliniMACS Prodigy device. UO supervised flow cytometry measurements. KH facilitated donor leukapheresis. EB and MB provided funding, supported the study and critically revised the manuscript. AF planned and supervised the study, contributed to Treg manufacturing, performed experiments, analyzed data, and wrote the manuscript.

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Conflict of Interest Statement: TB, A-CF, and JK are employees of Miltenyi Biotec.

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The Secretome Derived From 3D-Cultured Umbilical Cord Tissue MSCs Counteracts Manifestations Typifying Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is an autoimmune disorder whose treatment is mostly restricted to pain and symptom management and to the delay of joint destruction. Mesenchymal stem/stromal cells from the umbilical cord tissue (UC-MSCs) have previously been proven to be immunomodulatory and more efficient than bone marrow-derived MSCs in causing remission of local and systemic arthritic manifestations *in vivo*. Given the paracrine nature of UC-MSC activity, their application as active substances can be replaced by their secretome, thus avoiding allogeneic rejection and safety issues related to unwanted grafting. In this work, we aimed at demonstrating the viability of applying the 3D-primed UC-MSC secretome for the amelioration of arthritic signs. A proteomic analysis was performed to both, media conditioned by UC-MSC monolayer (CM2D) and 3D cultures (CM3D). The analysis of relevant trophic factors confirmed secretome profiles with very significant differences in terms of therapeutic potential. Whereas, CM3D was characterised by a prevailing expression of anti-inflammatory cytokines such as IL-10 and LIF, along with trophic factors involved in different mechanisms leading to tissue regeneration, such as PDGF-BB, FGF-2, I-309, SCF, and GM-CSF; CM2D presented relatively higher levels of IL-6, MCP-1, and IL-21, with recognised pro-inflammatory roles in joint disease and pleiotropic effects in the progression of rheumatoid arthritis (RA). Accordingly, different motogenic effects over mouse chondrocytes and distinct capacities of inducing glycosaminoglycan synthesis *in vitro* were observed between CM3D and CM2D. Finally, the evaluation of arthritic manifestations *in vivo*, using an adjuvant-induced model for arthritis (AIA), suggested a significantly higher therapeutic potential of CM3D over CM2D and even UC-MSCs. Histological analysis confirmed a faster remission of local and systemic arthritic manifestations of CM3D-treated animals. Overall, the results show that the use of UC-MSC CM3D is a viable and better strategy than direct UC-MSC administration for counteracting AIA-related signs. This strategy represents a novel MSC-based but nonetheless cell-free treatment for arthritic conditions such as those characterising RA.

Keywords: mesenchymal stem/stromal cells, umbilical cord tissue, 3D culture, secretome, arthritic signs, rheumatoid arthritis

INTRODUCTION

The destruction and functional disability of joint tissues caused by arthritis impart a massive burden to health services worldwide. There are two basic types of arthritis: osteoarthritis (OA), a degenerative condition that is the result of increased wear and tear on joints, and autoimmune-based arthritis, such as rheumatoid arthritis (RA), which produces systemic inflammatory joint symptoms, with a greater incidence in synovial tissues. OA may also produce inflammatory symptoms, but primarily destroys joint cartilage over time. Neither form of arthritis has yet a treatment, which is able to reverse joint tissue wear out. The available alternatives are restricted to pain and symptom management and/or to prevent or delay further joint destruction. Efforts to discover new target therapies have achieved some success. However, these new approaches are very expensive and none of the currently widely used biological agents reaches long-term drug-free remission (1, 2). RA is a systemic disease and the mechanisms behind its symptoms are complex and have not yet been fully uncovered. The innate immune system, through activation of Toll-like receptors, contributes to a joint pathophysiology characterised by the recruitment of aberrant inflammatory cells, such as T-cell, B-cell, and macrophages, that together with periarticular factors, such as adipocytokines, cause chronic joint inflammation (3).

Given the lack of expression of MHC Class II, and residual expression of MHC Class I, mesenchymal stromal/stem cells (MSCs) are thought to have immune-privileged properties and as such may be delivered in the absence of HLA matching and/or immunosuppression (4–6). Additionally, MSCs have immunomodulatory properties and as such have been successfully applied for the treatment of inflammatory and immune-mediated adverse reactions, such as graft vs. host disease (GVHD), organ rejection after transplantation, allergy, and autoimmune diseases (7–11).

Autoimmune-driven joint destruction, caused by persistent inflammation, renders RA a possible clinical target for cartilage and bone repair using MSCs (12). Indeed, previous results, using a mouse adjuvant-induced model for arthritis (AIA), showed that autologous bone marrow-derived MSCs (BM-MSCs), injected in the joints were able to reduce joint swelling and cartilage destruction, by decreasing the levels of TNF- α . These cells were shown to integrate into the synovium (13). More recently, conditioned medium from the same BM-MSCs was used for treatment (with increased levels of IL-10) significantly reducing histopathological signs of AIA, cartilage damage and suppressing immune responses by reducing aggrecan cleavage, enhancing Treg function and adjusting the Treg:Th17 ratio (14).

Alternatively to BM-MSCs, MSCs from the umbilical cord stromal tissue, or Wharton's jelly (UC-MSCs), can be safely used for allogeneic approaches given their lack of immunogenicity and their marked capacity for localised immunosuppression (15). This immunomodulatory effect is not contact-dependent and is thought to be mostly due to secreted paracrine factors. In addition, UC-MSCs have advantages over other MSCs given their relatively easy and non-invasive procurement, higher expansion potential and overall higher potency to differentiate into more

diverse specialised cells originating from the three germ layers (16, 17).

In our previous studies we have shown that the immunomodulatory properties of a particular population of human UC-MSCs, when compared to human BM-MSCs, were less immunogenic, suitable for xenotransplantation without inducing immunologic infiltrates, and had higher immunosuppression activity than BM-MSCs. Furthermore, unlike BM-MSCs, UC-MSCs did not need prior activation or priming to exert their immunomodulatory effects *in vivo*. Several gene and protein expression profile differences were found between UC-MSCs and BM-MSCs that could explain such observations, namely the increased expression of immunomodulatory surface proteins such as CD200, CD273, CD274, and cytokines such as IL-1 β , IL-8, LIF, and TGF- β 2 by UC-MSCs (6). In another comparative study, this time in the context of cutaneous wound healing, UC-MSCs have shown to secrete considerably higher amounts of G-CSF, EGF, FGF-2, and KGF than BM-MSCs, with concomitant improved motogenic effects over keratinocytes and fibroblasts, as well as enhanced pro-angiogenic activity (18). Accordingly, UC-MSCs were shown to potentially induce the regenerative capacity of tissues *in vivo* by attracting other endogenous MSCs via a unidirectional, UC-MSC-specific, G-CSF-mediated mechanism (18). More relevant to this study, such population of UC-MSCs when transplanted *in vivo*, in a rat AIA model, showed to be non-immunogenic, to have immunosuppressive properties through the inhibition of T-cell proliferation and induction of Tregs, and to promote an impressive remission of local and systemic arthritis manifestations (19, 20). Interestingly, by taking advantage of the 3D greater cell-to-cell communication and cell-to-extracellular matrix (ECM) interactions, the same cells cultured as self-aggregated spheroids could be primed toward a better therapeutic phenotype, as demonstrated in a wound healing context (21).

In this work, we aimed at demonstrating the feasibility of applying a 3D-culture-based UC-MSC priming strategy to improve the efficacy of the resulting secretome for the treatment of inflammatory arthritis. Differences in proteomic profiles and *in vitro* and *in vivo* therapeutic potentials were confirmed between secretomes produced in either 3D spinner flask bioreactors or UC-MSCs cultured under conventional two-dimensional (2D) monolayer conditions. The results clearly showed an improved efficacy of a UC-MSC 3D-derived secretome for the amelioration of experimental AIA manifestations, even when compared with the direct administration of UC-MSCs. The potential mechanisms behind our observations are discussed, as we disclose the potential use of a UC-MSC 3D-primed secretome, or some of its components, as active substances for *Advanced Therapy Medicinal Products* (ATMP) for the treatment of RA.

MATERIALS AND METHODS

Reagents

Antibodies and their respective isotypes were acquired from BioLegend (San Diego, CA, USA) unless stated otherwise.

Cell culture media and supplements were purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated. FBS, Formalin, Trypsin/EDTA (ethylenediamine tetra-acetic acid), Eosin Y and Harris's haematoxylin: Gibco (Life Technologies, Madrid, Spain). BCA protein assay kit: Novagen (San Diego, CA, USA). Blyscan™ Sulfated Glycosaminoglycan Assay kit: Biocolor (Carrickfergus, UK). Entellan®: Merck (Darmstadt, Germany). Flow-Cytomix™: eBioscience. TGF-β1: Tebu-bio (Le-Parray-en-Yvelines, France). Tissue Tek® O.C.T.™: Sakura (Zoeterwoede, The Netherlands). Xylene: EMD Chemicals, Inc. (Gibbstown, NJ, USA).

UC-MSC Isolation and Culture

UC-MSC Isolation

This study was approved by the Ethics Committee of the Hospital Dr. José de Almeida (Cascais, Portugal), in the scope of a research protocol between ECBio (Research & Development in Biotechnology, S.A.) and HPP Saúde (Parcerias Cascais, S.A.). Umbilical cord donations, with written informed consents, as well as umbilical cord procurement, were made according to Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurements, testing, processing, preservation, storage, and distribution of human tissues and cells. UC-MSCs were isolated from umbilical cords of healthy new-born babies, upon informed consent of healthy parturients, as previously described (22). Cells were cryopreserved in minimum essential medium Eagle alpha modification (α-MEM) containing 10% dimethyl sulfoxide (DMSO) stock solution and 20% foetal bovine serum (FBS), using a controlled rate of temperature decrease. When needed, UC-MSCs cryopreserved between passage 3 (P3) and P5 were thawed and further expanded during a maximum of 30 cumulative population doublings (cPDs), corresponding to P12 in culture. UC-MSCs are known to undergo at least 55 cPDs (P22) before reaching senescence, keeping MSC phenotype (21).

Three-Dimensional (3D) Culture

For 3D cultures, spinner vessels (125 mL) with ball impeller containing α-MEM supplemented with 15% FBS were inoculated with single cell suspensions at a concentration of 1×10^6 cells/mL. To promote cell aggregation spinner vessels were stirred at 80 rpm and kept at 37°C in a humidified atmosphere of 5% CO₂ for 24 h. After this period, half of the cell culture supernatant was replaced by fresh medium supplemented with 10% FBS (v/v). Culture medium was replaced every 3–4 days and the stirring rate was adjusted to 110 rpm to maintain spheroid size below 350 μm.

Two-Dimensional (2D) Monolayer Culture

For two-dimensional (2D), static monolayer, cultures, cells were seeded at a density of 1×10^4 cells/cm² in α-MEM supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere with 5% CO₂. Cell passage was performed by Trypsin/EDTA 0.05% incubation for 5 min every 72 h.

UC-MSC Characterisation

Flow Cytometry

Cell surface marker expression was analysed by flow cytometry in both 2D and 3D cultures. Cell detachment from culture t-flasks and dissociation from spheroids was performed by using 0.25% Trypsin/EDTA. The resulting single cell suspension was washed with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Detection of cell surface markers was performed with the following antibodies and their respective isotypes after incubation for 1 h at 4°C: phycoerythrin (PE) anti-human CD105 (eBioScience, San Diego, CA, USA); APC anti-human CD73; PE antihuman CD90; APC anti-human CD44; PerCP/Cy5.5 anti-human CD45; fluorescein isothiocyanate (FITC) anti-human CD34; FITC anti-human CD31; PerCP/Cy5.5 anti-human CD14; Pacific Blue anti-human CD19 and pacific-blue anti-human HLA-DR. All samples were acquired on a Gallios (Beckman Coulter, Pasadena, CA, USA) and the results analysed with Kaluza software (Beckman Coulter). A minimum of 1×10^4 events were acquired per surface marker. One replicate was analysed per independent experiment ($n = 4$).

Tri-lineage Differentiation

Spheroids were dissociated into a single cell suspension with 0.25% Trypsin/EDTA and transferred to appropriate culture t-flasks for cell proliferation and expansion. To induce adipogenic differentiation, UC-MSCs were incubated in α-MEM supplemented with 20% FBS, 10 μg/mL insulin, 200 μM indomethacin, 0.5 mM isobutylmethylxanthine, and 1 μM dexamethasone for 3 days and 1 day in medium supplemented with 20% FBS and 10 μg/mL insulin. To induce osteogenic differentiation, cells were incubated in α-MEM supplemented with 10% FBS, 10 mM β-glycerol phosphate, 100 nM dexamethasone, and 50 μg/mL ascorbate-2-phosphate. Finally, to induce chondrogenic differentiation, cells were maintained in suspension as pellets, incubated with Dulbecco's modified Eagle's medium (DMEM) with 4 mM glutamine and 1 g/L D-(+)-glucose, supplemented with 1% FBS, 6.25 μg/mL insulin, 10 ng/mL transforming growth factor (TGF)-β1, and 50 μM ascorbate-2-phosphate. For cytochemical staining, cells were fixed with paraformaldehyde 4% for 20 min. In adipogenic and osteogenic differentiation protocols, cells were stained with Oil Red O for 10 min and alkaline phosphatase for 30 min, respectively. For chondrogenic differentiation, the chondrospheres were fixed in formalin, embedded in paraffin and cut into sections of 5 μm and stained with alcian blue for 30 min. The presence of stained cells was confirmed by inverted microscopy with phase contrast (Leica, DMIL HC, Wetzlar, Germany).

Protein Quantification

For both 3D and 2D cultures, biomass was evaluated by total protein quantification using a BCA protein assay kit, after cell pellet lysis with 0.1 M NaOH at 37°C for 24 h.

UC-MSC Spheroid Visualisation and Measurement

Spheroids were observed by bright field microscopy (Olympus CK30, Olympus, Tokyo, Japan) and their average diameter

determined by a geometric mean of three diameters per spheroid as previously described, using the following equation: average diameter = $(d1 \times d2 \times d3)^{1/3}$ (17, 23). Diameters were measured using Motic Images Version 2.0 software (Xiamen, China).

Haematoxylin and Eosin Staining

Spheroids were suspended in Tissue Tek[®] O.C.T.[™] for preparing 10 μ m cryosections. Slides were first stained with Harris's haematoxylin for 10 min, followed by incubation with HCl 1% (v/v) in 70% EtOH, and by Eosin Y staining for 2 min. Slides were then submitted to increasing concentrations of ethanol and finally incubated in xylene. Samples were mounted with Entellan[®]. Images were acquired on an Olympus CK30 inverted microscope and processed using Motic Images Version 2.0 software.

Conditioned Media (CM) Preparation

Conditioned media (CM) were produced from cells having undergone the same number of cPDs. UC-MSC CM from 3D spinner flask cultures (CM3D) was obtained by cell inoculation as described above, subjected to successive medium adaptations: FBS concentration was reduced to 5% at culture day 2. After 3 days, medium was changed with α -MEM without FBS and volume adjusted to obtain a conditioning volume per cell equivalent to that in the 2D system. After 48 h of conditioning, CM3D was collected under sterile conditions. To produce UC-MSC CM in 2D monolayer cultures (CM2D), 1.75×10^6 cells were seeded in 175 cm² culture t-flasks and kept in medium supplemented with 5% FBS until they reached 90% confluence. At this point, cells were washed with fresh α -MEM and medium was replaced by α -MEM without FBS, to a final volume of 25 mL. After conditioning for 48 h, CM2D was harvested under sterile conditions. The control sample consisted of α -MEM which was never in contact with cells. CM3D, CM2D, and control were 10 \times concentrated using 3-kDa cut-off spin concentrators. Total protein content of CM2D, CM3D and control was quantified using a BCA protein assay kit. Samples were stored at -80°C until further use.

CM2D and CM3D Trophic Factor Quantification

Trophic factor concentrations within CM3D and CM2D samples were measured using the Human 64-Plex Cytokine/Chemokine Panel (Eve Technologies, Calgary, AB, Canada) or using Flow-Cytomix[™] according to manufacturer's recommendations. All cytokines/chemokines, except for IL-6 and MCP-1, were quantified by resorting to the multiplexing technology. IL-6 and MCP-1 detections were acquired on a Gallios (Beckman Coulter) and the results were obtained using FlowCytomix[™] Pro 3.0 Software. Data is expressed in terms of productivity: ng/mL/million cells/hour, normalised against the background (α -MEM that was never in contact with cells) threshold concentrations.

CM Testing *in vitro*

Mouse Chondrocyte (ATDC5) Cell Culture

Mouse chondrocytes (ATDC5) were seeded at 1×10^4 cells/cm² and cultured in DMEM-F12 supplemented with 5% FBS, at

37°C, in 5% CO₂ humidified atmosphere until reaching 70–80% confluence. Cell passage was performed by Trypsin/EDTA 0.25% incubation for 5 min every 72 h.

Scratch Assay

ATDC5 cells were seeded into 24-well plates at a density of 1.5×10^4 cells/cm² with DMEM-F12 supplemented with 5% FBS. Once at 90% confluence, scratches of ~ 0.5 mm in width were performed on the monolayer with a sterile 200 μ L pipette tip. Immediately after, the cell surfaces were washed with PBS and maintained in a final volume of 400 μ L of DMEM-F12 supplemented either with CM2D, CM3D, all 10 \times concentrated. DMEM-F12, DMEM-F12: α -MEM (1:1) and DMEM-F12 with 5% FBS were also tested as negative, solvent and positive controls, respectively. The area of the scratch, from the same field, was measured at 0, 3, 6, 8, 10, 20, and 24 h post-scratch to evaluate cell migration. Digital photographs were taken at an amplification of 40 \times on Olympus CK30 microscope. Cellular migration was analysed in the Motic Images Version 2.0 software by calculating scratch closure, given as the total area occupied by the cells after incubation with CM in relation to the initial scratch area at 0 h. Three independent experiments in triplicates were considered.

Glycosaminoglycan Quantification

Glycosaminoglycans (GAG) were quantified in ATDC5 cell culture supernatants. At a confluence of 60%, cells were incubated with DMEM-F12 supplemented either with CM2D or CM3D 10 \times concentrated. DMEM-F12, DMEM-F12: α -MEM (1:1) and DMEM-F12 with 5% FBS were also tested as negative, solvent and positive controls, respectively. After 24 h of incubation, GAGs were determined using the Blyscan[™] Sulfated Glycosaminoglycan Assay kit, according to the manufacturer's instructions. A total of three independent experiments were performed.

CM Testing *in vivo*

Adjuvant-Induced Arthritis (AIA) Model

All animal experiments were carried out with the permission of the local animal ethical committee in accordance with the EU Directive (2010/63/EU), Portuguese law (DL 113/2013) and all relevant legislations. The experimental protocol was approved by *Direção Geral de Alimentação e Veterinária* (DGAV). Animals were acclimatised before the experiments and housed in plastic cages under standard laboratory conditions, fed commercial chow, and acidified drinking water *ad libitum*.

Induction of the inflammatory reaction was achieved by injecting Wistar rats (365–480 g; Charles River Laboratories, France) with a single intradermal (i.d.) administration of 0.1 mL of a suspension of killed and dried *Mycobacterium butyricum* in incomplete Freund's Adjuvant—IFA (at 10 mg/mL) (Difco Laboratories), into the sub-plantar area of the right hind paw (24). Animals were randomly divided into groups ($n = 3-6$). Treatment was initiated at day 7 after induction. Induced animals were treated with different formulations: (i) one group received PBS by intraperitoneal (i.p.) injection (Sham UC-MSC group); (ii) another group received PBS by intra-articular (i.a.) route of administration (Sham CM group); (iii) a third

group received 2D-cultured UC-MSCs (1.7×10^6 cells per injection in 4 consecutive days) by i.p. injection (UC-MSC group); (iv) a fourth group received, by i.a. injection, $10 \times$ concentrated CM2D (CM2D group); (v) one group received, by i.a. injection, $10 \times$ concentrated CM3D (CM3D group); and lastly, (vi) three animals received neither inflammation induction nor any treatment and were used as a naïve control for histology analysis (Control). No experimental group received 3D-cultured UC-MSCs since full cell disaggregation from 3D aggregates has proven very difficult with consequent risk of acute inflammation and thrombosis. Animals treated intraperitoneally and the respective control group (Sham UC-MSC group) received a volume of 100 μ L of the tested formulations per injection (i.e., a total of 400 μ L). Animals treated intrarticularly and the respective control group (Sham CM group) received a volume of 200 μ L of the tested formulations per injection (i.e., a total of 600 μ L). I.p. injections were given for 4 consecutive days. I.a. administrations, in a total of three, were performed every-other-day. The experiment lasted for 57 days. To follow the course of the disease, body weight and volume of right and left paws measured by a water displacement method, using a plethysmometer (Ugo Basile, Italy), were measured. Blinding investigators to treatment groups were defined. Arthritis was evaluated in ankle joints in a blinded manner using a semiquantitative arthritic score based on the sum of the following grades: 0 = normal; 1 = for each inflamed paw; 1 = tail lesion; 1 = joint rigidity or deformity; 1 = wounded paw; 1 = infected paw; 1 = necrotic paw. The sum of the parameters is calculated as an arthritic index (AI) with a maximum possible score of 9. Photographs recorded the evolution of clinical signs in all experimental groups. Animals were sacrificed at day 57, necropsies and gross pathology examination was conducted. The experiment was performed twice with consistent observations, using two UC-MSC isolates (different donors).

Collection of Paw Samples and Histopathological Analysis

After the sacrifice, animal paws were collected, fixed in 10% buffered formalin and decalcified with 10% formic acid to undergo histopathological analysis. In order to evaluate the surroundings of the site of application, fixed decalcified paws were processed for embedding in paraffin wax by using routine protocol. Sections (5 μ m thick) were stained with haematoxylin and eosin (H&E). The slides were examined using light microscopy using an Olympus BX 40 microscope coupled with an Olympus DP 10 camera (Olympus, Shinjuku, Tokyo, Japan). Digital photographs were taken at an amplification of $100 \times$, except for the control that was acquired at an amplification of $40 \times$. The histological samples were evaluated for synovial inflammation and bone erosion. Synovial inflammation was scored as follows: 0- no inflammation; 1- slight synovitis with some cell infiltration; 2- moderate synovitis with moderate cell infiltration; 3- extensive synovitis with a moderate number of infiltrating cells; 4- extensive and severe synovitis, with the presence of numerous inflammatory cells. Bone erosion was scored as follows: 0-

no erosion; 1- small areas of resorption; 2- numerous areas of resorption; 3- extensive osteolysis; 4- extensive and severe osteolysis.

Statistical Analysis

Statistical analyses were performed in GraphPad Prism v6.0 software (La Jolla, CA, USA). To estimate the significance of the differences of trophic factor quantification and of the data obtained from GAG production *in vitro*, multiple *t*-tests and Student's paired *t*-test with one-tailed distribution were used, respectively. The two-way ANOVA with Tukey's *post-hoc* test was performed for the *in vitro* scratch assay data. Results are presented as means \pm standard error of the mean (SEM), except where indicated and *p*-values are presented for statistically significant results (**p* < 0.05, ***p* < 0.01, and ****p* < 0.001).

RESULTS

Pre-conditioning UC-MSCs in Tri-dimensional (3D) Culture Conditions Results in a Secretome Richer in Therapeutically Relevant Trophic Factors

Three-dimensional (3D) spheroids from UC-MSCs were obtained using a spinner flask suspension culture. Measurements of spheroids were performed by phase-contrast images throughout the whole culture period yielding the size-distribution plot shown in **Figure 1**. Firstly, UC-MSCs form small low-density cell aggregates of $\sim 100 \mu$ m diameter (Day 2, **Figures 1A,B**). After 4 days, spheroid diameters were, on average, $149.11 \pm 0.57 \mu$ m and $195.48 \pm 5.48 \mu$ m from day 5 to 7 of culture (**Figures 1A,B**). The results showed that the formation of more dense and viable 3D structures from day 4 onwards, with expected low diffusion rate of nutrients (**Figures 1B,C**). Nevertheless, a necrotic centre in spheroids was circumvented by maintaining the average spheroid size under 350μ m (**Figure 1C**).

In the present work, we firstly verified if the 3D culture conditions prompted the production of a secretome with higher potential to counter AIA signs than that obtained by UC-MSCs grown under 2D monolayer conditions. A comparative analysis of a representative pool of trophic factors involved in relevant immune-modulation and other relevant joint tissue regeneration events was performed between CM3D and CM2D. The relative productivity of such trophic factors was determined taking into consideration cell populations kept in similar conditions in terms of number of cells (with equivalent number of cPDs), same medium volume and conditioning time. The only variable in the experimental design was therefore the culture setup for modulating cell phenotype: 2D vs. 3D. **Figure 2** shows the logarithm (Log_{10}) of CM3D/CM2D ratio, representing the relative trophic factor productivity in 3D vs. 2D culture conditions normalised against the background medium that was never in contact with cells (α -MEM).

The results depicted in **Figure 2** clearly show some interesting differences in specific trophic factor productivities that could result different therapeutic activities between CM3D and CM2D.

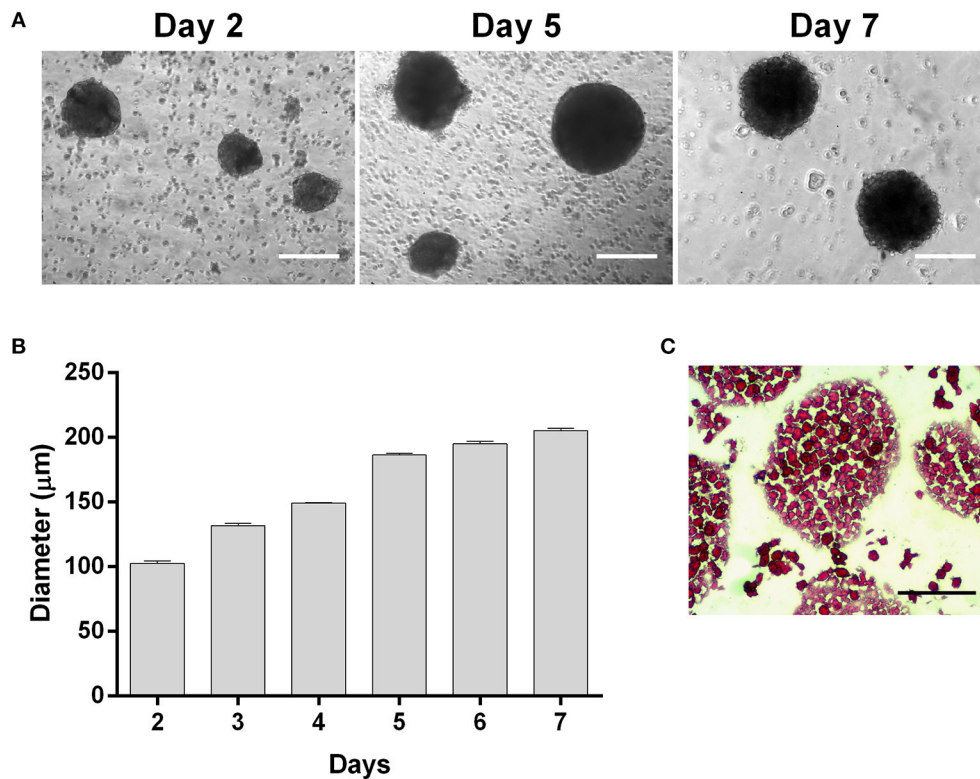


FIGURE 1 | UC-MSCs form viable three-dimensional spheroids. **(A)** Representative phase contrast images of UC-MSC spheroids from days 2, 5 and 7 of culture. **(B)** UC-MSC spheroid size during culture time. **(C)** Representative image of UC-MSC spheroid sections from day 7 of culture stained with H&E. Scale bar = 100 μm. $n = 7$.

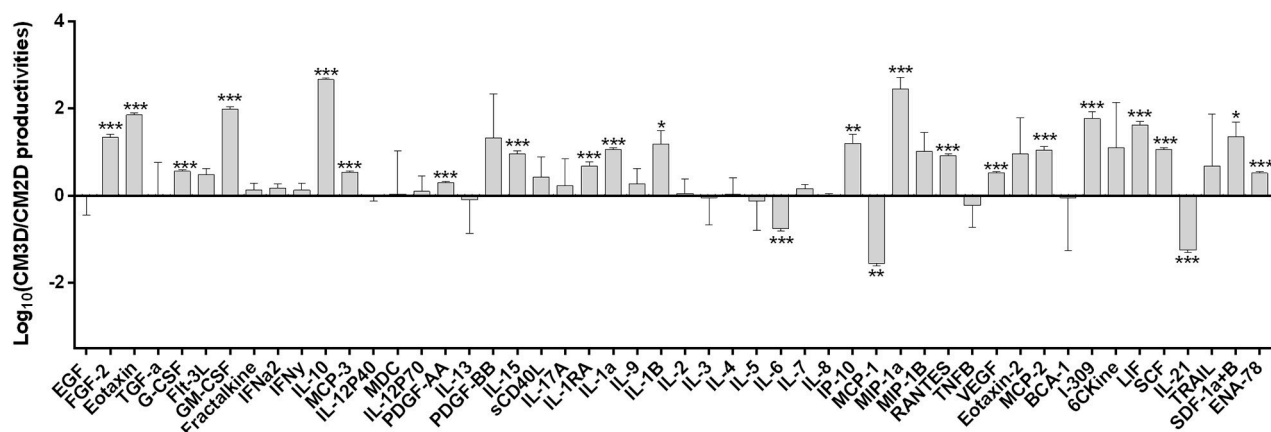


FIGURE 2 | UC-MSCs display differences in specific trophic factor productivities that could entail different therapeutic activities between CM3D and CM2D. Quantification in CM3D and CM2D of a representative pool of trophic factors involved in relevant immune-modulation and other RA-relevant regenerative cascades of events. Data is expressed as the logarithm of the ratio between CM3D and CM2D productivities (ng/mL of the growth factors or cytokines/million cells/hour) corrected by subtracting the background (α-MEM). Plotted ratios are mean ± SD. $n = 3$. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

CM3D was characterised by a prevailing expression of anti-inflammatory cytokines such as IL-10 and LIF, as well as trophic factors involved in different mechanisms leading to tissue regeneration, mainly PDGF-BB, FGF-2, I-309, SCF, and GM-CSF (25–29). In turn, CM2D was characterised by relatively

higher levels of cytokines with recognised pleiotropic roles in the progression of inflammatory arthritis, such as IL-6, MCP-1, and IL-21 (30–32). To confirm if the differences observed in trophic factor profiles would indeed translate into different paracrine activities *in vitro*, we set forward to evaluate (i)

the relative capacity of CM3D and CM2D to induce motility of joint chondrocytes and (ii) the relative capacity of CM3D and CM2D to induce glycosaminoglycan (GAG) synthesis, two important mechanisms connected to joint regeneration and arthritic aetiology.

CM3D Has a Higher Motogenic Activity Over Mouse Chondrocytes *in vitro*

The relative capacity of CM3D and CM2D to promote chondrocyte migration was evaluated by scratch assays. Scratch areas were monitored for 24 h post-scratch. The results depicted in **Figure 3** confirm significant differences between the paracrine activities of CM3D and CM2D. The CM3D supplement promoted a ~1.5-fold increase in chondrocyte migration capacity 24 h post-scratch when compared to CM2D, a fact that could be explained by e.g., relatively higher CM3D expression of PDGF-BB, IL-10, and FGF-2, all with recognised mitogenic, protective, and motogenic activities over chondrocytes (33–35).

CM2D Has a Higher Capacity of Inducing Glycosaminoglycan (GAG) Synthesis *in vitro*

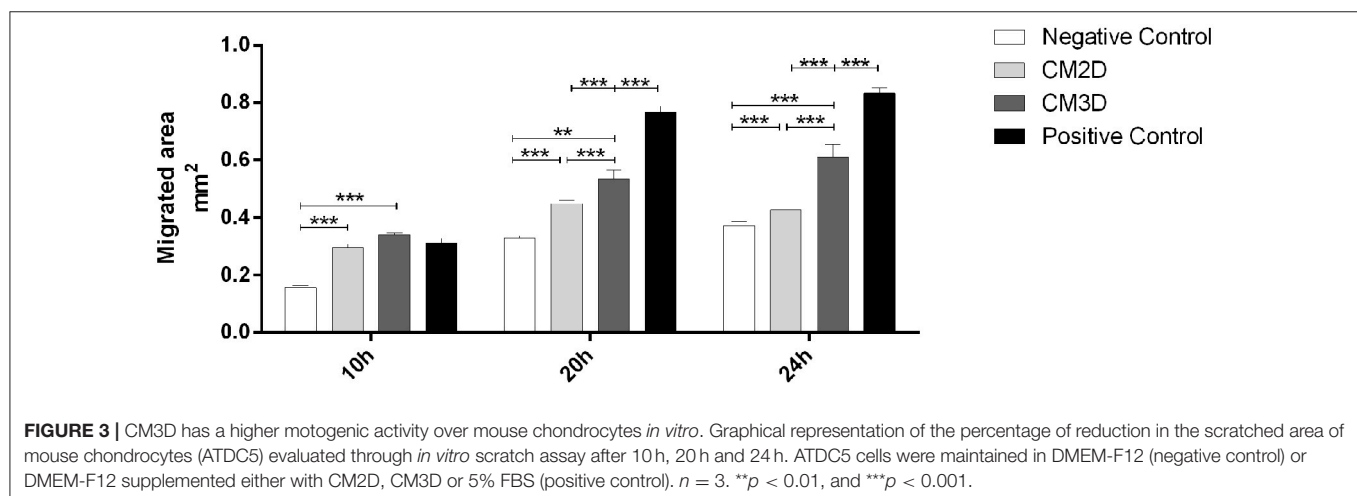
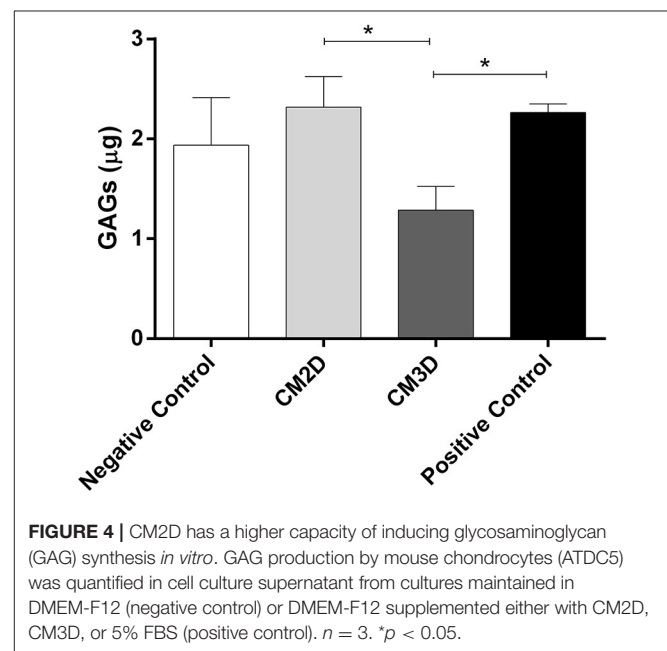
Differences between the paracrine activities of CM3D and CM2D were further confirmed *in vitro* by evaluating their relative capacities to induce GAG synthesis. GAG concentration was quantified in the supernatant after 24 h of incubation. The results depicted in **Figure 4** clearly show a ~2-fold increase in GAG induction by CM2D when compared to CM3D, a fact that could be explained e.g., by the relatively higher CM2D expression of IL-6 which has been shown to stimulate fibroblastic GAG synthesis and chondrocyte cartilage matrix production *in vitro* (36, 37).

CM3D Has a Higher Capacity for Both, Avoiding and Ameliorating Adjuvant-Induced Arthritis (AIA) Manifestations *in vivo*

Given the *in vitro* evidence that CM3D and CM2D had in fact different paracrine activities regarding important mechanisms

connected to joint regeneration and arthritic aetiology, we set out to evaluate the potential benefits of UC-MSCs primed by 3D culturing for reverting AIA signs *in vivo*.

The AIA model is useful for addressing the protective effects against manifestations typifying the late stage of entrenched chronic arthritis, sharing several pathological features with RA. The model is established by injecting *Mycobacterium butyricum* in incomplete Freund's Adjuvant into the sub-plantar area of Wistar rats' right hind paws. In our well-characterised setup, the disease shifts from a local arthritis stage to a systemic polyarthritis condition by day 13 after induction (20, 38). Indeed, we previously refined and characterised AIA by studying the time course of the disease, introducing new evaluation methods and identifying the main stages of the disease (24).



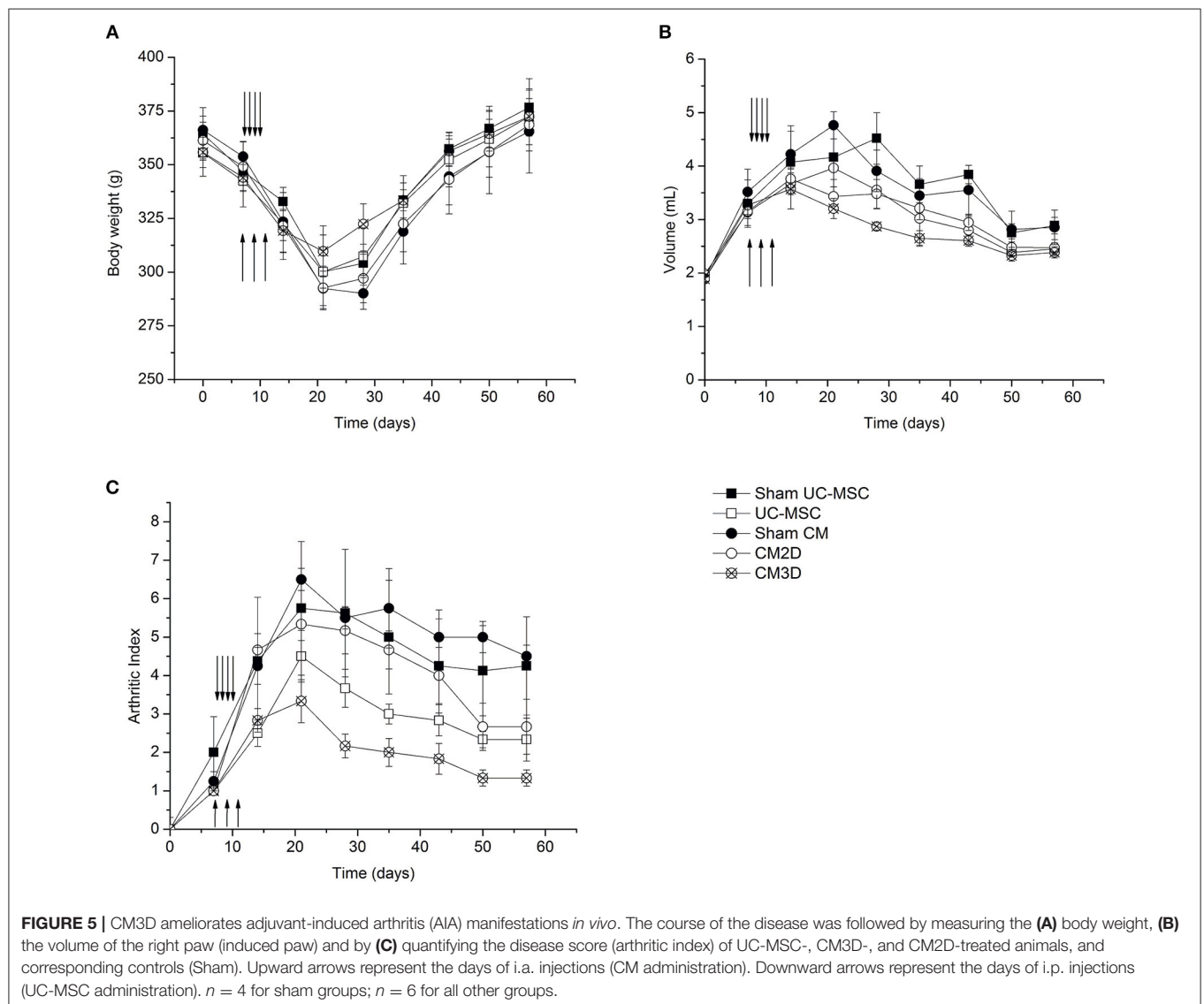
One day after the disease induction, the induced paw volume more than doubled and haematological parameters completely changed, corresponding to the first disease phase. Two weeks after induction, another stage occurred when the disease shifted from the local arthritis form toward a systemic polyarthritis along with an additional increase of the paw volume (20, 38). Animal body weight also reached the minimum values and radiographic observable joint lesions increased accordingly (24). Starting the treatment at day 13 or later hampers the possibility to stop and reverse joint erosion (data not shown). Early recovery on body weight was obtained for animals treated at day 7 when compared with animals treated later (data not shown). As such, our treatment protocol started at day 7 post-induction and lasted for 57 days. No adverse effects were observed during or after the treatment period.

I.a. administration of CM3D and CM2D was performed and compared to the better performing route for UC-MSC administration, which had been found previously to be i.p. in

the same AIA model (20). No experimental group received 3D-cultured UC-MSCs since full cell desegregation from 3D aggregates has proven very difficult with consequent risk of acute inflammation and thrombosis. The body weight, inflammatory swelling, clinical scoring through arthritic index (AI), and histopathological endpoints were measured.

The time course of body weight is illustrated in **Figure 5A**. Given the transient nature of the AIA model, animals in all groups ultimately recovered from AIA manifestations and regained their natural body weights. However, weight loss was less prominent in animals treated with CM3D, especially between days 20 and 30 where the arthritic signs reached their highest intensity.

The hind paw volume was monitored by water displacement as a function of time after disease induction (**Figure 5B**). Swelling of adjuvant-injected right paws with erythema became evident within 1 day after induction. As seen in **Figure 5B**, animals from all treatment groups (UC-MSC, CM3D, and CM2D) showed



ameliorated swelling. Nevertheless, the effect elicited by CM3D, as reflected by a considerable swelling reduction rate from day 15 onwards, conveys an unprecedented capacity for concomitantly prevent against the implantation and revert AIA inflammatory signs *in vivo*.

The development course of AIA signs was also evaluated by monitoring the evolution of the arthritic index (AI) as a function of time after disease induction (**Figure 5C**). All UC-MSC-based treatments resulted in amelioration of AIA manifestations when compared to the Sham controls as seen at day 20. Notably, treatment with CM3D resulted in more than a 2-fold reduction in AIA severity at day 20, culminating in a 5-fold difference at day 57, relatively to untreated animals. When compared to CM2D, CM3D was able to prevent the development of the AI more efficiently by a factor of ~ 1.8 -fold at day 20, and still culminating with a 1.5-fold amelioration at day 57. The amelioration effect of CM3D was not so pronounced when compared to the administration of UC-MSCs at day 20 (1.5-fold), although in the end the amelioration effect brought by CM3D was still significant throughout the full experimental time frame (**Figure 5C**).

The results of a more in-depth analysis of AIA manifestations at day 57 are depicted in **Figure 6**. Representative hind-paw photos of each experimental group clearly show that untreated animals, belonging to control (Sham) groups, still presented moderate-to-severe swelling encompassing the ankle, foot, and digits, with multiple foci of necrosis, inflammation, secondary infection, and joint deformity (**Figures 6A,B**). Notably, CM3D-treated animals exhibited only minimal paw swelling, with no signs of lesion (**Figure 6E**) when compared to CM2D- (**Figure 6D**) and UC-MSC-treated (**Figure 6C**) animals that still presented a moderate degree of swelling and moderate-to-negligible signs of lesion. Accordingly, the histopathological analysis of control (Sham) animals showed extensive osteoclastic activity along with the presence of granulomas, affecting the limits of the cartilage and bone tissues (**Figures 6F,G,K,L**). Bone necrosis appeared mainly in the periphery where numerous osteoclasts were noticed (**Figures 6K,L**). Synovitis was detected in all cases which represented the initial phase of RA (**Figures 6F,G**). No significant differences were observed between the two Sham groups (i.a. vs. i.p.). In turn, UC-MSC-treated animals still showed some signs of inflammation as seen by the presence of granulomas in the osteolytic area (**Figure 6M**) and of hyperplastic synovium membrane (**Figure 6H**). Finally, both animals treated with CM2D and CM3D presented small foci of synovitis with almost well-defined bone and cartilage tissue stratification (**Figures 6I,J**). Moreover, the osteolysis degree observed in CM3D-treated animals (**Figure 6O**) is lower than that of animals treated with CM2D (**Figure 6N**), in which granulomatous lesions are still present.

DISCUSSION

MSCs are known to modulate tissue regeneration through trophic effects exerted by secreted cytokines and growth factors. In fact, and contradicting the dogma that cells need to be

physically present to induce regeneration through mechanisms involving homing, engrafting, and secretion of trophic factors in response to local stimuli, our previous results have unlocked the possibility of using the MSC secretome as active substance for therapeutic formulations. By recreating a more physiological environment within our 3D culture system, characterised by tissue-like cell-to-cell and cell-to-ECM interactions, as well as the presence of stress signals discharged by MSCs within the core of self-aggregated spheroids (e.g., hypoxic and famine), we were able to mimic many stimuli found within the lesion niche.

In this work, we aimed at demonstrating the viability of applying this 3D-priming strategy to improve the efficacy of the resulting UC-MSC secretome for counteracting the signs caused by inflammatory arthritis. A comparative analysis of CM3D and CM2D proteomes, comprehending a significant set of relevant trophic factors, corroborated our previous finding that our 3D conditions promoted different trophic profiles. Within our pool of trophic factors, CM3D was marked by synthesis of mainly IL-10, LIF, FGF-2, I-309, GM-CSF, eotaxin, and MIP-1 α , and to a lower extent G-CSF, PDGF-BB, 6CKine, and SCF. In turn, CM2D was characterised by significantly higher expression of mainly IL-6, MCP-1, and IL-21.

The impact of such differences was validated *in vitro* where CM3D showed significantly higher motogenic activity over chondrocytes when compared to CM2D. This could be explained by e.g., relatively higher CM3D expression of e.g., IL-10, FGF-2, and PDGF-BB (33–35). IL-10 was found to directly protect chondrocytes *in vitro* through the inhibition of NOS2 and MMP-3 expression. In turn, FGF-2 and PDGF-BB are potent mitogens for articular chondrocytes which have also been found to promote chondrogenic differentiation (33, 35, 39).

Different paracrine activities between CM3D and CM2D were further validated *in vitro* by evaluating their relative capacities to induce GAG synthesis. This time CM2D proved to be a more potent inducer of GAG synthesis than CM3D, which could be justified by the relatively higher CM2D expression of IL-6. IL-6 was found to induce IL-1 β -related collagen and GAG biosynthesis and to stimulate cartilage matrix production *in vitro* (37, 40).

Although *in vitro* indications suggested differential capacities of CM3D and CM2D to induce important events connected to tissue regeneration, their impact in the specific context of AIA could not be directly extrapolated. For example, although apparent benefits should be drawn from FGF-2-induced chondrocyte motility in a OA context, FGF-2 expression in RA patients has been closely associated with disease severity (41). Recently, FGF-2 was even shown to cooperate with IL-17 in the pathogenesis of autoimmune arthritis (42). Similarly, while GAG production promoted by IL-6 is necessary for the regeneration of functional cartilage in osteoarthritic patients, self-antigenic GAGs were found to provoke autoimmune dysfunctions that involve the expansion of GAG-binding infiltrates, thus aggravating inflammatory conditions in RA (43). The amelioration effects of either CM3D or CM2D on arthritic signs would therefore be dependent on how the overall synergistic activity within each of their trophic factor compositions would interrelate with the AIA environment.

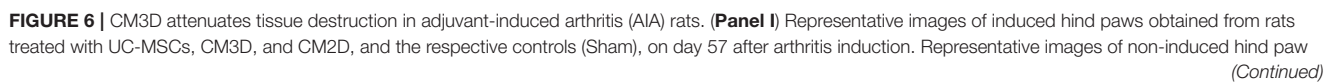


FIGURE 6 | (control) are also shown. **(A–E)** Macroscopic images of induced hind paws. **(F–O)** Representative photomicrographs from rat paw histological sections stained with H&E revealing signs of **(F–J)** synovitis and/or **(K–O)** osteolysis. Specifically: **(F)** sham UC-MSC histological sections show blood vessel congestion and severe synovitis with infiltration of lymphoid cells. **(K)** A pronounced osteolysis is also observed with the presence of granuloma, extensive neutrophil accumulation and bone necrosis. **(G)** Sham CM condition reveals synovial membrane with intense infiltration of inflammatory cells and capillary vessels congestion and **(L)** extensive number of osteoclasts. **(H,M)** UC-MSC-treated animals present milder granulomatous lesions than respective sham and hyperplasia of the synovial membrane **(N)**. **(I,J,N,O)** CM-treated animals exhibit lower inflammation and bone destruction level. **(I)** CM2D image shows infiltration of lymphoid cells in a lower extent than respective sham and **(N)** some degree of osteolysis. **(J,O)** CM3D image shows a synovial membrane with less blood vessels and cell infiltration than respective sham, being the changes located in the extra-articular space. **(P,Q)** Control paw from a naïve animal shows normal histological features, with no evidence of inflammation and with intact synovial membranes. **(Panel II)** Histological scores for synovial inflammation and bone erosion. Data represent the mean \pm SEM. $n = 3$ for control; $n = 4$ for sham groups; $n = 6$ for all other groups. AC, articular cartilage; B, bone; G, granuloma; LC, lymphoid cells; O, osteolysis; OC, osteoclasts; SC, synovial cavity; SM, synovial membrane.

Ultimately the results showed that CM3D has a clearly superior capacity for both, avoiding and ameliorating AIA manifestations *in vivo* when compared to CM2D or even UC-MSCs. CM3D treatment was able to both prevent and revert all major signs of AIA, including complete avoidance of necrotic foci around the joints, acute and chronic inflammation, joint deformity and secondary infection.

Mechanisms behind CM3D activity can also be extrapolated based on secretome profile features resulting from our comparative analysis. Mainly IL-10, a recognised potent anti-inflammatory type II cytokine, plays a central role in limiting host immune response to pathogens. Dysregulation of IL-10 is associated with enhanced immunopathology in response to infection as well as increased risk for development of many autoimmune diseases (34, 44, 45). IL-10 was found to be produced by innate cells, as well as CD4⁺ CD25[−] Foxp3[−] and CD4⁺ CD25⁺ Foxp3⁺ Tregs using a *Leishmania* chronic lesion model, which is consistent with our previous observations that UC-MSCs enhanced CD4⁺ CD25⁺ Foxp3⁺ Tregs in response to a AIA environment *in vivo* (20, 46). IL-10 was also shown to limit the inflammasome (NLRP3)-driven arthritic disease course and associated structural damage in an AIA model (47). Furthermore, reversion of arthritis by IL-10 was not limited to AIA. IL-10 produced by B cells was also found to be crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and the reduction of collagen-induced arthritis (CIA)-related signs, many common to AIA (48). The recognised IL-10 distinctive capacities to downregulate the production of pro-inflammatory cytokines meant that it has been regarded as a potential therapeutic agent for the treatment of arthritis (49). Besides IL-10, the highly expressed leukaemia inhibitory factor (LIF) can augment the immunosuppression capacity of CM3D through further induction of Tregs (50–52). LIF may also play an important role in regulating the neural-immune system interaction during early acute inflammatory stages of the disease and the subsequent healing and restitution process (53). Concomitantly, other CM3D highly expressed factors such as FGF-2 and I-309, have been found to be involved in different aspects of tissue regeneration. FGF-2 through mitogenic and motogenic activities over chondrocytes (39) and I-309 and SCF through promotion of angiogenesis (27, 54). Yet other factors produced in CM3D, such as SCF and G-CSF, have been found to support haematopoiesis and recruiting of other CD 34[−] endogenous MSCs to aid in regeneration (18, 28, 55). The remarkably low incidence of

secondary infection signs in animals treated with CM3D could have been due to the relatively higher expression of cytokines such GM-CSF, 6CKine, and eotaxin. Although these factors may contribute to pathogenic inflammatory infiltrate, GM-CSF for e.g., has even been used recently as primary immuno-target for treatment of specific groups of RA patients (56), their synergistic roles in stem cell stimulation and eosinophil recruitment may confer advantages in a AIA context; especially when coupled with the expression of anti-inflammatory cytokines like IL-10 and LIF and within an environment characterised by opportunistic secondary infection. Thus, in our experimental conditions, GM-CSF, together with other cytokines usually associated with RA pathogenesis, but with capacities to attract lymphocytes with distinct phenotypes, like MIP-1 α , MIP-1 β , 6CKine, and RANTES, could be modulating specific T-cell functions in favour of a wider host defence. In addition, eotaxin could be attracting eosinophils to provide further defence against infectious agents while producing antihistamines (57–63).

Finally, differences seen between CM3D and CM2D could also be explained by a distinctive pro-inflammatory character of CM2D, as patented by a comparatively higher expression of inflammatory MCP-1, IL-6, and IL-21 without counterbalancing expression of anti-inflammatory cytokines. Both MCP-1 and IL-6 have for long been found to be highly expressed in the synovial fluid of RA patients (64). MCP-1 and IL-6 have been consistently found to play critical roles in the development of AIA signs in several animal models (31, 37, 65). Post-onset treatment of AIA using endogenous MCP-1 inhibitors improved clinical signs of arthritis and histological scores measuring joint destruction, synovial lining, macrophage infiltration, and bone erosion (66). In turn, IL-6 is synthesised in response to many stimuli, including IL-1 β which is widely implicated in the pathogenesis of RA (36, 40). More recently, IL-21 has been found to be involved in several mechanisms related to RA pathogenesis being able to activate T cells, B cells, monocytes/macrophages and synovial fibroblasts through activation of JAK-STAT, MAPK, and PI3K/Akt signalling pathways, ultimately promoting osteoclastogenesis (32).

Overall the results demonstrate the viability of applying this 3D-priming strategy to improve the efficacy of the resulting UC-MSC secretome for counteracting the manifestations caused by inflammatory arthritis. On the path to simplify MSC-based therapeutic formulations more studies will now follow to discriminate what components within CM3D are exerting

the observed protective and therapeutic activities. This will involve a concerted action applying multi-faceted analyses involving exosome scrutiny, proteomics, metabolomics as well as epigenomics and miRNA regulomics. A more difficult task will be to define precise synergistic relationships between the different actors within the formulations and predict their synergistic effect within different disease environments. Nevertheless, we believe a novel path has been unleashed, involving the use of well-defined paracrine actors, instead of physical cells, as active substances for “off-the-shelf” *Advanced Therapy Medicinal Products* (ATMP).

AUTHOR CONTRIBUTIONS

JM, SS, and JS developed the study concept and the study design. SC, MG, JR, and MC performed the experiments and

data collection. SC, JR, and RB performed the data analysis and interpretation under the supervision of JM, SS, and JS. JM, SC, JR, SS, and JS drafted the manuscript. RB, PC, and HC provided critical revisions. All authors approved the final version of the manuscript for submission.

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Cell Based Therapy for Type 1 Diabetes: Should We Take Hyperglycemia Into Account?

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Diabetes mellitus is characterized by long standing hyperglycemia leading to numerous life-threatening complications. For type 1 diabetes mellitus, resulting from selective destruction of insulin producing cells by exaggerated immune reaction, the only effective therapy remains exogenous insulin administration. Despite accurate compliance to treatment of certain patients, transient episodes of hyperglycemia cannot be completely eliminated by this symptomatic treatment. Novel immunotherapeutic approaches based on tolerogenic dendritic cells, T regulatory cells and mesenchymal stem cells (MSCs) have been tested in clinical trials, endeavoring to directly modulate the autoimmune destruction process in pancreas. However, hyperglycemia itself affects the immune system and the final efficacy of cell-based immunotherapies could be affected by the different glycemic control of enrolled patients. The present review explores the impact of hyperglycemia on immune cells while providing greater insight into the molecular mechanisms of high glucose action and subsequent metabolic reprogramming of different immune cells. Furthermore, over-production of mitochondrial reactive oxygen species, formation of advanced glycation end products as a consequence of hyperglycemia and their downstream signalization in immune cells are also discussed. Since hyperglycemia in patients with type 1 diabetes mellitus might have an impact on immune-interventional treatment, the maintenance of a tight glucose control seems to be beneficial in patients considered for cell-based therapy.

Keywords: dendritic cells, immune tolerance, cell-based therapy, diabetes mellitus, hyperglycemia

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder resulting from immune-mediated destruction of insulin-producing cells in the islets of Langerhans (type 1 diabetes mellitus—T1D) or from the combination of insulin resistance and relative insulin deficiency (type 2 diabetes mellitus—T2D). Both types are associated with severe complications stemming from chronic hyperglycemia, the hallmark of diabetes mellitus (1). Hyperglycemia develops in T1D due to insufficient insulin production by pancreatic β cells. Transient episodes of hyperglycemia create an abnormal metabolic environment in various cell types leading to cell and tissue-specific metabolic reprogramming with subsequent macro- and microvascular complication in diabetic patients (2). Besides that, hyperglycemia also causes cumulative changes in long-lived macromolecules, which

persist despite restoration of normoglycemia. This phenomenon was described as a metabolic memory, meaning that early high glucose environment is remembered by the cells (3).

Regarding the immune system, defects in the immune defense against miscellaneous pathogens were detected in patients with DM (4). Hyperglycemia is associated with decreased function of innate immunity such as lower complement levels, impaired chemotaxis, phagocytosis, and decrease in diapedesis of polymorphonuclear cells and monocytes/macrophages (5, 6). There is also considerable evidence that hyperglycemia contributes to the partial breakdown of peripheral tolerance (7), modulates patients' leukocyte profile (8), affects the function of antigen presenting cells (9), facilitates higher proinflammatory Th1/Th17 cells differentiation and suppresses regulatory T cells (Tregs) (10–12).

The adaptive immunity plays an essential role in the pathogenesis of T1D, particularly due to the imbalance between the autoaggressive effector T cells and Tregs (13, 14). In recent years, huge effort has been undertaken to suppress the inadvertent immune response in T1D. Several clinical trials and *in vitro* studies focused on cell-based therapy were launched with the goal to directly modulate the autoimmune destruction process of pancreatic β cells and to regenerate lost islets (15–18). Tolerogenic dendritic cells (tolDCs) and Tregs especially represent a new promising therapeutic strategy, either alone or in combinatorial therapies. Next, human stem cell (SCs) therapy represent another therapeutic approach for both inducing tolerance and islet cell regeneration (19). Current status of cell-based therapy is summarized in **Table 1**. However, little is known about the impact of the patient's glucose level on the potential cell-based vaccine's functional characteristics and efficacy. The initial immune cells isolated from hyperglycemic patient for the vaccine generation could exhibit different properties compared to those ones from euglycemic patients. Thus, the subsequent cell-based vaccine may exhibit different tolerogenic properties than in euglycemic subjects and the autoimmune destruction process in pancreas might be more difficult to suppress in patients with suboptimal glycemic control.

This review will focus on the regulating effect of hyperglycemia on immune cells, with a particular emphasis on tolerogenic DCs, T cells and Tregs. In addition, we will more specifically explore the subsequent changes in immune cell metabolism, initiation of alternative metabolic pathways such as the advanced glycation pathway, the process of advanced glycation end products (AGEs) formation and their molecular signaling in different immune cells.

EFFECT OF HIGH GLUCOSE LEVEL ON DIFFERENT SIGNALING PATHWAYS VIA REACTIVE OXYGEN SPECIES AND ADVANCED GLYCATION END PRODUCTS

Hyperglycemia is associated with increased oxidative stress caused by over-production of nicotinamide

adenin dinucleotide (NADH) and mitochondrial reactive oxygen species (ROS), that inhibit glucose metabolism via glycolysis and tricarboxylic acid (TCA) cycle and consequently activate alternative glucose metabolic pathways including polyol pathway and hexosamine biosynthetic pathway (HBP). All these alternative pathways lead to increased ROS production, thus completing the vicious circle of cellular oxidative stress. Hyperglycemia-induced activation of protein kinase C (PKC) isoforms also strongly contributes to cellular and tissue damage by induction of proinflammatory gene expression and further ROS increase (30–32).

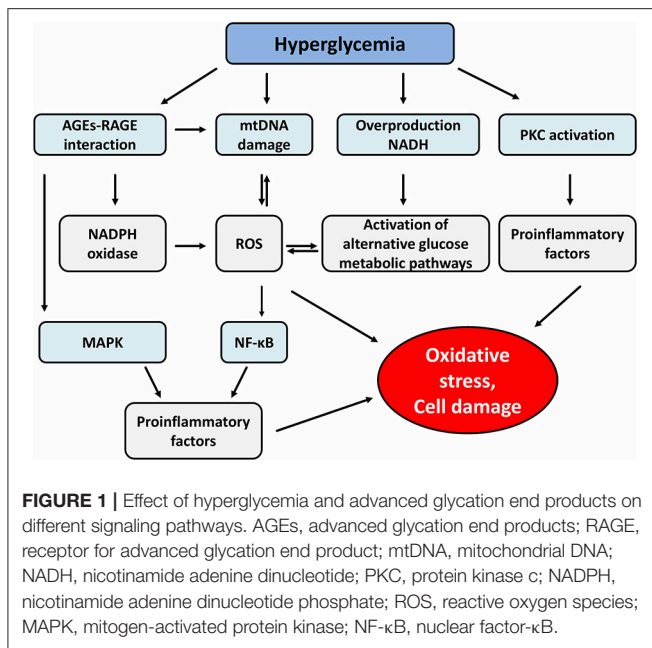
Last but not least, the formation of advanced glycation products (AGEs) is an equally important mechanism in the processes of tissue-damaging effects of hyperglycemia. AGEs are heterogeneous compounds arising from irreversible non-enzymatic glycation of proteins, nucleic acids and lipids (33). One of the major products is glycated hemoglobin (HbA1c) that has been used as a biomarker for diabetes because it reflects long-term glycemia (34). Formation of AGEs occurs in several steps. First, the condensation of carbonyl group with a free amino group of proteins forms Schiff bases, which transform to more stable covalently bound Amadori products. The final irreversible products of glycation—AGEs arise from slow Amadori product rearrangements (35). In diabetic patients, chronic hyperglycemia-driven accumulation of AGEs and its cognate receptor for advanced glycation end-product (RAGE) are involved in the pathogenesis of both micro and macrovascular complications (36). Indeed, the formation of AGEs interferes with cell integrity by irreversible glycation of various proteins. Furthermore, signaling via RAGE-mediated pathways increases ROS production via the activation of an NADPH oxidase system which contributes to further mitochondrial protein damage and DNA damage (37). RAGE consists of 5 domains (three extracellular, one transmembrane and one intracellular domain) (38). The intracellular domain is important for the activation of the transcriptional factor nuclear factor- κ B (NF- κ B), which leads to expression of growth factors, cytokines and RAGE itself in miscellaneous cells (39). In endothelial cells, downstream signaling via AGEs-RAGE receptor complex activates the signaling pathways of glycogen synthase kinase 3 β (GSK3 β), p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun amino-terminal kinase (JNK) and NF- κ B, all of which lead to endothelial cell dysfunction and diabetic vascular disease. Moreover, AGEs play an important role in β -cell failure by activating NADPH oxidase with a consequence of increased ROS generation and induction of β -cell apoptosis through the PKC β 2 pathway (40, 41) (**Figure 1**).

RAGE receptors are expressed on the surface membrane of various immune cells and can be found also in the cytosol. Several findings have been recently reported that AGEs-RAGE mediated signaling causes the induction of dendritic cell (DCs) maturation, Th1/Th17 polarization from naïve CD4⁺ T cells, activation and maturation of B cells and the reduction of the suppressor function of Tregs (9, 42–44).

TABLE 1 | Clinical studies (completed and with published results) for T1D treatment based on cells with regulatory properties including Tregs, tolerogenic DCs, and some examples of SCs.

	Tregs		DCs		SCs	
Trial ID	NCT01210664	ISRCTN06128462	NCT00445913	NCT01068951	NCT01374854	NCT00305344
Cell definition	CD4 ⁺ CD127 ^{lo/-} — CD25 ⁺ Polyclonal Tregs	CD4 ⁺ CD25 ^{high} CD127 ⁺ — Tregs	Immunoregulatory DCs	Autologous MSCs	Allogeneic UC-MSCs plus autologous BM-MNC	Autologous Umbilical Cord Blood Transfusion
Method of generation	Autologous Tregs isolated from the peripheral blood, expanded with anti-CD3/anti- CD28 beads in the presence of IL-2 and AB serum for 14 days	Autologous Tregs isolated from the peripheral blood, expanded with anti-CD3 and anti-CD28 antibodies, IL-2 and autologous serum for 7–14 days	Autologous DCs generated ex vivo from monocytes, modified using antisense oligonucleotides targeting primary transcripts of costimulatory molecules CD40, CD80 and CD86	MSCs aspirated from iliac crests and generated in growth media supplemented with lysed human platelets	Umbilical cord Wharton's jelly-derived MSCs generated in growth media supplemented with lysed human platelets; BM-MNCs aspirated from iliac crests	Umbilical cord blood as a source of immunomodulatory cells
Application route	Intravenously	Intravenously	Intradermal (peri-umbilical region)	Intravenously	Infusion through pancreatic artery	Intravenously
Cell number	1 0.05 × 10 ⁸ , 0.4 × 10 ⁸ , 3.2 × 10 ⁸ , or 26 × 10 ⁸	10 or 20 × 10 ⁶ /kg b.w., or 30 × 10 ⁶ /kg b.w.	10 × 10 ⁶	2.1–3.6 × 10 ⁶ autologous cells/kg	1 × 10 ⁶ /kg UC-MSCs plus 106.8 × 10 ⁶ /kg MNCs	–
Treatment application	1	1 (12 patients) or 2 (6/12 patients; 6–9 months apart)	4 (2 weeks apart)	1	1	1 or 2 (second after 3 months)
Results	No significant changes in C-peptide level (stable C-peptide level in 7/14 patients), HbA _{1c} level and insulin use after 2-year follow up; transiently ↑Tregs	↑C-peptide levels (8/12 and 4/6 patients after the first and the second dose, respectively), ↓insulin requirements (8/12, 2 patients insulin-independent) after 1-year follow up and ↓insulin requirements (4/12) after 2-year follow up; transiently ↑Tregs, ↓serum IL-1 and TNF-α	Partial ↑C-peptide (4/7); transiently ↑B220 ⁺ CD11c ⁺ regulatory B cells during 1 year follow up	Preserved or even increased C-peptide AUC (after meal tolerance test) during 1-year follow-up	↑C-peptide AUC (105.7%), ↑insulin AUC (49.3%) ↓fasting glycemia (24.4%), ↓HbA _{1c} (12.6%), ↓insulin requirements (29.2%) at 1-year follow-up	No metabolic improvement (C-peptide level, HbA _{1c} level, insulin requirements), ↑Tregs during 2-year follow-up
References	(20)	(17, 21, 22)	(16)	(23)	(24)	(25, 26)
						(27, 28)
						(29)

↑ Increase; ↓ Decrease; Treg, regulatory T cells; DCs, dendritic cells; IL, interleukin; BM, bone marrow; HbA_{1c}, glycated hemoglobin A1c; TNF, tumor necrosis factor; SCs, stem cells; MSCs, mesenchymal stem cells; UC-MSCs, umbilical cord mesenchymal stromal cells; BM-MNC, bone marrow mononuclear cell; AUC, area under curve; TGF, tumor growth factor; T_{EM}, effector memory T cells; T_{CM}, central memory T cells; T_{REG}, regulatory T cells; GAD, glutamic acid decarboxylase.



EFFECT OF HIGH GLUCOSE LEVEL ON TOLEROGENIC DENDRITIC CELLS

Dendritic cells, the most potent antigen-presenting cells (APC), are crucial for the induction of the pathological process in autoimmune DM. TolDCs represent immature or semi-mature DCs capable to promote immune tolerance by various mechanisms such as induction of autoreactive T cell hyporesponsiveness, anergy, or apoptosis and by induction of various types of T and B regulatory cells. These essential properties determine tolDCs as a new promising strategy for prevention of autoimmune DM in the subjects who are at risk or even for treatment of T1D. A human phase I trial of autologous monocyte-derived tolDCs in T1D has been completed (16, 45) and another trial based on proinsulin-loaded tolDCs has been recently opened (46).

Dendritic cell immune-metabolic state dictates the balance between immunity and tolerance. Immature DCs utilize oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) as their main energy sources, while DC maturation is followed by a pronounced switch to aerobic glycolysis (47, 48). TolDCs show a distinct metabolic profile. Generally, they exhibit catabolic and highly energetic profile favoring OXPHOS and FAO; however, metabolic plasticity can be observed regarding the protocol used for tolDCs generation (49, 50). Vitamin D3 (VitD3) and its derivatives, immunosuppressive drug dexamethasone (DEX) or Vit/DEX combination are widely used for tolDC generation (51, 52). Malinarich et al. reported high mitochondrial activity, increased OXPHOS, a shift in redox state and high glycolytic capacity as the metabolic signatures of VitD3/DEX generated tolDCs. In this study, inhibition of FAO caused increased expression of maturation markers on tolDCs and partially restored their T cell stimulatory capacity,

suggesting their dependence on FAO (53). Ferreira et al. showed that VitD3-induced tolDCs exhibited increased glucose metabolism, high mitochondrial activity and elevated OXPHOS profile with glucose as the main oxidative fuel compared to mature DCs. Interestingly, PI3K/Akt/mTOR driven glycolysis, but not glucose oxidation, was crucial for the maintenance of tolerogenic phenotype of VitD3-modulated tolDCs (54). Next, we documented in our study that increased glycolysis contributes to the stability of VitD2/DEX generated tolDCs in the inflammatory environment (55). Another study pointed out the profound metabolic changes toward increased OXPHOS and lipid metabolism while reducing amino acids and fatty acid synthesis in VitD3/DEX generated tolDCs compared to mature DCs (50). Such catabolic metabolic reprogramming with nutrient deprivation supports regulatory T cell induction (56).

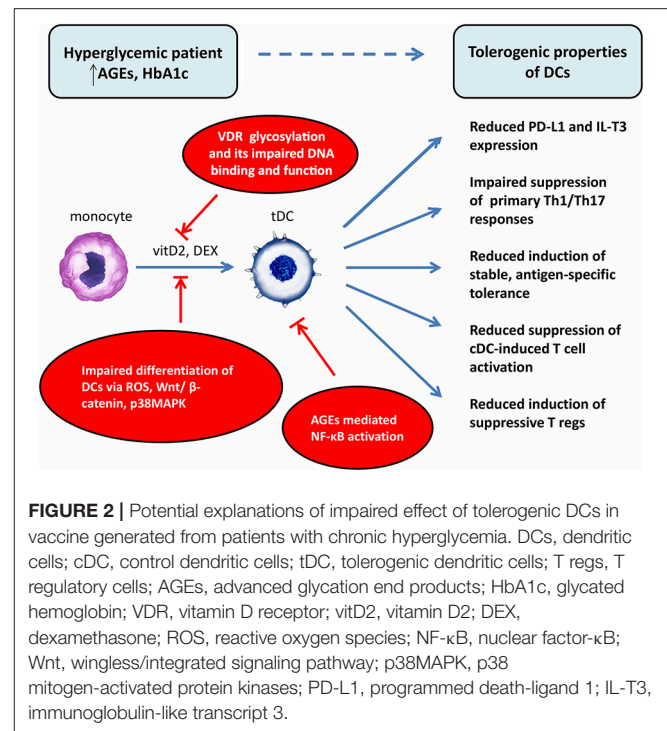
The studies assessing the potential effect of hyperglycemia, oxidative stress and AGEs in diabetic patients on tolDCs-based vaccine generation and its functional properties are limited. In our study we analyzed the impact of the metabolic state on functional properties of tolDCs generated from patients with T1DM, aiming to investigate the potential group of patients targeted for tolDCs immunotherapy. We showed that monocyte-derived tolDCs generated by DEX and VitD2 displayed better regulatory properties when prepared from the group of T1D patients with tight glucose control. TolDCs generated from T1D patients with a suboptimal level of glycated hemoglobin (HbA1c) exhibited down-regulation of immunoregulatory molecules PD-L1 and IL-T3 in comparison with tolDCs prepared from patients with optimal HbA1c level. Tolerogenic effect of tolDCs was better in normoglycemic patients in terms of more efficient suppression of Th1 and Th17 related cytokines production. Indeed, autologous primary culture of T cells and tolDCs from patients with optimal glucose control showed lower supernatant concentration of Th1-related cytokines IFN- γ and TNF- α and Th-17 related cytokine IL-17A, IL-23 and IL-9 and upregulated levels of IL-10 compared to cultures with control DCs. However, in patients with high level of HbA1c, we detected significantly lower levels of IFN- γ , TNF- α , and IL-9, but not IL-17A and IL-23 in comparison to control DCs. Overall, the ability of tolDCs to induce hyporesponsiveness of autologous T cells accompanied by a reduction of Th1 and Th17 cytokines was better in patients with tight glucose control. Importantly, improvement of glycemic control in T1D patients restores the ability of tolDCs to tolerize their autoreactive T cells (12).

One of the potential explanations for better effectiveness of DEX/VitD2-generated tolDCs from patients with tight glucose control is that hyperglycemia might attenuate the expression or function of vitamin D receptor (VDR) on monocytes used for generation of tolDCs. Indeed, hyperglycemia inhibited VDR expression in human vascular smooth cells, induced glycosylation of VDR in human monocytes and macrophages due to hexosamine pathway activation and interacted with VDR to impair its DNA binding and function. Thus, the high glucose level might exacerbate the function of VitD as a crucial tolerogenic agent indispensable for IL-T3 and PD-L1 expression on tolDCs (57–61). Next, high glucose, ROS, and AGEs have been previously shown to have an effect on *in vitro* DC

generation from blood monocytes. Indeed, high glucose impaired *in vitro* differentiation of monocytes from healthy donors into DCs by inducing ROS, activating Wnt/ β -catenin pathway and p38MAPK (62). Moreover, AGEs treatment led to persistent NF- κ B activation and abnormal NF- κ B function observed in T1D monocytes (63, 64). As Dex or Vitamin D receptor agonists have been described to generate tolDCs through NF- κ B down-regulation, it is possible that well-controlled patients have a better capacity to overcome sustained hyperglycemia driven NF- κ B activation in the process of tolDCs generation.

Once the immature or semimature tolDCs are applied to the patients' body, they will experience proinflammatory environment and high glucose milieu. Although the stability of various tolDCs in the proinflammatory environment is well documented, the data assessing the effect of high glucose are scarce (55, 65, 66). Regarding the effect of high glucose on immature DCs, short-term (24–48 h) high glucose treatment of monocyte-derived immature DCs generated from healthy donors accelerated the expression of co-stimulatory molecules, such as CD83 and CD86, and induced proinflammatory cytokine profile with up-regulation of IL-6 and IL-12 while the level of IL-10 was diminished (9, 67). Additionally, high glucose enhanced up-regulation of several DCs scavenger receptors, probably via increased production of intracellular ROS, and the activation of p38 MAPK pathway (67). Other studies demonstrated that AGE-modified serum molecules augmented the capacity of DCs to stimulate T cell proliferation and T cell cytokine secretion possibly through the up-regulation of RAGE on DCs. The subsequent activation of MAPK pathways and NF- κ B was crucial for this phenomenon (68, 69). Buttari et al. documented that polyphenolic antioxidant resveratrol prevented the immature DC maturation, IL-12, IL-1 β , TNF- α production and diminished the allostimulatory capacity of AGEs-treated DCs via abrogation of MAPK and NF- κ B activation (70). Overall, these findings highlight the role of ROS, MAPK, and NF- κ B as signaling molecules mediating the activating effect of high glucose in monocyte-derived DCs. Thus, the possibility exists, that tolDCs activated by high glucose conditions or AGEs might modify their tolerogenic profile into more matured and less potent phenotype due to the augmented DCs activation, presence of maturation markers and favorable cytokine profile. However, further studies are needed to fully elucidate the effect of high glucose levels, oxidative stress, and ROS on the stability of tolDCs.

So far, we can just speculate whether and how hyperglycemia can modulate bioenergetics and metabolism of tolDCs once they experience hyperglycemic conditions in T1D patients. As discussed above, hyperglycemia drives dysregulation of glycolysis as well as mitochondrial TCA cycle leading to mitochondrial respiratory chain complex dysfunction and the production of increased levels of ROS. Moreover, hyperglycemia driven AGEs formation and hexosamine biosynthetic pathway activation participate on the post-translational modification of protein subunits of mitochondrial electron transport chain (ETC) complexes leading to impaired mitochondrial function (31). Given the critical role of metabolic pathways in sustaining tolDCs function, exposure to hyperglycemia might affect the behavior of tolDCs and the outcome of the tolDCs therapy. On the other



hand, some data suggest that once the metabolic reprogramming takes place in particular tolDCs, they sustain their tolerogenic phenotype independently of the actual glycemia level (Figure 2).

EFFECT OF HIGH GLUCOSE LEVEL ON T-CELLS

Metabolically aberrant microenvironment caused by hyperglycemia in cooperation with chronic low-grade inflammation might affect T cell metabolism and immunological phenotype. Naïve T cells are in metabolically dormant state and primarily rely on OXPHOS and FAO to meet their bioenergetics and biosynthetic demands (71, 72). However, upon activation naïve T cells rapidly switch to high rates of glycolysis, glutaminolysis, and OXPHOS for cell growth and proliferation (73). Hyperglycemia drives increase in glycolysis in activated T cells thus leading to robust IFN- γ production by preventing glycolytic enzyme glyceraldehydephosphate dehydrogenase (GAPDH) from binding to and inhibiting the translation of IFN- γ mRNA (2, 74) and thus driving inflammation in a non-antigen specific manner. Moreover, hyperglycemia-driven oxidative stress exerts a global effect on T cells as shown by T cell hyperresponsiveness (75), induction of proinflammatory Th17 cells (10, 11) and diminished regulation of IL-7-mediated T cell survival and homeostatic expansion (76). Thus, it is plausible that “pre-activated” phenotype of T-cells detected in diabetic subjects (75) may affect the ability of immunoregulatory cell vaccine to induce the generation of antigen-specific tolerance in T cells.

In our study we reported that DEX/VitD2-generated tolDCs from patients with euglycemia, but not hyperglycemia, markedly reduced not only primary Th1/Th17 responses, but also mature DC-induced proinflammatory IFN- γ , IL-17, TNF- α , and IL-9 production. Suppressive effect of tolDCs on mature DC-induced cytokine production correlated negatively with HbA_{1c} level. Interestingly, T cells from hyperglycemic patients exhibited higher basal Th1/Th17 cytokine production and higher basal proliferation (antigen-independent) alongside with lower responses to antigen stimulation in primary cultures (12). The similar observation was reported on IL-10/TGF- β -induced tolDCs from T1DM patients (15, 77), where tolerance induction was dependent on the current activation state of T cells in each patient. In Segovia-Gamboa's study, antigen-specific T cell tolerance was lost in patients with high homeostatic T cell proliferation and low autoantigen-specific T cell response and the magnitude of T cell suppression inversely correlated with the level of HbA_{1c} (15). These data suggest that hyperglycemia reduces the ability of tolDCs to induce stable antigen-specific T cell tolerance by making autoreactive T cells more activated and thus less prone to be tolerated.

Nevertheless, the mechanism of increased T cell activation/proliferation in hyperglycemic diabetic patients is not fully understood. It has been assumed that hyperglycemia driven oxidative stress, AGEs formation, and AGEs-RAGE interaction is one of the possible cause as RAGE ligation affects T cell activation and controls T cell differentiation (44, 78). Mechanistically, Kumar et al. showed that hyperglycemia drives the expression of proinflammatory cytokines and chemokines, especially IL-6 and IL-17 family members transcriptionally through oxidative stress and NF- κ B activation via PKC and p38MAPK pathway in blood lymphocytes from diabetic subjects (11). Another study reported that AGEs dose-dependently promoted RAGE expression and induced differentiation of naïve CD4⁺ T cells into Th1/Th17 phenotype via down-regulation of transcription factor peroxisome proliferator-activated receptor gamma (PPAR- γ). RAGE knock-down abolished the AGEs-induced Th1/Th17 differentiation (10). Interestingly, RAGE is constitutively present intracellularly in both CD4⁺ and CD8⁺ T cells from diabetic subjects compared to healthy donors where RAGE was found to be expressed only following TCR activation. Moreover, RAGE⁺ T cells in diabetic patients display proinflammatory gene expression profile and express high levels of IL-17A. Elevated RAGE expression in diabetes is most likely due to the abundant overproduction of RAGE ligands including AGEs in diabetic patients (79) since it was detected on T cells in diabetic patients but not in T cells from patients with other autoimmune diseases such as Sjogren's syndrome and rheumatoid arthritis (80). Despite no correlation of RAGE expression with the level of glycemia in this study, the abundant overproduction of RAGE ligands in diabetic patients supports the hypothesis that glycated molecules forming AGEs could be contributing factor for exaggerated activation of T cells. On the other hand, higher expression of intracellular RAGE was shown also in high-risk euglycemic relatives (81). Thus, the direct impact of primary impaired T cell activation pathway in autoimmune diabetes cannot be ruled out.

Chronic hyperglycemia-induced "pre-activated" T cells phenotype was presented also in the mouse with streptozotocin-induced diabetes. Naïve T cells from hyperglycemic mice showed functional marks of antigen-experienced T cells with increased Th1, Th2, and Th17 cytokine production and higher proliferation upon TCR stimulation compared to euglycemic control, despite having the low CD44 expression. Simultaneously, they exhibited considerable chromatin decondensation which resulted in the facilitation of the transport of transcriptional factors to the DNA. These findings could be a consequence of inner modulation of TCR downstream regulators in high glucose milieu and are likely to be RAGE-dependent as RAGE deficiency reversed the hyperactivated phenotype of T cells in hyperglycemic mice. Of particular interest, all hyperglycemia-induced changes persisted after adoptive transfer into euglycemic hosts (75). This points to the phenomenon of metabolic memory and partly explains why the activating phenotype of T cells may persist even after glucose normalization.

EFFECT OF HIGH GLUCOSE LEVEL ON T REGULATORY CELLS

Both naturally occurring Tregs arising from thymus (nTregs) as well as induced T regs (iTregs) developing in the periphery are the key negative regulators of the immune response and play a crucial role in the maintenance of the peripheral tolerance. Administration of *ex vivo* expanded Tregs has been considered as another interesting strategy for the treatment of T1D. Early clinical studies utilizing polyclonally expanded autologous CD4⁺CD25⁺FoxP3⁺ Tregs demonstrated the safety and feasibility of this approach and supported their therapeutic potential for inhibition or delay of the destruction of pancreatic β cells in T1D patients (17, 20).

Recent studies utilizing more precise Tregs markers such as low CD127 expression or the selective demethylation of certain regions of the Foxp3 locus (82) show that T1D patients appear to have normal T regs frequencies compared to healthy donors, but they are often poorly functioning (83–85). The lower suppressive capacity of Tregs from T1D patients, their phenotypic shift accompanied by decreased production of anti-inflammatory cytokines IL-10, IL-35 and TGF- β and increased production of inflammatory cytokines IFN- γ and IL-17 (84, 86–89), unstable FoxP3 expression (90), and higher susceptibility to apoptosis were documented (91–95).

Whether the functional defect stems from the inner modulation of Tregs population in a subject with an autoimmune disease or whether it is partly caused or deepened by hyperglycemia remains to be clarified (96). Glycolysis was shown to orchestrate the generation and suppressive function of human iTregs by controlling of FoxP3 splicing variants through the glycolytic enzyme enolase-1 (97) that can bind DNA and regulate gene expression. Glycolysis was also shown to affect expression of Tregs' cell inhibitory and regulatory molecules CTLA-4, PD-1, GITR, CD37, and CD71 (97). Thus, impaired glycolysis observed in T1D might participate on defective induction/function of Tregs (98). Next, hyperglycemia

contributes to increased expression of IL-6 and TGF- β (11, 99) that synergistically down regulate FoxP3 at the post-translational level by promoting FoxP3 protein degradation in Tregs (100).

The negative effect of hyperglycemia on Tregs phenotype and function was observed also in streptozotocin (STZ) induced hyperglycemic mice, where long-term hyperglycemia changed the phenotype of CD4+CD25+FoxP3+ Tregs into activated/memory phenotype with lower CD62L, CD45RB, GITR, and higher CTLA-4 expression and reduced their suppressive effect on effector T cells proliferation. These properties were unambiguously reversed by insulin administration (101). In human studies from healthy donors, glucose-induced AGEs also reduced the suppressive function of Tregs and decreased the ratio of Tregs/Th17 cells (10). In T1D patients, the frequency of Tregs correlated negatively with the level of HbA_{1c} (102–104).

Next, Tregs defective function and number might result from defective signaling from dendritic cells which under hyperglycemia might exhibit weaker tolerogenic potential. Indeed, in our study, co-cultures of naïve T cells and antigen-loaded tolDCs from patients with high HbA_{1c} levels led to the induction of lower levels of proliferating CD4+CD25^{high}CD127^{low}FoxP3+ Tregs producing low IL-10 levels compared to euglycemic patients. Of a great interest, Tregs expanded by tolDCs from patients with high HbA_{1c} levels exhibited weaker suppressive abilities (12). In this case, it seems that reduced surface expression of ILT-3 and PD-L1 on dexamethasone/VitD2-generated tolDCs from poorly compensated T1D patients might play a role in defective Tregs induction and function (12).

From the present clinical studies is not clear whether the glycemia level on diabetic subjects influences the quality of resulting vaccine or not. Okubo et al. demonstrated in their study that T1D patients with tight glucose control (based on the HbA_{1c} level) showed higher percentage of activated Tregs (CD4+CD25+FoxP3+CD45RA+) in peripheral blood and lower percentage of resting Tregs (CD4+CD25+FoxP3+CD45RO+). As activated Tregs are the most suppressive Tregs, their higher percentage in primary culture could increase suppressive effect of the resulting vaccine in patient with optimal glucose control (105).

EFFECT OF HIGH GLUCOSE LEVEL ON STEM CELL-BASED THERAPY

Stem cells (SCs) are multipotent, self-renewing cells, with anti-inflammatory and immunomodulatory properties. Implantation of SCs to T1D patients might regulate ongoing autoimmune process by inhibition of T and B cell activation, DCs differentiation and NK cell activity, while induction of T cell anergy and T regs generation (106, 107). Moreover, SCs can differentiate into insulin-producing cells and revitalize the damaged pancreatic β -cells. Sources for SCs therapies in diabetes mellitus can be multiple, including embryonic stem cells (ESCs), cord blood stem cells, induced pluripotent stem cells (iPSCs), and adult stem cells derived from adult tissues. Among all

kinds of SCs, mesenchymal stem cells (MSCs) derived from the bone marrow or other sources, have been shown as an interesting viable approach for treatment of T1D and tested in human clinical studies aiming to prevent or arrest the onset and progression of T1D, inhibit β -cell destruction, and restore glycometabolic and immune homeostasis. Despite the favorable results indicating potential efficacy of MSCs to preserve β -cell function in some T1D patients demonstrated by the higher C-peptide level, decreased insulin doses and improved HbA_{1c} levels, there are still doubts on the long-term effectiveness of MSCs for the management of T1D (108, 109).

Regarding the hampering effect of hyperglycemia on MSCs therapies, several studies reported that hyperglycemic state and underlying defective microenvironment in diabetic patients impair SCs function. Indeed, high glucose can induce senescence of MSCs via Akt/mTOR signaling (110). Next, hyperglycemia and subsequent oxidative stress triggered by high glucose or chronic RAGE signaling might have a large negative effect on the differentiation, proliferation and regeneration capability of MSCs by affecting Wnt/ β -catenin pathway, PI3K/Akt pathway, MAPK signaling, PKC pathway or micro RNA expression (111–116). Study by Kornicka et al. documented that MSCs isolated from patients with T2D exhibited increased apoptosis, autophagy, ROS accumulation and mitochondria deterioration (117). In another study, hyperglycemic conditions in embryos hindered differentiation of human embryonic stem cells by changing their histone methylation pattern leading to pancreatic malfunction (118). Thus, it seems that hyperglycemic conditions in T1D patients might limit therapeutic potential of MSCs-based therapies.

CONCLUSIONS

In summary, new emerging evidence suggests that chronic hyperglycemia in patients with diabetes markedly influences their immune system. The rising incidence of T1D calls for new therapeutic options and the immune suppression strategy based on the tolerogenic DCs or T regs vaccines seems to be very promising. The purpose of our review was to summarize the effect of hyperglycemia on various cells of adaptive immunity aiming to clarify the link between the patient's metabolic status and different efficacy of immune interventional treatment. One of the important mechanisms mediating the effect of chronic high glucose level is AGEs formation and their signaling through a specific receptor—RAGE and other groups of scavenger receptors. In essence, AGEs-RAGE interaction leads to activation of transcription factor NF- κ B and ROS generation via diverse downstream signaling pathways including MAPK kinase signaling. Therefore, it is believed that this mechanism could contribute to hyperglycemia-induced proinflammatory environment in the body.

For the purpose of cell-based vaccine, tolDCs are generated in autologous system from monocytes of the peripheral blood of patients. Thus, the different status of patient's metabolic control may provide a different input for the cell-based vaccine resulting in a different final quality. Taking into account the

metabolic memory phenomenon together with the fact, that even transient episodes of hyperglycemia were associated with epigenetic changes in several cells including progenitor cells (119), the very early tight glucose control seems to be essential for adequate effect of resulting tolerogenic vaccination therapy. In fact, resistance to a further maturation stimulus such as proinflammatory environment and high glucose milieu is a prerequisite for tolerogenic DC clinical application, since a potential transformation of immature or semi-mature DCs into fully mature DCs would lead to the acquisition of the capacity to promote immunogenic instead of protective immune responses and thereby to exacerbation of patient's autoimmune condition. Similarly, stem-cell based vaccines might lose their immunomodulatory potential by facing hyperglycemia conditions at the time of cell administration. Controversially, our previous study showed that alteration of some immune cells (specifically tolDCs) caused by hyperglycemia may be reversed after improvement of glycemic control (12). Thus, the equal importance should be considered for optimal vaccine timing, preferably in the period of ideal metabolic control.

Not only patient's metabolic status, but also appropriate manufacturing protocols for generation of cell-based vaccines, especially with regard to glucose concentration, has to be managed as the applied culture media components may influence the tolerogenic properties of generated immune cells. Indeed, concentration of glucose in culture media above 10 mM are analogous to hyperglycemic conditions in diabetic patients and the cells growing under high-glucose conditions are modified by processes of glycation, glyoxidation, and subsequent oxidative stress. Several important media in bio-manufacturing contain diabetic levels of glucose supplementation (for example DMEM (Hi), GMEM, and IMDM media all contain 25 mM levels of D-glucose). For tolDCs manufacturing and/or T regs expansion Cell Gro GMP medium or X-VIVO 15 medium are widely used, however the precise glucose concentration in those media is unknown and should have been tested in house to avoid hyperglycemic culture conditions (21, 120, 121).

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The final question remains, how to circumvent the hyperglycemia issues in cell-based therapy for T1D. Based on the data mentioned above, special attention should be paid on managing patient's metabolic status. Precise adjustment of insulin regimen or conversion to insulin pump for tight glucose controlling seems to be the first step in consideration of cell-based therapy. Managing manufacturing process for generation or expansion of immune cells (appropriate culture conditions) and adjusting precise timing of cell-based vaccine application (euglycemia in patients) are equally important. Furthermore, it remains to be tested whether application of oxidative stress-reducing compounds such as resveratrol during *in vitro* immune cells generation /or expansion might in consequence improve the possible dysfunctionality of patient's immune cells and the efficacy of cell-based therapies (122). Regarding MSCs, considering allogeneic healthy donors as a source of MSCs for cell based therapies in diabetic patients might be another option.

To sum up, the precise effect of hyperglycemia on immune cells is not fully clear. More detailed studies should be performed to distinguish the genuine effect of high glucose from the changes in immune cells caused by autoimmune disease itself in T1D patients. Nevertheless, based on the recent knowledge, hyperglycemia in diabetic patients may have an impact on immune-interventional treatment and maintaining of a tight glucose control seems to be beneficial in patients considered for cell-based therapy.

AUTHOR CONTRIBUTIONS

AG and LP-J wrote the manuscript and KD and RŠ edited while adding additional insights. The final version was proofread and edited by RS.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tolerogenic Dendritic Cells and T-Regulatory Cells at the Clinical Trials Crossroad for the Treatment of Autoimmune Disease; Emphasis on Type 1 Diabetes Therapy

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Tolerogenic dendritic cells and T-regulatory cells are two immune cell populations with the potential to prevent the onset of clinical stage type 1 diabetes, and manage the beginning of underlying autoimmunity, at the time-at-onset and onwards. Initial phase I trials demonstrated that the administration of a number of these cell populations, generated *ex vivo* from peripheral blood leukocytes, was safe. Outcomes of some of these trials also suggested some level of autoimmunity regulation, by the increase in the numbers of regulatory cells at different points in a network of immune regulation *in vivo*. As these cell populations come to the cusp of pivotal phase II efficacy trials, a number of questions still need to be addressed. At least one mechanism of action needs to be verified as operational, and through this mechanism biomarkers predictive of the underlying autoimmunity need to be identified. Efficacy in the regulation of the underlying autoimmunity also need to be monitored. At the same time, the absence of a common phenotype core among the different dendritic cell and T-regulatory cell populations, that have completed phase I and early phase II trials, necessitates a better understanding of what makes these cells tolerogenic, especially if a uniform phenotypic core cannot be identified. Finally, the inter-relationship of tolerogenic dendritic cells and T-regulatory cells for survival, induction, and maintenance of a tolerogenic state that manages the underlying diabetes autoimmunity, raises the possibility to co-administer, or even to serially-administer tolerogenic dendritic cells together with T-regulatory cells as a cellular co-therapy, enabling the best possible outcome. This is currently a knowledge gap that this review aims to address.

Keywords: tolerogenic dendritic cells, T-regulatory cells, type 1 diabetes, clinical trials, immunotherapy, autoimmunity

INTRODUCTION

Type 1 diabetes (T1D) is a progressive autoimmune disease resulting in the impairment and loss of pancreatic insulin-producing beta cells via innate and adaptive leukocyte activity (1). The resulting dysregulation of, and eventual loss of controlled blood glucose variability, facilitates the onset of disease-associated complications like cardiovascular, neurologic, ophthalmic, and renal complications. T1D is a managed disease in need of a cure and despite the investment made in novel insulin formulations and glycemia level-activated pumps, pharmacologic insulin replacement fails to achieve a return to stable and long-term physiologic glycemic variability, to avoid the onset of the complications (2–7). Stem cell-based insulin-producing surrogate cells for transplantation are still far from being a realistic clinical option, also presenting their own challenges (8, 9). Similarly, islet allo- or xeno-transplantation, in spite of its clinical success, is applicable only for a select and very-restricted patient category (10, 11) with its own limitations conferred by an allogeneic or xenogeneic immune response on top of a latent autoimmunity that is readily re-activated.

THE POINTS OF ACTION OF TOLEROGENIC DENDRITIC CELLS

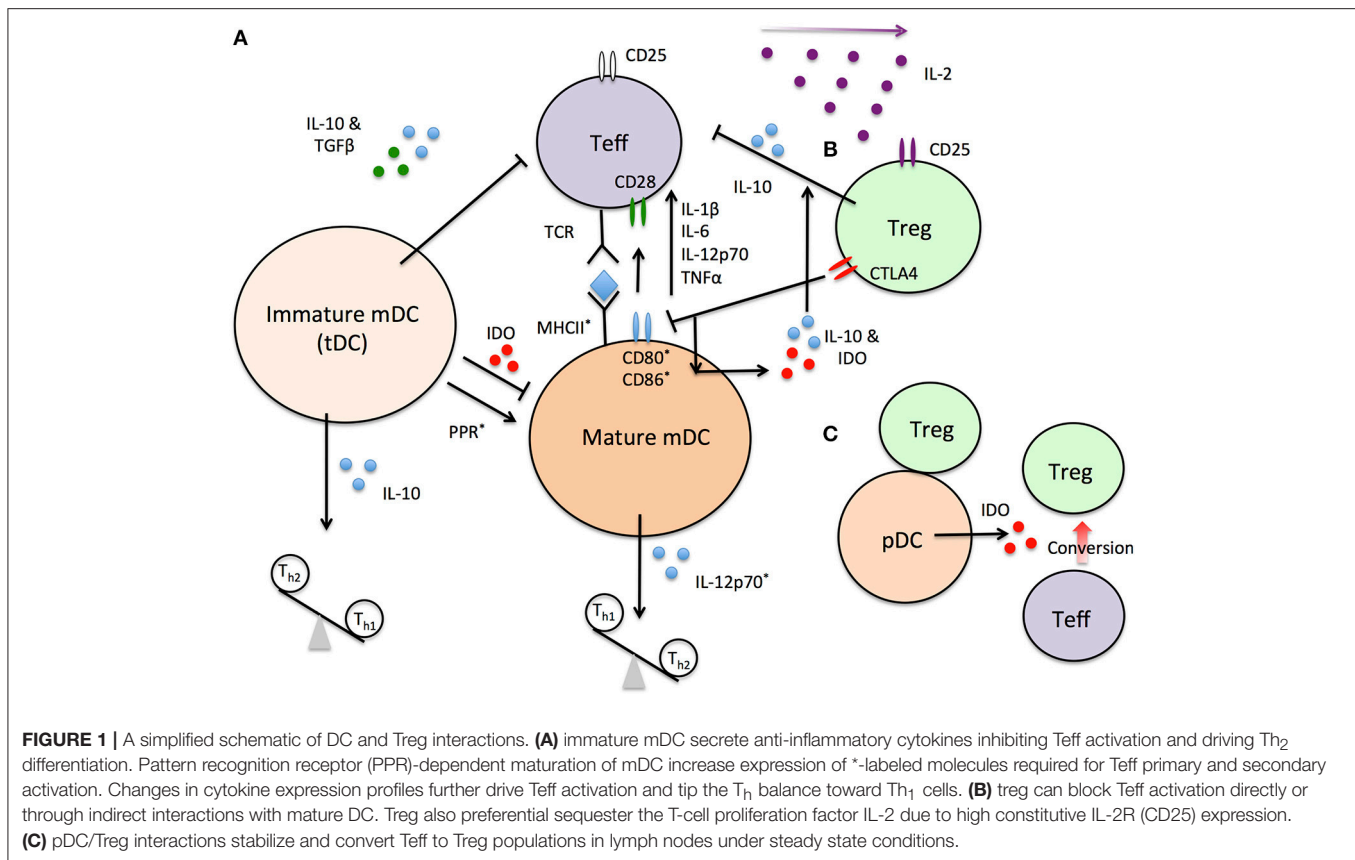
Dendritic cells (DC), alone or via T-regulatory cells (Tregs) and B-regulatory cells (Bregs), can determine the state of activation and can even direct the differentiation of pro-inflammatory and autoreactive CD8⁺ cytotoxic T-cells (CTL) as well as the balance of T-helper cell (TH)1, TH2, and TH17 populations (12–19) (**Figure 1**). Even though the different tolerogenic dendritic cell (tDC) populations used in clinical trials for autoimmunity thus far, including T1D, are mainly of the myeloid lineage (12–15), it has far from conclusively-demonstrated that they represent a completely-pure myeloid-derived lineage, following *ex vivo* generation, in the widely-used granulocyte-macrophage colony-stimulating factor (GM-CSF) + interleukin-4 (IL-4) cell culture medium (20). There is an important gap-in-knowledge concerning the actual balance of plasmacytoid DC (pDC), type 1 myeloid DC (mDC), and type 2 mDC (21–28) as well as what can be naturally-tolerogenic DC populations (20) inside the *ex vivo*-generated cell products immediately following generation under GMP conditions and even more

so immediately prior to the time of administration. It is also unclear if such a potential balance changes immediately following administration at the site of injection (usually intradermal/subcutaneous injection), or after DC migration into the lymphoid organs draining the site of injection. There are intriguing data indicating that *ex vivo*-generated tolerogenic DC (tDC) seeding and remaining inside the administration site are associated with the development of a neo-lymphoid stroma inside which Tregs, expressing the Foxp3 transcription factor (Foxp3⁺ Tregs), emerge (29). The significance of this event for the overall tolerogenic outcome, post-tDC treatment in autoimmunity, and in T1D in particular, remains to be determined. Studies in mice have largely focused on the expression at the cell surface of the common co-stimulation proteins on exogenously-administered tDC recovered from the lymph nodes draining the administration site, as well as the immunokines they produce, yet none of these phenotypes/activities have yet been associated with actual tolerogenic activities, resulting in direct or indirect suppression of autoreactive T-cells transiting through these organs. This, we believe is an important and unaddressed area of research which should be pursued to more-completely understand how tDC can affect the activation of effector T-cells inside the lymph nodes. It is possible that a common outcome on such effector T-cell activity could be identified and subsequently be associated with a measurable biomarker in peripheral blood or other easily accessible biofluid. Although a reasonable amount of data suggests tDC are able to maintain their tolerogenic state in the face of pro-inflammatory signals (30–33), there are some data that suggests that this is not always the case (34, 35). Whether low co-stimulation potential *in vivo* is *conditio sine qua non* for tDC, to confer some form of regulation and activity arrest in effector autoreactive T-cells inside the lymphoid organs, therefore remains an open question in terms of if it is critical in the mechanism of action of tolerogenic DC.

ARE THERE POINTS OF INTERSECTION IN PHENOTYPE AND TOLEROGENIC ACTIVITY AMONG THE DIFFERENT CLINICAL TDC PRODUCTS TESTED IN TRIALS TO-DATE?

In order to address this question, the different methods currently-used to generate tDC *ex vivo*, must be considered. Immature DC are generated from isolated monocytes with the addition of IL-4 and GM-CSF to the culture media, a method that is shared between clinical therapeutic techniques. To enforce or impart additional tolerogenic properties to the DC, various other agents have been used that impair DC maturation or specific pro-inflammatory functions (vitamin D3, immunosuppressive Dexamethasone and NF- κ B inhibitors, antisense oligonucleotides targeting co-stimulatory molecules) (20) (**Table 1**). tDC have been utilized to reduce tissue transplant rejection (36–39) and treat autoimmune disease (20, 40), the latter of which has utilized disease specific auto-antigens to enhance immune tolerance functions of tDC. To what extent these conditions

Abbreviations: APC, Antigen-Presenting Cells; ATG, Anti-Thymocyte Globulin; Breg, B-regulatory cells; CD, Cluster of Differentiation; CTLA-4, Cytotoxic T-lymphocyte-Associated Protein 4; DC, Dendritic Cells; DC-10, High IL-10 secreting Dendritic Cells; Foxp3, Forkhead box protein P3; G-CSF, Granulocyte Colony Stimulating Factor; GM-CSF, Granulocyte Macrophage Colony Stimulating Factor; HbA1c, Hemoglobin A1c; HLA-DR, Human Leukocyte Antigen-antigen D Related; IDO, Indoleamine 2,3-Dioxygenase; IFN γ , Interferon Gamma; IL, Interleukin; MHC, Major Histocompatibility Complex; MITAP, Minimum Information about Tolerogenic Antigen-Presenting cells; MPLA, Monophosphoryl Lipid A; NF- κ B, Nuclear Factor kappa-light-chain-enhancer of activated B-cells; PBMC, Peripheral Blood Mononuclear Cells; PRR, Pattern Recognition Receptors; T1D, Type 1 Diabetes; TCR, T-Cell Receptor; tDC, Tolerogenic Dendritic Cells; Teff, T-Effector Cell; TGF β , Transforming Growth Factor beta; TH, T-helper cells (1,2, or 17); TNF α , Tumor Necrosis Factor alpha; Tr1, IL-10 producing T-Regulatory cells; Treg, Foxp3⁺ T-Regulatory cells.



change cellular effectiveness and mechanisms of action of tDC to confer their potentially beneficial effects, is unclear at present.

A major coordinator of pro-inflammatory gene expression and DC maturation is the transcription factor NF- κ B. DC grown in the presence of NF- κ B-inhibiting compounds, displayed a reduced expression of CD40 and HLA-DR (human leukocyte antigen-antigen D related, a class II HLA molecule) (41–43). Generation of tDC under GM-CSF+IL-4 conditions, result in suppressed NF- κ B transcriptional activity (32, 43–47), which may be one avenue whose outcomes could identify a common tDC phenotype and the state of activity that informs mechanisms of the action of effector autoreactive T-cells. In the Rheumavax clinical trials (13) for rheumatoid arthritis, tDC generated in GM-CSF+IL-4 and the NF- κ B inhibitor Bay 11-7082, exhibited lower CD40, and HLA-DR on a per cell basis (13). In the AutoDECRA trials (12, 32), dexamethasone (Dex) and vitamin D₃-gerated tDC, were characterized with a low surface expression of the co-stimulation proteins CD40 and CD86 and the DC maturation marker CD83, with low levels of cell surface HLA-DR and very low concentrations of secreted IL-12p70 (32, 43, 45, 46). Instead, these tDC produced high concentrations of the immunosuppressive IL-10 immunokine (48). Interestingly, Vitamin D₃ in addition to DC *in vitro*, can also achieve similar outcomes (47, 49, 50). Another approach to generate tDC relied on the addition of

Dex, vitamin A, IL-1 β , IL-6, tumor necrosis factor alpha (TNF α), and prostaglandin E₂ in the culture medium (15, 51). The cell products exhibited elevated CD80 and CD86, and low CD83 expression. The MERTK gene product, a glucocorticoid-induced receptor that is prevalent in tDC, was also expressed at high levels. Production of IL-10 was detected in the cells with no detectable IL-12p70 or IL-23 in the cell culture media. Allogenic mixed lymphocyte reactions, performed in the presence of tDC, resulted in low T-cell proliferation and interferon gamma (IFN γ) production.

In our approach to treat T1D, we have generated tDC using a targeted approach; to directly impair the expression of three key co-stimulation proteins at the cell surface by *ex vivo* exposure of GM-CSF+IL-4-generated DC, to a mixture of antisense phosphorothioate DNA oligonucleotides, targeting the 5' end of the primary transcripts of CD40, CD80, and CD86 (14). Removal of these co-stimulatory molecules resulted in incomplete T-cell activation during DC antigen presentation inducing anergy (52–54). In addition to a significant reduction in cell surface levels of the proteins *ex vivo*, these tDC also exhibited some other interesting features, previously reported (55), that involve potential aptameric effects through secondary and tertiary nucleic acid structures on toll-like receptor 9 (TLR-9) signaling on the activity of phosphatidylinositol-3-kinase and glycogen synthase kinase-3 β , that are still under mechanistic investigation.

TABLE 1 | A comparison of current protocols for *ex vivo* generated tDC and Treg and their clinical application.

Cell source	PBMC	PBMC	PBMC	PBMC	PBMC	Umbilical cord blood	PBMC
Target cell	DC	DC	DC	DC	Treg	Treg	Treg/Tr1
Cell generation	GM-CSF+ IL-4 & Anti-sense CD40, CD80, CD86	GM-CSF+ IL-4 & BAY 11-7082 Auto-antigens	GM-CSF+ IL-4 & Dex Vitamin D3 MPLA	GM-CSF+ IL-4 & Dex Vitamin A Cytokines	IL-2 Anti-CD3 & CD28 Beads	IL-2 Anti-CD3 & CD28 Beads	IL-2 IL-4 Anti-CD3 antibody Ovalbumin
Added auto-antigens	No	Yes	No	Yes	No	No	No
<i>Ex vivo</i> Cell characterization	Low CD40 CD80 CD86 IL-12	Low CD40 CD80	Low CD83 IL-12	Low CD83 IL-12	Low CD127	Low CD127 IL-2 IFN γ	Low CD62L CD127 IL-4 IFN γ High Foxp3 CD25+ IL-10 IL-13
<i>In vivo</i> application	Increased Foxp3 Tregs IL-10 Bregs IL-4 IL-10 No Change DC	Increased Foxp3 Tregs Decreased IL-15 IL-29	No Change Foxp3 Treg	Increased Foxp3 Tregs	Increased Foxp3 Tregs Not Examined DC	X	X

A brief listing of reported cellular characteristics of generated cells are shown, but have not been uniformly examined across all studies. Post-administration changes in cell populations and plasma cytokines *in vivo*, in patients, are listed. Increased Treg numbers are reported in a majority of trials that utilize either *ex vivo* generated autologous tDC or Treg. Techniques marked as "X" are in clinical trials but have only been published under conditions with pre-clinical settings.

TABLE 2 | Cell marker and cytokine profiles for tolerogenic cell populations and mature dendritic cells.

Cell type	Makers	Cytokines	References
Immature mDC GM-CSF & IL-4	CD1c+ CD11c+ CD14- HLA-DR ^{Low} CD40 ^{Low} CD80 ^{Low} CD83 ^{Low} CD86 ^{Low}	IL-10, TGF β , IL12p70-	(81, 83–85)
Mature mDC	CD1c+ CD11c+ CD14- HLA-DR ^{High} CD40+ CD80 ^{High} CD83 ^{High} CD86 ^{High}	IL-12p70 ^{High}	(86–88)
DC-10	CD1c- CD14+ CD16+ CD11c+ HLA-DR+ CD83+ CD68- CCR7+	IL-10 ^{High} , IL-12p70-	(89, 90)
Treg	CD3+ CD4+ CD25+ CD127- Foxp3+ CTLA4+	IL-10 ^{Low}	(89, 90)
Tr1	CD4+ CD49b+ LAG-3+ CD226+	IL-10 ^{High} , TGF β	(70, 90)

In Treg cells, the lack of CD127 is used as a surrogate extracellular marker for the intracellular Foxp3 with 98% accuracy (68).

EXOGENOUSLY-SUPPLIED AUTOANTIGENS OR AUTOANTIGEN-DERIVED PEPTIDES: ARE THEY NECESSARY?

Autoimmune diseases each have their own unique auto-antigens and associated self-reactive T-cell populations. Preloading tDC with specific disease antigens, in some, but not all instances, enhance their ability to directly interact and inactivate self-reactive T-cells that cause tissue damage (56–58). Methodically this technique should reduce the chance of inducing tolerance to non-specific antigens and may provide a stronger suppressive

effect of tDC for disease treatment. In instances where autoantigens are well-defined, peptides could be used in their native or post-translationally-modified, autoantigenic form. For example, tDC for multiple sclerosis treatment considerations, have been generated in the presence of GM-CSF+IL-4, Dex (or Vitamin D3) and pre-loaded with myelin self-peptides (20). In one of the Rheumavax studies, GM-CSF+IL-4 culture medium was supplemented with the NF- κ B inhibitor Bay 11-7082 and then the cells were exposed to citrullinated peptides of aggrecan, vimentin, collagen type II and α and β fibrinogen, which are putative RA autoantigens (59). The rationale for this method and approach to generate rheumatoid arthritis-specific tDC, was based on the findings that anti-citrullinated

protein antibodies are found in 50–80% of patients over the lifetime of the disease (60). Not all patients, however, display uniform self-antigens for a given disease. In T1D, for example, a range of self-antigens and auto-antibodies are differentially-produced among patients and at different points during disease progression (61–64). Although most experts in the field of T1D autoimmunity pathogenesis agree that insulin and GAD65 are the major T1D auto-antigens, and therefore, by adding peptides from these proteins at the time of tDC generation could provide some level of antigen-specificity in terms of what populations of autoreactive T-cells are suppressed, the same experts note that by the time of disease onset, a significant degree of antigen spreading has occurred where other “late-antigen”-specific T-cells may in fact be driving autoimmunity. Targeting only common antigens may lead to reduced or abrogated effectiveness of tDC treatments, as has been demonstrated in at least one T1D animal model (57). Screening patient’s autoantigen and self-reactive T-cell profiles are possible, although expensive, and still does not guarantee that each individual’s responsible antigen is known, as the list of diabetes antigens continuously grows. The possibility of using individualized autoantigen profiles was addressed in the Newcastle study which used tDC for the treatment of rheumatoid arthritis. Synovial fluid contents from inflamed joints of each patient were added to the generated tDC, followed by *in situ* administration of the tDC into the inflamed space where, presumably, the cells would acquire patient-specific auto-antigens (12, 32). At this time though, the use of autoantigen loading in diabetic tDC treatments seems premature, with the ongoing discovery of new autoantigens and the lack of a concentrated biofluid that could serve as a natural reservoir of patient specific autoantigens.

NON-CELLULAR FACTORS AS TDC-RELATED DISEASE MODIFIERS

Two major differences among the clinical trials using tDC, lie in the manner in which they are administered. This could affect what kinds of mechanisms are activated to suppress autoreactive T-cells and to slow down, if not altogether halt disease progression. The first difference lies in the dose level administered. The second difference lies in the selection of the site of administration. This difference is important, we believe, in the kind of mechanism tDC activates, especially as the sites of inflammation and the cell populations constituting the inflammatory cells are different among autoimmune diseases. The majority of tDC clinical trials to date, consider local cell administration at the site which is subserved by lymph nodes that co-incidentally drain the site of inflammation, with the objective of facilitating tDC migration into the draining lymph node. Lymph nodes that drain the site of inflammation of an organ- or tissue-restricted autoimmune disease are characterized by a notable frequency of activated self-reactive T-cells, that are potential targets for anergy induction (65). Examples include the administration of tDC to an area subserved by the cervical lymph nodes in a recent multiple sclerosis trial (clinicaltrials.gov

identifier: NCT02618902) and abdominal administration of tDC proximal to the pancreas in our T1D trial (14). An alternative approach is to directly introduce tDC into the site of inflammation proper, bypassing any consideration of lymphoid organ drainage. An example that has been suggested is the direct administration of tDC to actual inflamed sites in Crohn’s disease (15). While the Newcastle University rheumatoid arthritis study introduced tDC directly at the site of inflammation, the intended goal was still for the migration of tDC to local draining lymph nodes. Even though the technique is more invasive than intradermal administration to facilitate tDC trafficking to the lymph nodes co-draining the inflamed tissue and the site of administration, the introduction of tDC producing IL-10 may have the added benefit of local immunosuppression inside the site of inflammation. This consideration is balanced by the possibility of an unwanted adverse effect where local inflammatory conditions may alter the phenotype of the *ex vivo* administered tDC, toward a more pro-inflammatory state.

WHAT, THEN, ARE THE COMMON PHENOTYPES AND ACTIVITIES?

In **Table 2**, we provide a list of markers that reliably distinguish the cells listed in the first column and that could be helpful to distinguish clinically-useful tDC from non-regulatory DC population during and after the cell generation process *ex vivo*. Of the tDC populations generated under different conditions, only four have entered clinical trials in autoimmune disease with outcomes publicly-reported (12–15). NF- κ B inhibition is the central feature of at least three of these tDC populations. Other features shared in common by these different tDC populations include decreased CD83 expression, decreased IL-12 secretion, and elevated IL-10 secretion. Even though a common phenotype, other than suppressed NF- κ B activity and perhaps low concentrations of pro-inflammatory immunokines, cannot be used as a distinguishing cell-inherent feature of tDC, all tDC share one mechanistic feature: increased regulatory lymphocytes (e.g., Foxp3+ Tregs and Bregs) *in vivo*, in animal models of autoimmune disease as well as in the peripheral blood of patients following administration (13–15). In addition to increased numbers of Foxp3+ Tregs in the circulation and inside the lymph nodes draining the injection site, there are reports of increased Bregs as well (17, 66). We noted that increases in patient C-peptide levels are correlated with B220+ CD19+ CD5+ CD1d+ IL-10+ B Bregs in the patients treated with our tDC (14).

Much of the current divergence among different tDC populations, in terms of phenotype and points of mechanistic intersection, other than their ability to confer an increased frequency of regulatory immune cells in the peripheral blood and/or the lymph nodes draining their site of administration, might also be due to the *ex vivo* upstream cell processing procedures prior to the addition of GM-CSF/IL-4. Examples include the degree of “contaminating” monocyte progenitors and granulocytes in the monocyte elutriation step(s). The effect of

the site of delivery (intravenous, subcutaneous, intradermal) on tDC mechanism of action (direct or indirect), at the lymphoid organs draining the inflamed tissues and/or the autoimmunity target tissues proper, remains to be better understood. In this light, establishment and retention of a tolerogenic phenotype can be a function of the *ex vivo* generation procedure and the method of/site of administration. Even once standardized methods are established to characterize an autologous *ex vivo*-generated cell population as tolerogenic, together with a set of biomarkers to confer such a designation, the ability of such cells to maintain proper function before and after administration will need to be verified and validated in human trials. Cellular therapies may require multiple injections over an extended period of time in some or all individuals. Generating and testing a single large batch of cells per patient could prove more cost effective than having several rounds of peripheral blood collection and differentiation, but storage methods, shelf-life, and frequency of retesting need to be determined. It is important, at the same time, to determine if freshly generated vs. thawed cryopreserved tDC are functionally-different *in vivo*. The objectives of international collaborations like the ones resulting in the proposal of tDC and Treg MITAP (Minimum Information about Tolerogenic Antigen-Presenting cells) are commendable steps in establishing uniform characterization of clinical tolerogenic cell products (67).

TREGS AS A COMMON MECHANISTIC OUTCOME OF TDC ADMINISTRATION AND AS A BASIS OF MANAGEMENT OF THE UNDERLYING AUTOIMMUNITY IN AUTOIMMUNE DISEASE, INCLUDING T1D

In autoimmunity, Tregs induce tolerance through indirection consequences of physical interaction with DC, or through direct regulation of autoreactive T-helper and/or T-effector cells. While representing a population of cells that are diverse in character and phenotype, Tregs largely refer to cells that are mainly CD4+ CD25+ CD127- Foxp3+ (68, 69) as well as CD4+ CD49b+ LAG-3+ CD226+ IL-10 producing cells (Tr1 cells) (19, 70, 71). Treg constitutively express the surface marker Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4), which is able to interact with DC co-stimulatory molecules CD80 and CD86. This not only acts as a competitive inhibitor blocking T-effector cell activation through CD28, but in a reciprocal manner on DC, which causes their expression of IDO, TGF β , and IL-10, further amplifying the tolerogenic state of DC and the suppressive activity of the Tregs (72, 73). IL-10 and TGF β are also produced from Treg cells blocking T-effector activation, with greater levels of production in Tr1 cells than Foxp3+ Treg cells. Mechanistically, Treg also compete with T-effectors for the cytokine IL-2, a necessary growth factor for cell proliferation and maintenance. Tregs constitutively express high levels of the IL-2 receptor α chain (CD25), which is the ligand-binding part of the IL-2 receptor complex. Thus, at limiting concentrations of IL-2, Tregs will sequester a greater amount of IL-2 away from T-effector cells.

EX VIVO-GENERATED TREGS FOR THE TREATMENT OF T1D AUTOIMMUNITY

Given the strong evidence demonstrating powerful suppressive activities of stably-expressing Foxp3+ Tregs on autoimmunity, their consideration for clinical translation was self-evident early on. The first major hurdle in cell-based therapeutics is coming to a consensus on what is known and what is yet to be clarified, in order to move forward in therapeutic development. Foxp3+ Tregs are better characterized than tDC, with a defined marker profile of CD3+ CD4+ CD25+ CD127^{low}. Furthermore, changes in CD25 expression levels and increased STAT5 pathway activity prior to administration to patients have been identified in clinical studies. Although their absolute numbers are low in the peripheral blood of humans, a number of techniques have evolved for their *ex vivo* expansion (74–77). Besides differences in the concentration of IL-2 supplied to the *ex vivo* Treg generation culture media, the current methods to expand Treg are consistently uniform and somewhat reproducible for future trials.

The greatest challenge and point of uncertainty is what happens to *ex vivo* generated Treg's after administration. Stability of the suppressive activity *in vivo*, post-administration is uncertain, given recent data that indicate unstable state *in vivo* (74, 78). Phenotypes in the *ex vivo*-generated Tregs that eventually-accumulate inside the disease target organ-draining lymph nodes, are also unclear. Are these Tregs directly involved in suppression of autoreactive T-effectors, or are they part of a network that responds to their presence whose comprehensive outcome is necessary to achieve some regulation of the underlying autoimmunity? Furthermore, *ex vivo*-generated Tregs, once administered into patients, begin to fall in numbers; circulating Treg levels fell to 25% maximal numbers in treated patients at 90 days in some studies (74). While some emerging planned trials are considering supplementing the Treg treatment with co-administration of IL-2 (NCT02772679), the level of CD25 on these cells (79) may limit the effect of the cytokine and instead further add to the survival and/or the stability of the Tregs once *in vivo*.

Two clinical trials have used *ex vivo*-expanded Treg cells for the treatment of new-onset disease, <2 months, in T1D patients (NCT01210664, ISRCTN06128462) (74–77). These studies relied on Tregs generated from patient CD4+ CD25+ CD127- cells isolated from peripheral blood by flow cytometry and followed patients for 24 months post administration (74–77). Both studies examined patient C-Peptide levels as a marker for maintained insulin production, hence preserved beta-cell mass in the pancreas, as C-Peptide is cleaved from the proinsulin when it is converted to its active insulin form. The first study maintained detectable C-peptide over the 2 year monitoring period, but revealed that circulating Treg levels fell to 25% at a peak of 90 days after infusion (74). During the same 90 day time frame the cell surface marker CD38, which has been associated with enhanced Treg function (80), dropped from >95% pre-infusion to <5% post infusion. An additional phase I study is being planned to combine Treg administration with low-dose IL-2 treatment to see if a greater number of Treg

can be maintained in T1D (NCT02772679). The second study displayed a transient increase in C-peptide with a reciprocal decrease in patient insulin usage. However, C-peptide values resumed a decline over the trial time-course (75–77). Plasma IL-6, a pro-inflammatory cytokine, was discovered to increase over the same 24 months in Treg treated patients, to levels detected in the untreated patients. A commercially-generated Treg population is also currently being tested in T1D (CLBS03; NCT02691247), however, at the time of our review, there were no results our outcomes publicly-disclosed. There are other reports indicating that autologous Treg therapy is in preparation for clinical trials in other conditions including autoimmune hepatitis (NCT02704338) and lupus (NCT02428309).

TWO IS BETTER THAN ONE: COMBINATION CELL THERAPY

While the possibility of combining tDC with Tregs as a co-administered or serially-administered cell therapy in autoimmunity, especially in new-onset T1D, would make scientific and therapeutic sense, thus far few if any have considered this. The inter-relationship of these cell populations on each other for functional outcomes, maintenance, stability, and “feed-forwarding” of a very powerful tolerogenic state should be self-evident. The autologous tDC, co-administered with the patient’s Tregs, would stabilize Foxp3 expression as well as its genomic locus from the standpoint of the epigenome and, as tDC have often been shown to produce IL-10, TGFβ, and retinoic acid (66, 81, 82), the stabilized Tregs would in turn impact the tolerogenic state of the tDC via cell-cell

interactions and paracrine immunoregulatory cytokines. In a potential treatment approach, the initial co-administration could be followed by periodic “boosters” of tDC and Tregs alone in serial administrations or be co-administered. While this makes mechanistic sense, the logistics to generate the cells would not necessarily be more challenging than they are now for the generation *ex vivo* of each product. For example, the leukapheresis that is part of the tDC generation protocols would cover the enrichment of monocytes, to generate the tDC as well as the initial step to collect the lymphocytes from which the Tregs would be expanded separately, in the same cell processing facility. As animal models of tDC and Treg cell therapy for autoimmunity including T1D are well-established, this possible co-therapy, we believe, is developed enough to investigate pre-clinically.

Considering the limitations and adverse events encountered using biologic agents and the need to move past systemically-acting immunosuppressives, the well-tolerated safety profile of tDC and Tregs, across a range of dose levels and administration sites, along with the evidence of increased regulatory cell frequency *in vivo* during treatment, strongly argues in favor of their further development, characterization and consideration, to fundamentally change how autoimmune diseases are treated, directly addressing the immune imbalance and moving away from disease and symptom management.

AUTHOR CONTRIBUTIONS

BP and NG wrote the manuscript. CE, MT, and YG edited the manuscripts and added additional insights. The final version was proofread and edited by NG.

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Conflict of Interest Statement: NG and MT hold equity in Diavacs Inc., which has licensed the intellectual property concerning the tolerogenic dendritic cells noted in the review under clinical trial numbers NCT00445913 and NCT02354911.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethyl Pyruvate Induces Tolerogenic Dendritic Cells

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Dendritic cells (DC) are professional antigen presenting cells that have a key role in shaping the immune response. Tolerogenic DC (tolDC) have immuno-regulatory properties and they are a promising prospective therapy for multiple sclerosis and other autoimmune diseases. Ethyl pyruvate (EP) is a redox analog of dimethyl fumarate (Tecfidera), a drug for multiple sclerosis treatment. We have recently shown that EP ameliorates experimental autoimmune encephalomyelitis, a multiple sclerosis murine model. Here, we expanded our study to its tolerogenic effects on DC. Phenotypic analysis has shown that DC obtained from mice or humans reduce expression of molecules required for T cell activation such as CD86, CD83, and HLA-DR under the influence of EP, while CD11c expression and viability of DC are not affected. Furthermore, EP-treated DC restrain proliferation and modulate cytokine production of allogeneic lymphocytes. These results demonstrate that EP has the ability to direct DC toward tolDC.

Keywords: dendritic cells, ethyl pyruvate, autoimmunity, tolerogenicity, immune-regulation

INTRODUCTION

Tolerogenic dendritic cells (tolDC) are promising candidates for the cell-based immunotherapy of autoimmune disorders, including multiple sclerosis (1). They are effective in restraining antigen-specific and allogeneic T cell responses *in vitro* and their beneficial effects have been demonstrated in the treatment of animal models of various autoimmune disorders (2). Moreover, their administration to humans has been shown safe and efficient by increasing the proportion of regulatory T cells in circulation (1). Vitamin D3 and dexamethasone are commonly used for induction of tolDC (1, 2), while a number of agents has been shown effective for the induction of tolerogenic properties over dendritic cells (DC). Ethyl pyruvate (EP) is a redox active compound that has been shown potent as an anti-inflammatory agent (3). It is a safe and simple chemical that has already been tested in humans (4). Importantly, it is a redox analog of dimethyl fumarate (Tecfidera), a drug that is approved for multiple sclerosis treatment (5). Our group has recently reported that EP ameliorates experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (6). The major pathogenic T helper (Th) cells in the central nervous system autoimmunity are interferon (IFN)- γ -producing Th1 cells and interleukin (IL)-17-producing Th17 cells (7). The beneficial effects of EP in EAE were paralleled with down-regulation of Th1/Th17 activity (6). Moreover, release/production of IL-6, tumor necrosis factor (TNF) and reactive nitrogen and oxygen species by macrophages were also inhibited by EP. Noteworthy, IL-6 is known to potentiate the resistance of effector T cells to regulatory T cells in multiple sclerosis (8), while TNF actively contributes to demyelination and axonal degeneration in neuroinflammation (9).

Correspondingly, reactive oxygen and nitrogen species participate in the loss of oligodendrocytes, blood-brain barrier dysfunction, T cell infiltration, and neurodegeneration (10). Effects of EP were also observed within the CNS, where reactivity of microglia and astrocytes was reduced (11).

We were also able to demonstrate that EP down-regulated the expression of antigen presenting molecules on macrophages (6) which led us to the investigation of the effects of EP on DC as the major professional antigen-presenting cells. Here, we present that EP exerts potent tolerogenic effect on murine and human DC. It down-regulates the expression of antigen-presenting molecules on DC, restricts the production of pro-inflammatory cytokines in DC and diminishes their T cell-activating function.

MATERIALS AND METHODS

Monocyte-Derived Human DC

Buffy coats, provided by the *Banc de Sang i Teixits* (Barcelona, Spain), were obtained from randomized healthy blood donors, following the institutional Standard Operating Procedures for blood donation and processing. Peripheral blood was obtained from untreated relapsing-remitting multiple sclerosis patients. The Ethical Committee of Germans Trias i Pujol Hospital approved the study, and all subjects gave their informed consent according to the Declaration of Helsinki (BMJ 1991; 302: 1994). Peripheral Blood Mononuclear Cells (PBMC) were isolated by Ficoll-Paque (Lymphoprep, Axis Shield, Oslo, Norway) density gradient centrifugation at $400 \times g$ for 30 min. Recovered cells were washed twice in PBS and counted using Perfect Count microspheres (Cytognos SL, Salamanca, Spain) following the manufacturer's instructions. Establishing Monocyte-derived DCs, PBMCs were first depleted of CD3⁺ T cells using the RosetteSepTM Human CD3 Depletion Cocktail (StemCell Technologies, Seattle, WA, United States). Afterwards, monocytes were obtained by positive selection using the EasySep[®] Human CD14 Positive Selection Kit (StemCell Technologies). For all samples, the purity and viability of the monocyte populations were >95 and 90%, respectively, as assessed by the expression of specific markers and Annexin V and 7-Amino-actinomycin D (7AAD) labeling (BD Biosciences). Monocytes were cultured at 1×10^6 /ml for 6 days in X-VIVO 15 culture medium (BioWhittaker[®], Lonza, Belgium) supplemented with 2% (vol/vol) heat inactivated AB human serum (BioWhittaker[®], Lonza, Belgium), 2 mM L-glutamine (Sigma-Aldrich Company LTD, Saint Louis, MO, United States), 100 U/mL penicillin (Cepa S.L, Madrid, Spain), and 100 µg/mL streptomycin (Laboratorios Normon S.A, Madrid, Spain) in

the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF: 1,000 U/ml; Peprotech, Freiburg, Germany) and interleukin 4 (IL-4: 1,000 U/ml; Peprotech). Cells were replenished on day 4 with fresh medium and cytokines. To induce mature DC (mDC), DC were treated with a cocktail of TNF (1,000 U/ mL), IL-1 β (10 ng/mL) (both from Peprotech); and PGE₂ (1 µM) (Pfizer, New York, NY, United States) on day 4. Treatment with Vitamin D3 (vitDC, 1 nM, Calcijex, Kern Pharma, Terrassa, Spain) was performed on days 0 and 4, while treatment with EP (EPDC) was performed on days 2 and 4. These cells were also stimulated as mature DCs at day 4 with the cytokine cocktail. Immature DC (iDC) were not treated with the maturation cocktail on day 4. On day 6, DC were harvested and washed extensively twice, before phenotype was determined and functional assays were performed.

Bone Marrow-Derived Murine DC

Murine DC were obtained from progenitor bone marrow cells that were flushed from femur of NOD and C57BL/6 mice (experiments were approved by the local ethics committee of the Institute for Biological Research "Sinisa Stankovic," in accordance with Directive 2010/63/EU, N^o 02-09/16). These cells were cultured in RPMI 1640 (Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 10% FCS (PAA Laboratories), 2 mM glutamine and 1 mM sodium pyruvate (both from Sigma-Aldrich) (1×10^6 /mL/well in 24-well plate). Bone marrow derived dendritic cells (BMDC) were cultivated for 8 days in the presence of 20 ng/mL of GM-CSF (Peprotech or Novus, Littleton, CO), with 100 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich) added for the last 24 h of cultivation for maturation. Treatment with EP was performed on days 3 and 6 (diffEP) or simultaneously with LPS (matEP). Number of viable cells was determined by trypan blue exclusion test on a LUNA-IITM Automated Cell Counter from Logos Biosystems (Gyeonggi-do, South Korea).

In vivo DC Application

Experiments were approved by the local ethics committee (Institute for Biological Research "Sinisa Stankovic," in accordance with Directive 2010/63/EU, N^o 03-01/17). Murine DC were prepared as described above. mDC or EPDC were injected subcutaneously into the hind paw of female 2–3 months old C57BL/6 mice (1×10^6 /80 µl/mouse). Complete Freund's adjuvant (CFA) was made from incomplete Freund's adjuvant (Difco, Detroit, MI) supplemented with *M. tuberculosis* (to 5 mg/ml, Difco). Each mouse was injected subcutaneously in the hind paw with 50 µl of emulsion made from CFA mixed with equal volume of phosphate buffer saline on the following day. Popliteal lymph nodes were isolated from the mice 3 days later and subjected to collagenase V (Sigma-Aldrich) digestion. Lymph nodes were gently minced with scissors and incubated with 1 mg/mL of Collagenase V solution in RPMI 1640 at 37°C with gentle shaking for 20 min. Subsequently, the digestion was stopped with FCS and the cells were pelleted by centrifugation. Number of viable cells was determined by trypan blue exclusion test on a LUNA-IITM Automated Cell Counter. For cell tracking, DC were stained with CFSE (2 µM, Invitrogen, Carlsbad, CA,

Abbreviations: 7AAD, 7-Amino-actinomycin D; BMDC, bone marrow derived dendritic; CD, cluster of differentiation; CFA, complete Freund's adjuvant; CNS, central nervous system; DC, dendritic cells; diffEP, DC treated with EP during differentiation/propagation; EAE, experimental autoimmune encephalomyelitis; EP, ethyl pyruvate; FCS, fetal calf serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; iDC, immature DC; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; matEP, DC treated with EP during maturation; mDC, mature DC; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline; SD, standard deviation; Th, helper T cells; TNF, tumor necrosis factor; tolDC, tolerogenic DC; Treg, regulatory T cells.

United States) prior to injection. Determination of CFSE⁺ cells among popliteal lymph node cells was performed on a CyFlow Space flow cytometer (Partec, Munster, Germany).

CD4⁺ T Cell Isolation

CD4⁺ T cells were purified from cervical lymph nodes obtained from BALB/c mice. For purification of CD4⁺ T cells from the lymph nodes, a biotin-conjugated antibody specific for CD4 (Invitrogen) and IMagSAV Particles Plus (BD Biosciences, San Diego, CA) were used in accordance with the manufacturers' instructions. CD4⁺ T cells were stained with CFSE (2 μ M) prior to cultivation with DC.

Allostimulatory Assays

Human DC were co-cultured with allogeneic human PBMC (10⁵ cells/well) in 96-well round-bottom plates. PBMC were co-cultured for 4 days (96 h) with MDDCs at a 1:20 ratio (DC: PBMC). Cell proliferation was determined by incorporation of 1 μ Ci [³H]-thymidine (PerkinElmer, Waltham, MA, United States) for 18 h on each well. Murine DC obtained from C57BL/6 mice were co-cultured with CFSE-labeled CD4⁺ T cells obtained from BALB/c mice (10⁵ cells/well) in 96-well round-bottom plates. CD4⁺ T cells were co-cultured for 5 days (120 h) with MDDCs at a 1:20 ratio (DC: CD4⁺ T cells). Cell proliferation was determined by the sequential loss of CFSE fluorescence, as detected by flow cytometry.

Immunostaining and Flow Cytometry

Human DC were washed, re-suspended in 50 μ L of PBS and incubated with mAbs for 20 min protected from light at room temperature (RT). After washing, acquisition was performed on a FACSCanto II flow cytometer using FACSDiva software (BD Biosciences, CA, United States). Subsequent analyses were done using FlowJo software (Tree Star, Inc., OR, United States). Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris. The following human mAbs were used: FITC-labeled mAbs: CD86 (BD Biosciences); PE-labeled mAbs: CD14 (ImmunoTools GmbH, Germany), CD40 (BD Biosciences); PE-Cyanine dye 7-labeled mAb: CD14 and CD11c (BD Biosciences); Allophycocyanin (APC)-labeled mAbs: CD83, APC-H7-labeled mAb: HLA-DR (BD Biosciences).

Murine cells were washed, re-suspended in 100 μ L of PBS supplemented with 2% of mouse serum and incubated with mAbs for 30 min at 4°C. After washing, acquisition was performed with a CyFlow Space flow cytometer. Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris, as well as using FSC and FSC-W to exclude cell doublets. The following murine mAbs were used: PE-Cyanine5 labeled CD11c and CD86 (eBioscience), FITC-labeled CD40 (eBioscience).

Prior to intracellular cytokine staining, cells were stimulated with eBioscienceTM Cell Stimulation Cocktail (plus protein transport inhibitors), containing phorbol myristate acetate, ionomycin and brefeldin A and monensin for 4 h, stained with anti-CD4 PerCP-Cy5.5 antibody (eBioscience), fixed and permeabilized with eBioscienceTM Intracellular Fixation and Permeabilization Buffer Set and then stained for the intracellular

cytokines with the following antibodies: anti-mouse antibodies against IL-17 or IFN- γ or IL-10 coupled with FITC or PE (all from eBioscience). After washing, acquisition was performed with a CyFlow Space flow cytometer. Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris, as well as using FSC and FSC-W to exclude cell doublets. Isotype-matched controls were included in all experiments (eBioscience).

ELISA

Cell culture supernatants were obtained and centrifuged to spin down the cells. Cell-free supernatants were used in sandwich ELISAs as instructed by the producers of the antibody pairs used. Samples were analyzed in duplicates for murine TNF, murine IL-6 (R&D Systems), murine IL-1 β , murine IL-12 (eBiosciences), murine IL-17 (eBioscience), human IFN- γ and human IL-10 (R&D Systems). Lower limit of detection was 30 pg/ml, whereas upper limit of detection was 10 ng/ml for all of the ELISA tests performed. Samples that showed values over the upper limit of detection were adequately diluted for the measurement. The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

Statistics

One-way ANOVA followed by Tukey's multiple comparison test or Student's *t*-test (two-tailed) were used as appropriate for statistical analysis. A *p* < 0.05 was considered statistically significant.

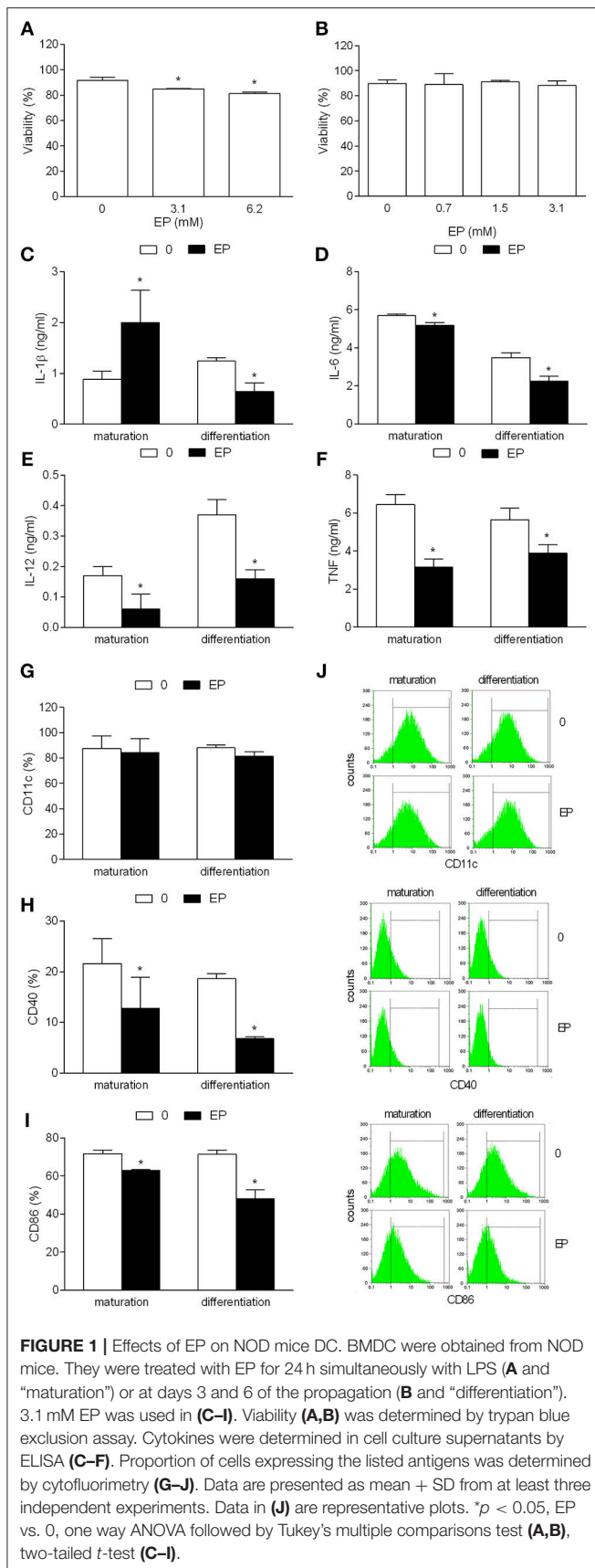
RESULTS

EP Exerts Tolerogenic Effects on Mouse BMDC

In order to determine if EP has tolerizing effects on NOD mice BMDC in the course of their maturation, the agent was applied during the last 24 h of their cultivation, simultaneously with the maturing agent, i.e., LPS. Alternatively, to assess EP effect on differentiation/propagation of DC from their bone marrow precursors, the agent was applied to BMDC cultures on days 3 and 6 of their cultivation. EP applied during the maturation had minimal effects on BMDC viability (**Figure 1A**), while no effects on the cell viability were observed with the alternative application of the agent (**Figure 1B**). While EP applied during the differentiation reduced IL-1 β production in BMDC, EP applied during maturation had the opposite effect (**Figure 1C**). Both ways of EP application led to the reduction of generation of IL-6, IL-12, and TNF in BMDC (**Figures 1D–F**). Also, CD11c expression was not affected, while both CD86 and CD40 expression were decreased in EP-treated BMDC, irrespectively of the way of its application (**Figures 1G–J**).

EP-Treated Mouse BMDC Are Inefficient Allogeneic Stimulators *in vitro*

To test if the tolerogenic effects of EP can also be observed in C57BL/6 BMDC, the same application protocols were used as with NOD mice BMDC. EP did not affect cell viability and CD11c expression, while it reduced generation of the examined



pro-inflammatory cytokines and expression of CD40 and CD86, irrespectively of the way of its application (**Figures 2A–J**). To examine if these effects of EP affected BMDC ability to activate T cells, EP-treated BMDC were co-cultured with allogeneic CD4⁺ T cells from BALB/c mice. Proliferation of CD4⁺ T cells (**Figures 3A,B**) and their capacity to produce IL-17 (**Figure 3C**) were reduced in co-cultures with EP-treated BMDC in comparison to those with the untreated BMDC.

EP Exerts Tolerogenic Effects on Human MDDC

EP was added to MDDC cultures in the process of their differentiation/propagation (EPDC) (**Figure 4A**). No effect of EP on cell viability was observed on human MDDC that were obtained from healthy subjects (**Figure 4B**) or individuals suffering from multiple sclerosis (**Figure 4C**). EP-treated DC were compared to vitamin D-treated DC (vitDC), non-treated mature DC (mDC) and immature DC (iDC) in the following experiments. There was no difference in the cell viability among the examined DC populations (**Figures 4D,E**). Also, no difference was observed in the level of CD11c expression (**Figures 4F–H**). However, both vitDC and EPDC reduced expression of HLA-DR, CD86, and CD83 in comparison to mDC, both in healthy and multiple sclerosis individuals (**Figures 4F–H**).

EP-Treated Human MDDC Are Inefficient Allogeneic Stimulators *in vitro*

Tolerogenic properties of EPDC were examined in allogeneic co-culture with PBMC. EPDC inhibited allogeneic cell proliferation in comparison to mDC to the similar extent as vitDC (**Figures 5A,B**). Effects of EPDC obtained with the highest EP dose applied (12.5 mM) were superior to those of vitDC (**Figures 5A,B**). There was no difference in the inhibitory activity of EPDC or vitDC obtained from healthy subjects or individuals affected with multiple sclerosis. Levels of IFN- γ and IL-10 in supernatants of the co-cultures were also determined. IFN- γ levels were reduced in the samples obtained from co-cultures performed with EPDC or vitDC in comparison to mDC (**Figures 5C,D**), irrespectively if MDDC were obtained from healthy individuals or the patients. However, IL-10 levels were higher with EPDC or vitDC in comparison to mDC, only if MDDC were obtained from healthy subjects (**Figures 5C,D**). Allogeneic cell proliferation and the cytokine levels determined with DC obtained from individual persons are presented, as well (**Figures 5E,F**) in order to enable discrimination of responses from different donors.

EP-Treated Mouse BMDC Show Immunomodulatory Function *in vivo*

EPDC were applied *in vivo* to mice immunized with CFA as depicted in **Figure 6A**. Their effects were compared to those of mDC-treated mice, as well as to mice injected with vehicle only (control). In order to track DC *in vivo*, the cells were labeled with CFSE. CFSE-labeled EPDC and mDC were detectable in popliteal lymph nodes 4 days after the injection (**Figure 6B**).

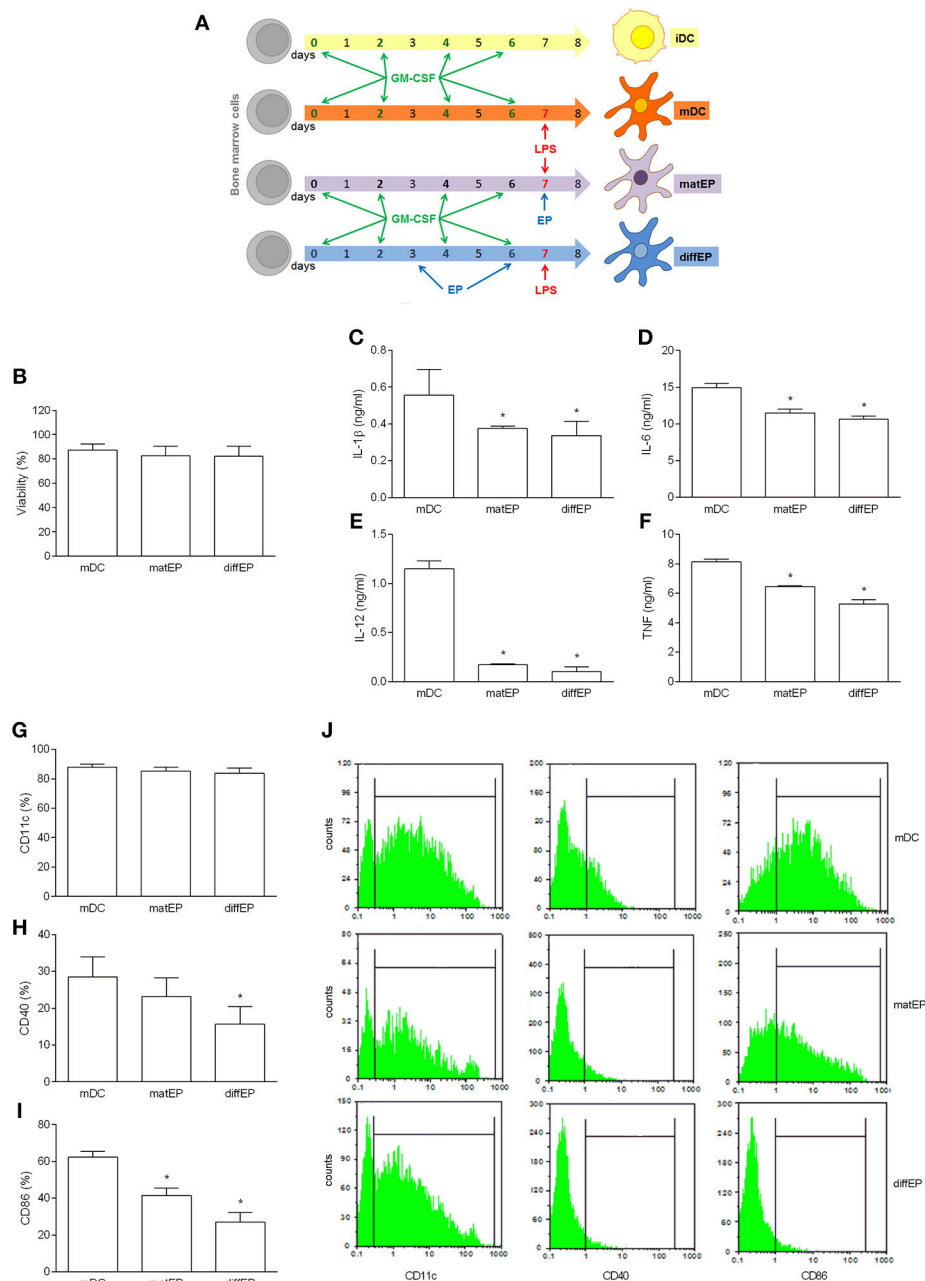


FIGURE 2 | Effects of EP on C57BL/6 mice DC. BMDC were obtained from C57BL/6 mice. They were treated with EP (3.1 mM) for 24 h simultaneously with LPS ("matEP") or at days 3 and 6 of the propagation ("diffEP") or they were untreated with EP (mDC), as schematized (A). Viability (B) was determined by trypan blue exclusion assay. Cytokines were determined in cell culture supernatants by ELISA (C–F). Proportion of cells expressing the listed antigens was determined by cytofluorimetry (G–J). Data are presented as mean + SD from at least three independent experiments. Data in (J) are representative plots. * $p < 0.05$, EP vs. mDC, one way ANOVA followed by Tukey's multiple comparisons test.

Popliteal lymph node cells were isolated for phenotypic analyses, at the same time. There were no differences in cellularity and proportion of CD4⁺ cells of popliteal lymph nodes isolated from vehicle-, mDC-, and EPDC-treated mice (Figures 6C,D). However, there were more IL-10⁺CD4⁺ cells in EPDC-treated than in control mice (Figure 6E) and less IFN- γ ⁺CD4⁺ cells in EPDC-treated than in mDC-treated mice popliteal lymph

nodes (Figure 6F). Both, IL-10⁺CD4⁺ cells and IFN- γ ⁺CD4⁺ cells were also more numerous in mDC-treated than in control mice popliteal lymph nodes (Figures 6E,F). There were no differences in proportion of IL-17⁺CD4⁺ cells among the groups (Figure 6G). Importantly, ratio of IL-10⁺CD4⁺ cells to IFN- γ ⁺CD4⁺ cells, as well as to IL-17⁺CD4⁺ cells was higher in popliteal lymph nodes of EPDC-treated mice in comparison to

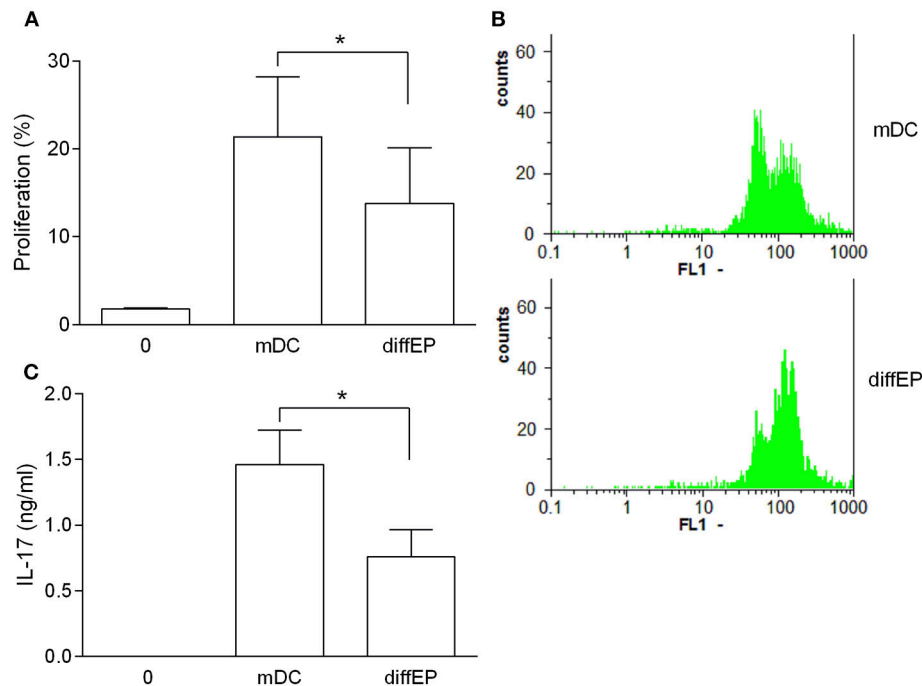


FIGURE 3 | Effects of murine EP-treated DC on allogeneic reactivity. BMDC were obtained from C57BL/6 mice. Their maturation was induced by LPS in the absence (mDC) or presence of 3.1 mM EP (diffEP). They were co-cultured with CD4⁺ T cells purified from BALB/C mice lymph nodes. Cell proliferation was determined by CFSE assay (A,B). IL-17 levels were determined in cell culture supernatants by ELISA (C). Data are presented as mean + SD from at least three independent experiments. Data in (B) are representative plots. * $p < 0.05$, diffEP vs. mDC, one way ANOVA followed by Tukey's multiple comparisons test.

controls (Figures 6H,I). Moreover, ratio of IL-10⁺CD4⁺ cells to IFN- γ ⁺CD4⁺ cells was higher in popliteal lymph nodes of EPDC-treated mice in comparison to mDC-treated mice.

DISCUSSION

Ethyl pyruvate modulates expression of antigen presentation-related molecules on DC. It also reduces release of pro-inflammatory cytokines from these cells. Moreover, it makes DC inefficient in T cell activation. Importantly, the effects are observed when EP is applied to DC during their differentiation/propagation from precursor cells, as well as when it is applied simultaneously with the maturation stimulus. EP-treated DC modulate immune response initiated by CFA *in vivo*. Thus, EP efficiently modulates immune activity of DC.

Our experiments on mice were performed with two mice strains, i.e., NOD and C57BL/6 mice. The reason to use both mice strains are our plans to expand the research to *in vivo* systems, where EAE and type 1 diabetes in mice will be analyzed. Both mouse strains are susceptible to EAE (12) induction, NOD mice develop diabetes spontaneously (13), while C57BL/6 mice are susceptible to multiple low dose streptozotocin-induced diabetes (14). Also, in this way we were able to exclude strain-specific effects of EP. The absence of genetic predisposition toward susceptibility of DC to EP were even more convincingly shown with human cells. There, the tolerizing effects of EP on DC were observed with the cells obtained from more than 10 different individuals. What is more, the effects were consistent

with the cells obtained from healthy individuals, as well as with those obtained from individuals affected by multiple sclerosis. This implies that EP-treated DC are good candidates for a tolerogenic cell-based therapy. Along this conclusion, EP-treated DC were comparable to vitamin D3-treated DC, regarding their immunomodulating properties. The highest dose of EP applied in our study (12.5 mM) was even superior to standard vitamin D3 dose used (1 nM) when T cell proliferation was determined. Having in mind that vitamin D3-induced tolDC have been investigated in details *in vitro* and *in vivo* in EAE and that they are currently tested in clinical trials in multiple sclerosis, the ability of EP to act on DC in similar fashion is an additional impetus for further studies toward clinical application of EP-treated DC as a therapy for autoimmunity.

Interestingly, there was a difference in IL-10 production in the co-cultures of tolDC with PBMC, depending on the source of DC, i.e., whether the cells were obtained from healthy or multiple sclerosis individuals. While IL-10 levels were increased in the co-cultures of tolDC from healthy individuals, they were not increased in the co-cultures of tolDC from multiple sclerosis individuals. This discrepancy was observed irrespectively of the tolerizing agent used (EP or vitamin D3). It is proposed that IL-10 plays the crucial role in immunomodulatory activity of tolDC, as it induces differentiation of regulatory T cells (15). Thus, the observed phenomenon is worthy of further investigation, as it could be potentially disadvantageous to therapeutic potential of EP- and vitamin D3-induced tolDC. Also, the possibility of inherent reduced capacity of DC and

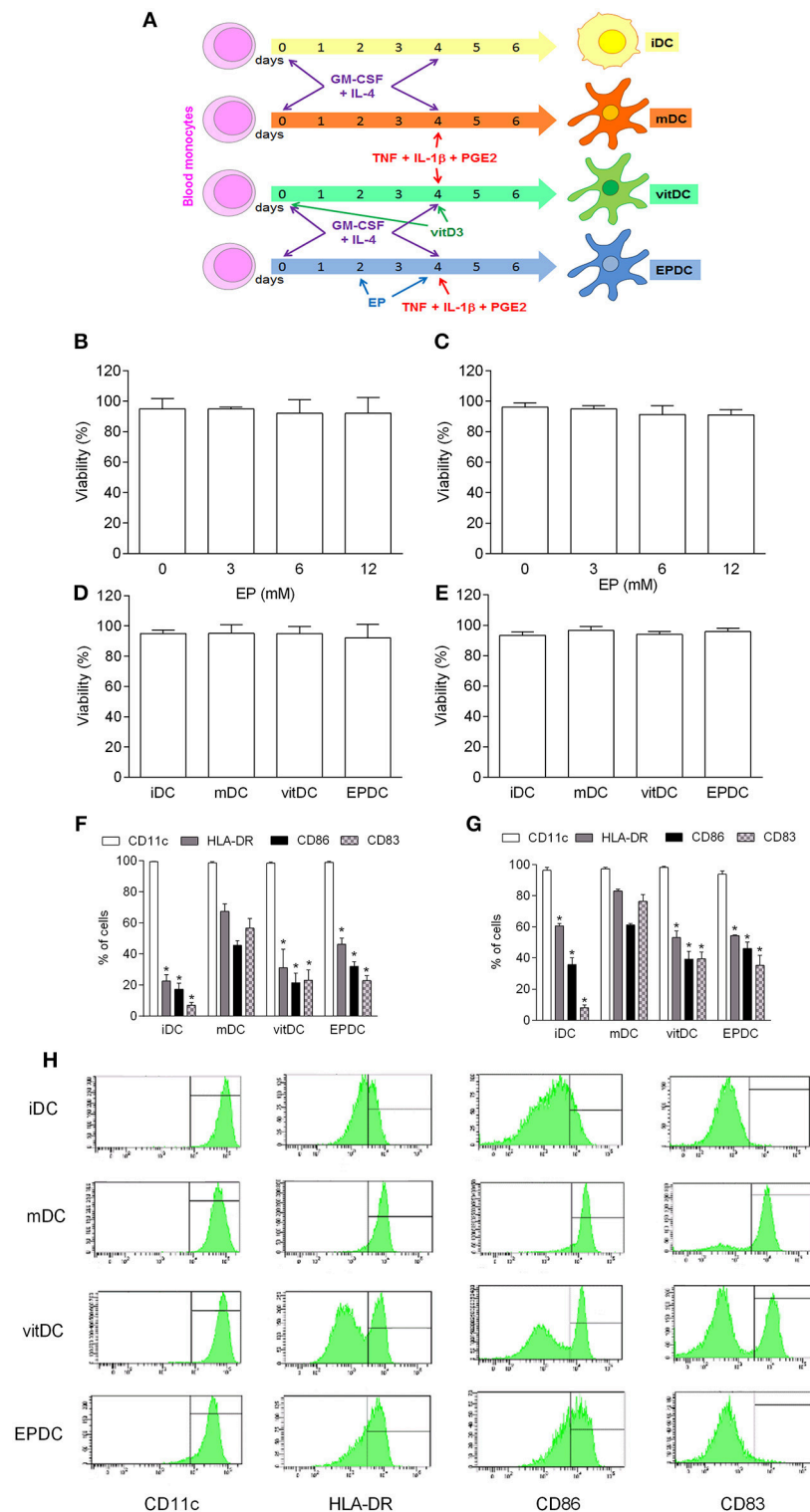


FIGURE 4 | Effects of EP on human DC. MDDC were propagated from peripheral blood monocytes and matured in the presence of TNF+IL-1 β +PGE₂ (mDC, vitDC, tEPDC) or were left immature without the treatment (iDC) as depicted (A). MDDC were obtained from healthy subjects (B,D,F,H) or from individuals affected by multiple sclerosis (C,E,G). EP was applied in various concentrations (B,C) or in concentration of 6.2 mM (D–H, tEPDC). Vitamin D3 (vitDC) was applied in concentration of 1 nM. Viability (B–E) was determined by 7AAD test. Proportion of cells expressing the listed antigens was determined by cytofluorimetry (F–H). Data are presented as mean + SD from at least five individuals. Data in H are representative plots. * $p < 0.05$ vs. mDC, two-tailed t -test.

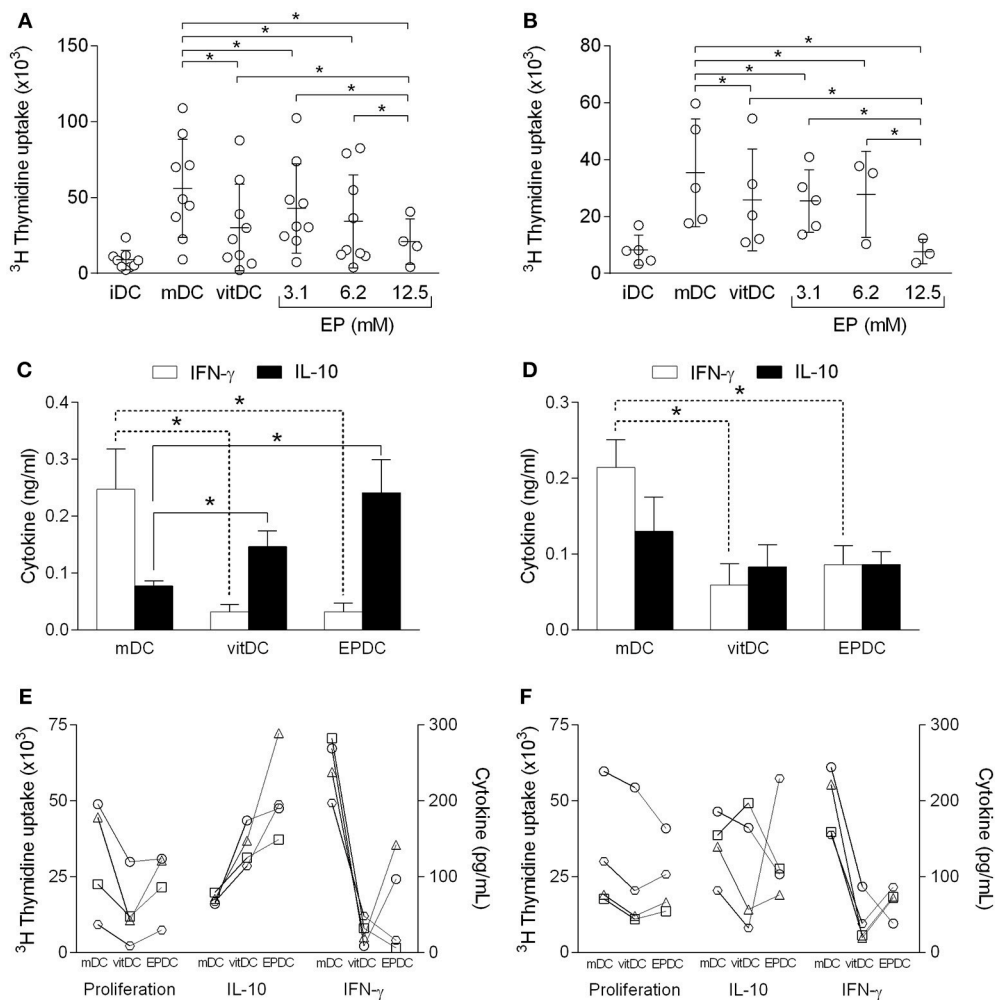


FIGURE 5 | Effects of human EP-treated DC on allogeneic reactivity. MDDC were obtained from healthy subjects (A,C,E) or from individuals affected by multiple sclerosis (B,D,F). The cells matured in the presence of TNF+IL-1 β +PGE₂ (mDC, vitDC, EPDC) or were left immature without the treatment (iDC). EP was applied in various concentrations (A,B) or in concentration of 3.1 mM (C–F). Vitamin D3 (vitDC) was applied in concentration of 1 nM. MDDC were co-cultured with allogeneic PBMC. Cell proliferation was determined by ³H incorporation test (A,B,E,F). Cytokines were determined in cell culture supernatants by ELISA (C–F). Data in (A,B) are presented as mean \pm SD from a number of individuals depicted as the open circles. Data in (C,D) are presented as mean \pm SD from four individuals. Data from four individuals are presented in (E,F), where the same person has a unique symbol (circle, square, triangle, or trapezoid). * p < 0.05, vs. mDC in (C,D), one way ANOVA followed by Tukey's multiple comparisons test.

other immune cells of multiple sclerosis patients to produce IL-10 warrants additional studies as it might be important for understanding the predisposition of humans toward the development of multiple sclerosis. Indeed, reduction in the proportion of IL-10-producing PBMC in multiple sclerosis patients (16), as well as of IL-10 mRNA expression in these cells (17, 18) were previously reported. However, multiple sclerosis patients and healthy subjects from whom blood samples were obtained were not adjusted to age, sex, and other relevant parameters that could bias our observation on the differential IL-10 response. Also, higher number of samples would be needed for the determination of the statistical correlation between the levels of cell proliferation and IL-10 or IFN- γ production within groups of healthy subjects and multiple sclerosis patients, as well

as between these two groups. Therefore, further studies enrolling higher number of individual samples for the exploration of the effects of EP on human DC, including their ability to generate IL-10 are needed. However, the fact that the samples were not adjusted between multiple sclerosis and healthy subjects did not jeopardize the general conclusion about tolerizing potency of EP-treated DC, i.e., EP-treated DC had the same, if not stronger tolerizing effects in comparison to classical tolDC induced by vitamin D3, irrespectively if they were obtained from the healthy subjects or the patients.

EP-treated DC applied to mice immunized with CFA shifted draining lymph node cytokine milieu from IFN- γ /IL-17- toward IL-10-dominated. EP-treated DC injected at the site of injection efficiently migrated toward regional lymph nodes and imposed

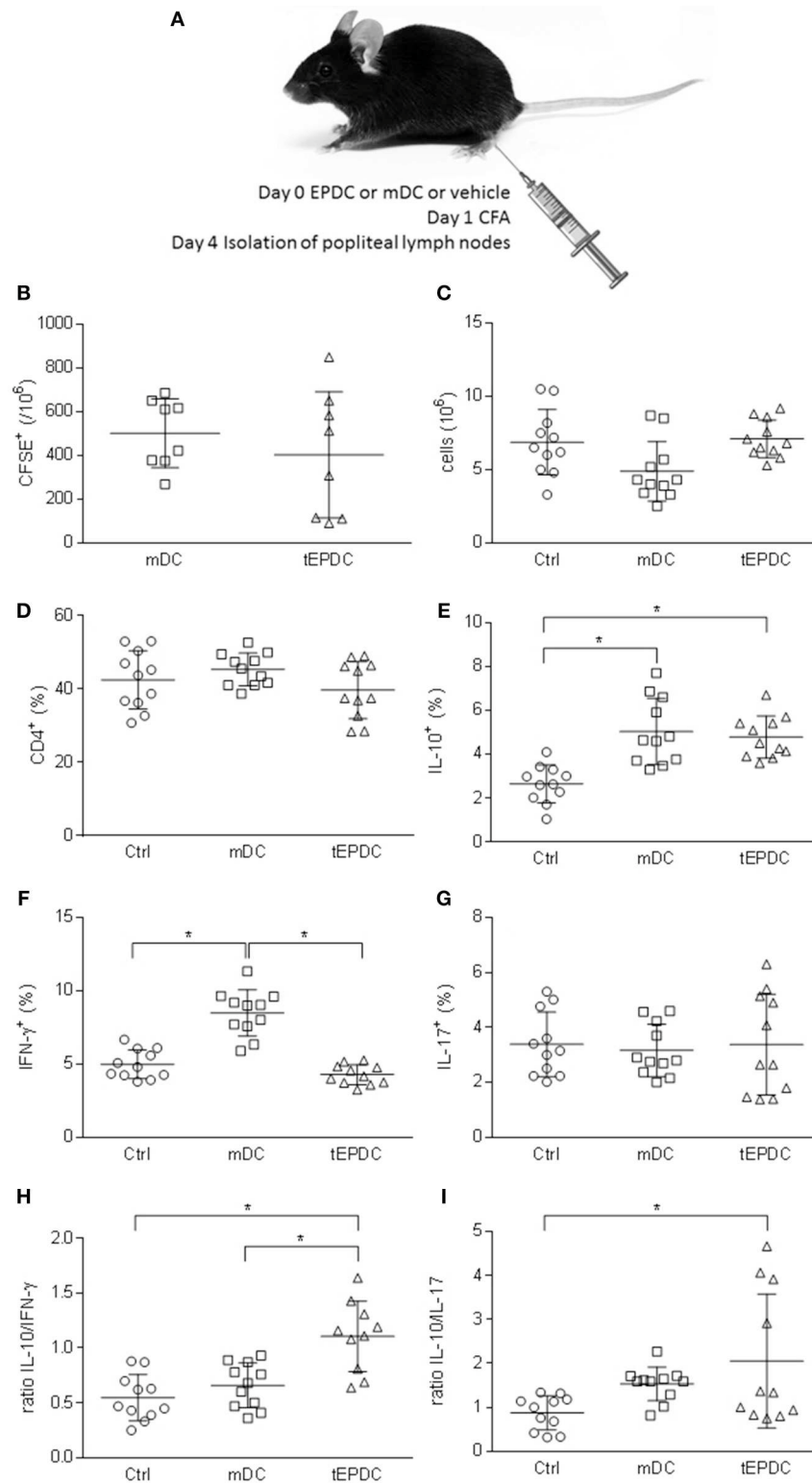


FIGURE 6 | Effects of murine EP-treated DC on CFA-induced immune response *in vivo*. Schematic representation of C57BL/6 mice treatment (**A**). Number of CFSE⁺ cells (**B**), total number of cells (**C**), and proportion of CD4⁺ cells (**D**) was determined in popliteal lymph nodes by flow cytometry (**B,D**) or cell counting (**C**). Proportion of IL-10⁺ (**E**), IFN- γ ⁺ (**F**), and IL-17⁺ (**G**) cells among CD4⁺ cells was determined by flow cytometry. Ratio of the cytokines expressing CD4⁺ cells is presented, as well (**H,I**). Data from three experiments are presented as mean \pm SD from a number of mice depicted as the circles, squares, or triangles. * $p < 0.05$, one way ANOVA followed by Tukey's multiple comparisons test.

the immunomodulatory effect. The observed modulation of the cytokine-generating T cell balance is particularly fascinating, as it was observed in strong inflammatory setting induced by CFA. Thus, immunomodulatory effects of EP-treated DC are rather potent and the one can expect that the effects would persist in antigen-specific setting, as well. Of course, in the context of a certain autoimmune disease it would be important to determine if EP-treated DC loaded with relevant antigens would acquire specificity of immunomodulatory actions. Thus, our results imply that EP-treated DC dampen initiation and propagation of an immune response in lymphoid organs, an effect that should be useful in restricting the immunopathology of autoimmune and chronic inflammatory diseases. This possibility will be explored in details in specific models of autoimmunity, such as type 1 diabetes in NOD mice and EAE in our future studies.

We have previously shown that EP exerts immunomodulatory properties in animal models of multiple sclerosis and type 1 diabetes (6, 19). Here we extend our observation and we introduce a novel biological effect of EP, i.e., its ability to induce tolerogenic DC *in vitro*. Moreover, EP-treated DC are efficient in modulating an immune response *in vivo*. EP applied to DC during the process of differentiation inhibits their response to the maturation stimulus, both regarding expression of antigen-presenting and co-stimulatory molecules and production of relevant pro-inflammatory cytokines. Also, EP-treated DC are impotent allogeneic stimulators of CD4⁺ T cells. Importantly, the effects are observed in human cells and not only murine cells. Further studies are needed for determination of molecular mechanisms behind the effects of EP. Also, *in vivo* studies on animal models of autoimmunity are necessary. These studies should help to elucidate if EP-treated DC are good candidates

for clinical trials of multiple sclerosis and other autoimmune disorders.

AUTHOR CONTRIBUTIONS

ND performed the main work, analyzed, interpreted, and critically revised the data. MM performed the work related to MDDC. BJ organ isolation, cell preparation, cytofluorimetry. JN-B performed the work related to MDDC. TS organ isolation, co-culture, CD4⁺ T cell purification. EM-C conception and design, supervised the work, and critically revised the manuscript. ĐM conception and design, supervised the work, analyzed and interpreted the data, drafted the work, and critically revised the manuscript. All authors finally approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Humanized Mouse Models of Rheumatoid Arthritis for Studies on Immunopathogenesis and Preclinical Testing of Cell-Based Therapies

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Rodent models of rheumatoid arthritis (RA) have been used over decades to study the immunopathogenesis of the disease and to explore intervention strategies. Nevertheless, mouse models of RA reach their limit when it comes to testing of new therapeutic approaches such as cell-based therapies. Differences between the human and the murine immune system make it difficult to draw reliable conclusions about the success of immunotherapies. To overcome this issue, humanized mouse models have been established that mimic components of the human immune system in mice. Two main strategies have been pursued for humanization: the introduction of human transgenes such as human leukocyte antigen molecules or specific T cell receptors, and the generation of mouse/human chimera by transferring human cells or tissues into immunodeficient mice. Recently, both approaches have been combined to achieve more sophisticated humanized models of autoimmune diseases. This review discusses limitations of conventional mouse models of RA-like disease and provides a closer look into studies in humanized mice exploring their usefulness and necessity as preclinical models for testing of cell-based therapies in autoimmune diseases such as RA.

Keywords: rheumatoid arthritis, humanized mice, transgenic mice, mouse/human chimera, preclinical model, cell-based immunotherapy

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder which affects the synovial tissue of the joints, causing articular pain and disability (1). Initially manifested locally, RA later develops into a systemic disease that involves major organ systems and reduces life expectancy (2). The disease is characterized by an infiltration of the synovium with inflammatory cells, proliferation of synovial fibroblasts, forming an invasive pannus that destroys the adjacent cartilage and bone, and progressive joint damage (3).

Rodent models have been used over decades to study the immunopathogenesis of RA and to test the efficacy of anti-rheumatic drugs (4). There are numerous rodent models of RA-like disease, each

mirroring certain aspects of the disease (4–6). Important findings have emerged from studies using these models, such as the need for CD4+ T cells and B cells for the development of RA (7, 8), the importance of pro-inflammatory cytokines in RA pathogenesis (9–11), and the discovery of RA-relevant autoantigens (12, 13). Nevertheless, mouse models of RA reach their limit when it comes to testing of new therapeutic approaches such as cell-based therapies. More than 80% of potential therapeutics, which have been shown to be safe and effective in animal studies, fail when tested in humans (14, 15). One example is the lack of therapeutic efficacy of interleukin (IL-) 17 inhibitors in RA patients (16, 17), although suppression of IL-17 signaling had been shown to reduce joint inflammation as well as cartilage and bone destruction in mice (18). Similarly, inhibition of IL-1 signaling had been demonstrated to ameliorate arthritis in mice (19), while anti-IL-1 therapy displayed limited efficacy in RA patients (4, 20). Differences between the human and the murine immune system make it difficult to transfer the results from the mouse model to patients (21, 22). Consequently, translational research needs to refocus on human, and not mouse, immunology (23, 24). This is particularly important for preclinical testing of therapeutic approaches based on dendritic cells (DCs). While monocyte-derived DCs are used for clinical applications in humans (25), DCs obtained from bone marrow precursors are administered in mouse models (26, 27), which considerably affects the transferability of the results from mice to men.

Since studies of human immune responses and disease *in vivo* are limited by ethical and technical constraints, there is a need for animal models that on the one hand accurately mirror the pathogenesis of the autoimmune disease, and on the other allow pre-clinical testing of cell-based therapeutic approaches targeting human cells and tissues *in vivo*. To tackle these issues, humanized mice have been developed using two different strategies: (i) the introduction of human RA-relevant transgenes such as human leukocyte antigen (HLA) molecules, T cell receptors (TCR), or autoantigens (28), and (ii) the generation of mouse/human chimera by the engraftment of human (RA-derived) cells and/or tissues into immunodeficient mice (29) (Figure 1). Combination of both approaches will lead to more

sophisticated humanized models of autoimmune diseases such as RA (32).

This review provides an overview of mouse models of RA-like disease and their limitations and discusses different humanization strategies for the generation of RA models, with focus on the use of humanized mice as tools for pre-clinical testing.

IMMUNOPATHOGENESIS OF RHEUMATOID ARTHRITIS AND THE PROSPECTS OF CELL-BASED THERAPY

Although the etiology of RA is not fully known, its autoimmune nature has been widely recognized. An interplay between genetic predisposition and environmental factors, such as smoking and microbial infections, are thought to trigger the development of the disease (33). The contribution of genetic factors to RA pathogenesis is mainly attributed to certain HLA alleles of the major histocompatibility complex (MHC) class II (34). Besides presenting antigen peptides to CD4+ T cells in the periphery, HLA class II molecules are also responsible for the selection of the CD4+ TCR repertoire in the thymus and thus control the release of autoreactive cells (35, 36). A consensus amino acid motif in the P4 peptide-binding pocket of the β 1 subunit of the HLA-DR molecule, denoted “shared epitope” (SE), is a major risk factor of RA (37, 38). Among HLA-DR alleles containing the SE, DRB1*0401, and *0101 have been described most extensively in the context of RA (39, 40). Various peptides derived from endogenous joint proteins, such as type II collagen (CII), cartilage proteoglycan aggrecan, and human cartilage glycoprotein (HCgp)-39, have been shown to bind to SE-containing HLA-DR molecules and to be specifically recognized by T lymphocytes from RA patients (13, 41–44). Particularly, peptides post-translationally modified by citrullination bind with high affinity to the SE, initiating citrulline-specific T and B cell responses (45, 46). Citrullination is catalyzed by peptidylarginine deiminases (PAD), which convert the amino acid arginine into citrulline, leading to a loss of positive charge that might render self-peptides immunogenic (47). Several citrullinated peptides, including fibrinogen, vimentin, α -enolase, aggrecan, and CII are present in RA joints and are targets of lymphocyte responses in RA patients carrying the SE (48, 49). Antibodies directed against citrullinated proteins/peptides (ACPAs) are specific to RA and associated with the presence of the SE and with increased disease severity (50, 51).

Antigen-presenting cells, particularly DCs, are key players in the initiation and maturation of the autoimmune response in RA (52). DCs can activate self-reactive CD4+ T cells by presenting autoantigens in the context of MHC class II molecules and providing costimulatory and pro-inflammatory signals (53–55). Activated autoreactive CD4+ T cells differentiate into inflammatory T helper (Th) cell subsets producing either interferon (IFN)- γ and tumor necrosis factor (TNF) (Th1), IL-17 and IL-21 (Th17), or a mixed cytokine profile (Th1/17), and accumulate in the inflamed joint (56–58). These autoreactive T cells drive the differentiation of B lymphocytes into plasma

Abbreviations: AA, adjuvant-induced arthritis; ACPAs, anti-citrullinated protein/peptide antibodies; AIA, antigen-induced arthritis; BLT, bone marrow-liver-thymus transplant; BRG, BALB/c-*Rag2*^{null} *IL2rg*^{null}; CII, type II collagen; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; FOXP3, forkhead-box-protein P3; gp, glycoprotein; GvHD, graft vs. host disease; HLA, human leukocyte antigen; HSCs, hematopoietic stem and progenitor cells; Ig, immunoglobulin; IL, interleukin; IFN, interferon; MHC, major histocompatibility complex; MMC, mutated mouse collagen; NK, natural killer; NOD, non-obese diabetic; NOG, NOD-SCID *Il2rg*^{tm1Sug}; NRG, NOD-*Rag1*^{null} *Il2rg*^{null}; NSG, NOD-SCID *Il2rg*^{tm1Wjl}; PAD, peptidylarginine deiminase; PBMCs, peripheral blood mononuclear cells; PG, proteoglycan; PGIA, Proteoglycan-induced arthritis; RA, rheumatoid arthritis; *Rag1/2*, recombination-activating genes 1/2; SCID, severe combined immunodeficiency; SE, shared epitope; SFMCs, synovial fluid mononuclear cells; T1D, type 1 diabetes; TCR, T cell receptor; Tg, transgenic; Th, T helper; TNE, tumor necrosis factor; tolDC, tolerogenic dendritic cell; Treg, regulatory T cell.

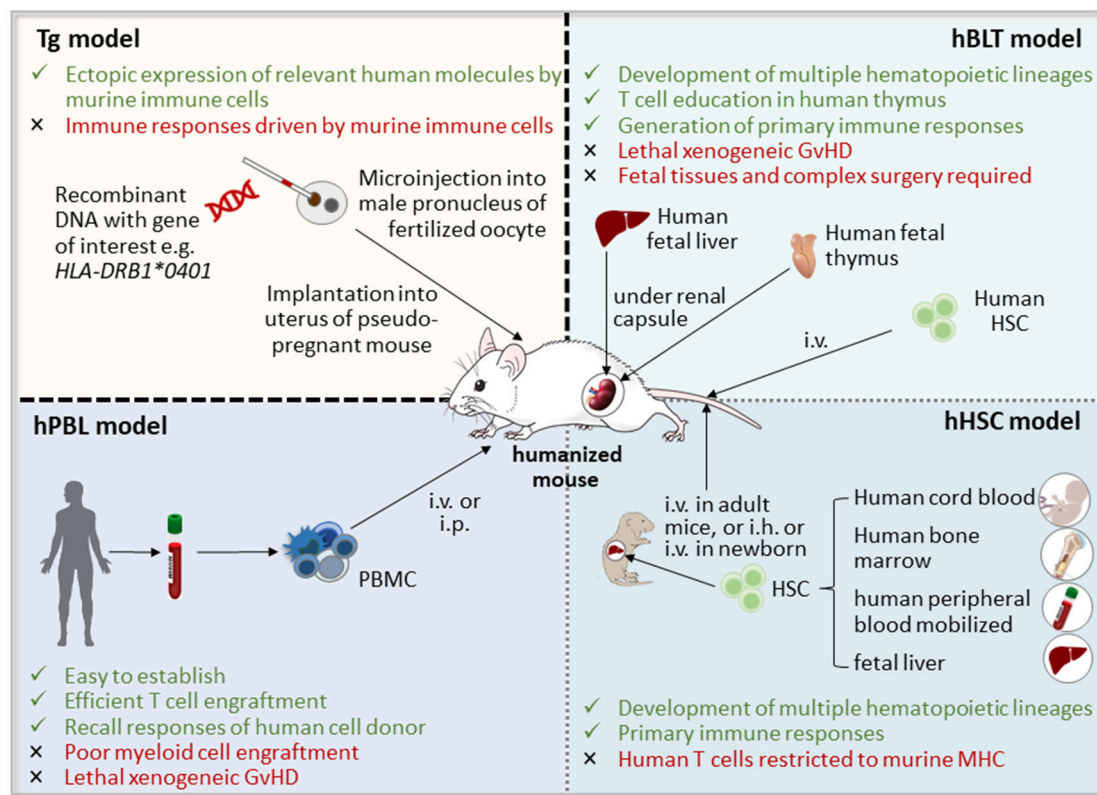


FIGURE 1 | Strategies for the generation of humanized mice [adapted from Shultz (30) and Hahn et al. (31)]. Humanized mice can be obtained by the introduction of human transgenes, such as human leucocyte antigen (HLA) class II molecules, which will then be expressed by mouse immune cells, or by the generation of mouse/human chimera through implantation of human cells, including hematopoietic stem and progenitor cells (HSCs) and peripheral blood mononuclear cells (PBMCs), and/or human tissues, such as fetal liver and thymus, into immunodeficient mice. GvHD, graft vs. host disease; hBLT, human bone marrow-liver-thymus-engrafted; hPBL, human peripheral blood lymphocyte-engrafted; i.h., intrahepatic; i.p., intraperitoneal; i.v., intravenous; Tg, transgenic mice.

cells producing autoantibodies such as ACPAs (59), which in turn promote osteoclast differentiation and activation, leading to cartilage and bone erosion (33). Autoreactive CD4⁺ T cells also stimulate macrophages and synovial fibroblasts to secrete pro-inflammatory mediators, including TNF, IL-1, and IL-6, which contribute to synovial inflammation through recruitment of immune cells and expansion of synovial fibroblasts, forming an invasive pannus (60, 61).

Current treatment options of RA involve non-steroidal anti-inflammatory drugs, glucocorticoids and disease-modifying anti-rheumatic drugs, either conventional, such as methotrexate, targeted, like Janus kinase inhibitors, or biologics, including antagonists of proinflammatory cytokines, modifiers of T cell co-stimulation and B cell-depleting antibodies (1, 62). Besides the fact that a proportion of patients remain refractory to treatment (63), none of these drugs provides a cure of RA, requiring life-long treatment, associated with a progressive loss of efficacy, toxicity, and the appearance of serious adverse effects (64–66). A promising strategy to restore self-tolerance and thereby achieve long-term remission, is the depletion or reprogramming of autoreactive T cells by regulatory T (Treg) cells or tolerogenic DCs (tolDCs) (25, 67, 68). Naturally

occurring CD4⁺ Treg cells are characterized by the constitutive expression of the transcription factor FOXP3 and IL-2 receptor α -chain (CD25). Treg maintain immunological tolerance and prevent autoimmune diseases by suppressing the activation and proliferation of self-reactive effector T cells (67). Although Treg cells are present in the synovial fluid of RA patients, these Treg cells fail to inhibit Th1 responses (69, 70). Strategies targeting Treg cells for the treatment of RA include (i) the expansion of autoantigen-specific naturally occurring Treg cells *in vivo*, (ii) their propagation *in vitro* and subsequent transfer back into the host, and (iii) the conversion of antigen-specific T cells into Treg cells *in vitro* or (iv) *in vivo* (67). Dendritic cells (DCs) are professional antigen-presenting cells that instruct T cells, according to the surrounding environment, to mediate immune responses or tolerance. TolDCs with immunoregulatory properties can be generated *in vitro* from monocytes or hematopoietic stem cells and are able to control aberrant CD4⁺ T cell responses through the induction of anergy, conversion of T effector into Treg cells, or deletion of autoreactive T cells (71–74). An important advantage of tolDC- or Treg-based therapy over conventional treatment of RA is its potential to modulate immune responses in an antigen-specific manner,

which might permit a selective downregulation of autoreactive lymphocyte responses while avoiding a general shutdown of immunity against pathogens. Both Treg cell and tolDC-based approaches have been extensively tested in conventional mouse models of RA-like disease (75) and the safety of tolDCs has even been approved in phase I/II clinical trials (76, 77). Nevertheless, sophisticated mouse models that accurately recapitulate human RA are still missing. Humanized mouse models of RA might help to predict the efficacy and side effects of cell-based approaches in further clinical trials, as well as to adjust parameters, such as dose, injection route, and required dosing interval.

CONVENTIONAL MOUSE MODELS OF RHEUMATOID ARTHRITIS AND THEIR LIMITATIONS

Numerous rodent models of RA are available, each of which mirrors particular aspects of the disease (4, 6). These conventional models represent classic hallmarks of RA, such as joint swelling, synovitis, pannus formation, and bone erosion, but differ in the mechanisms of induction and launched immune processes, as well as in their speed of onset, chronicity, and severity (6, 78). A distinction is made between induced and spontaneous models. In induced models, non-specific immune activation, cartilage-directed autoimmunity, or abundant exogenous/infectious triggers cause RA-like disease, while in spontaneous models, arthritis develops without deliberate immunization and is non-limiting, providing a chronic situation like in human RA (5, 79, 80). The most frequently used models are introduced below.

Induced Rodent Models of RA-like Disease

Adjuvant arthritis (AA) was the first described animal model of RA and can be induced by a single intradermal injection of complete Freund's adjuvant (CFA), containing heat-inactivated mycobacteria, at the base of the tail in Lewis rats (81) or by repetitive intra-articular CFA injection in DBA/1 or C57BL/6 mice (82). The hallmark of AA is its rapid onset and progression to polyarticular inflammation, leading to a chronic erosive disease with severe joint malformation (6). The disease is driven by CD4⁺ T cells (83) and susceptibility to develop AA is related to MHC and non-MHC genes (84). Originally, it was assumed that mycobacterial components, such as 65k heat shock protein, cross-react with self-antigens from joint cartilage in this model (85). However, it has been shown that nonimmunogenic adjuvants such as avridine, muramyl dipeptide, pristane, and incomplete Freund's adjuvant also induce AA in many rat strains and mice, indicating that adjuvants may enhance autoreactivity to articular antigens (83, 86–88). Unlike in human RA, the AA model displays not only bone erosion, but also bone apposition at early stages of the disease with limited to no cartilage damage (79).

Collagen-induced arthritis (CIA) is the most commonly used model of RA-like disease (89). In this model, severe joint inflammation is induced through immunization with CII, a major component of hyaline cartilage, together with CFA

(6, 90). Susceptibility to CIA is related to the murine MHC class II molecule H-2^q whose peptide-binding pocket has a similar primary structure like the SE of RA-associated HLA-DR molecules (91, 92). Although several mouse strains are susceptible to CIA, the DBA/1 strain is the “gold standard” of this model (90). Autoreactive CD4⁺ T cells are required for the induction of CIA (7, 93, 94), synovial proteins are subjected to PAD-induced citrullination and an association of anti-CII antibodies and ACPA to the development of arthritis has been described (95, 96). The passive transfer of polyclonal immunoglobulin (Ig) G from sera of CIA mice or monoclonal anti-CII antibodies induces arthritis even in mouse strains that are not susceptible to CIA, indicating an important role of autoantibodies in the effector phase of the disease (97, 98). Important limitations are that CIA is an acute model, in which remission occurs at 10–14 days after disease onset (99), extra-articular manifestations are due to CFA (100) and disease severity is highly variable and dependent on environmental factors, such as grouping stress (101). Joint inflammation in CIA mice is particularly mediated by Th17 cells (102), while the pathogenesis of human RA involves Th1, Th17, and Th1/17 cells (56–58).

Proteoglycan-induced arthritis (PGIA) is induced by immunization with human cartilage proteoglycan aggrecan in susceptible BALB/c or C3H mice strains (12). The progressive chronic polyarthritis in the PGIA model shares features with human RA such as deposition of immune complexes in the joint and the presence of rheumatoid factor autoantibodies (6, 12). Susceptibility to PGIA in BALB/c mice is associated with the presentation of the dominant epitope aggrecan 89–103 by I-A^d MHC class II molecules (103). PGIA development is dependent on B cells, which present antigen and produce anti-PG antibodies, as well as on CD4⁺ T cells, which provide help to B cells for antibody production. Antibodies are cross-reactive between human proteoglycan, used for immunization, and self (mouse) cartilage proteoglycan (104, 105). The choice of adjuvant and route of immunization determine the prevalent Th subset in the PGIA model. Immunization with CFA and subcutaneous injection route result in predominant Th17 responses, whereas dimethyldioctadecyl-ammonium bromide (DDA) as adjuvant and intraperitoneal injection induce more Th1 cells (106, 107).

Antigen-induced arthritis (AIA) is triggered by the injection of exogenous antigen, such as methylated bovine serum albumin (mBSA), into the joint (108). This robust model of destructive bystander arthritis develops almost independently of the genetic background of mice and leads to a moderate and limiting local disease involving both T cells and immune complexes (79, 109).

RA-like disease can also be passively transferred to naïve mice by injecting serum antibodies against endogenous CII, PG, or glucose-6-phosphate isomerase (GPI) (79, 98, 110, 111). These immune complex arthritis models are almost independent of the genetic background of mice and resemble the effector phase of the disease, in which excessive immune complex formation at joint tissues triggers complement activation and the release of inflammatory mediators through Fcγ receptor engagement on phagocytes, leading to rapid onset, but transient destructive

arthritis (78). Administration of pathogenic immunoglobulins is sufficient to confer disease and does not require T or B lymphocytes, nevertheless, T cells have an enhancing effect on autoantibody-induced arthritis (112).

Spontaneous Mouse Models of RA-like Disease

Spontaneous models include genetically modified mice, such as SKG, K/BxN, human TNF transgenic (Tg) and IL1ra^{-/-} mice (5, 80). In SKG mice, arthritis development is attributed to a missense mutation in the TCR signaling adaptor molecule ZAP70, leading to a defective negative selection in the thymus and the release of autoreactive T cells (113). SKG mice establish arthritis following stimulation of the innate immune system with zymosan or other microbial triggers, while housing under pathogen-free conditions or treatment with antibiotics completely block arthritis development (114, 115).

The K/BxN model was discovered coincidentally by crossing KRN mice, which express a transgenic TCR, specific for an epitope of bovine pancreas ribonuclease, with autoimmune-prone non-obese diabetic (NOD) mice. The offspring, referred to as K/BxN, spontaneously developed severe arthritis (116), driven by the activation of T cells expressing the KRN-derived TCR, which, due to cross-reactivity, recognize the ubiquitously expressed self-protein GPI bound to the NOD-derived MHC class II molecule I-A^{g7} (117, 118). Activated autoreactive T cells promote polyclonal B cell activation and T helper cell-dependent production of GPI-specific IgG autoantibodies (110, 119). Transfer of serum IgG from arthritic K/BxN mice has been shown to induce robust and reproducible arthritis in healthy mice, indicating that autoantibodies might directly trigger joint inflammation (110, 120). However, it must be considered that GPI might not be an essential or RA-specific autoantigen.

TNF transgenic (Tg) mice, which constitutively express human TNF, spontaneously develop inflammatory, highly erosive polyarthritis, similar to human RA, which can be completely prevented by treatment with monoclonal antibodies against human TNF (121). When these TNF-overproducing mice were crossed to a severe combined immunodeficiency (SCID) background, lacking mature B and T cells, inflammatory arthritis still develops, indicating that TNF acts downstream of lymphocyte responses in this model (122). Thus, TNF Tg mice are not suitable for testing of therapeutic approaches that target lymphocyte responses in RA.

Mice overexpressing human IL-1α Tg present chronic destructive polyarthritis, characterized by hyperplasia of the synovial lining, pannus formation and cartilage destruction (123). However, pathogenesis of arthritis in hIL-1α Tg mice is mediated by monocytes/macrophages and activated neutrophils, while T and B lymphocytes are sparse (123). In an opposite approach, deletion of IL-1 receptor antagonist (IL-1ra), an endogenous inhibitor of IL-1 signaling, leads to a spontaneous arthritis model in which T cells are main actors (10, 124). Increased levels of antibodies against IgG (rheumatoid factor), CII and double-stranded DNA also point to the development of an autoimmune response in IL1ra^{-/-} mice (124).

Achievements and Obstacles of Testing Cell-Based Strategies in Conventional Mouse Models of Rheumatoid Arthritis

Cell-based therapeutic approaches have mainly been tested in induced models of RA-like disease (75). Adoptive transfer of antigen-specific Treg, either expanded from natural occurring Treg, or induced from conventional T cells, e.g., by enforcing the expression of the Treg-specific transcription factor FoxP3 or reprogramming through tolDCs, has been shown to suppress the progression of arthritis in models of CIA, PGIA and AIA (125–129). Therapeutic effects of tolDCs, differentiated *in vitro* from precursors and genetically modified to express IL-4, or modulated by either short stimulation with bacterial lipopolysaccharide (LPS), vasoactive intestinal peptide, a combination of dexamethasone, vitamin D3 and MPLA, or the NF-κB inhibitor Bay11-7082, have been reported in mouse models with established CIA and AIA (26, 27, 130–132). However, testing of tolDC-based therapeutic strategies in conventional mouse models is limited by the fact that, due to technical constraints, administered murine DCs are generated from bone marrow progenitors, while monocyte-derived DCs are used for clinical applications in humans (25). It is evident that bone marrow- and monocyte-derived DCs represent different DC subsets which might have an altered or even opposite effect on the disease *in vivo*.

A common weakness of all conventional models of RA is that inflammatory responses are completely mediated by the murine immune system, which differs in its components and organization from the human immune system. Discrepancies in innate and adaptive immune mechanisms between both species include the composition of leukocyte subsets in peripheral blood, Toll-like receptors, Fc receptors, Ig subclasses, B cell and T cell signaling pathways, γδ T cells, response to IFN-γ, cytokines and cytokine receptors, expression of costimulatory and adhesion molecules, chemokine and chemokine receptor expression, as well as expression of receptors involved in the uptake of phagocytic cargo, production of reactive oxygen species and IL-1 by monocyte subsets, among others (21, 22, 133). While Foxp3 defines the Treg population in mice, human FOXP3+ T cells are heterogeneous in their function (67). In response to stimulation, human CD4+ effector T cells express MHC class II molecules (134) and upregulate FOXP3 without acquiring immunoregulatory functions (135), while mouse T cells do not. In CIA mice, γδ T cells are the predominant source of IL-17 in affected joints, but they are nearly absent in joints of RA patients, where Th1 cells dominate (136). Another difference between conventional animal models of RA-like disease and human RA is their lack of gender bias (137).

Due to these species-specific differences, it is not surprising that several promising therapeutic principles found in mouse models do not work in humans (15, 16, 20). This hampers the translation of experimental data obtained from conventional murine models of RA-like disease to clinical applications in patients and creates a demand for humanized models which accurately mimic the human disease (Table 1).

TABLE 1 | Comparison between conventional and humanized mouse models of rheumatoid arthritis.

Features	Model	Conventional mouse models of RA	Humanized mouse models of RA	
			Transgenic mice	Mouse/human chimera
Autoimmune response-driving cells		Mouse immune cells	Mouse immune cells (with ectopic expression of human Tg)	Human T and B cells, mouse macrophages and granulocytes
Relevant MHC molecules		Mouse MHC (CIA: H-2 ^d ; PGIA: I-A ^d ; K/BxN: I-A ^{g7})	Human MHC/HLA class II (DR1, DR4 or DQ8)	HLA alleles of the human cell donor
Involved antigen		Articular, non-articular or exogenous antigen	Restriction to already known antigen epitopes	Multiple synovial antigens
Autoantibodies		Only in induced models and K/BxN mice	Yes	Yes (except HSC-engrafted mice)
Disease development		Induced or spontaneous	Induced by immunization with antigen (and adjuvant)	Spontaneous (except HSC-engrafted mice)
Disease incidence		Moderate to high	Variable (14–100%), depending on the genetic background of mice	Variable, depending on the type of the human graft and disease status of the donor
Disease onset		Rapid (induced models, K/BxN) or slow (SKG, IL1ra ^{-/-})	Rapid	Rapid
Disease severity		Moderate to severe	Severe	Dependent on the human donor
Disease duration		Self-limiting (induced models, except PGIA) or chronic (spontaneous models)	Self-limiting or chronic	Limited by onset of GvHD
Gender bias		No (except CIA: male bias, in contrast to human RA)	Yes, more frequent in female mice	Yes, corresponding to human cell donor
Dependence on strain		Yes (except AIA, TNF Tg, IL1ra ^{-/-})	No	Yes, immunodeficient strains lacking T, B, and NK cells
Value as pre-clinical model for testing cell-based therapies		Testing of approaches based on the murine cell-equivalent with limited predictive value for clinical application	Testing of approaches based on murine cells expressing relevant human MHC class II or TCR; Restricted to well-defined antigen epitopes	Testing of human cell-based therapies in a human cell environment within mice; closest approximation to clinical application in patients

AIA, antigen-induced arthritis; CIA, collagen-induced arthritis; MHC, major histocompatibility complex; GvHD, graft vs. host disease; HLA, human leukocyte antigen; HSCs, hematopoietic stem and progenitor cells; PGIA, proteoglycan-induced arthritis; TCR, T cell receptor.

TRANSGENIC MICE AS HUMANIZED MODELS OF RHEUMATOID ARTHRITIS

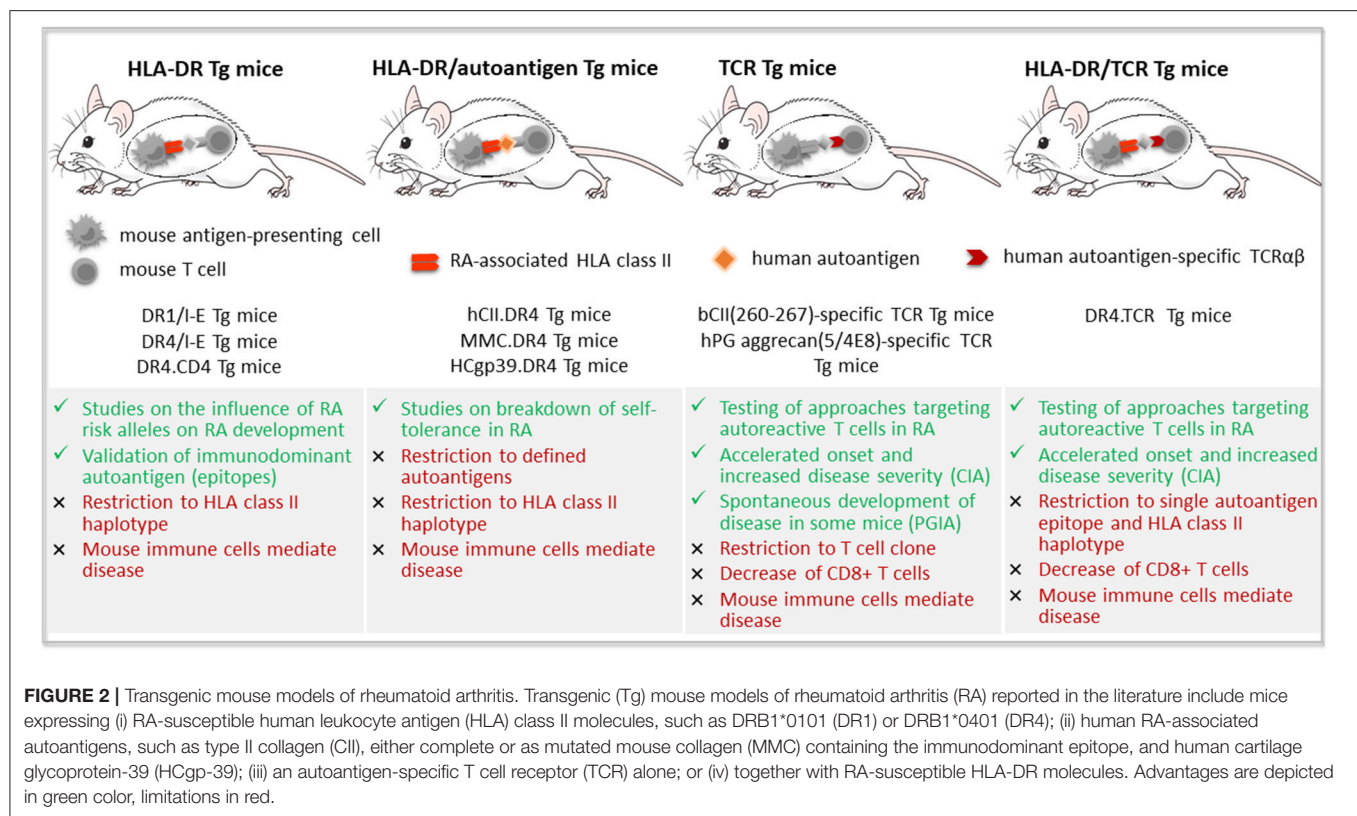
This humanization strategy is based on transgenic expression of human molecules, such as HLA class II, RA-associated synovial autoantigens and/or an autoantigen-specific T cell receptor, in immunocompetent mice (**Figure 2**).

Human Leukocyte Antigen Class II Transgenic Mouse Models

The advent of mice that lack endogenous MHC class II molecules and express human HLA transgenes instead has significantly advanced the understanding of the importance of certain HLA class II alleles, particular those containing the SE, in the development of RA (137). The basis for HLA class II transgenic mice was created in 1991, with the generation of a mouse with disrupted expression of the murine MHC class II genes I-A and I-E, lacking mature CD4⁺ T cells (138). First attempts to introduce human HLA class II transgenes in these murine MHC class II-deficient mice resulted in low CD4⁺ T cell selection, due to poor interaction between mouse CD4 and human HLA class II molecules (139). To circumvent this problem, two strategies have

been pursued: generation of double transgenic mice expressing the human HLA gene in all antigen-presenting cells and human CD4 in all T cells (140–142), or introduction of a chimeric HLA/I-E molecule composed of the peptide-binding domain from human DR and the CD4-binding domain derived from mouse I-E (143, 144). This way, the HLA class II transgene was able to positively select CD4⁺ T cells expressing diverse V β TCRs, resulting in normal development of the CD4⁺ T cell compartment and maintenance of peripheral tolerance to transgenic HLA molecules (137).

The first strategy was used by Fugger and colleagues, generating HLA-DRA1*0101/-DRB1*0401 (DR4)/human CD4 Tg mice which did not develop spontaneous autoimmune diseases, but were susceptible to CIA (140, 142). Rosloniec and coworkers followed the second approach to establish Tg mice expressing chimeric HLA-DRB1*0101 (DR1)/I-E or DR4/I-E, which developed severe autoimmune arthritis following immunization with bovine or human CII, accompanied by strong DR1 and DR4-restricted T and B cell responses, respectively (13, 145). The incidence of CIA varied from 14 to 100%, according to the introduced human HLA-DR transgene, the genetic background of mice, and the applied immunization



protocol (13, 142, 146). In both DR1 Tg and DR4 Tg mice, T cell response was focused to position 259–273 of human CII, suggesting that different RA-associated HLA-DR molecules containing the SE bind and present the same immunodominant epitope of CII (13, 145). Even though both HLA-DRB1*0401 (RA-susceptible) and *0402 (RA-resistant) molecules present CII and its derived peptide, only *0401 Tg mice develop a pro-inflammatory response (146). Furthermore, comparison of T cell polarization and immune responses between *0401 and *0402 Tg mice encouraged the hypothesis that the SE selects T cells with a predetermined Th17-biased cytokine profile that efficiently clear infections, but may, on the other hand, drive autoimmunity in response to pathogens or certain environmental factors (147). Interestingly, Tg mice carrying an RA-susceptible haplotype mimic the gender-bias of RA, displaying an increased susceptibility to develop the disease as well as a stronger cellular and humoral response to CII in females (146).

To date, DR4 Tg or DR1 Tg mice have been widely used to identify and validate immunodominant T cell epitopes of synovial autoantigens, such as CII, HCgp-39, proteoglycan aggrecan, fibrinogen, and vimentin, as well as to investigate the role of posttranslational modifications on epitope binding (13, 148–151). In DR4 Tg mice immunized with citrullinated (cit)-vimentin peptide aa 59–78, specific Th1 cells and strong proliferative recall responses were detected, however, mice did not show any signs of arthritis (150). In contrast, immunization of DR4 Tg mice with human cit-fibrinogen induced vigorous

citrulline-specific T and B cell responses in all DR4 Tg experimental mice and triggered arthritis, characterized by synovial hyperplasia and ankylosis, in 35% of these animals (149). Transfer of splenic lymphocytes from arthritic mice induced joint swelling in the human cit-fibrinogen-injected limb of all DR4 Tg recipient mice, but not in PBS- or unmodified human fibrinogen-injected limbs (152). The fact, that administration of cit-fibrinogen to wild-type C57BL/6 mice and immunization of DR4 Tg mice with native fibrinogen failed to induce arthritis and citrulline-specific reactivity, underscores the critical role of both SE-containing HLA-DR molecules and antigen citrullination in the development of RA (149). Another study in DR4 Tg mice corroborated that immune responses to homocitrulline-containing peptides are similarly dependent on the presence of the SE and later evolve into immune responses to citrullinated antigens (153).

With the advent of tetramer technology, it has become possible to identify, track, and characterize DR-restricted autoantigen-specific T cells in DR1 Tg and DR4 Tg mice, and to corroborate their pathogenic role in the development of RA (154–156). Activated CII-specific T cells expressing high levels of Th1, Th17 and pro-inflammatory cytokines have been detected in arthritic joints of DR1 Tg and DR4 Tg mice following immunization with CII (154, 155). Although CD4+ T cells represent only a minor population of the synovial infiltrate, they were shown to display a highly restricted TCR repertoire and limited clonality and their expansion correlated with onset and severity of arthritis as well as with anti-CII antibody levels

(154, 157). It is important to note, that, at the time of first clinical signs of arthritis, activated HLA-DR4:CII(158–170) tetramer-positive cells disappeared from synovial fluid and were rarely found in blood, while they persisted in lymph nodes, suggesting that autoreactive T cells play a role particularly in the early stages of arthritis, by triggering a local immune response (154, 157). This important finding, derived from studies in DR4 Tg mice also suggests a rationale for the lack of enrichment of antigen-specific T cells in the synovial fluid of patients with established RA (171).

DR1 and DR4 Tg mice have also been used to test alternative treatments for RA, such as synthetic analog peptides, which contain substitutions in critical positions of the CII immunodominant epitope and suppress inflammatory arthritis by promoting regulatory T cell responses (172, 173). Treatment of human cit-fibrinogen-immunized DR4 Tg mice with CTLA-4Ig fusion protein, a soluble form of the CTLA-4 receptor which inhibits T cell activation by competing with CD28 for binding to the costimulatory ligand CD80/CD86 (174), restrained the activation of cit-fibrinogen-specific T cell responses, and halted the progression of arthritis (152). The fact that splenocytes from cit-fibrinogen-immunized mice treated with CTLA-4Ig were unable to transfer arthritis to recipient mice supports a direct role of activated citrulline-specific T cells in arthritis development and progression (152).

The contribution of HLA-DQ polymorphism to RA susceptibility and severity has been explored by David's group, who established mice which express transgenic DQA1*0301/DQB1*0302 (DQ8) (175). In humans, HLA-DQB1*03 occurs in linkage with the RA risk locus HLA-DRB1*04 and has been suggested to affect the clinical expression of RA (176–178). Immunization with heterologous CII induced autoreactive T and B cell response and severe inflammatory arthritis in 70% of DQ8 Tg animals (179), while of mice expressing a protective DQ6 Tg, only 14% presented mild arthritis and 60% of Tg mice with mixed DQ8/DQ6 haplotype developed moderate CIA in response to CII (180). DQ8 Tg mice with deleted CD4 did not develop arthritis, while CD8 deficient DQ8 Tg mice developed severe CIA along with increased autoantibody levels, suggesting that CD4+ T cells, but not CD8+ T cells are indispensable for initiation of CIA (181). In the absence of B cells, DQ8 mice failed to develop CIA, suggesting that B cells are not only important for autoantibody production, but also for antigen presentation to autoreactive T cells (146). To simulate the situation in humans, where HLA-DR and -DQ occur in linkage, Taneja and coworkers established mice that express DR4 (either RA-susceptible *0401 or RA-resistant *0402) along with DQ8 transgene (182). Indeed, CIA incidence was increased in *0401.DQ8 double Tg mice compared to single Tg mice, while *0402.DQ8 Tg mice were resistant to CIA (182).

The link between smoking and the emergence of RA in individuals with an RA-associated HLA haplotype has been reproduced in DR4 and DQ8 Tg mice (183). Exposure to cigarette smoke augmented PAD enzyme expression and enhanced pro-inflammatory Th1 and antibody responses to native and citrullinated CII and vimentin in both DR4 and DQ8 Tg mice, while promoting Th2/Treg responses in Tg mice that

expressed protective DRB1*0402 (183, 184). An association between periodontal disease and RA has been studied on DR1 Tg mice, in which oral exposure to *Porphyromonas gingivalis* led to increased percentage of Th17 cells in blood and lymph nodes, systemic pro-inflammatory cytokine response, loss of bone density, and generation of anti-citrullinated protein antibodies (185).

Human Autoantigen Transgenic Mouse Models

The breakdown of immune tolerance to endogenous cartilage antigens is a central element in the development of RA. Immunization of CIA-susceptible mouse strains with non-self (e.g., human, bovine, chicken, or rat) CII induces severe arthritis whereas mice are less prone to CIA induction with self (mouse) CII (186). This is due to minor but decisive structural differences between mouse and human CII, which are located in the immunodominant T cell epitope CII(158–170, 187, 188), containing glutamic acid at position 266 in human (as well as bovine, chicken and rat) CII, whereas in mouse CII there is an aspartic acid instead (189).

In mice which express the entire human CII protein, or its immunodominant human (h)CII(158–170, 187, 188) epitope within a mutated mouse CII (MMC) protein, only autoreactive T cells should become activated upon immunization with human or rat CII. Indeed, although hCII Tg mice with cartilage-restricted expression of hCII generally mounted autoreactive T and B cell responses following immunization with hCII, only 10% of these mice developed arthritis (186). In this model, T cell tolerance was shown to depend on the expression level of hCII and its immunodominant epitope (186). In B10 mice expressing DR4 transgene together with either hCII or MMC, murine DR4-restricted T cells were tolerized against self-CII, and thus these mice were uniformly resistant to CIA (151, 190). In contrast, T cell tolerance to self-CII was incomplete in MMC.DR4 Tg mice with C3H background, rendering these mice prone to develop CIA (151). Tolerance to self-CII could be broken in B10.DR4 mice expressing MMC, but not hCII Tg, by introducing a mutation in the *Ncf1* gene, which encodes a subunit of the NADPH oxidase complex, resulting in reduced ROS production (151, 191).

Human Autoantigen-Specific T Cell Receptor Transgenic Mouse Models

Mice that express a transgenic TCR $\alpha\beta$ specific for an arthritogenic epitope of human autoantigen, such as CII or proteoglycan aggrecan, were used to gain more insight into the role of antigen-specific T cells in the development of autoimmune arthritis (192, 193). In such TCR Tg mice, more than 90% of CD4+ T cells express the antigen epitope-specific TCR transgene (192, 193).

TCR Tg mice, carrying the rearranged V α 11.1 and V β 8.3 chain encoding genes specific to bovine CII, respond vigorously to stimulation with bovine CII or its immunodominant determinant CII(158–164, 188) *in vitro*, but do not develop spontaneous arthritis (192, 194). However, an accelerated onset

and increased severity of arthritis was observed in CII-specific TCR Tg mice after immunization with CII in CFA, as compared to their non-Tg littermates (192, 194). Interestingly, Treg cell clones generated from splenocytes of CII-specific TCR Tg mice reduced the proliferation of CII-specific effector T cells *in vivo* and decreased the incidence and clinical symptoms of arthritis after adoptive transfer in CIA and collagen antibody-induced arthritis models (195).

Another TCR-Tg mouse model was generated by introducing TCR V α 1.1 and V β 4 chains of a T cell hybridoma with MHC class II-restricted specificity for the immunodominant epitope (5/4E8) of human cartilage proteoglycan aggrecan into PGIA-susceptible BALB/c strain (193). In these TCR Tg mice, a single dose of human proteoglycan, even in the absence of adjuvant, produced disease (193), which is in contrast to the conventional PGIA model, that requires multiple immunizations with human proteoglycan in adjuvant (12).

An elegant strategy has been to combine the expression of RA-associated DR1 and a transgenic V α 2/V β 8.1 TCR, recognizing the immunodominant determinant of bovine and human CII (196). These double Tg mice developed an accelerated and more severe form of CIA than their DR1 Tg littermates and provide a more reliable tool for testing novel therapeutic approaches (196).

TCR-Tg models support the hypothesis that antigen-specific T cells play a critical role in the initiation of arthritis. However, important limitations are the restriction to a single autoantigen epitope and the decrease of the CD8⁺ T cell pool in these mice (192–194).

In an ideal Tg mouse model of autoimmune arthritis, combined expression of RA-susceptible HLA-DR molecules, an RA-relevant autoantigen and a specific TCR, recognizing the immunodominant epitope of this autoantigen, would lead to spontaneous breakdown of self-tolerance and the development of arthritis. This has not been achieved so far, however, studies in Tg mouse models of RA have underscored the critical role of the SE, post-translational modification of antigen and self-reactive T cells in the development and progression of RA and contributed substantially to the identification and validation of immunodominant autoantigens and its T cell epitopes. Thus, Tg mice represent a decisive step forward in RA research and have undoubtedly enhanced the predictive value of mouse models in preclinical tests. However, it must be considered, that many of the herein presented models have been only established in one laboratory or published once. The poor reproducibility under different laboratory conditions might be at least partially explained by distinct microbial communities. Another problem is that human DR transgenes have been shown to be poorly expressed by DCs in some strains, resulting in poor antigen presentation. Finally, Tg mouse models still bear the same limitation as conventional models of RA-like disease, that is, inflammation is driven by the murine immune system that ectopically expresses SE-containing HLA class II molecules or a specific TCR but might contribute in a different way to the development of the disease.

MOUSE/HUMAN CHIMERAS AS HUMANIZED MODELS OF RHEUMATOID ARTHRITIS

Immunodeficient Mice as a Platform for the Engraftment of Human Cells and Tissues

An important advance in the generation of a preclinical mouse model of RA would be the establishment of a functional human immune system or some of its components in mice, in which they mount autoimmune responses and clinical features of RA. For this purpose, human hematopoietic stem and progenitor cells (HSC), peripheral blood mononuclear cells (PBMC), or tissue have been engrafted into immunodeficient mice, which lack the ability to reject xenografts and thus enable stable reconstitution with human cells (Figure 1).

Important achievements of the last decades have paved the way for the stable engraftment of human cells within the murine host. The *Prkdc^{scid}* (protein kinase, DNA activated, catalytic polypeptide) mutation in CB17 mice, commonly denoted “SCID” (severe combined immunodeficiency) (197), results in reduced numbers of functional T and B cells and thus enables limited and transient engraftment of human PBMCs, HSCs and fetal hematopoietic tissues (198–200). However, spontaneous generation of mouse T and B cells with aging, referred to as leakiness, as well as high levels of host natural killer (NK) cells and innate immune activity impede the stable engraftment of human cells and tissues in SCID mice (29). Alternatively, targeted mutations of the recombination-activating genes 1 (*Rag1*) and *Rag2* prevent the development of functional T and B cells in mice without causing leakiness (201, 202). Nevertheless, *Rag1/2*-deficient mice retain high levels of NK-cell activity, allowing only limited engraftment of human HSC (29).

An important step forward was accomplished by crossing NOD and SCID strains (203). NOD-SCID mice display additional defects in innate immunity, including the absence of complement C5, impaired macrophage cytokine production, antigen presentation and NK cell function (29, 203, 204). Although they have increased engraftment of human HSCs and PBMCs (205, 206), the limitations of NOD-SCID mice include relatively short life span due to thymic lymphomas, and residual activity of NK cells and innate immunity (29).

A decisive breakthrough was achieved by the generation of immunodeficient mice with a mutation in the *Il2rg* gene (207–210), encoding IL-2 receptor γ subunit (IL-2R γ), also denominated gamma-chain, required for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 signaling (211). An absent or truncated IL-2R γ -chain leads to defective T and B cell development, affects innate immunity and completely abolishes NK cell generation (212, 213). The main immunodeficient mouse strains bearing the *Il2rg* mutation are NOD-SCID *Il2rg^{null}*, including NOD-SCID *Il2rg^{tm1Wjl}* (NSG) and NOD-SCID *Il2rg^{tm1Sug}* (NOG), as well as NOD-*Rag1^{null} Il2rg^{null}* (NRG) and BALB/c-*Rag2^{null} Il2rg^{null}* (BRG) (208, 209, 214, 215). These strains support high engraftment of human tissue, HSCs and PBMCs (207, 208, 216), without the need for previous myeloablation through irradiation or drugs, thus reducing the required donor cell

number and prolonging survival of humanized mice (217). Engrafted HSCs differentiate into multiple lineages of human cells, including erythrocytes, platelets, T and B lymphocytes, NK cells, DCs, monocytes/macrophages and granulocytes (207–209, 215, 218). Reconstitution of the human immune system is most efficient, when CD34+ HSCs from cord blood or fetal liver (rather than from adult peripheral blood) are injected into newborn mice, where both donor cells and recipient are set for development and expansion of the hematopoietic system (215, 219). Co-transplantation of HSCs with fetal liver and/or fetal thymus (abbreviated BLT for bone marrow, liver, thymus) further improves the systemic repopulation with multilineage human cells, by providing an autologous thymic environment for proper T cell development (220, 221). The functionality of the reestablished human immune system in immunodeficient mice has been demonstrated by the presence of lymphoid tissues and the capacity to mount adaptive immune responses, including T cell-dependent antibody responses, cell-mediated cytotoxicity and delayed type hypersensitivity reactions (207, 217–219, 222).

However, absence of the IL-2R γ -chain results in a lack of some cytokines which are cross-reactive between human and mouse and are required for human cell differentiation and survival within the host (29, 223). Furthermore, cross-reactivity between murine and human cytokines does not necessarily imply a biological function, as demonstrated by the example of B lymphocyte survival factor/stimulator BAFF (224). To overcome this issue, human cytokines and growth factors, including IL-7, IL-6, BAFF, thrombopoietin, FLT3-ligand, IL-12, granulocyte-macrophage colony-stimulating factor and IL-3 can be either administered or provided by transgenic expression (208, 224–229).

A major obstacle to using humanized mice as a preclinical model is their susceptibility to xenogeneic graft-vs.-host disease (GvHD), which impedes the development of chronic disease (230). This applies rather to mice that have been engrafted with BLT or human PBMCs than to HSC-engrafted mice. In the first two models, mature CD4+ T cells have been educated in human thymic stroma and are therefore not tolerized to the murine antigenic environment, which leads to rapid-onset xenogeneic GvHD (231). Engraftment of human cells and GvHD onset and severity vary between donors and seem to depend on the dose of CD4+ and CD8+ T cells as well as of naïve CD4+ T cells within the transferred human cells (232, 233). In contrast, higher percentages of Treg cells delay xenogeneic GvHD (234). Importantly, human T cells in HSC-reconstituted mice are selected on murine MHC class II (H2) molecules in the mouse thymus and therefore might not be able to recognize antigens presented in the context of HLA-DR by human antigen-presenting cells in the periphery. This affects the induction of efficient immune responses, resulting in reduced Th1 activity and insufficient interactions between human T and B cells which are required for class-switch recombination (235, 236).

A significant improvement has been accomplished by the introduction of human HLA class II molecules into immunodeficient mouse strains. Transgenic expression of HLA-DR4 in NRG mice enabled proper development of CD4+ T cells and completely functional B cells from infused HSCs

of HLA-DR-matched donors (237). The additional removal of murine MHC class II molecules, which are the main target of human CD4+ T cell-mediated GvHD responses further improved the generation of human antigen-specific immune responses in immunodeficient mice reconstituted with human cells (238, 239), while reducing the risk of xenogeneic GvHD (240).

Attempts to Establish Autoimmune Diseases in Chimeric Humanized Mouse Models

While humanized mouse models based on mouse/human chimeras have been widely used for studies on cancer, human-specific infectious diseases and transplantation (216, 236, 241, 242), their great potential to recapitulate human autoimmune disorders has only recently been recognized and explored (243).

It has been shown that transfer of human HSCs with a psoriasis-prone HLA haplotype provokes a related disease in NSG mice (244). Recently it has been demonstrated that reconstitution of NSG-Ab⁰ DR1 mice (lacking murine MHC class II (Ab⁰) and expressing transgenic HLA-DR1) with HSCs from a patient with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, associated with FOXP3 dysfunction, spontaneously develop lethal autoimmune disease, involving multiple organs and the production of autoantibodies (245). In another study, autoimmune disease characterized by hepatitis, weight loss and anti-nuclear antibodies was induced in HSC-reconstituted NSG mice by inhibition of CTLA-4, a critical molecule for Treg suppressive function (246). A spontaneous humanized model of type 1 diabetes (T1D)-like disease was established by engrafting HSCs into NRG-Akita mice, which develop hyperglycemia due to a mutation in the insulin 2 gene (214). In NSG.DQ8 Tg mice reconstituted with human DQ8+ HSCs and fetal thymus, transfer of autologous human CD4+ T cells expressing an DQ8/insulin B chain peptide-specific TCR and immunization with the corresponding peptide induced hyperglycemia and diabetes (247).

Other investigators engrafted patient-derived PBMCs into immunodeficient mice to reconstitute T cell-mediated autoimmune disorders, such as Sjögren's Syndrome, Systemic Lupus Erythematosus (SLE), or T1D, involving the production of autoantibodies and tissue-specific autoreactivity (243, 248, 249). Engraftment of BRG mice with PBMC from SLE patients caused autoimmune-like disease with donor-dependent severity, characterized by nephritis, proteinuria, deposits of human IgG in kidneys and shorter life span compared to mice engrafted with PBMCs from healthy subjects (249). Similarly, engraftment of NSG.A2 mice, expressing T1D-associated HLA-A*0201 (A2) molecules, with HLA-A2 matched PBMCs from T1D patients resulted in islet infiltration by specific CD8+ T cells (243). Insulinitis and pancreatic β cell death, the characteristic hallmarks of T1D, were also induced by the transfer of autoreactive CD4+ T cell lines from T1D donors into NSG.DR4 mice (250). In these models, spontaneous development and severity of autoimmune-like disease are donor-dependent and mice that

have been reconstituted with PBMCs from healthy subjects fail to display an autoimmune phenotype (243, 249).

Five different approaches have been pursued to generate humanized mouse models of RA, using immunodeficient mice as host for (i) RA synovial tissue (251, 252), (ii) RA patient-derived synovial fibroblasts co-transplanted with normal human cartilage (253, 254), (iii) synovial fluid mononuclear cells (SFMCs) (255, 256), (iv) RA patient-derived PBMCs (233, 256), or (v) HSCs (257, 258) (Figure 3).

Transplantation of Rheumatoid Arthritis Synovial Tissue Into Immunodeficient Mice

It has been demonstrated that implanted human rheumatoid synovium maintains its histological and phenotypic properties in SCID mice and survives for more than 6 months (251). Synovial tissue transplants become vascularized by human endothelial cells and human vessels were connected to mouse vasculature (259, 260). To generate RA synovium/SCID mouse chimeras, pieces of rheumatoid synovium were placed into SCID mice subcutaneously (260, 261), under the renal capsule (251, 262) or into joints (263, 264). As a result, RA-like disease, characterized by inflammation, angiogenesis, pannus formation and cartilage infiltration by fibroblast-like cells developed (260, 262, 263). The implanted rheumatoid synovium even invaded and destroyed co-implanted normal human cartilage (262). Importantly, only synovial tissue from RA patients, but not healthy synovium, induced pannus formation and destruction of bone and cartilage, when transplanted into joints of beige SCID mice (263). Both human and murine macrophages as well as murine granulocytes contribute to synovial inflammation in this model (263, 264). Human TNF, IL-6 and all Ig subclasses were detected in the serum of RA synovium-grafted animals (260, 265, 266). However, in the absence of inflammatory stimuli present in the RA joint, human synovium returns to a “resting state” after transplantation into immunodeficient mice, as demonstrated by the decreased expression of cell adhesion molecules ICAM-1 and VCAM-1 and emigration of mononuclear cells (259, 267). To overcome the issue of decreased lymphocyte numbers within RA synovial grafts, human allogeneic or autologous PBMCs or T cells were injected into the peritoneal cavity, tail vein, or directly into the graft of RA synovium SCID mouse chimeras (251, 268, 269). However, only 1–5% of intravenously injected peripheral blood lymphocytes (PBL) were shown to reach the graft, while most human PBL were sequestered in spleen and liver (259). Other studies demonstrated that injected T cells specifically migrate into synovial grafts, though migration was not specific to the synovial origin or inflammatory state of the human graft (251, 260). Co-administration of pro-inflammatory mediators, such as TNF, the acute-phase protein serum amyloid A and IL-15, have been shown to play a critical role in maintaining an inflammatory milieu, which retains mononuclear cells within and enhances lymphocyte and monocyte migration toward human synovial grafts (252, 267–269). Elegant studies by Weyand's group, in which either T cells or B cells were depleted from the RA synovial grafts in NOD-SCID mice, revealed that Th1 cells drive pro-inflammatory cytokine and tissue-degrading enzyme expression

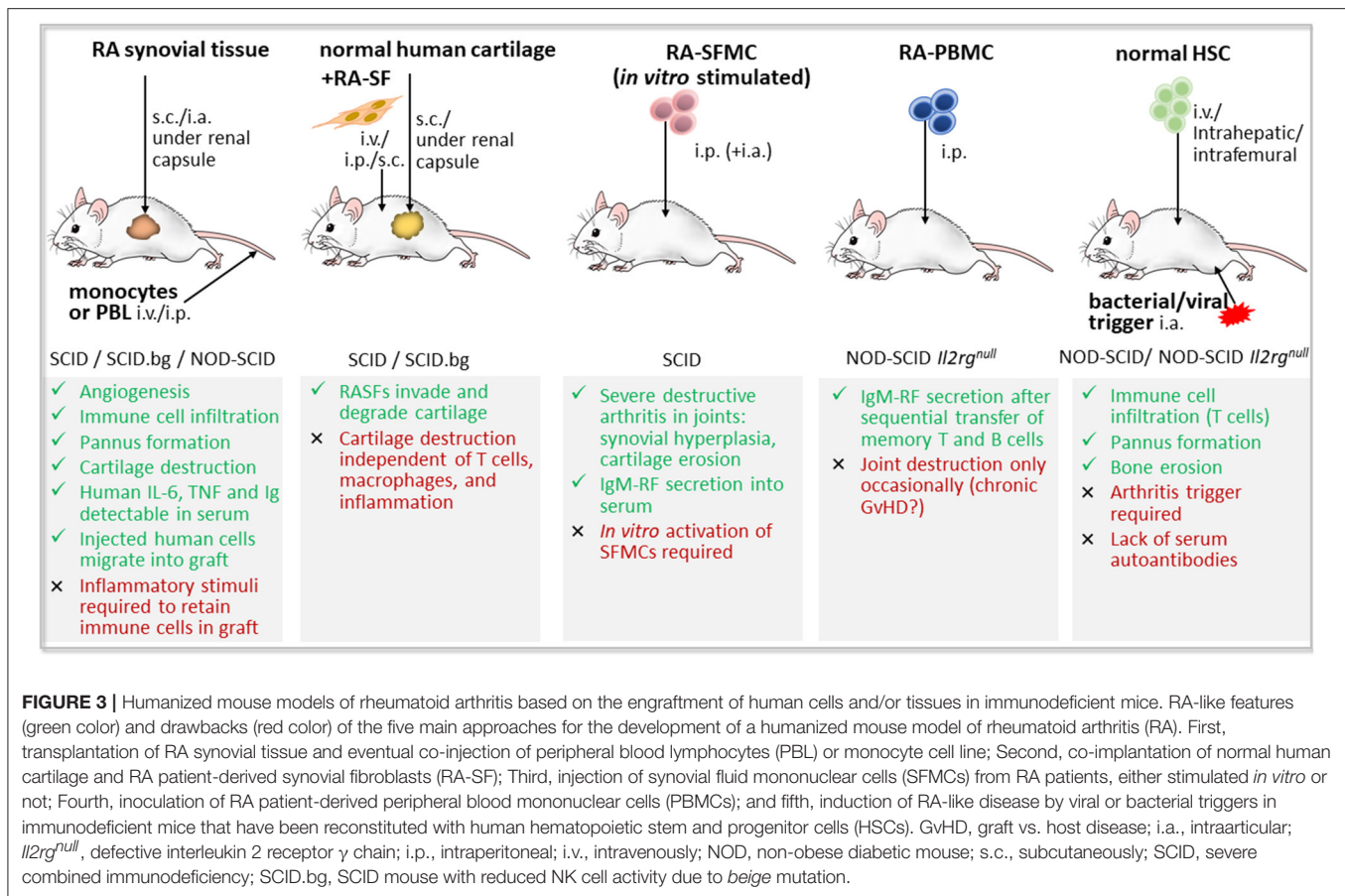
by synovial macrophages, and underlined a critical role of B cells in T cell activation and synovial inflammation (270, 271).

The RA synovium/SCID mouse chimera model has been widely used to study the properties of RA synovium *in vivo*, and to explore the effect of inflammatory mediators and its inhibitors on angiogenesis, cytokine secretion, and inflammatory cell infiltration (252, 266, 268, 269). The RA synovium/SCID mouse model was also used to investigate the mechanisms of action of anti-rheumatic drugs *in vivo* and to test novel biologic agents for the treatment of RA, such as monoclonal antibodies directed against human IL-6R, CD147 or Fas, as well as T and B cell-related therapies using CTLA-4Ig, anti-CD20, and anti-IL-17 antibodies (266, 272–275). For example, methotrexate decreased the number of inflammatory cells in RA synovial grafts through apoptosis (272), and anti-TNF antibodies, although reducing synovial inflammation, did not prevent bone and articular cartilage damage (276). This model also demonstrated inefficacy of anti-IL-1 and CTLA-4Ig therapy in the reduction of cellular infiltration and IL-6 secretion, and showed selective decreases in IL-6 secretion by anti-IL-17 only in those RA synovial tissues which contain high numbers of T cells (266).

Co-implantation of normal human cartilage with RA synovium into SCID mice resulted in pannus-like formation, cartilage invasion and perichondrocytic degradation for an extended period (261, 262). Intra-graft injection of IL-10 inhibited cartilage degradation and decreased ICAM-1 expression in and PBMC traffic toward RA synovial tissue in this model (261). In another approach, tissue derived from human RA pannus was implanted subcutaneously together with a slice of dentin into SCID mice (265). This work elucidated that only concomitant treatment with methotrexate and the TNF inhibitor infliximab suppressed pit formation in the dentin slice and thus detains bone destruction (265).

Implantation of RA Synovial Fibroblasts and Human Cartilage Into Immunodeficient Mice

In the SCID mouse co-implantation model of RA, synovial fibroblasts from RA patients (RASf) and normal cartilage were placed together in a gel sponge either subcutaneously or under the renal capsule (253, 254). The gel sponge replaced the synovial matrix as carrier for synovial fibroblasts, providing an environment devoid of stimulatory or inhibitory effects of other cellular and matrix components (253). This allowed study of the invasion of human RASf into human cartilage in a non-inflammatory environment. Using this model, RASfs were shown to invade and destroy human articular cartilage, independent of T cells, macrophages, and inflammation (253, 277). Activated RASfs specifically migrated toward, invaded and degraded implanted human cartilage, mirroring the progression from oligo- to polyarticular disease (254). However, due to the absence of inflammation and (antigen-specific) T and B cell responses, this SCID mouse co-implantation model of RA appears to rather reflect a facet of the disease, than the complete process of RA development.



Transfer of Rheumatoid Arthritis Peripheral Blood- or Synovium-Derived Mononuclear Cells Into Immunodeficient Mice

A first attempt to engraft mononuclear cells from peripheral blood, synovial fluid or synovial tissue of RA patients into immunodeficient mice was made in 1990 by Tighe and colleagues (255). Transferred PBMCs and SFMCs continued producing human IgG and IgM in SCID host mice, and IgM rheumatoid factor could be detected in mouse serum for more than 20 weeks after human cell engraftment (255). Later, Sakata and coworkers showed that previously stimulated synovial fluid-derived T cells from RA patients, simultaneously injected into knee joint and peritoneal cavity, caused severe destructive arthritis in SCID mice (256). Interestingly, arthritis occurred not only in the joint that received the cell injection but also in other joints, suggesting RA-like polyarthritis (256). In contrast, transfer of unstimulated synovial fluid-derived T cells or *in vitro*-activated PBMCs from RA patients failed to trigger arthritis in SCID mice (256, 263).

Almost two decades later, Ishikawa and colleagues engrafted RA patient-derived PBMCs into NOG immunodeficient mice (233). Since transfer of PBMC often causes lethal GvHD, the naïve CD4⁺ T cell fraction was removed from PBMCs, and RA patient-derived CD4⁺ memory T cells and B cells were transferred sequentially, resulting in sustained production of IgM rheumatoid factor autoantibodies in human cell-engrafted NOG

mice (233). However, only some mice reconstituted with RA patient-derived PBMCs displayed histological joint destruction, which was difficult to distinguish from alterations due to chronic GvHD (233). Nonetheless, it has been proven that human T cells engrafted in NOD-*scid* *Il2rg*^{null} mice migrate to air pouches containing RA synovial fluid and this recruitment was abolished by CXCR3 agonist (278).

The development of RA-like disease in immunodeficient mice engrafted with human cells seems to critically depend on the origin (peripheral blood vs. synovial fluid) and previous *ex vivo*-activation of transferred cells through mitogen or autoantigen, as well as on cell number and route of administration (intra-articular, intraperitoneal, or intravenous). Unlike other models of autoimmune diseases (243, 248, 249), injection of PBMCs of RA patients, even when activated, was insufficient to induce arthritis (233, 256, 263), indicating that pathogenic T cells might be concentrated in synovial fluid and tissue. Previous *ex vivo* activation of synovial fluid-derived T cells appears to facilitate the establishment of arthritis in immunodeficient mice through expansion of antigen-specific arthritogenic T cell clones and concomitant induction of growth factors such as IL-2 (256). Sole intra-articular injection of small numbers of mitogen-stimulated SFMCs or CII-specific T cell lines was not sufficient to induce joint destruction in beige SCID mice (263), while combination of systemic and local administration of synovial fluid-derived

cells appeared to concert inflammatory cell infiltration and local synovial cell proliferation and thus promote the development of RA-like lesions (256). It is of note that at 4 weeks after engraftment into NOD-*scid* *IL2rg*^{null} mice, almost all human cells were activated T cells and thus, it must be considered that the absence of myeloid cells may affect the development of a RA-like disease (278). The supply of antigen-presenting cells e.g., through repetitive injections of (auto-)antigen-loaded DCs or B cells, might be an option to circumvent this issue (158, 187, 188, 233).

Hematopoietic Stem Cell-Engrafted Mouse Models of Rheumatoid Arthritis

With the advent of immunodeficient mouse models that enable the complete reconstitution of the human immune system through engraftment of HSCs, researchers have also exploited this option to establish humanized mouse models of RA.

Injection of *Chlamydia trachomatis* into knee joints of NOD-SCID mice, that had been previously irradiated, treated with anti-CD122 Ab to block the IL-2R and repopulated with CD34+ bone marrow-derived HSCs from osteoarthritis patients, induced synovial inflammation with a predominance of human CD68+ macrophages (159). The main limitation of this model is the absence of mature human CD4+ and CD8+ T lymphocytes (159). To overcome this issue, later models used umbilical cord blood-derived HSCs which were able to give rise to all immune cell types, instead of HSCs from adult peripheral blood with restricted hematopoietic potential (210, 219).

Kuwana and coworkers used NOG mice transplanted with cord blood-derived HSCs to investigate the contribution of Epstein-Barr virus (EBV) infection to the development of RA (257). Erosive arthritis of the major joints, histologically characterized by pannus formation, bone marrow edema, synovial membrane proliferation and infiltration of inflammatory cells, mainly T cells, occurred in 65% of low dose EBV-infected, but not in non-infected, humanized NOG mice (257). However, rheumatoid factor and ACPA autoantibodies were not detected in peripheral blood of these mice (257). In another approach, acute inflammatory arthritis was induced in HSC-reconstituted NSG mice, through intra-articular injection of CFA (258). These mice developed clinical and histological signs of arthritis, such as swelling, erythema, decrease of function, immune cell infiltration, and bone erosion (258). Elimination of murine neutrophils by pre-treatment with anti-Gr-1 antibody did not affect arthritis development and human leukocytes were detected in inflammatory infiltrates, indicating that RA-like disease was mediated by engrafted human cells infiltrating the joint (258). Treatment with the TNF inhibitor Etanercept prior to the induction of CFA-triggered arthritis was shown to decrease scores of pannus formation, inflammation and bone erosion, as well as human cell counts in joints (258).

So far only healthy donor-derived HSCs have been used to establish the human immune system in immunodeficient mice. An ideal model of RA pathogenesis would utilize HSCs derived from RA patients, which has been non-feasible. On the other hand, stem cells obtained from adult peripheral

blood have proved to be unsuitable to completely reconstitute the immune system in immunodeficient mice (159, 219, 258). Induced pluripotent HSCs or cord blood-derived HSCs from relatives or offspring of RA patients, which might be equally predisposed to develop RA, or healthy individuals with RA-susceptible HLA-DR haplotype, might serve as source of HSCs for engraftment, but are difficult to obtain. Moreover, RA models based on immunodeficient mice, whose immune system has been reconstituted through engraftment of human stem cells, require a local inflammatory stimulus in the joint, such as viral or bacterial infection, to induce immigration of inflammatory cells and trigger RA-like disease.

Taken together, the ultimate humanized model of RA, mirroring all aspects of this chronic inflammatory autoimmune disease does not yet exist. Since T cells play a minor role in synovial tissue implant and RASF transfer models, these models are less suitable for testing cell-based therapeutic strategies targeting autoreactive T cells. Autoreactive lymphocytes are found in peripheral blood and, to a much higher extent, in synovial fluid and tissue of RA patients, which might therefore be the cellular source of choice to recapitulate arthritis in immunodeficient mice. However, *in vitro* T cell activation prior to transfer seems to be necessary, raising the question of antigen-specificity (256). Finally, development of arthritis-like symptoms in immunodeficient mice that have been engrafted with healthy donor HSCs requires pathogenic stimuli and thus does not involve RA-associated autoantigens (159, 258), unless HSC donors expressing RA risk alleles are used for the reconstitution of the human immune system.

THE CHALLENGE OF TRANSLATING CELL-BASED THERAPIES FOR RHEUMATOID ARTHRITIS IN HUMANIZED MICE

Humanized mouse models, particularly those reconstituted with human leukocyte populations, provide a promising tool for preclinical testing of novel therapeutic approaches, such as cell-based immunotherapy (Figure 4).

The clinical response of individual patients toward autologous cell-based immunotherapy has already been tested in humanized mouse models of diverse human cancers (160, 241). Adoptively transferred Treg cells, converted from CD4+ T cells through enforced expression of IL-10, were demonstrated to exert anti-tumor and anti-leukemic effects in NSG mice reconstituted with ALL-CM or THP-1 leukemia cell lines, through granzyme B-mediated lysis of myeloid tumor cells in a HLA class I-dependent but antigen-independent manner (161). These CD4^{IL10} cells were also able to contribute to graft vs. leukemia activity of injected allogeneic PBMCs, while preventing xenogeneic GvHD (161). Administration of autologous mature DCs, pulsed with tumor-associated antigen MART1 or WT1, was shown to induce antigen-specific cytotoxic T cell responses in NSG mice reconstituted with human PBL (158, 187). In a humanized mouse model of breast cancer, established by injecting breast cancer

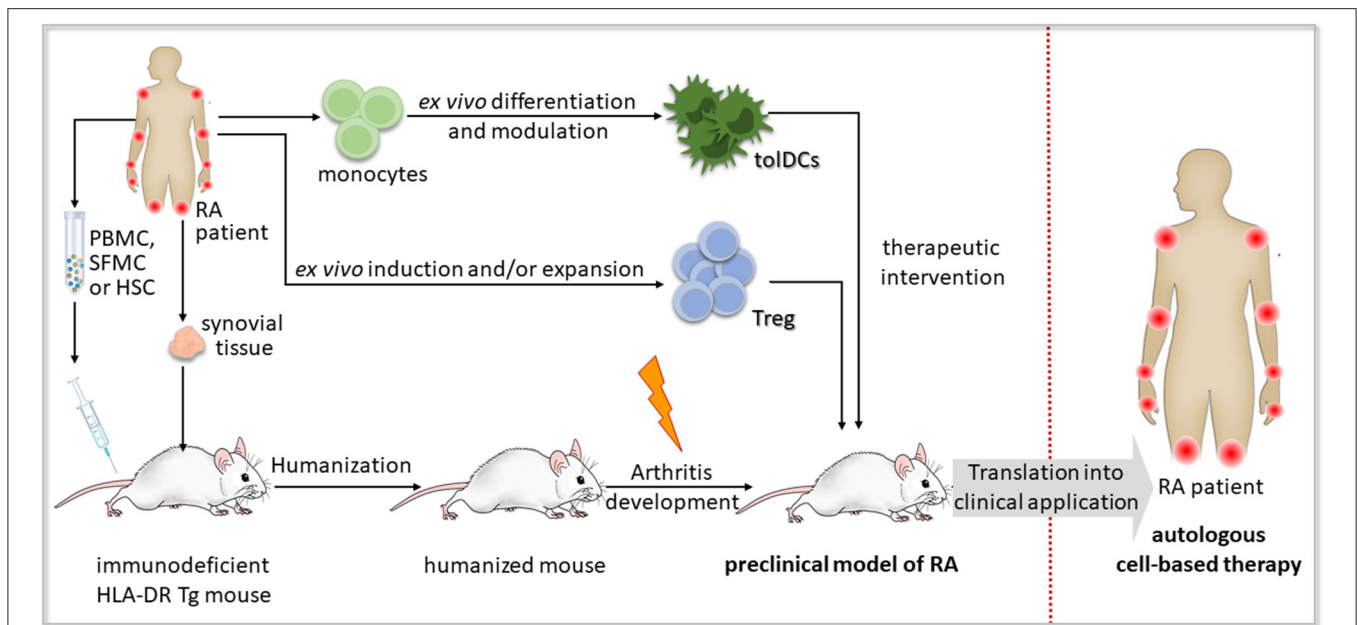


FIGURE 4 | Concept of preclinical testing of cell-based immunotherapy for rheumatoid arthritis in humanized mice. A humanized mouse model of rheumatoid arthritis (RA) could be established by the engraftment of human synovial tissue, hematopoietic stem and progenitor cells (HSCs), peripheral blood mononuclear cells (PBMCs) or synovial fluid mononuclear cells (SFMCs) from an RA patient bearing HLA-DR risk alleles such as HLA-DR*0401 or HLA-DR*0101 into immunodeficient mice expressing the respective transgenic (Tg) HLA-DR molecules. Autoimmune-like disease, if not developed spontaneously, might be induced by an additional trigger, such as RA-associated autoantigens or autoreactive T cell clones. The obtained humanized mouse model of RA enables preclinical testing of cell-based immunotherapeutic approaches applying autologous regulatory T (Treg) cells or tolerogenic dendritic cells (tolDCs) to recover self-tolerance, before their transfer to clinical application in RA patients.

stem cells into the mammary fat pad of HSC-reconstituted NOD-SCID mice, the application of autologous DCs loaded with breast cancer stem cell antigen reduced tumor size and prolonged survival (162).

Adoptive immunotherapy with human DCs, engineered to be long-lived and to co-express high levels of human IFN- α , human GM-CSF and cytomegalovirus pp65 antigen, regenerated immunity *de novo* in NRG mice after human allogeneic HSC transplantation (163, 164). *In vivo* targeting of Epstein-Barr virus nuclear antigen 1 (EBNA1) to the endocytic receptor DEC-205 expressed by CD141⁺ DCs, stimulated antigen-specific CD4⁺ T cell responses in NSG mice reconstituted with human fetal liver-derived CD34⁺ HSCs (165).

Promising cell-based approaches have also been achieved in humanized mouse models of transplant rejection. Injection of freshly isolated or *ex vivo*-expanded human CD4⁺ or CD8⁺ Treg cells into immunodeficient mice, that had been transplanted with human skin and reconstituted with allogeneic PBMCs, supported long-term survival of skin allografts (166–168). Similarly, transfer of *ex vivo* expanded autologous Treg cells prevented rejection of islet xenografts in mice reconstituted with human PBMCs or CD34⁺ HSCs by inhibiting immune cell infiltration and T effector cell differentiation in an IL-10 dependent manner (169, 170, 279).

In a humanized model of allergic airway disease, consisting of NSG mice reconstituted with PBMC from patients with allergic asthma and sensitized to birch pollen in the presence of IL-4, administration of autologous Treg cells or *in vivo* Treg

induction through sGARP or polyclonal activation via gp120, abrogated airway hyperresponsiveness and reduced airway inflammation in the lung in a TGF- β receptor 2-dependent manner (280, 281). In a similar model of allergen-specific gut inflammation, induced by rectal challenge with birch, grass pollen, or dust mite allergen, adoptive transfer of activated Treg cells decreased allergen-specific Th2 responses and IgE secretion (282).

Despite the great potential of humanized mice as preclinical models (241, 283, 284), there are only few studies that use mouse/human chimera to explore the efficacy of cell-based immunotherapies for autoimmune diseases *in vivo*, none of them in RA.

In humanized models of autoimmune T1D, autoantigen-specific vaccination strategies have been described, which aim at inducing Treg cells that prevent the destruction of insulin-producing β cells (285, 286). Systemic delivery of nanoparticles, coated with autoimmune disease-relevant peptides bound to MHC class II molecules, promoted the *in vivo* generation and expansion of antigen-specific FOXP3-CD49b+LAG-3+ Treg cells in different humanized mouse models, including NSG mice reconstituted with T1D patient-derived CD8⁺ T cell-depleted PBMCs, and ameliorated clinical and pathological signs of CIA and experimental autoimmune encephalomyelitis in *HLA-DR4-IE* Tg mice (285). In another study, sub-immunogenic vaccination of human HSC-engrafted NSG.DQ8 Tg mice with agonistic mimetopes of the T1D-relevant insulin B-chain epitope induced insulin-specific FOXP3⁺ Treg cells *in vivo* (286).

To explore therapeutic strategies to overcome the resistance of effector T cells toward Treg-mediated suppression in multiple sclerosis (MS) patients, a humanized model based on newborn Rag2^{-/-}γ^{-/-} mice engrafted with CD25-depleted PBMCs from different MS patients was applied (287). Transfer of gp120-activated Treg cells from healthy subjects into these mice prevented GvHD only when engrafted PBMCs were derived from MS patients that received disease-modifying therapy but not from those being therapy-naïve (287).

In a humanized model of myasthenia gravis (MG), generated by subcutaneous engraftment of thymic MG fragments into NSG mice, administration of *in vitro*-preconditioned human mesenchymal stem cells improved the disease by decreasing the serum level of acetylcholine receptor (AChR)-specific autoantibodies and restoring AChR expression at the muscle endplate (288).

Novel therapeutics for RA have been tested so far only in DR1 and DR4 Tg mice. Particularly, the capacity of CII analog peptide has been demonstrated to induce *in vivo* antigen-specific inhibitory T cells capable of suppressing CIA dependent on IL-10 and IL-4 secretion (173, 289). Nevertheless, it seems to be only a matter of time until humanized chimeric mouse models of RA will be available for preclinical testing of cell-based therapeutic strategies. But there are still some important obstacles to overcome (Table 2).

TABLE 2 | Challenges of recapitulating rheumatoid arthritis in chimeric humanized mice.

Problems	Possible solutions
Recipient gender-dependent engraftment of human cell populations	<ul style="list-style-type: none"> • Use of female mice only
Donor-dependent variations in PBMC engraftment	<ul style="list-style-type: none"> • Transfer of defined cell populations rather than whole PBMCs • Personalized model
Xenogeneic GvHD in PBMC-engrafted mice prevents development of a chronic disease model	<ul style="list-style-type: none"> • Use of human HSCs as cellular graft • Removal of naïve CD4⁺ T cells from human cell graft • Deletion of murine MHC class II • Expression of transgenic HLA-DR1 or -DR4 and engraftment of HLA-DR-matched donor cells
Disappearance of myeloid APCs in PBMC-engrafted mice	<ul style="list-style-type: none"> • Sequential transfer of (antigen-pulsed) APCs • Supply of survival factors, such as human GM-CSF
Difficulties in establishing autoimmune disease	<ul style="list-style-type: none"> • Removal of Treg cells from human cell graft • Transfer of autoreactive CD4⁺ T cell clones • Transfer of SFMCs and/or synovial tissue from RA patients
Poor autoantibody production	<ul style="list-style-type: none"> • Sequential transfer of B cells • Administration of BAFF
Unknown trigger of autoimmunity	<ul style="list-style-type: none"> • Transplantation of synovial tissue or administration of synovial fluid of RA patients

APCs, antigen-presenting cells; BAFF, B cell activating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; GvHD, graft vs. host disease; HLA-DR, human leukocyte antigen DR; HSCs, hematopoietic stem and progenitor cells; MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; SFMC, synovial fluid mononuclear cells; Treg, regulatory T cell.

Many of the RA models have been difficult to reproduce by other laboratories, requiring profound characterization of environmental and genetic susceptibility factors that interfere with, or promote, RA-like disease in humanized mice.

One major problem in humanized mice is that long-term evaluation of transferred human cells and the establishment of chronic disease is hampered by the development of xenogeneic GvHD, induced by cross-species interactions between mouse macrophages and human CD4⁺ T cells in skin and lymphatic tissues (290). HSC-reconstituted mice are less susceptible to GvHD than PBMC-engrafted mice, since T cell selection in this model occurs in the murine thymus. However, PBMCs might be the better choice for the establishment of an RA model, as autoimmunity develops due to the loss of peripheral but not central tolerance. Since it has been postulated that GvHD in human PBMC-engrafted SCID mice results from anti-mouse MHC class II reactivity of human CD4⁺ T cells (291), a possible solution would be to use immunodeficient mice that lack murine MHC class II molecules as platform for the engraftment of human cells (238). Additional expression of transgenic human RA-associated DR4 or DR1 molecules might further improve long-term engraftment of cells and tissues from HLA-DR-matched human donors (237, 239, 245). Alternatively, removal of the naïve CD4⁺ T cell fraction from transferred PBMC might reduce xenogeneic GvHD (233). Additional administration of purified autologous B cells might ensure efficient autoantibody production (233).

Another important issue is the rapid disappearance of myeloid antigen presenting cells after engraftment of PBMC into immunodeficient mice (29). Since almost all transferred T lymphocytes express HLA-DR after activation, it is conceivable that activated T cells could assume the role of APCs in humanized models (134). However, additional supply of survival factors or repeated administration of myeloid antigen presenting cells such as DCs pulsed with autoantigen might improve the propagation of autoantigen-specific T cell responses. Removal of Treg cells from the human cell graft might further facilitate the establishment of autoimmune responses in humanized mice (292).

A variety of potential RA-associated autoantigens have been described and T cell responses directed against them are extremely heterogeneous among different RA patients, requiring personalized models of RA. These can be easily established in immunodeficient mice by engrafting RA patient-derived PBMCs or SFMCs, which contain autoreactive T cells and are easier to obtain than patient-derived HSCs. Pathogenic T cell clones may be isolated from peripheral blood or synovial fluid of RA patients and expanded *ex vivo* before transferring them into mice. Eventually, it might be necessary to trigger arthritis by activation of human lymphocytes before transfer into mice or by co-administration of arthritogenic antigen.

CONCLUDING REMARKS

Since conventional mouse models of RA are only partially suited to preclinical testing of cell-based therapies, translational

research requires a humanized mouse model that accurately mirrors autoimmune processes of human RA and permits its modulation by the transfer of human immunoregulatory cells. Various approaches have been followed during the past decades to establish a humanized model of RA, with only partial success and poor reproducibility. Valuable lessons learnt from translational research models of other autoimmune diseases might help to improve current attempts. A combination of transgenic expression of RA risk alleles and the engraftment of RA patient-derived immune cells and/or RA synovial tissue seems a promising strategy to avoid GvHD and establish chronic autoimmune responses. Improved humanized mouse models of RA would provide a powerful tool for preclinical evaluation of cell-based immunotherapies.

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Ways Forward for Tolerance-Inducing Cellular Therapies- an AFACTT Perspective

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Clinical studies with cellular therapies using tolerance-inducing cells, such as tolerogenic antigen-presenting cells (tolAPC) and regulatory T cells (Treg) for the prevention of transplant rejection and the treatment of autoimmune diseases have been expanding the last decade. In this perspective, we will summarize the current perspectives of the clinical application of both tolAPC and Treg, and will address future directions and the importance of immunomonitoring in clinical studies that will result in progress in the field.

Keywords: tolerance, dendritic cells, regulatory T cells, autoimmunity, transplantation, cell therapy, clinic, immunomonitoring

INTRODUCTION

The number of patients with autoimmune diseases, severe allergies and recipients of organ or stem cell transplants is increasing worldwide. Currently, many of these patients require lifelong administration of immunomodulatory drugs, which cause generalized immunosuppression and hereby only partially alleviate the symptoms but do not cure the disease. Besides these drugs are inevitably associated with a risk of immediate or late-occurring severe adverse effects (e.g., life-threatening infections, cancer). Targeting the fundamental cause of autoimmunity, i.e., loss of tolerance to self-antigens, or inhibiting induction or execution of undesired immunity in transplantation and allergy will provide the next steps forward to avoid general immunosuppression. Accumulating knowledge on mechanisms of immune activation and tolerance has led to the development of tolerance-inducing cellular therapies with regulatory T cells (Treg) and tolerogenic antigen presenting cells (tolAPC), such as tolerogenic dendritic cells (tolDC) and regulatory macrophages (Mreg) [as reviewed in (1, 2)], with the specific objective to restrain unwanted immune reactions long-term.

The development of cell-based therapies is clinically attractive for many reasons, not in the least through their potential of being of low-toxicity, to simultaneously control many different inflammatory cells and induction of antigen-specific immunity. Since immunological tolerance is a self-reinforcing state (3), the therapeutic effects of cell therapy are expected to outlast the lifespan of the therapeutic cells themselves, opening the possibility of curative treatments. Production costs for these tolerogenic cell products range from 10,000 to 40,000 Euro depending on the therapeutic cell product and production site, which is relatively low considering that few injections of cells may be sufficient to induce long-lasting tolerance.

In recognition of the potential of tolerance inducing cell-based therapies and to join forces in the ongoing efforts in the field, A FACTT (Action to Focus and Accelerate Cell Based Tolerance-inducing Therapies (CTT) was initiated through EU COST Action Funding. Our goal was to initiate a network that would coordinate European CTT efforts to minimize overlap and maximize comparison of the diverse approaches across Europe. Now, looking back at 4 years of very active network interactions, we have evaluated our combined current stance in the field and defined avenues to support future directions.

TOLEROGENIC THERAPY WITH ANTIGEN PRESENTING CELLS IN CLINICAL PRACTICE

Over the past 20 years extensive experimental research has been invested in the generation and characterization of tolAPC, including tolDC and Mreg, with the aim to restore tolerance in autoimmune diseases (4–10) and transplant rejection (5, 11–14). To date, clinical trials exploring the safety, feasibility and efficacy of different types of tolAPC are a reality [reviewed in (1) and **Table 1**], and have confirmed so far that tolAPC therapy is safe, with no relevant side effects, and is well-tolerated by patients. Hence, to advance tolerogenic therapy with antigen-presenting cells (APC), we should stand on the shoulders of these pioneers and address remaining challenges, such as the optimal dose, injection route, frequency of administration, antigen-specificity, and the related issue of suitable biomarkers of cell therapy-induced reduction of general inflammatory state and induction of tolerance, in the design of the next-generation clinical trials.

Phenotypic and Functional Identification of *in vitro* Generated TolAPC

Both tolDC and Mreg can be generated *in vitro* starting from CD14⁺ monocytes and share some phenotypic and functional characteristics. Indeed, both tolAPC types express low to intermediate levels of T-cell costimulatory molecules, and secrete low amounts of pro-inflammatory cytokines, indicative of a partially matured APC. Similarly, immature DC (iDC) display minimal expression of costimulatory molecules and little secretion of inflammatory cytokines, demonstrating potential optimal requirements for tolerance induction *in vivo* (23, 24). However, iDC are unstable and may differentiate into immunogenic DC under inflammatory conditions (25, 26).

This invalidates their putative use as therapeutic products for tolerance induction. Therefore, different strategies to generate stable tolAPC have been explored, including treatment with pharmacological agents or cocktails of immunomodulatory cytokines, genetic engineering, and exposure to apoptotic cells (9, 27, 28). Most of these *in vitro* conditioning regimens aim at stabilizing a semi-mature state of tolDC, maintaining the capacity to induce immune hyporesponsiveness of T cells, even in presence of powerful pro-inflammatory signals.

Importantly, tolAPC inhibit T cell proliferation, albeit through different immunosuppressive mechanisms depending on the approach used to generate tolAPC *in vitro*. Induction of peripheral T cell anergy and apoptosis (29), attenuation of effector and memory T cell responses and the generation and activation of Treg populations (30, 31) result in part from presentation of low levels of antigen in the absence of costimulation; these are typical mechanisms attributed to a variety of tolerogenic subtypes (10, 32, 33). Additionally, tolerogenic DC may express various inhibitory receptors such as programmed death-ligand (PD-L)1, PD-L2 (34), immunoglobulin-like transcripts (ILT) (35), FasL (36, 37), and TRAIL (38). Secretion of anti-inflammatory cytokines such as IL-10 (39, 40) and TGF- β (41, 42), as well as reduced expression of pro-inflammatory cytokines, also may contribute to tolerance induction. A study comparing tolDC generated in presence of dexamethasone and rapamycin demonstrated that while both tolDC subsets were able to impair T cell proliferation, rapamycin-treated tolDC have a mature phenotype and are not able to produce IL-10 upon stimulation with LPS, as opposed to vitamin D₃- or dexamethasone-treated tolDC (27). Whereas, it was demonstrated that rapamycin-treated tolDC induce Treg (27), vitD₃-treated tolDC induce T-cell hyporesponsiveness and antigen-specific Treg (7, 43). Moreover, DC-10 induce Tr1 cells (44), while autologous tolDC have a weak capacity to stimulate allogeneic T cells and suppress T-cell proliferation and IFN- γ production (45). Mreg have been shown to convert allogeneic CD4⁺ T cells to IL-10-producing, TIGIT⁺, FoxP3⁺-induced Treg (46). Variations in the process to generate tolAPC may initiate regulation through distinctive mechanisms, making it difficult to compare these different types of tolAPC. Therefore, efforts have been made to find common features unique for tolerance-inducing cells (47). For example, since tolDC conditioned using vitamin D₃ and dexamethasone exhibit high cell surface expression of TLR2 (48) or CD52 (49), such markers might be considered to assess the quality and stability of tolDC in future cell-based clinical trial protocols. In addition, it was demonstrated that the expression of single immunoglobulin IL-1-related receptor has a role in maintaining low levels of costimulatory molecules and in regulation of Treg expansion (50). Others demonstrated that C-lectin receptor CLEC-2 upregulation by DC is associated with Treg induction (51). So far, however, gene expression studies comparing different tolDC and Mreg protocols have not been able to identify common biomarkers of tolerance induction [reviewed by (52, 53) and (54)].

The difficulty in comparing characteristics of different clinical tolAPC suggests the need for a uniform set of metrics for

TABLE 1 | Completed or ongoing trials with toAPC in autoimmunity and transplantation.

Study ID	Phase	Cell product	Indication	Dosing scheme	toIDC per dose	Route of administration	Outcome	Center	References
NCT00445913	I	Antisense ODN targeting CD40/CD80/CD86 toIDC	T1D	4 injections, bi-weekly	1×10^7	Intradermal	-No adverse effects (AE) -Increase of B220+CD11c+ B cells	Pittsburgh/USA	(15)
NCT02354911	II	Antisense ODN targeting CD40/CD80/CD86 toIDC	Recent onset T1D	4 injections, bi-weekly	1×10^7	Intradermal	-Evidence for C-peptide reactivation Not Recruiting	DiaVacs Inc. Pittsburgh/USA	
NTR5542	I	VitD3 and Dex toIDC pulsed with proinsulin peptide	T1D	2 injections, 4 weeks apart	Dose-escalation: 5, 10, or 20×10^6	Intradermal	Finished	Leiden/NL	
Rheumavax	I	NF- κ B inhibitor Bay 11-7082 toIDC pulsed with 4 citrullinated peptides	HLA-risk positive RA (minimal disease activity)	Single injection	Low dose $0.5-1 \times 10^6$ High dose $2-4.5 \times 10^6$	Intradermal	-Grade I AE. -Decrease DAS28 -Decrease eff T cells, ratio Teff/Treg -Decrease pro-inflammatory cytokines and chemokines	Brisbane/AUS	(16)
NCT01352858 (AutoDECRA)	I	Dex and VitD3 toIDC pulsed with autologous synovial fluid	Inflammatory Arthritis	Single injection	Dose-escalation: 1×10^6 3×10^6 10×10^6	Intraarticular	-Safe, feasible, acceptable -Knee symptomse stabilized in 2 patients receiving the highest doses	Newcastle upon Tyne/UK	(17)
CreaVax-RA CRIS KCT0000035	I	toIDC pulsed with recombinant PAD4, RA33, citrullinated, filagrin and vimentin	RA	5 injections	Low dose 0.5×10^7 High dose 1.5×10^7	Not indicated	-Treatment well tolerated -Antigen –specific autoantibodies decreased in 5/9 positive patients	Seoul/KOR	(18)
NCT03337165 (toIDCtoRA)	I	Dex and IFN- α toIDC	RA	Single injection	Dose-escalation: 1, 3, 5, 8, 10×10^6	Intraarticular	Recruiting	Novosibirsk/RUS	
NCT02283671	I	Dex toIDC pulsed with relevant disease peptides	MS and Neuro-myelitis optica	3 injections, bi-weekly	Not indicated	Intravenous	Recruiting	Barcelona/ES	
NCT02618902	I/IIa	VitD3 toIDC pulsed with myelin-derived peptides	Active MS patients	6 injections, 4 bi-weekly and 2 monthly	Dose-escalation: 5×10^6 10×10^6 15×10^6	Intradermal	Recruiting	Antwerp/BE	
NCT02903537 (Tolervit-MS)	I/IIa	VitD3 toIDC pulsed with myelin-derived peptides	Active MS patients	6 injections, 4 bi-weekly and 2 monthly	Dose-escalation: 5×10^6 10×10^6 15×10^6	Intranodal	Recruiting	Badalona, Pamplona/ES	

(Continued)

TABLE 1 | Continued

Study ID	Phase	Cell product	Indication	Dosing scheme	toIDC per dose	Route of administration	Outcome	Center	References
	I	Dex and ViTA toIDC	Refractory Crohn's disease	Single injection or 3 injections bi-weekly	Dose-escalation: 2 × 10 ⁶ 5 × 10 ⁶ 10 × 10 ⁶	Intra-peritoneal	-No AE (3 patients withdrew due to worsening of symptoms) -Clinical improvement in 3 patients -Increase of Treg and decrease of IFN- γ levels	Barcelona/ES	(19)
NCT02622763	I	Dex toIDC	Crohn's disease	Not listed	10 × 10 ⁶ 100 × 10 ⁶	Intralesional	Recruiting	Barcelona/ES	
NCT0252055 (ONEaDC)	I/II	Low-dose GM-CSF-recipient toIDC	Kidney Tx from living donor	Single injection, 1 day before Tx	1 × 10 ⁶ /kg bw	Intravenous		Nantes/FR	(20)
NCT03726307	I	ViTD3 and IL-10 donor toIDC (Dcreg)	Kidney Tx from living donor	Single injection, 7 days before Tx	Dose - escalation: 0.5 ± 0.1 × 10 ⁶ /kg bw, 1.2 ± 0.2 × 10 ⁶ /kg bw, 2.5 to 5 × 10 ⁶ /kg bw	Intravenous	Not yet recruiting	Pittsburgh/USA	
NCT03164265	I	ViTD3 and IL-10 donor toIDC (Dcreg)	Liver Tx	Single injection, 7 days before Tx	Not described	Intravenous	Enrolling by invitation	Pittsburgh/USA	
TAIC-I	I/II	Cell product containing donor Mreg	Kidney Tx from deceased donor	Single injection, 5 days after Tx	0.5–7.5 × 10 ⁶ /kg bw	Intravenous	Safe	Regensburg/DE	(21)
TAIC-II	I	Cell product containing donor Mreg	Kidney Tx from living donor	Single injection, 5 days before Tx	1.7–10.4 × 10 ⁷ /kg bw	Intravenous	-Safe -No acute or delayed AE were associated with the infusion. -3 of the 5 patients were able to tolerate low-dose tacrolimus monotherapy and one patient was withdrawn from all immunosuppression for over 8 months	Regensburg/DE	(22)
NCT02085629	I/II	Donor derived Mreg	Kidney Tx from living donor	Single injection, 7 days before Tx	2.5–7.5 × 10 ⁶ /kg bw	Intravenous	Completed, no results yet	Regensburg/DE	(20)

This table is based on information deposited on www.clinicaltrials.gov, www.clinicaltrialsregister.eu and/or indicated references. AE, adverse event; RA, rheumatoid arthritis; T1D, type 1 diabetes; Tx, transplantation; Dex, dexamethasone; MS, multiple sclerosis; /kg bw, per kg body weight; viTD3, vitamin D3.

their description, including full characterization of (at least) the immune phenotype and their functional activity (potency). Hence, a better identification of the characteristics that identify the tolerance-inducing properties of tolAPC, irrespective of the conditioning regimen, would be valuable for safe cell therapy delivery into patients. Joint efforts in translating tolAPC into the clinic by harmonizing protocols and defining functional quality parameters have been initiated (1, 55). A Minimum Information Model on Antigen-presenting cells (MITAP) has been defined to harmonize reporting on tolAPC therapy to ultimately allow the uncovering of commonalities between tolAPC and to define common quality control biomarkers and potency assays for the various tolAPC products for clinical use (55). Likewise, using similar immunomonitoring protocols in different clinical trials could help to better understand the *in vivo* mechanism of action of these cells (56).

Antigen Specificity of TolAPC-Based Immunomodulation

Targeted regulation of antigen-specific T cell responses would avoid generalized immunosuppression as observed with immunosuppressive drugs and monoclonal antibodies currently in use in the clinics and may thus overcome occurrence of impaired immune-surveillance leading to infections or development of malignancies. *Ex vivo* generated tolAPC have the potential to therapeutically induce, enhance, or restore antigen-specific tolerance. Indeed, after loading these cells with exogenous or endogenous antigens, one major advantage is their capability to act in an antigen-specific manner.

A number of *in vivo* studies demonstrate that antigen loading of tolAPC is indispensable to reach efficient clinical responsiveness following tolAPC therapy. For instance, a beneficial effect of vitamin D₃-tolDC loaded with MOG_{40–55} peptide was demonstrated in experimental autoimmune encephalomyelitis (EAE), whereas no clear beneficial effect on the clinical score of EAE mice was found when mice were treated with vitamin D₃- tolDC not loaded with myelin peptides (57, 58). Similar findings have been demonstrated in other animal models of autoimmune diseases, including collagen-induced arthritis and autoimmune thyroiditis (59–61). Altogether, these findings suggest that selection of the target self-antigen is critical for disease-specific tolerance induction *in vivo*. Suitable disease-associated self-antigens responsible for T cell priming have been identified for T1D and multiple sclerosis (MS). However, this is not the case for other autoimmune diseases, such as rheumatoid arthritis or Crohn's disease, for which specific disease-associated antigens are unknown or not tissue specific. Moreover, not all patients uniformly display the same set of self-antigens for a given disease. MS, for example, is associated with a range of self-antigens and auto-antibodies that are differentially expressed among patients and at different points during the disease (62). While the targetable autoreactive T cell populations may be limited to a select number of antigens at the onset of clinical disease, other "late antigen" or spread epitope autoreactive T cell populations may drive autoimmunity during progression of the disease. To overcome this hurdle, some groups have chosen to load the tolerance-inducing therapeutic cells with a broad

pool of distinct, candidate disease-related peptides (63–65) (NCT02283671, NCT02618902, and NCT02903537).

In contrast to autoimmunity, transplant rejection is mediated by an undesired immune response against epitopes that differ between the transplanted donor graft and the recipient host, so-called allorecognition (66). Specifically, recipient T cells may initiate a strong immune response leading to transplant rejection in the absence of adequate immunosuppression. To avoid transplant rejection, the induction of tolerance to donor-specific antigens has been coined as a therapeutic target for decades. For this, both donor tolAPC and recipient tolAPC loaded with donor-specific antigens are being considered for development of cell-based immunotherapeutic protocols in the transplantation setting (67). However, whereas the clinical use of donor-derived tolAPC is only feasible in the context of living donor transplantation and entails a risk of sensitization (including development of allo-antibodies), the use of autologous, i.e., recipient-derived, tolAPC is a less risky approach to begin with. Indeed, the use of recipient autologous tolAPC is likely to be more feasible than that of donor-derived tolAPC, since cell products can be prepared from the peripheral blood of the recipient before transplantation, stored while the patient is on the waiting list, and loaded with donor-derived antigens (such as HLA peptides or donor cell lysates) at the time of transplantation. In the context of the ONE study, two trials using tolDC and Mreg are being performed in living-donor kidney transplant recipients (Table 1).

Route of Administration of TolAPC

Although it is generally accepted that the route of administration is important for optimal tolAPC effector function, the best route of tolAPC administration is not known. To date, a variety of routes of administration have been used (see Table 1), including intradermal, intraperitoneal (19), intravenous and intra-articular (17). Different routes of administration may be required to allow tolDC to reach the relevant draining lymphoid tissue for T cell encounter or to end up at the site of inflammation. Especially since tolDC demonstrate a reduced expression of CCR7 and consequently a reduced (but not absent) ability to migrate in response to the CCR7 ligand CCL19 (68), the capacity of tolDC to reach the lymph nodes is a critical concern. While the migration of DC toward the lymph nodes increases following intradermal as compared to subcutaneous administration, only 2–4% of DC migrate to the draining lymph nodes after intradermal administration, but the situation may be different in patients with autoimmune diseases where monocyte-derived DC from MS patients have shown a significantly higher proportion of CCR7-expressing cells compared to healthy controls (69). Given these observations, and that in the setting of cancer vaccine development, DC injected into a lymphatic vessel showed a prolonged half-life as compared to DC injected intravenously (70, 71), direct intranodal injection of tolDC is being evaluated in a clinical setting (see Table 1).

As an alternative to lymph node targeting of tolAPC, tolAPC may also be introduced directly into the site of inflammation. Indeed, injection into the affected disease site (i.e., an inflamed joint) where the tolAPC could suppress auto-reactive effector T cell responses is logical. In this context, intra-articular injection

of tolDC differentiated using dexamethasone and vitamin D₃ and loaded with autologous synovial fluid in patients with rheumatoid arthritis was demonstrated to be safe and feasible. Hence, despite the fact that tolDC were directly injected at the site of inflammation, no adverse events were observed in most patients and hypertrophy, vascularity and synovitis were stable in all treated cohorts. Moreover, two patients receiving 10 million cells showed a decrease in synovitis score (17). Similarly, a phase I randomized clinical study currently evaluates the safety and efficacy of tolDC injected into the intestinal lesions in patients with refractory Crohn's disease (Table 1). In some conditions such as T1D, direct injection of cells in the inflammatory site, e.g., pancreas, might not be possible and require tolDC administration adjacent to the inflammation site. For the treatment of inflammatory diseases of the brain, the blood-brain barrier (BBB) may represent a major hurdle. Considering this potential problem, it was demonstrated that enhancing CCR5 expression in tolDC using mRNA electroporation endowed these cells with CCR5-driven migratory capacity. This enabled the cells to migrate to inflammatory sites, even when it required crossing of functional barriers such as the BBB (72). Similarly, introducing CCR7 expression in tolDC using the proposed approach of chemokine receptor mRNA electroporation could overcome the limited lymphoid homing capacity of tolDC. Indeed, DC transduced with lentiviral vectors coding for CCR7 and IL-10 genes were able to migrate to the lymph nodes and spleen, prolonging cardiac allograft survival in mice (73). However, there are still many unknowns and there is a clear clinical need to characterize the pharmacodynamics of tolDC in humans and relate this to clinical efficacy. Advances in cell imaging techniques, for example magnetic resonance imaging of 19F-labeled cells, have made it possible to address this question in future studies.

TolAPC Therapy: What Does the Future Hold?

In vivo Targeting

While our knowledge of tolAPC biology has expanded greatly, and *in vitro* generated tolDC and Mreg are currently being used in various clinical trials (Table 1), clinical-grade manufacturing of tolAPC is still a time-consuming and expensive process. It requires cell precursors that need to be isolated from the patient's blood, modulated *ex vivo* and reintroduced into the patient. Direct antigen delivery to tolAPC *in vivo* may limit the workload and costs. Indeed, specific antigen-targeting of DC-restricted endocytic receptors (DEC-205) with monoclonal antibodies has been shown to induce antigen-specific T cell hypo-responsiveness in experimental models (74). Interestingly, a phase I clinical trial demonstrated that *in vivo* targeting of human DC could be achieved by antibodies against DEC205 with subsequent antigen presentation and robust humoral and cellular responses (75). *In vivo* targeting of DC with biomaterials such as liposomes, microparticles and nanoparticles is also a promising approach [as reviewed in (76–78)]. This is exemplified by the fact that liposomes loaded with NFκB inhibitors targeting APC *in situ*, suppress the cellular responsiveness to NF-κB and induce

antigen-specific FoxP3⁺ regulatory T cells in an animal model of arthritis (79) and that administration of phosphatidylserine-rich liposomes loaded with disease-specific autoantigens lead to a beneficial effect in experimental models of T1D and MS (80, 81). Nevertheless, DC represent a heterogeneous cell population arising from bone marrow-restricted precursors identified in humans. While multiple subsets of DC have been found in the peripheral blood, lymphoid organs and tissues, most of the hallmark DC markers are promiscuously expressed making it difficult to unambiguously discriminate between DC subpopulations and specifically target those DC subpopulations that induce tolerance. Extensive phenotypic screening combined with gene expression profiling allows the identification of tolerance-inducing DC counterparts present *in vivo*. For instance, Gregori and co-workers identified a DC subset expressing HLA-DR⁺CD14⁺CD16⁺ that exhibits potent tolerogenic activity (44). In targeting only such tolerance-inducing DC cell type-specific targeting may emerge as another promising approach in DC-based immunotherapy.

Combination Therapy

Since a variety of often complementary mechanisms are involved in the maintenance of immune tolerance, a more complex therapeutic approach using combinations that target different pathways that contribute to induction and maintenance of tolerance may be required to fully control autoimmunity. For instance, combinations of tolAPC with disease-modifying treatments that reduce the frequency of disease-causing cells, e.g., alemtuzumab, should be explored as the latter therapy reduces the disease-causing cells to a number that may be more effectively controlled by antigen-specific tolerance-inducing strategies, such as tolDCs. Alternatively, therapies like fingolimod, an antagonist of sphingosine-1-phosphate receptor which retains naïve and central memory T cells in the lymph nodes, are promising as co-medication with tolAPC as it could increase the number of tolDC-T cell interactions in the lymph node thereby facilitating Treg priming and consequently the efficacy of tolDC-based strategies. Also in the context of solid organ transplantation, tolDC therapy could be improved by the addition of a complementary treatment. For instance, the combination of adoptive transfer of tolDC and CTLA-4 Ig, a fusion protein that blocks B7-CD28 costimulation, resulted in extended survival of MHC-mismatched heart allografts in mice (82, 83), while the pancreatic islet allograft survival improved by combination of autologous tolDC and CD3 targeting antibodies (84). With the recent data on the important role of other coinhibitory molecules for T cell-mediated inflammation such as CD96 the portfolio of combination therapies might increase in the next years (85). However, since antigens can easily trigger immunity instead of tolerance, a primary concern remains the safety of combining two immune-modulatory vaccination strategies in autoimmune diseases and in the prevention of transplant rejection. Although one can envisage that concomitant use of immunosuppressive therapies might synergistically reduce the risk to unexpectedly worsen antigen-specific reactions (86), any novel manipulation of the immune system may involve

an unpredictable risk. Furthermore, combination therapy may introduce confounding factors inducing additive, synergistic or antagonistic effects complicating the evaluation of the precise mode of action.

Conclusion

Several protocols to generate and administer tolAPC have been tested in phase I clinical trials with highly encouraging results from a safety point of view and in terms of adverse effects (Table 1). Further phase I/II studies are under way in Crohn's disease, T1D, rheumatoid arthritis, MS and kidney transplantation (Table 1). However, for the success of future tolAPC trials, there is great need to define the optimal vaccination protocol; to ensure optimal *in vivo*-acting of the tolAPC, future trials may require changes in administration route, dose or could demand repeated tolAPC administration. Furthermore, the identification of a common set of tolerogenic markers would enable optimized comparison of tolAPC products and their tolerance-inducing potential and provide an improved understanding of how these cells modulate the T cell response both locally and systemically. It would be of great help to analyze critical pathways contributing to programming and function of tolAPC. Ultimately, this may set the stage for new approaches improving the therapeutic potential of tolAPC for the future.

CD4⁺ REGULATORY T CELLS (TREG)

CD4⁺ Treg are recognized as a dominant cell population responsible for induction and maintenance of immune tolerance. They may be generated either in the thymus as natural regulatory T cells (nTreg or tTreg) or in the periphery as induced regulatory T cells (iTreg). Both subsets can induce tolerance toward auto- and alloantigens utilizing a variety of mechanisms including cell-to-cell contacts, secretion of immunosuppressive cytokines and inhibitory molecules (e.g., adenosine or prostaglandin E), local depletion of IL-2, or through killing of other cells (87). Treg actively traffic to inflammatory sites and the suppressive activity is usually localized without a significant impact on the general immunity. Since their more precise identification 2–3 decades ago in the mouse, and more recently in the human, steady advances in understanding Treg biology have eventually provided sufficient knowledge to culture, manipulate and expand the cells *in vitro* under Good Manufacturing Practice (GMP) conditions for therapeutic purposes. Indeed, Treg have become a promising cellular drug that can potentially be used to control disease-causing immune responses.

Treg in Clinical Practice

While the application of Treg for the treatment of autoimmune diseases is currently under intense investigation, Treg were first used in the clinic to treat patients with graft vs. host disease (GvHD) after hematopoietic stem cell transplantation (HSCT) (88) (Table 2). Results from the clinical trials in GvHD with polyclonal expanded Treg have suggested that altogether these cells are safe, but there is some concern about the occurrence of mild to moderate infections, and it still is unclear whether Treg treatment could promote cancer (92, 94). The latter problem has

been reported in only one trial to date, however it was concluded that the tumor was present before the therapy with Treg was applied (94). The safety and feasibility of adoptive transfer of *ex vivo* expanded Treg was further confirmed in T1D patients (2), which has driven the application of Treg therapy to clinical trials in other autoimmune conditions such as MS, autoimmune hepatitis, systemic lupus erythematosus, Crohn's disease, and autoimmune uveitis (102) (Table 2). Another clinical trial was recently published where polyclonal Treg were injected into T1D patients; results from this trial confirm the safety of this type of therapy and also show for the first time, by deuterium labeling of the Treg, that some of the injected Treg can be detected for up to 1 year after infusion (103).

Treg therapy is now being applied as a “tolerogenic” therapy to reduce dependency on immunosuppressant drugs in patients receiving solid organ transplants. The idea behind this strategy is very similar to the application of Treg in autoimmune diseases, namely to tilt the balance toward Treg dominance over rejection-causing Teff cells. The first reports using adoptive transfer of Treg in kidney transplant patients have been recently published demonstrating the safety of this strategy in the context of solid organ transplantation (103, 104). Recently, clinical trials are being completed using different variations of Treg products (*The ONE Study* and *ThRIL*) (Table 2). *The ONE Study* includes Phase I clinical trials comparing the safety of different types of regulatory cells, including polyclonal and donor-reactive Treg in patients receiving kidney transplants (www.onestudy.org) (20). The *ThRIL* trial is a Phase I/IIa dose-escalation clinical trial in the setting of liver transplantation. Results from the *ThRIL* and the various *ONE Study* trials are currently being prepared for publication. The impact of Treg on the recipient immune system will be revealed only when the very detailed immunomonitoring is completed, which is a major objective of the described clinical trials (105–107).

Altogether, from the outcomes of the completed clinical trials so far, it can be concluded that adoptive transfer of Treg is safe and technically feasible (Table 2). Therefore, increasing efforts are currently focusing on clinical trials to test their therapeutic efficacy. Importantly, several lessons have been learned from recent experiences with Treg to improve future trial designs. For example, the clinical state of the patients has been shown to influence the function and properties of Treg, and therefore can condition the success of *ex vivo* cell product expansion (108). Furthermore, the specific expansion protocol can affect Treg function and specificity, and can improve tissue targeting and suppression capacity (108). Finally, the immune modulatory therapies received by patients at the time of Treg adoptive transfer can positively or negatively impact therapeutic outcome (109, 110).

Treg Therapy: What Does the Future Hold? Antigen-Specificity of Treg Therapy

Studies in preclinical models using murine Treg have demonstrated that specificity for either the auto or allo (transplantation) -antigens may offer an advantage for Treg function compared to polyclonal Treg (111). Adoptively transferred allospecific murine Treg generated by using either

TABLE 2 | Completed or ongoing trials with Treg in autoimmunity and transplantation.

Study ID	Phase	Cell product	Indication	Dosing scheme	Tregs per dose	Outcome	Center	References
HSCt								
NKEBN/458-310/2008	I	Expanded polyTregs	GvHD treatment	Single injection or 3 injections	1 × 10 ⁵ /kg bw 3 × 10 ⁶ cells/kg bw	-Safe -Reduced immunosuppression in chronic GVHD -Only transient improvement in acute GVHD	Gdansk/PL	(88)
NCT00602693	I	Expanded OB polyTregs	GvHD prophylaxis	Single injection	Dose-escalation: 1, 3, 10, 30, 30+30, 100, 300, 1,000 and 3,000 × 10 ⁵ /kg bw	-Safe -Increased incidence of infections -Reduced incidence of acute GvHD/GvL effect	Minnesota/USA	(89, 90)
01/08	I	Fresh polyTregs	GvHD prophylaxis	Single injection	Dose-escalation: 0.5 × 10 ⁶ Tcons/kg bw with 2 × 10 ⁶ Tregs/kg bw 1 × 10 ⁶ Tcons/kg bw with 2 × 10 ⁶ Tregs/kg bw −2 × 10 ⁶ Tcons/kg bw with 4 × 10 ⁶ Tregs/kg bw up to 5 × 10 ⁶ /kg bw	-Safe -Reduced number of leukemia relapses -Reduced incidence of GVHD	Perugia/IT	(91, 92)
Treg002 EudraCT: 2012-002685-12 EK 206082008	I	Fresh polyTregs	GvHD prophylaxis	Single injection		Safe	Regensburg/DE	(93)
ALT-TEN	I	Expanded polyTregs	GvHD treatment	Single or 2 injections	0.97–4.45 × 10 ⁶ /kg bw	-Two cases of tumors -Stable chronic GVHD	Dresden/DE	(94)
	I	Tr1 (IL-10 DLI or DC-10 DLI)	GvHD prophylaxis	Single injection	Dose-escalation: 1 × 10 ⁵ , 3 × 10 ⁵ and 1 × 10 ⁶ , 3 × 10 ⁶ CD3+ T cells/kg bw	-Safe -Long-term free-disease survival in 4 patients	Milan/IT	(95)
NCT02749084	I/II	Multiple Treg DLI	Severe Refractory Chronic GvHD prophylaxis	3 injections	Dose-escalation: 1.7 × 10 ⁵ , 3.3 × 10 ⁵ and 6.6 × 10 ⁵ /kg bw per injection	Recruiting	Bologna/IT	
NCT02991898	II	Fresh CB polyTregs with IL-2	aGvHD prophylaxis after CB Tx	Single injection	No data	Suspended	Minnesota/USA	
NCT01911039	I	polyTregs	Steroid Dependent/Refractory Chronic GvHD treatment	Single injection	Dose-escalation: 1 × 10 ⁵ , 5 × 10 ⁵ , 1.5 × 10 ⁶ /kg bw	Unknown	Stanford/USA	
NCT02385019	I/II	Fresh donor polyTregs	Steroid-Refractory Chronic GvHD treatment	Single injection	Dose-escalation: 0.5 × 10 ⁶ , 1.0 × 10 ⁶ and 2.0–3.0 × 10 ⁶ /kg bw	Recruiting	Lisboa/PT	
NCT01937468	I	Fresh polyTregs with IL-2	Steroid -Refractory Chronic GvHD treatment	Unknown	Unknown	Active/not recruiting	Boston/USA	

(Continued)

TABLE 2 | Continued

Study ID	Phase	Cell product	Indication	Dosing scheme	Tregs per dose	Outcome	Center	References
NCT01903473	I	Fresh polyTregs with rapamycin	aGvHD and cGvHD treatment	Single injection	$\geq 0.5 \times 10^6$ /kg bw	Unknown	Liege/BE	
EudraCT: 2012-000301-71	I	Fresh polyTregs with rapamycin	Steroid -Refractory Chronic GvHD treatment	Single injection	$\geq 0.5 \times 10^6$ /kg bw	Recruiting	Liege/BE	
NCT01795573	I	Donor polyTregs expanded with recipient DC	aGvHD prophylaxis	Unknown	Unknown	Recruiting	Tampa/USA	
NCT01660607	I/II	Fresh polyTregs with Tconv	aGvHD prophylaxis	Single injection	initial doses will be 1×10^6 Treg/kg bw to 3×10^6 Tcon/kg bw (ratio 1:3)	Recruiting	Stanford/USA	
NCT02423915	I	Fucosylated fresh CB polyTregs	GvHD prophylaxis	Single injection	Dose-escalation: 1×10^6 /kg bw 1×10^7 /kg bw	Active/Not Recruiting	Houston/USA	
BMT Protocol 204 NCT01050764	I	Fresh allogeneic polyTregs with Tconv	GvHD prophylaxis	Single injection	Dose-escalation: 1×10^5 Treg and 3×10^5 T con/kg bw or 3×10^5 Treg and 1×10^6 Tcon/kg bw or 1×10^6 Treg and 3×10^6 Tcon kg/bw or 3×10^6 Treg and 1×10^7 Tcon/kg bw	Terminated, GvHD was within parameters to continue, but the study was terminated due to poor outcomes prior to sufficient accrual to set the MTD, even at the only dose tested (1×10^5 Treg and 3×10^5 T con/kg bw). Primary outcome result is null.	Stanford/USA	
NCT01634217	I	iTregs	GvHD prophylaxis	Unknown	Unknown	Active, not recruiting	Minnesota/USA	
TRANSPLANTATION								
NCT02129881	I/II	Expanded polyTregs	Living donor kidney Tx	Single injection	$1-10 \times 10^6$ /kg bw	Completed, no results yet	London, Oxford/UK	(20)
ONEnTreg13 NCT02371434 EudraCT: 2013-001294-24	I/II	Expanded polyTregs	Living donor kidney Tx	Single injection	Dose-escalation: 0.5×10^6 , 1×10^6 , and 3×10^6 /kg bw	Completed, no results yet	Berlin/DE	(20)
DART NCT02244801	I/II	Donor-Alloantigen-Reactive (dar) Tregs	Living donor kidney Tx	Single injection	Dose-escalation: 300×10^6 darTreg or 900×10^6 darTreg	No longer recruiting	San Francisco/USA	(20)
NCT02091232	I/II	Belatacept-conditioned Tregs	Living donor kidney Tx	Unknown	Unknown	No longer recruiting	Boston/USA	(20)
ThRIL NCT02166177	I	Expanded polyTregs	Liver Tx	Single injection	low dose and high dose	Completed, no results yet	London/UK	

(Continued)

TABLE 2 | Continued

Study ID	Phase	Cell product	Indication	Dosing scheme	Tregs per dose	Outcome	Center	References
NCT02188719	I	Donor-Alloantigen-Reactive Tregs	Liver Tx	Single injection	Dose-escalation: 0 × 10 ⁶ darTreg or 50 × 10 ⁶ darTreg or 200 × 10 ⁶ darTreg or 800 × 10 ⁶ darTreg	Recruiting	San Francisco/USA	
NCT02088931	I	Expanded polyTregs	Subclinical rejection in kidney Tx	Single injection	200 × 10 ⁶	Open/Not recruiting	San Francisco/USA	
NCT02474199	I	Donor-Alloantigen-Reactive Tregs	CNI reduction in liver Tx	Single injection	300–500 × 10 ⁶ /kg bw	Recruiting	San Francisco/USA	
NCT01624077	I	Donor-antigen expanded Tregs	Liver Tx	Multiple injections at several intervals	1 × 10 ⁶ /kg bw per injection	Unknown	Nanjing/CHN	
NCT01446484	I	polyTregs	Living donor kidney Tx	Single injection sub-cutaneous	2 × 10 ⁸ s.c.	Unknown	Moscow/RUS	
TRACT	I	Expanded polyTregs	Living donor kidney Tx	Unknown	Unknown	Active, Not recruiting	Chicago/USA	
NCT02145325								
NCT02711826	I/II	Donor-Alloantigen-Reactive Tregs vs. polyTregs	Subclinical rejection in kidney Tx	Single injection	400 ± 100 × 10 ⁶ darTregs	Recruiting	Birmingham, Los Angeles, San Francisco, Ann Arbor, Cleveland/USA	
AUTOIMMUNITY								
TregVAC	I	Expanded polyTregs	Recent T1D	Single or 2 injections	Dose-escalation: 10 × 10 ⁶ , 20 × 10 ⁶ , or 30 × 10 ⁶ /kg bw	-Completed -Safe -Reduced insulin consumption (insulin independence in 2 out of 12 patients) -Better stimulated C-peptide secretion profiles	Gdansk/PL	(96–98)
ISRCTN06128462								
NCT01210664	I	Expanded polyTregs	T1D	Single injection	Dose-escalation: 0.05 × 10 ⁸ , 0.4 × 10 ⁸ , 3.2 × 10 ⁸ , 26 × 10 ⁸	Completed/Safe	San Francisco/USA	(99)
CATS1	I/II	Ovalbumin-specific Tr1	Refractory Crohn's disease	Single injection	Dose-escalation: 1 × 10 ⁶ , 10 ⁷ , 10 ⁸ , or 10 ⁹	-Safe -Clinical response in 40% of patients	Lille/FR	(100)
CATS29	II	Ovalbumin-specific Tr1	Refractory Crohn's disease	Single injection	1 × 10 ⁶ /kg bw	Terminated/completed	Valbonne/FR	
EudraCT: 2014-001295-65							Multicenter: AT, BE, FR, GE, IT, UK	
NCT02327221								

(Continued)

TABLE 2 | Continued

Study ID	Phase	Cell product	Indication	Dosing scheme	Tregs per dose	Outcome	Center	References
TregVAC2.0 EudraCT: 2014-004319-35	II	Expanded polyTregs combined with antiCD20 antibody	Recent T1D	2 injections, 3 months apart	30 × 10 ⁶ /kg bw per injection	Recruitment closed/Follow up in progress	Gdansk/PL	
TregSM EudraCT: 2014-004320-22	I	Expanded polyTregs	MS	Single injection: Cohort I – <i>intravenous</i> Cohort II – <i>intrathecal</i>	Up to 40 × 10 ⁶ /kg bw	Recruiting	Gdansk/PL	
NCT02704338	I	Expanded polyTregs	Autoimmune hepatitis	Single injection	10–20 × 10 ⁶ /kg bw	Not yet recruiting	Nanjing/CHN	
NCT02772679	II	Expanded polyTregs with IL2	Recent T1D	Single injection	3 or 20 × 10 ⁶ /kg bw	Suspended	San Francisco/USA	
NCT02428309	II	Expanded polyTregs	Systemic lupus erythematosus	Single injection	Dose-escalation: 1 × 10 ⁸ or 4 × 10 ⁸ or 16 × 10 ⁸	Active/Not Recruiting	San Francisco/USA	
NCT02932826	I	Expanded third-party CB polyTregs	Recent T1D	Single injection	1–5 × 10 ⁶ /kg bw	Recruiting	Hunan/CHN	
NCT03011021	I	Expanded third-party CB polyTregs and Lirilutide	Recent T1D	Single injection	1–5 × 10 ⁶ /kg bw	Recruiting	Hunan/CHN	
T-Rex Study NCT02691247	II	Expanded polyTregs	Recent T1D	Single injection	low dose and high dose	Active/Not Recruiting	San Francisco, Aurora, New Haven, Gainesville, Miami, Indianapolis, Boston, Fargo, Kansas City, Portland, Sioux Falls, Nashville/USA	
OTHER								
NCT03101423	I	Donor polyTregs DLI	Beta Thalassemia Major	Unknown	Unknown	Active/Not Recruiting	Nanning/CHN	
NCT03241784	I	Donor polyTregs DLI	Amyotrophic Lateral Sclerosis (ALS)	8 injections iv in total concomitant with IL2 sc.: 4 injections over 2 months at early stage and 4 injections over 4 months at later stages	1 × 10 ⁶ /kg bw per injection	completed	Houston/USA	(101)

This table is an updated version of the table in (102) and is based on information deposited on www.clinicaltrials.gov, www.clinicaltrialsregister.eu and/or indicated references. Route of administration of the Tregs is intravenous, except where otherwise noted. AE, adverse event; T1D, type 1 diabetes; Tx, transplantation; MS, multiple sclerosis; /kg bw, per kg body weight; HSC-T, hematopoietic stem cell transplantation; CB, cord blood; gvHD, graft vs. host disease.

donor-derived APC or TCR transduction promote indefinite heart allograft survival, even in completely mismatched mouse strains (111). This strategy was subsequently applied to human Treg in a humanized mouse model, where Treg were generated in the presence of donor APC /DCs or B cells) and shown to be superior to polyclonal Treg in protecting from human skin graft rejection (112). More recently, by conferring specificity using a chimeric antigen receptor (CAR), human Treg transduced with a lentivirus encoding for HLA-A2-CAR were superior to polyclonal Treg in protecting HLA-A2⁺ human skin grafts (113–115). CAR constructs are now being developed to increase Treg stability and function.

Based on promising results with antigen-specific Treg in pre-clinical models, the use of alloantigen-specific Treg generated by culturing recipient Treg with donor-specific cells, either using activated donor-derived B cells (112) or donor-derived DCs is being tested in clinical trials (Table 2). Compared to the transplantation field where the antigens are known, generation of antigen-specific Treg in autoimmunity is more challenging because the inciting antigens are often not known (see also paragraph Antigen Specificity of tolAPC-Based Immunomodulation). New data suggest that regulatory and effector cell subsets are driven by different epitopes (116, 117). In addition, the auto-antigen triggering the autoimmune condition can change during disease progression due to epitope spreading and antigen-specific Treg may thus need to be tuned toward specific stages of disease.

Combination Therapy

Since adoptive transfer with Treg alone, particularly with a one-time infusion, may not be sufficient to control the immune response, combined or successive therapies are being tested. One approach is based on evidence that low doses of IL-2 can preferentially increase the endogenous pool of Treg; so far, low-dose IL-2 treatment has been safe in inflammatory conditions such as GvHD after HSCT (118). In a preclinical model it was demonstrated that IL-2/anti-IL-2 complexes not only promote Treg proliferation, they increase Treg survival and function while synergizing with calcineurin inhibitors to prolong graft survival (119). Recently, in a murine model of transplantation it was shown that by combining donor-specific Treg with the IL-2/anti-IL-2 complexes, a synergistic effect in extending skin transplant survival is observed (Ratnasothy et al., unpublished data). These results pave the way for the first clinical trial in liver transplant patients to combine Treg and low-dose IL-2 therapy (NCT02949492). More caution is being exercised regarding similar trials using low-dose IL-2 in autoimmune diseases, since in contrast to HSCT or solid organ transplantation, autoimmune patients are not lymphopenic and are likely to produce IL-2 themselves (98). However, in some conditions such as systemic lupus erythematosus an intrinsic defect in Tregs contributes to disease progression and here low-dose IL-2 therapy was shown to correct the defect (120, 121). Furthermore, in T1D settings low dose IL-2 treatment is currently being investigated (NCT02411253).

The positive effects of rapamycin on Treg *in vitro* or in a transplant setting (122–124) could not be effectively translated to

the *in vivo* treatment of autoimmune syndromes. For example, while rapamycin administered to T1D patients preferentially increased Treg levels, pancreas function deteriorated due to islet toxicity (125). Nonetheless, the complex and specific pathogenesis of autoimmune syndromes may provide hints toward the design of new combined therapies. Tandemly targeting different effector mechanisms involved in particular syndromes with Treg therapy may improve outcomes. For example, an ongoing trial in early phase of T1D (EudraCT: 2014-004319-35) supports the idea of a synergistic approach by combining Treg administration with B cell depletion.

New Technical Advancements

It is now accepted that the optimal way to manufacture Treg for clinical use requires an efficient *ex vivo* expansion rate while maintaining purity and suppression potency before GMP product release. Two main Treg isolation strategies are currently being used in clinical trials to purify the starting Treg, immunomagnetic selection and flow cytometry cell sorting. The magnetic platform (CliniMACS CD4⁺CD25⁺ selection) provides a highly automated GMP-grade approach which is easy to standardize across centers. However, the resultant Treg product does generally contain a minor population of CD127⁺ cells that could jeopardize product purity after expansion. Addition of rapamycin to the culture media has helped to maintain Treg purity and function, without reducing the expansion rate too much (126). The second method, which uses a flow cytometry approach, results in a highly pure Treg population due to CD127⁺ cell depletion ability. However, this cell-sorting strategy entails more complex protocols and challenges to maintain GMP grade. With the appearance of new GMP-compatible cell sorters (Tyto MacQuant, Miltenyi or Influx, BD Biosciences), this sorting approach is likely to become the preferential Treg isolation method.

Although cell-sorting of Treg increases their initial purity, a search for an optimally stable final Treg products is critical therapeutically. It is well known that prolonged *in vitro* culture results in epigenetic changes in Treg—methylation of TSDR region of *foxp3* gene—which in-turn reduces suppressive ability (127). The use of anti-methylation agents in cultures to prevent epigenetic changes has failed due to culture viability issues. The addition of rapamycin to the expansion culture, as used in both *The ONE Study* and *ThrIL*, was proposed as a remedy to such changes as the drug preferentially expands Treg both *in vitro* and *in vivo* (122). However, proper choice of media, addition of autologous serum, limited time frame for the expansion and temperature decreases during *in vitro* culture to ≈33°C, all impact the suppressive capacity of final Treg products (128–130).

Conclusion

Treg therapies are currently undergoing intense testing. An interest in therapeutic Treg preparations has resulted in several ongoing clinical trials in the transplantation setting, in autoimmune diseases, but now also in conditions such as beta thalassemia major and amyotrophic lateral sclerosis (101) (Table 2). We await new testing in the setting of hypersensitivity and cardiovascular disease (102), and anticipate that Treg therapy

will be considered for any condition where there is evidence for an immune regulation imbalance.

TIMING OF TOLERANCE-INDUCING CELL THERAPY

Timing of tolerance-inducing cell therapy in relation to the transplant or in the course of autoimmune disease development needs specific consideration. Clinical trials using Treg for GvHD indicate that Treg should be injected as early as possible, preferentially before disease onset (89, 91, 131). Early treatment is particularly important to achieve a high ratio between Treg and detrimental effector T cells, and thus prevent development of acute rejection or GvHD. In both HSCT and solid organ transplantation, tolerance-inducing cells are therefore mainly given around, just before or shortly after, the transplantation (see **Tables 1, 2**). Although early treatment is likely more effective, this approach encounters limitations such as increased immunosuppression doses and anti-CD25 treatment, which can interfere with the activity of infused cells. Thus, Tregs have also been infused at later time points e.g., 6 months after kidney transplantation in patients with biopsies showing evidence of inflammatory infiltrates to treat ongoing chronic rejection (103). It must also be considered that disease diagnosis timing is a factor in this respect. Although it is likely best to give the tolerance-inducing cell treatment as early as possible in disease development, it is currently not feasible in treating autoimmunity since a majority of autoimmune diseases start long before clinical symptoms and diagnosis, this brings in the added factor that the functional capacity of the attacked organ may already be irreparably damaged by the ongoing autoimmune process. Future insight into autoimmune disease development and early biomarkers will hopefully allow for earlier treatment with tolerance-inducing cell products.

REGULATIONS

The perspectives of tolerance-inducing cellular therapy depend also on recently introduced regulations. The majority of tested preparations in Europe are now classified as drugs under the 1394/2007 EU directive on advanced therapy medicinal products (ATMP). This significantly changes the legal path for their registration requirements, for manufacturing license and marketing authorization. While the idea of an ATMP in Europe is relatively nascent, and it is continuously evolving through public consultations with interested parties, cellular drugs have a distinct central paneuropean path for registration. While the Committee for Advanced Therapies (CAT) steers this process in Europe to optimize safety for patients, it would be useful to introduce wider rules allowing for introduction of the cells as a routine treatment. To accelerate the whole process, acknowledgment of flexible new types of manufacturing cGMP equipment and reagents could open the way to more widespread ATMP use. Furthermore, measures to reduce manufacturing costs would lessen this major limitation to new trials. When considering cell therapy, scientists, physicians and regulators

must keep in mind that ATMP must be affordable for patients and society. Interestingly, scientists are largely responsible for current guidelines, and should revisit those recommendations based on factors such as cost (55, 132).

IMMUNOMONITORING OF TOLERANCE-INDUCING CELLULAR THERAPIES

Tackling Immunomonitoring in Tolerogenic Therapies

Since cell-based therapies are becoming more common, it is important to reliably monitor the immune system for both desired and undesired immunological effects. Rigorous immunomonitoring will therefore provide information about the safety of these treatments, ideally at early time points after CTT administration. In addition it will give insight in the therapy-related mechanisms of tolerance-induction and maintenance and may aid in patient-tailoring of therapy. To accurately measure the effects, especially across different trials, it will require introduction of harmonized and validated immunomonitoring assays.

There are a number of possible assays that can determine cell therapy effects in humans. For instance, measurement of circulating cytokines, C-reactive protein or changes in antibody titer can determine immune status. Similarly, measuring CD4⁺ T cell responses after viral-antigen stimulation is possible by flow cytometry via CD40L expression or cytokine production in a functional assay; these assays could potentially identify non-specific immunosuppressive effects (133). Although the completed clinical trials have shown so far that cell-based tolerogenic therapies are safe and do not cause serious undesirable immune responses (96, 99, 103, 104), the extent to which these treatments achieve therapeutic efficacy remains largely undetermined. Current cell-based tolerogenic trials in autoimmunity and transplantation include clinical outcome measures such as C-peptide response, insulin consumption or reduction of immunosuppressive doses. However, clinical endpoints may not necessarily reflect the efficacy of cell-based therapies, since the tolerogenic effect of the transferred cells may not directly lead to immediate changes in systemic parameters such as inflammation, potentially underestimating a longer term effect. It is therefore important that future clinical trials incorporate suitable monitoring methods to assess the immunomodulatory effects of cellular therapies.

General vs. Specific Monitoring Assays

To assess therapeutic effectiveness, different methods have been proposed to identify tolerogenic responses. The assessment of *in vitro* autoreactive or donor-specific T cells responses prior to and after treatment could provide a precise evaluation of therapeutic efficacy. Antigen-specific assays allow discrimination between targeted tolerance to the induction of general immune suppression and loss of responses to pathogens. In addition, these methods provide an efficacy readout for antigen-specific therapies such as tolerogenic APC loaded with antigens or the

generation of donor-specific Treg. The identification of antigen-specific immune responses by ELISPOT (134) or through flow cytometry detection of CD40L upregulation (135) have shown promising results in predicting kidney and liver allograft rejection, suggesting potential applicability for the evaluation of tolerogenic therapies. Monitoring targets will be different depending on the main immune population involved in the disease (e.g., CD4, CD8T cells or antibodies). Unfortunately, the antigens mediating the immune responses in autoimmunity are not always available or identified (as discussed in paragraph Antigen Specificity of tolAPC-Based Immunomodulation); HLA antigens in the case of transplantation are known and can be used, or stimulation with donor or donor-matched cells is possible. Though less specific, identification of phenotypic or functional changes by flow cytometry on the total pool of cells targeted (e.g., Treg) by the tolerogenic treatment may reveal therapeutic effectiveness. Indeed, the acquisition of tolerance in animal models and in the clinic is associated with an increased number of regulatory cells and decreased pro-inflammatory function of innate and adaptive immune cells (5, 136, 137). Therefore, flow cytometry analysis to delineate the distribution and activation status of different cell types, or *in vitro* assays to evaluate the suppressive and inflammatory function of circulating cells can provide a non-specific approach to assess the development of tolerogenic properties (138). Other non-specific strategies such as gene expression profiling of circulating immune cells or tissue biopsies can add to the functional assessment of immune responses. Furthermore, there is a growing body of work focusing on the validation of transcriptional signatures to predict transplantation tolerance in liver and kidney transplant recipients (139–141), which may prove to be a valuable tool in assessing the efficacy of tolerogenic therapies.

Tracking of Cellular Product

The evaluation of homeostatic characteristics of infused cells such as survival, stability or tissue migration, constitutes another monitoring objective to assess therapeutic efficacy. Being able to track infused cells will help to determine the best site for administration/application of tolerogenic cell products. While simple phenotypic detection (flow cytometry) of the transferred cell subset after treatment suggests the presence of infused cells, it does not distinguish transferred from endogenous cells. Therefore, current techniques for tracking infused cells depend on direct cell labeling strategies, such as indium labeling or deuterium introduction during *ex vivo* expansion, with subsequent isotope detection in the different tissues or compartments (99, 103, 142). Unfortunately, this method is only semi-quantitative, since individual cells are not detected. Individual cells can be labeled with rare earth metals and detected with precision *in vivo* using laser ablation-inductively coupled plasma-mass spectrometry, but so far this has only been tested in mouse models (143, 144). Other emerging therapeutic approaches such as CAR-Treg could take advantage of genetic modifications to adapt reported gene imaging strategies to detect the transferred cells by non-invasive methods (e.g., MRI, PET, SPECT) (145, 146). Additionally, the use of T cell receptor (TCR) engineered Treg or *ex vivo* expanded antigen-specific Treg create the opportunity to track infused cells by predefining the TCR

clones and identifying them among the T cell compartment repertoire through TCR sequencing analysis (147).

Harmonization to Allow Comparison

Current tolerogenic cell-based trials are highly heterogeneous, comprising different cell types with variable manufacturing approaches, and targeting various autoimmune diseases and transplantation settings. Furthermore, by their very nature such trials tend to involve low numbers of patients, often participating in different centers or countries. It is therefore essential to establish common immunomonitoring strategies in the research community to achieve robust and reliable data which can be compared and combined between trials. First, not all the immunomonitoring methods are similarly standardisable, and second, not all the centers have the technical expertise or infrastructure to perform certain assays. Sample collection and storage of whole blood or tissue biopsies for transcriptional analysis does not involve extensive sample manipulation, making standardization of this method achievable. On the contrary, while flow cytometry analysis of circulating blood is an accessible technology, instrumentation must be calibrated appropriately to accurately define cell subsets, and analysis of the data needs to be strictly regulated. Although centralized phenotypic analysis in multicenter trials is feasible (105), this involves significant logistical challenges, including the decision to either test fresh or frozen samples; frozen samples sacrifice accuracy due to loss of certain cell populations during separation and freezing procedures. In general, flow cytometry standardization requires extensive cooperation between centers and precise planning. To further harmonize flow cytometry data implementation of automated gating approaches will be of outmost importance in the future (148–150). Finally, functional *in vitro* assays also represent a challenging method to standardize, involving different approaches depending of the cell type and function. Nevertheless, several efforts have been made to establish a minimal harmonization of antigen-specific functional assays (56). While these assays are likely to be the most informative in the assessment of therapeutic efficacy, it is unlikely that current assay results will be directly comparable between independent trials. Therefore, the inclusion of adequate control cohorts and reference groups in the assays, considering the specific cell therapy approaches and disease characteristics, remains an objective to achieve feasible comparisons (107).

FINAL CONCLUSION

Tolerance-inducing cellular therapies have great potential. Several cell types are now in early-stage clinical trials, including various types of Treg and tolAPC (including tolDC and Mreg) (Tables 1, 2). At the present time it is unclear which of these cell types will prove most suitable as a cell-based therapy; each likely has particular advantages that may be suitable for one particular disease or another. In Table 3, the main specific limitations to be considered for the treatment of autoimmunity or transplantation with tolerance-inducing cell therapies are summarized. It becomes clear that although we might treat these

TABLE 3 | Main specific differences in tolerance-inducing cell treatment between transplantation and autoimmune disease setting.

	Transplantation	Autoimmunity
Antigen	<ul style="list-style-type: none"> - Alloantigens (MHC alleles and other disparities) - Autoantigens in case of underlying or de novo developed autoimmune diseases 	<ul style="list-style-type: none"> - Autoantigen not always known - Epitope spreading might occur during disease progression
Pathogenic immune response	<ul style="list-style-type: none"> - Normal, but undesired, immune response against foreign antigen 	<ul style="list-style-type: none"> - Loss of tolerance to self-antigen
Timing	<ul style="list-style-type: none"> - Time point of antigen contact is known, treatment can be given around time point of transplantation 	<ul style="list-style-type: none"> - Disease already develops before clinical symptoms - Better diagnosis and biomarkers are needed to be able to intervene at earlier time point
Route of administration	<ul style="list-style-type: none"> - Intravenous 	<ul style="list-style-type: none"> - Intradermal - Local injection in affected tissue or draining lymph node of the tissue is to be considered
Co-medication	<ul style="list-style-type: none"> - High dose of conventional immune suppression (steroids, CNI, MMF) +/- antibody-based induction therapy at the moment of transplantation 	<ul style="list-style-type: none"> - Varies and is disease specific
Clinical efficacy evaluation	<ul style="list-style-type: none"> - Prevention of acute rejections - Reduction conventional immune suppression. It is difficult to lower co-medication without good markers to predict transplant tolerance 	<ul style="list-style-type: none"> - Disease-dependent. E.g. In T1D C-peptide response or insulin consumption can be determined. In other AID disease-specific scores can be used. - Depending on disease progression on moment of application. Irreversible tissues destruction will not improve. New relapses/lesions can be scored.
Immunomonitoring	<ul style="list-style-type: none"> - Donor antigen-specific T cells - Donor specific antibodies 	<ul style="list-style-type: none"> - Autoantigen not always known. - When known, T cell responses are difficult to detect, since they are very low frequent and of low affinity

diseases with the same manufactured cell product, the optimal treatment regime could be quite different.

Immunomonitoring is an indispensable aspect of current and future tolerogenic cell-based therapies that will provide fundamental information to understand and optimize cell therapies. Monitoring will also aid in identifying biomarkers with the capacity for early identification of therapy responders and non-responders and patient-tailoring of therapy. As part of the overall strategy to increase implementation of ATMPs, it will be critical to harmonize GMP manufacturing protocols, product characterization and immunomonitoring. Minimal information models such as MITAP and MiTREG (132, 151) will serve as important tools in this respect.

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Tolerogenic Role of Myeloid Suppressor Cells in Organ Transplantation

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Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature cells of myeloid origin with a specific immune inhibitory function that negatively regulates the adaptive immune response. Since MDSC participate in the promotion of tolerance in the context of organ transplantation, therapeutic strategies that regulate the induction and development of MDSC have been the center of scientist attention. Here we review literature regarding induction of MDSC with demonstrated suppressive function among different types of allografts and their mechanism of action. While manipulation of MDSC represents a potential therapeutic approach for the promotion of donor specific tolerance in solid organ transplantation, further characterization of their specific phenotype, which distinguishes MDSC from non-suppressive myeloid cells, and detailed evaluation of the inhibitory mechanism that determines their suppressive function, is necessary for the realistic application of MDSC as biomarkers in health and disease and their potential use as immune cell therapy in organ transplantation.

Keywords: MDSC, immune tolerance, transplantation, immunophenotyping, myeloid cells

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INTRODUCTION

Myelopoiesis is a regular process where the cells of the mononuclear phagocyte system (MPS) originate from common myeloid precursors (CMP) leading to monocytes, macrophages and dendritic cells (DC) under steady state. Under acute pathological conditions, myeloid cells respond to immunogenic signals like PAMPs (pathogen-associated molecular patterns) or DAMPs (damage-associated molecular patterns) showing multiple protective immune functions such as phagocytosis, pro-inflammatory cytokine secretion and activation of T cells. Under chronic pathological conditions, such as persistent inflammation or certain malignancies, myeloid cells are stimulated by continue immunogenic signals that have important effects on cell differentiation (1). The standard pathway for CMP cells differentiation is inhibited and the myelopoiesis is altered under chronic inflammation, which results in undifferentiated myeloid cells (2). Myeloid derived suppressor cells (MDSC) represent a mixture of myeloid progenitor cells at different stages of differentiation that may develop into macrophages, DC or granulocytes depending the microenvironment (3, 4).

Since the discovery of MDSC there has been an effort to define their heterogeneity, origin and function beyond cancer relates studies. The scientific interest of these cells have been spread through many fields where the immune system is altered due to chronic pathological conditions,

such as graft vs. host disease (GvHD), organ transplantation, infection, and autoimmune diseases. To better define the heterogeneity, murine MDSC were initially defined as myeloid cells expressing CD11b and GR-1. Based on these two markers and their morphology, MDSC were divided into two major groups: Granulocytic MDSC (G-MDSC) and monocytic MDSC (M-MDSC). Several studies included the differential expression of inducible nitric oxide synthase (iNOS) and arginase (Arg) and subdivided MDSC population based on the expression of Ly6C and Ly6G (5). CD11b+Ly6ChiLy6G- MDSC have a monocytic-like morphology express nitric oxide synthase 2 (NOS2), have increased T cell suppressive activity and are identified as M-MDSC. In contrast, CD11b+Ly6ClowLy6G+ MDSC have a granulocyte-like morphology and express high levels of arginase type 1 (Arg1) (5). Some authors recommend the term polymorphonuclear (PMN)-MDSC instead of G-MDSC attending to the differences in the phenotype from steady-state neutrophils. PMN-MDSC show less granules, reduced CD16 and CD62L and increased Arg1, peroxynitrite and CD11b expression (6). Additional MDSC populations have been described based on the intensity of the Gr-1 gene expression as Gr-1lo, Gr-1int, and Gr-1hi (7). Based on recent findings showing differences in modulation of the cell death pathway, the anti-apoptotic markers c-FLIP and MCL-1 could be also of help to respectively distinguish M-MDSC and granulocytic MDSC subsets. Different from granulocytic MDSC, the continuous expression of c-FLIP is needed by M-MDSC survival and function and defines them as the dominant immunosuppressive subset. This observation points out modulation of c-FLIP in monocytes to promote or block immunosuppressive cells for therapy purposes (8, 9).

The overlapping expression of phenotypic markers makes also difficult to distinguish MDSC from tumor-associated macrophages (TAM) and tumor-associated neutrophils (TAN). Mouse M-MDSC are phenotypically described as CD11b+Ly6ChiLy6G- myeloid cells expressing low levels of F4/80, while TAM express high levels of F4/80 (4). Human MDSC were initially described in cancer patients as lineage negative CD34+ cells (10). Thereafter, other myeloid markers such as the human leucocyte antigen (HLA)-DR was identified in a renal cell carcinoma study to define human MDSC as CD33+CD11b+HLA-DR- (11). Additionally, the use of CD14 expression is accepted for human M-MDSC, although it is still controversial for PMN-MDSC since granulocytes express low levels of this marker. As a result, M-MDSC are defined as CD33+CD11b+HLA-DR-CD14+ while PMN-MDSC are defined as CD33+CD11b+CD15+CD66b+ (6). Recently, another MDSC subpopulation was described as CD33+CD11b+HLA-DR-CD14-CD15-CD66b-, including a mixture of immature cells named early stage MDSC (e-MDSC). Recent findings suggest that PMN- and M-MDSC are the most potent immunosuppressive cells while e-MDSC show less Arg1 and iNOS amounts and may not inhibit T cells proliferation. More studies are needed to understand if e-MDSC are true MDSC precursors and evaluate their clinical significance (12). It is also recommended the use of additional phenotypic markers, such as CD62L, CD16 and the vascular endothelial growth-factor receptor1 (VEGFR1) to better define human MDSC (13).

Phenotypic characterization of MDSC remains controversial as MDSC are described as myeloid cells in different stages of differentiation associated with immune-regulatory molecules and receptors (Arg1, NOS2/NO, NOX2/ROS, PD-L1, and VEGF2), transcription factors (S100a8 and STAT3) and cytokines (IL-10, TGF β and IL4-R) (6). Since phenotypic characterization of MDSC is still debatable, MDSC are better defined as potent immunosuppressive myeloid cells characterized by less phagocytic activity or the production of high levels of reactive oxygen and nitrogen species and anti-inflammatory cytokines (14). The capacity to modulate T cells activity is the most often used immune suppressive feature of MDSC, which is also associated with their increased capacity to induce T cell apoptosis (15) and expansion of regulatory T cells (Treg) (16). Although immune modulation of T conventional cell activity is probably the main reported function of MDSC, the interaction between MDSC and other immune cells has been described in recent years. These include suppression of the B cells (17), dysregulation of T follicular helper cells (18, 19), loss of natural killer cell (NK) function (11) and suppression of DC development (20).

Focusing on the mechanism of MDSC induction, inhibitors of the mammalian target of rapamycin (mTOR), which represents a major immunosuppressive drugs for organ transplant recipients (21), plays a crucial role in promoting the development of MDSC. Using an immunological hepatic injury model, it was demonstrated rapamycin served as a functional immune modulator of CD11b+Gr1+ MDSC (22). Mechanistically, the authors demonstrated that mTOR down-regulation promotes CD11b+Gr1+Ly6ChiNOS+ M-MDSC recruitment to the inflammatory site that produced NO for tissue repair. Rapamycin also enhanced the suppressive function in murine PMN-MDSC after bone marrow transplantation, via up-regulation of Arg1 and iNOS (23). However, the effects of rapamycin and mTOR inhibition on MDSC remains controversial, as transgenic mice with a myeloid-specific deletion of mTOR display a decreased the number of M-MDSC *in vivo* after skin allograft transplantation (24).

MDSC IN ORGAN TRANSPLANTATION

The mononuclear phagocyte system (MPS), comprising DC, monocytes and macrophages, is implicated in many immunological mechanisms occurring during recognition of the non-self and the alloimmune response against the transplanted organ (25). Recipient DC infiltrate the allograft and form cognate contacts with T cells promoting effector T cell mediated rejection (26). In addition, donor DC derived exosomes promote an alloimmune response against the allograft by transferring functional MHC molecules to recipient DC (27). Acquisition of exosomes activates recipient DC that present donor MHC molecules to alloreactive T cells promoting T cell immunity. Monocytes also play a critical role in organ transplantation as they mediate the immune response against allogeneic non-self (28) and initiate allograft rejection by inducing T cell mediated immune responses (29). Macrophages act as effectors

of tissue damage in acute renal allograft rejection (30) and represent the majority of cells that infiltrate an allograft under severe rejecting conditions (31). Mechanistically, activated graft infiltrating macrophages increase their aerobic glycolysis metabolism and secrete pro-inflammatory cytokines associated with acute rejection (32). In addition to the MPS, neutrophils also play a critical role during organ rejection. The Lakkis laboratory demonstrated that depletion of neutrophils with anti-Ly6G significantly decreased inflammatory alloresponses (28). This is consistent with previous observations, which suggested that early neutrophil influx into the transplanted allograft favors organ rejection (33). Mechanistically, neutrophils may contribute to allograft rejection by different pathways that include the secretion of inflammatory cytokines (34), B cell stimulation (35) and through antigen presentation to T cells (36).

Since DC, monocytes, macrophages and neutrophils all the myeloid contribute to organ transplant rejection, MDSC must therefore prevent their immunogenicity against the allograft. Consequently, therapeutic protocols that prolong organ transplant survival may induce the development of MDSC, which inhibit myeloid cell derived graft reactive immune responses, such as antigen presentation and lymphocyte activation. Alternatively, experimental approaches that promote organ transplant acceptance may skew the differentiation of immunogenic DC, monocyte, macrophage and neutrophil precursors toward M-MDSC and G-MDSC favoring immune tolerance (**Figure 1**). Below we describe the role of MDSC in different organ transplant settings.

Kidney Transplantation

Vanhove's laboratory was the first to report the role of MDSC in kidney transplant recipient rats (37). In this experimental model, tolerance was induced by a costimulatory blockade with anti CD28 antibody. Myeloid cells expressing CD11b⁺CD80/86⁺Sirpα⁺ accumulated in the recipient allograft and were defined as MDSC for the first time in the context of organ transplantation. This study indicated that CD11b⁺Sirpα⁺ MDSC isolated from blood and bone marrow were able to suppress proliferation of anti CD3 anti-CD28 stimulated T cells. This suppressive mechanism of tolerance was in part mediated by iNOS, which was upregulated in graft infiltrating MDSC and by blood MDSC upon co-culture with activated effector T cells but not in Treg. The mechanistic role of NO in MDSC-mediated suppression was initially described by Mazzoni and colleagues using a NO synthase knockout mice (38). The authors demonstrated that CD11b⁺Gr-1⁺ MDSC from the spleens of immunosuppressed mice inhibit T cell proliferation in a NO-dependent manner, in response to signals from activated T cells that included IFN-γ. Another report from Vanhove's laboratory indicated that secretion of CCL5 by graft infiltrating MDSC was responsible for the accumulation of Treg into tolerized kidney allografts (39). In subsequent studies, Dilek and colleagues analyzed blood MDSC gene expression from kidney recipient showing that CCL5 was strongly downregulated after tolerant regimen. The amount of intra graft CCL5 protein was unchanged (40). The results indicate that a gradient of CCL5 between the

graft and peripheral blood might contribute to the intra graft localization of Treg in tolerant recipients controlled by MDSC.

In the clinical setting, Hock and coworkers showed significantly increased frequencies of total MDSC (CD33^{pos} HLA-DR^{neg} cells into the blood low density fraction), as well as both CD14^{pos} and CD14^{neg}-MDSC subsets in renal transplant recipients relative to normal donors. MDSC subsets frequencies and MDSC/DC ratios were higher in kidney recipients with or without current or prior squamous cell carcinoma than in healthy controls. *In vitro*, fMLP-activated MDSC from transplanted patients inhibited T cell proliferation (41). MDSC were shown to expand early after transplantation, independently of using basiliximab or thymoglobulin during induction (42) (and our unpublished observations).

Consistent with data from Vanhove's laboratory describing the presence of MDSC and of Treg in kidney transplanted rats, data from Murphy and colleagues reported presence of MDSC in human kidney transplant recipients. The study evaluated the capacity of blood derived CD11b⁺CD33⁺HLA-DR⁻ MDSC to suppress CD4⁺T cells proliferation *in vitro*. In addition, blood derived MDSC were able to expand Treg *in vitro* and correlated with increased Treg numbers *in vivo* (43). This was the first study where MDSC were associated with Treg in human transplant recipients. Consistent with these results, *ex vivo* experiments performed by Hoechst and colleagues demonstrated that blood derived CD14⁺HLA-DR^{-/-} myeloid cells isolated from hepatocellular carcinoma (HCC) patients induce CD4⁺CD25⁺Foxp3⁺ Treg when co-cultured with autologous T cells (44). A recent report from this group demonstrated that, while CD14⁺HLA-DR⁻ MDSC induce Foxp3⁺ Treg, CD14⁺HLA-DR⁺ myeloid cells from the same patient promote the generation of pathogenic Th17 cells when co-cultured with naive CD4⁺ T cells. Importantly CD14⁺HLA-DR⁻ MDSC modulate the trans differentiation of Foxp3⁺ Treg from monocyte-induced Th17 cells in a TGF-β and retinoic acid (RA) dependent mechanism (45).

More recently, the expression of myeloid-related S100A8 and S100A9 proteins was analyzed in two independent cohorts of patients with acute rejection. These proteins showed *in vitro* suppressive properties including inhibition of DC maturation and enhancement of ROS production. High S100A8 and S100A9 mRNA levels in biopsies predicted improved graft outcome and both proteins expression correlated with MDSC markers into PBMC and renal biopsies. Intra-graft, high amounts of S100A9 correlated with lower expression of T cell immunity (CD3e and CD4) and increased FoxP3, IL-10, and TGF-β regulatory markers (46). In a retrospective cohort of 50 renal recipients with biopsy-proven acute T-cell mediated rejection, patients with high MDSC in circulation (CD11b⁺CD33⁺HLA-DR⁻ cells) (MDSC > 10% into PBMC) showed increased estimated glomerular filtration rate and lower serum creatinine at the time of biopsy. Compared to low MDSC recipients, high MDSC patients showed less development of IFTA and significantly superior 1- and 5-year graft survival (47). However, prospective and randomized studies in large cohorts are still lacking to better understand the role of MDSC in clinical kidney transplant and their potential value as biomarker or therapy target.

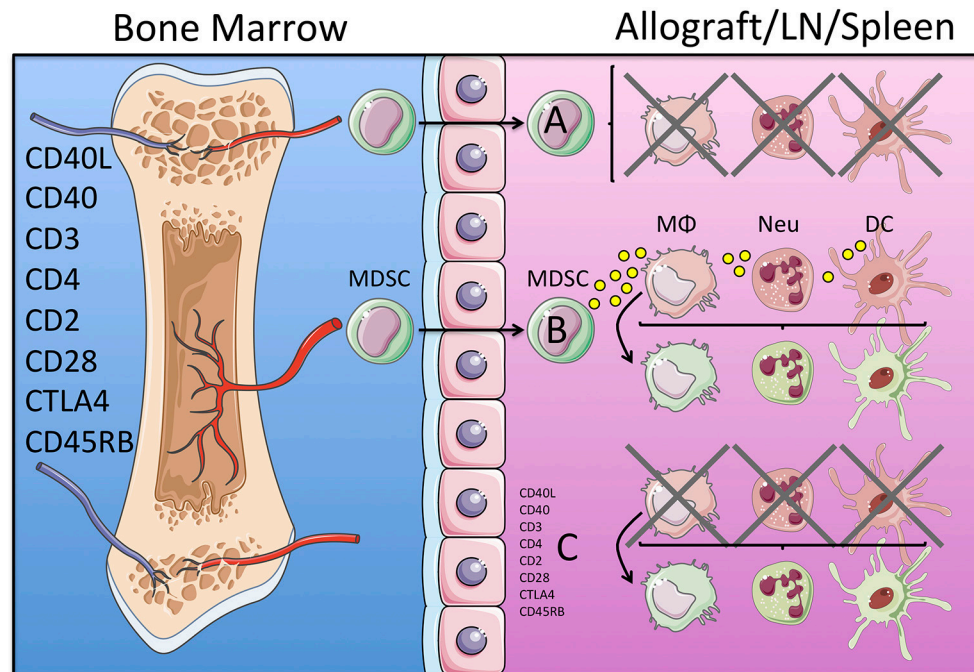


FIGURE 1 | Potential mechanisms of immune regulation mediated by MDSC in organ transplantation. Induction of transplantation tolerance in experimental murine models is achieved by targeting TCR and co-stimulatory blockade with monoclonal antibodies. These therapeutic treatments may induce the development of an MDSC precursor that leaves the bone marrow and may migrate into the allograft, lymph node (LN) and/or the spleen. Once in the tissue MDSC may mediate direct inhibition of immunogenic myeloid cells (macrophages, neutrophils and dendritic cells in red), as depicted in (A); or secrete cytokines and growth factors that convert immunogenic (red) into tolerogenic (green) myeloid cells, as depicted in (B). Alternatively, both processes (direct inhibition of immunogenic and/or conversion into tolerogenic myeloid cells) may be a direct effect of the tolerogenic regimen (monoclonal antibodies) independently of the MDSC, as depicted in (C).

Corneal Transplantation

Corneal allograft models in mice have been used to test the use or manipulation of MDSC as immunomodulatory strategies in transplantation. In two groups of mice receiving either cornea or cornea and skin allografts, longer grafts survival was observed in animals with prior transference of bone marrow MDSC induced in cecal ligated and punctured mice (48). In a different experimental model, B6 mice corneas were transplanted into BALB/c recipients who received intraperitoneal dexamethasone (dex) at decreasing doses from day 0 to 21 after surgery. Administration of dex significantly prolonged the allograft survival and correlated with decreased infiltration of CD3+ cells and low levels of IFN- γ and IL-1 β in the grafts, together with low IFN- γ CD4+ cells in draining lymph nodes, blood, spleen and bone marrow. Concomitantly, an expansion of MHC class II⁺CD11b⁺Ly6C⁺ monocytes (m-MDSC following Bronte and coworkers, ref 6) and increased iNOS were observed in the same compartments of dex-treated mice. FACS-sorted CD11b⁺Ly6C⁺ cells from bone marrow of dex-treated mice inhibited *in vitro* CD4+ T cells proliferation and prolonged the survival of corneal allografts when transferred into dex-untreated recipients. Depletion of MHC class II⁺CD11b⁺Ly6C⁺ monocytes abrogated the protective effect of dex on corneal allografts suggesting that these cells were required for mediating the induction of tolerance by glucocorticoids (49). Similar results

were obtained in corneal allograft recipient mice treated with a rapamycin nano-micelle (RNM) ophthalmic solution. Under this therapy, delay of rejection and expansion of Gr1^{int} CD11b⁺ MDSCs in allografts, cervical lymph nodes, blood and spleen were observed. The capacity of MDSCs from the RNM solution-treated mice to suppress proliferation of CD4+ T cells depended on iNOS and arginase-1, and the administration of anti-Gr-1 antibody or the pharmacological inhibition of iNOS abrogated the beneficial effects of rapamycin (50).

Pancreatic Islets Transplantation

In the Bronte's pioneer work murine MDSCs were obtained by treating bone marrow *in vitro* with GM-CSF + IL-6. When adoptively transferred into islet-allografted syngeneic mice, the GM-CSF + IL-6 induced MDSCs increased the survival of functional islets. Integrity of the C/EBP β transcription factors was needed to develop the tolerogenic MDSC (51).

In different experimental settings, prolonged islets allograft survival has been achieved by MDSC generated by hepatic stellate cells (HSC). CCR2 expression was needed to allow migration of HSC-induced MDSC into allografts (52), and their suppressive capacity relied on induction of apoptosis in T effector cells and B7-H1-mediated expansion of Tregs (53, 54). The induction of MDSC by HSC depended from soluble factors. Interestingly, SDS-PAGE and LC-MS analysis of the most bioactive fraction in

hepatic stellate cells culture supernatant identified complement component 3 (C3) as a key mediator, since depletion of HSC-derived C3 markedly reduced the ability to induce MDSC (53). Moreover, co-transplantation of BALB/c mice islets with wild type or C3^{-/-} HSC into diabetic B6 mice showed that 60% of mice receiving wild type HSC remained normoglycemic by post-transplant day 60 while all recipients of C3-deficient HSC lost islets under day 21. Draining lymph nodes and grafts from wild type HSC recipients showed increased frequency of Tregs and CD11c⁺ myeloid cells of immature phenotype, which were able to suppress proliferation of CD4⁺ and CD8⁺ cells and production of IFN γ (55). However, results in the opposite direction were obtained in a model of streptozotocin-induced diabetes in mice, in which expansion of MDSC and protection of pancreas against the autoimmune destruction were noticed in the absence of C3, and depletion of MDSC by anti-Gr1 led to diabetes in C3^{-/-} streptozotocin-treated mice (56). Concluding results to fully understand if and how C3 is required to promote generation of MDSC are still missing.

Skin Transplantation

Using an experimental skin transplant model where bm12 MHC-II minor mismatched graft were transplanted into C57BL/6 recipients, the Horuzsko laboratory demonstrated HLD-G expressing MDSC interacts with immune inhibitory receptors, such as immunoglobulin-like transcript 2 (ILT2), which induces the expansion of MDSC *in vivo* (57). The authors further demonstrated that MDSC from ILT2 transgenic mice exhibit an augmented suppressive function and were able to prolong skin graft survival following adoptive transfer into C57BL/6 recipients.

De Wilde and colleagues demonstrated that repetitive administration of lipopolysaccharide (LPS) *in vivo* induces the development CD11b⁺Gr-1⁺ MDSC (58). In a male-to-female mismatched skin transplantation model, the authors reported that *in vivo* transfer of MDSC treated with LPS significantly prolonged skin allograft survival. This was due to the expression of heme oxygenase-1 (HO-1), which impaired T cell activation. The authors also demonstrated that LPS induced MDSC suppresses Th1 and Th2 cytokine production, while produce large amounts of IL-10 as suppressive mechanism. Further, HO-1 inhibition by a specific inhibitor completely abolished T-cell suppression and IL-10 production. The importance of HO-1 during prolonged allograft survival was first described by Yamashita et al. (59). The authors demonstrated that cobalt protoporphyrin IX (CoPPPIX) treatment leads to a significant up-regulation HO-1 that was necessary for indefinite survival of fully mismatched heart allografts.

In a recent study, Zhao et al. established that the combination of M-CSF and TNF α efficiently induces functional MDSC *in vitro* (60). The resulting M-MDSC were characterized by the expression of F4/80, CD80, and PD-L1. Mechanistically, M-CSF + TNF α induced M-MDSC upregulated the expression of iNOS, which was necessary for suppression of T cell proliferation. Upon adoptive transfer, M-CSF+TNF α induced M-MDSC promoted immune tolerance in male-to-female skin transplanted mice. Consistent with Vanhove's observation, blockade of iNOS

activity failed to induce the graft acceptance, demonstrating that immunosuppressive ability of M-CSF+TNF α -induced M-MDSC is dependent on iNOS. The critical role of iNOS activity in the suppressive function of MDSC was also described by Wu et al. (61). The authors identified Smad3 as an intrinsic negative regulator of MDSC development and recognized that the immunosuppressive function of MDSC depends on NO production. Using *Smad3* deficient mouse recipients in a fully mismatched skin transplantation model, the authors observed an increase in both granulocytic and monocytic cells associated with less production of anti-donor IgG Abs and decreased IFN- γ production. Interestingly, L-NMMA significantly reduced NO production and efficiently blocked the immunosuppressive effects of Smad3-deficient G-MDSC on T cell proliferation.

In agreement with these results, Liao and colleagues demonstrated that MDSC development is induced by dexamethasone through the glucocorticoid receptor (GR) pathway (62, 63). Dexamethasone treatment significantly prolonged allograft survival in a fully allogeneic skin transplant model in mice through upregulation of iNOS and NO production in MDSC. These results validate the administration of glucocorticoids as a therapeutic approach that prolongs graft survival through the development of MDSC.

Further studies have also illustrated the immune-modulatory activity of IL-33 during the induction of iNOS expressing MDSC (64). Using both syngeneic and allogeneic skin transplants models, Pino-Lagos and colleagues demonstrated that IL-33 treatment up-regulated the number of Foxp3⁺ Treg and promoted the conversion of Foxp3⁻ T cells into Foxp3⁺ Treg in the periphery.

Heart Transplantation

Using a mouse heart transplantation model Rodriguez-Garcia and colleagues demonstrated the requirement of MDSC for the induction of transplantation tolerance (65). The authors treated recipient mice with anti-CD40L mAb costimulatory blockade and identified the critical role for tolerogenic CD11b⁺Ly6C^{low} expressing MDSC. Using depletional mAbs, clodronate-loaded liposomes, and transgenic mice specific for depletion of CD11b⁺ expressing cells the authors reported that monocytic precursors migrate from the bone marrow to the transplanted organ early after transplantation and prevent the initiation of adaptive immune responses that lead to allograft rejection.

Similar results were previously obtained from Terry Strom laboratory (66), which demonstrated that CD11b⁺Gr1^{low} MDSC exhibit high suppressive capacities and prevent grafts from prolonged cold ischemia-mediated injury. The authors induced the development of Ly6C^{low} MDSC by treating fully allogeneic recipient mice with rapamycin (3 mg/kg) and costimulatory blockade with anti-CD40L mAb. This combination therapy induced tolerance in C57BL/6 mice pre-sensitized by Balb/c skin grafts at day-7 that received BALB/c heart grafts in contrast to mice treated with either rapamycin or anti-CD40L mAb alone.

Consistent with these results, a recent report from Braza and colleagues described a novel nanoimmunotherapy based on high-density lipoprotein (HDL) that targets myeloid cell precursors *in vivo* (32). Using a fully allogeneic mouse heart transplant

model, the authors demonstrated that a rapamycin and CD40 costimulatory blockade combined nanotherapy (mTORi/Traf6i-HDL) favored the accumulation of CD11b⁺Ly6C^{low} myeloid cells, which prevented CD8⁺ T cell proliferation and promoted Treg expansion. Remarkably, a short-term treatment with nanobiologics during the first week after transplantation resulted in indefinite allograft survival of most transplant recipients with no signs of chronic rejection.

Considering mTOR inhibitors as a current immunosuppressive therapy for organ transplantation, Nakamura and colleagues demonstrated that treatment with rapamycin results in a significant prolongation of graft survival mediated by iNOS expressing MDSC in a murine cardiac transplantation model (67). The authors also confirmed that CD11b⁺ myeloid cells expressing lower levels of Gr-1 efficiently suppressed CD4⁺ T cell proliferation *in vitro*. Interestingly, adoptive transfer of rapamycin-induced MDSC and to a lesser extent G-MDSC, through the coronary arteries before organ reperfusion of transplant recipient mice significantly prolonged allograft survival. Graft survival prolongation of MDSC was associated with an increase of splenic Foxp3⁺ Treg. Mechanistically, the Nakamura group further reported that rapamycin treatment induces the expression of PD-L1 on MDSC that accumulate in the cardiac allograft following adoptive transfer (68).

A critical aspect in organ transplantation is the induction of donor-specific unresponsiveness. For this purpose, many authors use donor specific splenocytes in combination with tolerogenic therapy, such as co-stimulatory blockade (69, 70). In this respect, Luo and colleagues performed infusions of donor splenocytes treated with 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (ECDI-SPs) before and after transplantation (71, 72). The authors observed prolonged allograft survival associated with intragraft accumulation of CD11b⁺ MDSC that express high levels of indoleamine 2,3 dioxygenase (IDO). Furthermore, combination therapy of donor ECDI-SPs with systemic rapamycin induced indefinite cardiac allograft survival in 100% of the recipients for over 150 days.

Liver, Bowel, and Lung Transplantation

Whereas, liver transplants can be spontaneously accepted without the requirement of immunosuppression in different species (73, 74), the immune response acutely rejects hepatocyte transplants (75). This suggests that liver stromal cells protect parenchymal cells from rejection. Hepatic stellate cells have potent immune regulatory activity and they have been shown to promote MDSCs generation *in vivo* and *in vitro* (53, 76).

Kim et al. showed that in Rhesus macaques MDSC accumulate in high numbers in the liver when compared to blood, spleen and lymph nodes (77). In a model of allogeneic orthotopic liver transplantation in rats, the authors observed that the promotion of tolerance by treatment with rapamycin was associated with an increase of regulatory T cell phenotypes and accumulation of MDSC in spleen (78).

In a prospective cohort of 36 intestinal transplant recipients, the authors identified MDSC (lineage⁻HLADR⁻/lowCD33⁺CD11b⁺-expressing cells) and all

three M- (CD14⁺CD15⁻), PMN- (CD14⁻CD15⁺) and e-MDSC populations into PBMC. All three MDSCs subsets increased post-transplant although PMN-MDSC and e-MDSC did so immediately, while M-MDSC increased after 2 months post-transplant. All three MDSC types were able to suppress CD4⁺ and CD8⁺ T cells proliferation as well as IFN γ production. High plasma levels of IL-6 but not TNF α or GM-CSF, the use of exogenous steroids and low tacrolimus trough levels correlated with MDSCs numbers in PBMC. In agreement, IL-6 and methylprednisolone enhanced MDSC cells after culturing bone marrow cells from healthy controls in basic medium with GM-CSF and G-CSF. Intragraft MDSCs were low before transplantation but increased during a year after transplantation. The analysis of chemokines expression in intestinal grafts biopsies and of chemokines receptors expression in MDSC supported a role for CCL11 and CCL15 in recruiting CCR1- and CCR3- expressing M- MDSCs and e-MDSCs, and a role for CXCL6 in recruiting CXCR2- expressing PMN- MDSCs and e-MDSCs into the mucosa of intestinal allografts. Peripheral blood MDSCs were significantly lower in patients with acute rejection. The addition of MDSC into co-cultures of donor-reactive T cells with donor-derived intestinal epithelial organoids enhanced the organoids viability, suggesting that the accumulation of MDSC suppressed T cells alloresponse against the donor intestinal epithelium (79).

In the only publication regarding lung transplant at present, the authors observed that the phyla *Firmicutes* dominated the microbiome signature in the distal airways of subjects without bronchiolitis obliterans syndrome (BOS), while this shifted to a Proteobacteria-dominant signature in the BOS cohort. Suppressive MDSC predominated in the proximal airways and pro-inflammatory myeloid cells were more abundant in distal airways. These results suggested a functional link between the local microbiome and MDSC phenotype, which may play a role in the pathogenesis of BOS (80).

CONCLUSION AND PERSPECTIVES

Regulation of different MDSC subsets with distinct immune function may be used for future therapeutic approach that promotes tolerance in organ transplantation. In the setting of murine GVHD, the inflammasome activation in the transferred MDSC induced the loss of their suppressive capacity, thus, understanding the micro environmental signals that affect the stability of the MDSC suppressive capacity will be also critical for an optimal use in therapy (81). In addition, MDSC may be used as biomarkers that provide critical information regarding the functional immune status of organ transplant recipients. Non-invasive immune approaches that determine and characterize MDSC subsets in humans are urgently needed to move the field forward. Unfortunately, identification of specific mechanisms by which MDSC exhibit suppressive functions and contribute to the development of tolerance remains a difficult task. One of the main difficulties resides in a consensual classification and identification MDSC subset during pathological conditions. For that purpose, the COST action Mye-EUNITER was established in

2014 (<http://www.mye-eunit.eu/>) to create a general consensus to standardize the function and phenotype of MDSC across different species (82). Additional immune regulatory molecules, such as B7-H3 (83), may be validated in future experiments to further identify and characterize MDSC in transplant recipients. In this respect, the use of next generation genomic sequencing may help to identify the transcriptomic profile of different MDSC subsets and differentiate suppressor cells from normal myeloid cells (84).

One critical consideration to fully determine whether MDSC are indeed functionally inhibitory myeloid cell is the choice of the immune functional assay (85). While the clinical application of MDSC represents a promising therapeutic approach for the induction of organ transplant acceptance either as a cell therapy or by regulating their *in vivo* development from myeloid precursors, it requires consensus on markers that identify MDSC subsets, which limit our ability to specifically target MDSC *in vivo*.

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Prostaglandin-E2 Potentiates the Suppressive Functions of Human Mononuclear Myeloid-Derived Suppressor Cells and Increases Their Capacity to Expand IL-10-Producing Regulatory T Cell Subsets

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Myeloid-derived suppressor cells (MDSC) emerged as major factors driving the tumor progression due to numerous immunosuppressive mechanisms they possess. Prostaglandin (PG)E2 is shown critical for the induction of MDSC and their suppressive functions *in vivo*, but it is poorly understood how it affects the capacity of MDSC to induce different subsets of regulatory T cells (Treg). By using a novel protocol for the generation of mononuclear (M)-MDSC, we showed that PGE2 potentiates the GM-CSF/IL-6-dependent induction of CD33⁺CD11b⁺HLA-DR⁻CD14⁺ M-MDSC *in vitro*. PGE2 diminished the capacity of GM-CSF/IL-6 M-MDSC to produce proinflammatory cytokines upon activation and augmented their capacity to produce IL-27, IL-33, and TGF- β . These results correlated with an increased potential of GM-CSF/IL-6/PGE2 M-MDSC to suppress T cell proliferation, expand alloreactive Th2 cells, and reduce the development of alloreactive Th17 and cytotoxic T cells. Interestingly, GM-CSF/IL-6/PGE2 M-MDSC displayed a lower capacity to induce TGF- β -producing FoxP3⁺ regulatory Treg compared to GM-CSF/IL-6 M-MDSC, as a consequence of reduced IDO-1 expression. In contrast, GM-CSF/IL-6/PGE2 M-MDSC potentiated IL-10 production by CD8⁺T, Th2, and particularly CD4⁺FoxP3⁻ type 1 Treg, the latter of which depended on ILT3 and ILT4 expression. Cumulatively, PGE2 potentiated the suppressive phenotype and functions of GM-CSF/IL-6-induced M-MDSC and changed the mechanisms involved in Treg induction, which could be important for investigating new therapeutic strategies focused on MDSC-related effects in tumors and autoimmune diseases.

Keywords: myeloid derived suppressor cells, prostaglandin E2, type 1 regulatory T cells, FoxP3⁺ regulatory T cells, checkpoint blockade

INTRODUCTION

Cancer immunotherapy has been improved significantly by the discovery of checkpoint inhibitors targeting cytotoxic T leukocyte antigen (CTLA)-4 and programmed death (PD)-1 axis. Yet, one-third of cancer patients receiving checkpoint inhibitors relapse, and the mechanisms for resistance acquiring are poorly understood (1). Myeloid-derived suppressor cells (MDSC), which are heterogeneous cell population present in virtually all individuals with a diagnosed tumor (2, 3), have been recognized as major suppressors of the anti-tumor response, and a major limiting factor for the efficacy of checkpoint inhibition therapy (2, 4, 5). MDSC promote neoangiogenesis and tumor metastasis, by producing VEGF and metalloproteases, respectively (6). More importantly, MDSC utilize different suppressive mechanisms to limit the activation of immune cells, particularly of cytotoxic T cells (7), which are the major effector cells in anti-tumor response (8). Two major subtypes of MDSC were described in humans, both lacking lineage markers expression (CD3, CD19, CD20, CD56) and HLA-DR, while expressing myeloid markers CD33 and CD11b. The mononuclear subtype (M-MDSC) express a monocytic CD14 marker, whereas polymorphonuclear subtype (PMN-MDSC) express CD15 (9). Although these subtypes display different suppressive mechanisms, the studies on tumor models in mice suggested that M-MDSC exhibit a stronger immunosuppressive potential compared to PMN-MDSC (10). Additionally, a preferential accumulation of M-MDSC in the tumor of melanoma and prostate cancer patients (3, 11), suggests that M-MDSC are the key factors of immune suppression in some types of cancer. Besides tumor, M-MDSC appear an important factor in other chronic inflammatory processes, such as autoimmunity (12). From the clinical perspective, providing MDSC or their products may improve the efficacy of therapies for several autoimmune diseases (13). Moreover, MDSC are being advocated as promising therapeutic strategy in organ transplantation along with other myeloid suppressor cells, such as regulatory macrophages and tolerogenic dendritic cells (DC) (14). In line with this, it was shown that the induction of tolerance to kidney, skin and cardiac allografts is associated with infiltration of grafts by MDSC (15, 16).

Direct immunosuppressive mechanisms of M-MDSC have been studied extensively (7), and they include induction of M2 macrophages (17), suppression of NK cell-mediated cytotoxicity (18), and suppression of T cell activation by depletion of essential amino acids, such as arginine, tryptophan, and cysteine (19–21). In addition, M-MDSC were shown to recruit regulatory T cells (Treg) in the tumor (22), and promote *de novo* induction of FoxP3⁺ Treg (23), thus spreading the immune suppression further. Different mechanisms were described to contribute their capacity to induce Treg, including the involvement of CD80, TGF- β (24), PD1L (25), IDO-1 (26), ILT-3 (27), and ILT-4 (7, 28). However, it has been shown that these molecules are also involved in the induction of non-conventional Treg subsets, such as suppressor CD8⁺ and type 1 regulatory T (Tr-1) cells (29, 30). Our previous findings confirmed these mechanisms as well (31, 32). Moreover, non-conventional Treg subsets were shown to exhibit even stronger suppressive effects than the conventional

FoxP3⁺ Treg (33, 34). However, it remained unclear whether M-MDSC induce Treg other than FoxP3⁺, and which mechanisms are involved in their induction.

Detailed analyses of Treg-inducing mechanisms by human M-MDSC, enabling the development of new immunotherapeutic strategies in cancer and autoimmune diseases, is partially hampered by their relatively short half *in vivo*, and *in vitro* upon isolation (7). Consequently, several *in vitro* protocols have been proposed for the generation of M-MDSC *in vitro* (35–37). It was suggested that M-MDSC could be differentiated from monocytes *in vitro* by using GM-CSF and IL-4 in the presence of PGE2 (36, 38) or IL-10 (37), which shift the differentiation of monocytes away from DC, toward M-MDSC-like cells. However, similar protocols were described for the induction of tolerogenic DC (39, 40). To limit these controversies, Bronte et al. (9) suggested minimal phenotypic and functional criteria for defining M-MDSC. However, the majority of reported data did not show clearly whether the phenotypic and functional properties of obtained M-MDSC comply with these criteria. Lechner et al. (35) suggested that GM-CSF and IL-6 are the most potent cytokines for the induction of M-MDSC within PBMC, but the phenotypic and functional properties of these cells resembled more to PMN-MDSC. So, it remained unclear whether M-MDSC could be differentiated by using GM-CSF and IL-6. GM-CSF was demonstrated as a critical factor to maintain the myeloid cell viability in cancer (41), and IL-6 was shown as the most potent proinflammatory cytokine linked to MDSC accumulation and consequent tumor progression (42, 43). Besides, PGE2, and cyclooxygenase 2 (COX2) overexpression were shown critical for the differentiation of MDSC from mice bone marrow and tumor progression in animal models (44). Additionally, PGE2 was shown to induce M-MDSC (18, 45) and potentiate their suppressive properties in cancer patients (46), but no data reported how it affects the capacity of M-MDSC to induce different Treg subsets. Taking into account *in vivo* data on the importance of these inflammatory mediators, we hypothesized that the combination of GM-CSF and IL-6 enables the differentiation of M-MDSC from human monocytes and that PGE2 significantly potentiates their suppressive phenotype and functions *in vitro*. By doing so, PGE2 alters the capacity of M-MDSC to induce different subtypes of Treg. According the Minimum Information about Tolerogenic Antigen-presenting cells (MITAP) (47), the hypothesis was tested by analyzing viability, phenotype, cytokines production, suppressive capacities, and the mechanisms involved in the induction of Treg.

MATERIALS AND METHODS

Cells

All experiments involving human blood samples were approved by the Ethical Board of the Military Medical Academy, University of Defense (MMA), and carried out in accordance with the MMA Guidelines. PBMC were obtained from buffy coats of healthy volunteers, who signed the Informed Consent in accordance with the Declaration of Helsinki, using density gradient centrifugation on lymphocyte separation medium 1077 (PAA, Linz, Austria).

CD14⁺ monocytes and CD3⁺ T cells were isolated from PBMC with magnetic-activated cell sorting (MACS) of untouched cell populations, by using the Monocyte Isolation Kit II and Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. The purity of CD14⁺ monocytes and CD3⁺ T cells was higher than 85 and 95%, respectively, as evaluated by flow cytometry (Cube 6, Sysmex Partec GmbH, Görlitz, Germany; BD LSR II, San Jose, CA, USA).

Monocytes (1×10^6 /mL) were cultivated in CellGenix® GMP Dendritic Cell Medium (CellGenix, Freiburg, Germany) supplemented with 100 ng/mL of human recombinant granulocyte macrophages colony stimulating factor (GM-CSF; Novartis, Basel, Switzerland) and 20 ng/mL of human recombinant IL-4 (Roche Diagnostics, Basel, Switzerland) to induce immature DC. In some experiments, PGE2 (1 µg/mL, Sigma Aldrich Co.) was supplemented to the GM-CSF/IL-4 medium from the beginning of cells' cultivation to obtain GM-CSF/IL-4/PGE2 M-MDSC. The monocytes of the same donors were differentiated in the presence of GM-CSF (100 ng/mL) and human recombinant IL-6 (40 ng/mL, R&D Systems, Minneapolis, MN, USA) to obtain GM-CSF/IL-6 M-MDSC, or additionally with PGE2, to obtain GM-CSF/IL-6/PGE2 M-MDSC. After 5 days of cultivation at 37°C, 90% humidity, and 5% CO₂, the cells were either stimulated for the next 16 h with 50 ng/mL of interferon (IFN)-γ (R&D Systems, Minneapolis, MN, USA) and 200 ng/mL of LPS from *Escherichia coli* 0.111:B4 (Sigma-Aldrich Co.), or left unstimulated. Subsequently, the cells were analyzed for their phenotype or used in the functional assays with T cells, whereas cell-free supernatants were used for determination of the cytokines' levels.

Mixed Cell Cultures

Before the co-cultivation with allogeneic PBMC or T cells, M-MDSC were washed twice in RPMI medium to prevent the transfer of cytokines and stimuli. The capacity of M-MDSC and DC to suppress the proliferation of PBMC was tested by co-cultivating M-MDSC and DC ($2.5\text{--}0.62 \times 10^4$ /well) with allogeneic PBMC (2×10^5 /well) in the presence of phytohemagglutinin (10 µg/mL, Sigma Aldrich) for 5 days. Prior to the test, PBMC were labeled with carboxyfluorescein succinimidyl ester (CFSE, 0.5–2 µM, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. The suppressive effect of M-MDSC on T cell proliferation was tested by co-cultivating M-MDSC ($2.5 \times 0.62 \times 10^4$ /well) and MACS-purified allogeneic CFSE-labeled T cells (1×10^5 /well), in the presence of plate-coated anti-CD3 Ab (5 µg/mL, R&D Systems) and soluble anti-CD28 Ab (1 µg/mL, Miltenyi Biotec, Bergisch Gladbach, Germany). The proliferation of alloreactive T cells was tested in the absence of CD3/CD28 stimulation by co-cultivating LPS/IFN-γ-treated M-MDSC or DC with allogeneic CFSE-labeled T cells (1×10^5 /well) or T cells alone, for 5 days. To induce Treg, allogeneic T cells (1×10^5) were cultivated alone, or in the presence of LPS/IFN-γ-treated M-MDSC (2×10^3 /well) for 3 days, followed by 3-day stimulation with IL-2 (3 ng/mL, R&D Systems), as described previously for tolerogenic DC (31, 32). To assess the mechanisms of Treg induction, some M-MDSC/T cell co-cultures were supplemented with IDO-I

inhibitor 1-methyl-tryptophan (1-MT, 0.3 mM; Sigma-Aldrich Co.), blocking anti-ILT-3 or anti-ILT-4 Ab (both at 2 µg/mL; R&D Systems), or isotype control Ab (anti-rat IgG2b; Thermo Fisher Scientific). For cytokines' analysis in M-MDSC/T cell cocultures, the cultures were treated with PMA (20 ng/mL) and ionomycin (500 ng/mL) (both from Sigma-Aldrich Co.) for the last 4 h before harvesting the cell-free supernatants. For the flow cytometric detection of intracellular cytokines in T cells, the co-cultures were treated with PMA/ionomycin and monensin (3 µM; Sigma-Aldrich Co.) for the last 3 h of incubation.

Proliferation, Viability, and Cytokine Production

The proliferation of allogeneic CFSE-labeled PBMC and T cells co-cultivated with DC and M-MDSC, was analyzed by flow cytometry within the gated propidium (PI)⁺ population, by measuring CFSE dilution during the cells' divisions, as described (32). The percentage of proliferation was calculated using the proliferation fit statistics in FCS Express 4 software (De Novo Software, Glendale, CA, USA). The relative proliferation in suppression assays was calculated as the percentage of proliferation relative to control (i.e., without the presence of DC or M-MDSC, 100%). The apoptosis of M-MDSC and viability/cell count of T cells after the co-cultures with allogeneic M-MDSC was determined by staining the cells with Muse® Annexin V and Dead Cell Assay Kit and Muse® Count & Viability Assay Kit, respectively, followed by the analysis on Muse Cell Analyzer (Merk Millipore, Wien, Austria). The cytokine concentrations in cell culture supernatants were determined by appropriate ELISA kits (R&D Systems) spectrophotometrically, and bead-based immunoassays (Biolegend, San Diego, CA, USA) by flow cytometry.

Flow Cytometry

The phenotype analysis of M-MDSC, DC, and T cells was carried out by flow cytometry after staining the cells with the fluorescently labeled Abs (Clone) and reagents: IgG1 negative control-PE (MCA928PE), IgG1 negative control-FITC (MCA928F) (Bio-Rad); anti-CD1a-PerCP/Cy5.5 (HI149), anti-HLA-DR-APC/Cy7 (L234), anti-CD80-APC (2D10), anti-IL-4-PerCP/Cy5.5 (MP4-25D2), anti-IL-4-PE (42D1), anti-ILT-4-APC, anti-CD56-PerCP/Cy5.5 (MEM-188), anti-CD19-PerCP/Cy5.5 (HIB19), anti-CD25-PE (BC96), anti-CD25-PerCP/Cy5.5 (M-A251), anti-CD127-PE (A019D5), anti-CD11b-PE, anti-CD11b-PE/Cy7 (ICRF44), anti-IL-10-APC, anti-IL-10-PE (JES5-16E3), anti-TGF-β-APC (TW4-6H10), anti-IL17A-Alexa Fluor 488 (BL168), anti-IFN-γ-APC, anti-IFN-γ-FITC (4S.B3), IgG1 negative control-PerCP/Cy5.5 (HTK888), anti-CD73-PerCP-Cy5.5 (AD2) (all from Biolegend); anti-HLA-DR PerCP (L243), anti-IDO-1-APC (700838), anti-CD33-APC (6C5/2), anti-CD4-FITC, anti-CD4-APC (11830), anti-TGF-β-PE (9016) (all from R&D Systems), anti-CD14-FITC (TUK4) (Miltenyi Biotec), anti-CD86-PE (IT2.2), streptavidin-PerCP, streptavidin APC, anti-ILT3-PE (ZM4.1), anti-CD209-FITC (eB-h209), anti-CD206-APC (19.2), anti-CCR7-FITC (3D12), IgG1 negative control APC (MA5-18093), anti-CD39-PE (eBioA1),

anti-IL-17A-APC (eBio17B7) (all from Thermo Fisher); anti-CD40- APC (5C3), anti-IL-12 (p40/p70)-PE (C11.5), anti-CD3-PE (SK7), anti-FoxP3-PerCP/Cy5.5, anti-FoxP3-Alexa Fluor 488 (236A/E7) (all from BD Pharmingen, San Diego, CA, USA), anti-CD8-PerCP/Cy5.5 (HIT8a) (Elabscience), and anti-CD4-PE (MEM-241) (Partec Sysmex). Surface staining with primary Abs was conducted in PBS/0.1% NaN₃/0.5% FBS prior to intracellular staining that was carried out using the BD fixation/permeabilization kit (Becton Dickinson). The gates for cultivated M-MDSC and T cells were set according to their specific forward scatter (FS) and side scatter (SS) properties, thereby avoiding dead cells with low FS/SS signal. The gates, containing more than 97% of live cells, were confirmed by independent PI staining of non-permeabilized cells. The signal overlap between the fluorescent channels was compensated before each experiment using the single-labeled samples.

The non-specific fluorescence was determined by using the appropriate isotype control Abs and fluorescence minus one/two controls (FMO). The number of cytokine-producing cells was calculated according to the number of viable cells and the percentages of cytokine-producing cells detected after the co-cultures by flow cytometry.

Statistical Analysis

The results are presented as representative data or as mean \pm SD values of at least three independent experiments carried out with cells of different healthy donors. The differences between the treatments were analyzed by repeated measures (RM) ANOVA with Tukey's multiple comparison test or paired *T*-test, using the GraphPad Prism software (GraphPad, La Jolla, CA, USA). All tests were two-sided with the significance level of $p = 0.05$.

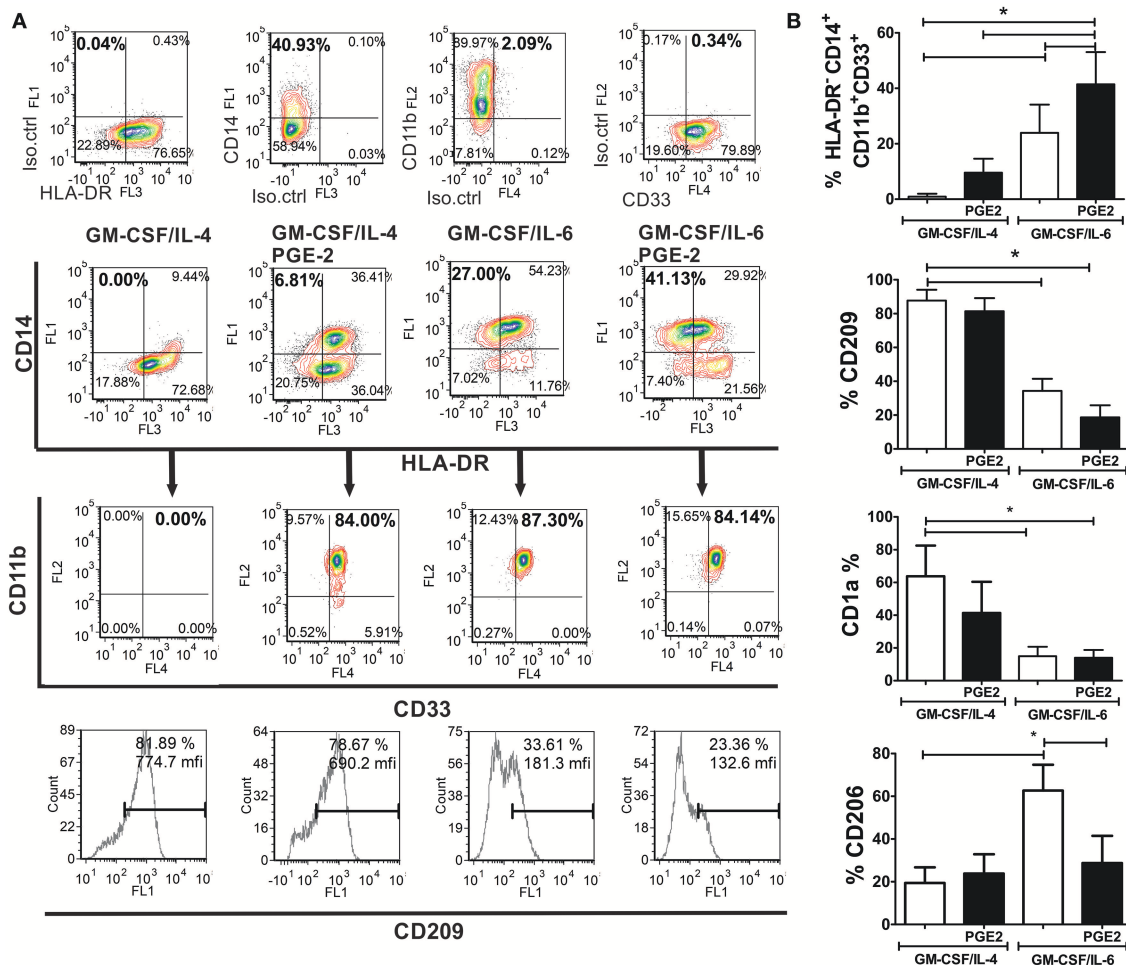


FIGURE 1 | Phenotypic analysis of M-MDSC and DC generated from human monocytes *in vitro*. **(A,B)** The monocytes were cultivated in the presence of GM-CSF/IL-4, GM-CSF/IL-4/PGE2, GM-CSF/IL-6, or GM-CSF/IL-6/PGE2 for 5 days, followed by their phenotypic analysis. **(A)** A representative analysis of the co-expression of HLA-DR, CD14, CD11b, and CD33 is shown. The doublets and the dead (FSC^{low}) cells were gated-out (not shown), and the quadrants were set according to the single-labeled samples (first row). CD11b/CD33 plots were gated from the HLA-DR⁺CD14⁺ region, and the percentage of HLA-DR⁺CD14⁺CD33⁺CD11b⁺ was calculated based on these plots. The expression of CD209 was analyzed within the total gated cell population. **(B)** The summarized results on % of HLA-DR⁺CD14⁺CD33⁺CD11b⁺ cells, CD209⁺, CD1a⁺, and CD206⁺ are shown as mean \pm SD from 4 independent experiments carried out with different donors. * $p < 0.05$ between the indicated samples (RM ANOVA, Tukey post-test).

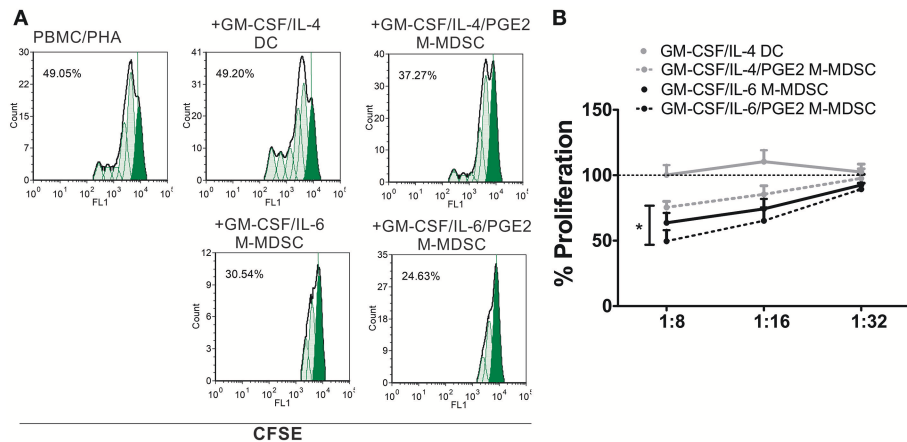


FIGURE 2 | Suppressive capacity of M-MDSC and DC. **(A,B)** The PHA-stimulated CFSE-labeled allogeneic PBMC (2×10^5 /well) were co-cultivated with M-MDSC or DC at different cell ratios (1:8–1:32, M-MDSC:T cells) for 5 days, followed by flow cytometry analysis. **(A)** A representative analysis of PBMC proliferation is shown with the G0 generation marked in full green. The % of proliferation was calculated by using the proliferation fit statistics. **(B)** The summarized results are shown as the mean relative proliferation % \pm SD ($n = 3$), i.e., % proliferation of control PBMC/PHA cultures in each experiment (100%). All M-MDSC suppressed the proliferation of PBMC at 1:8 cell ratio (not labeled). * $p < 0.05$ GM-CSF/IL-4/PGE2 M-MDSC vs. GM-CSF/IL-6/PGE2 M-MDSC at the 1:8 cell ratio.

RESULTS

Considering the important role of GM-CSF, IL-6 and PGE2 in M-MDSC induction and functions *in vivo* (41–46), we first sought to establish the model for the generation of M-MDSC from monocytes *in vitro* based on these factors. The phenotypic and functional properties of M-MDSC were assessed according to the criteria proposed by Bronte et al. (9). As a control, we also used the protocol for M-MDSC differentiation based on GM-CSF/IL-4/PGE2 (48), and as a negative control, we used GM-CSF and IL-4, which induce immature DC.

PGE2 Potentiates GM-CSF/IL-6-Mediated Induction of M-MDSC Phenotype

MACS-sorted CD14⁺ monocytes from healthy donors that were used for differentiation, contained <2 % of HLA-DR⁺ CD14⁺ CD33⁺ CD11b⁺ SSC^{low} (M-MDSC) and HLA-DR⁺ CD15⁺ CD33⁺ CD11b⁺ SSC^{low} (PMN-MDSC) cells, as expected for healthy donors (9). After their differentiation with GM-CSF and IL-6, up to 34% ($23.9 \pm 10.2\%$) of cells showed HLA-DR⁺ CD14⁺ CD33⁺ CD11b⁺ M-MDSC phenotype (Figure 1A), and no significant percentage of CD15⁺ cells was present in the population (data not shown). The addition of PGE2 to the GM-CSF/IL-6 cocktail induced a significantly higher percentage of HLA-DR⁺ CD14⁺ CD33⁺ CD11b⁺ M-MDSC cells ($41.23 \pm 11.6\%$) compared to GM-CSF/IL-6 (Figures 1A,B). GM-CSF/IL-4 induced almost complete down-regulation of CD14 on immature DC and no significant percentage of HLA-DR⁺ CD14⁺ CD11b⁺ CD33⁺ was detected. The addition of PGE2 to the GM-CSF/IL-4 cocktail induced a significantly higher percentage of CD14⁺ cells and majority of the cells expressed HLA-DR as well. The percentage of HLA-DR⁺ CD14⁺ CD33⁺ CD11b⁺ M-MDSC was not higher

than 15% ($9.5 \pm 5.0\%$) (Figure 1A). Besides, GM-CSF/IL-4-based protocols induced differentiation of CD209⁺ cells predominantly, unlike GM-CSF/IL-6-based protocols. The expression of CD1a was most prominent on immature DC, whereas other cell types displayed lower expression of this molecule, and the statistically significant reduction of CD1a was detected only in GM-CSF/IL-6-based protocols. Interestingly, the M-MDSC differentiated with GM-CSF/IL-6 alone, displayed significantly higher expression of CD206 compared to other tested cells. These results suggested that, according to the phenotypic criteria for M-MDSC (9), GM-CSF/IL-6-based protocols were more potent at inducing M-MDSC, and that PGE2 significantly potentiated this effect.

PGE2 Potentiates Suppressive Functions of M-MDSC

In addition to the phenotypic criteria, M-MDSC should display suppressive properties in one of the suggested assays (9). Except for DC, all cells suppressed significantly the proliferation of PHA-stimulated PBMC at higher cell-to-cell ratios, as compared to PHA/PBMC alone (Figures 2A,B). Thereby, GM-CSF/IL-6/PGE2 M-MDSC displayed the strongest effect, and GM-CSF/IL-4/PGE2-induced M-MDSC displayed the weakest suppression ($51.4 \pm 8.4\%$ and $24.6 \pm 4.6\%$ suppression at 1:8 M-MDSC:PBMC cell ratio, respectively). Therefore, according to the phenotypic criteria and functional assays, we decided to further assess the functions of GM-CSF/IL-6-induced M-MDSC *in vitro*, and in particular, how PGE2 affect their tolerogenic capacity.

PGE2 Induces a Stable Immature Phenotype of GM-CSF/IL-6 M-MDSC

The stimulation of M-MDSC with IFN- γ and TLR-4 agonists was shown to up-regulate their MHC class II expression and the capacity for Treg inductions (24, 49, 50). Therefore, we tested the

phenotype of GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC and cytokines production of M-MDSC stimulated with LPS/IFN- γ or unstimulated. The doses of LPS and IFN- γ applied for the stimulation of M-MDSC did not affect significantly their apoptosis (**Supplementary Figure 1**). Without the stimulation, both GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC displayed low surface expression of HLA-DR, CD86, CD80, and intracellular expression of CCR7 and p40 subunit. However, GM-CSF/IL-6/PGE2 M-MDSC contained a higher percentage of CD73⁺ cells compared to GM-CSF/IL-6 M-MDSC. Such a difference was found within both HLA-DR⁻/CD14⁺ and HLA-DR⁺/CD14⁺ subsets of non-stimulated M-MDSC (**Supplementary Figure 2**). After the stimulation with LPS/IFN- γ , both M-MDSC types upregulated significantly HLA-DR, CD80, CD40, and CCR7 expression. Thereby, GM-CSF/IL-6/PGE2 M-MDSC displayed significantly higher CCR7, CD39, and CD73 expression compared to GM-CSF/IL-6 M-MDSC. In contrast, GM-CSF/IL-6 M-MDSC stimulated with LPS/IFN- γ also upregulated significantly CD86 and p40 subunit, unlike LPS/IFN- γ -treated GM-CSF/IL-6/PGE2 M-MDSC (**Figure 3A**). Therefore, PGE2 reduced the maturation capacity of GM-CSF/IL-6 M-MDSC.

The analysis of cytokines production by GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC suggested significant differences between these cells, but predominantly after the LPS/IFN- γ stimulation. GM-CSF/IL-6/PGE2 M-MDSC displayed a significantly lower capacity to produce TNF- α , IL-1 β , IL-12p70, and IL-23 after the stimulation with LPS/IFN- γ , compared to GM-CSF/IL-6 M-MDSC (**Figure 3B**). These cells also displayed a lower capacity to produce IL-10 compared to GM-CSF/IL-6 M-MDSC. In contrast, GM-CSF/IL-6/PGE2 M-MDSC produced significantly more TGF- β , IL-27, and IL-33. By analyzing chemokines, we found that GM-CSF/IL-6/PGE2 M-MDSC produced significantly more CCL2, both in the presence and absence of stimulation with LPS/IFN- γ , as well as less CXCL8 after the stimulation. Cumulatively, these results suggested that GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC stimulated with LPS/IFN- γ differed significantly in their functional potential and could possibly display different Th polarization capacity in co-culture with T cells.

PGE2 Potentiates Suppressive Effects of GM-CSF/IL-6 M-MDSC on T Cells and Promote Their Th2 Polarization

To investigate how the results on M-MDSC phenotype and cytokines production correlate with the Th polarization capacity of these cells, we used LPS/IFN- γ -stimulated M-MDSC for cultivation with MACS-purified allogeneic T cells. Both GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC were able to induce alloreactive T cell proliferation after the stimulation (**Figure 4A**), although the response was much lower than the T-cell proliferation induced by LPS/IFN- γ -treated DC (data not shown). However, GM-CSF/IL-6/PGE2 M-MDSC displayed a significantly lower capacity to induce alloreactive T cells proliferation, compared to GM-CSF/IL-6 M-MDSC (**Figure 4A**). The viability of T cells in co-cultures did not

differ significantly, so the number of viable T cells in co-cultures with GM-CSF/IL-6 M-MDSC was higher than in co-cultures with GM-CSF/IL-6/PGE2 M-MDSC (**Figure 4B**). In the suppressive assay with CD3/CD28-stimulated allogeneic T cells, GM-CSF/IL-6/PGE2 M-MDSC displayed significantly stronger capacity to suppress the proliferation of T cells, compared to GM-CSF/IL-6 M-MDSC, especially when higher number of M-MDSC was present in the co-cultures (1:4, M-MDSC:T cell ratio, respectively) (**Figure 4C**).

When the cytokines produced in the co-cultures with T cells were analyzed, we found significantly lower levels of IL-17 and TGF- β , and significantly higher levels of IL-4 and IL-10 in the co-cultures with GM-CSF/IL-6/PGE2 M-MDSC, as compared to GM-CSF/IL-6 M-MDSC. In contrast, the levels of IFN- γ were similar between the two M-MDSC type containing co-cultures (**Figure 4D**). These results were confirmed by analyzing the intracellular levels of cytokines in CFSE-stained T cells from the co-cultures (**Figure 4E**), in which the most cytokine-producing cells were found within CFSE-low (proliferating) cells. Considering that the T cells cultivated without M-MDSC did not proliferate or display significant levels of intracellular cytokines (**Supplementary Figure 3**), these results suggested that the allogeneic proliferation was required for cytokine production by T cells. Additionally, we analyzed intracellular cytokines within CD4⁺ and CD8⁺ T subsets and found that a significantly lower percentage of CD4⁺IL-17⁺ T cells, and a higher percentage of CD4⁺IL-4⁺ T cells was induced by GM-CSF/IL-6/PGE2 M-MDSC, as compared to GM-CSF/IL-6 M-MDSC. These results were also confirmed by analyzing the expression of GATA-3 and ROR- γ t within CD4⁺ T cells (data not shown). The percentages of IFN- γ ⁺CD4⁺ T cells were similar between the tested groups. On the other side, the percentage of IFN γ ⁺ Granzyme B⁺ CD8⁺ cytotoxic T cells (CTL) was lower in the co-cultures with GM-CSF/IL-6/PGE2 M-MDSC, as compared to GM-CSF/IL-6 M-MDSC. The differences in the percentages of cytokine-producing cells correlated with the absolute number of cells in these co-cultures (**Figure 4F**). Therefore, GM-CSF/IL-6/PGE2-induced M-MDSC displayed an increased ratio of Th2/Th17 cells, and a reduced capacity to induce CTL compared to GM-CSF/IL-6 M-MDSC, pointing to their anti-inflammatory polarization potential.

PGE2 Reduces the Capacity of GM-CSF/IL-6 M-MDSC to Induce Conventional Treg

To study the capacity of GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC to induce conventional Treg, allogeneic T cells were co-cultivated with LPS/IFN- γ -stimulated M-MDSC at 1:50 (MDSC: T cell ratio) for 3 days and then expanded with IL-2, before the analysis of CD4⁺CD25^{hi}FoxP3⁺ T cells. These culture conditions enabled a higher viability of T cells and a lower difference in the total cell number between the co-cultures with GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC (**Supplementary Figure 4**). Interestingly, it was found that GM-CSF/IL-6 M-MDSC induced a significantly higher percentage of CD4⁺CD25^{hi}FoxP3⁺ Treg

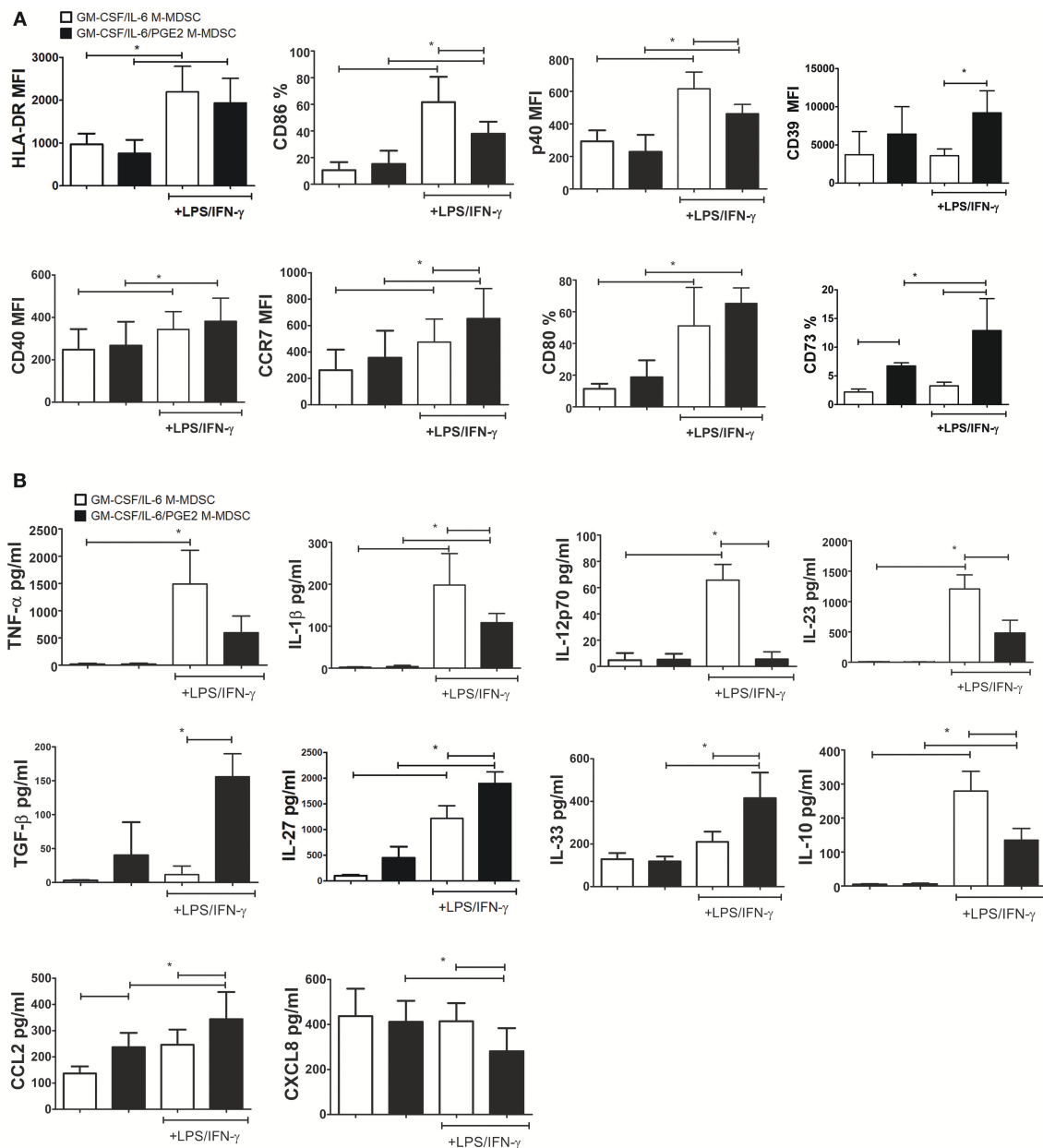


FIGURE 3 | Effects of LPS/IFN- γ on M-MDSC phenotype and cytokines/chemokines production. **(A,B)** The monocytes were cultivated in the presence of GM-CSF/IL-6, or GM-CSF/IL-6/PGE2 for 5 days, and then stimulated with LPS/IFN- γ , or left unstimulated, for the next 16 h. **(A)** The results on surface (HLA-DR, CD86, CD40, CD39, CD73, and CD80) and intracellular (CCR7 and p40) expression obtained by flow cytometry are presented as mean % or mean fluorescence intensity (MFI) \pm SD from 4 independent experiments (or 3 experiments in case of CD39 and CD73). **(B)** The levels cytokines/chemokines in cell-free supernatants from those cultures were analyzed by ELISA or beads-based immunoassay and the results are shown as mean pg/ml \pm SD ($n = 4$). * $p < 0.05$ as indicated by the line (RM ANOVA, Tukey post-test).

compared to GM-CSF/IL-6/PGE2 M-MDSC (Figure 5A). Similar results were observed when Treg were analyzed as CD4⁺CD25⁺CD127⁻FoxP3⁺ T cells (data not shown). Although the expression of FoxP3 within CD4⁺CD25^{hi} (or CD4⁺CD25⁺CD127⁻) Treg cells was similar between the two groups, TGF- β expression was reduced within FoxP3⁺ Treg co-cultivated with GM-CSF/IL-6/PGE2 M-MDSC. T cells cultivated without M-MDSC contained low percentage of total CD25⁺

cells ($2.4 \pm 1.3\%$), and no cells were found within CD4⁺CD25^{hi} region as set for the T cells co-cultivated with M-MDSC (data not shown).

To assess the mechanism by which GM-CSF/IL-6 M-MDSC induce a higher percentage of FoxP3⁺ Treg, the surface expression of PD-L1 and IDO-1 were analyzed, as these are critical molecules for the induction of FoxP3⁺ Treg (31, 51). It was found that PD-L1 was expressed similarly between the two

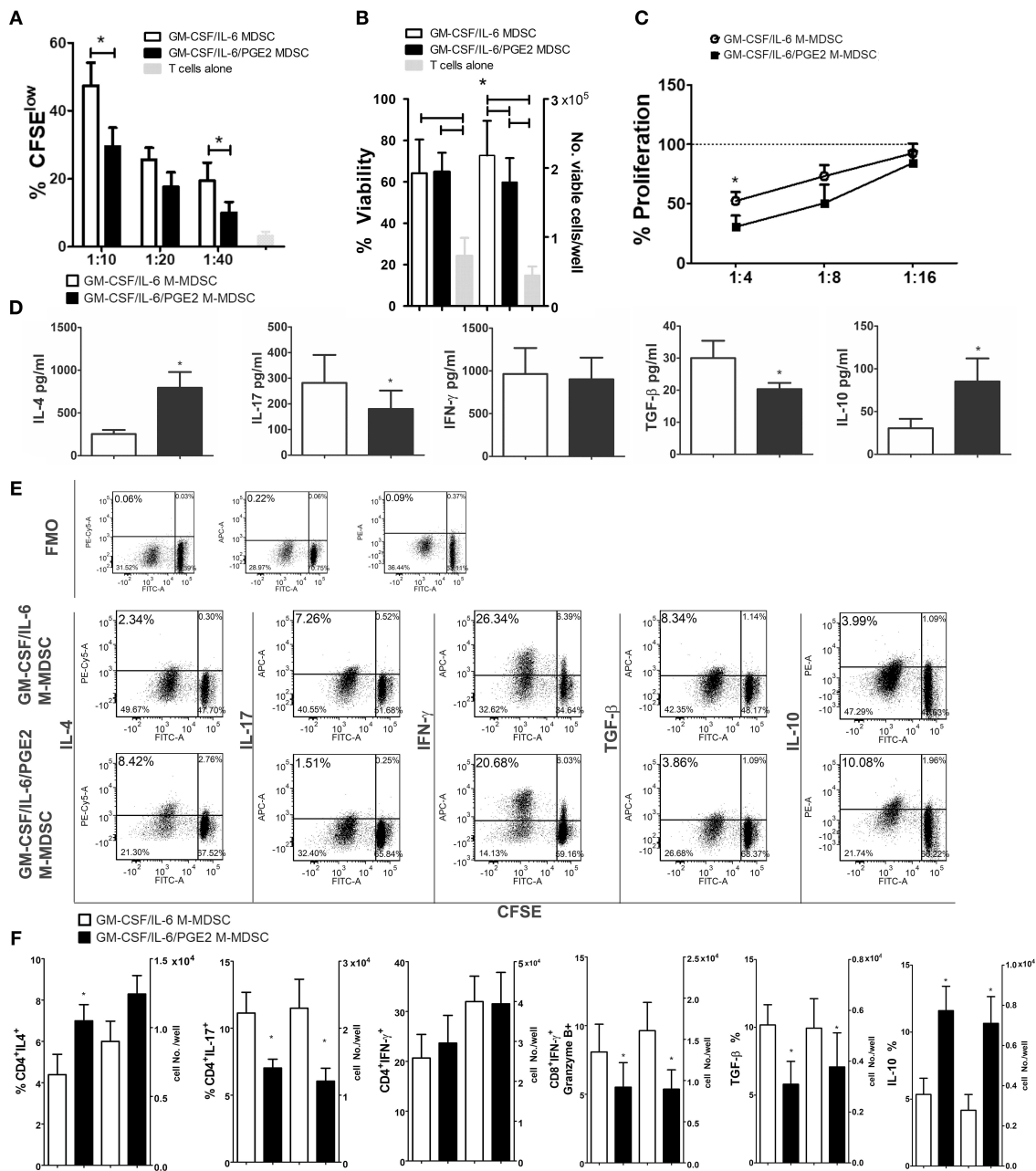


FIGURE 4 | Effects of LPS/IFN- γ -stimulated M-MDSC on proliferation and differentiation of allogeneic T cells. **(A)** The proliferation of MACS-purified allogeneic CFSE-labeled T cells (1×10^5 /well) in the presence or absence of different number of LPS/IFN- γ -stimulated M-MDSC (1×10^4 – 0.25×10^4 /well) was determined by flow cytometry after 5 days of co-cultivation, and the results from one representative experiment are shown as mean proliferation \pm SD of triplicates. **(B)** The viability and cell number of the T cells/well was determined on Muse Cell Analyzer, as described, and the data is presented as mean \pm SD of 5 independent experiments. **(C)** The proliferation of allogeneic T cells in the presence of CD3/CD28 stimulation and different number of LPS/IFN- γ -stimulated M-MDSC (2.5 – 0.62×10^4 /well) was determined by flow cytometry after 5 days of cultivation, and the results are shown as mean relative proliferation \pm SD, i.e., % proliferation of control CD3/CD28-stimulated T cells (100%) from 3 independent experiments. **(A,B)** $^*p < 0.05$ GM-CSF/IL-6 M-MDSC vs. corresponding GM-CSF/IL-6/PGE2 M-MDSC (RM ANOVA, Tukey post-test). **(D)** The levels of indicated cytokines, shown as mean pg/ml \pm SD, were determined from the supernatants of 1:10 M-MDSC/T cell co-cultures carried out as in **(A)** and treated for 4 h with PMA/Ca ionophore. The levels of cytokines were standardized to 1×10^5 of viable T cells from the co-cultures. **(E)** Expression of intracellular cytokines was determined within CFSE-labeled T cells co-cultivated at 1:10 cell ratio as in **(A)** and treated for 3 h with PMA/ionomycin/monensin. Data from one representative experiment are shown. **(F)** The percentages and cell number of CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺, CD4⁺IL-17⁺, CD8⁺IFN- γ ⁺ Granzyme B⁺, TGF- β ⁺, and IL-10⁺ cells were determined by flow cytometry from the M-MDSC/T cell co-cultures carried out at 1:10 cell-to-cell ratios as in **(A)** and treated for 3 h with PMA/ionomycin/monensin. The cell number was calculated from the absolute number of viable T cells after the cultures **(B)** and % of positive cells from flow cytometry. $^*p < 0.05$ paired *T*-test.

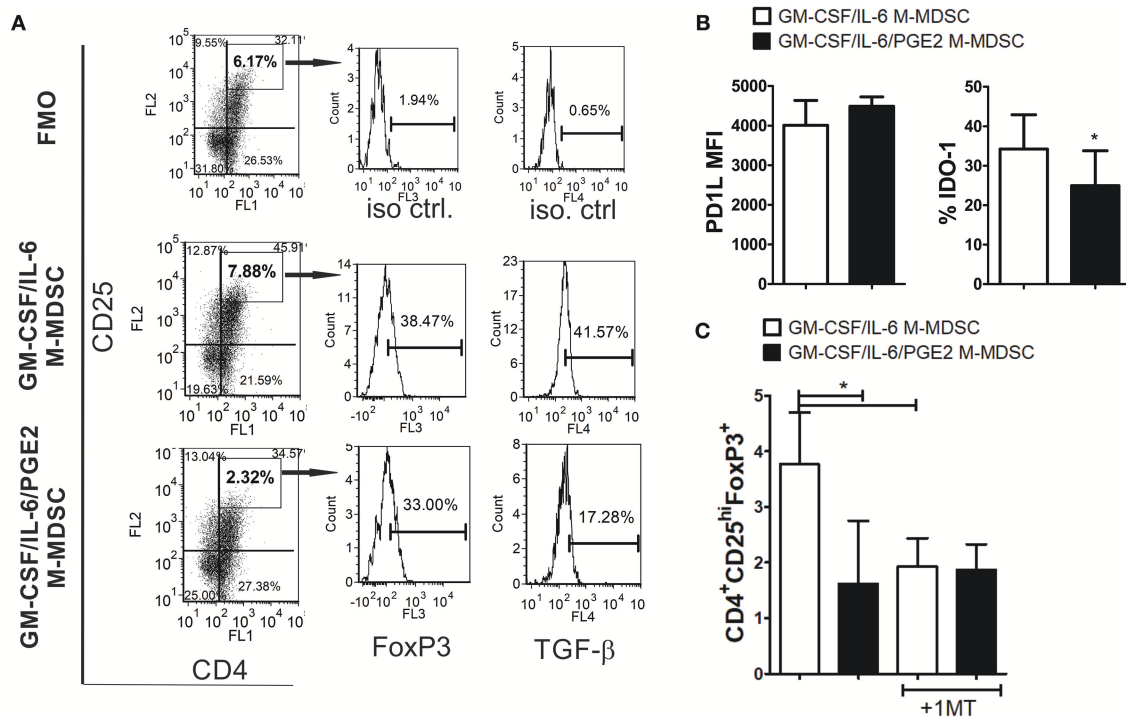


FIGURE 5 | The capacity of LPS-IFN- γ stimulated M-MDSC to induce FoxP3⁺ Treg. **(A)** A representative analysis of FoxP3⁺ Treg is shown from the experiments in which M-MDSC (2×10^3 /well) were co-cultivated with allogeneic T cells for 3 days, followed by IL-2 treatment for the next 3 days. The presented histograms of FoxP3 and TGF- β are shown from CD4⁺CD25^{hi} gates, and the markers were set according to FMO control. **(B)** The surface expression of PD-L1 and intracellular expression of IDO-1 were determined by flow cytometry after the staining of LPS/IFN- γ stimulated M-MDSC, and the results are shown as mean MFI or % \pm SD of 3 independent experiments. * $p < 0.05$ paired T -test. **(C)** The summarized data are shown on the % of CD4⁺CD25^{hi}FoxP3⁺ cells \pm SD ($n = 3$) induced in the co-cultures with M-MDSC that were carried out as in **(A)**, either in the presence or absence of 1-MT. * $p < 0.05$ as indicated by line (RM ANOVA, Tukey post-test).

M-MDSC types, whereas the expression of IDO-1 was higher on GM-CSF/IL-6 M-MDSC, as compared to GM-CSF/IL-6/PGE2 M-MDSC (**Figure 5B**). When IDO-1 inhibitor (1-MT) was used in the co-cultures with T cells, the percentage of FoxP3⁺ Treg induced by GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 MDSC was similarly lower (**Figure 5C**). These results suggested that increased IDO-1 expression by GM-CSF/IL-6 M-MDSC was responsible for a higher capacity of these cells to induce FoxP3⁺ Treg.

PGE2 Increases the Capacity of GM-CSF/IL-6 M-MDSC to Induce IL-10 Producing T Cells

The lower capacity of GM-CSF/IL-6/PGE2 M-MDSC to induce conventional Treg could explain the lower levels of TGF- β found in the co-cultures with GM-CSF/IL-6/PGE2 M-MDSC, but not the increased levels of IL-10 in the same co-cultures. To assess the source of increased IL-10 production by T cells we co-cultivated T cells with MDSC at 1:50 (M-MDSC:T) cell ratio and analyzed the expression of this cytokine within CD4⁺ and CD8⁺ T cell subsets by flow cytometry. Compared to GM-CSF/IL-6 M-MDSC, GM-CSF/IL-6/PGE2 M-MDSC induced significantly higher expression of IL-10 within CD4⁺IFN- γ ⁻ T cells, but not within CD4⁺IFN- γ ⁺ (Th1) cells (**Figures 6A,B**). In contrast,

GM-CSF/IL-6/PGE2 M-MDSC increased the expression of IL-10 within CD8⁺IFN- γ ⁺ T cells, and not within CD8⁺IFN- γ ⁻, as compared to GM-CSF/IL-6 M-MDSC. However, the presence of IL-10 in CD8⁺ T cells was much lower than in CD4⁺IFN- γ ⁻ T cells.

Therefore, we then analyzed the expression of IL-10 in CD4⁺IL-4⁺ (Th2) and CD4⁺IL-4⁻FoxP3⁻ T cells, also identified as Tr-1 cells (29, 40). We found that GM-CSF/IL-6/PGE2 M-MDSC induced significantly higher expression of IL-10 within both CD4⁺IL-4⁺ and CD4⁺IL-4⁻FoxP3⁻ T cells, as compared to GM-CSF/IL-6 M-MDSC (**Figure 6C**). In co-cultures with GM-CSF/IL-6/PGE2 M-MDSC, the percentage of IL-10⁺ Th2 cells was 2.1 ± 0.4 ($n = 3$) times higher than in co-cultures with GM-CSF/IL-6 M-MDSC, whereas the percentage of Tr-1 cells increased even higher [3.2 ± 0.6 ($n = 3$)]. Moreover, the total number of CD4⁺IL-4⁻FoxP3⁻ cells was about 4 times higher than the number of Th2 cells, suggesting that Tr-1 cells contributed more to the total number of IL-10⁺ T cells.

To analyze the mechanisms responsible for the Tr-1 induction by GM-CSF/IL-6/PGE2 M-MDSC, we focused on ILT-3 and ILT-4 expression, since these molecules were found critical for the induction of these cells (29, 30). Flow cytometry analysis showed that GM-CSF/IL-6/PGE2 M-MDSC expressed significantly more ILT-3 and ILT-4, as compared to GM-CSF/IL-6 M-MDSC (**Figure 6D**). Additionally, when M-MDSC/T cell co-cultures

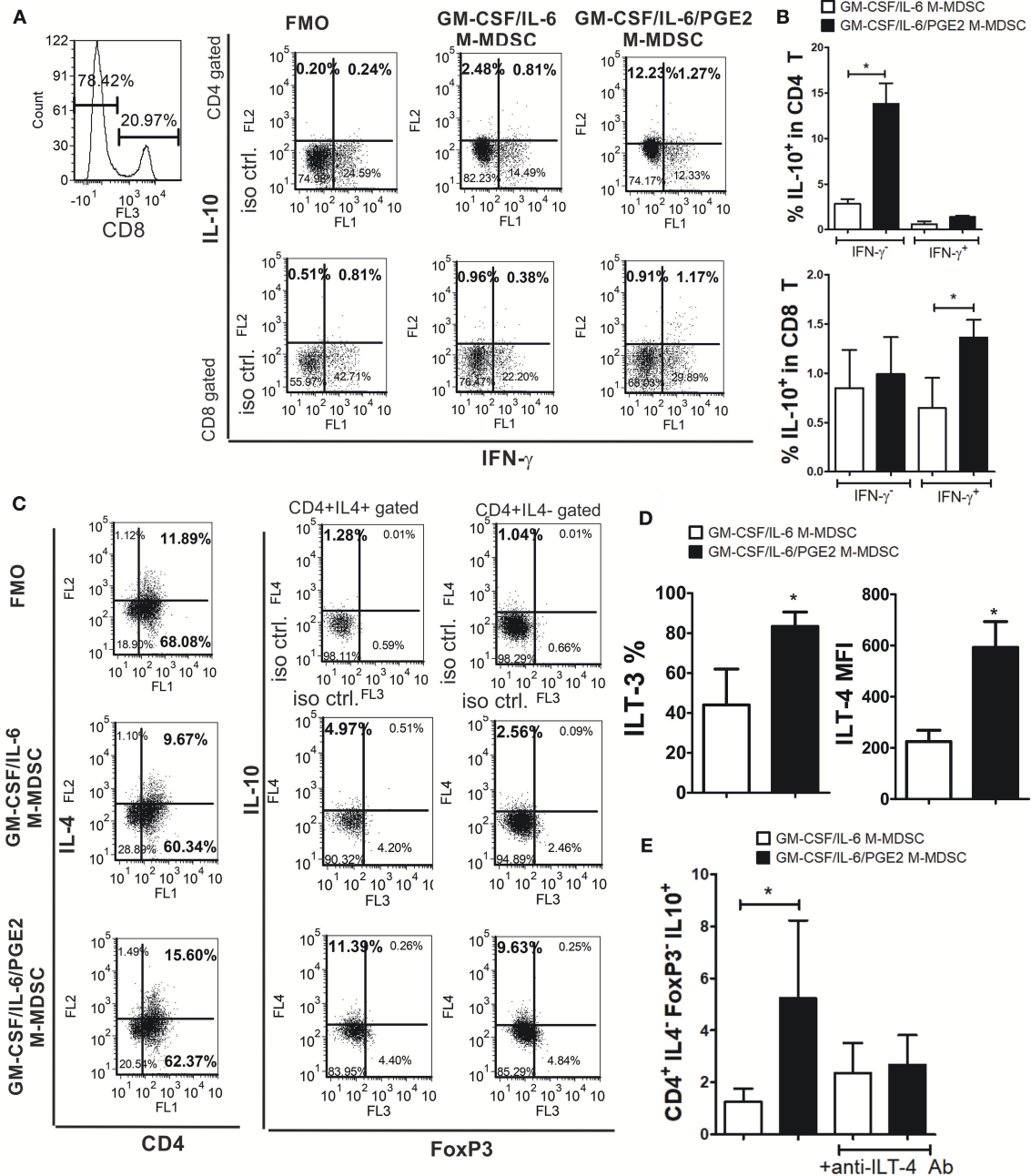


FIGURE 6 | The capacity of LPS/IFN- γ -stimulated M-MDSC to induce IL-10-producing T cells. **(A)** A representative analysis is shown of IL-10 and IFN- γ expression within gated CD8 $^{+}$ and CD8 $^{-}$ (CD4 $^{+}$) T cell populations after the co-culture with LPS/IFN- γ -stimulated M-MDSC (2×10^3 /well) for 3 days, followed by the IL-2 treatment for additional 3 days. **(B)** The summarized results from 3 independent experiments are shown as % of IL-10 $^{+}$ in CD4 $^{+}$ or CD8 $^{+}$ T cells co-expressing IFN- γ or not. **(C)** A representative analysis of IL-10 and FoxP3 expression within CD4 $^{+}$ IL-4 $^{+}$ and CD4 $^{+}$ IL-4 $^{-}$ T cells is shown from the experiments performed as in **(A)**. **(D)** The surface expression of ILT3 and ILT4 were determined by flow cytometry after the staining of LPS/IFN- γ -stimulated M-MDSC and the results are shown as mean MFI or % \pm SD of 3 independent experiments. * $p < 0.05$ paired T-test. **(E)** The summarized data are shown on the % of CD4 $^{+}$ IL-4 $^{-}$ FoxP3 $^{+}$ IL-10 $^{+}$ (Tr-1) cells \pm SD ($n = 3$) induced in the co-cultures with M-MDSC that were carried out as in **(A)**, either in the presence of anti-ILT-4 Ab or isotype control Ab. * $p < 0.05$ as indicated by the line (RM ANOVA, Tukey post-test).

were carried out in the presence of blocking anti-ILT-4 Ab (**Figure 6E**) or anti-ILT-3 Ab (data not shown), the percentage of Tr-1 cells in the co-cultures with GM-CSF/IL-6/PGE2 M-MDSC was similar to the percentage Tr-1 cells induced in

the co-cultures with GM-CSF/IL-6 M-MDSC. These results suggested that GM-CSF/IL-6/PGE2 M-MDSC utilize ILT-3- and ILT-4-dependent mechanisms to induce the development of Tr-1 cells.

DISCUSSION

MDSC, particularly M-MDSC, have been recognized as a major limiting factor for the efficacy of checkpoint inhibition therapy (2, 4, 5). A probable cause includes many mechanisms by which they suppress the activation of T cells (3, 10, 11) and induce FoxP3⁺ Treg (23). The relative contribution of these mechanisms, especially in the induction of different Treg subsets, remained poorly investigated. To facilitate such an inquiry, we have developed an original model for the generation of M-MDSC and demonstrated that the monocytes differentiated in the presence of GM-CSF and IL-6 acquire M-MDSC phenotype, produce IL-10, exhibit suppressive properties, and induce a higher percentage of FoxP3⁺ Treg via IDO-1-dependent mechanisms. PGE2, a key factor produced in chronic inflammation and tumor (44), potentiate GM-CSF/IL-6-dependent induction of M-MDSC, their suppressive potential and their capacity to induce the Th2 response *in vitro*. Moreover, we showed for the first time that PGE2 does not increase the capacity of GM-CSF/IL-6 M-MDSC to induce CD4⁺CD25^{hi}FoxP3⁺Treg, but rather augment their capacity to induce IL-10 production by CD8⁺ IFN- γ ⁺ T cells, Th2 cells, and particularly, by CD4⁺ IL-4⁻ FoxP3⁻ Tr-1 cells via ILT-4 and ILT-3 dependent mechanisms.

Previous reports suggested that M-MDSC could be differentiated *in vitro* from monocytes by using GM-CSF, IL-4, and PGE2 (36, 38). Our data confirmed that this protocol induces suppressive cells (especially compared to non-suppressive DC) with low CD1a expression and high CD14 expression. The described mechanisms behind the suppressive activity of GM-CSF/IL-4/PGE2 M-MDSC include EP2/EP4-dependent positive feedback loop between PGE2 and COX2, which drives an increased expression of suppressive markers on these M-MDSC, such as PD-L1 (36). However, unlike Obermajer et al. (36), the GM-CSF/IL-4/PGE2 M-MDSC obtained in our experiments expressed CD209. This could be due to different basal media used and lower doses of GM-CSF used in their experiments. DC-SIGN (CD209) is a hallmark of IL-4-induced effects on DC, and its expression is down-regulated in the presence of TGF- β (52). Unlike the other authors which used TGF- β -containing fetal calf serum (36, 37, 53), we used serum-free medium, which could be a probable reason for detecting a higher level of CD209 on GM-CSF/IL-4/PGE2 induced M-MDSC. Moreover, the percentage of HLA-DR⁻CD14⁺CD33⁺CD11b⁺ cells induced by GM-CSF/IL-4/PGE2, and the suppressive capacity of these cells, was lower than those induced by using GM-CSF/IL-6 or GM-CSF/IL-6/PGE2 cocktail.

GM-CSF/IL-6-based protocols induced much lower percentage CD209⁺ cells, and in the absence of PGE2, GM-CSF and IL-6 induced a higher expression of CD206. Although more specific in mice, some studies suggested that CD206 is a marker of human M2 macrophages generated from monocytes in the presence of M-CSF, but not in the presence of GM-CSF knowing to induce M1 type macrophages (54, 55). In contrast, other reports suggested that high expression of CD209 better correlates with the M2 phenotype of human macrophages (56). M-MDSC in our experiments were heterogeneous, and according to their phenotype and cytokines production, they hardly fit into the

standard M1/M2 macrophage model. Namely, GM-CSF/IL-6-induced M-MDSC displayed a higher capacity to up-regulate CD86, p40, a subunit of IL-12/IL-23 (57), and proinflammatory cytokines TNF- α , IL-1 β , IL-12, and IL-23, as well as a lower capacity to produce TGF- β in comparison to GM-CSF/IL-6/PGE2 M-MDSC. These properties resemble more to M1-like properties of GM-CSF/IL-6-induced M-MDSC. However, GM-CSF/IL-6 M-MDSC also produced increased levels of IL-10 and displayed suppressive capacity in the co-cultures with allogeneic PBMC and T cells, all of which mark M2 type macrophages (54, 55, 58). Unlike the standard protocols for macrophages differentiation (54–56), we used IL-6 and IL-6/PGE2 from the beginning of differentiation, and about 23% and 43% of these cells, respectively, lacked the expression of HLA-DR, which is the main reason for designating them as M-MDSC, rather than macrophages. It is not clear at the moment whether additional stages of myeloid cell differentiation are present in our M-MDSC population and to which extent. Therefore, additional phenotypic and functional characterization of *in vitro* induced M-MDSC are necessary to assess the heterogeneity of these cells and relate them to their *in vivo* counterparts.

The combination of GM-CSF and IL-6 was shown to potentiate the generation of IL-4R α ⁺ MDSC from bone marrow in mice and humans (59). Additionally, by analyzing the tumor-produced factors which induce suppressive CD33⁺ cells from PBMC, Lechner et al. (35) demonstrated that the combination of GM-CSF and IL-6 is more potent than the combination of GM-CSF and other mediators, such as PGE2. Although these authors suggested that GM-CSF/IL-6 induced CD33⁺ cells were mononuclear, the described phenotype was CD11b⁺CD66b⁺HLA-DR^{low}IL-13Ra2^{int}, which along with their increased NADPH oxidase activity (35), corresponds to PMN-MDSC (9, 60). It was left unclear, whether M-MDSC also contribute to the suppressive effects of CD33⁺ cells induced by GM-CSF and IL-6. To our knowledge, this is the first report showing the ability of GM-CSF/IL-6 combination to generate suppressive HLA-DR^{-/low}CD14⁺CD33⁺CD11b⁺ M-MDSC as well. Moreover, PGE2 potentiated significantly this induction. We found significantly higher percentage of CD39⁺CD73⁺ M-MDSC in cultures with GM-CSF/IL-6/PGE2 compared to GM-CSF/IL-6 alone. These molecules are involved in the adenosine-generating pathway, which was found critical for an increased infiltration and suppressive functions MDSC and tumor associated macrophages in cancer patients (61, 62). The fact that M-MDSC induced in presence of PGE2 contained a higher percentage of CD39⁺CD73⁺ cells within both HLA-DR^{-/low} and HLA-DR⁺ subsets, suggest that both populations contribute to the suppressive activity these cells. M-MDSC were shown to accumulate in tumor via CCL2-dependent mechanisms (63), whereas PMN-MDSC accumulate in a CXCL8-dependent manner (64). The phenomenon that PGE2 potentiates the production of CCL2 and simultaneously reduces CXCL8 production by GM-CSF/IL-6 M-MDSC, could explain the observation that M-MDSC accumulate preferentially in PGE2-rich tumor site, rather than PMN-MDSC (3, 11), although this hypothesis needs to be tested independently.

Significant functional differences were found between GM-CSF/IL-6 M-MDSC and GM-CSF/IL-6/PGE2 M-MDSC after their stimulation with LPS/IFN- γ , which could explain differences in their allostimulatory capacity, Th polarization, and Treg induction. IFN- γ and TLR4 agonists were shown to activate NF- κ B signaling in MDSC, up-regulate their MHC class II expression, and significantly contribute to the suppressive functions of MDSC in contact with T cells (24, 49, 50, 65). In respect to this, a lower allostimulatory capacity of LPS/IFN- γ -stimulated GM-CSF/IL-6/PGE2 M-MDSC could be explained by their lower capacity to up-regulate CD86 expression and pro-inflammatory cytokines, as well as a higher capacity to produce TGF- β . TGF- β was shown to exhibit direct anti-proliferative effects on T cells (66), and has a critical role in cancer driven immunosuppression (67). Moreover, an increased TGF- β production by CD14⁺HLA-DR^{low/-} M-MDSCs from patients with melanoma was shown to be PGE2 and COX2 dependent (18).

In contrast, GM-CSF/IL-6 M-MDSC produced higher levels of IL-10, which together with the up-regulated CD80 expression could have contributed to their increased capacity for inducing CD4⁺CD25^{hi}FoxP3⁺ Treg. Although CD80 can act as a co-stimulatory molecule, it ligates CTLA-4 with a higher affinity than CD28 (68). Accordingly, in an ovarian carcinoma-bearing mice model, IFN- γ -stimulated M-MDSC increased MHC class II, CD80, and IL-10 expression, and induced CD4⁺CD25⁺ Treg in a CTLA-4/CD80-dependent manner (65), which is in line with our results on IL-10-producing GM-CSF/IL-6 M-MDSC. Additionally, we found that GM-CSF/IL-6 M-MDSC display a higher IDO-1 expression, which could be involved directly in the induction of a higher percentage of CD4⁺CD25^{hi}FoxP3⁺ Treg by these cells, compared to GM-CSF/IL-6/PGE2. LPS and IFN- γ are strong inducers of IDO-1 (69) and IL-10 was shown to significantly potentiate IFN- γ -mediated IDO-1 expression (70). Therefore, a higher capacity of GM-CSF/IL-6 M-MDSC to produce IL-10, compared to GM-CSF/IL-6/PGE2, could be a reason for their higher expression of IDO-1. Other findings (69), including our own with nanomaterials or parasite products induced tolerogenic DC (31, 71–73), showed that FoxP3⁺ cells induced via IDO-1-dependent mechanisms express CD39, CD73, and TGF- β , which contribute to their suppressive functions in contact with allogeneic T cells. However, it should be noted that in the presence of IDO-1 inhibitor (1-MT), both M-MDSC types induced about 2% of FoxP3⁺ Treg. These results suggest that both M-MDSC types possess additional mechanisms by which they induce CD4⁺FoxP3⁺ Treg, independent of IDO-1.

Interestingly, GM-CSF/IL-6/PGE2 M-MDSC displayed a higher suppressive capacity in the co-culture with CD3/CD28-stimulated T cells, without inducing an increased percentage of FoxP3⁺ Treg. A probable reason for this finding is a higher capacity of these cells to induce IL-10-producing Th2 and Tr-1 cells. PGE2 potentiated IL-33 production by GM-CSF/IL-6 induced M-MDSC. This cytokine is highly produced by necrotic tumor cells as an alarmin, and its role in driving the recruitment and activation of MDSC was shown previously (74). Although IL-33 production by M-MDSC was not shown before, endogenous PGE2 was reported to amplify IL-33 production by macrophages

via EP2/EP4 cAMP-dependent pathway (75). Therefore, it is possible that similar mechanisms were involved in a higher capacity of GM-CSF/IL-6/PGE2 M-MDSC to produce IL-33. IL-33 is a potent inducer of Th2 cell differentiation and their maintenance, acting via ST-2 receptor (76). In line with this, we showed an increased capacity of IL-33-producing GM-CSF/IL-6/PGE2 M-MDSC to induce Th2 cells. Th2 cells were shown to promote tumor development, unlike Th1 cells specific for the same antigens (77), suggesting that GM-CSF/IL-6/PGE2 M-MDSC cells induced in our model display the functional resemblance to M-MDSC *in vivo* (7).

A lower percentage of IFN- γ ⁺ Granzyme B⁺ CTL in co-culture with GM-CSF/IL-6/PGE2 M-MDSC could be explained by a lower capacity of these cells to produce IL-12, as this cytokine is critical for CLT induction (78). In spite of this, we did not observe down-regulation of IFN- γ production by Th1 cells, and these cells did not produce a significant amount of IL-10. A possible explanation of this phenomenon could be a higher capacity of GM-CSF/IL-6/PGE2 M-MDSC to produce IL-27. Namely, this cytokine was shown to promote the differentiation of Th1 cells, but also to inhibit directly the development of Th17 cells (79). Therefore, both lower IL-23 production and increased IL-27 could explain the down-regulated capacity of GM-CSF/IL-6/PGE2 MDSC to induce Th17 cells, compared to GM-CSF/IL-6 M-MDSC. To our knowledge, this is the first report showing the production of IL-27 by M-MDSC, but the significance of this finding is still not clear. The role of IL-27 in cancer is still a matter of debate, considering that both pro-inflammatory and anti-inflammatory actions of IL-27 were demonstrated (80). In relation to our results, IL-27 was shown as an important inducer of Tr-1 cells, particularly in the presence of TGF- β (81). Moreover, it was reported that Tr1 cells can make up to 30% of all tumor-infiltrating lymphocytes in some tumors (33). Therefore, the roles of IL-27 and TGF- β produced by M-MDSC in the tumor microenvironment deserves further investigations.

In our study, Tr-1 cells were phenotypically identified as CD4⁺FoxP3⁺IL-4⁺IL-10⁺ according to other reports (29, 40). Moreover, in line with other findings on tolerogenic DC (40, 82), we showed that the induction of these cells is ILT3- and ILT4-dependent. PGE2 was shown previously to increase the expression of ILT4 and ILT3 by M-MDSC (36), but this is the first report to explain the role of these molecules in the induction of Tr-1 cells. Moreover, we showed previously that blockage of ILT-3 and ILT-4 on tolerogenic DC induced by cellulose nanomaterials (31) or mesenchymal stem cells (83) reduced both the percentage of induced Tr-1 cells, as well as the suppressive capacity of total T cell population containing Tr-1 cells. As before, it should be noted that even after blocking of ILT3 and ILT4, both M-MDSC types induced a low percentage of Tr-1 cells, suggesting that additional mechanisms could be involved in the induction of Tr1 cells by M-MDSC. Several studies suggested that Tr1 cells could induce stronger suppressive effects *in vivo*, compared to FoxP3⁺ Treg, due to their actions in both antigen-specific and antigen non-specific manner (33, 34). These results could also explain a higher *in vitro* suppressive capacity of GM-CSF/IL-6/PGE2 M-MDSC, as compared to GM-CSF/IL-6 M-MDSC. In addition, our preliminary experiments

in a rat model of experimental autoimmune encephalomyelitis (EAE) suggested that the application of both GM-CSF/IL-6- and GM-CSF/IL-6/PGE2-induced bone-marrow cells can suppress the development of EAE symptoms, and the duration of the disease. However, it remained to be investigated whether similar mechanisms of FoxP3⁺ and Tr-1 induction are involved in the observed *in vivo* effects. Besides, it should be investigated what is the potential of these M-MDSC in allogeneic transplantation models and whether similar mechanisms of immune suppression could be induced. These studies could provide important clues to which tolerogenic mechanisms should be targeted by *in vitro* generated M-MDSC in the development of a specific immunotherapy for autoimmunity and transplantation therapy.

In conclusion, we found that the combination of GM-CSF and IL-6 induce differentiation of monocytes into a heterogeneous population of M-MDSC which induce TGF- β -producing FoxP3⁺ Treg. PGE2 potentiated the suppressive phenotype and functions of GM-CSF/IL-6 induced M-MDSC and augmented their potential to induce IL-10-producing T cells, including Tr-1 cells. The mechanisms involved in these processes include, but are not limited to, IDO-1, ILT3, and ILT4, which represent potentially promising checkpoint inhibitors in cancer immunotherapy. The described model of human M-MDSC seems a good platform to study novel therapeutic strategies focused on M-MDSC-related effects in tumors and autoimmune diseases.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

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AUTHOR CONTRIBUTIONS

MČ, DV, and ST designed the study. ST, BJ, MV, MB, MM, and DV performed the experiments. ST and DV analyzed and interpreted the data. ST wrote the manuscript. MČ and DV supplied the materials and infrastructure. All authors revised critically and approved the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Role of Aryl Hydrocarbon Receptor (AhR) in the Regulation of Immunity and Immunopathology During *Trypanosoma cruzi* Infection

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Resistance to *Trypanosoma cruzi* infection is dependent on a rapid induction of Th1-type and CD8+ T cell responses that should be promptly balanced to prevent immunopathology. *T. cruzi*-infected B6 mice are able to control parasite replication but show a limited expansion of Foxp3+regulatory T (Treg) cells that results in the accumulation of effector immune cells and the development of acute liver pathology. AhR is a ligand-activated transcription factor that promotes Treg cell development and suppression of pro-inflammatory cytokine production in dendritic cells, altering the course of adaptive immune response and the development of immunopathology. Here, we used different AhR-dependent activation strategies aiming to improve the Treg response, and B6 congenic mice carrying a mutant AhR variant with low affinity for its ligands (AhRd) to evaluate the role of AhR activation by natural ligands during experimental *T. cruzi* infection. The outcome of TCDD or 3-HK plus ITE treatments indicated that strong or weak AhR activation before or during *T. cruzi* infection was effective to regulate inflammation improving the Treg cell response and regularizing the ratio between CD4+ CD25- to Treg cells. However, AhR activation shifted the host-parasite balance to the parasite replication. Weak AhR activation resulted in Treg promotion while strong activation differentially modulated the susceptibility and resistance of cell death in activated T and Treg cells and the increase in TGF- β -producing Treg cells. Of note, *T. cruzi*-infected AhRd mice showed low levels of Treg cells associated with strong Th1-type response, low parasite burden and absence of liver pathology. These mice developed a Treg- and Tr1-independent mechanism of Th1 constriction showing increased levels of systemic IL-10 and IL-10-secreting CD4+ splenocytes. In addition, AhR activation induced by exogenous ligands had negative effects on the development of memory CD8+ T cell subsets while the lack/very weak activation in AhRd mice showed opposite results, suggesting that AhR ligation restricts the differentiation of memory CD8+T cell subsets.

We propose a model in which a threshold of AhR activation exists and may explain how activation or inhibition of AhR-derived signals by infection/inflammation-induced ligands, therapeutic interventions or exposure to pollutants can modulate infections/diseases outcomes or vaccination efficacy.

Keywords: Chagas disease, TCDD, ITE, regulatory T cells, AhR

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that plays important roles in several biological processes, including development, detoxification and the immune response (1). AhR is expressed in immune system cells such as macrophages (Mo), dendritic cells (CDs), NK cells, B lymphocytes and certain subtypes of T cells as Th17 and Treg cells. When inactive, AhR is located in the cytoplasm as part of a protein complex which translocate to the nucleus after ligand activation (1). Genomic AhR signaling pathways involve the interaction of AhR with other transcription factors to directly regulate the transcription of target genes through AhR-responsive elements (AhREs) (1, 2). In immune cells, AhR can be activated by several physiological ligands that include many derivatives of tryptophan (Trp) such as L-kynurenine (Kyn) and 3-hydroxy-kynurenine (3-HK) generated by indoleamine 2,3 dioxygenase (IDO) activity (3, 4) and other endogenous ligands as the Trp photoproducts 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1*H*-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE) (5). Also exogenous xenobiotics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) triggers AhR-mediated signaling (5). AhR activation can regulate innate and adaptive immune responses via regulation of multiple AhREs present in the promoter regions of several genes, such as those implicated in the regulation of NF- κ B (6) and the development of regulatory T cells (Treg) (Foxp3, TGF- β and IL-10) and Th1 (IL-12), and Th17 (IL-21 and IL-23) cells (1, 2, 7). In particular, AhR has been shown to regulate the inflammatory response at different levels, for example imprinting tolerogenic properties to DCs and promoting the development of regulatory T cells (Tr1, and CD4+CD25+Foxp3+ Treg cells) (7–9). In that way, AhR plays an important role in the regulation of autoimmune inflammatory diseases as rheumatoid arthritis (10), psoriasis (11) and experimental autoimmune encephalomyelitis (EAE) (12–14).

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, represents a major public health problem in the Americas from Mexico to southern Argentina. Each year there are approximately 12,000 deaths which are attributable to Chagas disease, typically due to severe chronic Chagas disease cardiomyopathy (CCC), which affects approximately 30% of infected individuals and occurs decades after acute infection (15). The remaining patients develop digestive disorders (5–10%) or persist asymptomatic (Asy) and free from cardiac or digestive disorders (60–70%). Although the mechanisms underlying the differential progression to CCC are still not fully understood, it is clear that CCC patients display a more intense inflammation

than Asy patients, who appear to have a more regulated immune response. The former show increased circulating levels of pro inflammatory cytokines (16), together with increased numbers of IFN- γ producing T cells and reduced numbers of IL-10-producing CD4+ T cells (17–19) and Treg cells (20–22) in peripheral blood compared with patients having the Asy form of Chagas disease. In addition, a Th1-rich inflammatory infiltrate predominantly secreting IFN- γ and TNF is found in the heart tissue of CCC patients (17, 23–27). Taken together, these results suggest that, in addition to parasite-mediated damage, the unbalanced T cell response plays a role in CCC development.

Host resistance during experimental *T. cruzi* infection is dependent on a rapid induction of a Th1 inflammatory response and CD8+ T cell mediated immunity. Th1 mediators are essential for pathogen control during the acute phase of the infection while CD8+ T cells are important throughout all the stages of the infection, although not sufficient for complete parasite elimination (28). In addition, diverse regulatory mechanisms should promptly balance the inflammatory response to prevent the immunopathology. Studies in which C57BL/6 (B6) mice are infected with the Tulahuén strain of *T. cruzi* revealed an acute disease accompanied by splenomegaly and liver damage (29). Likewise to that observed in CCC patients, B6 mice have great difficulty in controlling the inflammatory response leading to the premature death of these animals by liver failure. In this experimental setting, the increased morbidity was associated to high levels of TNF and low levels of IL-10 (29). Interestingly, it was demonstrated that B6 mice do not expand the population of Treg cells in parallel with the large expansion undergone by the T cell compartment, resulting in an increased ratio of T effector/Treg cells (30, 31). These results suggest that, as observed during human Chagas disease, the fatal outcome in B6 mice may be linked to an unbalanced Th1 response by poor Treg cell induction. In this way, the severity of *T. cruzi*-induced immunopathology may be ameliorated by regulating the balance of CD4+ effector and Treg cells, and procedures that change this balance could represent a promising approach for therapy.

We have previously demonstrated that IDO activity is up-regulated after *T. cruzi* infection in mice, being the Trp catabolite 3-HK toxic for *T. cruzi* amastigotes and trypomastigotes (Tps) (32, 33). We assayed the treatment of *T. cruzi* infected BALB/c mice with 3-HK and observed that, in addition to control the parasite load, the treatment was able to modulate the immune response at the acute phase of the infection impairing the Th1- and Th2-type specific immune response, inducing TGF- β -secreting cells, promoting the emergence of Treg cells and markedly reducing the incidence and the severity of the

inflammatory pathology (32, 34). Because there is a well-established connection between inflammation, AhR expression and IDO induction, and considering that Trp-derived IDO-induced catabolites Kyn and 3-HK AhR ligands may signal AhR to promote Treg cell induction (3, 4, 35–37), here, we evaluated the role of physiological AhR signaling and the effect of the treatment with different AhR agonists on the regulation of the immune response and the outcome of *T. cruzi* infection in B6 mice.

MATERIALS AND METHODS

Mice and Parasites

All animal experiments were approved by and conducted in accordance with guidelines of the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba (Approval Number HCD 743/18). C57BL/6 (B6) was obtained from School of Veterinary, La Plata National University (La Plata, Argentina) and B6.D2N-Ahrd/J (AhRd), kindly provided by Dr. Francisco Quintana (Ann Romney Center, Boston, USA). All animals were housed in the Animal Facility of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba (OLAW Assurance number A5802-01). The Tulahuen strain of *T. cruzi* was used, which was maintained by weekly intraperitoneal (ip) inoculations in mice.

Treatments and Parasite Load

Groups of B6 mice (6–8 weeks old) maintained under standard conditions were ip injected with 1 µg of TCDD (AccuStandard, New Haven, CT, USA) or vehicle (DMSO, Sigma-Aldrich) 24 h before to be infected with 50,000 bloodstream trypomastigotes (Tps) of *T. cruzi*. To evaluate the effect of weak AhR activation on acute and chronic phase of the infection, B6 mice were infected with 50,000 *T. cruzi* Tps, and 5 days post-infection (pi), were ip injected with 3-HK (1 mg/kg/day, Sigma Aldrich) for 5 consecutive days (5–10 post-infection) (32, 34) and with ITE (200 µg Tocris Bioscience, R&D Systems) on days 7, 9, and 11 pi. 3-HK and ITE were resuspended in 0.1 M PBS and DMSO, respectively, with these vehicles also being employed as control. AhRd mice and its B6 counterpart were ip infected 50,000 Tps of Tulahuen strain. The levels of parasitemia were monitored in blood collected at different times pi as previously described (32). For determination of tissue parasitism, genomic DNA was purified from liver, heart and skeletal muscle using TRIzol reagent (Life Technologies) and following manufacturer's instructions. Satellite DNA from *T. cruzi* (GenBank AY520036) was quantified by real time PCR using specific Custom Taqman Gene Expression Assay (Applied Biosystem) using the primer and probe sequences described by Piron et al (38). A sample containing 200 ng of genomic DNA was amplified and considered positive for *T. cruzi* when the threshold cycle (CT) for the *T. cruzi* target when $CT < 45$. Abundance of satellite DNA from *T. cruzi* was normalized to GAPDH abundance (Taqman Rodent GAPDH Control Reagent, Applied Biosystem) and expressed as arbitrary units.

Transaminase Activity

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using commercial kits (Wiener Lab) following manufacturer instruction.

Cell Preparations and Culture

Spleen was obtained and homogenized through a tissue strainer. Erythrocytes in cell suspensions were lysed for 5 min in Tris-ammonium chloride buffer (Sigma Aldrich), washed and resuspended in complete medium, containing RPMI 1640 (Gibco,) supplemented with 2 mM GlutaMAX (Gibco, ThermoFisher), 10 µg/ul gentamicin (Richet S.A, CABA, Argentina) and 10% FBS (NATOCOR, Cordoba, Argentina). Viable cell numbers were determined by trypan blue exclusion using a Neubauer counting chamber. For cytokine determinations, spleen mononuclear cells (SMC) were cultured with or without *T. cruzi* lysate (1 mg/ml) at 2×10^6 cells/ml in complete RPMI medium in 24-well plates (Costar) for 72 h. Supernatants were collected and the secreted cytokines measured.

Cytokine Quantification

Cytokines were measured in sera and cell culture supernatants by capture ELISA using antibodies and protocols suggested by the manufacturer (eBiosciences, ThermoFisher). Cytokine concentration in serum samples was expressed as pg/ml, while the cytokine levels in culture supernatants were represented as Index obtained by dividing the cytokine concentration in supernatant of *T. cruzi*-stimulated cultures/cytokines concentration in supernatants of non-stimulated cultures (medium).

Flow Cytometry

Cell suspensions were washed in ice-cold FACS buffer (PBS-2% FBS) and incubated with fluorochrome labeled-Abs for 20 min at 4°C. Different combinations of the following Abs were used: anti-CD25-PeCy7 (eBiosciences, ThermoFisher), anti-CD25-APC (eBiosciences, ThermoFisher), anti-CD4-PerCp5.5 (eBiosciences, ThermoFisher), anti-CD44-PECy7 (eBiosciences, ThermoFisher), anti-CD62L-APCCy7 (BD Bioscience), anti CD8-FITC (BD Bioscience), anti-FR4-FITC (eBiosciences, ThermoFisher), anti-LAP-PeCy7 (eBiosciences, ThermoFisher) and an H-2K(b) *T. cruzi* trans-sialidase amino acids 569-576 (ANYKFTLV) (TSKB20) APC-Labeled Tetramer (NIH Tetramer Core Facility), which was used for staining 15 min before the addition of the rest of the surface markers. Intracellular cytokines were detected after stimulating cells during 4 h with 50 nM PMA (Sigma Aldrich) and 0.5 µg/ml ionomycin (Invitrogen, ThermoFisher), or 10 µg/ml *T. cruzi* lysate in the presence of GolgiStop (BD Biosciences). Cells were surface-stained, fixed, and permeabilized with BD Cytofix/Cytoperm and Perm/Wash (BD Biosciences) according manufacturer's instruction and intracellular stained with anti-IL-10-APC (eBiosciences, ThermoFisher), anti-IL-10-PE (eBiosciences, ThermoFisher), anti-IL-17-PE (eBiosciences, ThermoFisher), anti-IFN-γ-APC (eBiosciences, ThermoFisher), and anti-Foxp3-PE (eBiosciences, ThermoFisher). For Annexin

V and 7-AAD staining, the Apoptosis detection kit I (BD Pharmingen) was used. Then, were washed and acquired in FACSCanto II (BD Biosciences). For LAP staining the cells were activated with PMA and ionomycin during 4 h in the absence of GolgiStop.

Statistical Analysis

Data distribution was analyzed with Shapiro-Wilk test. Normally distributed data were presented as means \pm SD. Differences between the mean values were assessed using Student's *t*-test. Differences on survival were analyzed applying Gehan-Breslow-Wilcoxon test. Results were considered significantly different when $p < 0.05$. Statistic was performed using GraphPad Prism7 software.

RESULTS

Activation of AhR With TCDD Impairs Host Resistance to *T. cruzi* Experimental Infection

Evidence from studies to control autoimmunity and graft-vs.-host disease, even virus induced inflammatory lesions indicate that the regulation of the immune response to avoid immunopathology could be achieved by the administration of the stable agonist of AhR, TCDD (7, 39–41). TCDD is a non-degradable high affinity ligand for AhR and most studies using this ligand have reported inhibitory effects on inflammatory reactions (40, 41).

To evaluate the role of AhR engagement by TCDD on the outcome of *T. cruzi* infection, a single intraperitoneal (ip) administration of TCDD was given to mice 1 day prior to infection and the effects on parasite load, host survival and protective immune response were compared with vehicle-treated controls. TCDD treatment impaired resistance to infection as treated mice showed increased parasitemia and serum levels of ALT and AST that resulted in decreased survival compared to control mice (Figures 1 A–C). In addition, the treatment with TCDD did not affect survival or adversely affect hepatic enzymes levels in uninfected mice (Figure 1C and data not shown), suggesting that the dose of TCDD used did not present toxic effects per se.

To measure the consequences of TCDD treatment on the specific immune response, mice were sacrificed during the acute phase of infection (day 10 post-infection). At the time of sacrifice TCDD-treated mice showed spleens markedly decreased in size and cell numbers compared with non-treated infected mice, which showed the characteristic infection-induced splenomegaly (Figure 1D).

To determine the effect of TCDD treatment on Th cell polarization, SMC from treated and control mice were stimulated *in vitro* for 4 h with PMA and ionomycin to enumerate cells that produced either IFN- γ or IL-17. In addition, the frequencies of CD8+ T cells specific for the immunodominant epitope TSKB20 (ANYKFTLV) (42) and Foxp3+ CD25+ CD4+ Treg cells were also evaluated. Compared with non-infected controls, *T. cruzi* infected mice showed significantly increased

percentages and absolute numbers of CD4+ cells producing IFN- γ and IL-17 while the TCDD treatment resulted in marked decrease in the percentage and absolute number of these effector cell subsets (Figure 1E). In addition, TCDD-treated mice showed a significant reduction of specific CD8+ TSKB20/Kb+ cells (Figure 1F).

To investigate whether TCDD treatment differentially affected cell survival of infection-activated *T. cruzi*-specific T cells, we compared the level of cell death in the CD8+ TSKB20/Kb+ population from TCDD-treated and control mice by assaying Annexin V binding and 7-AAD permeability. *T. cruzi*-specific CD8+ T cells from TCDD-treated mice showed lower frequency of live cells (Annexin V-, 7-AAD-) and higher frequency of dead cells (Annexin V+, 7-AAD+/-) than those from control mice (Figure 1G), suggesting a direct toxic effect of TCDD on parasite-activated cells.

When the effect of TCDD treatment on CD4+ CD25+ Foxp3+ Treg population was evaluated, it was observed that although the numbers of splenic Treg cells were not significantly different between both experimental groups, the percentage of Treg cells and the number of Treg cells producing the immunoregulatory cytokine TGF- β were significantly higher in TCDD-treated compared to control mice, while no differences were observed for IL-10-secreting Treg cells (Figures 2A,B). In addition, TCDD treatment decreased the ratio between total numbers of CD4+CD25- (conventional CD4+ T cells), Th1 cells (IFN- γ -producers) and Th17 cells (IL-17-producers) and total numbers of Treg cells (Figure 2C). Of note, TCDD treatment normalized the ratio of total and effector cytokine-producing conventional CD4+ T cells and Treg cells to values similar to those observed in uninfected mice (Figure 2C). These findings suggested that Treg cells are much less sensitive to TCDD-induced death than conventional T cells. To evaluate this hypothesis, we analyzed comparatively the level of cell death in the CD4+ FR4^{hi} cell population [phenotype compatible with Treg cells (43)] from treated and control mice. Figure 2D shows that the level of cell death in the Treg cell population from TCDD-treated and control mice was similar.

Given the results described above, TCDD might be effective to induce regulation of the inflammatory response in *T. cruzi* infected B6 mice but would not be recommended for usage during the induction of the immune response triggered by the initial parasite replication. With this in mind, we performed additional experiments in which TCDD treatment was given on day 7 pi, a time point in which the innate and effector T cell response is ongoing and the inflammation-induced damage may begin to become apparent. Although TCDD administration on day 7 pi prolonged the survival of TCDD-treated mice, it still increased mice mortality in comparison to controls (not shown).

Combined AhR Agonists Promote Treg Cell Differentiation and Have Detrimental Effects on Parasite-Specific Immunity

As TCDD, the endogenous AhR ligand ITE is able to induce Treg cells (12, 44, 45), but is nontoxic and could, therefore, be useful to modulate the inflammatory response without a

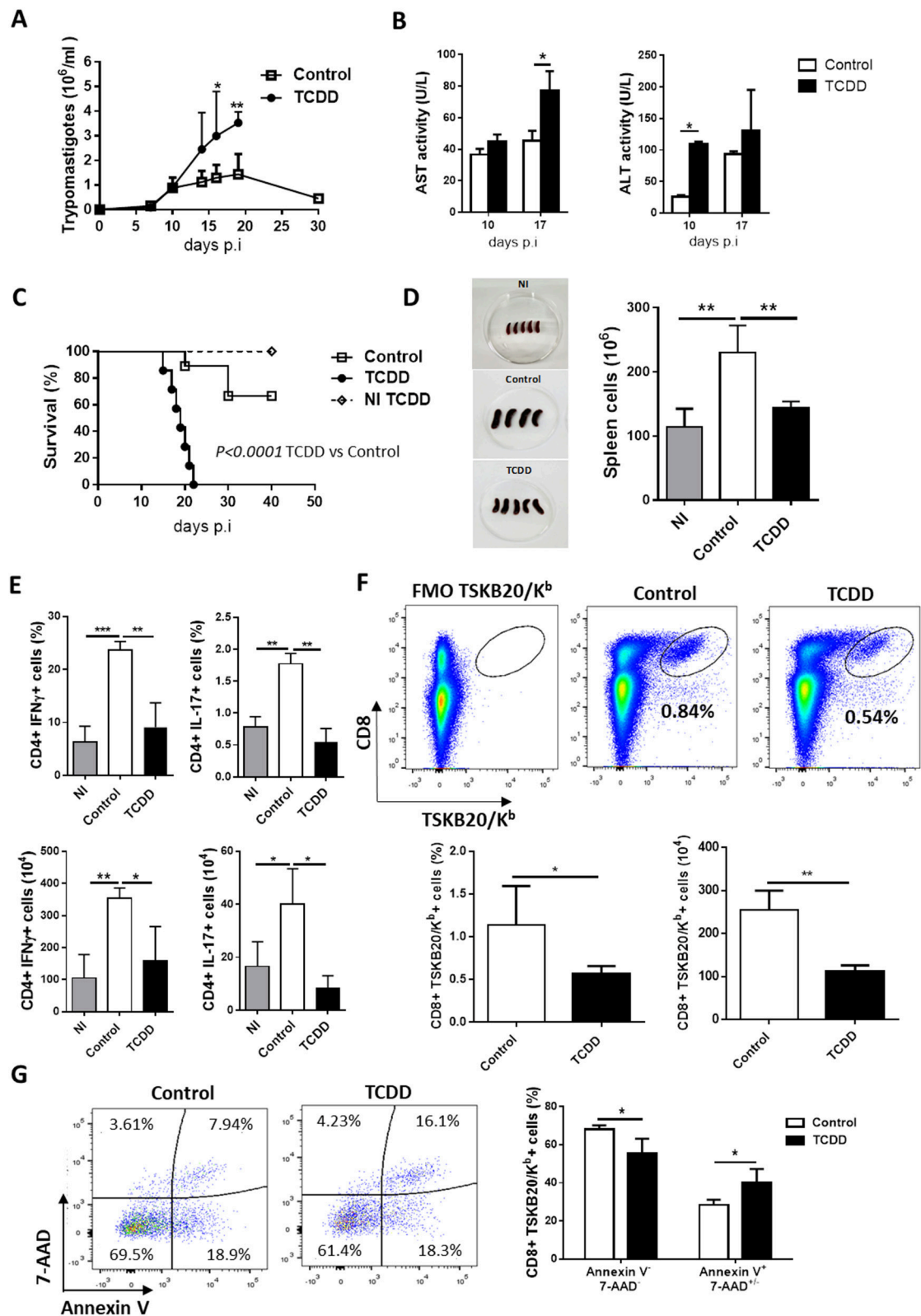


FIGURE 1 | TCDD treatment suppresses T cell expansion, cytokine production, and impairs resistance to *T. cruzi* infection. C57BL/6J mice were ip injected with TCDD or DMSO (Control) 24 h before being infected with 50,000 Tps of *T. cruzi*. **(A)** Parasitemia (Tps/ml blood). Data are shown as mean \pm SD of 7–9 mice per group. (Continued)

FIGURE 1 | (B) ALT and AST activity determined in plasma at day 10 and 17 pi. Data are shown as mean \pm SD, $n = 3$ mice per group. **(C)** Survival rate of non-infected TCDD treated (NI TCDD), infected TCDD-treated and infected vehicle-treated (control) mice. P -values were calculated with the Gehan-Breslow-Wilcoxon test. **(D)** Images of spleens from NI and infected TCDD-treated and control mice in one representative experiment. The bars represent the number of total spleen cells from NI and infected TCDD-treated and control mice \pm SD. **(E)** Splenocytes taken from NI ($n = 4$) and *T. cruzi*-infected mice treated (TCDD, $n = 5$) or not (Control, $n = 4$) with TCDD at day 10 pi were *in vitro* stimulated with PMA/ionomycin in the presence of Golgi stop. The bars represent the percentage and absolute number of CD4+ splenocytes producing IFN- γ or IL-17 cytokines. **(F)** Representative dot plots (top) and bars showing the percentage and absolute number (bottom) of splenocytes CD8+ TSKB20/Kb+ from control and TCDD-treated mice at day 10 pi. **(G)** Representative dot plots (left) and bars (right) showing the percentage of Annexin V- 7-AAD- and Annexin V+ 7-AAD+/- splenic cells within the CD8+ TSKB20/Kb+ population at day 10 pi. Bars represent the mean values \pm SD. Data in A-C and in D-G are representative of three and two independent experiments, respectively. P -values were calculated with two-tailed Student's t -test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

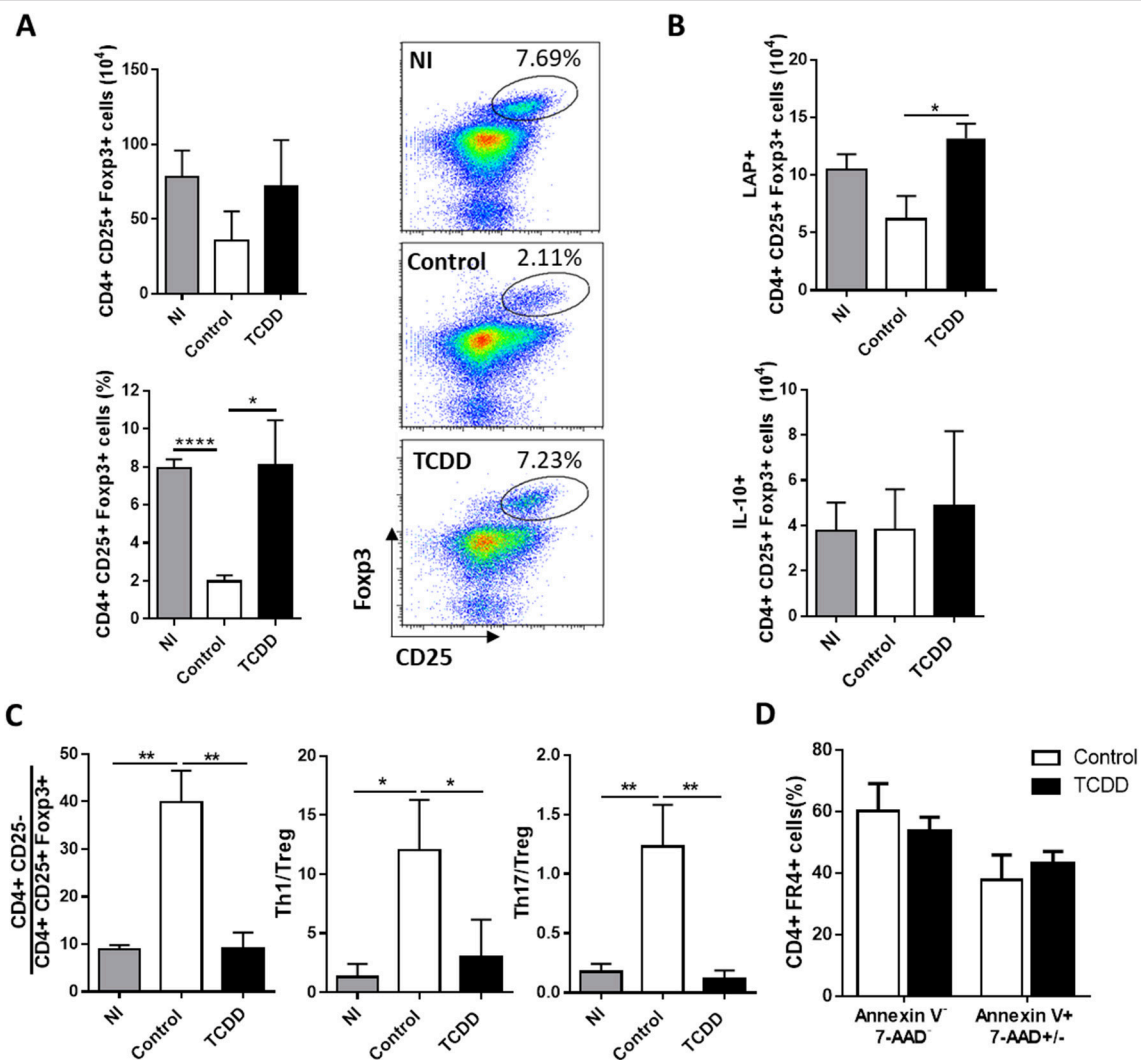


FIGURE 2 | TCDD treatment shifted the balance between inflammatory and CD4+ CD25+ Foxp3+ Treg cells. **(A)** Representative dot plots (right) and bars (left) showing the percentage and absolute number of splenocytes from NI, control and TCDD-treated mice expressing CD25+ Foxp3+ within CD4+ population at day 10 pi. Bars are shown as mean \pm SD, $n = 3$ mice per group. **(B)** Absolute number of CD4+ CD25+ Foxp3+ IL-10+ and CD4+ CD25+ Foxp3+ LAP+ splenocytes after PMA/ionomycin stimulation at day 10 pi. Bars are shown as mean \pm SD, $n = 3$ mice per group. **(C)** Ratio of splenic effector T cells (CD4+Foxp3-IFN γ -) and Th17 cells (CD4+ IL-17+) to Treg cells (CD4+CD25+Foxp3+). Bars represent mean values \pm SD with $n = 3$ mice per group. **(D)** Percentage of Annexin V- 7-AAD- and Annexin V+ 7-AAD+/- splenic cells within the CD4+ FR4+ population. Data in A-C and in D are representative of three and two independent experiments, respectively. P -values were calculated with two-tailed Student's t -test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

cytotoxic effect on parasite-specific T cells. Furthermore, it has been demonstrated that 3-HK is a weak AhR agonist (3) and also it is active against the amastigote and trypomastigote forms of

T. cruzi (32, 34). Considering the background mentioned above, we developed a treatment scheme to simultaneously activate AhR and inhibit *T. cruzi* replication that consisted in the daily

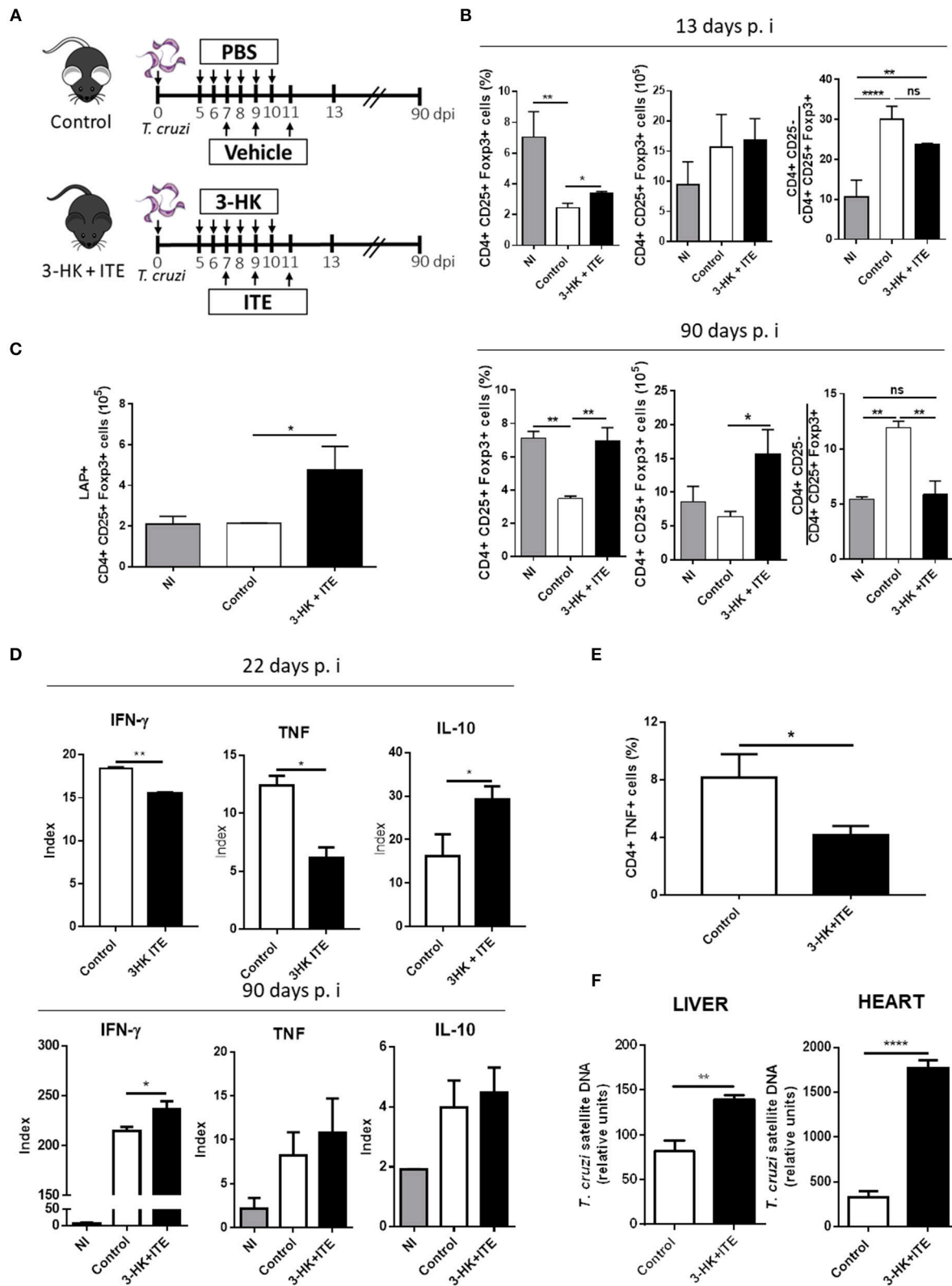


FIGURE 3 | 3-HK plus ITE treatment promotes the differentiation of Treg cells with detrimental effects on protective immune response. **(A)** *T. cruzi*-infected B6 mice were treated with 3-HK plus ITE with vehicle-treated mice used as control (PBS+DMSO). **(B)** Percentage, absolute number of splenic CD25+ Foxp3+ cells within
(Continued)

FIGURE 3 | CD4+ population and ratio of effector T cells (CD4+CD25-) to Treg cells (CD4+CD25+Foxp3+) at days 13 and 90 pi. Bars are shown as mean \pm SD with $n = 3$ mice per group. **(C)** Absolute number of splenic CD4+ CD25+ Foxp3+ LAP+ cells at 90 days pi after PMA/Ionomycin stimulation. Bars are shown as mean \pm SD with $n = 3$ mice per group. **(D)** Cytokine levels assayed in supernatants of splenocytes taken at 22- and 90-days pi and cultured with medium alone or total *T. cruzi* lysate for 72 h. The results are represented as an Index which is the ratio between cytokine concentration in supernatant of *T. cruzi*-stimulated cultures and cytokine concentration in the corresponding not stimulated culture. **(E)** Percentage of splenic cells CD4+ TNF+ cells at 22 days pi after 4 h of PMA/Ionomycin stimulation. **(F)** Relative amount of *T. cruzi* satellite DNA in heart and liver at day 90 pi. GAPDH was used as endogenous control for normalization. One experiment representative of two independent is shown. *P*-values were calculated with two-tailed Student's *t*-test. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001.

administration of 3-HK from days 5 to 10 pi together with ITE injection at days 7, 9, and 11 pi, as depicted in **Figure 3A**. The cellular immune response and the levels of parasite load in the target tissues were then investigated. Acutely infected mice treated with 3-HK plus ITE did not show differences in parasitemia and survival compared with control mice (**Figure S1** in Supplementary Material). However, *T. cruzi*-infected B6 mice treated with ITE plus 3-HK had a significant increase in the % (day 13 and 90 pi) and number (day 90 pi) of splenic Treg cells (**Figure 3B**), and in the number of Treg cells producing TGF- β (day 90 pi) (**Figure 3C**) when compared with controls. Moreover, 3-HK plus ITE treatment reversed the characteristic unbalanced ratio between conventional CD4+ T cells to Treg cells to values like those observed in uninfected mice (**Figure 3B**).

To evaluate the cellular immune response developed in 3-HK plus ITE-treated mice, the Ag-specific cytokine production was analyzed in SMC taken at different times pi after *ex vivo* restimulation with parasite lysate. *T. cruzi*-stimulated SMC taken from mice treated with 3-HK plus ITE at the acute phase of the infection (day 22 pi) secreted significantly lower amounts of TNF and IFN- γ and higher amounts of IL-10 than those from untreated mice (**Figure 3D**). In agreement, treated mice showed a significant lower percentage of TNF-producing spleen CD4+ T cells than control mice (**Figure 3E**). In addition, only a small increase in the production of IFN- γ by *T. cruzi*-stimulated SMC was observed in treated vs. control mice during the chronic phase of the infection (90 days pi).

Finally, to determine whether the 3-HK+ITE-induced regulation of the specific response compromised the protective immunity to *T. cruzi*, the parasite load was determined in liver and heart at 90 days pi. Compared with untreated, the mice that had been treated with 3-HK plus ITE showed higher parasite load in both liver and heart (**Figure 3F**). This increased parasite burden could underlie the higher production of IFN- γ by SMC from chronically infected 3-HK plus ITE-treated mice.

Together, our results suggest that 3HK plus ITE might be a novel therapeutic treatment able to control the inflammatory response. However, considering our results and a recent report (31) the expansion of Treg cells during the acute phase of *T. cruzi* infection with *T. cruzi* may also prevent the emergence of protective anti-parasite immunity and critically influence host resistant.

AhRd Mice Develop a Proper Inflammatory and Anti-inflammatory Response Able to Restrict Parasite Replication

To investigate further on the role of AhR activation during the *T. cruzi* infection and considering that endogenous AhR ligands

are generated during this infectious process, we took advantage of AhRd mice, a congenic B6 mice expressing a mutant AhR protein with reduced affinity for its ligands (46). AhRd and WT mice were infected and parasitemia, tissue parasite load, survival and the splenic T cell populations were studied during the acute and the chronic phase of the infection. Infected AhRd mice presented significantly lower peak of parasitemia (**Figure 4A**), had prolonged survival compared to WT mice (**Figure 4B**) and displayed significantly lower levels of the hepatic transaminase ALT in sera (**Figure 4C**). AhRd mice also showed at day 10 pi an expansion of CD4+ IFN- γ producing cells compared with WT mice and (**Figure 4D**), somehow contrasting previously reported association of high Th1-type response and liver tissue damage in B6-infected mice (29). In addition, we found a strong increase in the number of splenic as well as IFN- γ -producing cells between days 10 and 17 pi in WT, but not in AhRd, mice (**Figure 4D**). As expected, and consistent with the low strength of AhRd signaling (3, 7), the number of splenic Treg cells was significantly lower in AhRd compared to WT mice (**Figure 4D**). Also, B6- and AhRd-infected mice showed similar levels of CD4+ IL-17-producing cells (not shown). Interestingly, whereas the levels of pro-inflammatory cytokines in sera were similar in both mice groups (data not shown), the levels of IL-10 in sera and the % of IL-10-producing cells were significantly higher in AhRd vs. WT mice during the acute phase of the infection (**Figures 4E,F**), being a CD4+ cell population the main producer of this cytokine at day 10 pi (**Figure 4F**). In addition, AhRd mice exhibited lower number of parasite-specific IFN- γ -producing splenocytes during the chronic phase of the infection, in concordance with lower parasite load in liver, heart and skeletal muscle compared with WT mice (**Figures 4G,H**). Taken together, these results indicate that AhR signaling is critically involved in the development of robust parasite-specific Th1 responses and immunoregulatory mechanisms during *T. cruzi* infection.

AhR Signaling Restricts the Differentiation of Memory CD8+ T Cell Subsets During *T. cruzi* Infection

The acquisition of memory T cells is defined by the generation and persistence of T cells that can provide long-lasting protection against pathogens. Signals given by DC as TCR engagement, costimulation and cytokines co-participate in the induction of memory T cells. Thus, changes in any of the factors controlling the activation of T cells during the antigen presentation can regulate T effector and memory cell differentiation. Different observations suggest that AhR, directly or through its effects on antigen presenting cells modulates critical events in the

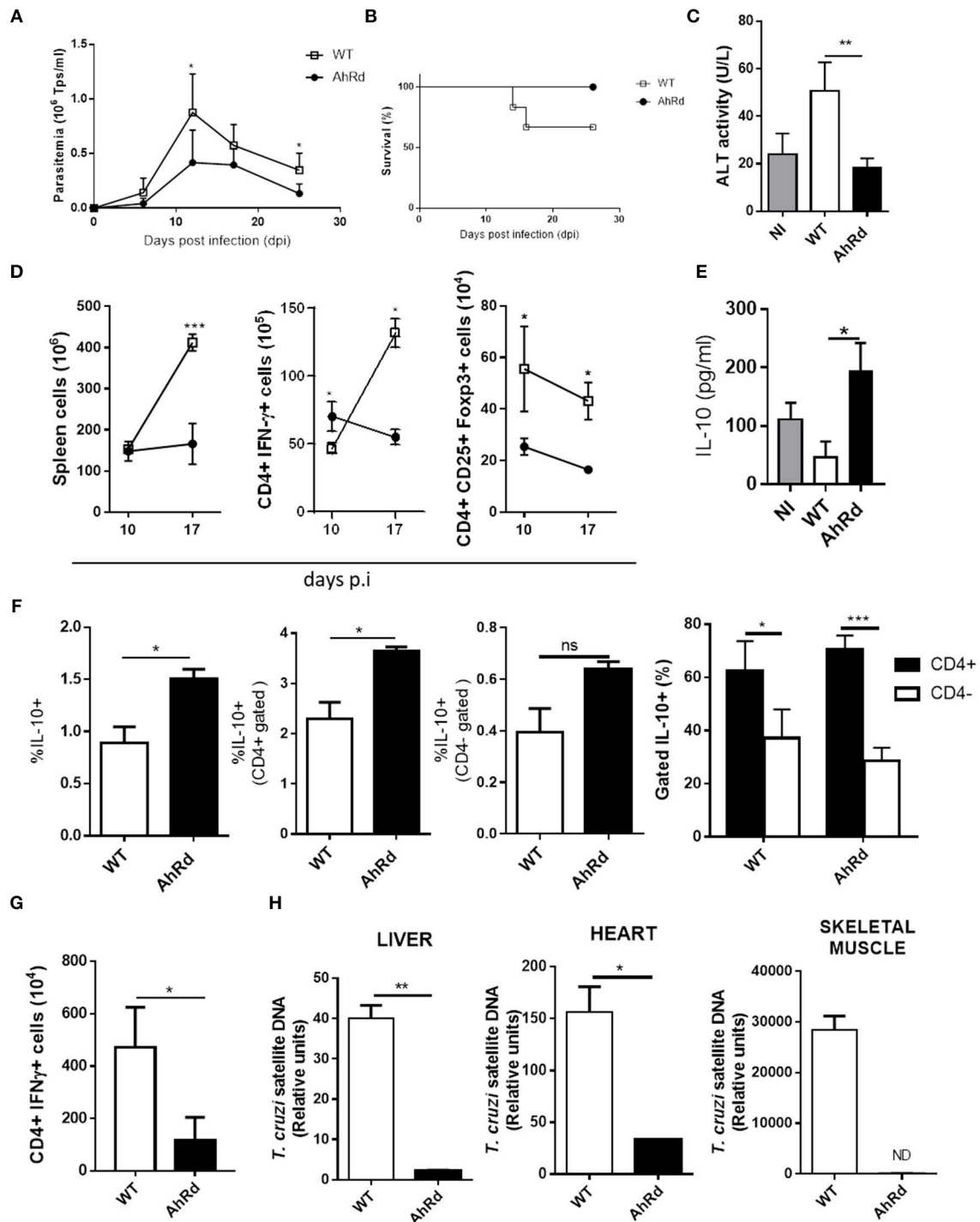


FIGURE 4 | AhRd mice develop a proper inflammatory and anti-inflammatory response able to restrict parasite replication. AhRd and WT mice infected with 50,000 Tps of *T. cruzi* were evaluated for parasitemia, survival and other groups sacrificed at days 10, 17, and 170 pi. **(A)** Parasitemia (Tps/ml blood). Data are shown as mean \pm SD, $n = 5$ mice per group. **(B)** Survival rate of *T. cruzi* infected WT and AhRd mice, $n = 5$ mice per group. **(C)** ALT activity determined in sera at day 17 pi. **(D)** Absolute number of CD4+ IFN γ + and CD4+ CD25+ Foxp3+ cells in spleen from WT- and AhRd-infected mice at day 10 and 17 pi. **(E)** Levels of IL-10 in sera from NI, and infected WT and AhRd mice at day 10 pi. **(F)** Percentage of total, CD4+ and CD4- splenocytes that produce IL-10 after PMA/Ionomycin stimulation at day-17 pi. **(G)** Absolute number of CD4+ IFN γ -producing splenocytes at day 170 pi after *in vitro* stimulation with *T. cruzi* total lysate (10 μ g/ml). **(H)** Relative amount of *T. cruzi* satellite DNA in heart, liver and skeletal muscle from WT and AhRd mice at day 170 pi. GAPDH was used as endogenous control for normalization, $n = 4$ mice per group. One experiment representative of three independent experiments is shown. P values were calculated with two-tailed Student's t -test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

activation of naive T cells that could modify the development of effector and memory T cells (47, 48). To evaluate the role of AhR activation on T cell differentiation of different memory subsets, we evaluated the effect of different strength of AhR ligation on the frequencies of CD8+ total and specific (TSKB20/Kb+) cell subsets defined by CD62L and CD44 expression. **Figure 5A** shows that strong AhR ligation with TCDD significantly decreased the frequency of CD8+ CD44^{hi} CD62L^{lo} cells (effector/effector memory cells, EM) and the number of both EM and CD8+ CD44^{hi} CD62L^{hi} cells (central memory cells, CM) in spleen at day 10 pi. Moreover, the number of splenic *T. cruzi*-specific (TSKB20/Kb+) EM and CM subpopulations showed a significant decrease in treated compared with control mice. These results suggest a negative effect of a strong AhR activation on the development of effector and memory CD8+ T cells precursors. However, the fact that antigen-experienced cells suffer the direct effect of TCDD toxicity (**Figure 1G**), does not allow to conclude clearly on the consequence of AhR ligation on the development of memory precursor cells. Indeed, CD8+ TSKB20/Kb+ cells with EM phenotype from TCDD-treated mice showed higher percentage of Annexin V+ 7-AAD+ cells than those from control mice (**Figure 5B**).

To test the hypothesis that the activation of AhR might alters the *in vivo* distribution and frequency of CD8+ total and parasite-specific T cells subsets, we analyzed the effector phase of memory induction in *T. cruzi* infected B6 mice that were treated with 3-HK plus ITE as depicted in **Figure 3A**. **Figure 5C** shows that weak AhR ligation with 3HK plus ITE during *T. cruzi* infection decreased the percentage of total and specific CD8+ splenic T cells that acquired EM and CM phenotype.

To support these findings, we compared the frequency and number of total and *T. cruzi*-specific CD8+ T cells with EM and CM phenotype in AhRd and WT mice after 17 and 170 days pi. Similar frequency and number of CD8+ T cells specific for the immunodominant epitope TSKB20 were observed for AhRd and WT mice at day 20 pi (not shown). Moreover, and in contrast to that observed when AhR was activated by exogenous ligands (**Figures 5A,C**), the frequency of CD8+ total and *T. cruzi*-specific T cells with EM phenotype was higher in AhRd than WT mice at 17 days pi (**Figure 6A**) and during the chronic phase of the infection (day 170 pi) (not shown). Interestingly, AhRd mice showed higher percentages and absolute number of CD8+ total and *T. cruzi*-specific T cells than WT mice at the chronic phase of the infection (day 170 pi) (**Figure 6B**). Analysis of CD44 and CD127 expression, that distinguish central memory cells (CD44^{hi} CD127^{hi}) from effector memory cells (CD44^{hi} CD127^{lo}), in subpopulations of CD8+TSKB20/Kb+ T cells revealed that the frequency and number of splenic cells with CM phenotype was significantly higher in AhRd than in WT mice during the chronic phase of the infection (**Figure 6C** and not shown). Taken together, these results suggested that during *T. cruzi* infection physiological AhR ligation restricts the differentiation of CD8+ memory T cells impacting in the magnitude of the long-term parasite-specific immune response and the chronic control of tissue parasitism.

DISCUSSION

It has been demonstrated that AhR activation can be induced by diverse ligands in the environment, however, Trp metabolites have emerged as key family of physiological agonistic ligands (49). During *T. cruzi* infection, the up-regulation of the enzyme IDO that catalyzes the Trp degradation along the Kyn pathway has at least two main roles, the control of pathogen growth by producing the catabolite 3-HK and the immune regulation facilitated by the 3-HK-mediated expansion of Treg cells (32, 34). IDO induction depends on AhR expression, and Kyn as well as 3-HK are both AhR ligands that signal for Treg induction (3, 4, 35). Thus, the AhR agonist 3-HK used as a therapy in *T. cruzi*-infected BALB/c mice was able to increase the Treg population and to modify the outcome of the infection (34).

After *T. cruzi* infection, B6 mice develop an important Th1-type response able to control the parasite replication (29). However, and likewise to that observed in CCC patients, *T. cruzi*-infected B6 mice have great difficulty in controlling the inflammatory response, resulting in the premature death by liver failure, being the increased morbidity associated to high levels of TNF and low levels of IL-10 in sera and the incapacity to expand Treg population (29–31). It is known that immune responses can be enhanced or dampened by differential manipulation of Treg cells vs. other naive or activated T cells. In this study, we have assayed different AhR activation-dependent strategies aiming to specifically boost Treg cells in B6 mice infected with *T. cruzi*. The results obtained by TCDD treatment indicated that strong AhR activation during *T. cruzi* infection can normalize the ratio between Th1 and Th17 to Treg cells. Our results showed that the balance of the inflammatory response observed after TCDD treatment was the result of the increased death of activated T cells, the increase in the number of Treg cells producing the immunoregulatory cytokine TGF- β and the resistance of these cells to TCDD-induced death, with no significant changes in the frequency or number of IL-10 producing cells between control and treated mice. It is worth to mention that the direct toxic effect of TCDD on activated T cells has been previously demonstrated (50). In addition, Winans et al. (51) have shown that CD8+ T cells exposed to TCDD exhibit methylation patterns similar to those of exhausted CD8+ T cells suggesting that a strong AhR ligation has an important impact on basal functionality and initial responses to antigenic stimulation, and also explains the decreased memory cell subsets showed in this paper for *T. cruzi*-infected TCDD-treated mice. As far as we know, the resistance to TCDD toxicity by Treg cells has not been described. Taking into account that murine and human Treg cells are more resistant than activated non-Treg CD4+ T cells to apoptosis induced by different pathways such as irradiation, Fas ligation and viral infection (52–55), it should not be surprising that these cells would be also resistant to strong AhR activation. Thus, strong AhR activation with TCDD was effective to regulate inflammation in *T. cruzi* infected B6 mice, but the TCDD-induced immunosuppression contributed to parasite replication, and the treatment resulted in increased parasitemia and death of the treated mice.

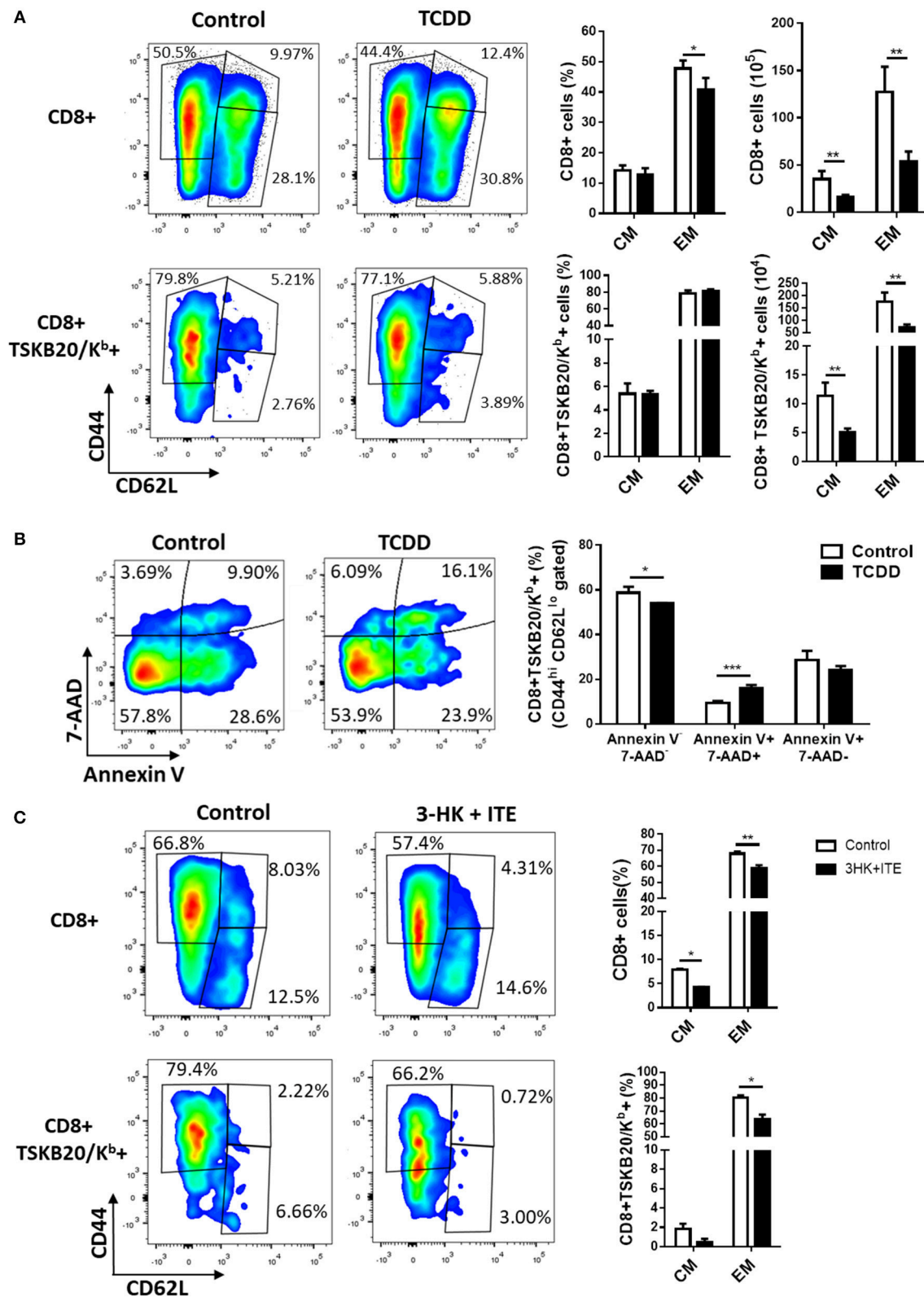


FIGURE 5 | AhR ligation regulates the development of memory CD8+ T subsets during *T. cruzi* infection. Splenocytes from TCDD- and 3HK + ITE-treated mice and its respective controls were isolated at day 10- and 22-days pi to evaluate total CD8 and TSKB20/K^b-specific memory subpopulations by flow cytometry.

(Continued)

FIGURE 5 | (A) Representative plots, percentage, and absolute number of splenocytes from control and TCDD-treated mice at day 10 pi showing effector/memory (EM, CD44+CD62L-) and central memory (CM, CD44+CD62L+) phenotype on CD8+ and CD8+ TSKB20/K^b+ cell populations gated as described in **Figure S2A** in Supplementary Material. Bars represent the mean values \pm SD. Control, $n = 5$ and TCDD, $n = 4$. **(B)** Representative plots and bars showing the percentage of splenocytes expressing Annexin V- 7-AAD-, Annexin V+ 7AAD- and Annexin V+ 7AAD+ cells within the CD8+ TSKB20/K^b+ EM cell population of Control and TCDD treated mice at day 10 pi. Bars represent the mean values \pm SD. Control, $n = 5$ and TCDD, $n = 4$. **(C)** Representative plots and bars showing the percentage of splenocytes from control and 3-HK plus ITE-treated mice with EM and CM phenotype within CD8+ and CD8+ TSKB20/K^b+ cell populations (gated as indicated in **Figure S2B** of Supplementary Material) at day 22 pi. Bars represent the mean values \pm SD. Control, $n = 3$ and 3-HK+ITE, $n = 3$. One experiment representative of three independent experiments is shown. *P*-values were calculated with two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The other strategy used to induce Treg cells in B6 mice was to treat the mice with two weak AhR ligands, ITE and 3-HK, to simultaneously activate AhR and concomitantly inhibit parasite replication (32–34). Once more, although this treatment was able to induce Treg cells and improve the unbalanced ratio between CD4+ CD25- to Treg cells during the chronic phase of the infection, the fact that 3-HK is only partially efficient in controlling the parasitemia and unable to eradicate the parasite (32), shifted the host-parasite balance to the parasite replication. Indeed, although 3-HK plus ITE-treated and control mice did not have significant differences in parasitemia and survival during the acute phase of the infection, treated mice showed higher parasite load in both liver and heart during the chronic phase. Considering that TNF and IFN- γ are both critical cytokines to mediate protection while IL-10 mediates susceptibility in *T. cruzi* infection (56), the increase in parasite load was in line with the lower IFN- γ and TNF and higher IL-10 production by splenocytes from 3-HK plus ITE treated mice during the acute phase of the infection.

Thus, the results obtained by using two different experimental strategies to promote Treg development by AhR activation demonstrated that the increase of the number of Treg did not ameliorate the immunopathology issue, since limited Treg response during *T. cruzi* infection in B6 mice is critically required to allow the development of protective parasite specific T cell immunity, as was recently reported by Araujo et al. (31). Decrease host resistance to a variety of infectious agents has been reported when TCDD exposure occurs prior to infection, however, whether the AhR ligation contributes to improve or worsen host resistant depends on the pathogen (48). Thus, in contrast to what is observed for *T. cruzi* infection, AhR activation by TCDD in *Leishmania major* infection reduces the parasite burden (57, 58).

It has also been reported that AhR signaling under Tr1-skewing conditions (IL-27 or TGF- β plus IL-27) promotes the differentiation of Tr1 cells (CD4+ IL-10+) (59). However, neither TCDD nor 3-HK plus ITE treatments were able to significantly increase Tr1 population [CD4+, Foxp3-, IL-10+ Lag-3+ cells (60)] in *T. cruzi*-infected B6 mice (data not shown), in spite of both treatments up-regulated TGF- β production and *T. cruzi* infection induces IL-27 production in B6 mice (30).

By using AhR-/- (KO) mice, Barroso et al. have examined the role of AhR in the immune modulation and development of myocarditis during *T. cruzi* infection (61), and some of the findings reported in their work correlate with our findings in AhRd mice. It has been reported that besides being refractory to most of the TCDD toxic effects, AhR KO mice exhibit multiple physiological abnormalities that are independent of xenobiotic

exposure (62). The fact that AhR KO mice present an abnormal development of the immune system and the liver (63), should be an important constraint for evaluate the role of this receptor in modulating immune response and liver pathology in a model of infection in which the main target of pathological inflammation is the liver, and not the heart as in infected human patients or BALB/c mice. For that reason, we decided to evaluate the role of AhR activation by natural ligands (as Trp metabolites) generated during *T. cruzi* infection by using B6-AhRd congenic mice. AhRd mice express an AhR with a 10-fold lower binding affinity for TCDD, and required a 10-fold higher dose of TCDD than AhRb-1 allele, present in B6 mice, to elicit similar effects (46). We have found that these mice suffer a more pronounced collapse of the Treg population than WT mice, which is consistent with weak/lack of AhR activation (3, 7). As expected, low Treg levels were associated to a stronger Th1 response which was able to control the parasite burden during the acute and the chronic phase of the infection. Significantly reduced parasitemia and cardiac pathology with increased levels of splenic IFN- γ -producing cells were also observed by Barroso et al. during the acute phase of AhR KO mice infected with Y strain of *T. cruzi*, although they not observed significant differences in Treg cells compared with WT mice (61). Surprisingly, in our experimental model AhRd mice were protected from the Th1-dependent liver pathology. Here we showed that these mice developed a Treg-independent mechanism of Th1 constriction between 10- and 17-days pi. After day 10 pi, serum levels of IL-10 were increased and spleen CD4+ cells secreting IL-10 were significantly up-regulated in AhRd vs. WT mice. Increased levels of CD4+ IL-10 producing T cells were also observed in AhR KO mice during the acute phase of *T. cruzi* infection (61). Likewise, IL-10 production by liver CD4+ T cells and increased levels of serum IL-10 were also found after *Plasmodium berghei* Anka infection in AhR KO mice (64). We speculate that the population of CD4+ IL-10-secreting T cells that is increased in *T. cruzi*-infected AhRd mice are different from Tr1 cells, because the *in vivo* generation of Tr1 cells is strongly dependent on AhR signals and these signals are very weak in AhRd mice (65). Moreover, in our model we have observed that Lag-3 [a marker of Tr1 cells (60)] is not up-regulated in the IL-10-producing CD4+ cells, and that many of these cells are also IFN- γ producers (data not shown). IL-10 production from effector T cells represents an essential negative feedback mechanism in the self-limitation of excessive inflammatory responses in many infections (66–69). Thus, the relative amounts of IL-10 and IFN- γ produced by Th1 cells may influence the balance between clearance, persistent chronic infection or immunopathology (70, 71). It has been demonstrated that IL-10 producing Th1 cells,

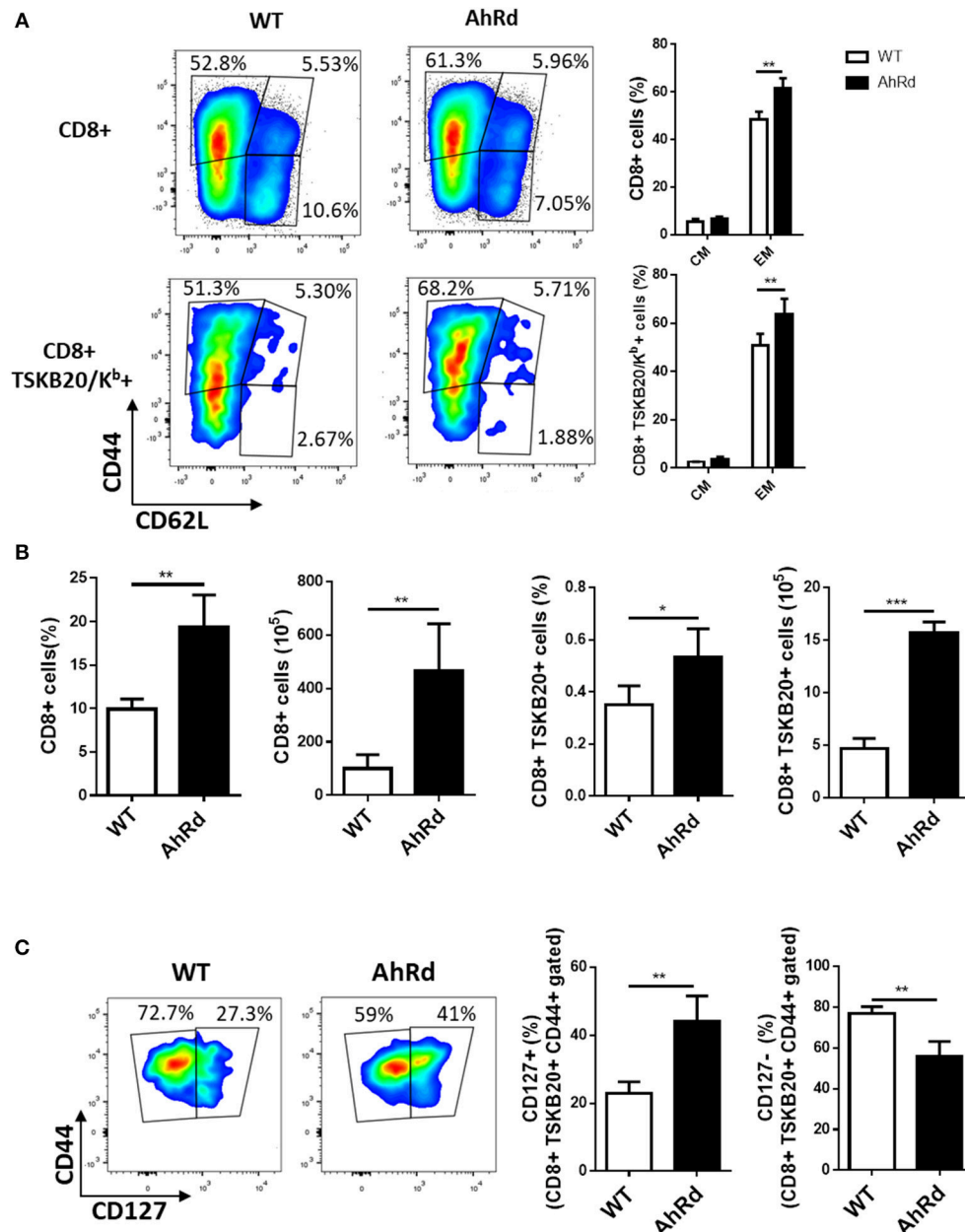


FIGURE 6 | AhR activation restricts the differentiation of CD8+ EM cells. Splenocytes from infected AhRd and WT mice were isolated at day 17 and 170 pi to evaluate total CD8 and TSKB20/kb-specific memory subpopulations by flow cytometry. **(A)** Representative plots and bars showing the percentage of splenocytes from WT and AhRd mice bearing EM and CM phenotype on CD8+ and CD8+ TSKB20/kb+ T cell populations at day 17 pi. Bars represent the mean values \pm SD with WT, $n = 3$ and AhRd, $n = 4$. **(B)** Percentage and absolute number of splenocytes CD8+ and CD8+ TSKB20/kb+ from WT and AhRd mice at day 170 pi. Bars represent the mean values \pm SD with WT, $n = 4$ and AhRd, $n = 4$. **(C)** Representative plots showing CD44 and CD127 expression on splenocytes from WT and AhRd mice gated on CD8+ TSKB20/kb+ cell populations at day 170 pi gated as indicated in **Figure S3** of Supplementary Material. Percentage of splenocytes from WT and AhRd mice showing CD127+ and CD127- expression within CD8+ TSKB20/kb+ CD44+ cell populations. Bars represent the mean values \pm SD with WT, $n = 4$ and AhRd, $n = 4$. One experiment representative of at least three independent experiments is shown. P -values were calculated with two-tailed Student's t -test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

also called self-regulatory Th1 cells, are activated early in a strong inflammatory environment by repeated TCR triggering (high antigen dose) and continued IL-12 action (72). Because fully differentiated Th1 cells fail to up-regulate AhR after activation and, therefore, cannot be directly modulated by AhR ligation, we hypothesize that weak/lack AhR activation in DCs induces

differentiation signals that promote IL-10 production by CD4+ T cells. It has been demonstrated that AhR activation induces tolerogenic properties to DCs. In fact, Quintana and others have reported that AhR DC activation reduces the expression of the pro-inflammatory cytokines IL-1 β , IL-6, and IL-12 (12, 13). In agreement, we have observed a higher IL-12 production by

splenocytes from *T. cruzi*-infected AhRd vs. WT mice (data not shown), and this has also been reported in *T. cruzi*-infected and OVA-immunized AhR KO mice (61, 73).

Few studies have examined whether AhR signaling affects the acquisition of immunological memory, but several epidemiological studies have revealed that exposure to dioxins and PCBs conditions low response to routine childhood vaccinations (74–76). In accordance with this, our results demonstrated a negative effect of a strong AhR activation on the development of memory subsets, since the administration of single dose of TCDD before *T. cruzi* infection decreased the specific cellular response and prevented the accumulation of memory cells by a mechanism that involve the death of activated cells. Diminished memory response was also observed by TCDD administration before primary influenza virus infection, (47, 77). Even so, when TCDD is administered after the immunological memory is established, there are no detectable effects on the magnitude of the recall CD8+ T cell responses, suggesting that AhR activation modulates critical events for the activation of naïve T cells (47, 77), and as far as we know there are no reports about the effect of AhR activation with ligands other than TCDD on memory subsets development. Because antigen-experienced cells suffered the direct effect of TCDD toxicity, no conclusions can be drawn regarding the effects of AhR ligation on the development of memory subsets during the acute phase of *T. cruzi* infection. Therefore, we considered that weak AhR ligation with 3-HK plus ITE and AhRd mice are good models to study the development of CD8+ T cell memory subsets in this infection. Interestingly, 3HK plus ITE activation of AhR decreased the percentage of total and specific CD8+ splenic T cells that acquired EM and CM phenotype while the lack/very weak activation of AhR in AhRd mice showed opposite results. Certainly, at the chronic phase of the infection, AhRd mice had significantly higher frequency of splenic cells expressing IL-7 receptor (CD127), a marker for long-living memory T cells that would allow their vigorous proliferation driven by the homeostatic cytokine IL-7 (78). Together, these results suggest that AhR ligation restricts the differentiation of CD8+ memory T cells. Like other persistent infections, during *T. cruzi* infection the CD8+ T cells are maintained primary by the presence of antigen, and thus have phenotype of EM over CM as observed in transient infections (79). Interestingly, 3-HK plus ITE treated mice showed diminished number of EM cells compared with untreated mice, although the former have more parasite load able to drive preferably EM phenotype in CD8+ T cells, thus supporting the role of AhR activation on restricting memory development.

Different studies suggest that, similar to its indirect role on Th1 cells, AhR would likely regulate DCs rather than directly CD8+ T cells (47, 80). The very low expression of AhR in most CD8+ T cell populations might support the lack of direct regulation of CD8+ T cells by the AhR signaling. Thus, DCs may be the main cell population capable of sensing and secreting AhR ligands during *T. cruzi* infection, and the lack, weak or strong activation of DC AhR might modulate their activation status, inducing either proinflammatory or tolerogenic net effects on

T cells and modulating memory subsets development (3, 4, 81, 82). Taken together, our results allow us to propose a model in which a threshold of AhR activation exist. Thus, signals above the threshold (corresponding to WT B6 mice) would induce tolerogenic properties in DCs (12, 13), restrict the development of memory CD8+ T cells and promote Treg cells (as observed here by treatment of B6 mice with 3-HK plus ITE or TCDD) able to prematurely control Th1-type response. On the contrary, signals below threshold would promote an early inflammatory Th1-type response able to restrict the parasite replication and its timely contraction by non-Tr1 IL-10 producing cells. Thus, AhR induce diverse regulatory pathways that finally impacts on parasite replication and infection outcome.

ETHICS STATEMENT

All animal experiments were approved by and conducted in accordance with guidelines of the Institutional Animal Care and Use Committee (IACUC), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba (Approval Number HCD 743/18).

AUTHOR CONTRIBUTIONS

LA, LC, and CM designed the experiments. LA, CI, XV, and EAR performed the experiments, data analysis and interpretation. LA, EAR, LC and CM wrote the manuscript. FJQ and HMS revised critically the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00631/full#supplementary-material>

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Shikonin Prolongs Allograft Survival via Induction of CD4⁺FoxP3⁺ Regulatory T Cells

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A transplanted organ is usually rejected without any major immunosuppressive treatment because of vigorous alloimmune responsiveness. However, continuous global immunosuppression may cause severe side effects, including nephrotoxicity, tumors, and infections. Therefore, it is necessary to seek novel immunosuppressive agents, especially natural ingredients that may provide sufficient efficacy in immunosuppression with minimal side effects. Shikonin is a bioactive naphthoquinone pigment, an ingredient originally extracted from the root of *Lithospermum erythrorhizon*. Previous studies have shown that shikonin regulates immunity and exerts anti-inflammatory effects. In particular, it can ameliorate arthritis in animal models. However, it is unclear whether shikonin inhibits alloimmunity or allograft rejection. In this study and for the first time, we demonstrated that shikonin significantly prolonged the survival of skin allografts in wild-type mice. Shikonin increased the frequencies of CD4⁺Foxp3⁺ regulatory T cells (Tregs) post-transplantation and induced CD4⁺Foxp3⁺ Tregs *in vitro* as well. Importantly, depleting the Tregs abrogated the extension of skin allograft survival induced by shikonin. It also decreased the frequencies of CD8⁺CD44^{high}CD62L^{low} effector T cells and CD11c⁺CD80⁺/CD11c⁺CD86⁺ mature DCs after transplantation. Moreover, we found that shikonin inhibited the proliferation of T cells *in vitro* and suppressed their mTOR signaling. It also reduced the gene expression of pro-inflammatory cytokines, including IFN γ , IL-6, TNF α , and IL-17A, while increasing the gene expression of anti-inflammatory mediators IL-10, TGF- β 1, and indoleamine-2, 3-dioxygenase (IDO) in skin allografts. Further, shikonin downregulated IDO protein expression in skin allografts and DCs *in vitro*. Taken together, shikonin inhibits allograft rejection via upregulating CD4⁺Foxp3⁺ Tregs. Thus, shikonin is a novel immunosuppressant that could be potentially used in clinical transplantation.

Keywords: allograft rejection, immunosuppressant, immunoregulation, Treg, T cell, transplant immunology

INTRODUCTION

Organ transplantation is an effective and final treatment for patients undergoing end-stage organ diseases (1, 2). However, almost all transplantation patients require continuous treatment with immunosuppressive drugs to prevent allograft rejection. Thus, immunosuppressive agents play an essential role in maintaining allograft survival. Conventional immunosuppressive drugs can effectively suppress allograft rejection and improve the outcome of transplantation through different mechanisms (3). However, global immunosuppression may also cause severe side effects. Adverse effects of an calcineurin inhibitor, such as cyclosporine (CsA), include nephrotoxicity, malignancies, hypertension, and infections (3–5) while rapamycin, an mTOR inhibitor, may result in hyperlipidemia, impaired glucose tolerance and diabetes, and acute renal toxicity (6, 7). Hence, it is necessary to search for novel immunosuppressive agents that may provide high efficiency in immunosuppression with minimal side effects.

Shikonin is a bioactive naphthoquinone pigment originally extracted from the root of *Lithospermum erythrorhizon* Sieb et Zucc. Shikonin has been utilized as an ingredient of the traditional Chinese herb to treat macular eruption, measles, sore throat, carbuncle, and burns in China for several centuries (8). Recent studies have also revealed that shikonin can exert anti-inflammatory, anticancer, and antimicrobial effects (9–11). In particular, shikonin has been demonstrated to inhibit the development of some immune-based inflammatory diseases, such as arthritis and asthma, in animal models (12, 13). Shikonin significantly decreased the severity of murine collagen-induced arthritis, alleviated the joint swelling and cartilage destruction. Further, it suppressed the production of matrix metalloproteinase (MMP)-1 and increased expression of tissue inhibitors of metalloproteinase (TIMP)-1 in this model (12). Shikonin suppressed allergic airway inflammation in a murine model of asthma by inhibiting the maturation of bone marrow-derived DCs (13). It also reduced IL-4, IL-5, IL-13, and TNF- α release in bronchial alveolar lavage fluid and lowered IL-4 and IL-5 production in lung cells and mediastinal lymph node cells. Importantly, shikonin has been proved to suppress human T lymphocyte activation via suppressing JNK signaling and IKK β activity (14). Although shikonin has been shown to regulate immunity and inflammatory responses (10, 14, 15), it remains unknown whether shikonin modulates alloimmunity and suppresses allograft rejection. Here we hypothesized that shikonin could suppress alloimmune responses.

In current study, we found that shikonin significantly prolonged the survival of murine skin allografts. To our knowledge, this was the first evidence that shikonin inhibits allograft rejection in an experimental animal model. Shikonin significantly increased the frequencies of CD4⁺Foxp3⁺ regulatory T cell (Tregs) *in vivo* and induced CD4⁺Foxp3⁺ Tregs *in vitro* as well. Shikonin also decreased the frequencies of CD8⁺CD44^{high}CD62L^{low} effector T cells and CD11c⁺CD80⁺/CD11c⁺CD86⁺ mature DCs after transplantation. Moreover, we demonstrated that

shikonin inhibited the proliferation of T cells *in vitro* and suppressed their mTOR signaling. Finally, shikonin reduced the gene expression of proinflammatory cytokines in skin grafts while increasing IDO and FoxP3 protein expression in the grafts. Therefore, shikonin may represent a novel immunosuppressant that can be potentially applied to clinical transplantation.

MATERIALS AND METHODS

Animals

C57BL/6 and BALB/c mice (6–8 week-old, weighing 20 \pm 2 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All mice were housed in a specific pathogen-free room with controlled conditions. All experiments were approved by the Institutional Animal Ethical Committee of Guangdong Provincial Academy of Chinese Medical Sciences.

Skin Transplantation

Skin donors were 6–8 week-old wild-type BALB/c mice while skin graft recipients were 6–8 week-old C57BL/6 mice. Full-thickness trunk skin with an approximate size of 10 mm² was transplanted to the dorsal flank area of recipient mice and secured with a bandage of Band-Aid (Johnson Johnson, New Brunswick, NJ). The bandage was removed 8 days after transplantation. Skin allograft rejection was monitored daily and defined as graft necrosis >90%, as also described in our previous publication (16).

Administration of Drugs

Mice were randomly grouped into control groups, and experimental groups that were administrated with shikonin (*p.o.* 20 or 40 mg/kg body weight) or cyclosporine (CsA, *i.p.* 20 mg/kg, or 10 mg/kg for joint treatment with shikonin) for a period of 2 consecutive weeks or until graft rejection/sample collection. Anti-CD45RB Ab (Clone MB23G2; Bio X Cell, NH, USA) was injected *i.p.* at 0.1 mg/day on days 0 and 5. CsA (Sigma, USA) was dissolved in saline while shikonin (Push Bio-technology, China) was prepared with sodium carboxymethyl cellulose (CMC-Na; Sigma). Control groups were administrated orally with equivalent CMC-Na solution only. Our previous study has shown that oral administration of CMC-Na (vehicle) did not alter allograft rejection compared with untreated groups (unpublished observation). To deplete Tregs, recipient mice were administrated *i.p.* with anti-CD25 Ab (eBioscience, USA) at 0.2 mg on days 0, 4, and 8 post-transplantation as described in our previous publication (17).

Histological Analysis

Skin grafts of recipient mice were harvested, fixed with 4% paraformaldehyde for more than 24 h and then embedded in paraffin. The sections (3 μ m) of skin tissues were then made and stained with Hematoxylin and Eosin (H&E). In addition, sections were incubated first with primary anti-CD3 antibody (1:100, Abcam, USA) at 4°C overnight, then with secondary antibody HRP-anti-Rabbit IgG (Maxim,

China) at 37°C for 30 min, and finally colored with diaminobenzidine at room temperature without light for immunohistochemical analysis.

Immunofluorescence

Skin grafts of recipient mice were harvested, embedded in OTC and frozen. Skin tissue sections were cut with a thickness of 3 µm using freezing microtome. Then they were incubated in 0.3% Triton X-100 and 10% bovine serum albumin for 1 h, following by incubation overnight at 4°C with primary mouse anti-indoleamine-2, 3-dioxygenase (Millipore, USA) and rabbit anti-CD11c (Cell Signaling Technology, USA) or rabbit anti-Foxp3 (Cell Signaling Technology) antibody at a concentration of 1:100. Sections were then stained with a secondary antibody Alexa Fluor® 555-conjugated anti-mouse IgG or Alexa Fluor® 488 conjugated anti-rabbit IgG (Cell Signaling Technology). These cryosections were finally mounted using DAPI-Fluoromount-G clear mounting agents (SouthernBiotech, Birmingham, UK). All of the images were obtained randomly using a fluorescence microscope (magnification 200×).

Flow Cytometric Analysis

Draining lymph node (LN) and spleen cells were harvested and stained with anti-CD4-PE (Clone H129.19), CD8-FITC (Clone 53-6.7), CD44-V450 (Clone IM7), CD11c-PE (Clone HL3), CD80-FITC (Clone 16-10A1), CD86-FITC (Clone GL1), and anti-CD62L-APC (Clone MEL-14) antibodies (all from BD Biosciences, USA) to analyze surface markers. Intracellular FoxP3 staining was performed using Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent Kit (eBioscience, USA) according to manufacturer's instruction. Then, some cells were stained with anti-FoxP3-APC antibody (Clone FJK-16s, eBioscience). The stained cells were washed and finally analyzed using a flow cytometer (FACSCalibur, BD Biosciences). To isolate CD3⁺ T cells and CD4⁺CD25⁻ T cells, spleen cells were isolated and stained with anti-CD3-APC (Clone 145-2C11), CD4-FITC (Clone H129.19), and anti-CD25-PE (Clone 3C7) Abs (BD Biosciences). CD3⁺ T cells and CD4⁺CD25⁻ T cells were then purified using FACSARIA III cell sorter (BD Biosciences). The purity of the sorted cells was typically >96%.

Induction of Tregs *in vitro*

CD4⁺CD25⁻ T cells from C57BL/6 mice were sorted out using FACSARIA III cell sorter. Then, cells (4×10^5 cells/well) were cultured in 96-well plates (200 µl/well) in complete RPMI-1640 medium (Gibico, USA) containing 10% fetal bovine serum (Gibico), 100 U/ml penicillin and 100 µg/ml streptomycin, and stimulated with anti-CD3/anti-CD28 Abs (2.5 µg/ml) and IL-2 (10 ng/ml, Peprotech) in the absence or presence of TGF-β1 (positive control, 5 ng/ml, Peprotech) or shikonin (0.25 or 0.5 µM) for 4 days. The frequency of CD4⁺Foxp3⁺ Tregs was finally determined using a FACSCalibur.

T Cell Proliferation Assay and Measurement of Cytokines *in vitro*

FACS-sorted CD3⁺ T cells derived from C57BL/6 mice were labeled with 3 µM CFSE (Invitrogen, Germany) at room temperature without light for 15 min. Then, cells (4×10^5 cells/well) were cultured in 96-well plates in complete RPMI-1640 medium as described above and stimulated with anti-CD3/anti-CD28 Abs (2.5 µg/ml) plus IL-2 (10 ng/ml, Peprotech) at 37°C with 5% CO₂ for 4 days. These cells were also treated with either shikonin (0.25 or 0.5 µM) or CsA (0.1 µM) for 4 days. Finally, cell proliferation was measured using a FACSCalibur. On the other hand, the levels of IFN-γ, IL-10, TGF-β1, and IL-17A in the supernatant were also measured using ELISA according to the manufacturer's instructions (Boster, China), and the absorbance was read at 450 nm in a microplate spectrophotometer (Thermo Fisher Scientific, USA).

Assays of T Cell Cytotoxicity *in vitro*

T cell cytotoxicity was detected using CCK-8 assays. FACS-sorted CD3⁺ T cells derived from C57BL/6 mice were cultured in 96-well plates in complete RPMI-1640 medium (Gibico, USA) containing 10% fetal bovine serum (Gibico), 100 U/ml penicillin and 100 µg/ml streptomycin, and stimulated with anti-CD3/anti-CD28 Abs (2.5 µg/ml) and IL-2 (10 ng/ml, Peprotech). Shikonin was added to each well at different concentrations (0.1, 0.25, 0.5, 1, and 2 µM, respectively) with four wells *per* concentration. After 24, 48, and 96 h, 20 µL of CCK-8 was added to each well and incubated at 37°C for 4 h. The absorbance was measured by a microplate spectrophotometer (Thermo Fisher Scientific, USA) at the wavelength of 450 nm. Control without shikonin was set as 1.0.

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

Total mRNA from a skin graft was isolated using Trizol reagents (Invitrogen, USA) and mRNA was then transcribed to cDNA using a PrimeScript™ RT reagent kit (Takara Bio Incorporation, Kusatsu, Japan) according to the instructions of the manufacturer. The cDNA was analyzed for the expression of cytokines using a Quantifast SYBR Green PCR kit (Takara Bio Incorporation) via an ABI 7500 Fast RealTime PCR System (Thermo Fisher Scientific). The primer sequences were shown in Table 1. The relative mRNA expression levels of cytokines were normalized against β-actin, and analysis was performed through a comparative 2^{ΔΔCT} method. All data are shown in the form of relative expression as fold changes.

Generation of Dendritic Cells *in vitro* From Bone Marrow

Bone marrow cells were harvested from femurs of C57BL/6 mice. Cells (1×10^6 /mL) were cultured overnight in 12-well plates with complete RPMI-1640 medium in the presence of recombinant mouse rGM-CSF (20 ng/ml, Peprotech) plus rIL-4 (10 ng/ml, Peprotech). Adherent cells were further incubated

TABLE 1 | Primer sequences of target genes.

Target gene	Primer sequence (5' → 3')
IFN- γ (forward)	CACGGCACAGTCATTGAAAG
IFN- γ (reverse)	CATCCTTTTGCCAGTTCCTC
TNF- α (forward)	ACGGCATGGATCTCAAAGAC
TNF- α (reverse)	GTGGGTGAGGAGCACGTAGT
IL-10 (forward)	CCAAGCCTTATCGAAATGA
IL-10 (reverse)	TCCTGAGGGTCTTCAGCTTC
IL-17A (forward)	GTCCAAACACTGAGGCCAAG
IL-17A (reverse)	ACGTGGAACGGTTGAGGTAG
IL-6 (forward)	ACTTCCATCCAGTTGCCTTCTGG
IL-6 (reverse)	TTAAGCCTCCGACTTGTGAAGTGG
Foxp3 (forward)	CCCATCCCAGGAGTCTTG
Foxp3 (reverse)	ACCATGACTAGGGGCACTGTA
IDO (forward)	GCTTTGCTCTACCACATCCAC
IDO (reverse)	CAGGCGCTGTAACCTGTGT
TGF- β 1 (forward)	CAATTCCTGGCGTTACCTTG
TGF- β 1 (reverse)	AGCCCTGTATTCCGCTCCT
β -actin (forward)	TGTCCACCTTCCAGCAGATGT
β -actin (reverse)	TGTCCACCTTCCAGCAGATGT

with the complete medium containing fresh rGM-CSF and rIL-4 for 5 days before suspension cells were removed. Finally, these dendritic cells (DCs) were treated with either shikonin (0.25 or 0.5 μ M) or CsA (0.1 μ M) for 48 h. Total protein samples from DCs were obtained and then measured for IDO protein expression.

Western Blotting

Total protein samples from T cells or DCs were obtained in RIPA lysis buffer followed by centrifugation (12,000 rpm and 10 min) in 4°C. Then, the protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific). Samples were run on 10% SDS-PAGE gels and electro-transferred onto a PVDF membrane. TBST with 5% (w/v) BSA was used to block non-specific binding to the membrane at room temperature for 1 h. The membrane was then incubated with a primary antibody anti-phospho-P70S6K, anti-P70S6K, anti-phospho-mTOR, anti-mTOR (1:1,000; Cell Signaling Technology, USA), or anti-IDO (1:1,000; Millipore, USA) at 4°C overnight. After incubation, the membrane was washed and incubated with a secondary antibody, HRP-conjugated goat anti-rabbit IgG (1:2,000) for 1 h. GAPDH (1:2,000; Cell Signaling Technology) was also used for loading controls. Finally, blots were visualized by an ECL method (Beyotime, China) and analyzed using an Image J Program software.

Statistical Analysis

Comparisons of means were conducted using Student's *t*-test and one-way ANOVA. Data were presented as the mean \pm SD and analyzed through GraphPad Prism 6 (GraphPad Software, USA). The analysis of graft survival was performed using Kaplan–Meier method (log-rank test). A value of *P* < 0.05 was considered statistically significant.

RESULTS

Shikonin Significantly Prolongs Skin Allograft Survival and Reduces CD3⁺ T Cell Infiltration in the Skin Allografts

To investigate whether shikonin would suppress allograft rejection, we used BALB/C mice as skin donors and C57BL/6 mice as skin recipients that were then treated with shikonin or CsA for 14 consecutive days following transplantation. As shown in **Figure 1A**, we found that both low and high doses of shikonin significantly prolonged skin allograft survival compared to the control group [median survival time (MST) = 16 (low dose) vs. 12 days and 22 (high dose) vs. 12 days, both *P* < 0.05]. Interestingly, treatment with high doses of shikonin was nearly as effective as CsA (20 mg/kg) in prolongation of skin allograft survival (MST = 22 vs. 26 days, *P* > 0.05). On the other hand, the skin allografts were analyzed via H&E or immunohistochemical (IHC) staining 10 days post-transplantation. We found that treatment with either shikonin or CsA obviously reduced overall cellular infiltration (**Figure 1B**) as well as CD3⁺ T-cell infiltration (**Figure 1C**) in the skin allografts compared to the control group, suggesting that shikonin prolongs skin allograft survival and reduces CD3⁺ T-cell infiltration in the allografts. Moreover, we examined the effects of combined treatments with shikonin and low doses of CsA (10 mg/kg) or anti-CD45RB Ab (0.1 mg at day 0 & 5), and found that the double treatment was more effective in suppression of allograft rejection than shikonin alone while the triple treatment with all of three agents further prolonged allograft survival compared to the double treatment with either Shikonin+CsA or Shikonin+anti-CD45RB Ab (**Figure 1D**). However, most of the allografts (7/9) in recipient mice with the triple treatment were still rejected within 60 days.

Shikonin Induces CD4⁺ Foxp3⁺ Tregs *in vivo* and *in vitro*

Regulatory T cells play a key role in maintaining immune homeostasis and tolerance (18–20). Thus, we examined whether shikonin would prolong skin allograft survival by inducing CD4⁺Foxp3⁺ Tregs *in vivo* and *in vitro*. Draining lymph node and spleen cells were isolated 8 days after transplantation, and CD4⁺Foxp3⁺ Tregs were detected using flow cytometric analysis. As shown in **Figure 2A**, shikonin significantly increased the frequencies of CD4⁺Foxp3⁺ Tregs in draining lymph nodes compared with the control group while CsA did the opposite. Moreover, high doses of shikonin increased the Treg frequencies more drastically than did its low doses. In contrast, shikonin at high doses, but not low doses, significantly augmented the frequencies of splenic Tregs post-transplantation. Shikonin also increased the Treg frequencies in recipient mice 14 days after skin transplantation (data not shown). Since shikonin increased CD4⁺Foxp3⁺ Tregs in recipient mice, we asked if shikonin would also promote the induction of CD4⁺Foxp3⁺ Tregs *in vitro*. FACS-sorted CD4⁺CD25[−] T cells from C57BL/6 mice were activated by anti-CD3/CD28 Abs in the presence of IL-2 without or with

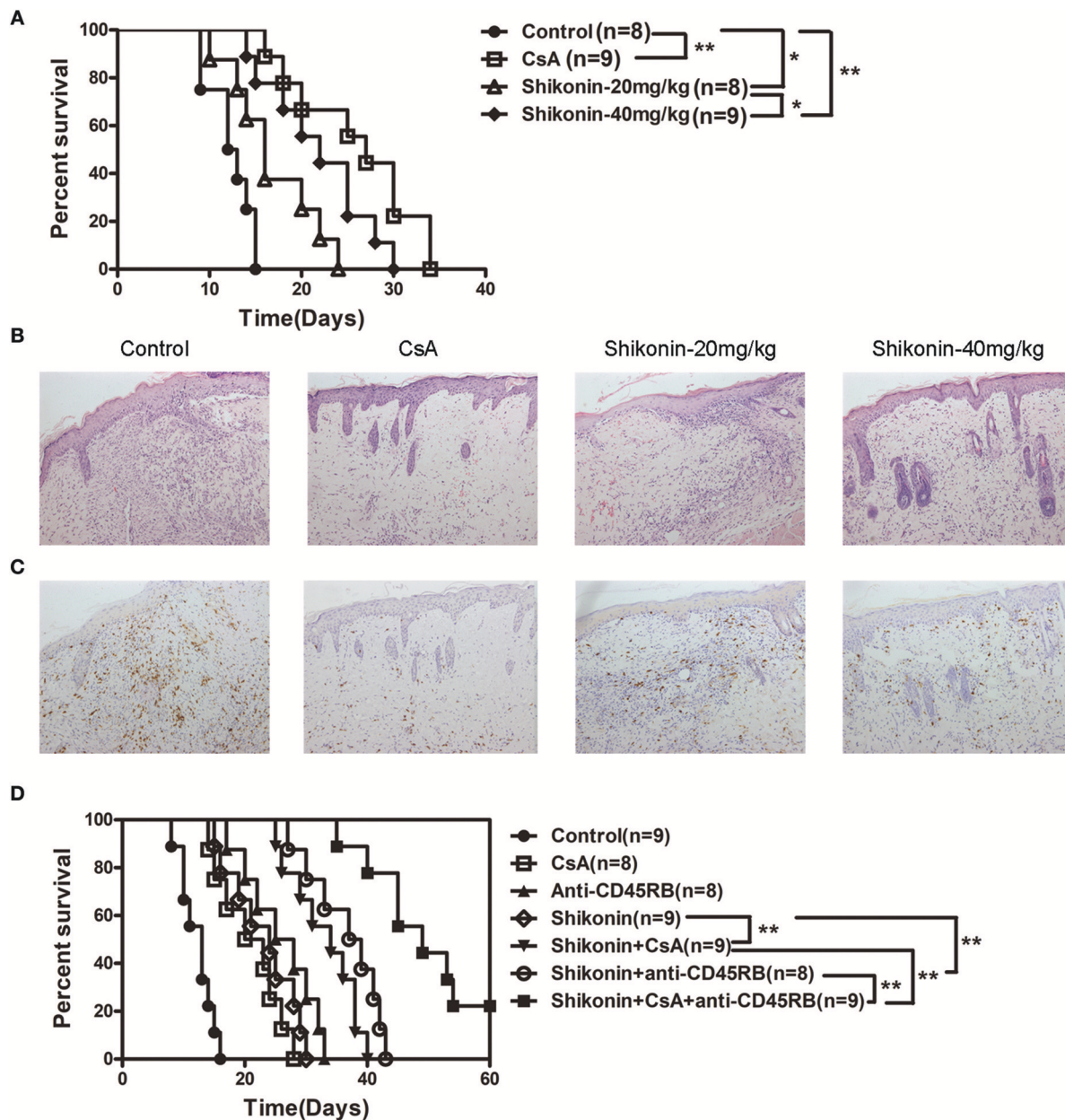
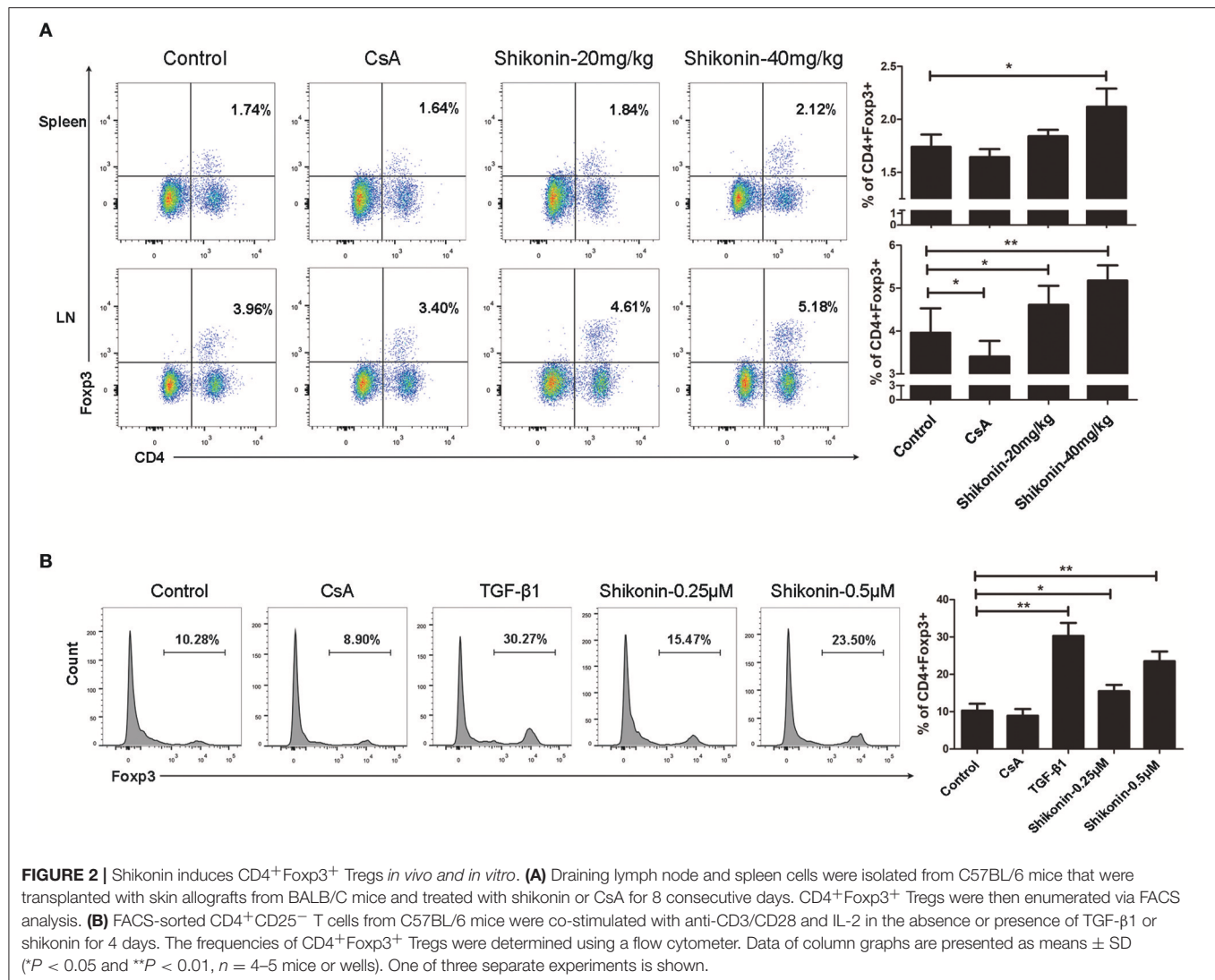


FIGURE 1 | Shikonin prolongs skin allograft survival and reduces CD3⁺ T cell infiltration in skin allografts. **(A)** C57BL/6 mice were transplanted with a skin allograft from a BALB/C donor and treated without or with shikonin or CsA (20 mg/kg). The survival times of the skin grafts were observed (* $P < 0.05$ and ** $P < 0.01$, $n = 8-9$). H&E **(B)** and immunohistochemical staining for CD3 **(C)** were performed 10 days post-transplantation. **(D)** Transplanted mice were treated with shikonin plus low doses of CsA (10 mg/kg) and/or anti-CD45RA mAb (0.1 mg) to observe the effects of the double or triple treatments on skin allograft survival (** $P < 0.01$, $n = 8-9$).

TGF- β 1 or shikonin for 4 days, and CD4⁺Foxp3⁺ Tregs were then quantified via FACS analysis. As shown in **Figure 2B**, shikonin, at both low and high concentrations, significantly increased the percentages of Foxp3⁺ Tregs *in vitro*, and so did TGF β 1, a positive control. However, CsA did not augment the Treg frequency. Taken together, our data suggest that shikonin promotes CD4⁺Foxp3⁺ Treg generation both *in vivo* and *in vitro*.

Shikonin Increases Both IDO and FoxP3 Expressions in Skin Allografts While Augmenting IDO Expression by DCs *in vitro*

It is generally accepted that IDO can induce CD4⁺Foxp3⁺ Tregs. We then asked whether shikonin would increase IDO protein expression in skin allografts. Immunofluorescence staining of IDO and FoxP3 on skin allografts was performed 10 days following skin transplantation. As shown in **Figure 3A**, shikonin,



but not CsA, augmented FoxP3 expression in skin allografts. Furthermore, it also increased IDO expression in the grafts while IDO was mainly expressed by DCs, as demonstrated by the double stainings of both IDO and CD11c (**Figure 3B**). On the other hand, bone marrow-derived DCs were cultured in the absence or presence of shikonin for additional 2 days. IDO protein expression by DCs was then determined via Western blot analysis. As shown in **Figure 3C**, it was shikonin, but not CsA, that enhanced IDO protein expression in DCs *in vitro*, suggesting that shikonin upregulates IDO protein expression *in vivo* and *in vitro*.

Shikonin Hinders DC Maturation Post-Transplantation

To determine if shikonin would also have an impact on DC maturation in the context of allotransplantation, splenocytes, and lymph node cells were analyzed for the markers of DC maturation 10 days after skin transplantation. As shown in **Figure 4A**, either CsA

or shikonin significantly reduced the percentage of CD11c⁺CD86⁺ mature DCs in both the spleen and lymph nodes. As for CD11c⁺CD80⁺ cells, another subset of mature DCs, shikonin only decreased their frequency in the draining lymph nodes, but not the spleen (**Figure 4B**). These findings indicate that shikonin does interfere with DC maturation.

Depletion of CD25⁺ Tregs Reverses Allograft Survival Prolonged by Shikonin

To investigate whether the effects of shikonin on allograft survival were dependent on its induction of CD4⁺CD25⁺ Tregs, C57BL/6 mice transplanted with BALB/C skin were treated with shikonin (40 mg/kg). They were then depleted of CD25⁺ Tregs through injection of anti-CD25 mAb (PC61). As shown in **Figure 5**, we found that depleting CD25⁺ Tregs mostly reversed skin allograft survival prolonged by shikonin but not CsA. Isotype control Ab did not reverse it (our unpublished observation).

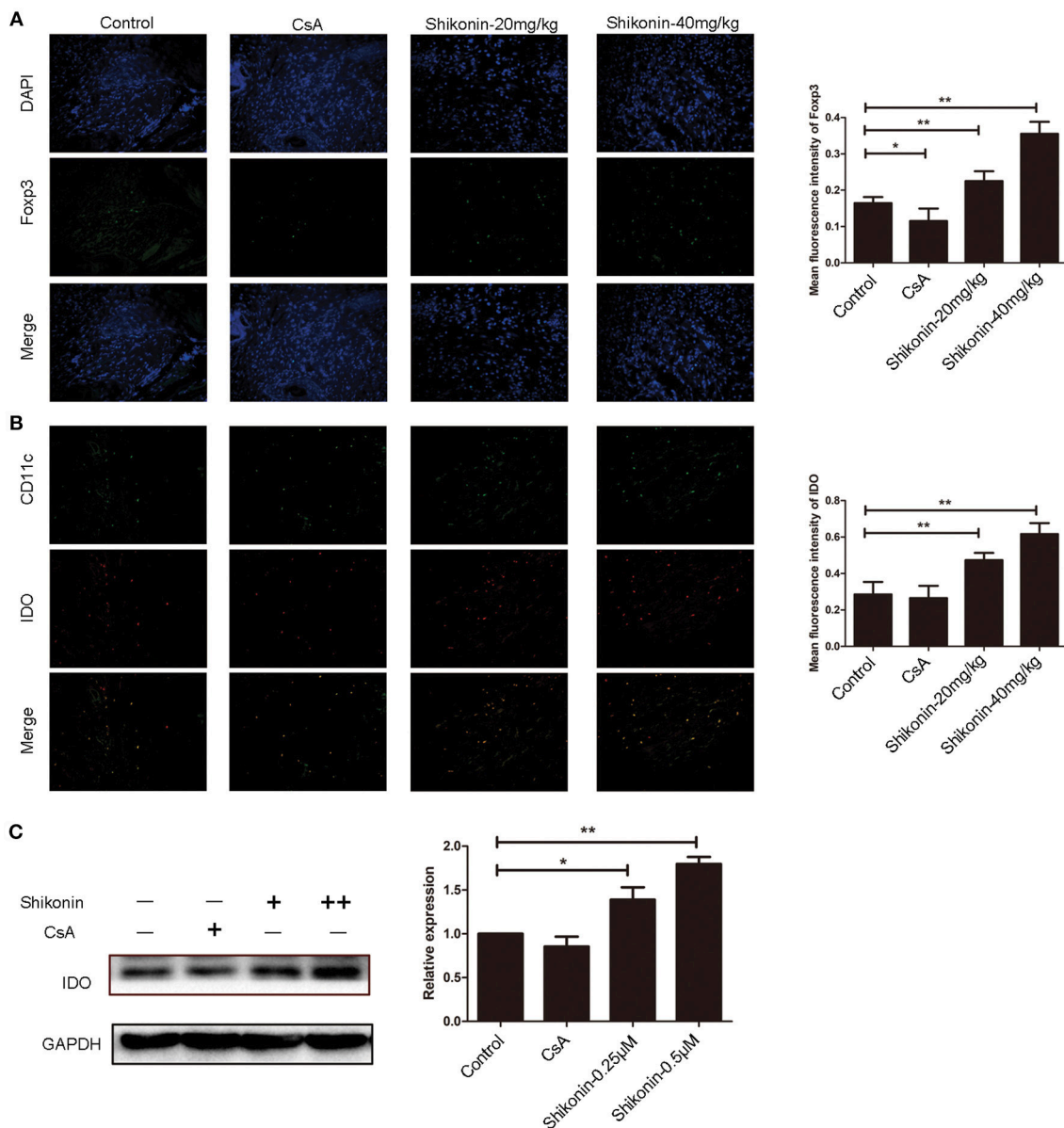


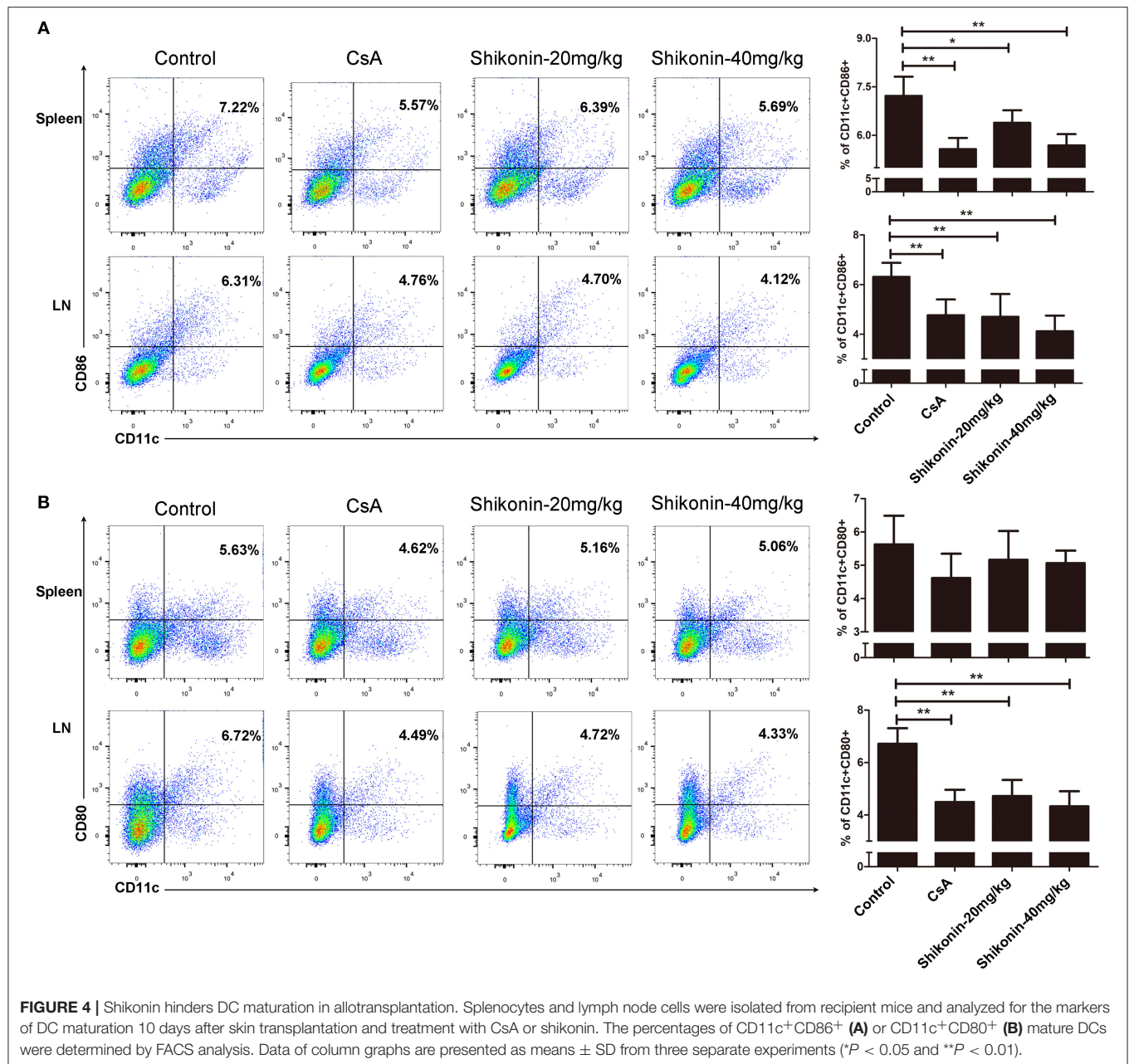
FIGURE 3 | Shikonin increases IDO expression in skin allografts and DCs *in vitro*. Immunofluorescence staining of Foxp3 (A) and IDO (B) on skin allografts was performed 10 days following skin allotransplantation. Merged images from double stainings of CD11c/IDO or DAPI/Foxp3 are also shown. Data of column graphs on right panels are presented as means \pm SD of mean fluorescence intensity from three separate experiments (** $P < 0.01$). On the other hand, bone marrow-derived DCs were cultured in the absence or presence of shikonin for 2 days. IDO protein expression by DCs was then determined via Western blot (C). One of three sets of images is shown. Data of column graphs are presented as means \pm SD relative to control from three separate experiments (* $P < 0.05$ and ** $P < 0.01$).

These findings suggest that extension of allograft survival by shikonin is largely dependent on its induction of CD4⁺CD25⁺ Tregs.

Shikonin Reduces Effector CD8⁺ T Cell Frequency *in vivo*

To determine whether shikonin would suppress effector CD8⁺ T cells *in vivo*, draining lymph node and spleen cells were isolated from C57BL/6 recipients treated with

shikonin or CsA. The cells were then analyzed for the frequency of CD8⁺CD44^{high}CD62L^{low} effector T cells (Teff) through a flow cytometer 10 days after transplantation. As represented by Figure 6, shikonin, at either low or high doses, significantly reduced the percentage of CD8⁺CD44^{high}CD62L^{low} effector T cells in both lymph nodes and spleens of the recipients compared with the control group. As a control, CsA also did the same. These findings suggest that shikonin suppresses the expansion of



CD8⁺CD44^{high}CD62L^{low} effector T cells in transplanted recipient mice.

Shikonin Suppresses T cell Proliferation and Production of Proinflammatory Cytokines *in vitro*

Given that shikonin significantly prolonged skin allograft survival, we then asked whether it would suppress T cell proliferation and alter cytokine secretion *in vitro*. FACS-sorted CD3⁺ T cells from naïve C57BL/6 mice labeled with CFSE were co-stimulated with anti-CD3/CD28 Abs and IL-2 in the absence or presence of shikonin or CsA for 4 days. Cell division was

measured via FACS analysis. As shown in **Figure 7A**, shikonin, at both low and high concentrations, significantly suppressed T cell proliferation. As expected, CsA did the same. On the other hand, shikonin moderately promoted IL-10 secretion in the supernatant but dramatically inhibited production of IFN- γ (**Figure 7B**). Interestingly, CsA did not significantly alter IL-10 secretion although it largely blocked IFN- γ production. In contrast, shikonin at a high, but not low, concentration significantly reduced IL-17A level compared with control group. Further, CsA or high concentration of shikonin increased TGF- β 1 level. These findings suggest that shikonin suppresses T cell proliferation and their production of IFN- γ and IL-17A while increasing IL-10 and TGF- β 1 levels *in vitro*. To rule out

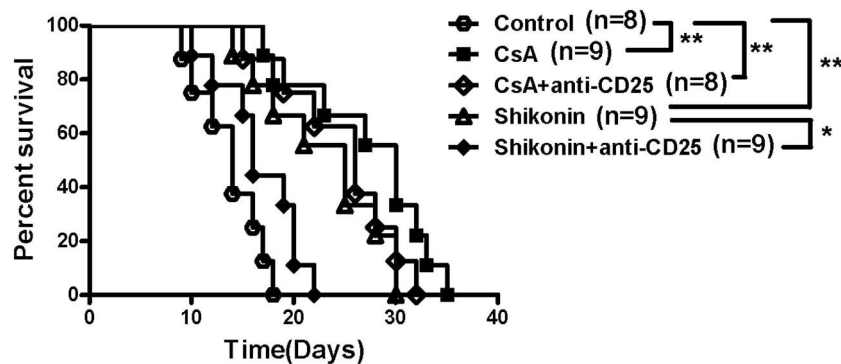


FIGURE 5 | Depleting CD25⁺ Tregs reverses skin allograft survival prolonged by shikonin. C57BL/6 mice were transplanted with BALB/C skin and treated with shikonin (40 mg/kg) or CsA (20 mg/kg) for 14 consecutive days or until allograft rejection. They were also depleted of CD4⁺CD25⁺ Tregs through injection of anti-CD25 Ab (PC61), as described in the methods. Skin allograft survival times were observed (* $P < 0.05$ and ** $P < 0.01$, $n = 8-9$ mice).

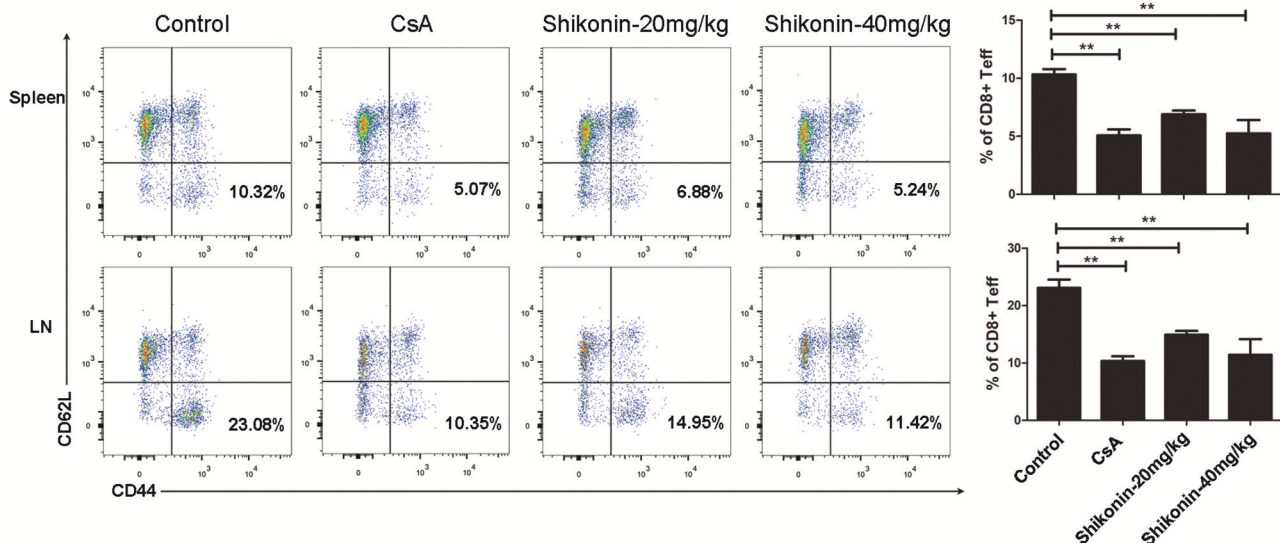


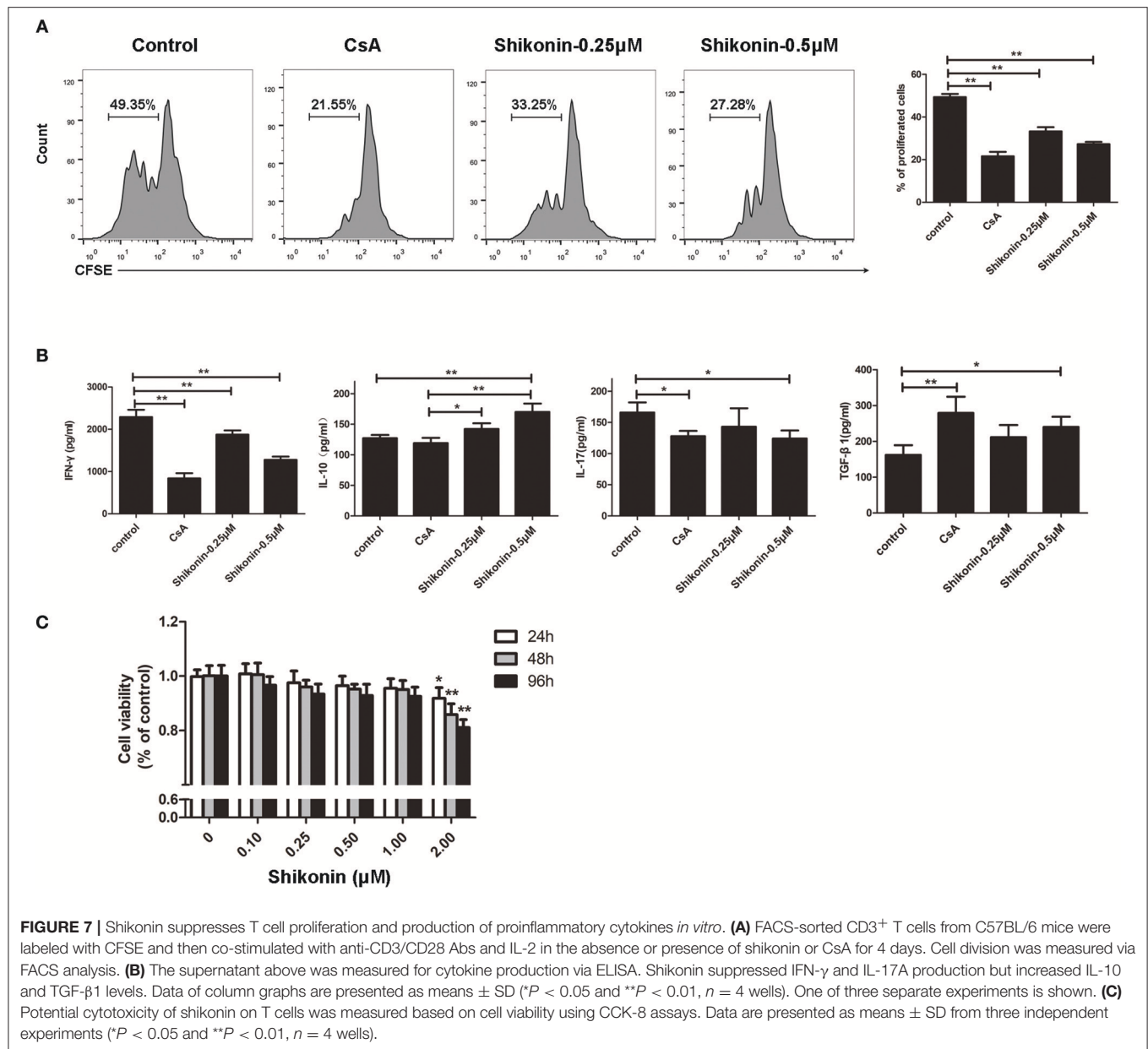
FIGURE 6 | Shikonin decreases effector CD8⁺ T cell frequency *in vivo*. C57BL/6 mice were transplanted with BALB/C skin and treated with shikonin or CsA daily. Draining lymph node and spleen cells were isolated from the recipient mice 10 days after transplantation, and the percentages of CD8⁺CD44^{high}CD62L^{low} effector T cells (Teff) were determined using a flow cytometer. Data of column graphs are presented as means \pm SD (** $P < 0.01$, $n = 4-6$ mice). One representative of three separate experiments is shown.

the possibility that suppressive effects of shikonin on T cells were caused by its cytotoxicity, we measured cell viability using CCK-8 assays. As shown in **Figure 7C**, cell viability was not compromised when up to 1.0 μ M shikonin was used in the cell culture, suggesting that suppression of T cell proliferation and activation by shikonin was not attributed to its cytotoxicity.

Shikonin Suppresses mRNA Expressions of Proinflammatory Cytokines but Increases IDO and FoxP3 Gene Expressions in Skin Allografts

To further examine the anti-inflammatory effects of shikonin, mRNA expressions of IFN- γ , TNF- α , IL-6, IL-10, IL-17A, FoxP3,

IDO, and TGF- β 1 in skin allografts were determined by RT-PCR 10 days after skin transplantation as well as related treatments. As shown in **Figure 8**, the mRNA expressions of these proinflammatory cytokines in skin allografts were significantly downregulated after the treatment with either CsA or shikonin, especially at high doses, whereas shikonin, but not CsA, upregulated IL-10 and FoxP3 mRNA levels compared with control group. On the other hand, both CsA and high doses of shikonin augmented TGF- β 1 gene expression while either low or high doses of shikonin, but not CsA, increased IDO gene expression. These findings indicate that shikonin upregulates gene expressions of IDO and FoxP3 as well as immunosuppressive cytokines IL-10 and TGF- β 1 in allografts and is indeed anti-inflammatory in the context of alloimmunity.



Shikonin Inhibits mTOR Signaling in T Cells

The mTOR signaling is closely associated with the generation of Tregs since rapamycin, an mTOR inhibitor, has been shown to induce CD4⁺Foxp3⁺ Tregs (21). Given that shikonin induced CD4⁺Foxp3⁺ Tregs *in vivo* and *in vitro*, we determined whether shikonin would have an effect on T-cell mTOR signaling. In our study, the expressions of phosphorylated p70S6K (P-p70S6K), p70S6K, P-mTOR, and mTOR proteins in T cells were measured using western blotting analysis 2 days after T cells were stimulated *in vitro* with anti-CD3/CD28 Abs in the absence or presence of shikonin. As shown in **Figure 9**, shikonin significantly inhibited the expression of phospho-p70S6K (both low and high concentrations: *p* < 0.01) and phospho-mTOR (high concentration: *p* < 0.01) compared to the control group. As

a positive control, rapamycin largely blocked expressions of both phospho-p70S6K and phospho-mTOR. These findings indicate that induction of Tregs by shikonin is likely due to its blockade of mTOR signaling pathway.

DISCUSSION

An allograft is always rejected by a recipient in the absence of any treatment with a conventional immunosuppressant. However, continuously global immunosuppression usually causes side effects, including nephrotoxicity, tumors and infections. Therefore, it's necessary to seek a natural product or ingredient with potentially less side effects. Shikonin, an ingredient originally extracted from a traditional Chinese herb,

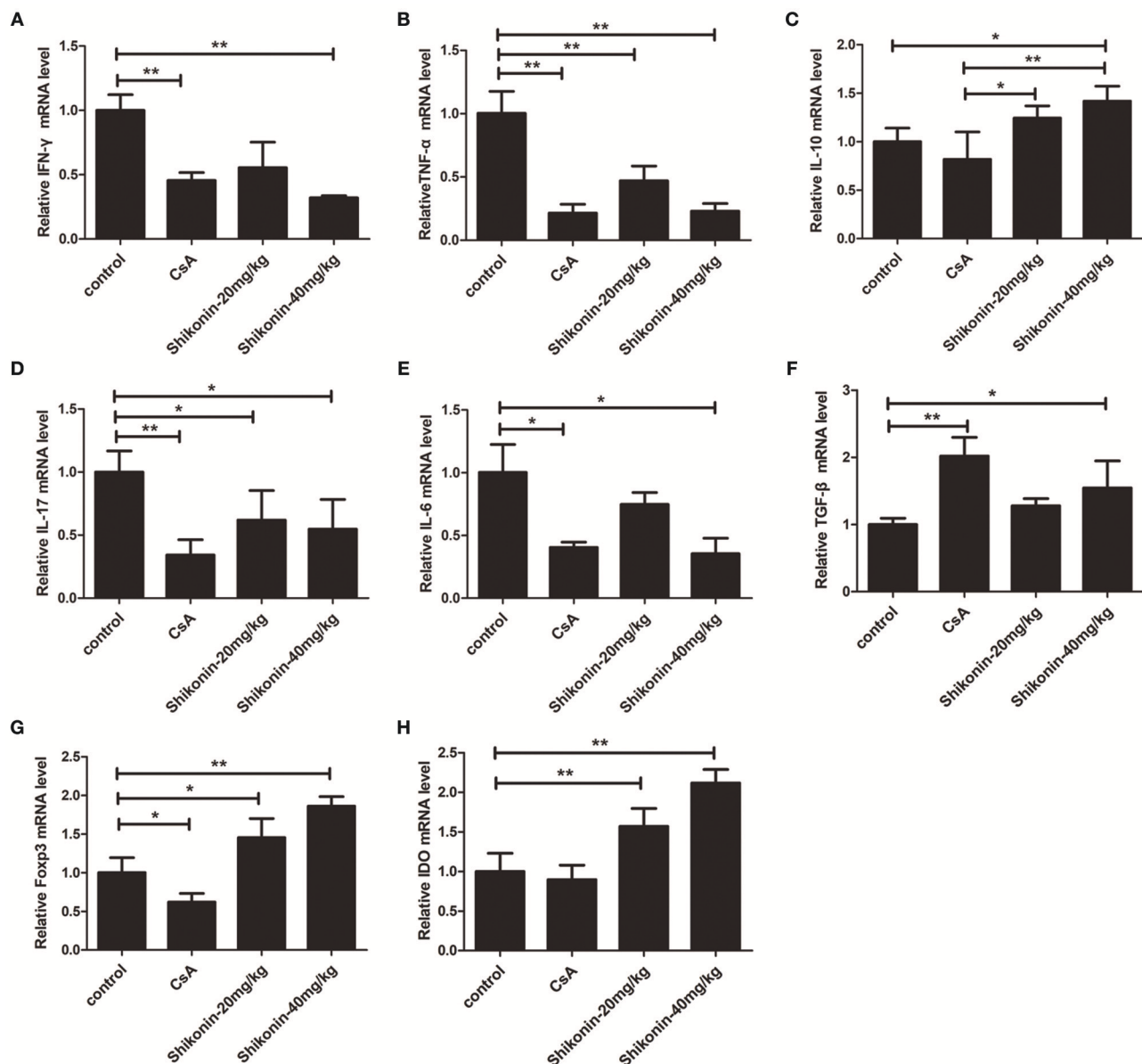


FIGURE 8 | Shikonin suppresses the mRNA expressions of proinflammatory cytokines while increasing IL-10 expression in skin allografts. The mRNA levels of IFN- γ (A), TNF- α (B), IL-10 (C), IL-17A (D), IL-6 (E), TGF- β 1 (F), FoxP3 (G), and IDO (H) in skin allografts were measured by RT-PCR 10 days after skin transplantation and treatment with shikonin or CsA. Data of column graphs are presented as means \pm SD (* P < 0.05 and ** P < 0.01, N = 4–5 mice). One of three separate experiments is shown.

has been shown to regulate immune responses and ameliorate inflammatory diseases (10, 12, 13). However, it's unknown whether shikonin has a significant impact on alloimmunity or allograft rejection. In this study, we have provided the first evidence that shikonin prolongs allograft survival in a murine model of skin allotransplantation and suppresses T cell proliferation and mTOR signaling while inducing CD4⁺Foxp3⁺ Tregs and upregulating IDO.

Tregs play an important role in maintaining immunological homeostasis and tolerance (18–20). A small subset of CD4⁺

T cells expressing the α chain of IL-2 receptor (CD25) and specifically fork head family transcription factor 3 (Foxp3) are typical Tregs (22, 23). Either induction of endogenous CD4⁺CD25⁺ Tregs or adoptive transfer of exogenous Tregs prevents autoimmune diseases and allograft rejection in many animal models (24–26). Deletion of the transcription factor Foxp3 (23, 27) or the growth factor IL-2 (28) diminishes the development or function of CD4⁺CD25⁺ Tregs. Our results showed that shikonin significantly increased the frequencies of CD4⁺Foxp3⁺ Tregs in recipients compared with

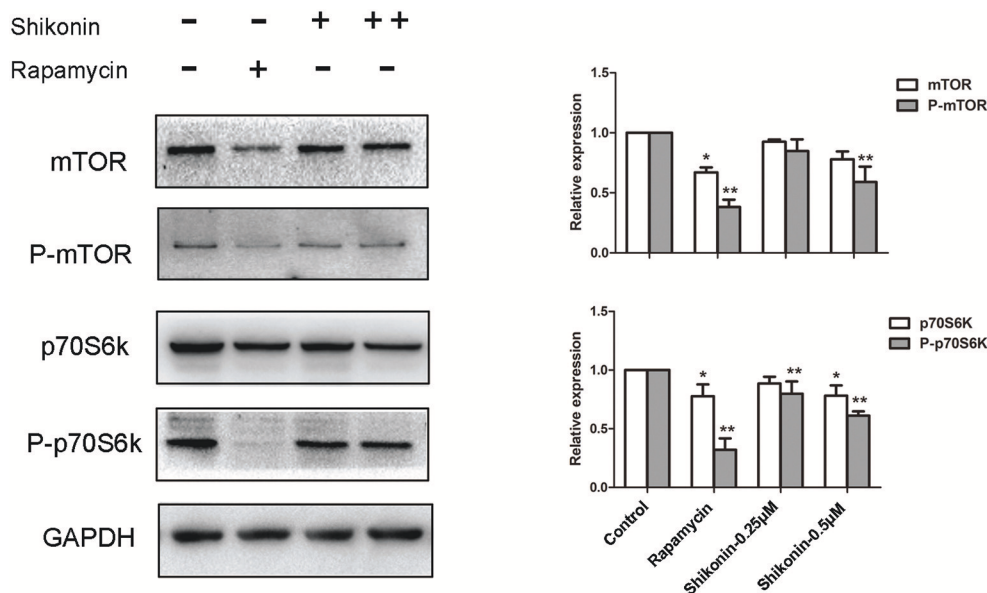


FIGURE 9 | Shikonin inhibits T-cell mTOR signaling *in vitro*. The protein expressions of phosphorylated p70S6K (P-p70S6K), p70S6K, P-mTOR, and mTOR in T cells were measured using western blotting analysis 2 days after T cells were stimulated *in vitro* with anti-CD3/CD28 Abs in the absence or presence of shikonin. A representative of Western blot images of P-p70S6K, p70S6K, p-mTOR, and mTOR expressions was shown (left panel). GAPDH was used as a loading or internal control. OD values (relative to GAPDH, right panel) in column graphs are presented as means \pm SD from three separate experiments (* $P < 0.05$ and ** $P < 0.01$, $n = 3$).

control while CsA did the opposite, suggesting that shikonin and CsA suppress allograft rejection via totally differential mechanisms. As a conventional immunosuppressive drug, CsA also suppresses allograft rejection by inhibiting T cell activation (3). Interestingly, it has been shown that CsA, a widely used conventional immunosuppressant, prevents tolerance induction by costimulatory blockade (29–31) since it inhibits the expression of IL-2 (32–34), an important growth factor for Treg development (28, 35, 36). Perhaps, this is why CsA did not promote Treg generation in our study although it increased TGF- β 1 expression. Therefore, shikonin rather than CsA may be used to induce allograft tolerance in futuristic study.

IDO has been shown to play an important role in Treg generation and function. DC-derived IDO is essential for Treg generation (37), expansion (38), and activation (39). IDO also suppresses allograft rejection by inducing Tregs (40, 41). Moreover, IDO-expressing MDSCs inhibits islet xenograft rejection (42) while DCs expressing IDO also suppresses allograft rejection by expanding Tregs (43). Here we found that shikonin treatment increased IDO expression in skin allografts and DCs *in vitro* and induced Tregs as well. Therefore, it's possible that shikonin promotes Treg generation via upregulating IDO expression in DCs. On the other hand, we demonstrated that shikonin hindered DC maturation post-transplantation. Thus, it's also possible that shikonin induces Tregs via suppression of DC maturation.

In present study, FACS-sorted CD4⁺CD25⁻ T cells were stimulated with anti-CD3/CD28 Ab and recombinant IL-2 or TGF- β 1 in the absence or presence of shikonin for

4 days. Our data demonstrated that shikonin promoted CD4⁺Foxp3⁺ Treg differentiation nearly as effectively as TGF- β 1 *in vitro*. Previous studies have shown that TGF- β 1 dramatically promotes naïve CD4⁺ T cell differentiation into Tregs (44). Thus, shikonin appears to be a potent inducer of Tregs. Additionally, depleting CD25⁺ Tregs mostly reversed skin allograft survival prolonged by shikonin, suggesting that shikonin extends allograft survival likely through induction of CD4⁺CD25⁺ Tregs.

T cells play a key role in the initiation of immune responses (45). Autoreactive T cells can induce autoimmune diseases while alloreactive T cells cause graft rejection (46, 47). T cells activated in draining lymph nodes migrate to a transplanted organ/tissue and orchestrate the process of graft rejection. Then activated effector T cells produce large amounts of proinflammatory cytokines, leading to the tissue destruction and ultimate allograft rejection. Therefore, it is imperative to inhibit T cell activation in order to suppress allograft rejection. In our studies, we found that shikonin significantly suppressed the proliferation of T cells and their production of IFN- γ and IL-17A *in vitro* while increasing IL-10 and TGF- β 1 levels. Further, shikonin inhibited gene expressions of proinflammatory cytokines, including IFN- γ , TNF- α , IL-6, and IL-17, in skin allografts. Thus, shikonin inhibits allograft rejection by suppressing T cell activation and expressions of proinflammatory cytokines in an allograft.

Rapamycin, an inhibitor of mTOR signaling, suppresses T cell proliferation by preventing cells from entering G1-phase (48, 49). It has been shown that rapamycin can promote CD4⁺Foxp3⁺ Treg generation (50–52). In present

study, we demonstrated that shikonin induced CD4⁺Foxp3⁺ Tregs *in vivo* and *in vitro* and that depletion of the Tregs reversed allograft survival induced by shikonin. We then asked whether shikonin induced Tregs also by blocking mTOR signaling pathway. Indeed, shikonin effectively inhibited the protein expression of phospho-p70S6K and phospho-mTOR in T cells. Therefore, shikonin turns out to be a novel mTOR inhibitor.

In conclusion, shikonin significantly prolonged skin allograft survival in a mouse model of skin allotransplantation by promoting CD4⁺Foxp3⁺ Treg differentiation. It also suppressed T cell proliferation *in vitro* while reducing their mTOR signaling. Furthermore, shikonin inhibited expressions of proinflammatory cytokines while increasing expression of immunosuppressive cytokines and IDO in skin allografts. Our data revealed a novel role of shikonin in inducing Tregs/IDO and suppressing allograft rejection. These findings indicate that shikonin, an originally natural product, may be utilized as a potent immunosuppressive drug for the prevention of human transplant rejection in the future.

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DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

QiZ performed experiments and wrote the manuscript. FQ and YC performed some experiments. CL, HL, and C-LL analyzed the data. QuZ and ZD edited the manuscript.

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Transiently Activated Human Regulatory T Cells Upregulate BCL-XL Expression and Acquire a Functional Advantage *in vivo*

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Regulatory T cells (Tregs) can control excessive or undesirable immune responses toward autoantigens, alloantigens, and pathogens. In transplantation, host immune responses against the allograft are suppressed through the use of immunosuppressive drugs, however this often results in life-threatening side effects including nephrotoxicity and an increased incidence of cancer and opportunistic infections. Tregs can control graft-vs.-host disease and transplant rejection in experimental models, providing impetus for the use of Tregs as a cellular therapy in clinical transplantation. One of the major barriers to the widespread use of Treg cellular therapy is the requirement to expand cells *ex vivo* to large numbers in order to alter the overall balance between regulatory and effector cells. Methods that enhance suppressive capacity thereby reducing the need for expansion are therefore of interest. Here, we have compared the function of freshly-isolated and *ex vivo*-manipulated human Tregs in a pre-clinical humanized mouse model of skin transplantation. Sorted human CD127^{lo}CD25⁺CD4⁺ Tregs were assessed in three different conditions: freshly-isolated, following transient *in vitro* activation with antiCD3/antiCD28 beads or after *ex vivo*-expansion for 2 weeks in the presence of antiCD3/antiCD28 beads and recombinant human IL2. While *ex vivo*-expansion of human Tregs increased their suppressive function moderately, transient *in vitro*-activation of freshly isolated Tregs resulted in a powerful enhancement of Treg activity sufficient to promote long-term graft survival of all transplants *in vivo*. In order to investigate the mechanisms responsible for these effects, we measured the expression of Treg-associated markers and susceptibility to apoptosis in activated Tregs. Transiently activated Tregs displayed enhanced survival and proliferation *in vitro* and *in vivo*. On a molecular level, Treg activation resulted in an increased expression of anti-apoptotic *BCL2L1* (encoding BCL-XL) which may be at least partially responsible for the observed enhancement in function. Our results suggest that *in vitro* activation of human Tregs arms them with superior proliferative and survival abilities, enabling them to more effectively control alloresponses. Importantly, this transient activation results in a rapid functional

enhancement of freshly-isolated Tregs, thereby providing an opportunity to eliminate the need for *in vitro* expansion in select circumstances. A protocol employing this technique would therefore benefit from a reduced requirement for large cell numbers for effective therapy.

Keywords: regulatory T cells, Tregs, tolerance, rejection, BCL-XL, *in vivo*, humanized mouse model, apoptosis

INTRODUCTION

Regulatory T cells (Tregs) are critical in the control of immune homeostasis as demonstrated by the development of autoimmune pathologies following their elimination (1) and the resolution of disease following their adoptive transfer (2). However, due to the high precursor frequency of alloreactive T cells in transplantation, unless the balance of Tregs to T effector cells (Teffs) is significantly altered, transplants are rejected despite the presence of functional Tregs (3). Current clinical practice is focused mainly on disarming the effector arm of the alloresponse using immunosuppressive drugs to deplete T cells or inhibit their proliferation and function (4). Such an approach leads to generalized immunosuppression, exposing patients to the cytotoxic effects of these drugs and increasing the risk of cancer and opportunistic infections. There is therefore increasing focus on studying the biology and function of Tregs for their ability to control graft-vs.-host disease (GVHD) and allograft rejection with the aim of utilizing them as a cellular therapy (5, 6).

Tregs are commonly described as CD25⁺CD4⁺ T cells in mice and CD127^{lo}CD25⁺CD4⁺ T cells in humans, and exhibit sustained expression of the master regulator transcription factor, FOXP3. Thymically-derived, naturally-occurring Tregs, tTregs, are the population most studied as a possible source of therapeutic cells, with the majority of protocols utilizing *in vitro* expanded tTregs [reviewed in (7, 8)]. So far, both freshly isolated (9) and *in vitro* expanded tTregs (10) have been tested in phase I clinical trials as a prevention of GVHD after HSC transplantation and proved to be safe, however their comparative efficacy is unclear and has not been tested so far.

Humanized mouse models provide a useful pre-clinical tool to study *in vivo* effectiveness of human Treg populations. Using these models, expanded human CD127^{lo}CD25⁺CD4⁺ Tregs have been shown to control rejection in vessel (11), islet (12) and skin (13, 14) transplantation and to prevent GvHD (15). However, the direct comparison of the *in vivo* potency of freshly isolated and *in vitro* expanded human Tregs is lacking. In this study, we compare the ability of suboptimal doses of freshly sorted and *in vitro* expanded human CD127^{lo}CD25⁺CD4⁺ Tregs to promote human skin allograft survival and demonstrate that higher effectiveness of expanded Tregs can be compensated by transient activation of freshly isolated Tregs. Recently-activated Tregs are characterized by an increased expression of Treg functional markers and better *in vitro* and *in vivo* survival, correlating with an increased expression of anti-apoptotic BCL-XL. The ability to enhance Treg function without long *in vitro* culture may be of value in the treatment of specific immunopathological situations.

MATERIALS AND METHODS

Mice

Immunodeficient BALB/c Rag2^{-/-} IL2rγ^{-/-} mice were purchased from Jackson Laboratories (Maine, USA) and housed under specific pathogen-free conditions in the Biomedical Services Unit at the John Radcliffe Hospital (Oxford, UK). Animals were treated with strict accordance to the UK Animals (Scientific Procedures) Act of 1986 and under PPL P8869535A. Mice between ages of 6 and 12 weeks were used.

Procurement of Human Skin and Blood

Healthy skin and blood was donated from patients undergoing plastic surgery procedures as previously described (13) and with full informed consent under approval number 07/H0605/130 from the Oxfordshire Research Ethics Committee B. PBMCs were isolated from buffy coats or leukocyte cones from healthy volunteers (NHSBT, UK).

Skin Grafting

Skin grafting was performed as previously described (13). Briefly, 1 × 1-cm piece of human skin was fashioned and sutured to the mouse recipient skin on the left dorsal thorax over the costal margin. Grafts were left to heal for 35 days, before receiving an intraperitoneal injection of 5 × 10⁶ human peripheral blood mononuclear cells (PBMCs) allogeneic to the graft donor. Skin grafts were monitored regularly until loss. In experimental groups with Treg cells, 1 × 10⁶ Tregs from the same donor as PBMCs were coinjected with PBMCs. In all mice the degree of human leukocyte reconstitution was measured by flow cytometry at the time of harvest. Mice with human leukocyte chimerism levels of >0.1% in the blood or >1% in the spleen were defined as reconstituted and included in the study (13). Skin allograft survival time was calculated from the point of PBMC injection to the point of complete graft loss/visible rejection.

Sorting and Expansion of Human Tregs Cells

Human Tregs were sorted and expanded as previously described (16) with minor modifications. Briefly, CD25⁺ cells were bead-enriched (CD25 Microbeads, Miltenyi Biotech) from PBMCs isolated from buffy coats from healthy volunteers (NHSBT, UK). CD127^{lo}CD25⁺CD4⁺ Tregs were sorted using a BD FACSAria cell sorter (Becton Dickinson) after staining with anti-CD127 PE, anti-CD25 PE-Cy7 (both Becton Dickinson) and anti-CD4 ECD (Beckman Coulter). Sorted cells were either used unmanipulated, activated overnight (15 h in 37°C 5%CO₂ with anti-CD3/anti-CD28 beads (Invitrogen) at 1:5 bead:cell ratio), or expanded *in vitro* with 1000U/ml recombinant human IL-2 (rhIL-2,

Chiron) and anti-CD3/anti-CD28 beads (Invitrogen) during two, 7 days long, expansion rounds, followed by resting after beads removal. Cells were cultured in RPMI-1640 medium (Sigma) supplemented with L-glutamine, penicillin-streptomycin (both Sigma), sodium pyruvate (Gibco) and 10% human AB pooled serum. In some experiments, expanded Tregs were activated overnight in a similar manner to sorted Treg (15h in 37°C 5%CO₂ with anti-CD3/anti-CD28 beads at 1:5 bead:cell ratio).

***In vitro* Suppression Tests**

To assess Treg suppressive capacity, 5×10^4 PBMC were incubated with 1×10^5 irradiated allogeneic PBMC and co-cultured with decreasing number of Tregs. ³H-thymidine (Perkin Elmer) was added for the last 16–18 h of the 7 day culture. All experimental conditions were done in 4–6 replicates. Results were obtained as cpm (counts per minute) and normalized to positive control (PBMC+allo) with positive control values setup as maximum (100%) proliferation and all other values recalculated accordingly. Cpm values over 10,000 for positive control (PBMC+allo) were required to classify test as passing quality control for proliferation.

***In vitro* Cellular Proliferation and Apoptosis Assays**

To measure proliferation and apoptosis, freshly isolated Tregs were stained with 10 μM Cell Trace Violet (CTV; Invitrogen), activated for 15 h with anti-CD3/anti-CD28 beads (at a ratio of 1 bead to 5 cells ratio) or left untreated. After activation, beads were carefully removed and cells plated at 10^5 onto 96 well U bottom plates in the presence or absence of rhIL-2 at 250 U/ml. After 5 days of culture, cells were washed with Annexin V binding buffer and stained with Annexin V and 7-AAD (both eBioscience). Stained cells were resuspended in Annexin V binding buffer and FACS analyzed within an hour.

***In vivo* Survival and Proliferation**

To measure *in vivo* survival and proliferation, freshly isolated Tregs were stained with 10 μM CTV (Invitrogen), activated for 15 h with anti-CD3/anti-CD28 beads (at a ratio of 1 bead to 5 cells) or left untreated. After activation, beads were carefully removed and 1×10^6 Treg cells were mixed with 5×10^6 PBMCs, labeled with 1 μM CFSE (eBioscience), from the same donor. PBMCs and Tregs were injected intraperitoneally into BALB/c Rag2^{-/-} IL2rγ^{-/-} mice and human cells isolated from peritoneal lavage on d5 for analysis as previously described (17).

Flow Cytometry

For analysis of *in vivo* experiments, cells were stained with antibodies against human CD45 APC (Invitrogen), CD4 ECD (Beckman Coulter), CD3 Pacific Blue (eBioscience) CD8 PE and CD25 PE-Cy7 (BD) and the viability dye 7-AAD (eBioscience). To analyse expression of Treg-associated markers, freshly sorted or expanded Tregs were stimulated for 15h with anti-CD3/anti-CD28 beads at a ratio of 1 bead to 5 cells, or left untreated. Cells were stained with 7-AAD and antibodies against GITR FITC (R&D Systems), CTLA-4 PE, CD69 APC-Cy7, CD25 PE-Cy7 (BD), TIGIT PE, OX-40 FITC, TIM-3 APC, CD39

PE, FOXP3 eFluor 450, Perforin APC (eBioscience) Helios AlexaFluor 647 (Biolegend), and CD4 ECD (Beckman Coulter). For intracellular antigens (FOXP3, Helios, Perforin, CTLA-4), cells were fixed and permeabilized using a Foxp3 Staining Buffer Set (eBioscience). Samples were acquired using a BD FACSCanto (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences). For staining for BCL-XL and MCL1, Abcam anti-BCL-XL FITC (7B2.5; ab26148) and anti-MCL-1 Alexa Fluor 488 (Y37, ab197529) antibodies were used, respectively, following manufacturer's instructions. Briefly, the cells were fixed with 4% paraformaldehyde and permeabilized with PBS/0.1% Tween. The cells were then blocked with 10% normal goat serum/0.3 M glycine, followed by incubation with the antibody.

Real-Time PCR

Total RNA (including small RNA) was isolated from cell pellets using a mirVana PARIS Kit (Ambion, Applied Biosystems). For mRNA analysis, cDNA was generated with High Capacity RNA-to-cDNA Kit (Applied Biosystems) followed by real-time PCR using *HPRT* primers and probe as described previously (18) and TaqMan Gene Expression Assays (Applied Biosystems) for the following genes: Hs99999146_m1 (*BCL2L1*, coding BCL-XL), Hs00608023_m1 (*BCL2*), Hs99999001_m1 (*BAX*) and Hs01083836_m1 (*BCL2L11*, coding BIM). Stratagene Mx3000P thermo cycler (Agilent Technologies) was used for qPCR. Delta Ct values were calculated using *HPRT* as an endogenous control and converted to $2^{-\Delta\Delta Ct}$ values.

For microRNA analysis, samples were analyzed for expression of RNU48 (assay ID 001006), hsa-miR-16-5p (assay ID 000391) and hsa-let-7c (assay ID 000379) using TaqMan MicroRNA Assays (Life Technologies). cDNA reactions were performed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). Subsequent qPCR was performed using TaqMan Universal Master Mix II and MicroRNA Assays using a Stratagene Mx3000P thermo cycler (Agilent Technologies). Delta Ct values were calculated using RNU48 as an endogenous control and converted to $2^{-\Delta\Delta Ct}$ values.

Tissue Typing

Blood from buffy coats and skin donors was analyzed at the Oxford Transplant Center Transplant Immunology and Immunogenetics laboratory. A full typing was performed for HLA-A, -B, -Cw, -DR and -DQ using PCR-SSP method.

Statistical Analysis

Statistical evaluations were performed using Graphpad Prism software (GraphPad Software Inc, California). Survival data were analyzed using log-rank tests. Groups of three or more were compared using a non-parametric Kruskal-Wallis ANOVA with Dunn's *post-hoc* multiple comparisons test. For comparison of two groups non-parametric Mann-Whitney *U*-tests were applied. To assess differences between *in vitro* suppressive capacities of different Treg populations the area under the curve (AUC) method was used as described by previously (19). *p*-values <0.05 were considered significant.

RESULTS

***Ex vivo* Expanded Human Tregs Cells Have Increased *in vitro* and *in vivo* Suppressive Capacity**

The main aim of expanding Tregs *ex vivo* is to provide a sufficient number of cells for clinical application, altering the balance between Tregs and Teffs (20). However, the process of expansion has the potential to impact Treg biology and function. To determine whether expansion of Tregs influences their suppressive function, Tregs were sorted from a single donor, rested overnight and assessed directly in a suppression test, or *in vitro* expanded in the presence of anti-CD3/anti-CD28 beads and rhIL-2 before also being rested and then subjected to the same suppression assay assessment. At the higher Treg to responder ratios, expanded Tregs have shown a trend toward higher suppressive capacity than freshly sorted Tregs (**Figure 1A** top panel, **Figure 1B**). We next assessed whether this observation was also reflected *in vivo*. We have previously shown that treatment with *ex vivo* expanded Tregs results in long-term graft acceptance when adoptively transferred at a ratio of 1:1 Tregs to PBMCs (13), whereas a reduced (or “suboptimal”) number of Tregs (1:5 Tregs:Teff or below) results in graft prolongation (21). To assess the *in vivo* suppressive capacities of freshly isolated and *ex vivo* expanded Tregs, each of these populations was adoptively transferred together with PBMCs at the suboptimal 1:5 dose. As expected, expanded Tregs provided significant allograft prolongation with 75% grafts being accepted long-term (**Figures 1C,D**, median survival time (MST) >100 days, $p = 0.0013$ vs. PBMC group). However, in line with the *in vitro* data, freshly isolated Tregs only temporarily extended graft survival (MST 51 days compared to 27 days in the PBMC alone group) with 2 out of 6 grafts accepted long-term (**Figures 1C,D**, $p < 0.0001$ vs. PBMC group).

Transient Activation Increases Suppressive Abilities of Freshly Isolated Tregs

Having observed trend toward enhanced suppressive capacity of expanded human Tregs over their freshly isolated counterparts, we next asked whether *ex vivo* expansion is necessary for Tregs to increase their regulatory capacity or whether activation without expansion would be sufficient. We therefore activated freshly isolated human Tregs for 15 h in the presence of anti-CD3/anti-CD28 beads and compared their suppression *in vitro* to non-activated freshly isolated Tregs. This transient activation notably increased the regulatory capacity of Tregs, an effect which was especially pronounced at higher Treg to PBMC ratios (**Figure 1A** bottom panel, **Figure 1B**). In support of the *in vitro* suppression data, transient anti-CD3/anti-CD28 activation of Tregs also resulted in excellent regulation of alloresponses *in vivo*, leading to long-term acceptance of all skin allografts (**Figures 1C,D**, MST >100 days, $p < 0.0001$ vs. PBMC group). Transiently activated Tregs were more effective *in vivo* than freshly isolated cells ($p = 0.0137$), however there was no statistically significant difference between *in vivo* function of short-term activated and expanded Tregs ($p = 0.2207$).

***In vitro* Activation Increases Expression of Treg Markers**

Next, we examined the expression of Treg-associated markers in Tregs either following activation for 15 h, after *in vitro* expansion, or immediately following fresh isolation (**Figure 2**). An additional group of transiently reactivated expanded Tregs was also examined to determine whether restimulation following expansion promotes the upregulation of the same molecules as in stimulated freshly isolated cells. The classical Treg markers GITR and CTLA-4 were up-regulated both after transient activation and *in vitro* expansion as compared with freshly isolated Tregs, although the difference was only statistically significant for transient activation (**Figures 2A,E**). A similar pattern of expression was also observed for the Ig family member with immunomodulatory function, TIGIT (22, 23) (**Figures 2A,E**). As expected, the early T cell activation marker, CD69, was induced upon activation (**Figures 2B,E**). Similarly, OX-40 and perforin expression was induced on transiently activated Tregs, both freshly isolated and expanded with statistically significant differences observed for both markers, both after transient activation of freshly isolated cells and restimulation post-expansion (**Figures 2B,E**), suggesting different expression kinetics compared with GITR, CTLA-4 and TIGIT. T cell immunoglobulin and mucin domain-3 protein (TIM-3), which is expressed on fully differentiated effector T cells [mainly Th1 (24) and Th17 (25) cells], and was described as a marker of short-lived but highly suppressive Tregs (26) was expressed by about 20–30% of expanded Treg but not by freshly isolated or transiently activated Tregs (**Figures 2C,E**). In contrast, expression of CD39 and Helios was not changed upon activation or expansion (**Figures 2D,E**). There was a trend toward an increase in FOXP3 expression levels per cell after activation, although this was not statistically significant (**Figure 3A**). The proportion of FOXP3+ cells was also not different after activation (**Figure 3B**). Overall, the phenotypic analysis of *in vitro* stimulated Tregs demonstrated significant differences in expression of functional Treg markers, in agreement with their increased suppressive function.

Transient Activation Promotes Treg Proliferation and Survival

As demonstrated above, short-term transient activation and, to a certain degree, 2-week *in vitro* expansion of human Tregs influence the function of freshly isolated cells (**Figure 1**). The improved suppressive activity of both activated and expanded Tregs correlates with increased expression of Treg markers and functional molecules (**Figure 3**), however their differential expression pattern suggests further differences between both groups. To further explore the differences between freshly isolated and activated or expanded Tregs, we investigated the ability of Tregs to survive *in vitro* and *in vivo*. First, we measured the effect of transient activation on apoptosis of freshly isolated and expanded Tregs cultured for 5 days in the presence or absence of IL-2 without further stimulation. Without IL-2, the majority of expanded Tregs and about 30% of freshly isolated Tregs underwent apoptosis (**Figure 4A**, left panels), whereas the addition of exogenous IL-2 decreased the frequency of cells

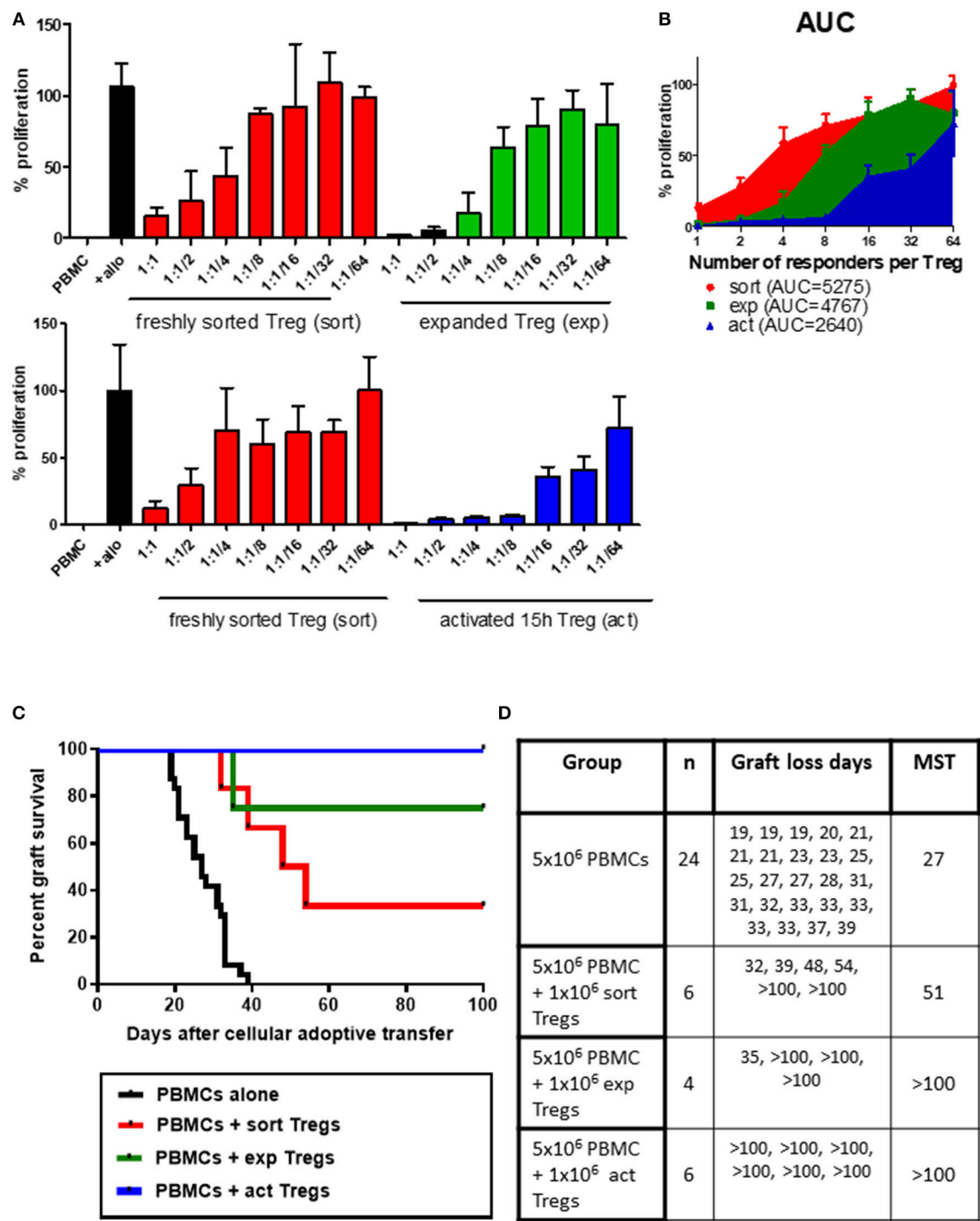


FIGURE 1 | *Ex vivo* expanded or transiently activated human Tregs have superior *in vitro* and *in vivo* suppressive capacity. **(A)** 5×10^4 PBMCs were incubated with 1×10^5 irradiated allogeneic PBMCs and co-cultured with a decreasing number of freshly isolated (sort) or *ex vivo* expanded (exp) (top panel) or transiently activated (act) (bottom panel) human Tregs. Ratios represent ratio of responder PBMC to Tregs. Data represented as percentage of proliferation of allogeneically-stimulated PBMC. **(B)** Area under curve for the frequency of proliferating responders in the presence of Tregs at different ratios. **(C)** Mice received a skin graft and 5×10^6 allogeneic PBMCs together with 1×10^6 freshly isolated, transiently activated (15 h) or *ex vivo* expanded Tregs. Data are pooled from different skin/cell donor combinations. **(D)** Table representing data from B, MST—median survival time. *p*-values using the log-rank test for each comparison are shown.

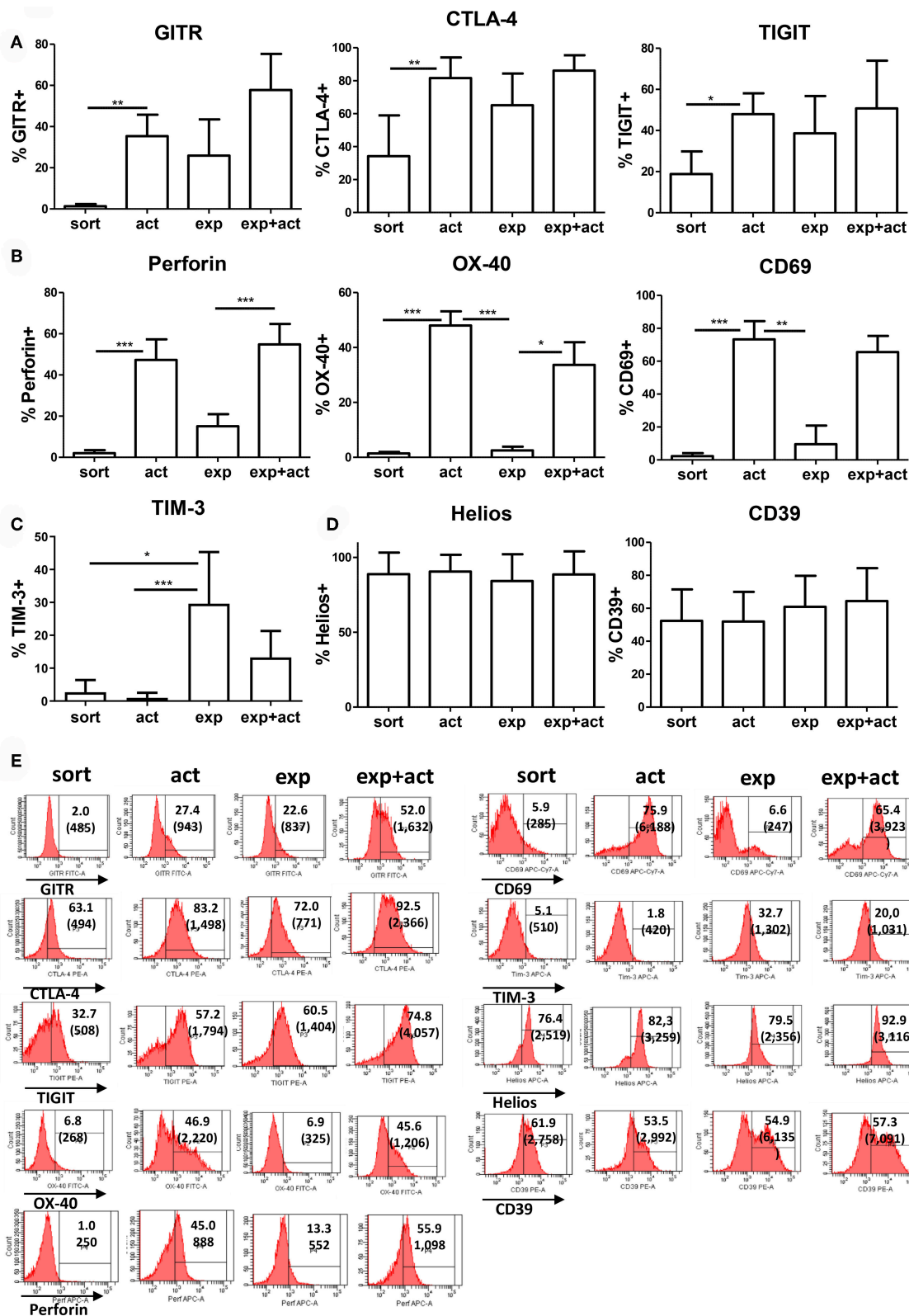
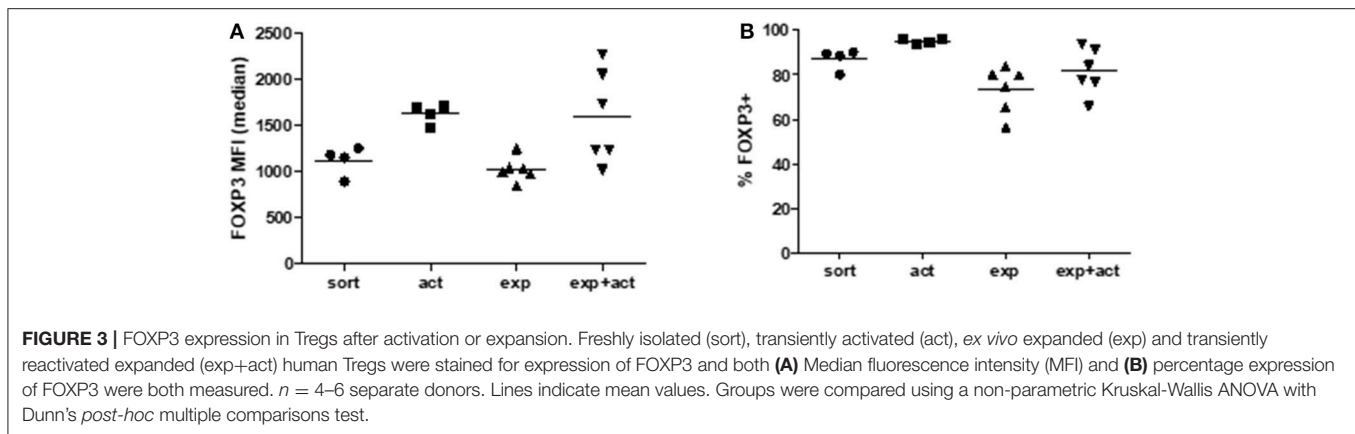


FIGURE 2 | Expression of Treg-associated markers. (A–D) Freshly isolated (sort), transiently activated (act), ex vivo expanded (exp) and transiently reactivated expanded (exp+act) human Tregs were stained for expression of Treg-associated markers. (E) Representative FACS plots of the Treg-associated markers shown in (A–D). $n = 4–7$ separate donors. * $p < 0.05$ Error bars represent the means and standard deviations (SD). Groups were compared using a non-parametric Kruskal-Wallis ANOVA with Dunn's *post-hoc* multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$.



with apoptotic features (Figure 4A, right panels). More detailed analysis of cells undergoing apoptosis in the presence of IL-2, which is a major survival factor for Tregs (27), revealed that the frequency of late apoptotic (7-AAD⁺AnnV⁺) cells was decreased in activated freshly isolated Tregs but increased in activated expanded ones (Figure 4B). In line with this finding, the percentage of live cells (7-AAD⁻AnnV⁻) increased in stimulated freshly isolated cells but was reduced in activated expanded Tregs (Figure 4C). Importantly, when live cells were enumerated, there was a more than two-fold increase in cell number in activated freshly sorted Tregs as compared with expanded Tregs (Figure 4C, right panel).

The 2–3 fold increase in the number but not percentage of live cells in activated Tregs suggested intense proliferation. Indeed, staining with the proliferation dye CTV revealed that close to 70% of activated Tregs activated with IL-2 divided at least once, with up to 6 divisions observed (Figure 5A). This was despite the stimulatory signal being removed after 15h. Conversely, unstimulated Tregs remained mainly undivided (Figure 5B). Next, we assessed whether activated Tregs are more likely to survive and expand *in vivo* by co-injecting BALB/c Rag2^{-/-}IL2r γ ^{-/-} mice with 5×10^6 CFSE-labeled PBMCs and 1×10^6 CTV-labeled Tregs and examining the number of CTV⁺ cells recovered in a peritoneal lavage after 5 days. In agreement with the *in vitro* data, we observed more Tregs in the activated group, with these cells displaying enhanced proliferation (Figure 5C). Importantly, activated Tregs were also found to be more suppressive *in vivo* than freshly isolated cells, as demonstrated by their ability to inhibit proliferation of CD8⁺ T cells in the co-injected PBMCs (Figure 5D).

Activation Enhances Tregs *BCL2L1* (*BCL-XL*) Expression

To further explore the finding that the transient activation of Tregs results in their enhanced survival, we measured the expression of pro-apoptotic [*BAX* and *BCL2L11* (encoding BIM)] and anti-apoptotic [*BCL2* and *BCL2L1* (coding BCL-XL)] genes in activated Tregs. Our real-time PCR data showed that *BCL2L1* but not *BCL2* expression was upregulated upon stimulation (Figure 6A). At the same time there was no difference in the

expression of pro-apoptotic *BAX* and *BCL2L11* (Figure 6A), confirming that activated Tregs are less prone to apoptosis. Interestingly, when we incubated Tregs in the presence of IL-2 for 4 further days after removal of the activation stimulus, there was a trend toward upregulated expression of *BCL2L* and downregulated expression of *BCL2* when compared with unstimulated Tregs (Figure 6B). In order to further explore the possible mechanisms regulating *BCL2* and *BCL2L1* expression in activated Tregs, we measured the expression of miR-16 and Let-7c microRNAs, which have been implicated in the regulation of *BCL2* (28) and *BCL-XL* (29, 30), respectively. Let-7c has been demonstrated to regulate *BCL-XL* expression in human hepatocellular carcinoma (30) and endothelial cells (29). Activated Tregs downregulated Let-7c expression (Figure 6C) while upregulating *BCL2L1* (*BCL-XL*) (Figure 6B), suggesting that Let-7c may also be engaged in the control of *BCL-XL* expression in Tregs. Conversely, there was no difference in miR-16 expression after Tregs activation (Figure 6C). Gene expression levels of *BCL2L1* correlated with increased *BCL-XL* levels on activated Tregs by FACS (Figure 6D). Levels of another apoptosis regulator, MCL1, were also increased (Figure 6E).

DISCUSSION

In recent years, the potential clinical application of Tregs both in transplantation and autoimmunity has attracted a great deal of enthusiasm. Here, we describe a feasible method for significantly enhancing human Treg suppressive capacity and demonstrate the efficacy of this approach in a pre-clinical *in vivo* transplantation model. We demonstrate that transient (15h) *in vitro* activation of freshly isolated human Tregs is sufficient to provide a functional advantage over unmanipulated freshly isolated Tregs and may therefore eliminate the need to expand Tregs *in vitro* to achieve clinical efficacy. This provides a useful optimization technique for current protocols that employ Tregs as a cellular therapy to prevent GVHD or graft rejection [reviewed in (5, 6, 31)]. This may be of particular importance in clinical situations where expansion is impractical or where time restrictions on cellular manipulation exist.

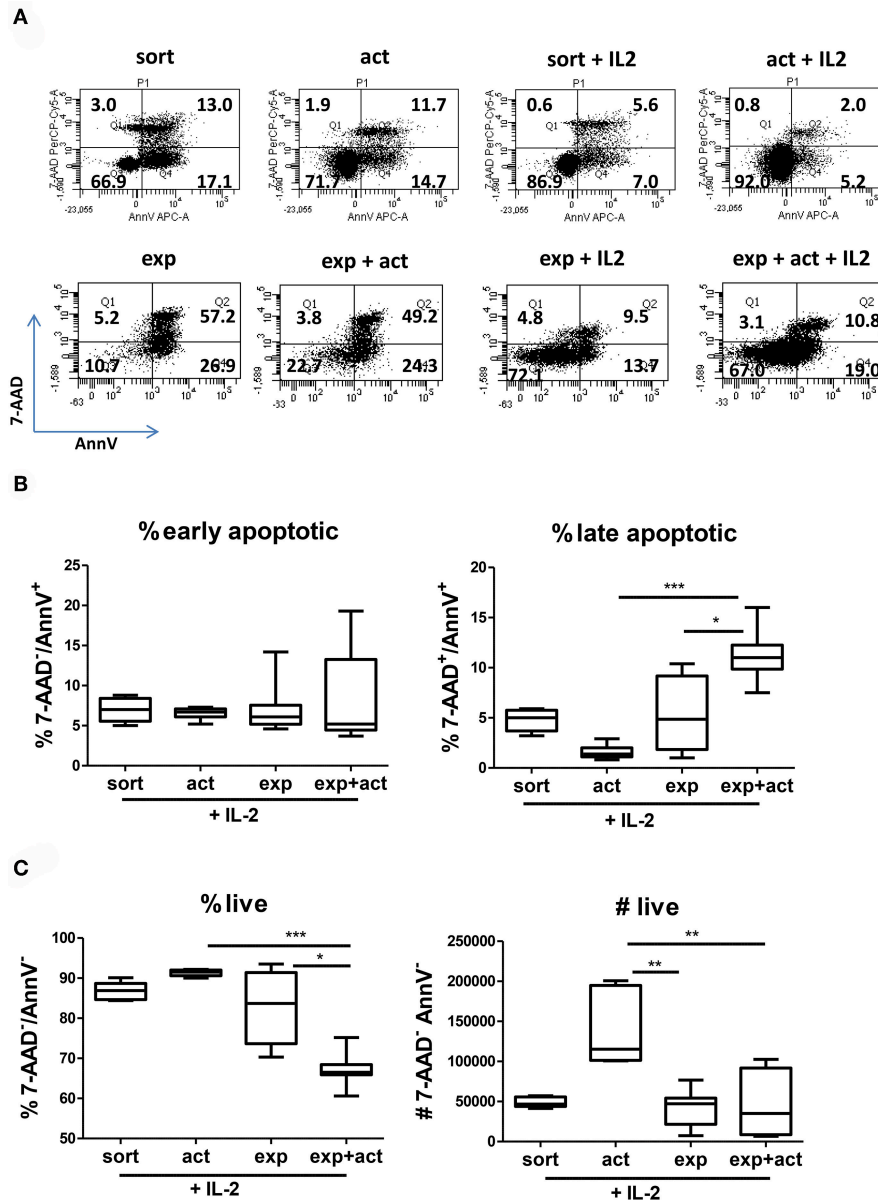


FIGURE 4 | Transient activation promotes Treg survival and responsiveness to IL-2. **(A–C)** CTV-labeled Tregs (10^5 per well) were activated *in vitro* for 15 h with anti-CD3/anti-CD28 beads and cultured for 5 days without further stimulation or cultured without any TCR stimulation at all. Where indicated (+IL-2), exogenous IL-2 was added. **(A)** Representative dot plots of cells stained with 7-AAD and Annexin V and the percentage of cells in each quadrant are depicted. **(B)** Percentage of early apoptotic (7-AAD⁺/AnnV⁺), late apoptotic (7-AAD⁺/AnnV⁺) and **(C)** percentage and number of live cells (7-AAD⁺/AnnV⁺) are shown. Data for **(A–C)** were obtained from 2 or 3 separate donors, each donor with 2–5 biological repeats (separate wells). Data from multiple cell donors were pooled. **(B,C)** min-max with median and interquartile range is shown. Groups were compared using a non-parametric Kruskal-Wallis ANOVA with Dunn's *post-hoc* multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

There is no consensus at present as to whether freshly isolated or expanded Tregs should be used in clinical cellular therapy; each option having its distinct advantages. Freshly isolated Tregs were used by Martelli and colleagues as a strategy for GVHD prevention after HSC transplantation with no adverse effects and moderate clinical efficacy (9). Meanwhile, Blazar and his team used cord blood-derived expanded Tregs (10), again with no adverse effects and a reduction in the incidence of higher grade

acute GVHD. Other groups have utilized expanded Tregs in T1D (32, 33) and kidney transplantation (34), whilst others yet have generated the cells from whole lymphocyte populations *ex vivo* (35). The advantage of using expanded Tregs is the ability to obtain large numbers of cells, however *ex vivo* expansion can be restrictively expensive and creates legitimate concerns regarding the safety of the clinical product as cells are subjected to a relatively long period (at least 2 weeks and in most cases 4–6

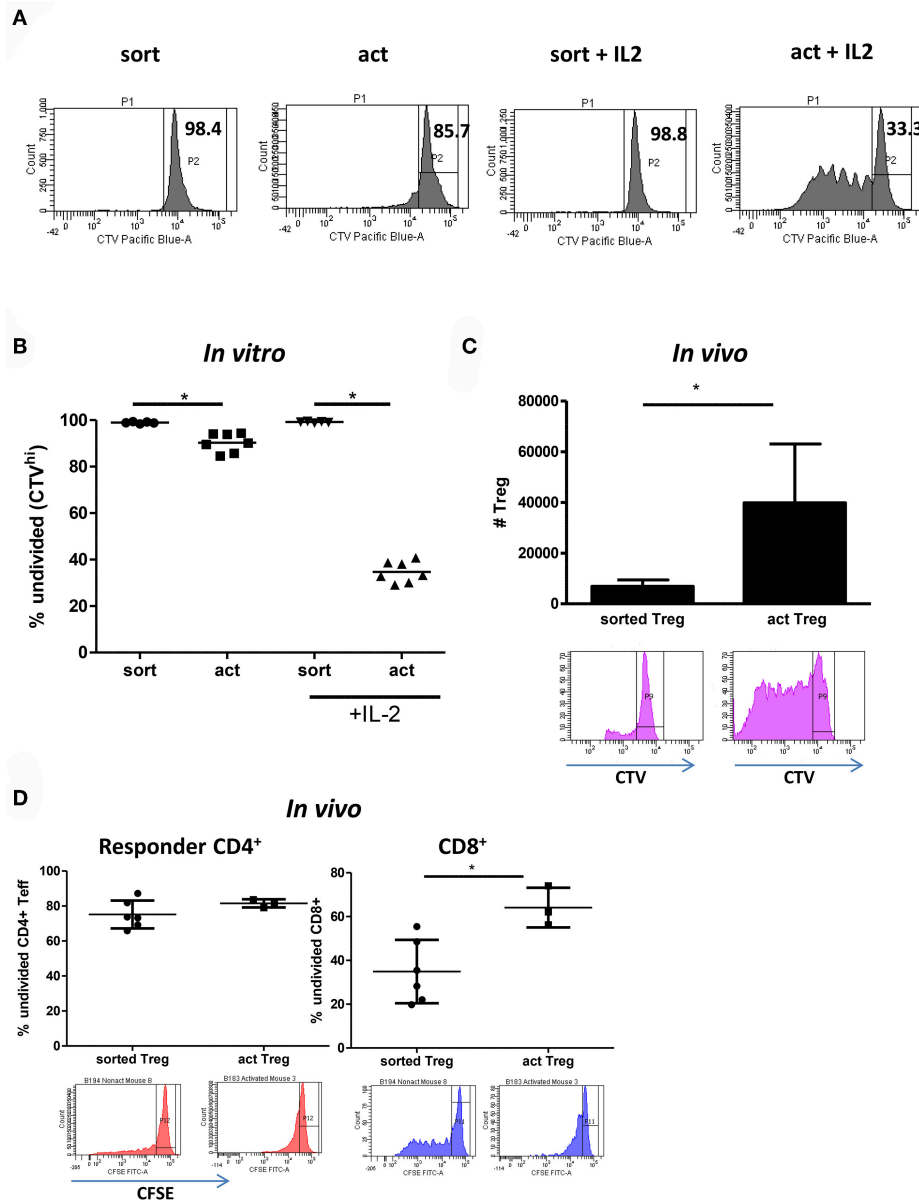


FIGURE 5 | Transient activation is sufficient to induce Treg proliferation if IL-2 is present. **(A)** Representative histograms and **(B)** graph depicting the percentage of undivided (CTV^{hi}) Tregs is shown. **(C)** 5×10^6 CFSE-labeled PBMC and 1×10^6 CTV-labeled freshly isolated Tregs ($n = 6$) or transiently activated Tregs ($n = 3$) were injected intraperitoneally into immunodeficient BRG mice. The number of CTV⁺ cells in the peritoneum on day 5 is depicted on the graph. Histograms represent CTV dilution in gated Tregs from freshly isolated and transiently activated Treg groups respectively. **(D)** Cells were prepared and injected as in **(C)**. The percentage of undivided (CFSE^{hi}) responder CD4⁺ and CD8⁺ lymphocytes is shown. CFSE dilution in gated responder CD4⁺ and CD8⁺ T cells in mice receiving freshly isolated and transiently activated Tregs is shown. **(C)** mean with SD is depicted, **(D)** each point represents separate mouse. Groups were compared using a non-parametric Mann-Whitney *U*-test. * $p < 0.05$.

weeks) of *in vitro* culture and manipulation. From this aspect, the possibility to activate freshly isolated Tregs overnight before use as a clinical product may be particularly helpful in situations where obtaining relatively large numbers of non-expanded Tregs is feasible, such as in HSC transplantation where donor Tregs may be isolated from a leukapheresis product. Other uses include situations in which treatment with the regulatory cellular therapy

must be instigated promptly and without prior notice, for example during an acute and unexpected autoimmune flare-up.

It is widely accepted that Tregs need to be activated by interaction of their TCR receptor with antigen presented in the context of MHC in order to elicit their suppressive ability. Additionally, it has been suggested that expanded Tregs have superior *in vitro* suppressive function compared with freshly

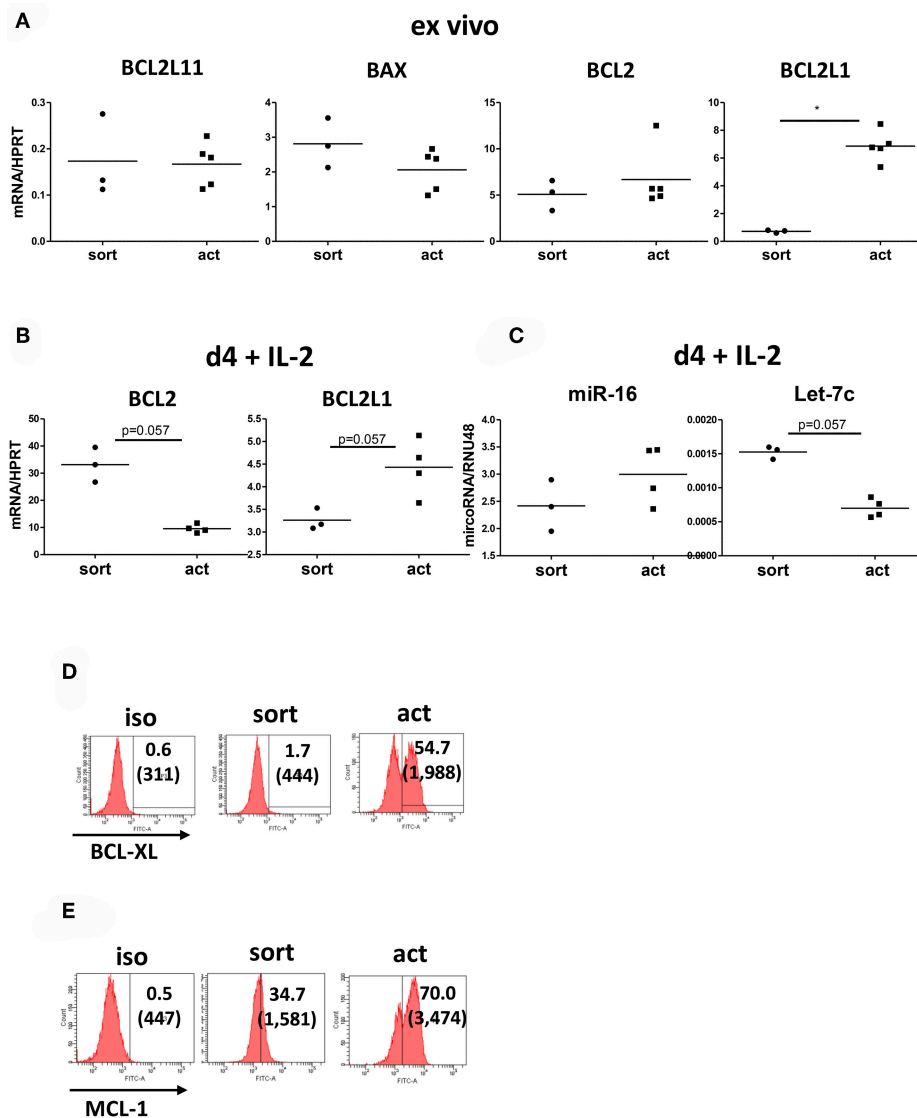


FIGURE 6 | Transiently activated Treg upregulate *BCL2L1* (*BCL-XL*) expression. **(A)** Real-time PCR analysis of expression of genes encoding pro-apoptotic [*BAX*, *BCL2L11* (*BIM*)] and anti-apoptotic [*BCL2*, *BCL2L1* (*BCL-XL*)] proteins in freshly isolated and transiently activated (15 h) Tregs. $n = 3-5$, data normalized to *HPRT*. **(B)** Real-time PCR analysis of *BCL2* and *BCL2L1* (*BCL-XL*) expression, normalized to *HPRT*, from day 4 cultures of unstimulated and 15 h transiently activated Tregs incubated with IL-2. **(C)** Real-time PCR analysis of miR-16 and Let-7c microRNA expression, normalized to RNU48, from cultures described in **(B)**. $n = 3-4$ donors, groups were compared using a non-parametric Mann-Whitney *U*-test, $*p < 0.05$. Additionally, levels of **(D)** BCL-XL and **(E)** MCL-1 were measured by FACS. Histograms from one representative donor are shown. $*p < 0.05$.

isolated cells (36, 37). Indeed, in our study *ex vivo* expanded Tregs showed a tendency toward being more suppressive than freshly isolated cells. However, simply activating freshly isolated Tregs for 15 h increased their suppressive capacity significantly. This finding was supported by the observation that freshly isolated Tregs are less prone to apoptosis; a feature that is even more pronounced after transient activation.

While improvements in Treg survival are important, it is clear that this is unlikely to be the only mechanism accounting for the functional changes observed. Both transiently activated and expanded (but not activated) Tregs upregulate the expression of

a number of Treg-associated markers including CTLA-4, GITR, TIGIT, and to a lesser degree, perforin. TCR activating signals are clearly important for Treg function, yet the duration and strength of these signals may result in different outcomes (38). Tregs likely require progressive signals provided at the appropriate time, to ideally result in enhanced survival and subsequent effective suppressive activity (39). Transiently activated Tregs may be at the first step of this sequential process. We also show that OX40 is induced on transient activation of Tregs but not expanded Tregs. The effects of OX40 expression or ligation on Tregs are complex and not completely understood (40). Some studies have

demonstrated that OX40 stimulation can negatively regulate the induction of Tregs from naïve or effector T cells (41, 42), yet OX40 signaling has also been shown to be important in the generation of Tregs during the TCR-independent phase of Treg development, with OX40^{-/-} mice having significantly reduced numbers of Tregs (43). Given the potential for OX40 to be a therapeutic target, its function on T cells and Tregs remains under intense investigation.

Apoptosis is regulated through a balance between the Bcl-2 family of proteins, such as pro-apoptotic BAX and BIM and anti-apoptotic BCL2 and BCL-XL (44). BAX acts as pro-apoptotic effector mediating mitochondrial outer membrane permeabilization and is regulated by sequestration by anti-apoptotic BCL2 and BCL-XL (45). BIM acts as a sensor of growth factor deprivation and can activate BAX by its release from sequestration. Importantly, the pro-survival proteins BCL2 and BCL-XL play distinct roles in regulating survival of quiescent and activated lymphocytes, respectively (46). After *in vitro* culture with IL-2 we observed a decrease in BCL2 but increase in BCL-XL mRNA in activated Tregs which may be at least partially responsible for the enhanced cell proliferation and survival, although other mechanisms are also likely to also be active. Such an inverse correlation between levels of BCL2 and BCL-XL has been observed in a number of cancers (47). Notably, increased levels of BCL-XL but not BCL2 are observed in T cells in response to CD28 costimulation (48), in line with our observation in Tregs. The precise molecular mechanisms regulating apoptosis in Tregs are still under active investigation. When compared to conventional CD4⁺ T cells, freshly isolated human Tregs have been shown to be more sensitive to apoptosis than freshly isolated Teffs, but this is reversed after *in vitro* culture in the presence of CD3 and CD28 stimulation and exogenous IL-2 (49). Importantly, enhanced Treg survival correlated with the 2-fold increase in the expression of BCL2 and six-fold increase in BCL-XL expression (49). In mice, increased Bcl-xL expression has been demonstrated both in naturally-occurring (50) and induced (51) Tregs and ectopic co-expression of FoxP3 and Bcl-xL in CD4⁺ T cells induces regulatory cells with improved persistence and function *in vitro* and *in vivo* (52).

Regulation of BCL-XL is complex and is controlled on both transcriptional and post-transcriptional levels. miRNA Let-7c has been demonstrated to inhibit BCL-XL expression in hepatoma (30) and endothelial (29) cells. In our study increased BCL-XL expression in cultured Tregs negatively correlated with Let-7c expression, suggesting a similar control mechanism in Tregs. Let-7c miRNA belongs to the ubiquitously-expressed prototypical

family of miRNAs (53). Targets for Let-7 include several genes involved in cell cycle and mitotic signaling such as HMGA2 (54), CDC25A and CDK6 (55), therefore its role in controlling Treg proliferation and survival is likely to be more complex and further studies are planned to explore this finding.

This is the first study to demonstrate and provide a potential mechanism for enhanced suppressive function in freshly isolated human Tregs following a short period of activation. This has important clinical implications, as it may provide a simple, efficient, rapid and cost-effective method for enhancing the suppressive capacity and survival of Treg cellular therapy.

ETHICS STATEMENT

This study was carried out in accordance with the Helsinki Declaration and approved by the Oxfordshire Research Ethics Committee B (approval number 07/H0605/130) with written informed consent from all subjects.

The animal studies were carried out in strict accordance to the recommendations of the UK Animals (Scientific Procedures) Act of 1986. The protocol was approved by the University of Oxford Animal Care and Ethics Review committee and all procedures were carried under PPL P8869535A.

AUTHOR CONTRIBUTIONS

JH, FI, KM, RG, and GB performed the experiments. JH, FI, KM, and KW conceived and designed the experiments. JH and FI analyzed the data. JH, FI, and KW wrote the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Tolerogenic Function of Regulatory T Cells in Pregnancy and Cancer

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Regulatory T cells, a subpopulation of suppressive T cells, are potent mediators of self-tolerance and essential for the suppression of triggered immune responses. The immune modulating capacity of these cells play a major role in both transplantation, autoimmune disease, allergy, cancer and pregnancy. During pregnancy, low numbers of regulatory T cells are associated with pregnancy failure and pregnancy complications such as pre-eclampsia. On the other hand, in cancer, low numbers of immunosuppressive T cells are correlated with better prognosis. Hence, maternal immune tolerance toward the fetus during pregnancy and the escape from host immunosurveillance by cancer seem to be based on similar immunological mechanisms being highly dependent on the balance between immune activation and suppression. As regulatory T cells hold a crucial role in several biological processes, they may also be promising subjects for therapeutic use. Especially in the field of cancer, cell therapy and checkpoint inhibitors have demonstrated that immune-based therapies have a very promising potential in treatment of human malignancies. However, these therapies are often accompanied by adverse autoimmune side effects. Therefore, expanding the knowledge to recognize the complexities of immune regulation pathways shared across different immunological scenarios is extremely important in order to improve and develop new strategies for immune-based therapy. The intent of this review is to highlight the functional characteristics of regulatory T cells in the context of mechanisms of immune regulation in pregnancy and cancer, and how manipulation of these mechanisms potentially may improve therapeutic options.

Keywords: regulatory T cells, immune tolerance, cancer, immunotherapy, pregnancy, preeclampsia, HLA class Ib

INTRODUCTION

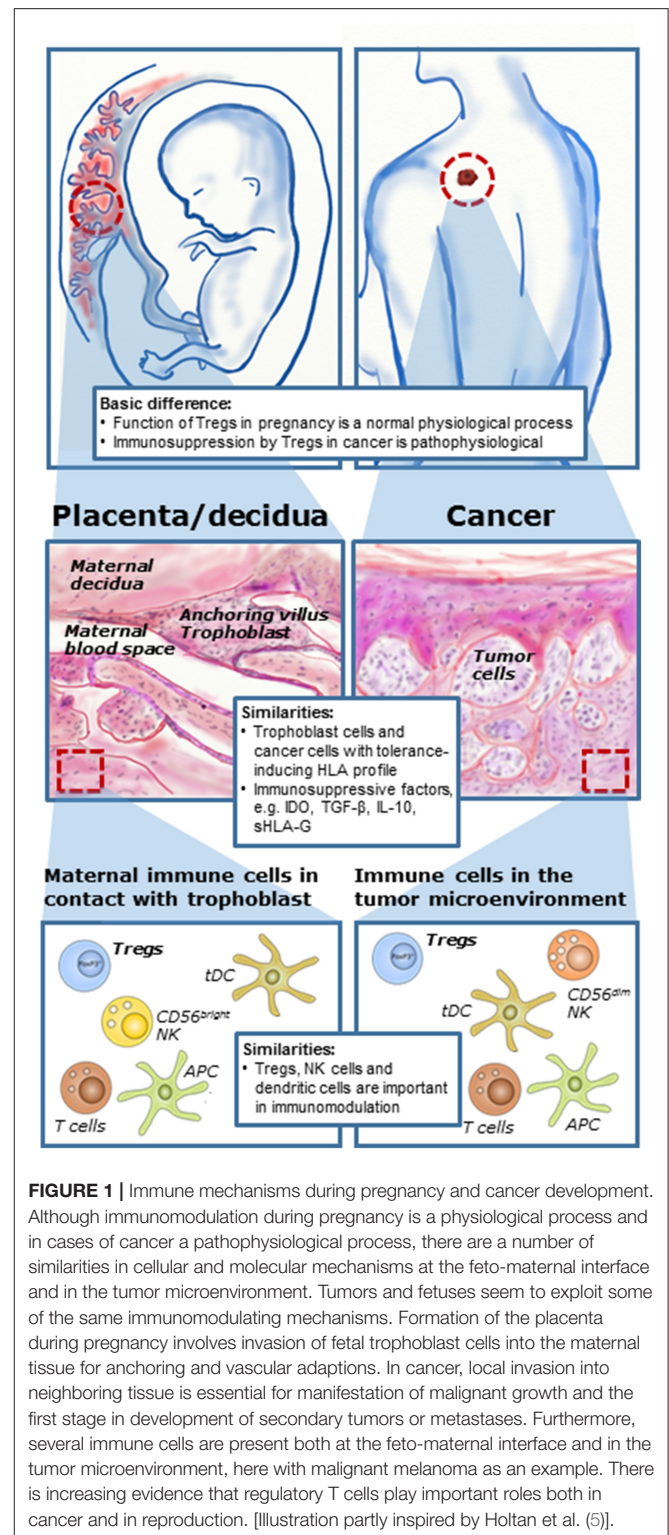
Regulatory T cells (Tregs) constitute a dynamic and diverse T cell population composed of several subsets distinguished by phenotypic and functional characteristics. With their immunosuppressive properties, Tregs are central to the maintenance of immune homeostasis. They are implicated in critical immunoregulatory functions in several physiological conditions such as inflammatory responses, tissue repair, and reproduction. Furthermore, Tregs also play an important role in the pathophysiological immune tolerance induced by tumors (1–4). Hence, selective immunological tolerance is essential during any of these processes, and the mechanisms by which immune

tolerance is sustained by Tregs might be similar. Some of the mechanisms responsible for induction of maternal immune tolerance during pregnancy may be the same as those involved in controlling an inflammatory response from not exaggerating beyond control, and furthermore the same mechanisms that may provide a pro-tumorigenic environment which allows cancer development. The role of Tregs is somewhat opposing in relation to a role in protecting the body and preventing disease development. Tregs must allow protective immune responses against pathogens and tumors, but simultaneously prevent inflammatory diseases by restraining aberrant responses to self and innocuous antigens with pregnancy as a borderline condition, where Tregs contribute to the establishment of active immune tolerance toward the fetus (Figure 1).

The similarities between reproductive biology and cancer development in terms of immunology is not that implausible. During pregnancy, the formation of the placenta involves the invasion of the semi-allogeneic fetal trophoblast cells into the maternal tissue for anchoring and vascular adaptations, such as formation of spiral arteries providing nutritional support for the growing fetus. The maternal immune system has to allow this invasion of partly foreign cells to ensure a successful pregnancy. Thus, cancer cells and cells of the developing placenta both share the capacity to invade normal tissue and create a microenvironment that support immunologic privilege and angiogenesis (Figure 1). The proliferation and migration of cancer cells at a distant site mediated in part by modulation of a tolerogenic immune response in the tumor microenvironment may be compared to the situation in pregnancy, in which the developing placenta invades the uterus and a semi-allogeneic fetus escapes rejection from the maternal immune system (5–7). A prominent hypothesis states that the failure to establish immune tolerance during pregnancy may lead to pregnancy complications or pregnancy loss. However, this may indicate that it should be possible to exploit the same mechanisms responsible for immune regulation during pregnancy in treatment of cancer and to reject cancer cells by immunological mechanisms (5). Finally, it is important to remember that immunomodulation and immunosuppression during pregnancy are physiological mechanisms but in cases of cancer they are pathological and in most cases unfavorable.

The function of Tregs as potent anti-inflammatory cells has led to considerable interest in their therapeutic potential. In cancer, there has been much progress within the field of immunotherapy within the last decade. Especially, cancer therapy by inhibition of negative immune regulation is already used in the clinic. Manipulation and propagation of Tregs and their therapeutic application is a promising approach in order to reach a clinical benefit for affected patients (8–10).

As briefly mentioned above, while pregnancy is a physiological process in which the presence of Treg cells is favorable, cancer is a pathophysiological scenario in which the suppression of a potential anti-tumor response is undesirable. However, as will be discussed in later sections, this distinction is not always obvious, and in some cancer settings, the presence of Treg cells and thus the control of the inflammatory environment can probably be advantageous seen from an anti-tumor perspective.



This highlights the importance of broadening our understanding of the function of Treg cells across different physiological and pathophysiological settings, such as pregnancy, pregnancy complications, and cancer, in order to develop and offer the right therapeutic treatment. This review provides an overview

of current knowledge on the tolerogenic function of Tregs in immunological mechanisms during pregnancy and cancer, and in relation to possible therapeutic intervention of both human malignancies and reproduction.

REGULATORY T CELLS

Regulatory T cells are a T lymphocyte population with immune suppressive properties responsible for maintaining antigen-specific T cell tolerance. Tregs comprise both CD4⁺ and CD8⁺ subtypes. Whereas, CD4⁺ Treg cells have been extensively studied, lack of clear markers to distinguish CD8⁺ Tregs from conventional CD8⁺ T cells has led to unsatisfactory characterization of origin, function and phenotype (11, 12). Therefore, this review will focus mainly on CD4⁺ regulatory T cell subsets, and “Treg” or “regulatory T cell” will refer to CD4⁺ regulatory T cells, unless stated otherwise.

Normally, CD4⁺ Tregs constitute 5–10% of the total CD4⁺ T cell population and are derived from thymic precursors (13). Regulatory T cells were first described in 1972, where Gershon et al. showed that T cells were capable of suppressing the antigen-induced response of other T cells directly without the mediation of B cells and their production of antibodies (14). However, it was not until 1995 that Tregs were identified as a specialized CD4⁺ T cell population expressing CD25 (15). Subsequently, several *in vitro* studies showed that CD4⁺CD25⁺ T cells represent a distinct lineage of naturally anergic and suppressive cells (16, 17). The original studies on characterization of Tregs were performed in mice. However, in 2001 a T cell population with identical immunosuppressive properties was identified in humans (18–21). In 2003, the transcription factor forkhead box protein P3 (FoxP3) was identified as a potent marker for Tregs in several mouse studies. FoxP3 deficiency caused a fatal lymphoproliferative disease demonstrating that the transcription factor was essential for development of Tregs and for their immunosuppressive function (22–24). The requirement of FoxP3 expression for immunosuppression was later demonstrated in humans (25).

Based on these discoveries, expression of CD25 on the cell surface and presence of the intracellular transcription factor FoxP3 became the key characteristics of the Treg population. The mutual expression of these markers is commonly used for identification of Tregs in experimental settings. Conversely, some studies suggest a lack of correlation between CD25 and FoxP3 in human and mice CD4⁺ T cells (24, 26). Alternatively, Liu et al. found that low expression of CD127 serves as a good biomarker for human Tregs together with CD25 expression (26), although other studies have not been able to find a clear correlation between CD127^{lo} and FoxP3 expression (27). In addition, several sub-populations of CD4⁺CD25⁺FoxP3⁺ Tregs have also been identified (28). Hence, the most specific marker still remains a matter of debate. Nevertheless, as expression of FoxP3 has been shown to correlate with suppressor activity irrespectively of CD25 expression many consider FoxP3 as the most specific Treg marker (29).

Regulatory T Cell Subsets

Tregs are found throughout the body, where they modulate activities of cellular components of both the innate and adaptive immune system. CD4⁺ Tregs can be divided into distinct subsets according to unique functional and homeostatic properties (Figure 2). FoxP3⁺ Tregs originating from the thymus, where they have differentiated during T cell ontogenesis, are referred to as natural or thymic (t) Tregs, and Tregs developed in the periphery or *in vitro* from conventional CD4⁺ T cells are referred to as peripheral or induced (i) Tregs (30, 31). Furthermore, there are two phenotypically distinct immunosuppressive subtypes of the iTregs, namely the IL-10 producing T regulatory type 1 (Tr1) cells and the TGF- β -producing Th3 cells (32, 33). It remains to be determined, whether the different subsets of Tregs belong to unique cell lineages, or whether they only reflect the plasticity of the Treg population and represent an altered state of differentiation (34). Furthermore, it is debated, whether iTregs can arise from any conventional T cell or from a pre-committed cell lineage (35).

Both thymus-derived tTregs and peripheral iTregs are characterized by high expression of CD25, FoxP3, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor-related receptor (GITR), but iTregs have been shown to express reduced levels of programmed cell death protein 1 (PD-1), CD73, the transcription factor Helios and the surface antigen neuropilin-1 (Nrp1) (36). Both Helios and Nrp1 have been suggested as markers for distinguishing between tTregs and iTregs, but the specificity of these markers is a current matter of debate (36–39). Mice studies have suggested that GITR is involved in the generation and maturation of FoxP3⁺ tTregs and Tr1-like cells (40, 41). Furthermore, it has been suggested that GITR is a marker of active Tregs (42). In addition to the above mentioned markers, expression of the ATP-degrading enzymes CD39 and CD73 on the surface of Tregs have been increasingly used as markers of Tregs and might contribute to the suppressive activity together with expression of the immunoglobulin-like transmembrane protein LAG3 (Figure 3) (43–46).

Thymic CD4⁺CD25⁺ tTregs are developed in the thymus from CD4⁺ precursors. Development of tTregs or conventional CD4⁺ T cell populations from the CD4⁺ precursor depends on the affinity of the T cell receptor (TCR) for self-antigens: low affinity leads to positive selection of conventional CD4⁺ T cells, whereas medium affinity interactions with thymic epithelial cells lead to development of CD4⁺CD25⁺ tTregs (47–49). Immunosuppression by tTregs require activation via their TCR. When activated, the suppressor effector function is independent of antigen-specificity. Conversely, inhibition of the effector T (Teff) cell population is mainly depending on cell contact and independent of suppressive cytokines (18, 50). The result of tTreg mediated immune regulation is reduced number of Teff cells and altered activity and trafficking pattern of activated Teff cells (37).

In vitro or *in vivo* induced iTregs can be differentiated from naïve CD4⁺ T cells in response to antigen, CD28, TGF- β and IL-2 stimulation, and mediate their suppressive activity mainly via secretion of cytokines such as IL-10 and TGF- β that reduce the capacity of dendritic cells (DCs) to present antigen (37).






Subtype	Markers	Origin	Activation/ differentiation factor	Main suppressive mechanism
	CD25 ⁺ CD127 ⁺ CTLA-4 ⁺ GITR ⁺ Nrp-1 ⁺ Helios ⁺	Thymus	TCR/CD28 Affinity-dependent IL-2	Cell contact
	HLA-G ⁺ CD25 ⁺ FoxP3 ⁺	Thymus	TCR/CD28 Affinity-dependent	Cell contact, IL-10, sHLA-G5
	CD25 ⁺ /hi CTLA-4 ⁺ GITR ⁺ Nrp-1 ⁺ /+? Helios ⁺ /+? FoxP3 ^{hi}	Periphery	Antigen-dependent	IL-10, TGF-β
	CD25 ⁺ CTLA-4 ⁺ GITR ⁺ FoxP3 [?] IL-10 ⁺	Periphery	Antigen-dependent, IL-10	IL-10
	CD25 ⁺ /+ CTLA-4 ^{lo} GITR ⁺ FoxP3 [?] TGF-β ⁺	Periphery	Antigen-dependent, TGF-β	TGF-β

FIGURE 2 | Characteristics of CD4⁺ regulatory T cell subsets. Different subsets of CD4⁺ regulatory T (Treg) cells exist and play a role in the establishment of tolerance in different physiological and pathophysiological settings. Thymic (t)Tregs and HLA-G⁺ Tregs are developed in the thymus in response to self-antigen, whereas induced (i)Tregs, Tr1 cells and Th3 cells are developed in the periphery in response to antigen presentation and cytokines. Natural Treg and iTregs are characterized by CD25 and FoxP3 expression, while HLA-G⁺ Tregs, Tr1, and Th3 cells are CD25⁺ FoxP3⁺, although controversies do exist (see the text for details). The thymus-derived Treg cells mediate their effect mainly through cell contact. In contrast, immune suppression by peripheral induced iTreg, Tr1, and Th3 cells are mediated mainly via secretion of the anti-inflammatory cytokines TGF-β and IL-10.

As for the iTregs, the peripheral Tr1 and Th3 subsets are also induced in the periphery from the conventional CD4⁺ T cells. In contrast to tTregs and iTregs, expression of CD25 and FoxP3 in Tr1 and Th3 cells are controversial (51–53). Tr1 and Th3 have been identified as FoxP3⁺ and CD25⁺, although it seems that expression of both markers can be upregulated in response to activation (53, 54). The Tr1 cells were first described by Groux et al. (55), who found that Tr1 cells are activated by IL-10 and suppress the proliferation of CD4⁺ cells in response to antigen (55). Presence of IFN-α further enhances IL-10-mediated induction of Tr1 activation and differentiation (56). The Tr1 cells constitute a low proliferating subset that produces high levels of IL-10, low levels of TGF-β and marginal or no IL-2 and IL-4 (55, 57). Th3 cells are activated upon antigen stimulation (58). However, TGF-β also promote the induction of Th3 cells from CD4⁺ T cells, which can be further enhanced by the presence of IL-10 and IL-4 (32). When active, the Th3 cells have suppressive properties for Th1 and Th2 cells through secretion of TGF-β (59, 60).

A new subset of regulatory T cells have emerged during the recent years defined by expression of the immunosuppressive Human Leukocyte Antigen (HLA) molecule HLA-G (Figure 2). In 2007, Feger et al. identified HLA-G⁺ T cells among CD4 and CD8 single-positive cells in the peripheral blood and thymus from healthy individuals (61). The cell population showed reduced proliferation to allogeneic and polyclonal stimuli and

the suppressive effect of HLA-G expression was confirmed by neutralization of HLA-G on CD4⁺HLA-G⁺ cells, which reduced their suppressive capacity. The cells were, however, not expressing CD25 and FoxP3, like previously described Tregs. When comparing the properties and molecular characteristics of CD4⁺HLA-G⁺ cells and CD4⁺CD25⁺FoxP3⁺ cells, there is a clear distinction between the phenotype and the cytokine profile of the two cell populations (62). The suppressive function of CD4⁺HLA-G⁺ cells is mediated mainly by secretion of soluble HLA-G and high levels of IL-10 and IL-35, while CD4⁺CD25⁺FoxP3⁺ cells seems to work mainly in a cell-contact dependent manner. Furthermore, CD4⁺HLA-G⁺ cells are clearly distinct from Tr1 cells as they do not require the presence of other cell types (63). The identification of a novel T cell population with regulatory properties expressing HLA-G on the surface has led to the notion of a new subset belonging to the repertoire of suppressor T cells (64). As these cells have a similar function as CD4⁺CD25⁺FoxP3⁺ Tregs, their role in peripheral immune regulation is increasingly recognized. However, whether they should be identified as traditional regulatory T cells as the classical Tregs is somehow controversial.

No universal agreement on which factors that can be used to differentiate tTregs from iTregs seems to exist. Moreover, it is important to note that most studies have used shared markers such as FoxP3, CD25, and CD127 for identification of Treg cells, thus do not differentiate between tTregs and iTregs,

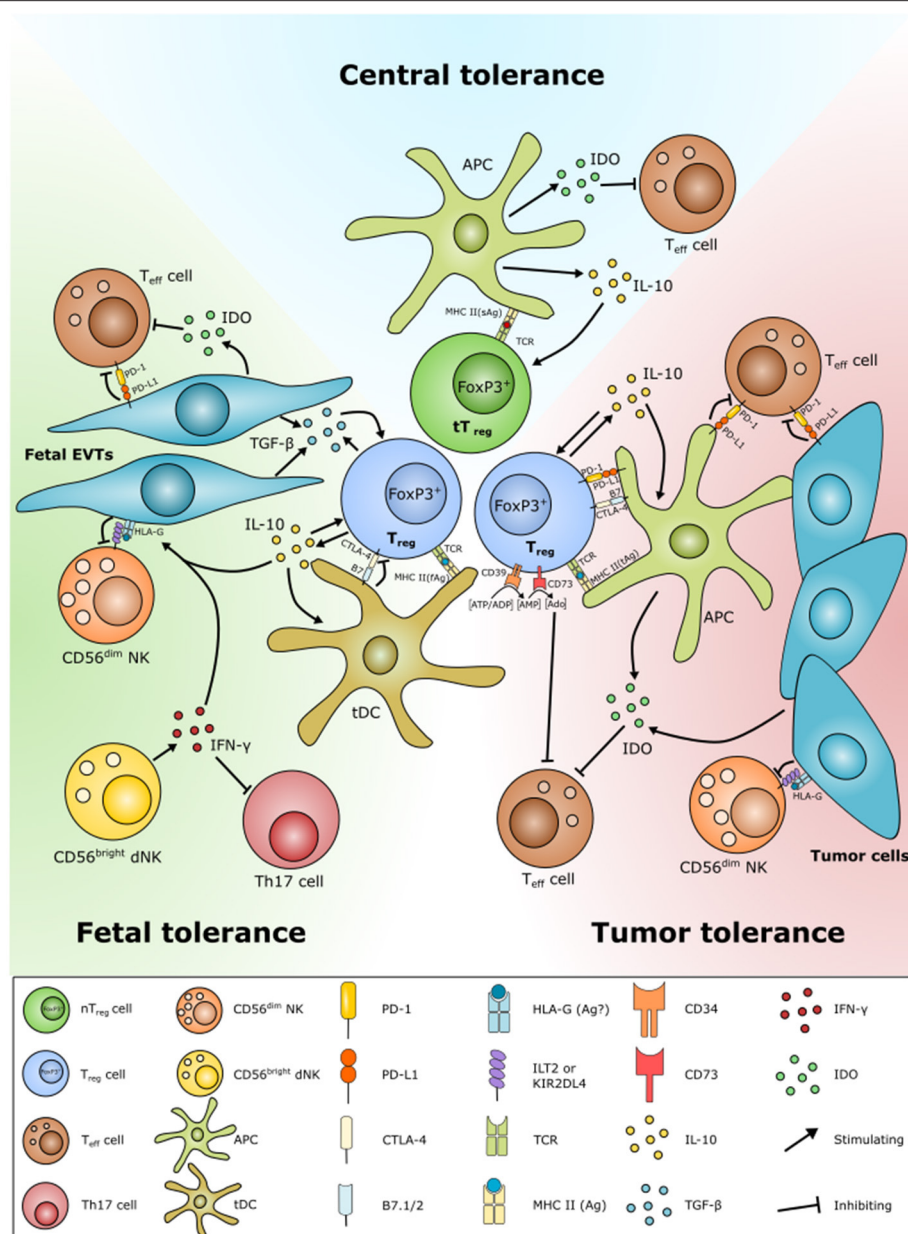


FIGURE 3 | Schematic overview of similarities in Treg function in central tolerance, fetal tolerance, and cancer tolerance. Tolerance play an important role in both fetal and cancer tolerance. Tregs are developed by presentation of antigens of fetal (fAg) or tumor (tAg) origin. Many tumor cells and fetal extravillous trophoblast (EVT) cells have both diminished or no expression of MHC class II and classical MHC class I molecules. Instead, the EVT cells and some cancer cells express HLA class Ib molecules, e.g., the immune modulatory non-classical HLA-G. HLA-G is able to protect fetal and tumor cells from NK cell lysis, as well as according to a few studies to induce Treg formation. Fetal EVTs and tumor cells are also able to contribute to Treg homeostasis by inhibiting effector T cell activation and proliferation through PD-L1/PD-1 and indoleamine-2,3-dioxygenase (IDO) expression. Decidual (d)NK cells further contribute by inhibiting Th17 responses by IFN-γ expression. Fetal EVTs also express cytokines, e.g., IL-10 and TGF-β that induce Treg development. Tregs limit Teff cells and promote their own proliferation and survival through direct engagement with Teff cells, e.g., via PD-L1/PD-1, by the conversion of ATP to Adenosine (Ado) and cytokine secretion.

and by that definition also exclude any immunosuppressive FoxP3⁺ T cells, such as the Tr1, Th3, and HLA-G⁺ Tregs. The following section will focus on studies using FoxP3, CD25, and CD127, and the term “Treg” will therefore refer to regulatory CD4⁺ T cells regardless of origin, unless specifically stated otherwise.

THE ROLE OF TREGS IN CANCER

The progression of cancer is controlled by a complex biologic system that is highly dependent on interaction between the malignant cells and the surrounding tumor microenvironment comprising the immune cells. Various types of immune cells

can infiltrate the tumor and may influence tumors differently depending on their histological and molecular type, their stage, the microenvironment of the organ in which they occur, and the nature of the tumor (**Figure 1**) (65). Immune effector cells can detect and destroy cancer cells preventing tumor formation by means of both the innate and adaptive immune compartments. However, the anti-tumor activity of the immune cells are often downregulated by tumor-derived signals leading to immune escape. A large number of preclinical models have demonstrated the influence of Tregs in development and progression of several types of malignancies, and Tregs are generally believed to be a significant contributor to tumor immune escape (66, 67). A widely accepted hypothesis is that recruitment of tumor-infiltrating Tregs with immunosuppressive properties enable the malignant cells to evade the host immune response (68).

Increased numbers of tumor-infiltrating Tregs have been demonstrated in patients with ovarian (69), liver (70), melanoma (68), gastric, and esophageal cancer (71), in chronic lymphocytic leukemia (72), and in breast cancer, where it is associated with a more aggressive phenotype (73). The same is seen in gastric and esophageal cancers, where Tregs increase with disease stage suggesting induced expansion of Tregs by tumor-related factors (74). Furthermore, Treg numbers are also increased in the peripheral blood of patients with breast, pancreatic (75), liver (70), gastric, and esophageal cancer (71) in comparison with blood from healthy individuals.

Various studies have tried to identify the role of Tregs in immune evasion, and as it has become clear that the effect of Tregs on tumor progression vary according to the tumor type, the prognostic significance of Treg infiltration remains a matter of debate. An overview of the clinical significance in a range of cancers is provided in **Table 1**. In connection to the role of Tregs in evading immune recognition, a common presumption is that high numbers of Tregs within lymphoid infiltrates can be predictive of relapse and death. However, the prognostic value of Tregs is somehow controversial as in some cancers Tregs infiltration may exert a beneficial role or can have both a negative and positive effect on disease progression and survival. The negative effect on survival is observed in pancreatic (87), liver (90), gastric, and esophageal cancer (74). It is though more likely to observe opposing roles of Tregs in terms of survival in a wide range of cancer types such as cases of ovarian carcinoma (69, 76), colorectal cancer (85, 86), melanoma (88), breast cancer (77–84), head and neck squamous cell carcinoma (91, 92), and lymphoma (93–96), where a high frequency of Tregs improve disease-specific survival in some patients and in others favors immune escape and tumor growth. Furthermore, in some patients there is no correlation between Treg infiltration and disease progression at all (89). The reason for this discrepancy in the prognostic value of Treg infiltration might be related to the different nature of the cancers and the effect of inflammation on tumor growth, but could also be dependent on the presence of different Treg subsets in the different malignancies.

Mechanisms of Treg-Mediated Immunosuppression in Cancer

Several mechanisms contribute to the accumulation of Tregs within neoplastic lesions including increased infiltration, local expansion, survival advantage, and development from conventional CD4⁺ cells (30). All of these mediated through signaling with other cells and through different signaling molecules (**Figure 3**).

Studies on mice deficient of key markers of Tregs, including IL-10, CTLA-4, GITR, or PD-1 that develop severe immune-related disorders indicate that these molecules are crucial for Treg function in a cancer setting. The CTLA-4 receptor is a negative regulator of T cell responses functioning as an immune checkpoint. Leach et al. was the first to show that blockades of the inhibitory signals of CTLA-4 enhance antitumor immunity in mice (97). Proof that this was also the case in humans came in 2003 in a clinical investigation, where CTLA-4 blockade increased tumor immunity in some previously vaccinated melanoma and ovarian carcinoma patients (98). Much research have been performed on the mechanism of antitumor immunity elicited by CTLA-4 blockade and one study has shown that Treg-specific CTLA-4 deficiency results in downregulation of CD80 and CD86 on DCs leading to loss of immunosuppression (99). This happens in part through abrogated expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) by the DCs (100). When it comes to cancer, IDO is expressed also within solid tumors from both tumor and stromal cells, where it under normal conditions restrains an inflammatory reaction against cancer cells by degradation of tryptophan and recruitment of Tregs (101, 102). Another commonly known checkpoint molecule is PD-1. PD-1 is a receptor expressed on activated T cells, B cells, and myeloid cells. One of the early proofs of PD-1 being involved in maintenance of self-tolerance came in 1999, where a knock-out mouse model showed that a defect in the PD-1 gene specifically predisposes to development of lupus-like autoimmune disease suggesting that PD-1 serves as a negative regulator of immune responses (103). The same was seen in humans, where a study by Freeman et al. revealed that engagement of PD-1 by its ligand PD-L1 led to inhibition of T cell receptor-mediated lymphocyte proliferation and cytokine secretion (104). Furthermore, blockade of PD-1 seems to enhance recruitment of Teff cells in intrasplenic tumors and prevent metastatic spread of several different cancers (105). The crucial role of CTLA-4 and PD-1 in regulation of a tolerogenic immune response opens up for a blockage of both checkpoint molecules that may have great therapeutic potential in terms of activating an immune response against the cancer cells. Whereas, both CTLA-4 and PD-1 function as negative regulators, GITR function as a co-stimulatory receptor, leading to activation, proliferation and cytokine production in both Teff and Treg cell populations (106–108). As mentioned, GITR is expressed in high levels by Tregs, and has been shown to be increased in several cancer forms including breast cancer (42, 109, 110). Engagement of GITR on Treg cells has been shown to inhibit their suppressive function, and rendering Teff unresponsive to Treg-mediated suppression (106, 107). However, it has also been shown that

TABLE 1 | Examples of clinical significance of Tregs in the tumor microenvironment.

References	Cancer	Presence of Tregs	Effect on clinical outcome		Comment
			Good/bad	How	
Curjel et al. (69)	Ovarian carcinoma	High	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	Bad	Reduced survival
Milne et al. (76)	Ovarian carcinoma	High	FoxP3 ⁺	Good	Increased disease-specific survival
Gobert et al. (77)	Breast cancer ^a	High	CD4 ⁺ CD25 ^{hi} CD127 ^{lo} FoxP3 ⁺	Bad	Higher risk of relapse and death
Demir et al. (78)	Breast cancer ^a	High	FoxP3 ⁺	Bad	Shorter overall survival
Sun et al. (79)	Breast cancer ^a	High	FoxP3 ⁺	Bad	Shorter disease-free survival
West et al. (80)	ER ⁻ breast cancer	High	FoxP3 ⁺	Good	Prolonged recurrence-free survival
Bates et al. (81)	ER ⁺ breast cancer	High	FoxP3 ⁺	Bad	Shorter relapse-free and overall survival
Liu et al. (82)	ER ⁻ breast cancer	High	FoxP3 ⁺	-	No impact
	ER ⁺ breast cancer			Bad	Poor survival
	ER ⁻ breast cancer			Good	Improved survival
Lee et al. (83)	Triple-negative breast cancer	High	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	Good	Improved survival
Liu et al. (84)	Triple-negative breast cancer	High	FoxP3 ⁺	Good	Better overall and disease-free survival
Frey et al. (85)	Colorectal cancer	High	FoxP3 ⁺	Good	Improve disease-specific survival
Chang et al. (86)	Colorectal cancer	High	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	Bad	Favor tumor growth
Kono et al. (74)	Gastric and esophageal cancer	High	CD4 ⁺ CD25 ^{hi}	Bad	Poor survival rate
Hiraoka et al. (87)	Pancreatic ductal adenocarcinoma	High	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	Bad	Poor prognosis
Miracco et al. (88)	Primary cutaneous melanoma	High	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	Bad	Predictive of recurrence
Ladányi et al. (89)	Primary cutaneous melanoma	High	FoxP3 ⁺	-	No prognostic impact
Kobayashi et al. (90)	Hepatocellular carcinoma	High	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	Bad	Lower survival
Badoual et al. (91)	Head and neck squamous cell carcinoma	High	CD4 ⁺ FoxP3 ⁺	Good	Favorable

(Continued)

TABLE 1 | Continued

References	Cancer	Presence of Tregs	Effect on clinical outcome		Comment
			Good/bad	How	
Drennan et al. (92)	Head and neck squamous cell carcinoma	High	Bad	Favor tumor progression	Elevated frequency and suppressive activity of CD25 ^{hi} Tregs is associated with advanced tumor stage and metastasis to lymph nodes
Tzankov et al. (93)	Lymphomas	High	Good	Improved survival	Increased number of FoxP3 ⁺ cells positively influence survival in follicular lymphoma, germinal center-like diffuse large B cell lymphoma, and Hodgkin's lymphoma
Carreras et al. (94)	Follicular lymphoma	High	Good	Improved overall survival	Patients with low Treg numbers presented more frequently with refractory disease
Alvaro et al. (95)	Hodgkin's lymphoma	Low	Bad	Unfavorable	Low infiltration of Tregs in conjunction with cytotoxic lymphocytes is predictive of unfavorable outcome
Schreck et al. (96)	Hodgkin's lymphoma	High	Bad/–	Shorter disease-free survival	A high ratio of Treg over Th2 cells is associated with shortened disease-free survival. Tregs have no prognostic impact alone

^a Cohort of patients with advanced/invasive breast cancer irrespective of molecular subtype.

GITR induces IL-10 production, that if blocked leads to further GITR-mediated proliferation (108), leaving the exact role of GITR controversial. As shown in a study, the function of GITR on Treg cells is most likely context-dependent and rely on the model used to study its function, as well as the immunological milieu (111). Nevertheless, GITR is, like CTLA-4 and PD-1, an attractive target for immunotherapy, and GITR triggering using agonist antibodies and Fc-GITRL abrogates Treg-mediated suppression (106).

Whereas, the function of tTregs is mainly cell-cell contact-dependent, the secretion of soluble factors, such as cytokines by iTregs and other Treg subtypes is essential for their function (Figure 2). IL-10 is a cytokine produced by CD44^{hi} Tregs and plays a central role both in parasitic infections (112), intestinal inflammation (113), and cancer (114) again emphasizing the involvement of similar mechanisms in different pathophysiological conditions. In addition to IL-10, TGF-β is also produced by peripheral Tregs. Both IL-10 and TGF-β have pleiotrophic functions and have been implicated in both cancer progression as well as clearance [reviewed by (115)]. The effect of IL-10 and TGF-β therefore most likely depends on the specific cancer type, and therapy targeting these cytokines should be done with careful considerations. Considering that Treg cells are defined as CD25^{hi}, the high affinity IL-2Rα chain, IL-2 is another cytokine central to both thymic and peripheral Treg development, function and homeostasis (116, 117). In contrast, the IL-7 receptor α chain, CD127, is low or absent in human Tregs, indicating that IL-7 is not required for Treg function, although a study in mice has suggested that IL-7 might be involved in early Treg development and in development of CD4⁺FoxP3^{lo} Tregs (116, 117).

Another increasingly acknowledged mechanism involved in development of cancer is regulation of the expression of HLA molecules in the tumor microenvironment. Increasing evidence suggest that expression of the classical and non-classical HLA class Ia (HLA-A, HLA-B, HLA-C) and class Ib (HLA-E, HLA-F, HLA-G) molecules influence immune regulation in a coordinated action with Tregs. This influence of HLA molecules is seen in multiple physiological and pathophysiological processes as the antigen-presenting capability of HLA molecules play a crucial role in infectious diseases, graft rejection, autoimmunity, reproduction, and cancer. Deregulation of the HLA class I molecules on the cancer cells leads to evasion of the host immune system (118). In a recent study on the prognostic value of tumor-stroma ratio combined with the immune status of the tumor, Vangangel et al. showed that breast cancer patients with a stroma-low tumor and expression of classical HLA class I molecules have a better prognosis compared to patients with a stroma-high tumor and downregulation of HLA class I (119). Furthermore, when expression of HLA class Ia molecules are concomitantly lost, high expression of HLA-G is associated with a worse relapse-free period in breast cancer (120) and is suggested to facilitate invasion and increase the metastatic capacity of invasive ductal breast carcinoma (121–123). In gastric cancer, HLA-G expression significantly correlates with the presence of Tregs and is predictive of poorer survival (124). Expression of HLA-G and the presence of FoxP3⁺

tumor-infiltrating lymphocytes is also believed to contribute to the suppression of effective T cell immune responses in melanoma (68, 125). We have recently shown an association between high HLA-G expression and a high frequency of FoxP3⁺ tumor-infiltrating lymphocytes in malignant melanoma patients (126). Furthermore, in an *in vitro* study we have demonstrated that the HLA-G⁺ choriocarcinoma cell line JEG-3 originating from placenta upregulates Tregs, and that the level of pro-inflammatory cytokines is modulated through HLA-G (127).

A subset of HLA-G-expressing T cells have also been shown to play a role in promoting a tolerogenic tumor microenvironment. A recent study found a population of CD4^{lo}HLA-G⁺ T cells associated with development of castration-resistant prostate cancer in prostate cancer patients after treatment with androgen deprivation therapy. Expansion of the CD4^{lo}HLA-G⁺ cells resulted in impaired immune surveillance and a tumor microenvironment that were permissive of tumor growth (128). In pregnancy, CD4⁺HLA-G⁺ T cells have been reported and may be reduced in pre-eclampsia, although knowledge of a possible role of this subset is currently very limited (129).

We are currently investigating how expression of HLA class Ia and Ib expression modulate the immune response in breast cancer with emphasis on Tregs and Natural Killer (NK) cells. By studying molecular and genetic changes of the immune cells in contact with tumor cells we aim to identify molecular markers associated with the regulatory function of the immune cells and clinical outcome. Identification of regulatory immune cell gene signatures in tumors can be important and relevant when assessing the clinical course of the disease and prognosis. A recent study focusing on immunogenic gene signatures in triple-negative breast cancer found a high expression of tumor-infiltrating lymphocyte gene signatures in the tumor compared to normal tissues and that elevated levels of Treg gene sets were consistently associated with better overall survival and disease free survival (84). This confirms the controversy about the prognostic significance of Tregs in the tumor microenvironment and emphasizes the importance of research that can elaborate on the role of Tregs in a specific cancer setting and for the individual patient.

Substantial redundancy may exist in the mechanisms essential for establishment and maintenance of immune tolerance (46). Hence more research is necessary to identify mechanisms that could constitute the best targets for immunotherapeutic treatment strategies.

Antigen-Specific Tregs

With the aim to elucidate the role of Tregs in cancer development, several studies have found that the Treg response is an early event preceding the activation of Teff cells (130, 131). It was seen many years ago in mice, that a regulatory immune response are present early followed by a decrease in cellular reactivity against the tumor cells and a progressive loss of immune recognition correlated with progression of tumor growth (132). A mechanism by which Tregs are stimulated by the presence of the tumor is via recognition of antigens.

Tumors are believed to present tumor-specific antigen in the form of neo-epitopes, sometimes known as tumor-associated

antigens. Since tumor cells originate from normal cells and develop within the context of self-tissue, most cancer antigens are self-antigens, and the immune mechanisms that prevent immune recognition of the tumor cells might function in similar ways as those that prevent autoimmune attack of normal tissue (133, 134). This is contrary to pregnancy, where both foreign and self-antigens are present from the semi-allogenic fetus and immune suppression is necessary in order to avoid fetal rejection. However, it may be emphasized that cancer cells might eventually also, due to high mutational rate, express antigens foreign to the body that can be recognized by Teff cells.

A previous study by Wang et al. characterized tumor-specific CD4⁺ T cells derived from a melanoma patient and were the first to isolate antigen-specific Tregs, and further showed that cell-cell contact was required for T cell-mediated immune suppression in agreement with previous studies (135). The group identified Tregs specific for LAGE1 and afterwards the ARCT-1 peptide (136). Tregs specific for a broad range of tumor antigens including melanoma tissue differentiation antigens and cancer-testis antigen, have been identified in patients with metastatic melanoma (137), and following studies performed in colorectal cancer have also revealed tumor antigen-specific Tregs (138). In colorectal cancer patients undergoing resection, a high level of FoxP3⁺ Tregs specific for tumor antigens drives immunosuppression and correlates with tumor recurrence and relapse (139). Studies in diabetic mice have revealed a superior immunosuppressive activity for antigen-specific Tregs compared to non-specific Tregs (140, 141). Furthermore, Tregs responding to self-antigens have also been shown to suppress anti-tumor immune responses (142, 143). Indications are that Tregs are likely to play an important role in cancer immunology and elaborating on the specificity of Tregs involved in antitumor responses could be beneficial from a therapeutic perspective.

Immunotherapeutic Intervention in Cancer

Given the role of Tregs in immune evasion and tumor progression, several studies have already suggested that they are promising as therapeutic targets (31). Initially, studies focused almost exclusively on the cancer cells as targets for therapeutic interventions. However, cancer cells frequently acquire therapeutic resistance because of inherent genetic instability. Hence, working toward manipulation, propagation, and therapeutic application of Tregs will provide new and improved treatment options. The prognostic effect of Tregs in different cancer types is important to take into consideration when selecting a treatment strategy, and even though Tregs appear as an obvious target for anti-tumor treatment, manipulation of Treg mechanisms is not that simple and more selective approaches for therapeutic strategies are needed. This involves targeting of specific Treg subsets and the inhibition or activation of Tregs depending on the type of cancer (30). Furthermore, the composition of other immune cells in the tumor microenvironment must also be taken into account when assessing whether a patient will benefit from immunotherapy. Recently an immune biomarker task force elicited by the Society for Immunotherapy of Cancer (SITC) sought to make recommendations of immune-related

biomarkers that can predict the outcome of immunotherapy in cancer patients (144). They focus on biomarkers in the tumor microenvironment, gene expression at the tumor site, tumor antigens, mutational load, peripheral biomarkers, and host-related genetic biomarkers. Overall, this suggests that a combination of personalized diagnostics is necessary in order to assess immunocompetence of the individual. In terms of this, an analysis of immune gene signatures should be feasible to determine the potential for immunotherapy. Liu et al. performed an extensive analysis on immunogenic signatures in triple-negative breast cancer on two large-scale breast cancer genomic datasets. They demonstrated that this type of breast cancer has a strong tumor immunogenicity, which suggested that these patients could benefit from immunotherapy (84).

Even though treatment by activation of the immune system have proved to be successful it is not without side effects. One of the biggest challenges of targeting Tregs and blocking immune checkpoints is the development of severe system immune-related side effects. Releasing the brake on the immune system can lead to a systemic immune activation and might cause extensive autoimmune reactions (31).

One branch of immunotherapy evolves around the idea of activating the immune system targeting the regulatory mechanisms that suppress an immune response against the cancer. Especially, cancer therapy by inhibition of negative immune regulation has proved very successful within recent years in the form of immune checkpoint inhibitors and are currently used in cancer immunotherapy. Discovery of the two checkpoint molecules CTLA-4 and PD-1 that function as brakes on the immune system has led to a new approach for treating cancer patients. Ipilimumab and tremelimumab are two well-characterized anti-CTLA-4 antibodies, the first approved for treatment of malignant melanoma, colorectal cancer, and renal cell carcinoma and the second being tested in clinical trials on colorectal cancer and lung cancer patients (145–151). Pembrolizumab is an anti-PD-1 drug approved for treatment of multiple cancers including cervical cancer and melanoma (152–155). Nivolumab is another anti-PD1 drug that in combination with ipilimumab is used as first-line treatment of melanoma being more effective than either agent alone (156). Furthermore, nivolumab is shown to have a higher efficacy as compared to chemotherapy in patients with melanoma, who progressed after CTLA-4 treatment (157). These immunotherapies have emphasized how manipulation of immune regulation is essential for eradicating tumors.

Another strategy of breaking the tolerance to tumor tissue is to inhibit the IDO pathway. Studies show that elimination of IDO-positive immunosuppressive cells change the regulatory microenvironment (158). Furthermore, it was found that 1-methyl-tryptophan isomers capable of blocking IDO activity is effective in reversing the suppression of T cells promoted by DCs (159). Combined with other immune activating drugs, IDO might also enhance the efficacy of immunotherapy by preventing counter-regulation in response to immune activation (160). Combining induction of IDO-specific immune responses with anti-cancer immune therapy has the synergistic potential to both eliminate cancer cells and immune suppressive cells expressing

IDO (158). Hence, clinical trials have been initiated to evaluate the efficiency of IDO inhibitors and IDO-based vaccinations. A combination of pembrolizumab and the selective IDO inhibitor Epacadostat initially showed promising results increasing the anti-tumor activity in patients with advanced solid tumors in a phase I/II study (NCT02178722) (161). Unfortunately, no benefit in survival was observed with the combined treatment compared to pembrolizumab alone in the following phase III clinical trial (NCT02752074) (162). A clinical phase I study have shown that a vaccine with an epitope derived from IDO is well-tolerated in patients with metastatic non-small cell lung cancer (NCT01219348) (163). Currently, a clinical phase 2 study is testing a combination therapy of the PD1 antibody Nivolumab and a vaccine consisting of PD-L1 and IDO (NCT03047928).

A third way to enhance anti-tumor effects is to deplete Tregs in the tumor microenvironment. Mouse studies have proven the effectiveness of eliminating Tregs by administration of IFN- γ and the use of IL-2 antibodies in combination with stimulation of effector immune cells (140, 164). An ongoing clinical trial is investigating a combination of pembrolizumab and low-dose IL-2 in patients with advanced melanoma or renal cell cancer (NCT03111901). Furthermore, a phase I/II study have shown that CD4⁺CD25⁺ Treg depletion improves the graft-vs.-tumor therapeutic effect of donor lymphocyte infusion in patients suffering from hematopoietic malignancies and relapse after standard allogeneic hematopoietic stem cell transplantation (NCT00987987) (165).

Another branch of immunotherapy focuses on targeting tumor antigens. Recognizing an increased activity for Tregs that are antigen-specific gave the idea that Tregs could also be exploited to target cancer cells. Expression of chimeric antigen receptor (CAR) T cells to engineer T cells with antigen-specificity toward cancer cells have already offered a promising strategy to target diseases with extensive immune activation. This directs the attention to a similar approach for Tregs with the possibility that CAR Tregs could be used in Treg-mediated therapy reducing a generalized immunosuppression (35). In terms of this, studies have shown that it is possible to isolate CD4⁺CD25⁺ cells with immunosuppressive function from peripheral blood and expand them *in vitro* without loss of function, which represent a major advance toward the therapeutic use of these cells in T cell-mediated diseases (166). So far, engineered Tregs have been shown to target the central nervous system reducing symptoms of multiple sclerosis by suppression of inflammation and in colitis patients CAR T cells could hinder development of colorectal cancer (167, 168). This indicates that the use of engineered Tregs is preferred in cancers with prominent inflammation and where immune suppression will have a beneficial role in preventing tumor progression. Moreover, a new study suggests a promising role for CAR T cells in delivery of checkpoint inhibitors. Mouse CAR T cells was modified to secrete PD-1 blocking single-chain variable fragments and was shown to enhance the anti-tumor function in mouse models of hematologic and solid tumor (169). Hence, the targeted delivery of immune checkpoint inhibitors or expression of other immunomodulatory molecules could prevent systemic blockade, eventually improving treatment and minimizing adverse side effects.

THE ROLE OF TREGS IN REPRODUCTIVE BIOLOGY

With the inheritance of half of the genes from the father, the fetus is considered to be semi-allogenic in an immunological sense. This results in the immunological paradox in which the maternal immune system has to be able to tolerate the presence of the foreign paternally derived antigens for a successful pregnancy to take place. Initially, a shift from a Th1 pro-inflammatory response toward an anti-inflammatory Th2 response has been the central paradigm to explain the generation of fetal tolerance (170). However, during normal pregnancy the decidua contains a decreased CD4⁺/CD8⁺ ratio compared to the peripheral blood, and decreased numbers of CCR6⁺ Th1, Th2, and Th17 cells, while CCR6⁻ Th1 cells and CD4⁺CD25^{hi}FoxP3^{+/hi} Tregs are increased (171, 172). This reflects a much more complex scenario, and are now explained as a balance between Th1, Th2, Th17, and regulatory responses involving both innate and adaptive immune cells (173, 174). Moreover, recently it has been proposed that the immune system plays different roles in the different phases of pregnancy; an inflammatory response seems necessary for the implantation of the blastocyst, while there is an establishment of a tolerogenic milieu for maintenance of the pregnancy, and yet another shift toward inflammation at parturition (174, 175). To constrain inflammation and avoid fetal rejection, several mechanisms have developed in which increasing focus has been giving to the role and function of the anti-inflammatory properties of the regulatory Tregs (10, 173, 174, 176), which is described in the next section.

Although maternal Treg cells are fully capable of recognizing paternal antigens and become activated, this does not lead to rejection of the fetus (177, 178). Tafuri et al. were also able to show that paternally derived tumor cells were able to persist during pregnancy independent of antibody response, but was rejected after parturition (178). This indicates a pivotal role for establishment of a temporal state of tolerance against the paternal antigens during pregnancy, and thus an important role for Tregs (178, 179). Several mechanisms have been identified that protect the fetus from immune attack, including attenuated expression of polymorphic Major Histocompatibility Complex (MHC)/HLA proteins as well as expression of the nearly monomorphic HLA class Ib molecules, release of anti-inflammatory hormones, cytokines, and immunomodulatory molecules by the placenta, and suppression of allo-reactive responses (173). Fetal tolerance during pregnancy seems to be a balance between clonal exhaustion (i.e., deletion or inactivation) of allo-reactive T cells and immune regulation—a phenomenon also seen in transplantation (180–182).

During the formation of the maternal-fetal interphase fetal trophoblast cells will invade into the maternal decidua harboring maternal immune cells to form the placenta. In parallel, the tumor microenvironment can be seen as a pathological situation with tumor cells with a distinct and possible non-self-phenotype in close contact with immune cells (**Figure 1**). The placenta is regarded as an immunological privileged site and is the source of many immunomodulatory molecules, hormones and cytokines that contributes to establishment of fetal tolerance

(183). Roughly speaking, there are two compartments in the placenta in which maternal immune cells interact with the fetal cells; the intervillous space and the decidua. The interactions between the fetal trophoblast cells and the maternal T cells will be different at the two places. The intervillous space is the space between the anchoring villi, flooded with maternal blood that allows exchange of nutrients. The syncytiotrophoblast cells here lack the expression of all MHC/HLA molecules and should, in theory, not be able to interact with the maternal T cells (184). It has been suggested that the main role of the T cells located here is to protect mother and fetus against infectious pathogens (185). However, it should be noted that maternal antigen presenting cells (APCs) are still able to induce an adaptive immune response by presenting paternal antigens despite the lack of MHC/HLA on the syncytiotrophoblast cells (186). In contrast, invading extravillous trophoblasts (EVTs) present in the decidua express a unique combination of HLA-C and the non-classical HLA-E, -F, and -G molecules, enabling them to elicit immunosuppression and induce tolerance. The expression of a polymorphic paternally inherited HLA-C molecule on EVT has the potential to induce alloreactivity toward the fetal-derived cells. However, HLA-C is only expressed at a level of ~10% of HLA-A and -B, and HLA-C interacts both with T cells and NK cells through KIRs (7, 184). In addition to the local immune changes happening in the placenta during pregnancy, peripheral tolerogenesis is also observed (187). It is not yet fully understood whether the peripheral changes reflects the local changes or if there is a separate systemic response to pregnancy, e.g., through interaction with shed trophoblast debris or exosomes.

Many studies have shown the importance of Tregs for pregnancy (10, 173, 174, 176). Tregs and FoxP3 mRNA have been found in the endometrium throughout the menstrual cycle, increasing in the follicular/estrus phase and thereby the receptive phase, suggesting that the uterus is preparing for pregnancy also involving immunomodulatory changes (188, 189). Some studies might also indicate that the female immune system is primed for pregnancy through contact with antigens and immunomodulatory molecules present in the seminal plasma during coitus (190). In mice, the CD4⁺ and CD8⁺ Treg populations expand immediately after mating due to activation by paternally derived antigens present in the seminal fluid (186). Pregnant women have a higher level of peripheral Tregs compared to non-pregnant women with Treg numbers peaking during first and second trimester (191–194). In parallel, higher levels of Tregs can also be observed in certain cancer patients compared with healthy individuals as discussed briefly previously. Moreover, it has been shown that women with infertility problems and women experiencing recurrent pregnancy loss (RPL) in first trimester have reduced number of Tregs and FoxP3 mRNA, indicating an early role for Tregs in the establishment of pregnancy (188, 195). The role of Tregs in connection with the uterine (u)NK cells in the endometrium of infertile women has been thoroughly described in a recent review by Kofod et al. (196). Reduced numbers of Tregs, and increased number of CD8⁺ T cells and Th17 cells, have also been associated with pregnancy complications such as pre-eclampsia (PE) and RPL (191, 192, 197). In mice, depletion of Tregs using anti-CD25

monoclonal antibodies at the time of implantation resulted in poor implantation and fetal reabsorption in allogeneic, but not in syngeneic pregnancies. In contrast, no effect was observed on either pregnancy outcome, blood pressure or urinary protein levels, when Tregs were depleted later in pregnancy (182). This confirms the proposed role for the Tregs in creating a tolerogenic environment toward the paternal allo-antigens early in implantation and pregnancy. It has been suggested that both thymic and induced peripheral Tregs play important roles in pregnancy. Mice studies have shown that pre-existing thymic memory/activated Tregs specific for self-antigens are present very early in pregnancy and thus play a role in implantation, whereas depletion of peripheral Tregs leads to increased abortion later in pregnancy (198). In human first trimester decidua, FoxP3^{hi} Tregs with a similar phenotype (CD45RO⁺HLA-DR⁺CTLA-4⁺) have been identified (171). Analysis of Treg cells from term placenta tissue also showed that these cells expressed GITR and had higher expression of CD25, CTLA-4, and CD69 in comparison to their peripheral counterparts, indicating an activated phenotype (194). Lastly, recent studies have shown that pregnancy also leads to the generation of both effector memory and central memory CD4⁺ and CD8⁺ T cells that persist after pregnancy (199). The development of memory Tregs after pregnancy and their possible role for subsequent pregnancies remains to be elucidated.

Despite these observations, the exact role of the Tregs are still poorly understood. Also, the activation and generation of Tregs are dependent on recognition of antigen. Although mice studies have shown that allo-reactive T cells are clonally deleted and inactivated in a paternal antigen-specific manner, and like-wise, that Tregs recognizing paternal antigens are generated during pregnancy, the exact nature and origin of the antigen responsible for generation of pregnancy-specific Tregs in natural settings are sparse (180, 182). More studies are needed to understand, whether the role of the Tregs is specifically to limit harmful pro-inflammatory/Th1 and allo-reactive immune responses toward the fetus, or whether the generation and function of the Tregs are to limit general inflammatory responses in an environment of tissue repair owed to the growing placenta (176).

Mechanisms of Treg-Mediated Immunosuppression in Pregnancy

The mechanisms of fetal tolerance in pregnancy are many and cannot exclusively be attributed to the generation of fetal-specific Tregs. Tolerance include a balance between clonal deletion and/or inactivation of allo-reactive effector cells and immune suppression mediated by regulatory subsets comprising both innate cells, such as tolerance-inducing DCs, alternatively activated macrophages (M2) and the cytokine-producing CD56^{bright}CD16⁻ decidual (d)NK cells, and adaptive cells, including CD4⁺ and CD8⁺ Tregs as well as regulatory B cells. All working together in an impressive network that secures a successful pregnancy (200–202).

Cells of the endometrium and placenta release numerous chemokines that play a role in orchestration of immunomodulatory cells (203). In contrast to dNK cells, which can be generated from CD34⁺ hematopoietic precursors present

in the human decidua (204, 205), Tregs seem to be recruited to the uterus during estrus and in early pregnancy by chemokines such as CCL1, CCL4, CCL17, and CCL22 (171, 189, 206). In the pregnant mouse, the chemokine receptor CCR5 recognizing CCL4 is expressed by 70% of the CD4⁺CD25⁺ Tregs, and interaction of CCR8 with CCL1 has been shown to enhance the immunosuppressive function of the Tregs by inducing FoxP3 expression and IL-10, TGF- β and Granzyme B production (189, 207).

The fetal trophoblast cells also express and release a number of immunomodulatory molecules that contribute to the Treg balance. Importantly, as seen in cancer and discussed above, the attenuated expression of polymorphic HLA molecules in addition to the expression of the non-classical HLA class Ib, which show very limited polymorphism, are believed to protect the fetal trophoblast cells from a direct cytotoxic response by maternal Teff and NK cells (7, 208–210). Moreover, interactions with HLA-G have been shown to induce the development of immunosuppressive CD4⁺ T cells and suppress APCs (211, 212). A special CD8 α ⁺ Treg cell that specifically identifies Qa-1a (equivalent to the human MHC class Ib molecule HLA-E), has been found to control activated CD4⁺ T cells in mice (213). Furthermore, CD8 α ⁺ cells have been shown to infiltrate the ovaries during ovulation. Although the origin and characterization of the nature of the CD8 α ⁺ cell was unclear, the CD8 α ⁺ cells seemed to originate from the thymus and responded to the thymus-expressed chemokine (TECK), which is important for T cell development. Importantly, it was found that depletion of the CD8 α ⁺ cells resulted in impaired fertility of the female mice, suggesting a role in the establishment of pregnancy (214). The role of CD8⁺ Tregs in pregnancy is unclear, however, it would be interesting to study if any similar cell populations are important for pregnancy in humans.

Negative regulators such as PD-L1 (215), the TNF family members FasL (CD95L or Apoptosis Antigen (APO)-1L) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL; CD235/APO-2L) (216–218) and IDO (219, 220) are also expressed by the trophoblast cells. These molecules, as described in previous sections, contribute to T cell homeostasis by inducing apoptosis in allo-reactive Teff cells. Moreover, the trophoblast cells also secrete IL-10 and TGF- β that contribute to Treg recruitment and differentiation (221, 222), of which IL-10 also has been shown to upregulate HLA-G, thus further contributing to the Treg balance (223). As mentioned earlier, IL-10 and TGF- β play an equally important role during cancer development. However, in a cancer setting their pleiotrophic function imply a more unclear effect on the Treg balance depending on cancer type.

The function of Tregs during pregnancy mirror those occurring in the tumor microenvironment, in which Tregs regulate other immune cells present in the maternal-fetal interphase (**Figures 1, 3**). Tregs limit the effect of allogene-specific Teff cells by the expression of CD25, CTLA-4, and the PD-L1 pathway and the secretion of IL-10 and TGF- β that induce apoptosis and suppress cytotoxicity in recipient cells (171, 173, 176, 197). PD-L1 expression by Treg cells has been found to inhibit proliferation of CD4⁺CD25⁻ T cells and suppress

expression of the pro-inflammatory cytokines IFN- γ and TNF- α (224). PD-1 expression on T cells seem to be increased in healthy pregnancy compared to non-pregnant women (225), while reduced levels of PD-1 and PD-L1 have been suggested to promote Th17 proliferation, thus causing the Treg/Th17 imbalance observed in PE (226). Consistent with this, mice studies have shown that blocking of PD-L1 results in lower numbers of Tregs and increased Teff and Th17 populations, as well as increased fetal resorption and reduced litter size (227, 228). Moreover, engagement with PD-L1 and secretion of TGF- β promote the development of Tregs by increasing FoxP3 expression, and reducing Teff cell development (227). The immunosuppressive function of the PD1 pathway seems to work by similar mechanisms in cancer and pregnancy, though with opposite effect in terms of prognosis. Whereas, inhibition of the pathway is desirable for activating the immune response against cancer cells, activation and high PD-L1 expression is important in terms of promoting a healthy pregnancy.

The DCs are central for activation and differentiation of T cells by presenting antigen and providing co-stimulatory signaling. Formation of the placenta in early pregnancy is associated with increased number of tolerogenic immature (i)DCs. These cells have been shown to produce increased levels of IL-10 and induce Treg formation during pregnancy (229–231). Further, Tregs have been shown to induce the formation of anti-inflammatory alternatively activated macrophages (M2), partly by IL-10 (232). Moreover, Tregs secrete heme oxygenase-1 (HO-1) that keep DCs in an immature state in which they secrete higher amounts of IL-10 that further induce the formation of Tregs (233). In turn, these cells secrete IDO and TGF- β and engage with the CTLA-4 receptor on Tregs that together impairs allogene-specific T cell activity and induce Treg formation, further affecting the Tregs/Teff balance (234, 235).

Uterine and decidual NK cells play important regulatory functions for the vascularization and formation of the placenta in early pregnancy (236, 237). Like Tregs, a balance between cytotoxic CD56^{dim} and regulatory CD56^{bright} NK cells seems important for a successful pregnancy. Pregnancy complications such as RPL and PE have also been linked to a reduced CD56^{bright}/CD56^{dim} NK cell ratio (238, 239). Tregs might also be important in regulation of the dNK cell phenotype. It has been shown that Tregs reduce cytotoxicity of NK cells in an TGF- β -dependent fashion and inhibit the release of IL-15 from DCs that are important for the generation of dNK cells (240, 241). Likewise, TGF- β secreted from decidual stroma cells has been shown to change the peripheral CD56^{dim} toward a decidual-like CD56^{bright} NK cell phenotype (242). It is likely that TGF- β secreted from Tregs will have a similar effect on the NK cell phenotype. On the contrary, NK cells are also able to contribute to the Treg homeostasis by reducing Th17 cell responses through the production of IFN- γ and inducing CD25⁺FoxP3⁺ Treg development (235, 243).

Apart from the classical CD4⁺CD25⁺/hiFoxP3⁺ T cells described above, other types of Tregs have also been associated with pregnancy. As briefly addressed in previous sections recent studies have identified an HLA-G-expressing CD4⁺ T cell population with immunosuppressive functions. The HLA-G⁺

Tregs show an activated/memory phenotype (CD25⁺CD45RO⁺) as the classical Treg cells, but lack the expression of FoxP3 (129). The CD4⁺HLA-G⁺ T cells are found at increased levels in peripheral blood in pregnant women compared with non-pregnant women. Additionally, one study reported that the placenta was enriched in CD4⁺HLA-G⁺ T cells compared to the peripheral compartment, and cases of PE have been associated with reduced levels of the CD4⁺HLA-G⁺ T cell subpopulation in both the decidua and in peripheral blood, indicating an important role for pregnancy (129). As mentioned earlier, HLA-G-expressing T cells are also observed in the tumor microenvironment promoting a tolerogenic immune milieu, but as with other immunological mechanisms having the same effect during pregnancy and cancer development, a favorable effect is actually opposite in the two settings. Immune suppression by HLA-G is crucial in terms of a healthy pregnancy, but unwanted in a cancer setting where it promotes tumor growth.

Taken together, it has become increasingly clear that Tregs are an important player in the complex network of immune cells that secure a healthy pregnancy. Regulatory T cells are central regulators at the maternal-fetal interphase, as well as in induction of peripheral tolerance during pregnancy. However, it is also evident that the Tregs cannot stand alone. The Treg cells regulate and are regulated by a variety of cells and immune modulatory molecules. Their exact role and the precise mechanism by which they exert their immune regulation needs to be further elucidated.

Immunotherapeutic Intervention in Pregnancy Complications

Clinical treatments based upon immunomodulating Treg function in cases of infertility, pregnancy loss and pregnancy complications have not yet been implemented in routine settings.

Regarding the use of Treg measurements as a diagnostic or prognostic marker, Winger and Reed have reported an interesting but small study of 54 pregnant women with a history of infertility and/or pregnancy loss (195). In a new pregnancy, 23 of the women experienced another pregnancy loss in the first trimester, and 31 women were still pregnant after 12 weeks of gestation. The percentage of CD4⁺CD25⁺FoxP3⁺ Tregs in peripheral blood was significantly higher in the still pregnant >12 gestational week compared with the pregnancy loss group at mean day 49.2 \pm 36.1 of the pregnancy. Based on the results from this pilot study the authors propose that measurements of Tregs may serve as a biomarker for the assessment of risk of pregnancy loss in newly pregnant women. Clearly, larger studies are needed to validate this.

In a rat model of pregnancy loss induced by the administration of lipopolysaccharide (LPS) resulting in maternal inflammation Renaud et al. showed that pregnancy loss could be prevented by immunomodulation (244). This was either accomplished by administration of IL-10 or by blockade of TNF- α by a TNF- α inhibitor (Etanercept). As discussed previously, studies especially in mice have shown the importance of the presence of Tregs for a successful pregnancy. In one study by Heitmann et al. a targeted depletion of Tregs was performed

using a transgenic mouse model (245). It was observed that embryo implantation in syngenic matings was defective after Treg depletion. However, it was possible to restore embryo implantation by the transfer of Tregs into the mating mice. It can be speculated that administration or induction of pregnancy-related Tregs resembling engineered T cells used in cancer treatment could rescue some unsuccessful pregnancies caused by abnormal Tregs function either by aberrant number of cells or a functional defect. There might also be therapeutic potential in blockage or the administration of specific cytokines or HLA class Ib molecules locally in the female reproductive tract. In theory, such immunomodulation might be able to affect numbers or functionality of regulatory T cell subsets beneficial for a successful pregnancy. However, this therapeutic area clearly needs more studies primarily to clarify the basic mechanisms upon which new therapeutic strategies may be based on.

A reason as to why treatment based on immune modulation is not extensively studied in terms of pregnancy complications compared to the field of cancer immunotherapy, might be that the focus on the cause of pregnancy complications such as PE has been directed toward several different factors besides immune regulation.

CONCLUSIONS AND PERSPECTIVES

Many similarities exist in the regulatory immune landscape of the tumor microenvironment and at the feto-maternal interface during pregnancy (**Figure 1**). While trophoblast cells possess both maternal and paternal antigens, cancer is also a kind of a chimera consisting of cells presenting both self and tumor-associated antigens. Furthermore, it seems that the role of Tregs in pregnancy and cancer, modulating the host response directed toward foreign antigens in the placenta and the tumor, respectively, may not be very different. Keeping this in mind, the immunosuppressive role of Tregs in pregnancy is a physiological process, while the inhibitory role of Tregs in cancer is pathophysiological, which nevertheless also makes the elaboration of immune modulating capacity in both cases even more appealing. The apparent role of Tregs in early tolerance induction is another issue also important in both cancer and pregnancy. The early Treg response to embryo implantation is similar to those in a cancer setting with Tregs being activated within the first days of implantation and tumor emergence, respectively (5, 198). Most essential in reproduction and cancer immunology is the similar mechanisms of escape from host immunosurveillance mediated by Tregs in combination with other immune cells and immune factors. Therefore, investigating mechanisms engaging Tregs and their regulation in apparently

distant fields like pregnancy and cancer have close connections and could be highly beneficial (246). This would involve a better mapping of cytokine networks and e.g., interactions with HLA class Ib molecules in both situations.

Investigating the similarities in immunity through the different trimesters in pregnancy and in advanced malignancies has the potential to advance the knowledge of mechanisms involved in Treg function and eventually help to overcome the burden of long-term antigen exposure and immunologic exhaustion. Treatment strategies can be aimed at aspects such as invasion, angiogenesis, immune privilege, and malignant proliferation (5). We can take advantage of the knowledge from the two different fields of cancer and pregnancy complications and potentially use it to facilitate the search for novel treatment strategies in either of them.

Modification of the presence of Tregs and the function of these cells have been studied more extensively in relation to cancer than in the case of pregnancy complications, and treatment strategies targeting immunosuppressive pathways are already established for some cancers. However, more discoveries on Treg regulation is essential for the exploitation of these cells both in the field of cancer and reproductive immunology in order to improve immunotherapy and to help prevent pregnancy complications. Similar for both fields, future research in interactions of Tregs with other cells, molecules responsible for recruitment of Tregs into the maternal-fetal interface and tumor site, and intracellular pathways of regulatory signaling in Treg cells, will be highly valuable. Especially knowledge about the interactions of Tregs with other immune cells is needed to provide safe treatment and to reduce immune-related side effects (246).

AUTHOR CONTRIBUTIONS

NJ, GP, and TH participated in the design and draft of the manuscript. NJ is the main author of sections dealing with Tregs in cancer, while GP drafted sections regarding Tregs in reproductive immunology. TH was responsible for overall supervision and did the final proofreading of the draft. All authors have read and accepted the final version of the manuscript. The figures and the table included in the article are made by the authors (**Figure 1**: TH and GP, **Figures 2, 3**: GP, **Table 1**: NJ), and the figures and the table have not been published before.

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Optimal Tolerogenic Dendritic Cells in Type 1 Diabetes (T1D) Therapy: What Can We Learn From Non-obese Diabetic (NOD) Mouse Models?

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Tolerogenic dendritic cells (tolDCs) are explored as a promising standalone or combination therapy in type 1 diabetes (T1D). The therapeutic application of tolDCs, including in human trials, has been tested also in other autoimmune diseases, however, T1D displays some unique features. In addition, unlike in several disease-induced animal models of autoimmune diseases, the prevalent animal model for T1D, the NOD mouse, develops diabetes spontaneously. This review compares evidence of various tolDCs approaches obtained from animal (mainly NOD) models of T1D with a focus on parameters of this cell-based therapy such as protocols of tolDC preparation, antigen-specific vs. unspecific approaches, doses of tolDCs and/or autoantigens, application schemes, application routes, the migration of tolDCs as well as their preventive, early pre-onset intervention or curative effects. This review also discusses perspectives of tolDC therapy and areas of preclinical research that are in need of better clarification in animal models in a quest for effective and optimal tolDC therapies of T1D in humans.

Keywords: type 1 diabetes, cell therapy, animal models, tolerogenic dendritic cells, NOD mouse, protocol optimization

INTRODUCTION

Type 1 diabetes (T1D) is a multifactorial, organ/cell-specific disease resulting from an autoimmune destruction of insulin-producing β cells of the endocrine pancreas by CD4⁺ and CD8⁺ T cells, as well as macrophages infiltrating the islets. The insulin deficiency together with suboptimal insulin replacement result in a complex metabolic derangement with abnormal metabolome (1, 2). The number of children and adolescents with T1D is estimated at 1,106,500 worldwide. The incidence of T1D is increasing more rapidly than expected and is causing a significant health problems and economic burden, also due to severe complications (e.g., diabetic retinopathy, neuropathy, kidney failure), (3). At present, no effective cure or secondary prevention of T1D exists. Although heavy immunosuppression, or a reset of the immune system by immunoablative therapy followed by autologous or allogeneic bone marrow transplantation, were able to stop/prevent recurrence of β -cell destruction, they have not been considered acceptable as treatments for T1D (4–7). Nevertheless, with recent advances, non-ablative autologous hematopoietic stem cell

transplantation may yet come into its renaissance as a cure of T1D [reviewed (8)]. Apart from the above mentioned cases, no clinical trial has so far been able to establish remission of T1D in patients.

Dendritic cells (DCs) are specialized, potent antigen-presenting cells (APCs) that represent key regulators of immune responses, both innate and adaptive, effector and tolerance (9). Dendritic cells were first discovered by Steinman et al. in 1973, who described their immunostimulatory effect on T cells (10, 11). Under physiological conditions, when antigen presentation occurs without additional “danger” stimuli, DCs displaying immature character, steadily migrate to lymph nodes (LNs) and maintain peripheral tolerance in various tissue-specific environments (12, 13). Dendritic cells induce peripheral tolerance by various mechanisms including T-cell deletion, T-cell anergy and hyporesponsiveness, and the expansion of natural Tregs, inducible Tregs (14, 15), and Bregs (16). Although several different human and mouse DC subsets have been identified, and functionally specialized subsets exist [reviewed (17, 18)], it seems that their tolerogenic functions are not linked to a specific lineage or tissue subset and several micro-environmental factors (e.g., microbiom, apoptosis) may contribute to maintaining their tolerogenic character (19, 20).

Cell-therapies comprising tolerogenic DCs (tolDCs), Tregs or bone marrow transplantations represent novel emerging strategies for the treatment of autoimmune diseases (8, 21, 22). They also hold promise for the treatment of allergies (23–25), and may also improve transplantations (26). Both tolDCs and Tregs as *ex vivo* cell-therapies share certain disadvantages. For example, a requirement for extensive manipulations *in vitro* or their patient-specific, tailor-made character, makes their preparation laborious and expensive. Tolerogenic DCs display some specific advantages compared to Tregs. First, they act as central regulators of immune responses and may thus target Tregs at various check points [reviewed 17] lacking the clonality issues of T-cells (27), they possess good potential to migrate to immune inductive sites [reviewed (28, 29)]. Second, DCs are relatively easier to differentiate and to expand from peripheral blood monocytes separated by leukapheresis (30). Tolerogenic DCs are being tested in clinical trials as a potential cell-therapy for autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, T1D and Crohn’s disease [reviewed (31)]. At this moment, there is one completed and one ongoing phase I clinical trial with autologous tolDCs in patients with type 1 diabetes (32–34).

For T1D, the beginning of dendritic cell-based therapies goes back to the study by Clare-Salzler et al. who documented that DCs isolated from pancreatic lymph node (PLN), but not T cells or DCs from other lymph nodes, of 8–20-week-old NOD females prevented diabetes in 4-week-old recipient NOD mice (35). This study still poses questions requiring follow-up experimentation e.g., does the age of DC donors alter their disease-preventive effects, or do environmental factors influencing the penetrance of T1D modify the disease-preventive capacity of PLN DCs. Nevertheless, and more importantly, this study paved the road to DC-based cell therapies in T1D. Since then, several protocols of tolerogenic DCs have been developed, many using tolDCs without *in vitro* supplied antigen (36–42),

although antigen-loaded tolDCs protocols have also been tested (42–46). These protocols have been applied to animal models of T1D, preferentially the non-obese diabetic (NOD) mice (21).

Animal models represent an irreplaceable tool in preclinical tolDCs testing. Many studies tolDCs studies have been carried out in the NOD mouse model, as it represents a very close (genetically, immunologically, and environmentally) and spontaneous model of the human disease, allowing one to study therapeutical interventions in the context of the natural history of type 1 diabetes (47, 48). NOD mice however display also several suboptimal features, among them defects in maturation of the myeloid lineage and myeloid DCs are indeed the most related to tolDCs testing (49, 50). Several other mouse models have been employed, albeit less frequently, such as the NOD-SCID model of adoptive cotransfer of diabetes (51), the NOD RIP-IFN- β mouse (44), the LCMV-RIP induced model (52), or humanized HLA-DQ8/RIP-B7.1 or HLA-DR4 mice (39, 46).

Because a wide array of protocols for tolDCs exists, preclinical testing of multiple parameters is both difficult and necessary. Various parameters of safe and effective tolDCs for T1D should be optimized in *in vitro* and in animal models (e.g., tolDC stability, homogeneity, survival, migration capacities). In addition to optimal antigen form and dose in case of antigen-loaded tolDCs, an optimal combination of cell dose, application scheme and application route should be determined. Only a few tolDC protocols, e.g., IL-4 transduced tolDCs, were able to cure or revert diabetes in NOD mice, thus other protocols should be tested in more animal models and attempts should be made not only to prevent, but also to stop the diabetogenic process before disease onset and/or to cure already diabetic animals (53, 54).

Compared to e.g., mucosal delivery of autoantigens as prevention/therapy of T1D (55, 56), using a cell entity for *in vivo* therapeutic effects represents a much more challenging scenario that requires thorough preclinical testing. Efforts have been made to standardize information provided for various protocols, models and data from preclinical testing of tolDCs in autoimmune diseases (57, 58).

While the therapeutic use of *in vivo* targeted tDCs via DEC-205 (9) or the use of plasmacytoid DCs (59) in T1D have already been reviewed, this review deals with animal testing of tolDCs prepared *in vitro* from mouse bone marrow precursors, that are almost exclusively used as a mouse parallel to human monocyte-derived tolDCs from peripheral blood mononuclear cells (PBMCs) (30). In this review we discuss the parameters of *in vitro* generated tolDCs in mouse models of T1D, the importance of protocol optimizations and what aspects are desirable to be further addressed in preclinical testing in animal models of T1D.

CULTURE CONDITIONS OF tolDCs IN T1D

TolDCs *in vitro* Propagation

Most of the protocols applied in T1D use propagation of tolDCs from bone marrow progenitors in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (36, 37, 42, 45, 46, 53, 54, 60–66), while two groups reported GM-CSF and IL-10 (39, 43, 52). A few studies employed GM-CSF alone for *in vitro* generation of diabetes-preventive tolDCs

(38, 44, 67). An overview of tolDCs protocols is provided in **Table 1**. Adoptively transferred *in vivo* generated GM-CSF DCs (69) decreased diabetes incidence in NOD mice as well. There are also reports supporting the role of TGF- β in DC-mediated disease protection. Thus, in another study targeting DCs *in vivo*, *s.c.* microparticle-encapsulated TGF- β was used to enhance diabetes protection of NOD mice (70) and *in vitro* GM-CSF generated bone marrow-derived DCs (BMDCs), conditioned for 24 h with TGF- β , prolonged islet graft survival in diabetic mouse recipients (71).

Although both GM-CSF+IL-4 propagated tolDCs, and to a lesser extent also GM-CSF tolDCs, were shown to prevent diabetes, comparison of unloaded GM-CSF/IL-4 vs. GM-CSF/IL-10 vs. GM-CSF alone-cultured tolDCs carried out by Tai et al. documented diabetes-preventive effect only for GM-CSF+IL-10 cultured tolDCs (39). Two other studies testing IL-10 showed that the GM-CSF+IL-10 protocol is effective only for antigen-loaded tolDCs cultured in autologous serum (43, 52). GM-CSF tolDCs were inferior to GM-CSF+IL-4 generated ones in diabetes prevention in NOD mice, especially when cultured without antigen (37, 72). The importance of IL-4 in the propagation of effective tolDCs was further documented by several studies (54, 73, 74), including gene array analyses, mapping increased expression of co-stimulatory molecules and differences in cytokine/chemokine signatures (75). BMDCs cultured in GM-CSF showed suboptimal characteristics compared to cells generated in combination with IL-4, especially in serum-free conditions (73) that are more relevant for human DC-preparations. Markedly enhanced trafficking and functional capacities were reported for IL-4 and GM-CSF propagation of DCs (76). These data may thus explain the less satisfactory results obtained with GM-CSF prepared tolDCs. Later a more worrying message on the heterogeneity of bone marrow derived DCs appeared (77).

Homogeneity of tolDCs

Helft et al. provided detailed and comprehensive data documenting that mouse BMDCs prepared by culture with GM-CSF consist of a heterogeneous cell population comprising both immature DCs but also monocyte-derived macrophages that are found within the CD11c⁺MHC-II⁺ cells (77). While a similar study on GM-CSF+IL-4 or GM-CSF+IL-10 propagated cells using gene expression profiling is not available, one can perhaps speculate that an addition of IL-4 or IL-10 is unlikely to fully overcome this problem. Not only bone marrow lymphoid precursors, but also early progenitors of mouse conventional and plasmacytoid DCs, express Flt3 (78). Monocytes, macrophages, osteoclasts and DCs share a common progenitor (MDP). Compared to human lineage commitment, a monocyte/macrophage and osteoclast bipotent progenitor (MDP) is described in the mouse, but a dedicated DC progenitor is currently not clearly identified (79). Apart from these inherent homogeneity issues at the level of bone marrow progenitors used for generation of tolDCs, contaminating cells (T cells and/or B cells and MHC II⁺ cells) were depleted by complement/Abs in some protocols (37, 43, 52, 54) or by MACS depletion using

e.g., anti CD3, B220, and Gr-1 mAbs (40). Several other tolDCs protocols however did not employ this purification step.

Another, more technical aspect of BMDC cultures for tolDCs, is the cell adherence and the fact that non-adherent and/or loosely adherent cells are harvested. This gray zone is further augmented by the tolerogenic protocols used. Various tolerogenic protocols or their modifications, such as the lengths of dexamethasone and vitamin D3 or D2 exposure, influence both *in vitro* adherence/yield of the tolDCs, but also the level of expression of their characteristic surface markers e.g., CD11c, CD40, CD80, CD86 (80, 81). Similarly, when reporting surface characteristics of *in vitro* generated tolDCs by flow cytometry in relation to diabetes prevention, modest or no SSC-A vs. FSC-A pre-gating should be used to document heterogeneity of cells that are actually injected to animals. Careful gating strategies have been documented, such as in the comparative study of clinical grade human tolDCs (80). Thus, mouse tolDCs protocols, especially those showing promising results in disease prevention, should be tested for cell homogeneity (**Figure 1**). This would also allow better comparison of their efficacies. Interestingly, preparation of mouse tolDCs from PBMCs was reported already back in 2000 (82). Although more laborious and demanding, perhaps the promising protocols should be re-tested with PBMC-derived mouse tolDCs for easier translation to clinical trials. No such attempt seems to have been published.

Some of these above described issues are indeed not present in human tolDCs that are prepared from PBMCs, and where closed culture systems/bags are preferred; their different materials were extensively tested and current best practice methods for preparation of immature or mature DCs do not rely on cell adherence to surfaces (83).

Tolerogenic Protocols in T1D

An overview of tolerogenic protocols for *in vitro* generation of tolDCs in T1D is provided in **Table 1**. While some of the protocols were tested in several modifications and experimental set ups, others were reported only as a standalone study. Anti-sense oligonucleotides targeting expression of co-stimulatory molecules CD40, CD80, and CD86 led to induction of tolDCs with immature phenotype. Single *i.p.* administration of 2×10^6 cells delayed diabetes onset and led to induction of splenic Tregs in NOD mice (36). Weekly injections from age of 8 to 12 weeks then completely prevented diabetes onset in NOD mice, possibly by enhanced expression of IL-7 as a survival factor for Tregs (84). The anti-sense oligonucleotides were tested in a phase 1 clinical trial (32, 33). In another study by this group, a microsphere delivery system of the anti-sense oligonucleotides was able not only to prevent diabetes but repeated (twice weekly) *s.c.* administrations also reversed hyperglycemia in new onset diabetic NOD mice (85). The second opened phase I clinical trial (34) is based on a protocol of antigen (peptide)-loaded tolDCs prepared in the presence of vitamin D3 with final lipopolysaccharide (LPS) activation (46). We have used a tolDCs protocol based on vitamin D2 and dexamethasone for diabetes prevention in NOD-SCID and NOD models of T1D, both with antigen-loaded and unloaded tolDCs prepared in FBS-supplemented or serum-free conditions, and with final

TABLE 1 | Example protocols of tolDCs in T1D and extent of their preclinical testing.

tolDC propagation	Modification	Culture condition	Stabilization	Unloaded/Ag1/Ag2	Ag dose	Cell dose	App. route and scheme	Model	Prevention	Pre-diabetic	Cure	References
GM-CSF+IL-4	No	FCS	No	Yes/insulin B9-23 /GAD6578–97 /GAD65260–279	3 μ M	1 \times 10 ⁵	S.c. (footpad) 3 times, weekly or 3 times, weekly + every other week	NOD	Yes	Yes	–	(65)
GM-CSF+IL-4	Vitamin D2/Dex on day 6	FBS/serum-free	MPLA	Yes/GAD65/OVA/GAD65 peptide no. 35	1 μ g or 2 μ g/ml	3 \times 10 ⁶	I.p.	NOD	Yes	–	–	(42)
GM-CSF+IL-4	Microspheres with antisense oligos. CD40/CD80/CD86	FBS	No	Yes/insulin B9-23	5 μ g	2 \times 10 ⁶	S.c. 8 times, weekly	NOD-SCID	–	–	Yes	(64)
GM-CSF+IL-4	Antisense oligos. CD40/CD80/CD86	FBS	LPS	Yes/no	n.a.	2 \times 10 ⁶	S.c. (abdominal) single or 8 times, weekly	NOD	–	–	Yes	(63)
GM-CSF+IL-4	No	FBS/serum-free	No	No/GAD65217–236	10 μ g/ml	1 \times 10 ⁵	I.v. 5 times, weekly	NOD	Yes	–	–	(66)
GM-CSF	No	FBS	No	Yes/NIT-1 apoptotic bodies	3 \times 10 ⁵ cells	1 \times 10 ⁶	I.p.	NOD	–	–	No	(68)
GM-CSF	No	FCS	No	Yes/no	n.a.	1 \times 10 ⁶	I.p. i.v. 3 times, weekly	NOD-DQ8 RIP-B7.1	Yes	–	–	(39)
GM-CSF+IL-4												
GM-GSF+IL-10						3 \times 10 ⁶	I.v.	NOD-SCID				
GM-CSF+IL-4	IL-4 transduced DCs (electroporated)	FCS	No	Yes/no	n.a.	1 \times 10 ⁶	I.v.	NOD	–	Yes	Yes	(53)
GM-CSF+IL-10	No	FBS/normal mouse serum	No	Yes/2 peptides (insulin B9-23+insulin B15-23)	10 μ g/ml	1 \times 10 ⁶	I.p.	NOD	Yes	–	–	(43)
GM-CSF	No	FBS	No	Yes/NIT-1 or SV-T2 apoptotic bodies	3 \times 10 ⁵ cells	1 \times 10 ⁶	I.p.	NOD RIP-IFN β	Yes	–	–	(44)
GM-CSF+IL-4	No	FCS	No	Yes/insulin B9-23 /proinsulin C19-A3/GAD6578–97	3 μ M	1 \times 10 ⁵	S.c. (footpad) 3 times, weekly	NOD	Yes	–	–	(45)
GM-CSF+IL-4	Antisense oligos. CD40/CD80/CD86	FBS	LPS	Yes/NIT-1 lyzate	n.a.	2 \times 10 ⁶	I.p.	NOD	Yes	–	–	(36)
GM-CSF+IL-4	IL-4 transduced DCs (adenoviral vector)	FBS	No	Yes/no	n.a.	4–5 \times 10 ⁵	I.v. single or 2 times, weekly	NOD	Yes	Yes	–	(54)
GM-CSF or GM-CSF+IL-4	No	FBS	No	Yes/3 peptides (hsp60437–460 + GAD65509–528 + GAD65524–543)	3 \times 60 μ g/ml	4–8 \times 10 ⁵	I.v. 3 times, weekly	NOD	Yes	–	–	(37)

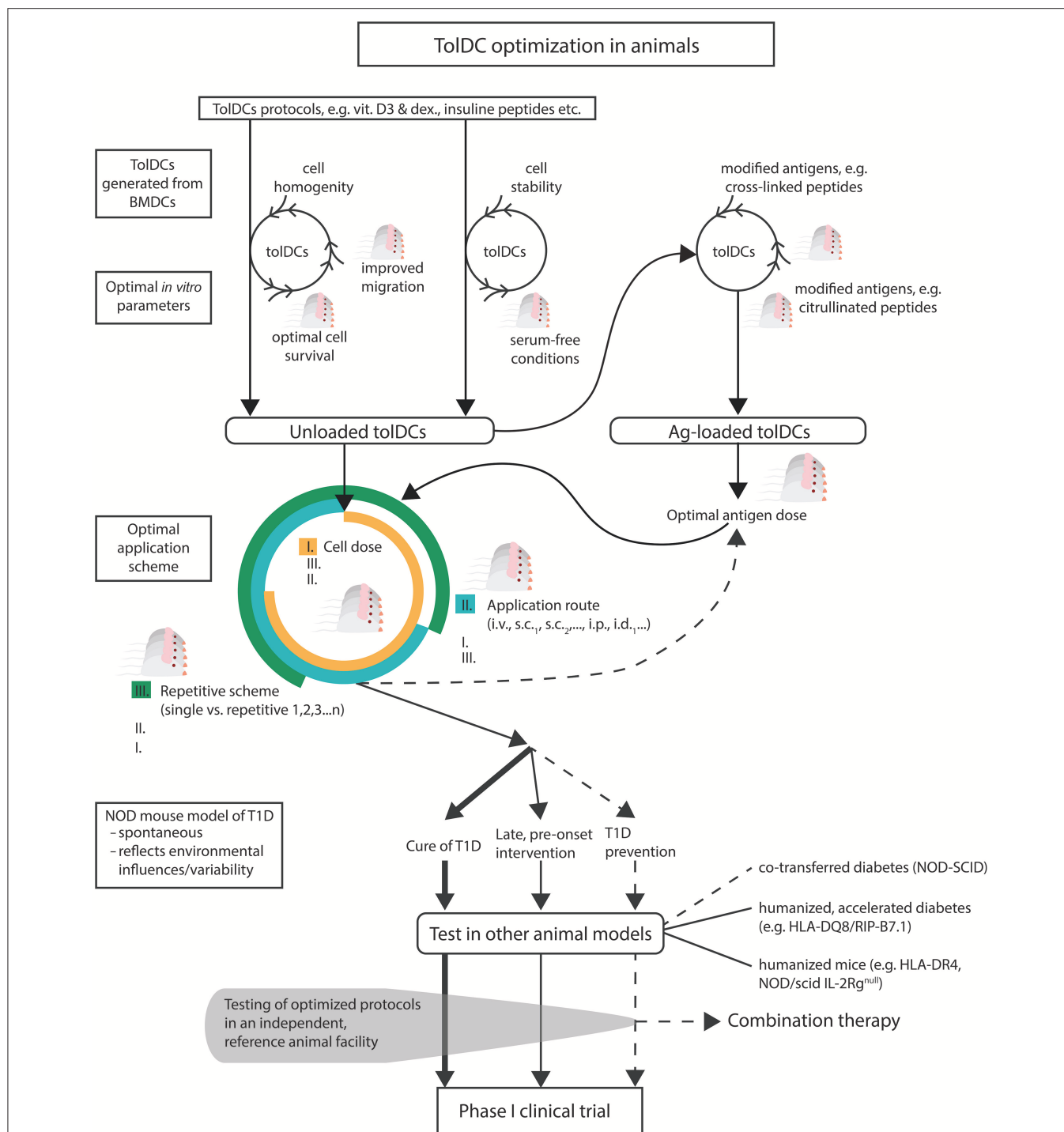


FIGURE 1 | A scheme of suggested preclinical optimizations of tolDCs in T1D. Existing protocols of tolDCs should be optimized first *in vitro* for parameters such as cell homogeneity, serum-free conditions (to mimic closer human tolDCs protocols), enhanced stability, lengths of survival *in vivo*, and improved mucosal migration. The same parameters together with improved antigen modifications should be tested for antigen-loaded tolDCs, including optimal antigen doses. Next, combination of optimal tolDCs dose, regimen and application route should be determined. Effective and fine-tuned tolDC protocols should be tested in the spontaneous (NOD mouse) and also humanized models of T1D for not only prevention but also for their effect at the late prediabetic age or for the cure of diabetes. Finally, when possible, independent testing in a reference animal facility would be desirable before undertaking difficult translation from *in vitro* and mice to humans.

tolDC stabilization by MPLA (42, 86). Using human DCs, we documented that the tolerogenic effect of such dendritic cells is controlled by p38, MAPK, ERK1/2 mTOR, and STAT3 signaling pathways (87). Comparable tolerogenic properties of vitamin D2 and vitamin D3 on *in vitro* cultured tolDCs were documented (88). Ferreira et al. showed that in NOD mice, vitamin D3 treated tolDCs migrate to PLNs and suppress T cell proliferation *in vivo* (61). The effect of vitamin D3 and dexamethasone on DCs was assessed at the transcriptome level and 11 genes that confer increased risk for T1D were found differentially expressed in tolDCs (89). It has been documented that vitamin D3 controls tolerogenic properties of DCs via a glucose metabolic pathway (61, 90). Morel's group showed that immature tolDCs were effective in disease prevention and associated with a Th2 cytokine shift (37, 60). Interestingly, IL-4 transduced tolDCs were reported by several studies to prevent diabetes in NOD mice, including at later pre-onset age (40, 54, 91) or even restore normoglycaemia in diabetic animals (53).

Several other protocols of tolDCs were reported in disease prevention, however they often included immature or semi-mature tolDCs without final stabilization (**Table 1**). Thus, immature DCs pulsed with ignored GAD65 antigen determinant GAD65_{78–97} (45), conditioning with IL-10 (43, 52), IL-25 (92), fungal extracts (93), *Lactobacillus casei* (67), carbon monoxide (94), or anti-CTLA4 Ab (95) were reported to prevent diabetes in animal models whereas DCs treated with PEGylated TLR7 ligand reduced diabetes and insulinitis (38). Similarly, DCs pulsed with apoptotic bodies from the β cell line NIT-1 prevented diabetes in transgenic RIP-IFN- β NOD mice (44). Li et al. reported genetically modified DCs expressing T-cell co-inhibitory receptor BTLA that induced CD8⁺ T-cell tolerance and decreased diabetes in NOD mice (62). Thus, many promising protocols of tolDCs in T1D have been published, but often not further developed and/or optimized.

Serum-Free, FBS, and Autologous Serum Conditions

Most of the published protocols of mouse tolDCs in T1D use fetal bovine serum (FBS) in the cell culture media (**Table 1**). However, Hasse et al. showed that DCs prepared in the presence of heterologous FBS and also pulsed with FBS on day 8, induced a Th2 cytokine shift in CD4⁺ T helper cells and increased Th2 cytokine production to FBS epitopes, including BSA, whereas tolDCs prepared in the presence of autologous mouse serum regulated immune responses in an antigen-specific manner. While unloaded tolDCs prepared in autologous mouse serum were ineffective in diabetes prevention in the induced RIP-LCMV mouse model, peptide-loaded tolDCs displayed some level of prevention. On the other hand, much better disease prevention was observed with tolDCs (irrespective of a peptide-loading) prepared in the presence of heterologous FBS (52). Later they reported similar results in NOD mice; antigen-loaded tolDCs were effective in disease prevention when cultured in autologous serum and this effect was accompanied with an increase of Foxp3⁺ Tregs in peri-insulinitic infiltrate (43). These data, together with data by Feili-Hariri et al. who reported

no additional beneficial effect of antigen-loading for tolDCs prepared in the presence of FBS, point to the possible role of FBS in immune mechanisms (Th2 vs. Tregs) by which tolDCs may operate (37). Nevertheless, human cell therapies are manufactured in serum-free conditions. Tolerogenic DCs cultured in serum-free conditions were shown to display superior characteristics compared to FBS-prepared ones as regards their tolerogenic phenotype, induction of Tregs in PLNs and also disease prevention in already prediabetic 8–9 weeks old NOD mice (66). While we observed almost no effect on phenotypic differences in vitamin D2 and dexamethasone generated tolDCs, both unloaded and antigen-loaded tolDCs displayed a tendency to better disease protection when prepared in serum-free conditions (42). Interestingly, while tolDCs propagated by GM-CSF alone displayed similar properties in serum-free vs. FBS-supplemented media, serum-free conditions were superior for GM-CSF+IL-4 generated tolDCs (73). Mouse DCs generated as an antitumor vaccine also possessed better phenotypic and functional characteristics when generated in serum-free conditions (96). Based on this evidence as well as the fact that it would bring animal experiments one step closer to clinical testing, mouse protocols of diabetes-preventive tolDCs should be tested and optimized in serum-free conditions (**Figure 1**). However, only a few studies included or compared serum-free culture conditions (**Table 1**). Testing of promising protocols in serum-free media is not difficult and in our opinion is necessary in preclinical studies.

TolDCs Stability

Among many parameters of tolDCs, their stability is of utmost importance (**Figure 1**). DCs are sufficient for CD8⁺ T-cell priming *in vivo* (97) and in pathogenesis of T1D they are instrumental for mounting effector T-cell responses involved in β -cell destruction (98, 99). Tolerogenic DCs used as a therapy for autoimmune diseases or allergies are likely to encounter inflamed environments and their compromised stability could lead to a change toward immunostimulation with possible dangerous consequences, especially in case of antigen-loaded tolDCs. Similarly, hyperglycemia and consequent oxidative stress may alter tolDCs effectiveness by reducing their T regulatory capacity (86). Naranjo-Gómez et al. compared the stability of clinical grade human tolDCs stabilized by a cytokine mix and showed that vitamin D3, rapamycin or dexamethasone conditioning suppressed allogeneic proliferations and IFN- γ production and led to stable tolerogenic phenotype *in vitro* (80). Similarly, dexamethasone-treated tolDCs further stabilized by monophosphoryl lipid A (MPLA) displayed a stable and enhanced migratory phenotype (100). We have reported stable mouse tolDCs prepared with vitamin D2/dexamethasone and exposed for 24 h to MPLA (42). Other protocols used LPS (36, 46) or its combination with IL-10 (69) or IFN- γ (81). Both protocols that progressed to clinical trials used stabilized tolDCs in animal experiments (36, 46).

There are also reports of unstable tolDCs not suitable for cell therapies (101). Attempts to further harness stability of tolDCs (**Figure 1**) were undertaken e.g., DCs transduced with human 25-hydroxyvitamin D 1 α hydroxylase (102), whereas Chai et al.

reported that recombinant OCILRP2-Fc (Osteoclast inhibitory Lectin-related Protein 2) inhibits LPS-driven maturation and differentiation of BMDCs (103). The modulating effects of vitamin D3, rapamycin dexamethasone, TGF- β and IL-10 were also assessed in a comprehensive study with clinical grade tolDCs by Boks et al. who nicely documented superior tolerogenic effect of IL-10, including stability of DCs and induction of Tregs with strongest suppressor activity on T cells (104). Thus, propagation of tolDCs with IL-10 (39, 43, 52) might be superior to GM-CSF+IL-4 only. Unfortunately, many of the animal protocols use immature DCs without terminal differentiation and/or their stability was not adequately addressed (Table 1). Since tolDCs stability is critically important for translation to humans, it should be thoroughly addressed for any protocol developed with this intention.

Optimal tolDCs Dose, Application Schemes

While many different tolDCs protocols were tested in animal models of T1D for their diabetes preventive or even curative effect, experiments optimizing such protocols are either missing or not published. Only a few studies actually reported testing multiple doses of tolDCs and/or single vs. multiple repetitive schemes or their combinations (Table 1). Cell doses varied from 2×10^5 to 3×10^6 tolDCs. More than one application scheme have also been reported (39, 54, 60, 63, 65) (Table 1). Although human clinical trials are usually only inspired by doses from animal testing and more data are available from the use of DCs in cancer immunotherapy, more optimizations of the promising tolDCs protocols in T1D should be carried out in preclinical testing (Figure 1). Such optimization is perhaps not scientifically very appealing and may be difficult to get published, yet such important animal data are lacking.

ROUTE OF ADMINISTRATION AND tolDCs MIGRATION

Several application routes including *i.p.*, *i.v.*, *s.c.*, and also *i.d.* were used in animal studies of tolDCs in T1D (Table 1). Many animal studies of diabetes prevention used *i.p.* (36, 39, 42–44, 52, 61, 87) or *i.v.* (37–40, 53, 54, 60, 66, 69) routes of administration. Preferred migration to PLN compared to mesenteric lymph node (MLN), spleen and inguinal lymph node (ILN) (29, 42), and increased accumulation of bone marrow-derived DCs in pancreas and liver (61) was documented after *i.p.* administration. Intravenous application also targeted PLNs but together with the spleen (29, 37, 54). Creusot et al. provided a very elegant study comparing *in vivo* homing of bone marrow-derived DCs after *i.v.* and *i.p.* administrations. While *i.p.* administration led to accumulation of DCs preferentially in PLNs and also omentum, the *i.v.* route targeted spleen as well as PLNs and lung-draining LNs but very few cells were detected in MLNs, ILNs, and LNNs (29). Intraperitoneal and intravenous application routes are also being used in current human trials with tolDCs [reviewed (31)]. The two tolDCs clinical trials in T1D (32, 34) are using subcutaneous and intradermal application routes,

respectively. While the *i.d.* application was reported in a proof-of-concept animal study without diabetes incidence testing (46), *s.c.* applications at locations such as the abdominal flank overlying the pancreas (63, 64) the footpad (45, 65) or unspecified (62) were referred for tolDCs in T1D. Tolerogenic DCs were nicely documented in the subcutaneous compartment after *s.c.* injection and their accumulation in PLNs was reported (64). The *s.c.* abdominal application close to the pancreas projection was described as preferable for accumulation in PLNs [reviewed (30)]. Most of the animal studies in T1D included *i.p.* and *i.v.* applications of tolDCs. A comparison of all currently used application routes carried out with the same tolDCs protocol is however missing (Figure 1). TolDCs were reported to survive about 1–2 weeks *in vivo* (29). We detected live tolDCs *in vivo* for up to 12 days following *i.p.* administration (42). Nevertheless, more experiments on the lengths of *in vivo* survival of tolDCs are needed, as application routes may also influence survival of tolDCs (Figure 1).

Increased migration of tolDCs to PLNs, pancreas and/or other mucosal LNs is a highly desirable feature of therapeutic tolDCs in T1D, so that they have a better access to T1D-related antigens. Priming of diabetogenic T cells in NOD mice occurs in PLNs and gut-associated LNs (105). The importance of PLNs in T1D may be also supported by a study showing that surgical removal of PLNs at 3 weeks (but not 10 weeks) prevents development of diabetes in NOD mice (106). Among molecules that may improve migration and mucosal homing of tolDCs, CCR7 expression was documented as critically important [reviewed (28, 107)] while L-selectin may be engaged for entering the LNs (108). Migration pattern is an important parameter for tolDCs. While tolerogenic agents (e.g., dexamethasone, vitamin D3) may decrease CCR7 expression [reviewed (109)], MPLA activation and terminal differentiation of tolDCs increases CCR7 and CXCR4 expressions and thus improves their migratory capacity (100). Similarly, rapamycin was reported to increase CCR7 expression in human DCs (110). Final stabilization of clinical grade human tolDCs by exposure to TNF- α , IL-1 β , and PGE (2) also increased their migration efficacies (104). On the other hand while IL-10 was reported to improve tolDC stability and Treg-mediated tolerogenic capacity, it also impairs mucosal migration of DCs by downregulation of their CCR7 expression (111). Interestingly short but not continuous exposure to IL-4 toward the end of GM-CSF propagation of DCs was reported to enhance their trafficking efficacy (76). Retinoic acid, but not expression of CD103 by DCs, was shown to be critical for mucosal $\alpha 4\beta 7$ -mediated homing of T cells (112). Protocols of tolDCs in T1D should be thus optimized not only for their stability and tolerogenic capacity but also migratory efficacy to secondary lymphoid organs. However this is rarely the case (Table 1).

ANTIGEN-UNSPECIFIC vs. ANTIGEN-SPECIFIC APPROACH, ANTIGEN DOSE

Several studies reported diabetes prevention by unloaded tolDCs (Table 1). Feili-Hariri et al. reported tolDCs generated without

an antigen, to prevent diabetes after *i.v.* administration to NOD mice (37). Pulsing tolDCs with a mixture of hsp60 and two GAD65 peptides did not augment their ability to prevent diabetes development. Diabetes prevention was possibly mediated by an induced Th2 shift in treated animals (60). Later, they also reported tolDCs transduced for IL-4 expression to prevent diabetes in NOD mice when applied *i.v.* at the age of 5 weeks but also at 7–8 and 10 weeks, i.e., in animals just before the onset of diabetes and with progressed insulinitis (54). In both studies, migration of tolDCs to spleen and PLNs was nicely documented. Another approach was documented in the study by Ma et al. who also used antigen-unloaded tolDCs that were treated *in vitro* with NF- κ B-specific oligodeoxyribonucleotide (ODN) for diabetes prevention in older 6–7-week-old NOD mice (41). Interestingly, when these tolDCs were used in an antigen-specific manner i.e. pulsed *in vitro* with islet lysate, the diabetes preventive effect was lost. Unloaded tolDCs treated with antisense oligonucleotides against costimulatory molecules (CD40, CD80, and CD86) delayed diabetes in NOD mice, but not if pulsed *in vitro* with cell lysate from the NIT-1 β -cell line. The diabetes prevention was associated with increased numbers of CD4⁺CD25⁺ T cells (36). Creusot et al. then published a study using IL-4 transduced tolDCs. When applied *i.v.* to 12-week-old NOD mice, these antigen-unspecific tolDCs migrated to spleen and PLNs and were able to significantly delay or prevent onset of diabetes in pre-diabetic animals (40). Tolerogenic DCs propagated with GM-CSF+IL-4 from BMDCs isolated from GM-CSF-treated NOD mice also decreased development of diabetes when applied to 3-week-old NOD recipient mice (69). An elegant study by Tai et al. in which multiple parameters were assessed, showed that tolDCs propagated with GM-CSF+IL-10 suppressed diabetes and insulinitis in two animal models, the NOD and HLA-DQ8/RIP-B7.1 mice (39). While *in vivo* stimulation of DCs with PEGylated TLR7 ligand delayed diabetes and reduced insulinitis upon transfer to prediabetic NOD mice, when these DCs were pulsed with GAD65_{515–524} peptide they significantly increased insulinitis compared to both controls but also unloaded PEGylated TLR7 ligand-treated DCs (38). Vitamin D2 and dexamethasone conditioned tolDCs also prevented diabetes in NOD-SCID and NOD models, but this effect was lost if tolDCs were loaded with mouse GAD65, its immunodominant peptide no. 35 or even with a control protein - OVA (42, 86). Remarkably, multiple *s.c.* (abdominal flank) injections of immature DCs treated with antisense oligonucleotides against costimulatory molecules restored normoglycaemia in already diabetic NOD mice (63).

This review is not listing all tolDCs studies in animal models of T1D, but the above described examples well-document that unloaded tolDCs, often without stabilization, or immature DCs were effective in disease prevention, in stopping clinical onset of diabetes at 12 weeks of age or even restoring normoglycaemia in already diabetic NOD mice. The last two stages may correspond to individuals that could be diagnosed as having high risk of progression to T1D or new onset T1D patients. On the other hand, modifications with antigen rendered these protocols ineffective or even worsened insulinitis (36–38, 41, 42). This scenario is surprisingly different from

the expectations with antigen-loaded tolDCs that are being developed aiming for a more specific and efficient tolDCs therapy in T1D.

Among studies dealing with an antigen-specific approach, Marin-Gallen et al. showed that immature DCs loaded with apoptotic bodies from the NIT-1 β -cell line, but not from control SV-T2 embryonic cell line, prevented diabetes. Unloaded control immature DCs (iDCs) had no preventive effect as well (44). The importance of using autologous serum and not heterologous antigens (i.e., FBS serum) for antigen-loaded (insulin B9-23 and B15-23 peptides) tolDCs was clearly documented both by diabetes prevention and Tregs induction (43). Nevertheless, when splenocytes from disease-protected animals using insulin peptide-loaded tolDCs were retested for their regulatory potential in the adoptive NOD-SCID co-transfer model, they caused more rapid and a 100% onset of diabetes compared to controls (43). Later Looney et al. investigated the effect of serum-free vs. FBS-supplemented culture condition on tolDCs loaded with GAD65217-236 peptide. They demonstrated that only tolDCs cultured in serum-free medium prevented diabetes in NOD mice, induced Tregs and lasting β -cell specific T-cell responses (66). Recently, Lo et al. showed that immature DCs cultured in the presence of FBS and pulsed with subdominant or ignored peptide determinants, but not with immunodominant insulin peptide B9-23, decreased diabetes incidence in already 9-week-old NOD mice (65). Thus, this is another example of antigen-specific iDCs being less effective in disease prevention.

At present, unloaded tolDCs seem to represent more suitable choice for clinical testing, both from the point of their efficacy as well as safety. More research is needed in the field of antigen-loaded tolDCs, as delivery of immunodominant epitopes may pose an increased risk of disease acceleration. Such protocols should be well-optimized in animal models of T1D (**Figure 1**). One risk factor may represent the after death fate of antigen loaded tolDCs. Antigens from therapeutic tolDCs may cause sensitization via processing and presentation by recipient APCs (113). Another parameter to consider is the antigen dose (**Figure 1**). An elegant study by Smyth et al. documented that low doses of antigen presented by both immature and mature DCs, but also unloaded mature DCs, induced weak TCR signaling via Akt/mTOR pathway and expansion of Foxp3⁺ Tregs. On the other hand, high antigen doses led to strong Akt/mTOR signaling and expansion of Foxp3⁺ Th cells. This effect was modulated by T-cell-produced IL-6. The DC phenotype was thus less important than antigen dose (114). This finding corresponds with data from other immunointervention strategies such as mucosal delivery of autoantigens in which lower autoantigen doses were often associated with more satisfactory results (56, 115, 116).

A new perspective for antigen-loaded tolDCs in T1D is perhaps represented by post-translationally modified T-cell epitopes. Increasing evidence suggests that post-translationally modified epitopes may play a role in autoimmune diseases including T1D, especially during the not so well understood initial phases of autoimmune responses (117). Enzyme modifications such as citrullination by peptidyl deiminases

or deamidation by tissue transglutaminases, as well as cross-linked peptides or aberrant mRNA translation, were described as sources of neo-epitopes relevant in T1D (118–120). The neo-epitopes may explain how T-cell tolerance (T-cell deletion and peripheral tolerance) is circumvented in autoimmunity and also represents an interesting link to an initial environmental insult such as stress or viral infection that triggers their increased genesis (117), which has been implicated in pathogenesis of T1D. In type 1 diabetes cross-linked peptides of proinsulin to other β -cell peptides (HIPs) were reported to be recognized by pathogenic CD4⁺ T cells (121). Autoreactive CD4⁺ T cells have been implicated in the initial breakdown of tolerance by providing help to autoreactive CD8⁺ T and B cells. In a rheumatoid arthritis human phase I trial with four cintrullinated peptides, there was documented a reduction in effector T cells in 11 out of 15 patients and, to a lesser extent, also an increase of Tregs (122). Thus, T1D-related neo-epitopes represent interesting and promising antigens to be tested in antigen-specific tolDCs therapies for T1D (**Figure 1**).

PREVENTION vs. TREATMENT

Several tolDCs protocols prevented diabetes in animal models (mostly NOD mouse) of T1D diabetes (36–39, 42, 44, 66, 69) (**Table 1**). There are however a few tolDCs protocols that prevented diabetes in older NOD mice with advanced insulinitis or at the age just before usual clinical onset of diabetes. Thus, Feili-Hariri et al. reported prevention of diabetes in 10-week-old NOD mice, Lo et al. showed in 2 papers diabetes prevention in NOD mice that were treated from 9 weeks of age (45, 65), and Creusot et al. prevented diabetes in already 12-week-old NOD mice using immature DCs transduced to express IL-4 (40). A few tolDCs protocols have been shown to cure diabetes/restore normoglycaemia in already diabetic NOD mice. Single *i.v.* administration of DCs electroporated with IL-4 mRNA reversed hyperglycemia in diabetic NOD mice to fluctuating levels for up to 300 days and prevented diabetes in 12-week-old prediabetic animals (53). Later Di Caro et al. restored normoglycaemia in diabetic NOD mice by eight *s.c.* injections of immature DCs treated with antisense oligonucleotides against costimulatory molecules (63). The same group then showed reversal of hyperglycemia with antisense oligonucleotides, and also in combination with insulin B9-23 peptide, for at least 24 weeks (64). Indeed, these are the good candidate protocols for translation to clinical testing (**Table 1**; **Figure 1**).

Diabetes preventive protocols should be further optimized and also tested as early pre-onset interventions or for diabetes reversal. In addition they should be also tested in combination therapies (**Figure 1**). There are however very limited published data on tolDCs protocols that tested, but did not prevent, diabetes in the late pre-onset age, or failed to cure already diabetic animals. One such published study is by Pujol-Autonell et al. who showed DCs loaded with apoptotic bodies from the NIT-1 β -cell line did not reverse diabetes in NOD mice (68). In addition, this study also probed a combination approach with rapamycin and reported a negative outcome. Nevertheless, tolDCs were

propagated in GM-CSF alone, and thus optimized variants of this protocol may still have a different outcome. More attempts of combination therapy, especially when using well-optimized diabetes preventive tolDCs, should be undertaken (**Figure 1**). There are examples of combination therapies tested in closely related applications e.g., prevention of T1D by acetylated dextran microparticles with rapamycin and pancreatic peptide P31 (123).

ANIMAL MODELS

The most common animal model in T1D research is the NOD mouse. It displays several important similarities, but also some differences, compared to human T1D. While multiple manipulations have been reported to prevent disease in NOD mice (2), this goal has not yet been achieved in humans. The main advantage of this model is that unlike in many other autoimmune diseases, it spontaneously develops the disease with incomplete penetrance, thus reflecting well the contribution of environmental factors in T1D. Similar to human T1D, NOD mice possess polygenic genetic susceptibility with prevalence of MHC genes. Furthermore, diabetes onset is preceded by an increased number of circulating autoreactive T cells and autoantigens, including the most important ones to (pro) insulin, GAD65, IA-2, and others. In the NOD mouse, the initiating antigen seems to be (pro) insulin, whereas in human T1D more antigens can give rise to autoimmune reactivity [reviewed (124)]. Although the cellular composition of pancreas infiltrating cells is also similar, the histological characters of insulinitis differ, being more severe and frequent than in human T1D (48). NOD mice also have the advantage of less severe ketoacidosis and thus relatively long survival after diabetes onset, allowing easier set-up of experiments involving insulin treatment and reversal of diabetes. NOD mice also have the advantage of less severe ketoacidosis and thus relatively long survival after diabetes onset, allowing easier set-up of experiments involving insulin treatment and reversal of diabetes. Thus, the NOD mouse has been established as the most frequently used proof-of-concept animal model in T1D.

There are however also differences and weaknesses of the NOD mouse model specifically applying to DC therapies. Several studies reported abnormalities in the development of myeloid cells in NOD mice (125, 126), including defective maturation of myeloid DCs via IDD10/17/18 (50), while a later gene profiling study revealed over 300 differences in NOD DCs upon LPS stimulation, including expression from a cluster of 16 INF- α/β target genes (127). Apart from the defect in the maturation of NOD DCs (128), a lower responsiveness of bone-marrow progenitors to GM-CSF propagation was also described (72). Other studies documented that BM-derived DCs from NOD mice possess a hyperinflammatory profile with elevated NF- κ B levels, increased IL-12 production and reduced ability to induce proliferation of the Treg population (129). A lower stimulatory T-cell capacity and a defect in CD8⁺ dendritic cells have been reported more recently (130). These characteristics may indeed negatively influence cell yields as well as sensitivity for tolDC protocols tested. Nevertheless, despite the above mentioned DC defects, various tolDC protocols tested in NOD mice have

yielded fully functional tolerogenic DCs with capacity to prevent T1D (36, 42, 61) or even reverse hyperglycaemia in recent onset diabetic recipients (53, 63, 64), indicating that NOD mice represent a satisfactory model for preclinical testing of tolDCs.

The other spontaneous rodent model, the BB rat, displays a defect in thymic epithelial cells and severe lymphopenia as well as altered maturation of DCs (1, 131, 132) and no preclinical testing of tolDCs seems to have been reported in this model. Very recently, a tolDCs and cDCs comparison was carried out in autoimmune-prone and resistant rats, but not in BB rats (133).

The NOD-SCID mouse model is used for adoptive co-transfer of diabetes (51). While the observation period for diabetes incidence is much shorter than in NOD mice, a titration of diabetogenic splenocytes and their capacity to transfer diabetes across different experiments should be controlled to ensure similar sensitivity of the model. In addition, because of the relatively small number of T cells, homeostatic expansion of T cells may also influence this model. Nevertheless, comparable data in tolDCs therapies were obtained using both models (39, 42). Other models of accelerated diabetes e.g., LCMV-RIP and NOD RIP-IFN- β have also been used in tolDC-based therapies of T1D (44, 52). The induced animal models represent a more challenging scenario for diabetes prevention or treatment and should therefore be included in preclinical optimization of tolDCs.

In addition, humanized mouse models were employed to bring testing closer to clinical trials and to assess immune responses in the context of risk human HLA molecules such as HLA-DQ8/RIP-B7.1, or HLA-DR4 transgenic mice (39, 46). The NOD/scid IL-2Rg^{null} humanized mouse developed as a preclinical model for rapid *in vivo* evaluation of human DCs-based therapies, including *ex vivo* T-cell responses with recovered human T cells (134). The humanized mouse models bring preclinical optimization of tolDCs one step closer to translation to clinical trials (Figure 1).

CONCLUDING REMARKS

The development of DC-based therapies consists of multiple steps and involves many parameters. While it is difficult to optimize all of them given their interplay, we think it is important to assess single parameters side by side, not only *in vitro* as it has been done for example with some protocols and clinical grade tolDCs, but also in animal models of T1D. Published animal studies on tolDCs in T1D do not address this issue sufficiently. Since tolDCs therapies will probably evolve incrementally, optimization of various parameters and better understanding how they influence efficacies of tolDCs *in vivo* in animal models is important.

In this review we have assessed tolDCs protocols reported in animal models of T1D for parameters such as culture conditions comprising tolDCs propagation, homogeneity, serum vs. serum

free conditions, and stability or terminal differentiation. Next we have discussed how cell dose, single vs. repetitive application schemes, routes of administration including migration properties of tolDCs, unspecific vs. antigen-specific approach were researched and optimized in animal, mainly NOD, models of T1D. Effective and fine-tuned protocols should be then tested and reported not only for prevention but also as an intervention at the age of advanced pre-diabetes or cure of T1D in multiple animal models including humanized mice. Modifications of autoantigens and combinatorial approaches were briefly mentioned.

After decades of research to find a cure or effective secondary prevention for type 1 diabetics, DC-therapies represent a relatively new approach with remarkable achievements. A translation to humans seems optimistic as a few tolDCs protocols even reversed diabetes in NOD mice. This most frequently used spontaneous model of T1D is sometimes criticized for the easiness to prevent diabetes. Nevertheless, this is only easy at age of 3–4 weeks or even prenatally and thus no comparative human data exists. Another lesson from animal models is that among the main two approaches of using unloaded or antigen-loaded tolDCs, more data are at present available for an antigen-unspecific approach, yet this may change in the near future. As discussed in several subchapters of this review, almost all parameters of tolDCs would benefit from a more thorough optimization for translation to a clinical testing, starting from *in vitro* parameters, such as serum-free conditions (42, 52, 66) and stability testing, to optimal application scheme (e.g., multiple doses were used for reversal of diabetes by tolDCs (63), to the use of various mouse models in preclinical experiments. There are also some unexpected factors such as the increased effectiveness of lower tolDCs doses (114). In addition, some other parameters not yet tested in animals could be important in patients e.g., the effect of glycaemia control on functional properties of patient-prepared tolDCs [reviewed (86, 135)].

Although many tolDCs protocols in T1D were reported, we think they should be thoroughly optimized in animal models as tolDCs therapies in patients comprise not only safety issues, but also involve significant time, costs and a great deal of the patient's hopes.

AUTHOR CONTRIBUTIONS

DF wrote the first draft of the manuscript. DF, LP-J, JG, ZK, AF, TH, and RŠ contributed in design, scientific insights, manuscript writing, editing and proof reading.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Physical Exercise Induces Immunoregulation of TREG, M2, and pDCs in a Lung Allergic Inflammation Model

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The benefits of moderate aerobic physical exercise for allergic asthma are well-known, particularly that of the anti-inflammatory effect that occurs by reducing Th2 responses and lung remodeling. However, the mechanisms of this immunoregulation are still under investigation. In this study, we investigated the possible immunoregulatory mechanisms of lung inflammation induced by moderate aerobic exercise in an experimental asthma model. BALB/c mice were distributed into Control, Exercise (EX), OVA, and OEX groups. OVA and OEX groups were sensitized with ovalbumin (OVA) on days 0, 14, 21, 28, and 42 and were challenged with OVA aerosol three times a week from days 21 to 51. The EX and OEX groups underwent moderate aerobic physical exercise from days 21 to 51 (5 d/w, 1 h/d). The mice were euthanized on day 52. We evaluated pulmonary cytokine production, serum immunoglobulin levels, and the inflammatory cell profile in lung and mediastinal lymph nodes. OVA mice showed increased expression of IL-4, IL-6, IL-10, and TGF- β and decreased macrophage type 2 (M2) recruitment. Physical exercise did not affect the increased antibody production of IgG2a, IgG1, or IgE induced by OVA. Of note, physical exercise alone markedly increased production of anti-inflammatory cytokines such as IL-10 and TGF- β . Physical exercise in OVA-mice also increased the recruitment of M2 in the lungs, as well as the influx and activation of regulatory T cells (Tregs) and CD4 and CD8 lymphocytes. In the draining lymph nodes, it was also observed that physical exercise increased the activation of CD4 T cells, regardless of the presence of OVA. Notably, physical exercise decreased common dendritic cells' (cDCs; pro-inflammatory) expression of co-stimulatory molecules such as CD80, CD86, and ICOSL in the draining lymph nodes, as well as increased ICOSL in plasmacytoid dendritic cells (pDCs; anti-inflammatory). Together, these findings show that physical exercise modulates pulmonary allergic inflammation by increasing Treg and M2 recruitment, as well as pDCs activation, which leads to an increase in anti-inflammatory cytokines and a decrease in pro-inflammatory cells and mediators.

Keywords: asthma, physical exercise, immunoregulation, Treg, M2, dendritic cell

INTRODUCTION

Asthma is a heterogeneous disease characterized by the chronic inflammation of the airways and variable remodeling (1). It is defined by a history of respiratory symptoms, such as wheezing, difficulty breathing, chest tightness, and cough that varies in time and intensity, along with a variable airflow limitation (1, 2). Aerobic exercise has been used in several rehabilitation programs for asthmatic patients, resulting in decreased dyspnea, airway hyperresponsiveness, the induction of bronchospasm and even corticosteroid use. In addition, these patients demonstrate improvements in aerobic capacity and quality of life (3, 4).

Some authors have already shown that aerobic physical exercise may decrease allergic lung inflammation in sensitized animals and have suggested that this reduction may occur by inhibition of nuclear factor activation (NF- κ B) or by the increased expression of anti-inflammatory cytokines such as IL-1 α and IL-10 (3, 5, 6). Mackenzie et al. (7) have shown that animals sensitized to ovalbumin (OVA) and submitted to an experimental exercise protocol demonstrate reduced IL-4, IL-5, and IL-13 levels in the mediastinal lymph nodes. Furthermore, this same study demonstrated that physical exercise inhibits maturation and the activation of dendritic cells, indicating that the decrease in Th2 response in sensitized and trained animals may be due to some interference in the maturation of the OVA antigen presenting cell during the exercise (7).

In view of this context, we hypothesize that moderate physical exercise generates greater activation of regulatory cells, thus reducing pulmonary inflammation. To investigate our hypothesis, we conducted this study to investigate the role of some immunomodulatory cells, such as Treg, M2, and pDCs, in the immunomodulation induced by aerobic physical exercise in OVA-sensitized animals.

MATERIALS AND METHODS

Animals

This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals—NIH (8). The protocol was approved by the Ethics Committee of the School of Medicine of the University of São Paulo (Protocol number 067/16). Male Balb/c mice (6–8 weeks old) were purchased from the University of São Paulo (São Paulo, Brazil) and were divided into four experimental groups ($n = 10$ per group): SAL (non-sensitized and non-trained), EX (non-sensitized and trained), OVA (sensitized and non-trained), and OEX (sensitized and trained).

Sensitization Protocol

Animals from the OVA and OEX groups were sensitized with five intraperitoneal injections containing 20 μ g/mL of OVA (Grade V, Sigma Chemical Co., MO, USA) absorbed in 3 mg/mL of aluminum hydroxide (Pepsamar gel, Snofi-Synthelabo S.A., RJ, Brazil). These animals received a total of 0.2 mL on days 0, 14, 21, 28, and 42. Animals from the SAL and EX groups received five intraperitoneal injections of saline solution (NaCl 0.9%) and aluminum hydroxide on the same days as the OVA and OEX

groups. Three times a week, from days 21 to 51, the OVA-sensitized groups were challenged with OVA aerosol at 1%, and non-sensitized groups were aerosolized with saline solution. On day 52, the animals were studied (Figure 1).

Aerobic Exercise Protocol

The EX and OEX groups underwent an aerobic exercise protocol lasting 5 weeks on a treadmill (KT 400, Imbramed, RS, Brazil), 1 h per day starting on day 21 until day 52 of the experimental protocol. Before starting the training protocol, the mice underwent an exercise test. The test consisted of an initial 5 min running at a speed of 0.2 km/h, and then the speed was gradually increased by 0.1 km/h every 2.5 min until the animal was exhausted. Exhaustion was considered at the time when the animal was unable to remain running even after 10 mechanical stimuli. The training intensity during the experimental protocol was 50% of the average maximum speed reached in the exercise test (Figure 1).

Specific Antigen-Antibody and Cytokines Analysis

Blood (0.3 mL) was collected from the inferior vena cava of anesthetized animals and diluted with 600 μ L of saline solution. Then, it was centrifuged at 3,000 rpm for 10 min at 4°C to obtain serum. The serum was maintained at -70°C until immunoglobulin analysis of OVA-specific (Ig)E (IgE), by Passive Cutaneous Anaphylaxis reaction, as well as IgG1 and IgG2a analysis, by immuno-enzymatic assay (ELISA) according to the manufacturer's instructions (R&D Systems, MN, USA). Both lungs were removed and homogenate adding 600 μ L of saline solution, according to manufacture instructions (PowerLyser, MoBio Laboratories Inc, CA, USA). Interleukin (IL)-4 (IL-4), IL-6, IL-10, and the transforming growth factor (TGF)- β 1 levels in the lung homogenate were measured using an ELISA kit according to the manufacturer's instructions (R&D Systems, MN, USA).

Passive Cutaneous Anaphylaxis (PCA) Reaction

The determination of OVA-specific IgE anaphylactic antibodies was performed by the passive cutaneous anaphylaxis (PCA) reaction, according to the technique described by Mota and Wong (9). Rats were shaved on the back and were sensitized intradermally with serial dilutions of the serum from experimental mice. After a period of 18 to 24 h of sensitization, the animals were challenged intravenously with 0.25% Evans blue containing 500 μ g of OVA. The result was determined 30 min after the challenge, measuring the diameter of the positive reaction (blue spots) in the inverted skin of the animals. The probable IgE titers were expressed as the reciprocal of the highest dilution of the serum that resulted in a positive reaction of more than 5 mm in diameter. All tests were done in triplicate, and differences between PCA titers >2 times were considered significant (10, 11).

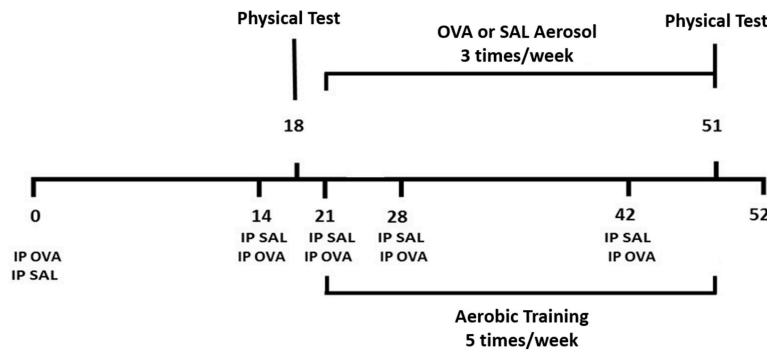


FIGURE 1 | Experimental protocol. Balb/c mice were sensitized with ovalbumin and alumen on days 0, 14, 21, 28, and 42 and were challenged with an aerosol of OVA three times per week, from day 21 until day 51 [OVA and OVA + exercise (OEX) groups]. The EX (exercise) and OEX groups practiced moderate exercise from day 21 until 51. Twenty-four hours after the last challenge the animals were analyzed.

Cell Phenotype

Mice (ten per group) were euthanized, and the lungs and mediastinal draining lymph nodes were removed. Lymph nodes were harvested and homogenized using saline solution and a 40 μ m cell strainer. The single cell suspension was centrifuged, and the cell pellet was re-suspended in 1 mL of saline for total cell counting. The lungs were cut in small pieces with scissors and incubated in a solution containing collagenase (07 mg/mL, Sigma-Aldrich, MO, USA) and DNase 1 (30 μ g/mL, Sigma-Aldrich, MO, USA) for 30 min at 37°C. After incubation, the single cell suspension was filtered through a 40 μ m strainer, and the enzymes were blocked with phosphate buffered saline (PBS) supplemented with fetal bovine serum (FBS) at least three times of the initial volume. Then, the cell suspension was centrifuged at 1,500 rpm for 10 min and re-suspended in 1 mL of saline, centrifuged, and the cell pellet was re-suspended in 1 mL of saline for total cell counting. The cells were stained for cell surface and intracellular markers (Saponine 0.05%, Sigma-Aldrich, MO, USA). The phenotype of the cells was evaluated using the following antibodies: anti-CD3—PERCP CY5.5 (17A2), anti-B220—PERCP (RA3-6B2), anti-MHC-II—PE (M5/114.15.2) or PE Cy7.7 (M5/114.15.2), anti-SIGLECF—PE CF594 (E50-2440), anti-CD11b—PE CY7 (M1/70) or BV605 (M1/70), anti-F4/80—eFluor 450 (BM8), anti-CD11c—FITC (HL3), anti-CD4—V500 (RM4-5), anti-CD69—FITC (H1.2F3), anti-CD8a—APC CY7(53-6.7), anti-CD25—PE CY7 (PC61), anti-FOXP3—V450 (MF23), anti-IL-10—APC (JESS-16E3), anti-latency related peptide (LAP)—PE (TW7-16B4), anti-CD24—PERCP CY5.5 (M1/69), anti-ICOSL—PE (HK5.3), anti-PDL2—APC (TY25), anti-CD86—A700 (GL1), and the viability marker—Texas Red. All antibodies were purchased from BD Biosciences, NJ, USA and eBioscience, SD, USA. A total of 1×10^5 live events were acquired with LSR Fortessa (BD, San Jose, CA, USA) and analyzed with Flow Jo 10.0.6 software (Tre Star, OR, USA). Fluorescence was performed for all antibody panels minus that of one control. The analysis strategy is provided in **Supplementary Figures 1–6**.

Statistical Analysis

Statistical differences between experimental groups were detected by analysis of variance (two-way ANOVA) followed by the Holm-Sidak *post hoc* test for multiple comparisons (SigmaStat 2.03, SPSS, Chicago, IL, USA). We considered significant values $p < 0.05$.

RESULTS

The Antigenic Exposure Associated With Physical Exercise Maintains Elevated Levels of Antibodies

We analyzed OVA-specific IgG1 and IgG2a levels by ELISA. The results showed an increase of IgG1 and IgG2a levels in ovalbumin-sensitized groups compared to non-sensitized animals. When we evaluated the production and functionality of anti-OVA IgE by PCA, we notice that sensitized animals had higher IgE-related mast cell degranulation titer than 1/160. Similar to IgGs, physical exercise did not modify the intense production of IgE induced by OVA (**Figure 2**).

Physical Exercise Reduces Eosinophil Influx to the Lung of Sensitized Animals

The antigen-sensitized group (OVA) showed a high influx of eosinophils in lung tissue that was diminished in OVA-sensitized mice that also underwent aerobic exercise (OEX; **Figure 3**). In order to explain better anti-inflammatory effect of physical exercise, we measured some cytokines in the lung homogenate.

Physical Exercise Leads to an Anti-inflammatory Cytokine Release in OVA-Sensitized Animals

The quantification of IL-4, IL-6, TGF- β , and IL-10 cytokines from lung homogenate ($n = 10$ per group) demonstrated that sensitization to OVA increased IL-4 and IL-6 levels accompanied by an increase in the levels of anti-inflammatory cytokines IL-10

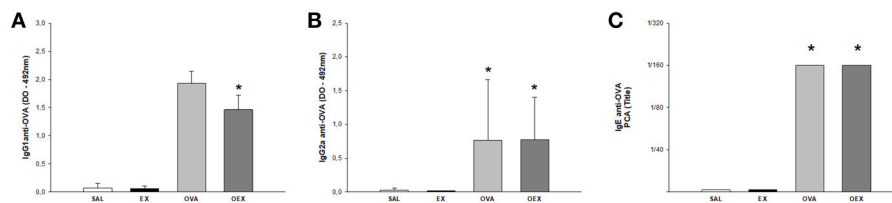


FIGURE 2 | Specific antibodies induced by OVA-sensitization. The results are expressed as the means of the absorbance values (\pm SE) obtained by ELISA with serum from animals of the various experimental groups (A) anti-OVA IgG1 antibodies (B) anti-OVA IgG2a. * $p = 0.047$ compared to the SAL group. (C) The mean values of the titer of IgE anti-OVA. *Statistically different values in relation to the control group, SAL or EX.

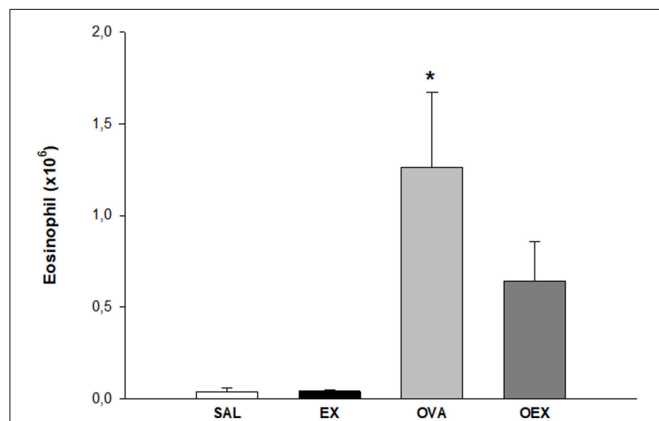


FIGURE 3 | The practice of physical exercise reduces the influx of eosinophils in the lung of OVA-sensitized animals. The mean values of eosinophil counts (\pm SE) obtained in lung tissue. * $p < 0.001$ compared to the SAL group. $N = 10$ per group.

and TGF- β (Figure 4). In addition, physical exercise reversed this elevation in the OEX group (Figures 4A,B, respectively) whereas increased the production of IL-10 and TGF- β (Figures 4C,D, respectively). We observed that physical exercise alone elevated the levels of IL-4 and also anti-inflammatory cytokines, such as those of TGF- β and IL-10 (Figures 4A,C,D). This anti-inflammatory pattern of cytokines observed in OEX group corroborate with the reduction in eosinophil influx observed in the lung and also with other cellular finds showed above.

Trained Mice Show an Increased Influx of Lymphocytes and a Greater Activation of These Cells

To identify the cellular effects of aerobic exercise, we analyzed the inflammatory cell profile in the lungs and mediastinal lymph nodes. Animals that were sensitized and underwent the physical exercise protocol (OEX) had an increase in CD4 and CD8 T cells numbers and activation in the lungs (Figure 5). In the lymph nodes, sensitized animals showed a significant increase in the number of CD4 T cells. Interestingly, we observed that the CD4 T cells in the trained group (EX) showed greater expression of activation markers (Figure 6). This data showed that physical exercise in sensitized mice induced the more pulmonary influx of T cells such as increasing its activation.

Physical Exercise Induces an Increase of Treg and M2 Regulatory Cells in the Lungs of Sensitized Animals

Physical exercise significantly increased activated Tregs (CD4⁺, CD25⁺, FOXP3⁺, LAP⁺) in the lungs of OVA-sensitized and challenged mice. These data suggest that Tregs, which are the main source for TGF- β and IL-10 (Figure 7) are responsible for the local counter regulation of OVA allergen-driven inflammation induced by physical exercise.

We also evaluated the macrophages present in lung tissue and observed that OVA sensitization and physical exercise increased the levels of total macrophages, both macrophages type 1 (M1) and M2 (Figures 8A,B).

Physical Exercise Reduces M2 Migration to Draining Lymph Nodes

The evaluation of macrophages presents in the lymph nodes showed that the OVA sensitization increased M2 recruitment and that physical exercise reduced these numbers (Figure 8C). Of note, M1 numbers were not affected by physical exercise or OVA-sensitization (Figure 8D). Taken together with lung analysis, further studies are needed to explain the mechanisms by which physical exercise reduces the presence of M2 in the mediastinal lymph nodes.

Exercise Leads to an Anti-inflammatory Profile of DCs in the Lymph Nodes

By evaluating the subtypes of dendritic cells present in the lymph nodes and the expression of co-stimulatory molecules, we found that common DCs (cDCs) from sensitized animals showed increased expression of co-stimulatory molecules such as CD80, CD86, and ICOSL, demonstrating a pro-inflammatory profile of these cells. Physical exercise reverted this activation pattern in cDCs (Figure 9). In addition, there was a significant increase of plasmacytoid DCs (pDCs) in the OVA group that also presented the elevated expression of CD80, indicating a higher activation of these cells. Although the OEX group showed a reduction of CD80 expression by pDCs, these cells were highly activated by increased expression of ICOSL, clearly demonstrating an anti-inflammatory profile in these cells (Figure 10).

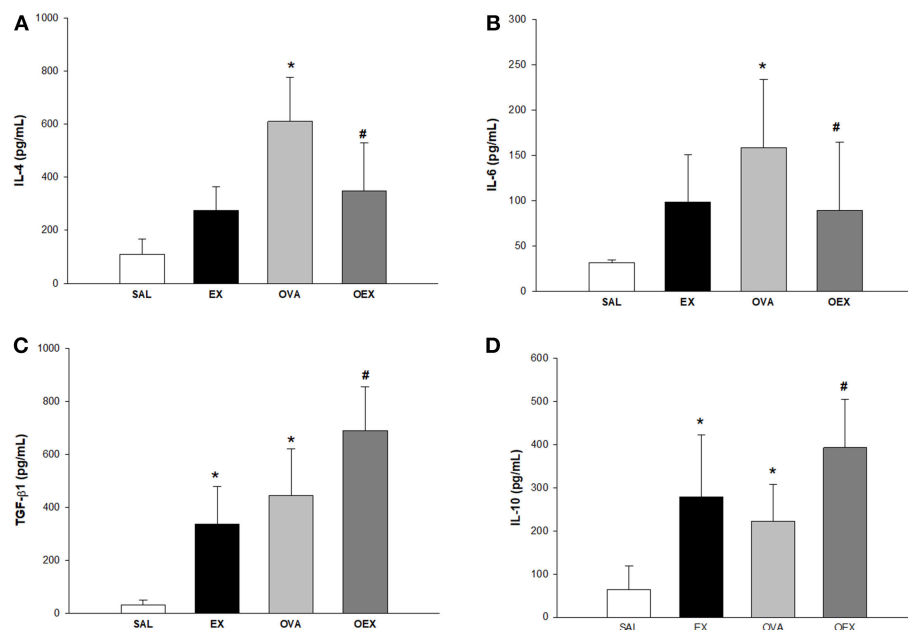


FIGURE 4 | Physical exercise decreases pro-inflammatory cytokines and increases anti-inflammatory cytokines in the lungs of OVA-sensitized animals. The mean values (±SE) obtained in the pulmonary homogenate of (A) IL-4 (* $p < 0.001$ and 0.023 , respectively when compared EX or OVA to the SAL group and # $p = 0.035$ compared to the OVA group), (B) IL-6 (* $p = 0.008$ compared to the SAL group and # $p = 0.035$ compared to the OVA group), (C) TGF- β and (D) IL-10 (* $p = 0.001$ compared to the SAL group and # $p = 0.035$ compared to the OVA group). $N = 10$ per group.

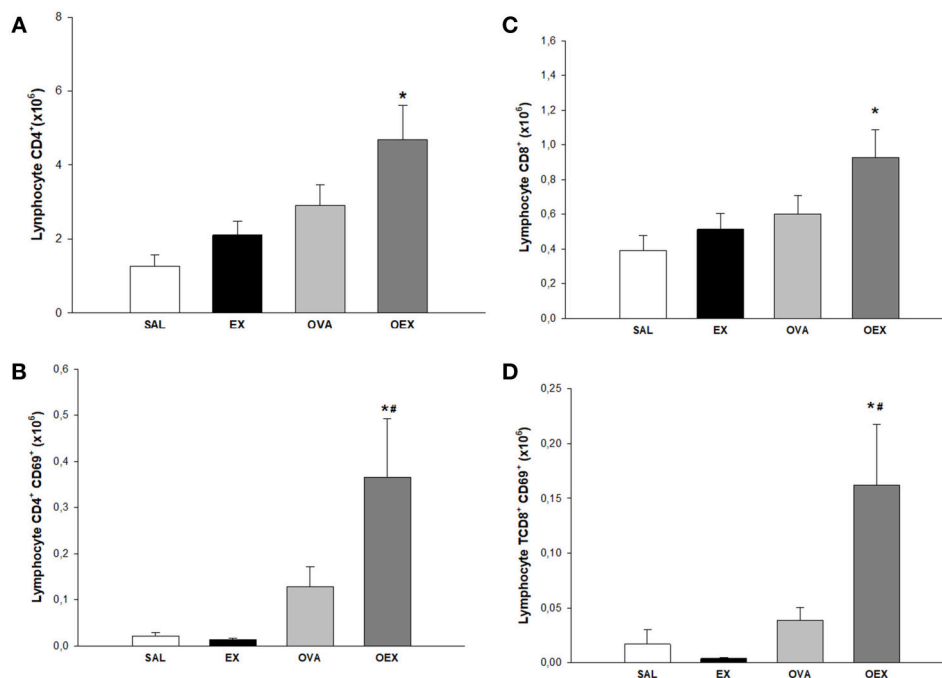


FIGURE 5 | Lymphocytes in lung tissue. (A) The mean values of CD4⁺ T cell numbers. * $p = 0.01$ compared to the SAL group. (B) Mean values of CD4⁺ T cells expression of CD69 (±SE). * $p = 0.006$ compared to EX and # $p < 0.001$ when compared to OVA. (C) Mean values of CD8⁺ T cell numbers (±SE). * $p = 0.026$ compared to the EX group. (D) Mean values of CD8⁺ T cells expression of CD69 (±SE). * $p < 0.001$ compared to the EX group # $p = 0.004$ compared to the OVA group. $N = 10$ per group.

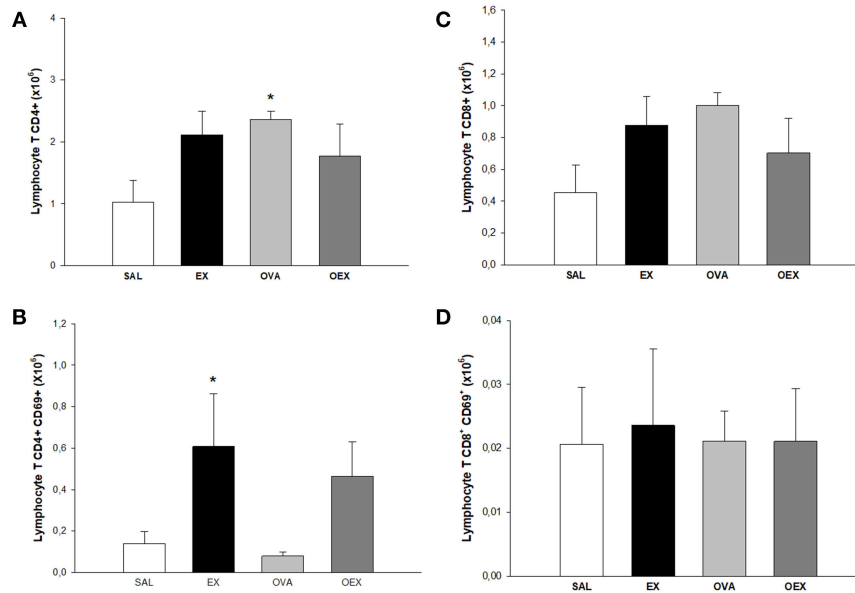


FIGURE 6 | Lymphocytes in lymph nodes. **(A)** The mean values of CD4 T cell numbers. * $p = 0.036$ compared to the SAL group. **(B)** The mean values of CD4 T cell expression of CD69 (\pm SE). * $p = 0.036$ compared to EX. **(C)** The mean values of CD8 T cell numbers (\pm SE). **(D)** The mean values of CD8 T cell expression of CD69 (\pm SE). $N = 10$ per group.

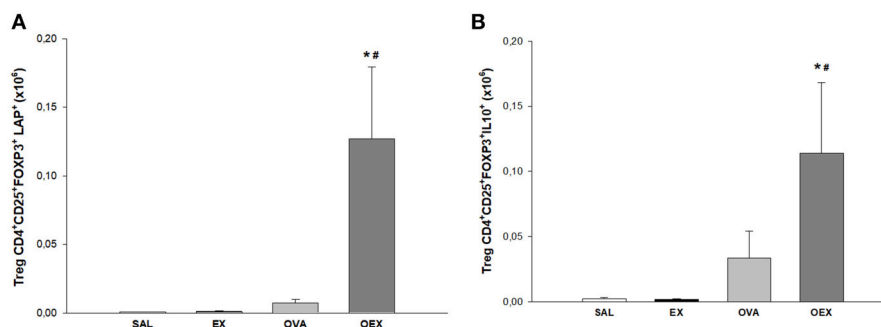


FIGURE 7 | Physical exercise induces higher production of TGF- β and IL-10 by pulmonary Tregs in allergic animals. **(A)** The mean values of TGF- β ⁺ (\pm SE) Tregs obtained from lung tissue. ** $p < 0.001$ compared to the EX group or the OVA group. **(B)** The mean values of IL-10⁺ Tregs (\pm SE) obtained in lung tissue. * $p = 0.011$ compared to EX and # $p = 0.049$ to OVA). $N = 10$ per group.

DISCUSSION

The OVA-specific immunoglobulin dosage in the serum of the OVA group demonstrated a significant increase in IgG1, IgG2a, and IgE levels, which were not altered by physical exercise. This suggests that the effects of physical exercise do not act on plasma cells or B cells, which are producers of these immunoglobulins (12).

To evaluate the cellular profile presented in chronic allergic pulmonary inflammation, we quantified and characterized the main cells involved in this inflammatory process (13). We observed that OVA animals showed an increased number of eosinophils present in lung tissue, and physical exercise markedly reduced eosinophilia, indicating that exercise is protective in

lung inflammation. The reduction of pulmonary eosinophilia by physical exercise has already been demonstrated by some authors, who demonstrated that there is a reduction in the number of eosinophils in the bronchoalveolar lavage and pulmonary tissue of asthmatic animals following physical exercise (14, 15).

Elevated levels of IL-4 and IL-6 were detected in the OVA group, and interestingly, moderate exercise reduced the levels of these cytokines in the lung of sensitized mice. Some studies have shown that animals sensitized to OVA show a significant increase in the levels of these cytokines and that there is a reduction when the animals undergo a exercise protocol, corroborating our findings (7, 15–17). We could also compare the reduction of the IL-4 levels with lower lung eosinophilia once there is evidence, suggesting that this cytokine increases airway eosinophilia (18).

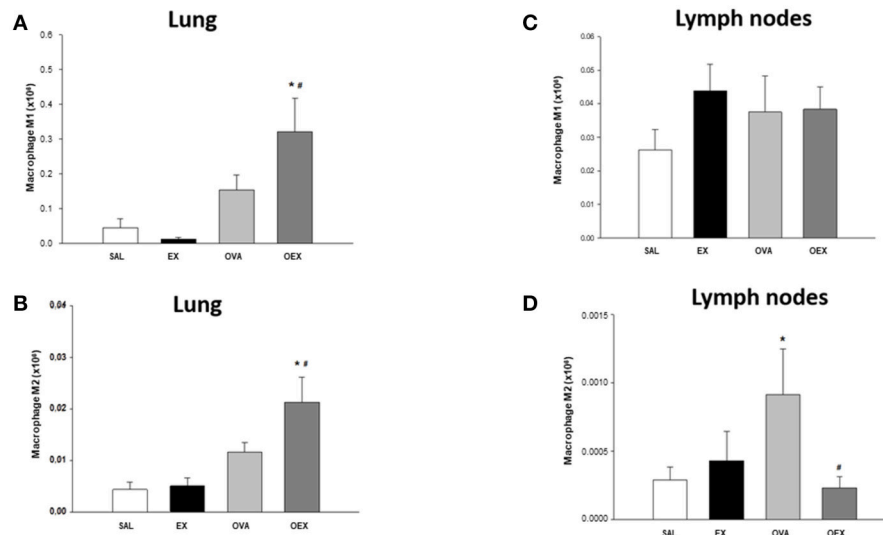


FIGURE 8 | Physical exercise increases the amount of M2 regulatory cells in the lung of sensitized and trained animals as well as M1 but also reduces the presence of M2 in mediastinal lymph nodes. **(A)** The mean values of lung M1 macrophages (\pm SE) obtained in lung tissue. * $p = 0.036$ when compared to the EX group and # $p = 0.020$ when compared to the OVA group. **(B)** The mean values of lung M2 macrophages (\pm SE) obtained in lung tissue. * $p = 0.001$ compared to the EX group and # $p = 0.030$ when compared to the OVA group. **(C)** The mean values of macrophages M1 (\pm SE) obtained in lymphoid tissue. **(D)** Mean values of M2 macrophages (\pm SE) obtained in lymphoid tissue. * $p = 0.036$ when compared to the EX group and # $p = 0.020$, compared to the OVA group. $N = 10$ per group.

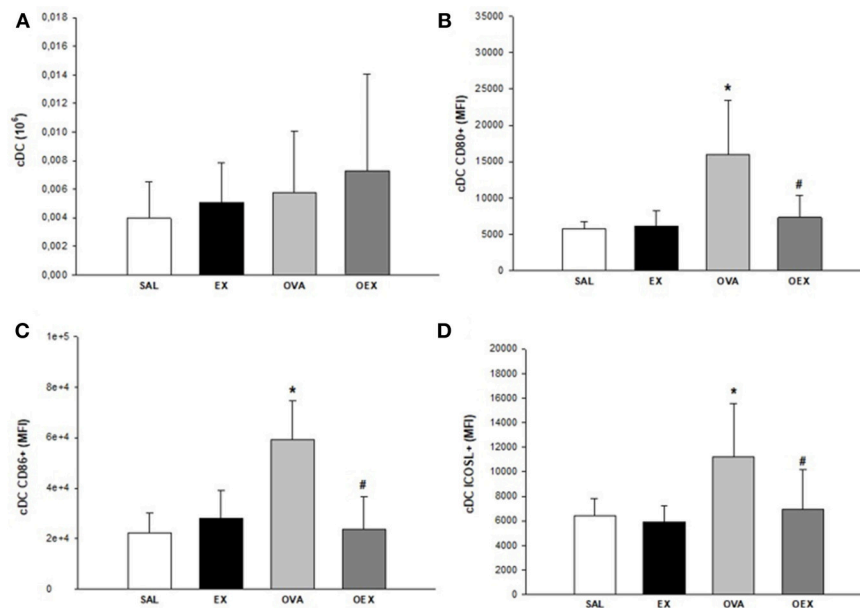


FIGURE 9 | Physical exercise reduces OVA-induced activation in cDCs in lymphoid tissue. **(A)** Quantification of cDCs in lymphoid tissue. Mean values of cDCs (\pm SE) obtained in lymphoid tissue. **(B)** Sensitization to OVA elevated CD80 expression in cDCs and physical exercise reduced this expression. Mean values of CD80⁺ cDCs (\pm SE) obtained in lymphoid tissue, * $p < 0.001$ compared to SAL group, # $p = 0.001$ if compounded to OVA group. **(C)** Sensitization to OVA elevated CD86 expression in cDCs and physical exercise reduced this expression. Mean values of CD86⁺ cDCs (\pm SE) obtained in lymphoid tissue, * $p < 0.001$ compared to SAL group, # $p = 0.001$ if compounded to OVA group. **(D)** Sensitization to OVA increased ICOSL expression in cDCs and physical exercise reduced this expression. The mean values of ICOSL⁺ cDCs (\pm SE) obtained in lymphoid tissue, * $p < 0.007$ compared to SAL group, # $p = 0.012$ if computed to OVA group. $N = 10$ per group.

Physical exercise promotes anti-inflammatory responses in a lung inflammation model by increasing anti-inflammatory cytokines such as IL-10 and IL-1-Ra (3, 19, 20). In addition to the increased levels of IL-10, we also noted an increase in

TGF- β 1 levels in lung homogenate from groups that underwent physical exercise, indicating that there is another regulatory cytokine co-participating in the anti-inflammatory process of physical exercise. *In vitro* experiments have shown that TGF- β 1

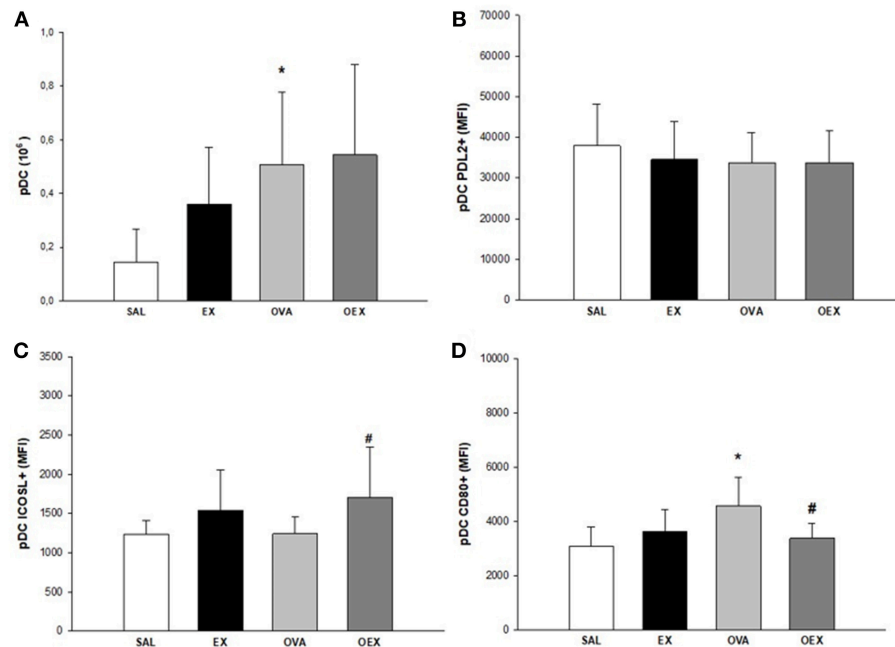


FIGURE 10 | Quantification of pDCs in lymphoid tissue. **(A)** Sensitization to OVA increased numbers of pDCs. The mean values of pDCs (\pm SE) obtained in lymphoid tissue, $*p = 0.024$ when compared to SAL group. **(B)** Levels of PDL2 expression in pDCs. The mean values of PDL2⁺ pDCs (\pm SE) obtained in lymphoid tissue. **(C)** Sensitization to OVA associated with exercise increased the expression of ICOSL⁺ in pDCs. The mean values of ICOSL⁺ pDCs (\pm SE) obtained in lymphoid tissue, $\#p = 0.029$ when compared to the OVA group. **(D)** Sensitization to OVA elevated CD80 expression in pDCs and physical exercise reduced this expression. The mean values of CD80⁺ pDCs (\pm SE) obtained in lymphoid tissue, $*p < 0.005$ compared to SAL group, $\#p = 0.021$ if compounded to OVA group. $N = 10$ per group.

has a regulatory function in both innate and adaptive immune cell function (21). Furthermore, TGF- β 1 is associated with suppressor T cell immune function in that it suppresses immune responses through inhibition of inflammatory cell function and promotion of Treg production, as a Treg inducer (22). Of note, Tregs are the main source for TGF- β and IL-10. We observed an increase in Treg numbers in the lungs. In addition, the high expression of CD25 and LAP reinforces the hypothesis that these cells are regulating the lung inflammation. LAP is an important marker of TGF- β excretion in Tregs. TGF- β is produced as an inactive latent complex made up of LAP (23).

We observed an increased influx of lymphocytes, both of CD4 and CD8 T cells, in the lungs and lymph nodes in the OEX group. CD8 T cells show immunoregulatory/immunosuppressive capacities in a CD4 T cell-associated inflammatory disease such as asthma (24, 25). Activated CD8 T cells can generate a regulatory microenvironment through adhesion molecules and release of cytokines, indicating a potential to induce regulation in DCs and finally, activation and proliferation of CD4 T cells. In this same context, DCs play a central role in the activation and proliferation of CD4 T cells. It has also been shown that CD8 T cells have the capacity to negatively regulate splenic DC phenotypes, such as the expression of CD86, CD80, and MHC-II (26). Of note, it is known that the tolerogenic functions of DCs are closely related to immunosuppressive cytokines such as TGF- β and IL-10 (26, 27). In the lymph nodes, we observed a decrease of CD80, CD86, and ICOSL

expression in cDCs of trained and sensitized mice when compared to the sensitized and challenged group. When we examined pDC in the same compartment, we noticed the increase of pDCs in mice only OVA-sensitized, whereas the exercise increased ICOSL expression and decreased CD80. *In vivo* and *in vitro* studies have both clearly shown that pDCs can stimulate the induction of Treg cells, possibly in an ICOSL-dependent way (28). Taken together, these data indicates an anti-inflammatory pattern induced by exercise on DCs. Increased levels of IL-10, observed both in the sensitized and trained animal samples, are linked with decreased expression of the co-stimulatory molecules CD80 and CD86. Studies have already shown that IL-10 has the ability to decrease the expression of these co-stimulatory molecules, such as MHC-II and, therefore, reduce the DC pro-inflammatory phenotype (29).

We also analyzed macrophages in the lung tissue. In the OEX group, the increase in the amount of M2 macrophages could be directly linked to the increase in IL-10 measured in the lung tissue (27). Interestingly, the reduction in the numbers of M2 macrophages in the lymph nodes of the sensitized and trained mice (OEX) indicates the interference of physical exercise in the migration of these cells. Additional studies must be performed to clarify this issue.

Thus, we conclude that physical exercise induces an increase of M2 macrophages in the lungs, in addition to an anti-inflammatory profile of DCs and an increase of Treg cells in

OVA-sensitized and challenged mice. These findings help to better understand some of the mechanisms underlying how exercise regulates chronic allergic inflammation.

ETHICS STATEMENT

The Ethics Committee of the School of Medicine of the University of São Paulo approved all animal experiments (Protocol number 067/16). Male Balb/c mice (6–8 weeks old) were purchased from the University of São Paulo (São Paulo, Brazil) and maintained as described in the Guide for the Care and Use of Laboratory Animals—NIH (8).

AUTHOR CONTRIBUTIONS

PF performed the experiments, analyzed all data, and drafted the manuscript text. LO and MS helped plan the flow

cytometry experiments and LO performed them. TB helped with data analysis and manuscript writing. CO helped with the experimental exercise protocol. FA-C designed the study, reviewed the data, and helped write the manuscript text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00854/full#supplementary-material>

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Regulation of Tolerogenic Features on Dexamethasone-Modulated MPLA-Activated Dendritic Cells by MYC

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The potential of tolerogenic dendritic cells (tolDCs) to shape immune responses and restore tolerance has turned them into a promising therapeutic tool for cellular therapies directed toward immune regulation in autoimmunity. Although the cellular mechanisms by which these cells can exert their regulatory function are well-known, the mechanisms driving their differentiation and function are still poorly known, and the variety of stimuli and protocols applied to differentiate DCs toward a tolerogenic phenotype makes it even more complex to underpin the molecular features involved in their function. Through transcriptional profiling analysis of monocyte-derived tolDCs modulated with dexamethasone (Dex) and activated with monophosphoryl lipid A (MPLA), known as DM-DCs, we were able to identify MYC as one of the transcriptional regulators of several genes differentially expressed on DM-DCs compared to MPLA-matured DCs (M-DCs) and untreated/immature DCs (DCs) as revealed by Ingenuity Pathway Analysis (IPA) upstream regulators evaluation. Additionally, MYC was also amongst the most upregulated genes in DM-DCs, finding that was confirmed at a transcriptional as well as at a protein level. Blockade of transactivation of MYC target genes led to the downregulation of tolerance-related markers IDO1 and JAG1. MYC blockade also led to downregulation of PLZF and STAT3, transcription factors associated with immune regulation and inhibition of DC maturation, further supporting a role of MYC as an upstream regulator contributing to the regulatory phenotype of DM-DCs. On the other hand, we had previously shown that fatty acid oxidation, oxidative metabolism and zinc homeostasis are amongst the main biological functions represented in DM-DCs, and here we show that DM-DCs exhibit higher intracellular expression of ROS and Zinc compared to mature M-DCs and DCs.

Taken together, these findings suggest that the regulatory profile of DM-DCs is partly shaped by the effect of the transcriptional regulation of tolerance-inducing genes by MYC and the modulation of oxidative metabolic processes and signaling mediators such as Zinc and ROS.

Keywords: tolerogenic dendritic cells (tDC), tolerance mechanism, DC transcription factors, zinc metabolism in DC, dexamethasone-modulated and MPLA-activated DC, ROS metabolism in DC

INTRODUCTION

The ability of dendritic cells (DCs) to modulate immune responses and educate the immune system to induce tolerance, has led to a wide range of studies of these cells as targets for cellular therapy in autoimmunity and other disorders where immune tolerance is broken. These tolerogenic DCs (tolDCs), are capable of inducing anergy or deletion of effector T cells, as well as the differentiation and/or proliferation of regulatory T cell (Treg) subsets and the establishment of a local anti-inflammatory milieu. Regulation may result from various processes, including deficient antigen presentation, reduced co-stimulatory molecules, expression of inhibitory molecules and/or secretion of anti-inflammatory cytokines such as IL-10 and TGF- β (1, 2). To this date, many *in vitro* differentiation protocols of tolDCs from blood monocytes have been published, which include the use of a wide variety of immunomodulatory stimuli to induce a regulatory profile on DCs (3–7). Although some features may differ between tolDC subsets, all are endowed with the capacity to exert regulatory functions (8, 9). The main idea is to *ex vivo* differentiate precursor cells from peripheral blood of patients to DCs, endow them with regulatory features, load them with a specific antigen, and then administrate them to the patient, in order to restore immune tolerance in an antigen-specific manner.

Keeping this on mind, our group developed a protocol for the generation of tolDCs from peripheral blood monocytes further modulating DCs with dexamethasone (Dex) to induce a tolerogenic phenotype, followed by an alternative activation with the non-toxic LPS analog monophosphoryl lipid A (MPLA), named DM-DCs. These cells display reduced levels of surface markers CD83 and CD86, secrete high amounts of IL-10 and TGF β , show lymph node homing capacity and exhibit a reduced capacity to promote effector Th1 and Th17 cell proliferation, besides being able to render these cells hypo-responsive in an antigen-specific manner while remaining stable in front of pro-inflammatory stimuli (10, 11).

While the mechanisms by which tolDCs can exert their immunomodulatory actions have been broadly studied, the molecular setup that leads to the differentiation of DCs into a regulatory profile, is much less understood, and the fact that different tolerogenic stimuli can generate different tolDC subsets makes it even harder to identify the molecular components accountable for immune regulation in tolDCs, since different stimuli activate different signaling pathways that can lead to tolDCs differentiation. Recent technological advances in the last few years mostly in the “omics” field, along with the advent of multiparametric flow cytometry combined with

bioinformatics analyses, have made it possible to acquire a deeper insight into the molecular characterization of DC biology. Using these techniques, through genome-wide transcriptional analysis complemented by multi-parametric flow cytometry, we demonstrated that DM-DCs exhibited a transcriptional and phenotypic profile that clearly distinguished them from other monocyte-derived DC (moDC) subsets, such as MPLA-matured DC (M-DCs), Dex-modulated DC (D-DCs) and untreated/immature DC (DCs) (2, 12). These cells were further characterized by the upregulation of several tolerance-related molecules such as IDO1 (indoleamine 2,3-dioxygenase 1), IL-10, MERTK (receptor tyrosine kinase), FCGR2B (Fc fragment of IgG, low affinity IIb), C1q (complement C1q) and JAG1 (Jagged 1); and the downregulation of maturation/inflammation associated markers CD1c, IL-12, FCER1A (Fc fragment of IgG, alpha polypeptide), and DC-SCRIPT (DC-specific transcript protein) (12).

In this work, using the same experimental approach, we focused on the identification of molecular regulators of DM-DCs profile as well as the main biological functions represented on these cells, which might lead to the regulatory phenotype of DM-DCs. We further identify MYC as a key regulator of tolerance-related genes in DM-DCs, and ROS production and zinc homeostasis as main metabolic processes activated on these tolDCs.

MATERIALS AND METHODS

Blood Samples

Fifteen buffy coat samples from healthy subjects were obtained from the Clinical Hospital of the University of Chile. All subjects signed an informed written consent and all procedures were approved by the Ethics Committees for Research in Human Beings from the Faculty of Medicine and from the Clinical Hospital of the University of Chile. Demographic characterization of healthy controls is detailed in **Table S1**.

Generation of Monocyte-Derived Dendritic Cell Subsets

Human moDCs were generated from monocytes as previously described (10). Monocytes were isolated from peripheral blood by negative selection using RosetteSep Human Monocytes enrichment cocktail (Stemcell Technologies, Vancouver, Canada) according to manufacturer's instructions. Monocytes were cultured at 2×10^6 cells/ml in serum-free AIM-V medium (Gibco BLR, Grand Island, NY, USA), supplemented with 500 U/ml of recombinant human GM-CSF and IL-4 (eBioscience, San Diego, CA, USA) for 5 days at 37°C and 5% CO₂.

At day 3, culture medium was replenished and cells were incubated with dexamethasone (Sigma-Aldrich, St. Louis, CO, USA) at a final concentration of $1\ \mu\text{M}$. At day 4, cells were stimulated with $1\ \mu\text{g/ml}$ of cGMP-grade MPLA (Avanti Polar Lipids Inc., Alabaster, AL, USA) (DM-DCs). Unstimulated cells (DCs) and MPLA-matured DCs (M-DCs) generated in the absence of dexamethasone were used as controls of immature and mature DCs, respectively. On day 5, cells were harvested and characterized by flow cytometry. Monocyte purity and gating strategy for DC characterization are shown on **Supplementary Figure S1**.

Flow Cytometry

Antibodies used for analysis were anti-human CD11c BUV395 (clone B-ly6), CD83 BUV737 (clone HB15e), STAT1 Alexa Fluor 647 (clone 1/Stat1), STAT3 PerCP-Cy5.5 (clone M59-50) (BD Biosciences); IDO1 PECy7 (clone eyedio), TSC22D3/GILZ PE (clone CFMKG15) (eBioscience); CD86 BV650 (IT2.2), CD11c BV510 (clone L161), MERTK BV421 (clone 590H11G1E3), ZBTB16/PLZF PE (clone Mags.21F7), CD3 BV711 (clone SK7), CD4 Alexa Fluor 700 (clone OKT4), CD25 PE (clone M-A251), IFN γ PE/Cy7 (clone 4S.B3), and TNF α BV605 (clone Mab11) (BioLegend) and P65/RELA PE (APC) (clone 14G10A21); JAG1 Fluorescein (clone 188331) and MYC PerCP (clone 9E10) (R&D Systems). Prior to antibody staining, cells were labeled with Fixable viability dye FVD eFluor 780 (eBioscience). Cells were resuspended in PBS supplemented with 10% of fetal bovine serum (FBS) (HyClone Thermo Scientific, Logan, UT, USA), stained with specific antibodies, fixed with IC fixation buffer (eBioscience) and resuspended in FACSFlow buffer (Becton Dickinson, San Diego, CA, USA) for subsequent analysis. For intracellular cytokine secretion, cells were treated with $50\ \text{ng/ml}$ PMA, $1\ \text{ug/ml}$ ionomycin and $1\ \text{ul/ml}$ brefeldin A for 5 h. After harvest and surface staining, cells were fixed with IC fixation buffer prior to incubation of antibodies against IFN γ and TNF α in permeabilization buffer (eBioscience). After washing, cells were resuspended in FACSFlow buffer (Becton Dickinson, San Diego, CA, USA) for analysis in the flow cytometer. Data were acquired on a LSR Fortessa X-20 with FACSDiva v6.1.3 software (both Becton Dickinson) and analyzed by FlowJo software (Treestar, USA).

RNA Isolation

RNA was isolated from 5×10^5 DCs on day 5 using total RNA isolation RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Yield and quality of RNA samples were evaluated with NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA integrity (RIN score) was analyzed with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) or LabChip GX/GX II (Caliper LifeSciences, Hopkinton, MA, USA).

Microarray Analysis

A total of 40 samples, corresponding to 10 healthy donors under 4 experimental conditions, were considered for microarray analysis. Design and data preparation and analysis are detailed in García-González et al. (12).

Confirmation of Gene Expression by qRT-PCR

cDNA was prepared from moDCs RNA samples using the Superscript II Reverse Transcriptase kit (Thermo Fisher Scientific). Quantitative RT-PCR was performed in Stratagene Mx300P, using Brilliant II SYBR Green QPCR Master Mix (Agilent Genomics) with primer sets from IDT. The housekeeping genes Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and 18S ribosomal RNA (*r18S*) were used as internal controls and target gene expression was normalized using untreated DCs expression values. Primer sequences for each target gene are described on **Table S2**.

MYC Blockade on DCs

To study the role of MYC on DC phenotype, on day 3 of culture, 3 h after Dex treatment, DCs were incubated with the small MYC inhibitor 10058-F4 (Sigma Aldrich), $30\ \mu\text{M}$ for 48 h until the end of culture. After harvest, cells were washed and stained with antibodies specific for tolerogenic and inflammatory markers analysis through flow cytometry. As control, DCs were also incubated with DMSO.

Functional Assay

Functional regulatory capacity of DM-DCs was assessed by co-culturing DCs with allogeneic CD4 $^{+}$ T cells, which were isolated from peripheral blood using the RosetteSep CD4 $^{+}$ human T cell enrichment cocktail (Stemcell Technologies, Vancouver, Canada) and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich) at a concentration of $5\ \mu\text{M}$. On day 5 after harvest, 10058-F4-treated and untreated DM-DCs were seeded in a 96-well U-bottom plates and co-cultured with CFSE-labeled CD4 $^{+}$ T cells at a 1:2 (DC:T cell) ratio in RPMI medium supplemented with 10% heat inactivated fetal calf serum, at 37°C and 5% CO_2 for 6 days, after that, CFSE dilution was analyzed by flow cytometry as a measure of proliferation, in addition to IFN γ and TNF α production.

Determination of Zinc Intracellular Levels in DCs

Zinc influx in DCs was assessed using the cell-permeant zinc fluorescent indicator Newport Green DCF (NG-DCF). Once harvested, cells were washed then fixed and permeabilized prior to staining with $3\ \mu\text{M}$ of Newport Green DCF and an anti-human CD11c antibody. Cells were immediately analyzed at the flow cytometer FACSaria III with FACSDiva v6.1.3 software.

ROS and Superoxide Detection in DCs

Total ROS and superoxide production in cells was assessed with two fluorescent cell-permeable reagents using the kit Cellular ROS/Superoxide Detection Assay (Abcam). Cells were first stained with CD11c as described above and then fixed. The kit Cellular ROS/Superoxide Detection Assay (Abcam) was then used to stain the cells following the manufacturer's instructions. After staining, cells were immediately analyzed at the flow cytometer FACSaria III with FACSDiva v6.1.3 software.

Data Exploration and Statistical Analyses

For flow cytometry and qPCR data, Friedman repeated measures test and Dunn's *post hoc* test were used for data comparison between moDC culture conditions. For functional analyses we used a parametric *t*-tests for data comparison. Analyses were performed using Prism 5.01 software (Graphpad, San Diego, USA).

For microarray data analysis differentially expressed genes in modulated DCs relative to unstimulated DCs, were identified with the Maanova package v1.36.0 *t*-test for gene pairwise comparisons (13) and *p*-values were adjusted using false discovery rate method (FDR). Genes with adjusted $p \leq 0.05$ were considered differentially expressed. Upstream regulators analysis as well as overrepresentation of biological functions was assessed using Ingenuity Pathway Analysis (Ingenuity Systems, Qiagen, Hilden, Germany).

RESULTS

Identification of Transcription Factors Governing Gene Expression on DM-DCs

We have previously shown that treatment of human monocyte-derived DCs with dexamethasone and MPLA (DM-DCs) induces a tolerogenic profile, that distinguishes them from other DC subsets at both phenotypic and transcriptional level, and provides them with the potential to exert immune regulatory functions (10, 12). Through genome-wide transcriptional analysis of these cells we found many tolerance-related genes such as IDO1, MERTK, JAG1, FCGR2B, IL-10, and C1Q to be amongst the most upregulated genes in DM-DCs when compared to MPLA-matured DCs and untreated/immature DCs (DCs) (12). Further analysis of differentially expressed genes on DM-DCs using Ingenuity Pathway Analysis allowed us to identify transcription factors acting as upstream regulators in these cells (Table 1). In addition to act as upstream regulators of a large number of genes expressed on DM-DCs, several transcription factors shown on Table 1, are also upregulated on DM-DCs, with fold change values above 1.2, such as *gilz* (glucocorticoid-induced leucine zipper), *myc* (avian myelocytomatosis viral oncogene homolog), *stat1* (signal transducer and activator of transcription 1), *stat3* (signal transducer and activator of transcription 3), *plzf* (promyelocytic leukemia zinc finger) and *nfk1b* (nuclear factor kappa b subunit 1). Real time PCR analysis of these genes confirmed their differential expression on DM-DCs, which presented higher mRNA levels than the mature MPLA-DCs (M-DCs) (Figure 1) and immature/untreated (DCs) controls (data not shown).

Except for MYC, almost all the other transcription factors identified have a known association with immune regulation; mainly induction of expression of tolerance-related genes, Treg induction, inhibition of DC maturation or promotion of regulatory responses (14–17). Interestingly, MYC experimental Fold change value was shown to be the highest after GILZ in both microarray analysis and qPCR data (Table 1 and Figure 1).

Regulation of Immune Regulatory Markers on DM-DCs by MYC

Since IPA analysis revealed that a large number of molecules from the dataset were predicted to be targets of this transcription factor and, since MYC gene expression was also predicted be regulated by several other transcription factors described as main upstream regulators of DM-DCs transcriptome (Table 1), we decided to evaluate its involvement in DM-DCs biology.

As depicted in Figure 2 and Supplementary Figures S2, S3, in concordance with its gene expression, MYC protein levels were shown to be higher in DM-DCs than in M-DCs.

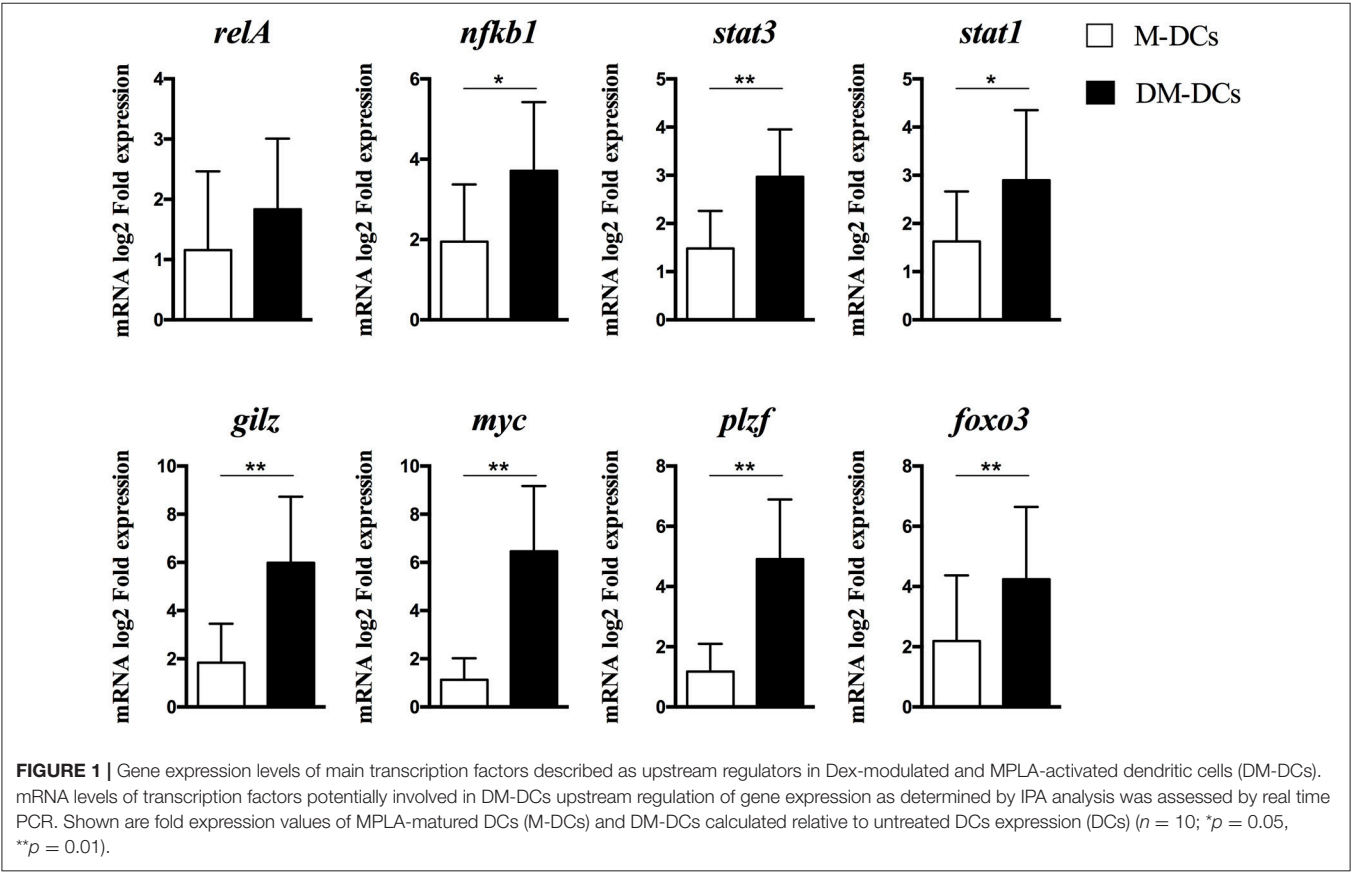
MYC overexpression and association with differentially expressed genes in DM-DCs suggests a potential involvement in DM-DCs regulatory function, so we set to determine if its blockade affected DM-DCs tolerogenic phenotype. For this purpose, we used the small cell-permeable inhibitor 10058-F4, which blocks the transactivation of MYC target genes by specifically blocking the interaction of Myc with its partner Max to form heterodimers which drive gene transcription. Myc blockade for 48 h did not exert any effect on DC maturation/inflammation markers CD83, CD86 and CD1c (Figure 3A and Supplementary Figure S3), indicating that this transcription factor is not involved in the regulation of gene transcription of these molecules. However, we found a robust reduction on protein levels of the tolerance markers JAG1, IDO1 and MERTK, in some cases reaching levels similar to M-DCs (Figure 3A). Gene expression analysis also showed a reduction in mRNA levels of these genes, implying that their expression could be under the control of Myc (Figure 3B). Furthermore, the expression of other transcription factors upregulated on DM-DCs and associated with immune regulation such as PLZF and STAT3, was also affected by the inhibition of Myc (Figure 3B and Supplementary Figure S3). Thus, Myc expression on DM-DCs associates with their tolerogenic phenotype by directly modulating the expression of tolerance markers, and indirectly by regulating the expression of other transcription factors involved in immune regulation.

Modulation of DM-DCs Regulatory Function by MYC

Given that genes found to be modulated by Myc are proposed to contribute to the tolerogenic profile of DM-DCs, we set out to determine whether Myc inhibition could also affect the functional regulatory features of these cells. To evaluate this, we co-cultured DM-DCs that were differentiated in the presence of the Myc inhibitor 10058-F4 with allogeneic CD4⁺ T cells for 6 days and analyzed their ability to affect T cell activation and proliferation. DM-DCs normally display a reduced capacity to promote effector T cell proliferation and pro-inflammatory cytokine secretion (10, 11). Blockade of Myc during DM-DCs differentiation affects DM-DCs tolerogenic function, leading to an increase in CD4⁺ T cell proliferation (Figure 4A and Supplementary Figure S4), in addition to higher levels of IFN γ and TNF α expression in the proliferative CD25-activated CD4⁺ T cell population (Figure 4B and Supplementary Figure S4).

TABLE 1 | Main transcription factors identified as upstream regulators of differentially expressed genes in DM-DCs.

Upstream regulator	Exp fold change	p-value of overlap	Target molecules in dataset
GILZ	2,332	3,48E-02	CCL5, DUSP1
CEBPD	1,985	1,05E-02	IL1B, MYC
MYC	1,881	6,50E-03	BCL6, GADD45B, ID2, IL17RB, JUN, MYC, SAT1, SHMT1, SPP1, TMSB10/TMSB4X
FOXO3	1,579	4,20E-03	CCND2, CCNG2, CLDN1, CXCL10, EGR2, GADD45B, IER3, JAG1, SGK1, SOD2
STAT1	1,312	8,41E-08	CD14, CD40, CXCL10, CXCL9, IDO1, IFI27, IFI6, IFIT1, IFITM1, IRF7, MX1, MYCSTAT1
TCF7	1,277	6,66E-03	CEBPA, CEBPD
STAT3	1,212	1,26E-02	CD40, CD83, CDH2, CXCL10, CXCL9, EGR2, FSCN1, , JAG1, MUC1, MYC, NAMPT, PTAFR, SGK1, SLAMF1, STAT1
VDR	1,177	4,16E-03	CD14, IFI44L
NFKB1	1,115	1,52E-03	CCL5, CD59, CXCL10, CXCL9, FSCN1, IL1B, IL2RA, IRF4, MYC
SMARCA4	1,079	8,56E-07	CCR6, CD52, DHRS9, ESPNL, FCGR2A, FCGR2B, GPR183, IFIT1, IFITM1, IFITM3, IL1B, JUN, MFGE8, MT1H, MUC1, MYC, TACSTD2, TREM2
CTNNB1	1,132	1,67E-02	ECM1, F13A1, IDO1, IFITM1, MMP7, MYC, SGK1
SMAD4	1,079	1,97E-02	CCL20, CCNG2, FSCN1, GADD45B, IRAK3, JAG1, NAMPT, THBS1
E2F3	1,068	4,04E-06	CCL20, MT1F, MT1G, MT1H, MT1X, MYC, S100A8, THBS1
HIF1A	1,110	1,41E-03	CD24, CLDN1, FSCN1, GADD45B, IRS2, MYC, SDC4, SOD2
EZH2	1,101	3,40E-02	CRISPLD2, CXCL10, GPR68, MYC, RAP1GAP, SLC1A3
STAT5A	1,076	8,26E-04	CD24, CXCL5, GSTT1, MMP7, TACSTD2, TNFRSF6B
NUPR1	1,040	1,60E-02	CXCL5, FOXO3, IRS2, MMP12, MT1X, MX2, MYC, RILPL2, SLC16A10, SLC39A8, XBP1
RELA	1,017	3,87E-04	CCL20, CCL5, CD59, CEBPB, CXCL10, CXCL9, FSCN1, IL1B, IRF4, MYC, NAMPT, SOD2



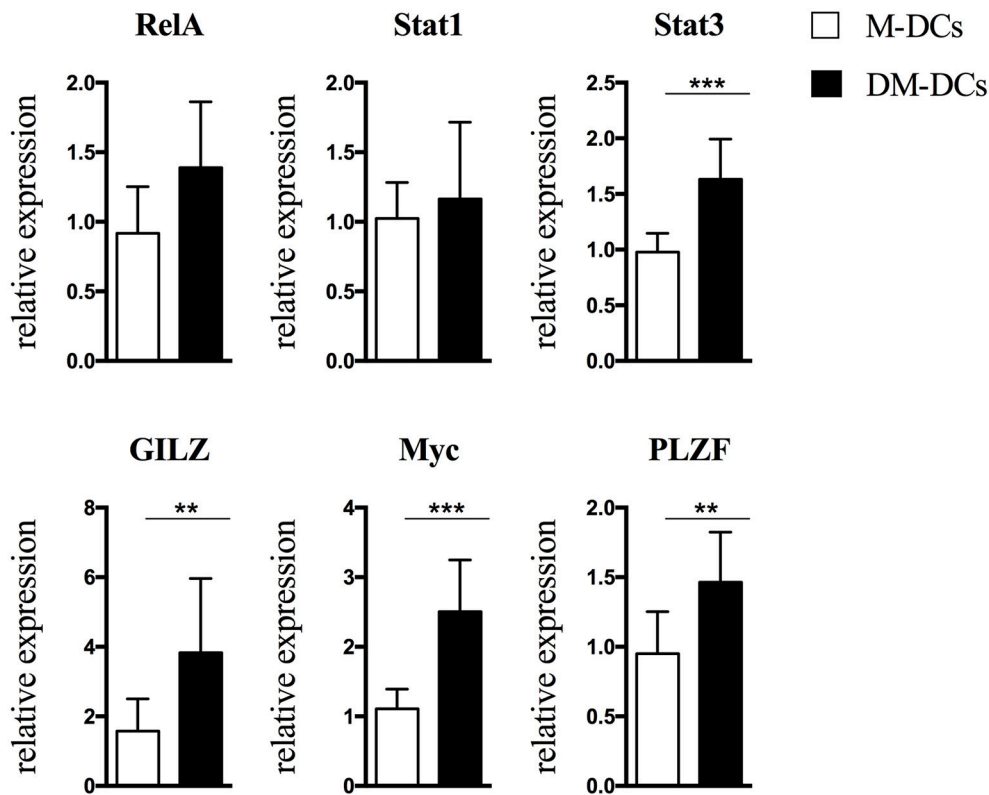


FIGURE 2 | Protein levels of transcription factors involved in regulation of Dex-modulated and MPLA-activated dendritic cells (DM-DCs) gene transcription correlate with gene expression. Protein level of transcription factors potentially involved in upstream regulation of gene expression of DM-DCs was determined by flow cytometry analysis. Shown are relative expression data of MFI values of M-DCs and DM-DCs respect to untreated DCs MFI values ($n = 10$; ** $p = 0.01$; *** $p = 0.001$).

These results support a role for Myc as an important modulator of DM-DCs tolerogenic features.

Metabolic Pathways Enriched in DM-DCs

Myc is known to play an important role in many cellular processes, and more importantly, in metabolism. In the immune system, Myc acts as a key player coordinating metabolic reprogramming in different cell types (18, 19), and in DCs is a major regulator of energetic processes (19). Moreover, it could also be that a high expression of Myc in DCs is related with activation of metabolic processes.

We have previously shown that several differentially expressed genes on DM-DCs are associated with functions guarding metabolic responses, mainly fatty acid metabolism, ROS production and ion homeostasis (12), all contributing in cellular balance. Several genes related to redox homeostasis and ROS production were amongst the most upregulated genes in DM-DCs [(12) and **Supplementary Tables S3, S4**], and as shown in **Figure 5A**, analysis of mRNA expression of different DC subsets using real time PCR confirms microarray analysis results, with DM-DCs exhibiting higher mRNA levels of these genes compared to the mature M-DCs control. To further confirm that this upregulation of redox-related genes has a functional outcome, we set to determine ROS intracellular levels on DCs, as

a measure of evaluating cellular metabolic responses. As shown in **Figure 5B**, ROS levels are higher in DM-DCs than in M-DCs. Additionally, superoxide levels were also found to be higher in DM-DCs than M-DCs (**Figure 5B**), although the latter was not statistically significant; supporting an important role of redox metabolism in these cells.

Zinc Influx Is Highly Regulated on DM-DCs

Besides being a metabolic product and therefore act as indicator of the cell's metabolic state, reactive oxygen species can act as second messengers in different signaling pathways controlling cellular proliferation and differentiation (20). One way by which ROS can drive changes in the cell is by increasing intracellular zinc levels through the modulation of the proteins involved in its cellular availability (21). In addition to ROS production, enrichment analysis of biological functions revealed that ion homeostasis, and cellular response to zinc ion in particular, were also highly represented on DM-DCs transcriptome (12), with several heavy metal scavengers and zinc binding proteins upregulated within these cells. Moreover, several genes involved in the regulation of this biological process, were not just upregulated, but remained amongst the most upregulated genes in DM-DCs transcriptome [**Supplementary Tables S3, S4** and (12)]. Real time PCR analysis corroborated a higher expression

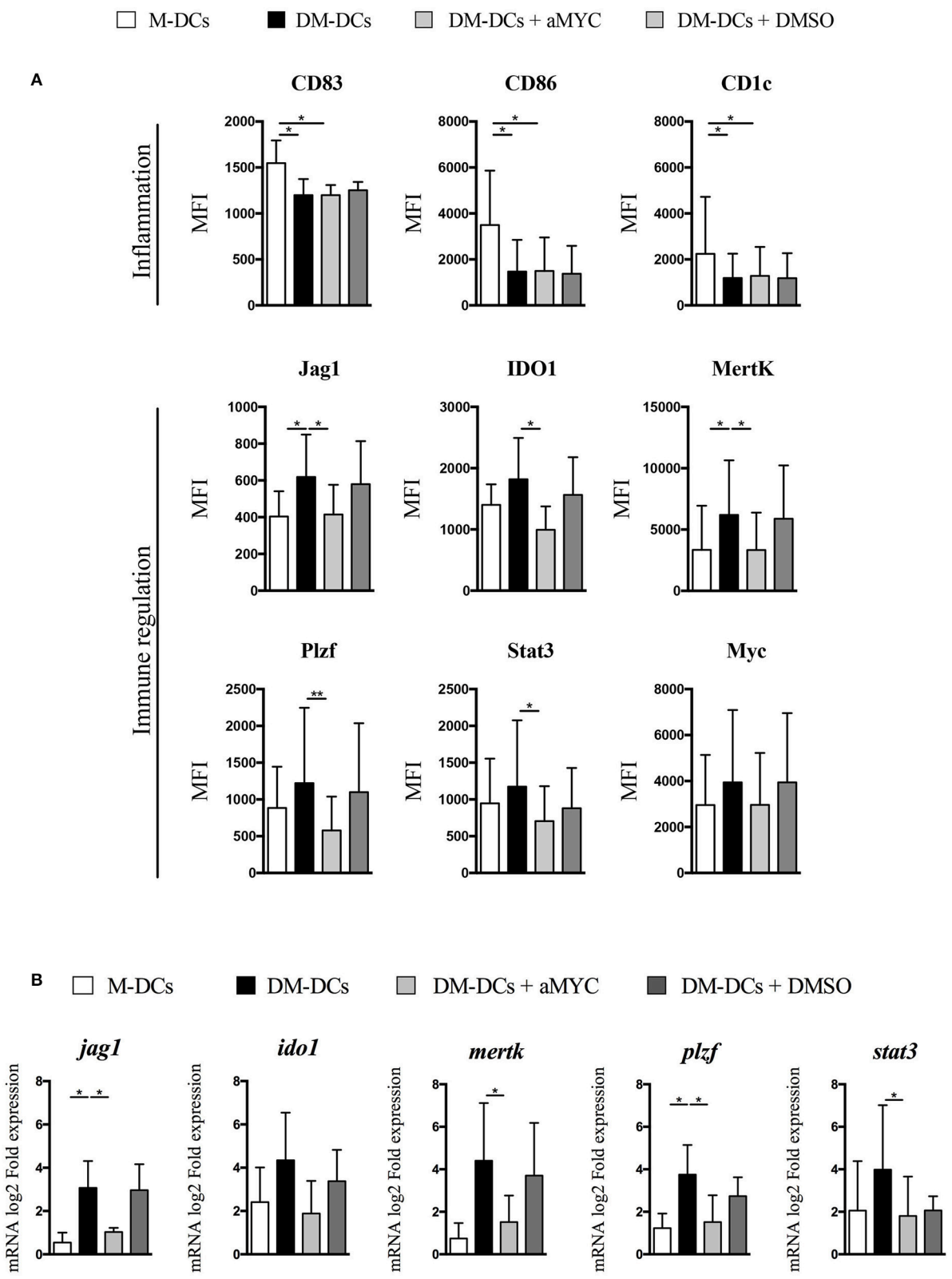
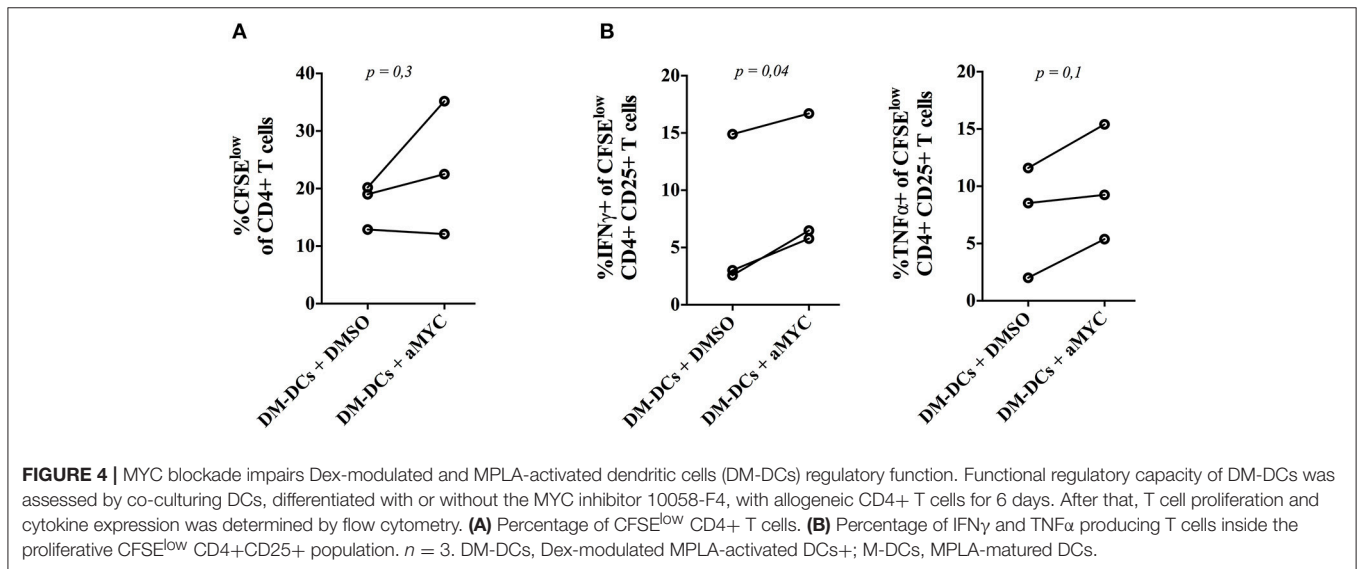


FIGURE 3 | Blockade of MYC leads to inhibition of tolerance-associated gene expression. MYC transactivation of target gene was blocked with the small inhibitor 10058-F4. On day 3 cells were incubated with 10058-F4 (30 μ M) for 48 h, after which immune tolerance markers expression was evaluated at the transcriptional and (Continued)

FIGURE 3 | protein level. (A) Flow cytometry analysis of molecules involved in immune response. Shown are MFI values of 5 independent experiments ($n = 5$). **(B)** Gene expression of tolerance-related genes was assessed through real time PCR. Shown are fold expression values of Dex-modulated and MPLA-activated dendritic cells (DM-DCs) with or without 10058-F4 calculated relative to untreated/immature DCs (DCs) expression. M-DCs and DMSO-cultured DM-DCs were used as controls ($n = 3$) (* $p = 0.05$, ** $p = 0.01$). DM-DCs, Dex-modulated MPLA-activated DCs+; M-DCs, MPLA-matured DCs.



of these genes in DM-DCs related to M-DCs and untreated DCs (Figure 6A), in most cases doubling mRNA levels, denoting an important role of these molecules in DM-DCs phenotype. Since all these genes coordinate zinc availability in the cell, we analyzed zinc intracellular levels in DCs using a cell-permeant fluorescent zinc probe (Supplementary Figure S5), and found that compared to M-DCs and untreated DCs, DM-DCs exhibited higher concentrations of the ion within its cytosol (Figure 6B).

DISCUSSION

The molecular setup driving the immune regulatory functions of tolerogenic DCs is still poorly understood. Even more, the transcriptional regulation of the genes involved in tolerance induction and regulatory responses of tolDCs remains to be fully comprehended. However, technological advances in the last years within the “omics” field as well as the development of multi-parametric flow cytometry analyses bring forward a deeper insight into the molecular characterization of DC biology (22–24). Using these techniques, we have previously described a set of tolerance-related markers found to be upregulated in Dex-modulated tolDCs, alternatively activated with MPLA (DM-DCs) at a transcriptional and at a protein level (12). Here, following the same approach, we identified a set of transcription factors differentially expressed in DM-DCs and potentially involved in the upstream modulation of tolerance markers upregulated in DM-DCs. In particular, we have found that the proto-oncogene MYC, is highly expressed in these cells and modulates the expression of the regulatory markers IDO1, JAG1 and MERTK, as well as other transcription factors STAT3

and PLZF, thus suggesting an important role of these molecule in DM-DCs tolerogenic features. Although the role of MYC in DC development and differentiation is well known (19, 25), a role for MYC in the establishment of immune regulatory responses had not been described before, except for the group of Casey et al. (26) whom showed that MYC promotes tumor growth and development through the modulation of the regulatory molecules CD46 and PD-L1 expression on tumor cells as a means to evade anti-tumor responses. PD-L1 is not expressed in our tolDCs (10), so we could not confirm its association with this regulatory marker, but we did find downregulation of other regulatory molecules, such as IDO1, JAG1 and MERTK when MYC was blocked. To our knowledge, this is the first time that a direct association between MYC and the immune regulatory phenotype of DCs has been established. Besides its effect on the expression of the three tolerance markers mentioned above, MYC blockade also led to downregulation of STAT3 and PLZF, transcription factors associated with immune regulation as well as inhibition of DC maturation (14), pointing out that MYC does not only directly controls the expression of tolerance genes on DM-DCs, but it could also indirectly modulate other regulatory genes and features through the modulation of these transcription factors. Even more, MYC inhibition also led to an impairment of DM-DCs functional features, further supporting its role as a modulator of DM-DCs tolerogenic profile.

In addition to upstream regulators analysis, IPA Functional Enrichment Analysis allowed us to discover that metabolic processes, mainly redox-related metabolic functions were highly represented on our cells, in particular ROS production and zinc homeostasis (12). Changes in cellular metabolism are key for

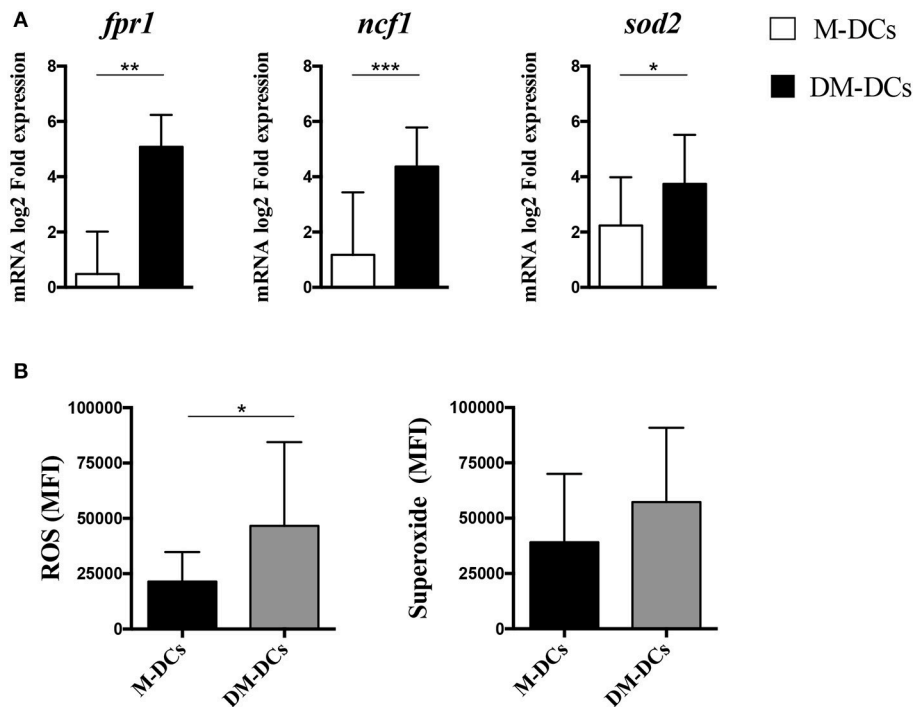


FIGURE 5 | Intracellular concentration levels of reactive oxygen species and superoxide anions is higher in Dex-modulated and MPLA-activated dendritic cells (DM-DCs) than in other DC subtypes. Enrichment of ROS production in DM-DCs compared to DCs and M-DCs was confirmed through gene expression analysis of associated molecules and determination of intracellular levels of free radicals. **(A)** Determination of mRNA levels of genes related to ROS and superoxide production and upregulated on DM-DCs was determined by real time PCR. Data shows fold expression values of DM-DCs and M-DCs relative to untreated DCs expression ($n = 10$; * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$). **(B)** Total ROS and superoxide production in cells was assessed with two fluorescent cell-permeable reagents and flow cytometry analysis. Shown are MFI values for each probe reacting with ROS and superoxide ($n = 3$; * $p = 0.05$, ** $p = 0.01$). DCs, untreated/immature DCs; DM-DCs, Dex-modulated MPLA-activated DCs+; M-DCs, MPLA-matured DCs; M-DCs, MPLA-matured DCs.

certain aspects of DCs, and tolDCs have been described to display a different metabolic profile than other DC subtypes (27, 28). While inflammatory DC exhibit an anabolic type of metabolism which favors glycolysis, tolDCs show a highly energetic and catabolic profile, which in turn favors oxidative phosphorylation and fatty acid oxidation. Furthermore, it has been described that IL-10, a major hallmark of tolDCs, highly produced by our DM-DCs, can also favor this process through inhibition of the metabolic changes induced by TLR activation in DC (27, 29). In accordance to our previous IPA analysis findings (12), we not only confirmed a higher expression of genes involved in ROS and zinc homeostasis, but also found that these cells exhibited higher concentrations of both ROS and zinc in the cytosol, when compared to their mature (M-DCs) and immature counterparts (DCs).

ROS are known to participate in different physiological processes, and even though usually the presence of ROS and other free radicals is considered to promote pro-inflammatory responses, there are instances in which ROS has been shown to promote immune regulatory responses such as to exert a suppressive effect on immune cells (30). While effector T cells are sensitive to oxidative conditions, which if sustained leads to impaired proliferation and death of these cells, Treg can retain their suppressive features under the same circumstances. In fact,

this is one of the strategies used by Treg as well as macrophages and other cells to suppress effector T cell responses and promote immune regulation (31–33). Even more, macrophages have been shown to induce Treg differentiation and proliferation in a ROS-dependent fashion (33), and both, macrophages and moDCs treated with Dex, an immunomodulatory drug used for immune modulation of inflammatory responses, also used by our group and others to generate tolDCs, are found to increase their ROS production, suggesting an association between the induction of a regulatory profile and ROS concentrations (34).

Zinc is known to modulate immune responses through its availability, while zinc deficiency can lead to increased inflammation and inflammatory diseases, zinc supplementation in DCs has been shown to interfere with their maturation, blocking surface expression of MHC II and co-stimulators, and promoting the expression of tolerance markers (35, 36). Yamasaki et al. (37) and Murakami and Hirano (38) propose zinc to act as an intracellular secondary messenger, which can affect other signaling molecules to modulate the final output triggered by extracellular stimuli. This effect depends on zinc availability, which in turn depends on two factors which differ in time, an early component such as a zinc wave that is induced by an extracellular stimuli, and a second component which depends on the transcriptional regulation of the expression

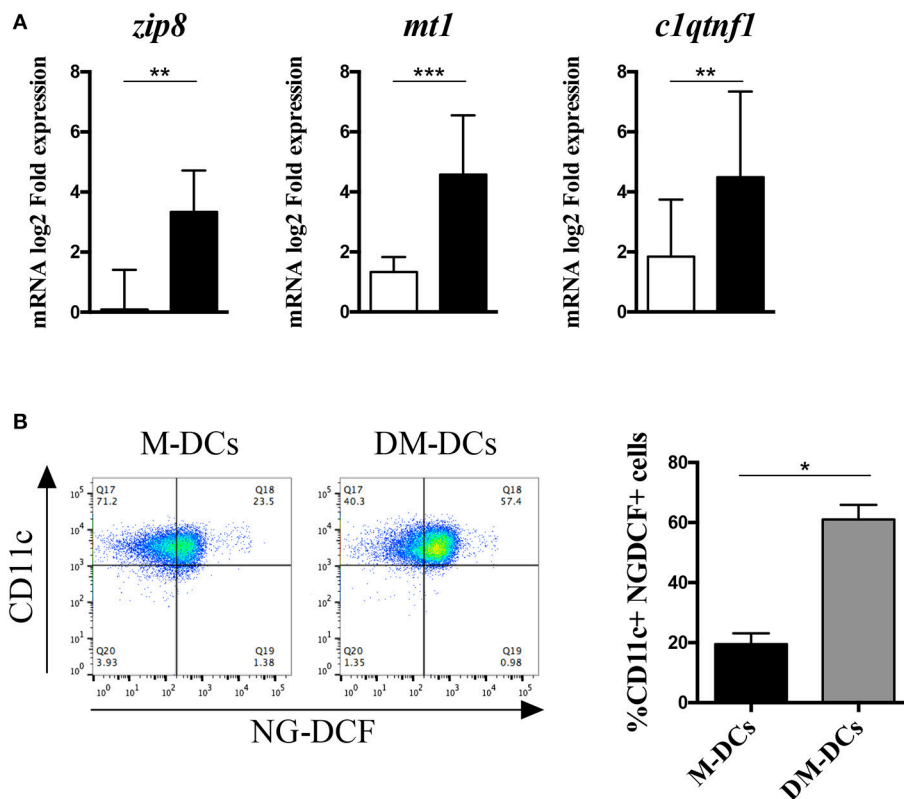


FIGURE 6 | Dex-modulated and MPLA-activated dendritic cells (DM-DCs) show high expression of zinc transporters and zinc intracellular levels. Enrichment of zinc homeostasis in DM-DCs compared to DCs and M-DCs was evaluated. **(A)** Gene expression of zinc binding molecules upregulated in DM-DCs microarray analysis was determined by real time PCR. Data shows fold expression values of DM-DCs and M-DCs relative to untreated DCs expression ($n = 10$; * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$). **(B)** Determination of zinc intracellular levels in DCs was assessed with the zinc fluorescent indicator Newport Green DCF (NG-DCF) and analyzed by flow cytometry. Shown are percentages of CD11c+ cells positive for NG-DCF intracellular staining ($n = 4$; * $p = 0.05$, ** $p = 0.01$). M-DCs, MPLA-matured DCs.

of zinc transporters (38). Zinc availability in the cytosol is partly modulated by metallothioneins (MTs) in addition to other proteins such as zinc transporters, zinc-binding proteins and sensors (38). The treatment of DCs with dexamethasone and MPLA lead to the upregulation of several of the genes involved in zinc homeostasis, and gene expression analysis allowed us to confirm a higher expression of some of these molecules in DM-DCs compared to M-DCs and untreated DCs. Furthermore, to confirm a functional relation between zinc-binding proteins and zinc availability, we determined zinc intracellular levels on different DCs subtypes, finding a higher zinc influx on DM-DCs than in the mature (M-DCs) or immature control (DCs).

Several forms of MTs, cysteine-rich metal binding proteins involved in the homeostasis of zinc and other heavy metals at a cytoplasmic level, displayed the highest fold change values and were highly represented in DM-DCs transcriptomic profile [Tables S3, S4 and (12)]. Both, MTs expression and function, is modulated by intracellular ROS. Oxidation of MTs leads to release of zinc ions into the cytosol, increasing the influx of this cation in the cell. Thus, MTs fulfill an important role as signal transducers, turning ROS signals into transient increases of zinc (21) which then can act as an

intracellular secondary messenger as described by Yamasaki et al. (37).

Thus, our work shows that the tolerogenic features of DM-DCs are partially modulated by the expression of MYC, as well as ROS production and zinc influx. This proposed regulatory role of MYC on DM-DCs could not only be occurring on our DCs. A review made by our group regarding transcriptional and proteomic research developed in human tolDCs, revealed that this transcription factor was expressed in almost all tolDCs studied (23), further supporting our findings for an important role of this transcription factor in tolDCs.

MYC is a key regulator of many biological processes within the cell, mainly metabolic programs, and it has been described that its expression increases intracellular ROS levels (39–43). In addition to their potential role in immune regulation and apart from acting as an indicator of the cell's metabolic state, ROS can act as second messengers in different signaling pathways controlling cellular proliferation and differentiation (20). One way by which ROS can drive changes in the cell is by increasing intracellular zinc levels through the modulation of proteins involved in its cellular availability (21). In DCs, besides its importance in development and differentiation, MYC has an

important role in DC:T cell priming and DC metabolism (18, 43, 44), so we propose that there is a close association between all findings of this work, where MYC expression not only leads to transcriptional regulation of tolerance markers, but also to increase in ROS levels, which in turn is translated into a zinc signal. However, further studies will be required to confirm this.

ETHICS STATEMENT

All subjects signed an informed written consent and all procedures were approved by the Ethics Committees for Research in Human Beings from the Faculty of Medicine and from the Clinical Hospital of the University of Chile.

AUTHOR CONTRIBUTIONS

PG-G, KS, JA, RV, RT, and DC participated in the conception and design of the study. PG-G, KS, JA, RV, AS-G, DC, OA, and MM worked on analysis and interpretation of data. PG-G and JA participated in manuscript preparation and redaction. PG-G, KS, AM, HN, AS-G, and JM performed most experiments and data acquisition. LS, ON, and JM participated in the recruitment

of subjects for this study. BP collaborated in data acquisition and analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01171/full#supplementary-material>

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MAP7 and MUCL1 Are Biomarkers of Vitamin D3-Induced Tolerogenic Dendritic Cells in Multiple Sclerosis Patients

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The administration of autologous tolerogenic dendritic cells (tolDC) has become a promising alternative for the treatment of autoimmune diseases, such as multiple sclerosis (MS). Specifically, the use of vitamin D3 for the generation of tolDC (vitD3-tolDC) constitutes one of the most widely studied approaches, as it has evidenced significant immune regulatory properties, both *in vitro* and *in vivo*. In this article, we generated human vitD3-tolDC from monocytes from healthy donors and MS patients, characterized in both cases by a semi-mature phenotype, secretion of IL-10 and inhibition of allogeneic lymphocyte proliferation. Additionally, we studied their transcriptomic profile and selected a number of differentially expressed genes compared to control mature and immature dendritic cells for their analysis. Among them, qPCR results validated *CYP24A1*, *MAP7* and *MUCL1* genes as biomarkers of vitD3-tolDC in both healthy donors and MS patients. Furthermore, we constructed a network of protein interactions based on the literature, which manifested that *MAP7* and *MUCL1* genes are both closely connected between them and involved in immune-related functions. In conclusion, this study evidences that *MAP7* and *MUCL1* constitute robust and potentially functional biomarkers of the generation of vitD3-tolDC, opening the window for their use as quality controls in clinical trials for MS.

Keywords: tolerogenic dendritic cells, multiple sclerosis, biomarkers, vitamin D3, immune tolerance

INTRODUCTION

The role of dendritic cells (DC) within the immune system is crucial, since they are in charge of orchestrating immune responses and maintaining the homeostasis between immunogenicity and tolerance. Under normal conditions, DC remain in an immature status (iDC), characterized by their ability to capture and present antigens and other signals in their environment. However, these cells are not stable, and when exposed to a danger signal, iDC become activated and differentiate into professional, antigen-presenting mature DC (mDC). This pro-inflammatory status is

characterized by an increase in the expression of MHC and co-stimulatory molecules, thus enabling mDC to induce an efficient and potent immunogenic response (1–4).

If the immune homeostasis is lost, and a breach of tolerance causes mDC to recognize and present specific self-antigens to T cells, different autoimmune pathologies may develop depending on which protein or tissue is targeted, such as multiple sclerosis (MS), rheumatoid arthritis or type 1 diabetes. These complex disorders involve many innate and adaptive mechanisms of the immune system, and their etiology still remains unknown. For this reason, a cure has not been found yet, and the currently available treatments consist in strong immunomodulatory or immunosuppressive drugs. In general, these are focused on addressing the symptoms in a poorly effective and unspecific manner, with potentially severe side effects. Hence, there is an increasing need for new, more specific and effective therapies. Over the last years, tolerogenic DC (tolDC) have been postulated as a novel and promising alternative to treat these disorders (5). In fact, several approaches have already been tested in Phase I clinical trials for autoimmune diseases, as reviewed by ten Brinke et al. (6), and other clinical studies are still ongoing. In all cases, these treatments have demonstrated to be safe, with no relevant side effects on the patients. Consequently, many initiatives are now focused on assessing the actual efficacy of tolerogenic cell therapies.

In general, tolDC are defined as a stable and semi-mature subset of DC with the potential to restore immune tolerance in an antigen-specific manner if loaded with one or more determined peptides, thus not compromising the protective immunity of the patients. Compared to mDC, these cells are typically characterized by their low —or lower— expression of MHC and co-stimulatory molecules (such as CD40, CD80, CD83 or CD86), and by their reduced or null secretion of IL-12, IFN- γ and other pro-inflammatory cytokines, combined with an increment in the production of IL-10 or TGF- β . These features confer to tolDC a reduced capability to induce T cell proliferation and the possibility to prime regulatory T cell (Treg) responses, thus potentially directing the immune response toward a regulatory context (7–9). However, these characteristics can sometimes be very variable, since there is a wide variety of protocols to generate tolDC *in vitro* from human peripheral blood monocytes. These approaches include the use of several compounds, cytokines and immunomodulatory drugs such as IL-10 (10, 11), dexamethasone (11–15) or rapamycin (11, 12, 16), as well as different genetic engineering techniques (17, 18).

Among all of them, the use of 1,25-dihydroxyvitamin D3 —the active form of vitamin D3— to generate tolDC is one of the most widely established protocols. Specifically, vitamin D3-induced tolDC (vitD3-tolDC) present a semi-mature profile, accompanied by an ability to inhibit allogenic T cell proliferation and to polarize the immune response toward an anti-inflammatory T_H2 profile (12, 19–28). Furthermore, several studies using animal models of autoimmune diseases have demonstrated their functionality *in vivo* (29–31). In general, these cells are characterized by the suppression of the NF- κ B pathway (21, 32), accompanied by an increased activity of the oxidative metabolism of glucose, and indeed the glucose

availability and the glycolytic activity mediated through mTOR signaling are crucial for the induction and maintenance of their tolerogenic function (27). However, despite the identification of several pathways involved in the anti-inflammatory role of vitD3-tolDC, the specific mechanisms for the induction of immune tolerance by these cells have not been clearly identified yet.

Previously, our group has successfully generated human vitD3-tolDC —demonstrating their tolerogenic properties *in vitro* using cells generated from both healthy donors and MS patient samples—, and has studied their transcriptomic profile compared to other tolDC protocols (12, 25, 26, 33). Additionally, in further *in vivo* studies, we also reported a positive and beneficial effect of antigen-specific vitD3-tolDC treatment over the course of the murine model of MS, the experimental autoimmune encephalomyelitis (30, 31). Altogether, these results have led to the development of an ongoing Phase I clinical trial in MS patients with peptide-loaded vitD3-tolDC (<http://www.clinicaltrials.gov>, NCT02903537).

However, for the full translation of an autologous, antigen-specific, tolerogenic cell therapy into the common clinical practice, several additional steps yet need to be taken. Among them, the definition of adequate, robust and objective biomarkers constitutes one of the priorities. These markers would, on the one hand, guarantee the proper generation and functionality of tolDC, without compromising the safety for the patients. On the other hand, these biomarkers would enable the comparison of results with other research groups, hereby accelerating the translation of tolDC therapies into the clinic in a collaborative endeavor (6). However, although many efforts have been made in this regard, and despite several genes and molecules have been identified for a variety of tolDC protocols separately, such as *IDO1*, *GILZ*, or *ANXA1*, the definition of universal biomarkers of tolerance-inducing cell products has not been possible so far, and it seems unlikely provided the wide heterogeneity of approaches being used (34). For this reason, in this study we have analyzed the transcriptomic profile of vitD3-tolDC in order to select and validate several differentially expressed genes (DEG) that may be used as transcriptomic biomarkers of these cells in a clinical trial for MS patients.

MATERIALS AND METHODS

Sample Collection

Buffy coat samples from 24 randomized healthy donors were obtained from the *Banc de Sang i Teixits* (Barcelona, Spain), following the institutional Standard Operating Procedures for blood donation, which included a signed informed consent. Whole blood samples from 10 MS patients were collected by standard venipuncture in lithium heparin tubes. Patients did not receive any corticoid or disease-modifying therapy during at least the previous 2 months, and both relapsing and progressive forms of the disease were considered. The same procedure was followed for whole blood obtention from 34 healthy donors for the functional assays (see below). This study was approved by the Germans Trias i Pujol Hospital ethical committee, and all patients and healthy controls signed an informed consent.

Complementary DNA (cDNA) of paired IL-10-induced tolDC (IL10-tolDC) and mDC samples from 5 healthy donors were obtained from Sanquin Bloodbank (Amsterdam, The Netherlands) after informed consent. These samples were generated as described in Boks et al. (11).

Monocyte Isolation

Samples from healthy donors were processed first depleting CD3⁺ cells using the RosetteSep[®] Human Monocyte Enrichment Cocktail kit (StemCell Technologies, Vancouver, Canada) prior to a ficoll-hypaque (Rafer, Zaragoza, Spain) density gradient separation. Subsequently, CD14⁺ cells were isolated by positive selection using the EasySep[®] Human CD14 Positive Selection Kit (StemCell) following manufacturer's instructions. For the isolation of monocytes from MS patients, peripheral blood monocytes were isolated from 50 mL of whole blood by ficoll-hypaque density gradient separation, followed by the abovementioned CD14 positive selection step. The initial CD3⁺ cells depletion step was not performed due to the limited amount of blood. Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and cell viability was determined using 7-amino-actinomycin D (7-AAD) (BD Biosciences) and phycoerythrin (PE)-conjugated annexin V (Immunotools, Friesoythe, Germany) staining for 20 min at 4°C, protected from light. Cell counts were quantified using PerfectCount microspheres (Cytognos, Salamanca, Spain) and monocyte purity was determined using forward and side scatter gating strategies on FACSDiva software (BD Biosciences).

VitD3-tolDC Generation

Monocytes from both healthy donors and MS patients were cultured at a density of 1×10^6 cells/mL in 24-well plates at 37°C and a 5% CO₂ atmosphere for 6 days in 1 mL X-VIVO 15 medium (Lonza, Basel, Switzerland), supplemented with 2% heat-inactivated human AB serum, 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin (Reig Jofre, Sant Joan Despí, Spain) and 100 µg/mL streptomycin (Normon, Tres Cantos, Spain). For the generation of iDC, monocytes were differentiated in the presence of 200 U/mL granulocyte macrophage colony-stimulating factor (GM-CSF) and 250 U/mL IL-4 (both from Peprotech, London, UK). Whole volume of fresh medium and cytokines was replenished on day 4. In addition, a maturation cocktail containing 1,000 U/mL IL-1β, 1,000 U/mL TNF-α (both from Peprotech) and 1 µM prostaglandin E2 (PGE2) (Pfizer, New York, NY, USA) was added on day 4 to both mDC and vitD3-tolDC conditions. Finally, for the induction of vitD3-tolDC, these cells were besides treated with 1 nM vitamin D3 (Calcijex, Abbott, Chicago, IL, USA) on days 0 and 4. On day 6, all three conditions were harvested following an incubation with accutase (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C to detach the cells from the plate, and washed twice. Cell counts and viability were determined by flow cytometry, as shown above, and after the phenotypical and functional characterization, dry pellets of each condition were generated by centrifugation and stored at -80°C.

Phenotype Analysis

Surface protein expression of CD11c, CD14, CD25, CD83, CD86 and HLA-DR of iDC, mDC and vitD3-tolDC was determined by flow cytometry. For each measurement, DC suspensions were incubated for 20 min, protected from light, with the adequate amounts of monoclonal antibodies anti-: CD11c PE-Cyanine dye 7 (PE-Cy7), CD14 Violet 450 (V450), CD25 allophycocyanin (APC), CD83 APC, CD86 fluorescein isothiocyanate (FITC) and HLA-DR Violet 500 (V500) (all of them from BD Biosciences). Afterwards, at least 10,000 CD11c⁺ events of each sample were acquired in a FACSCanto II flow cytometer and analyzed using FACSDiva software.

Functionality Test

Allogeneic peripheral blood mononuclear cells (PBMC) from whole blood of healthy donors were isolated by ficoll-hypaque density gradient separation. Cells were washed twice afterwards, and their absolute number and viability was determined as shown above.

Subsequently, a proliferation assay was performed in 96-well round bottom plates with co-cultures of 10⁵ allogeneic PBMC and 5,000 iDC, mDC, or vitD3-tolDC (1:20 ratio) in a total volume of 200 µL of supplemented X-VIVO 15 medium. The same number of PBMC cultured in the presence of either supplemented X-VIVO 15 medium or 50 ng/mL phorbol 12-myristate-13-acetate (PMA) and 500 ng/mL ionomycin were used as negative and positive controls, respectively. Six replicates of each condition were performed. Cells were incubated for 4 days at 37°C in a 5% CO₂ atmosphere.

Finally, 1 µCi [³H]-thymidine (PerkinElmer, Waltham, MA, USA) was added to each well and the plate was incubated for further 18 h under the same conditions. Cells were then collected using a HARVESTER96 2M cell harvester (Tomtec Inc, Hamden, CT, USA) and read on a 1450 MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland).

Cytokine and Soluble Protein Production

The production of granzyme B (GZMB) and vascular endothelial growth factor (VEGF), as well as IL-10, IFN-γ and IL-12p70 cytokines, was quantified in the culture supernatants of tolDC using the Human Soluble Protein CBA Flex Set (BD biosciences) according to manufacturer's instructions. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FACSDiva software.

The production of TGF-β was determined using the Human/Mouse TGF beta 1 Uncoated ELISA kit (Invitrogen) in 100 µL of supernatant samples, again following manufacturer's instructions. The optical density of each well was measured at 450 nm, and the optical density at 570 nm was then subtracted as background signal, using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Differential Expressed Genes Selection and Generation of a Network of Protein Interactions

The data of a comparative transcriptomic analysis of vitD3-tolDC, iDC and mDC from 5 healthy donors, previously

performed by our group in a microarray study (33), was used to select several DEG. For that, the mean difference of expression (MeanDiff) of each gene was evaluated, and only those genes that were specifically induced or repressed in vitD3-tolDC vs. both iDC and mDC conditions (either $\text{MeanDiff}_{\text{vitD3-tolDC vs. mDC}} > 0.5$ —first criterium—, while $\text{MeanDiff}_{\text{iDC vs. mDC}} < 0.5$, —second criterium—; or $\text{MeanDiff}_{\text{vitD3-tolDC vs. mDC}} < -0.5$, while $\text{MeanDiff}_{\text{iDC vs. mDC}} > -0.5$), with a statistically significant differential expression in the first criterium ($p < 0.01$), were selected in order to validate them. Unlike in our previous study, B-statistic —an indicator of the likelihood of the results— was not considered for the selection of candidate genes this time. Microarray raw and processed data were deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6937.

Additionally, a bioinformatic analysis was performed in order to characterize the molecular mechanisms involved in the tolerogenicity of vitD3-tolDC. Briefly, the Therapeutic Performance Mapping System technology (35, 36) was used to generate a mathematical model of protein interactions from our transcriptomic microarray data, based on an effectors database of tolDC biology and functionality (Anaxomics, Barcelona, Spain). This database was generated using information available in the literature as well as public and private repositories. From this model, a network of protein interactions between all the effectors found in our microarray data was built using an Artificial Neural Networks analysis and represented using Cytoscape 3.6.1 software.

RNA Extraction and qPCR Validation

Total RNA was isolated from dry pellets using the RNeasy Mini Kit (Qiagen, Hilden, Germany) along with a complementary DNase treatment with the RNase-free DNase Set (Qiagen), following manufacturer's instructions. Quantity and purity of the samples was then determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and the RNA was subsequently retrotranscribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Finally, 250 ng cDNA were preamplified using the TaqMan™ PreAmp Master Mix Kit (Applied Biosystems).

The expression of genes *CA2*, *CAMP*, *CLEC5A*, *CYP24A1*, *DHRS9*, *GAPDH*, *GZMB*, *IL1R1*, *MAP7*, *MUCL1*, *OS9*, *PPIA*, *SNORD30*, *SPARC*, *ST6GAL1*, *TBP* and *THBS1* was determined by quantitative PCR (qPCR) using the respective TaqMan Gene Expression Assays (Applied Biosystems) shown in **Supplementary Table 1**, following the instructions provided by the manufacturer, in a LightCycler 480 System thermocycler (Roche, Basel, Switzerland). Housekeeping genes *CYP24A1*, *TBP* and *GAPDH* were used as controls. The quantitative expression of each gene was calculated based on the $2^{-\Delta C_p}$ method (37), using the mean C_p values of the 3 housekeeping genes. The decimal logarithm of fold change (logFC) expression values of each gene were considered for the definition of validation criteria. Similar to the MeanDiff parameter from the microarray, in this case genes were considered as differentially expressed in vitD3-tolDC

vs. both iDC and mDC when either $\logFC_{\text{vitD3-tolDC vs. mDC}} > 0.5$ —first criterium—, while $\logFC_{\text{iDC vs. mDC}} < 0.5$, —second criterium—; or $\logFC_{\text{vitD3-tolDC vs. mDC}} < -0.5$, while $\logFC_{\text{iDC vs. mDC}} > -0.5$, if statistical significance was reached for the first criterium ($p < 0.05$).

Immunocytochemistry Validation

In order to confirm the qPCR results and analyze the protein expression and distribution of MAP7 and MUCL1 molecules, an indirect immunocytochemistry (ICC) staining was carried out in vitD3-tolDC, mDC and iDC from healthy donors and MS patient samples. Cell culture differentiations of vitD3-tolDC, mDC and iDC were performed in 24-well plates, following the same protocol described above, over 12 mm round coverslips. After 6 days of culture, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% TWEEN20 (Sigma-Aldrich) and subsequently blocked with 10% goat serum for 15 min. Afterwards, samples were incubated for 1 h at room temperature or overnight at 4°C with the primary antibodies mouse anti-human α -tubulin (Invitrogen) and either rabbit anti-human MAP7 (Invitrogen) or rabbit anti-human MUCL1 (Sigma-Aldrich). Next, cells were washed and later incubated with AlexaFluor (AF) 488 goat anti-mouse IgG and AF 594 goat anti-rabbit IgG secondary antibodies (both from Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) for 30 min at room temperature, protected from light. Cells were washed again, and the coverslips were finally mounted using ProLong® Gold Antifade Mountant medium with DAPI (Invitrogen) for nucleus staining. Samples were analyzed on an Axio Observer Z1 fluorescence microscope (Zeiss, Oberkochen, Germany) with a 63x objective, using ZEN software (Zeiss), and the expression of MAP7 and MUCL1 was calculated as the corrected total cell fluorescence (CTCF) of each protein (38, 39) using the FIJI distribution of ImageJ software (40, 41).

Statistical Analysis

All the statistical analyses were performed with either parametric or non-parametric tests depending on the normality of each compared data set, as determined by the D'Agostino & Pearson test, using Prism 6.0 software (GraphPad, La Jolla, CA, USA). For multiple comparisons, either the one-way ANOVA test with Geisser-Greenhouse correction or the non-parametric Friedman test with Dunn's correction were used depending on the normality of the groups. Analogously, for comparisons between two groups, either the t-Student or the Wilcoxon test were used if the samples were normally distributed or not, respectively. When $N \leq 6$, parametric tests were used anyway due to the small sample size (42). Results were expressed as mean \pm standard deviation (SD), unless noted otherwise, and they were considered statistically significant when $p < 0.05$.

RESULTS

VitD3-tolDC Show Phenotypical Characteristics of tolDC

TolDC were generated from samples of 24 healthy donors and 10 MS patients with $83.1 \pm 0.01\%$ purity of monocytes and $98.4 \pm$

0.03% viability after CD14⁺ cells positive selection. After *in vitro* differentiation, DC were harvested, and their phenotype, purity and viability were characterized by flow cytometry following the gating strategy depicted in **Supplementary Figure 1a**. As determined by the percentage of CD11c⁺ cells, purity of DC was always >90%, with $91.1 \pm 0.04\%$ viability in both healthy donor and MS patient cells (**Supplementary Figure 1b**). When we analyzed the phenotype of the cells, the median fluorescence intensity (MFI) values for CD83, CD86 and HLA-DR on each condition were considered. As displayed in **Supplementary Figures 1c–e**, vitD3-tolDC from healthy donors showed reductions of $83 \pm 34\%$, $59 \pm 15\%$ and $71 \pm 15\%$ in the expression of CD83, CD86 and HLA-DR, respectively, compared to mDC, and similar to the expression levels showed by iDC. In the case of MS patient-derived vitD3-tolDC, a similar behavior was observed, with reductions of $77 \pm 30\%$, $60 \pm 13\%$ and $61 \pm 14\%$ in CD83, CD86 and HLA-DR expression, respectively, compared to mDC, and similar to iDC. All the results reached statistical significance for both healthy donors and MS patients ($p < 0.05$).

VitD3-tolDC Induce Allogeneic Hyporesponsiveness and Produce Anti-inflammatory Cytokines

The functional assay results evidenced that vitD3-tolDC significantly inhibited the proliferation of allogeneic PBMC. As shown in **Supplementary Figure 2a**, a $73.6 \pm 16.6\%$ reduction compared to mDC was observed in healthy donors, similar to the $84.1 \pm 10.7\%$ reduction exhibited by iDC. Analogously, although in a less strong degree, for MS patient-derived DC, a $47.9 \pm 25.6\%$ and a $46.3 \pm 28.9\%$ reduction in the proliferation induction was observed for vitD3-tolDC and iDC, respectively, in comparison to mDC. In all four comparisons, statistical significance was reached ($p < 0.05$).

When the cytokine secretion profile of vitD3-tolDC was compared to mDC and iDC, an increase in the production of the anti-inflammatory cytokines IL-10 (**Supplementary Figure 2b**) and TGF- β (**Supplementary Figure 2c**) was detected in vitD3-tolDC differentiated from healthy donors (IL-10_{vitD3-tolDC}: 166.0 ± 287.7 pg/mL vs. IL-10_{mDC}: 44.0 ± 51.4 pg/mL; $p = 0.003$; and TGF- β _{vitD3-tolDC}: 306.5 ± 159.5 pg/mL vs. TGF- β _{mDC}: 188.1 ± 165.3 pg/mL; $p = 0.046$), but only of IL-10 in the case of vitD3-tolDC generated from MS patients (IL-10_{vitD3-tolDC}: 148.6 ± 141.7 pg/mL vs. IL-10_{mDC}: 62.1 ± 54.1 pg/mL; $p = 0.043$). Furthermore, IL-12 production could not be detected in any condition (data not shown). Finally, no statistically significant changes were found in the production of GZMB (**Supplementary Figure 2d**), nor IFN- γ (**Supplementary Figure 2e**), between the different conditions.

CYP24A1, MAP7 and MUCL1 Genes Are Induced in vitD3-tolDC From Healthy Donors and MS Patients

Following the study previously performed by our group in a comparative microarray study of vitD3-tolDC, iDC

and mDC from 5 healthy donor samples (33), we applied the filtering criteria described in the methods section to the data results (either MeanDiff_{vitD3-tolDC vs. mDC} > 0.5 —first criterium—, while MeanDiff_{iDC vs. mDC} < 0.5, —second criterium—; or MeanDiff_{vitD3-tolDC vs. mDC} < -0.5, while MeanDiff_{iDC vs. mDC} > -0.5, with a statistically significant differential expression in the first criterium). Briefly, these parameters spotted those genes that were specifically induced by the effect of vitamin D3 over DC according to our microarray analysis, since they were differentially expressed in vitD3-tolDC compared to both mDC and iDC, and therefore could be considered as potential transcriptomic biomarkers of vitD3-tolDC (**Table 1**). As a result, we selected *CA2*, *CAMP*, *CLEC5A*, *CYP24A1*, *GZMB*, *IL1R1*, *MAP7*, *MUCL1* and *SNORD30* genes for validation.

The subsequent qPCR analysis of the actual expression of these genes in healthy donors evidenced that all of them showed an expression pattern compliant with the expression thresholds that were established for the validation —analogously to those from the microarray but depicted in logFC, as defined in the methods section—, except for *CA2* (uncompliant with the second criterium), *GZMB* (uncompliant with the second criterium) and *SNORD30* (uncompliant with the first criterium), as shown in **Table 1** and **Figure 1A**. Consequently, these 3 genes were discarded from further analysis and the expression of the remaining genes was tested on MS patient samples. In this case, **Table 1** and **Figure 1B** show that, however, neither *CAMP*, nor *CLEC5A*, nor *IL1R1* fulfilled our expression criteria. As a result, only *CYP24A1*, *MAP7* and *MUCL1* could be validated as DEG for our vitD3-tolDC product —reaching statistical significance for the first criterium, as required, in all cases ($p < 0.05$)—, and therefore were selected as transcriptomic biomarkers of our tolDC-inducing protocol.

MUCL1 Is Also Induced in tolDC Generated With IL-10

Given the positive results, we therefore intended to test the expression of some of these genes in 5 samples of IL10-tolDC differentiated from healthy donors, in order to assess their potential value as biomarkers of a different tolDC-inducing protocol. While the expression of *CA2*, *CAMP*, *CLEC5A*, *IL1R1* (data not shown) and *MAP7* (**Figure 1C**) was not altered in these cells, we observed the up-modulation of *MUCL1* gene expression in IL10-tolDC compared to mDC (logFC_{IL10-tolDC vs. mDC} = 0.960 ± 0.395 ; $p = 0.034$), similar to that observed for vitD3-tolDC (**Figure 1C**). The expression of *CYP24A1* was not analyzed, since this gene is directly related to the response of the cells to vitamin D3, which was not present in these specific cultures.

MAP7 and MUCL1 Are Functionally Related in vitD3-tolDC Through a Network of Protein Interactions

In order to find potentially common genes related to *CYP24A1*, *MAP7* and *MUCL1* that could provide a mechanistic insight

TABLE 1 | Expression by microarray and qPCR of the selected genes in dendritic cells differentiated from healthy donors and multiple sclerosis patients.

MICROARRAY DATA FROM HEALTHY DONORS [FROM NAVARRO-BARRIUO ET AL. (33)]					
Gene	iDC vs. mDC		vitD3-tolDC vs. mDC		
	MeanDiff	p-value	MeanDiff	p-value	
CA2	1.044	0.097	1.680	0.010	
CAMP	0.337	0.279	1.351	<0.001	
CLEC5A	0.015	0.975	1.573	0.003	
CYP24A1	−0.585	0.264	2.271	<0.001	
GZMB	0.097	0.582	0.734	<0.001	
MAP7	0.075	0.663	0.880	<0.001	
MUCL1	0.084	0.857	2.132	<0.001	
SNORD30	−0.040	0.949	1.616	0.016	

HEALTHY DONORS						
Gene	iDC vs. mDC		vitD3-tolDC vs. mDC		vitD3-tolDC vs. iDC	
	logFC ± SD	p-value	logFC ± SD	p-value	logFC ± SD	p-value
CA2	0.566 ± 0.391	0.001	0.858 ± 0.445	<0.001	0.292 ± 0.276	0.042
CAMP	0.290 ± 0.397	0.042	0.957 ± 0.447	<0.001	0.668 ± 0.433	0.003
CLEC5A	−0.254 ± 0.371	0.063	0.602 ± 0.324	0.001	0.856 ± 0.346	<0.001
CYP24A1	−1.680 ± 0.422	0.008	1.532 ± 0.637	0.004	3.212 ± 0.914	0.004
GZMB	0.588 ± 0.426	0.001	0.607 ± 0.554	0.001	0.019 ± 0.515	>0.999
IL1R1	−0.133 ± 0.259	0.250	−0.662 ± 0.353	<0.001	−0.528 ± 0.293	<0.001
MAP7	0.473 ± 0.353	0.002	1.015 ± 0.220	<0.001	0.542 ± 0.308	0.007
MUCL1	−0.318 ± 0.453	0.130	1.511 ± 0.419	<0.001	1.829 ± 0.405	<0.001
SNORD30	−0.025 ± 0.204	0.056	−0.001 ± 0.207	0.233	0.024 ± 0.278	>0.999

MULTIPLE SCLEROSIS PATIENTS						
Gene	iDC vs. mDC		vitD3-tolDC vs. mDC		vitD3-tolDC vs. iDC	
	logFC ± SD	p-value	logFC ± SD	p-value	logFC ± SD	p-value
CAMP	0.431 ± 0.342	0.042	0.441 ± 0.331	0.076	0.010 ± 0.459	>0.999
CLEC5A	−0.837 ± 0.539	0.002	0.111 ± 0.249	0.668	0.947 ± 0.490	0.002
CYP24A1	−0.762 ± 0.739	0.160	1.465 ± 0.908	<0.001	2.227 ± 1.448	<0.001
IL1R1	0.143 ± 0.158	0.103	−0.307 ± 0.179	0.002	−0.450 ± 0.214	0.008
MAP7	0.320 ± 0.296	0.081	0.506 ± 0.297	0.003	0.187 ± 0.292	0.174
MUCL1	−0.283 ± 1.209	0.221	0.790 ± 0.325	0.042	1.073 ± 1.362	<0.001

Gene expression values from a microarray study with healthy donors ($n = 5$) or from qPCR analysis from healthy donors ($n = 24$, except for CYP24A1, in which $n = 10$, and GZMB and SNORD30, in which $n = 20$) and multiple sclerosis patients ($n = 10$). Data presented as either the mean difference of expression (MeanDiff) for the microarray data or as the mean decimal logarithm of fold change (logFC) ± SD for the qPCR results. Housekeeping genes GAPDH, TBP and CYPA were used as controls. One qPCR experiment was performed for each donor or patient, with triplicated measurements for each sample. Microarray p-values < 0.01 and qPCR p-values < 0.05 are highlighted in bold. iDC, immature dendritic cells; mDC, mature dendritic cells; vitD3-tolDC, vitamin D3-induced tolerogenic dendritic cells. Friedman test with Dunn's correction or one-way ANOVA test with Geisser-Greenhouse correction.

in their functionality and the metabolic pathways triggered in vitD3-tolDC, we constructed a network of protein interactions. Briefly, previously reported data from the literature and several public and private databases were crossed with our microarray results in order to link the function of each gene of our transcriptomic study between them. This approach would allow us to reveal potential interactions between our biomarkers that might explain their involvement in immune regulation. Our study revealed that, while CYP24A1 seemed to be functionally separated from the rest of the genes, MAP7 and MUCL1 were closely related through common immune-related mechanisms

—such as HLA class II antigen presentation and different anti-inflammatory mediators— and other cellular processes and pathways —highlighting the metabolism of retinoic acid and the oxidative metabolism—, as shown in Figure 2. Furthermore, CAMP, CLEC5A, GZMB and IL1R1 genes appeared closely related to MAP7 and MUCL1 in our network.

To validate these results and confirm the close functional relation between MUCL1 and MAP7, we studied the expression of DHRS9, OS9, SPARC, ST6GAL1 and THBS1 genes. As shown in Table 2, their expression in our microarray indicated an overexpression of DHRS9, SPARC and ST6GAL in vitD3-tolDC

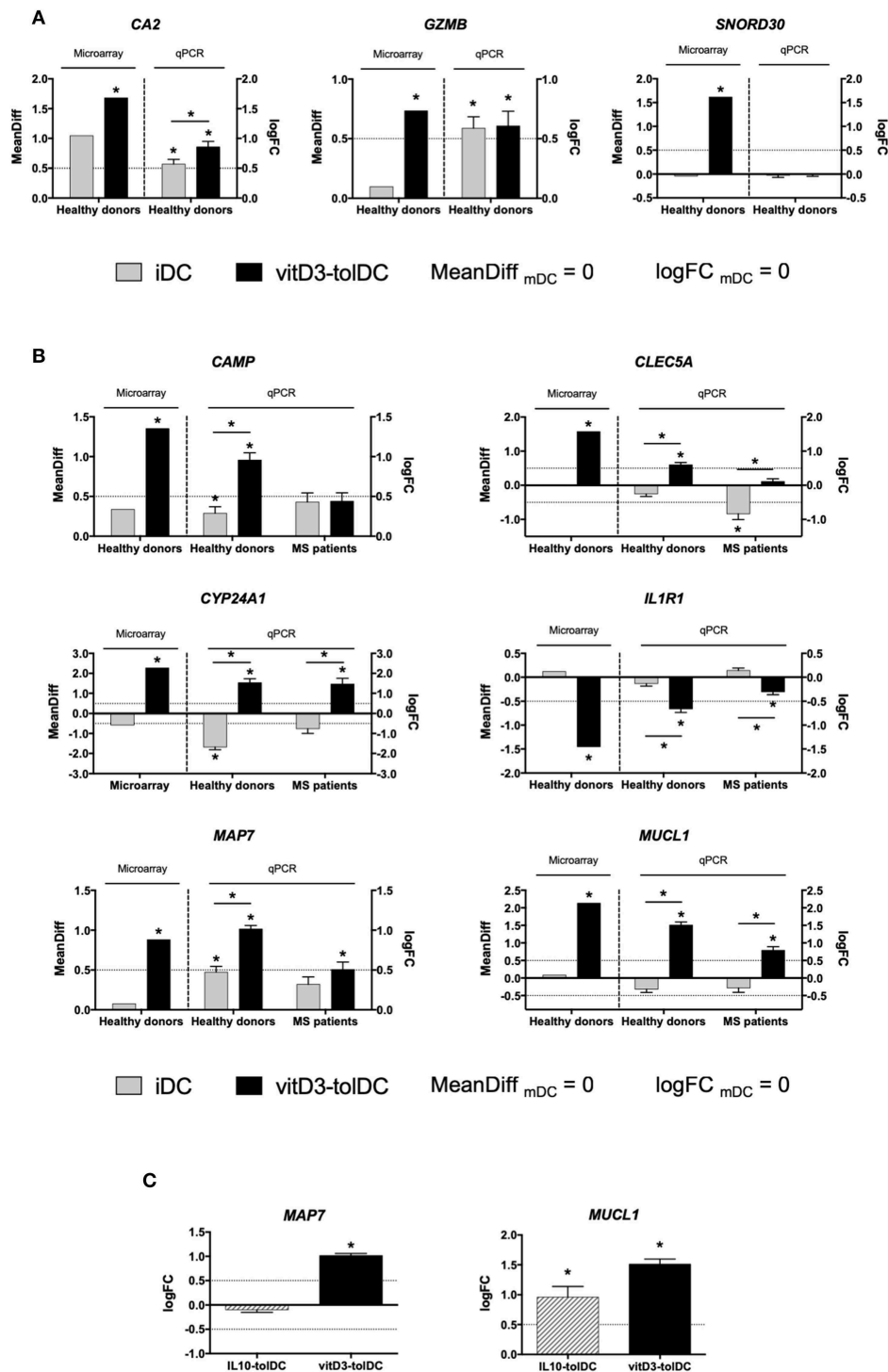


FIGURE 1 | Expression of the selected genes as candidate biomarkers of vitD3-toIDC. **(A)** Expression of *CA2*, *GZMB* and *SNORD30* in healthy donors by microarray ($n = 5$) and quantitative PCR (qPCR; $n = 20$, except *CA2*, in which $n = 24$) in immature dendritic cells (iDC), mature DC (mDC) and vitD3-toIDC. **(B)** Expression of *CAMP*, *CLEC5A*, *CYP24A1*, *MAP7* and *MUCL1* in healthy donors both by microarray ($n = 5$) and qPCR analysis ($n = 24$, except *CYP24A1*, in which $n = 10$) in iDC, mDC and vitD3-toIDC, and in multiple sclerosis (MS) patients by qPCR only ($n = 10$). **(C)** Expression of *MAP7* and *MUCL1* in vitD3-toIDC ($n = 24$) and in IL10-toIDC ($n = 5$) by qPCR. Data presented as the mean difference of expression (MeanDiff) or the decimal logarithm of fold change (logFC) expression for the microarray and qPCR results, respectively, in both cases normalized to mDC expression. Housekeeping genes *GAPDH*, *TBP* and *CYP11B* were used as controls. One qPCR experiment was performed for each donor or patient, with triplicated measurements for each sample. Error bars corresponding to SEM. Dotted lines represent the logFC = 0.5 or -0.5 expression threshold. * $p < 0.05$. Friedman test with Dunn's correction, one-way ANOVA test with Geisser-Greenhouse correction or paired t -test.

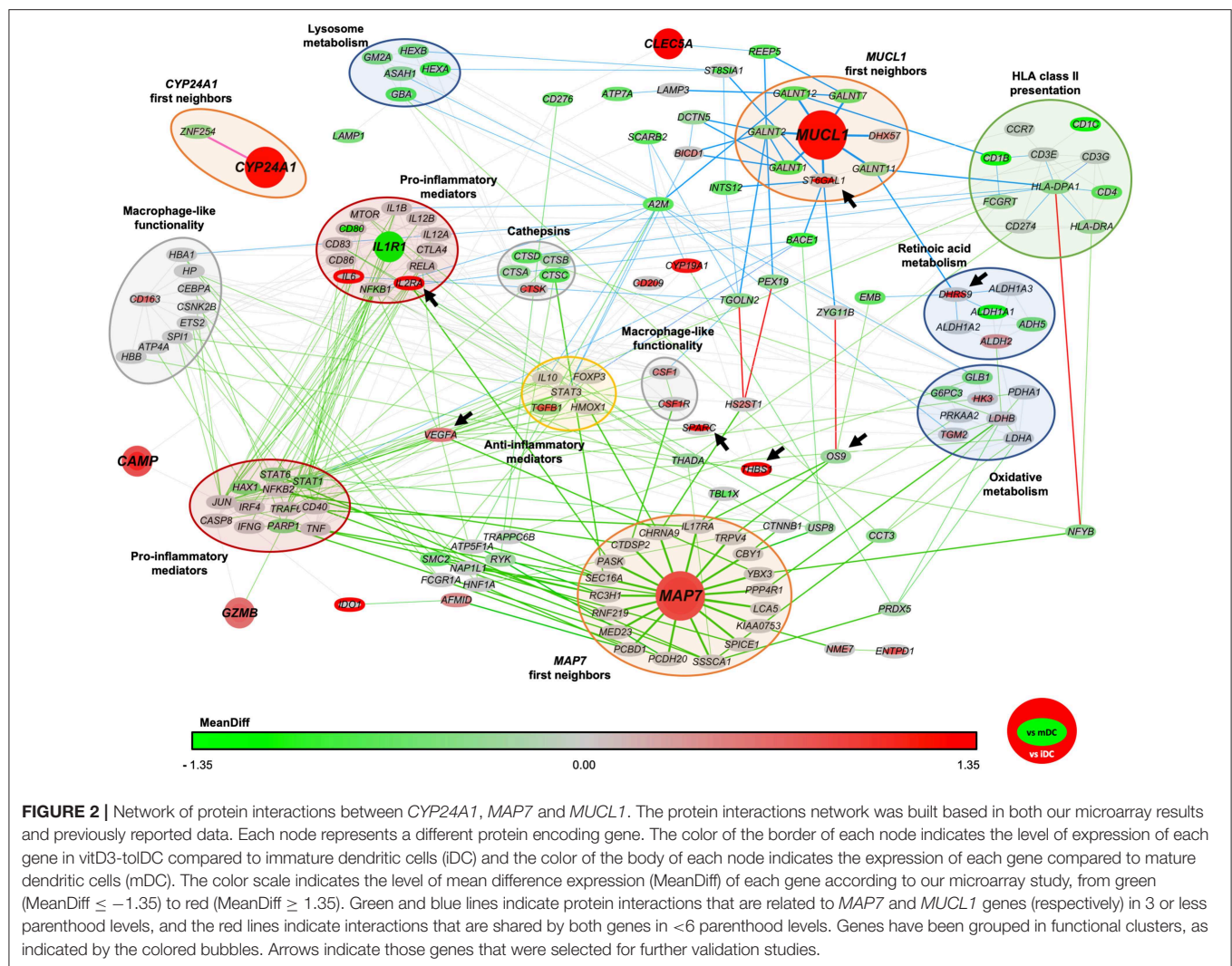


FIGURE 2 | Network of protein interactions between CYP24A1, MAP7 and MUCL1. The protein interactions network was built based in both our microarray results and previously reported data. Each node represents a different protein encoding gene. The color of the border of each node indicates the level of expression of each gene in vitD3-tolDC compared to immature dendritic cells (iDC) and the color of the body of each node indicates the expression of each gene compared to mature dendritic cells (mDC). The color scale indicates the level of mean difference expression (MeanDiff) of each gene according to our microarray study, from green (MeanDiff ≤ -1.35) to red (MeanDiff ≥ 1.35). Green and blue lines indicate protein interactions that are related to MAP7 and MUCL1 genes (respectively) in 3 or less parenthesis levels, and the red lines indicate interactions that are shared by both genes in <6 parenthesis levels. Genes have been grouped in functional clusters, as indicated by the colored bubbles. Arrows indicate those genes that were selected for further validation studies.

vs. mDC, but not iDC, while *THBS1* and *OS9* appeared induced and slightly down-modulated, respectively, in vitD3-tolDC compared to iDC. We, therefore, validated their expression in 10 samples from healthy donors and 10 samples from MS patients, and observed that the expression pattern of these 5 genes in healthy donor samples was in accordance to that shown in our microarray data (Table 2). Furthermore, and with the only exception of *DHRS9* and *OS9*, these expression patterns were also confirmed in DC samples obtained from MS patients, as shown in Table 2.

Additionally, *VEGFA* and *IL2RA* were also studied due to their relevance in our network, but their expression was validated at the protein level. Their gene expression in our microarray study is shown in Table 2. VEGF production (encoded by the *VEGFA* gene) was analyzed by cytometric bead array (CBA) in supernatant samples from 10 healthy donors and 8 MS patients, and the surface expression of CD25 (encoded by the *IL2RA* gene) was assessed by flow cytometry in 6 healthy donor samples. First, in accordance with the results from the microarray (Table 2), an increase in the production of VEGF

was evidenced in vitD3-tolDC from healthy donors compared to iDC—since it could not be detected in this condition—, but no statistical significance could be reached in the reduction found compared to mDC (VEGF_{vitD3-tolDC}: 205.5 ± 276.9 pg/mL; VEGF_{mDC}: 422.2 ± 497.6 pg/mL). Furthermore, similar results were evidenced in samples from MS patients, and VEGF production could not be detected on iDC either (VEGF_{vitD3-tolDC}: 331.8 ± 321.5 pg/mL; VEGF_{mDC}: 369.2 ± 243.1 pg/mL). On the other hand, the study of the expression of CD25 in healthy donor samples from the microarray evidenced a strong up-modulation of the *IL2RA* gene in vitD3-tolDC compared to iDC only. The expression of this gene in vitD3-tolDC compared to mDC, however, did not reach statistical significance (Table 2). Accordingly, our results in samples from 6 healthy donor DC cultures showed a statistically significant increase ($p = 0.040$) in the MFI values of this marker in vitD3-tolDC compared to iDC, but not mDC, probably due to the high variability observed in this specific condition (CD25_{vitD3-tolDC}: 433.8 ± 194.9 ; CD25_{mDC}: 796.3 ± 621.2 ; CD25_{iDC}: 175.0 ± 16.8).

TABLE 2 | Expression by microarray and qPCR of the selected genes from the protein interaction network in dendritic cells differentiated from healthy donors and multiple sclerosis patients.

MICROARRAY DATA FROM HEALTHY DONORS [FROM NAVARRO-BARRIUO ET AL.(33)]				
Gene	vitD3-tolDC vs. mDC		vitD3-tolDC vs. iDC	
	MeanDiff	p-value	MeanDiff	p-value
DHRS9	1.160	0.007	−0.161	0.685
IL2RA	−1.539	0.040	2.297	0.003
OS9	−0.014	0.926	−0.478	0.004
SPARC	1.347	<0.001	0.506	0.101
ST6GAL1	1.564	<0.001	0.189	0.570
THBS1	−1.052	0.048	1.944	<0.001
VEGFA	0.200	0.509	0.696	0.028
qPCR DATA FROM HEALTHY DONORS				
Gene	vitD3-tolDC vs. mDC		vitD3-tolDC vs. iDC	
	logFC ± SD	p-value	logFC ± SD	p-value
DHRS9	0.683 ± 0.387	0.002	n/a	
OS9	−0.011 ± 0.172	0.917	−0.265 ± 0.182	0.015
SPARC	1.210 ± 0.346	0.003	n/a	
ST6GAL1	0.571 ± 0.223	0.002	n/a	
THBS1	−0.023 ± 0.285	0.694	2.508 ± 0.303	0.004
qPCR DATA FROM MULTIPLE SCLEROSIS PATIENTS				
Gene	vitD3-tolDC vs. mDC		vitD3-tolDC vs. iDC	
	logFC ± SD	p-value	logFC ± SD	p-value
DHRS9	0.264 ± 0.358	0.557	n/a	
OS9	−0.036 ± 0.152	>0.999	−0.190 ± 0.222	0.076
SPARC	0.552 ± 0.194	0.011	n/a	
ST6GAL1	0.403 ± 0.331	0.001	n/a	
THBS1	−0.027 ± 0.216	0.420	2.391 ± 0.381	0.002

Gene expression values from a microarray study with healthy donors ($n = 5$) or from qPCR analysis from healthy donors ($n = 10$) and multiple sclerosis patients ($n = 10$). Data presented as either the mean difference of expression (MeanDiff) for the microarray data or as the mean decimal logarithm of fold change (logFC) ± SD for the qPCR results. Housekeeping genes GAPDH, TBP and CYPA were used as controls for the qPCR experiments. One qPCR experiment was performed for each donor or patient, with triplicated measurements for each sample. Microarray p-values < 0.01 and qPCR p-values < 0.05 are highlighted in bold. iDC, immature dendritic cells; mDC, mature dendritic cells; vitD3-tolDC, vitamin D3-induced tolerogenic dendritic cells. Friedman test with Dunn's correction, one-way ANOVA test with Geisser-Greenhouse correction, Wilcoxon matched-pairs signed rank test or paired t-test.

Protein Expression of Both MAP7 and MUCL1 Is Strongly Induced in vitD3-tolDC

Since MAP7 and MUCL1 proved themselves as transcriptomic biomarkers of vitD3-tolDC in both healthy donors and MS patient samples and, additionally, they were closely related between them in our functional network, we further analyzed them and determined the actual expression of their respective encoded proteins in order to provide more reliability to our qPCR results. The ICC analysis in 4 healthy donor samples evidenced that, in fact, a strong up-modulation of these proteins was observed in vitD3-tolDC compared to both iDC and mDC conditions. As shown in Figure 3A, the microtubule-associated protein 7, encoded by MAP7, showed a 2.81-fold and a 4.00-fold higher CTCF expression in vitD3-tolDC compared to iDC and mDC, respectively ($\text{MAP7}_{\text{vitD3-tolDC}} = 198.35 \pm 40.45$

$\times 10^3$; $\text{MAP7}_{\text{mDC}} = 49.58 \pm 17.16 \times 10^3$; $\text{MAP7}_{\text{iDC}} = 70.54 \pm 36.39 \times 10^3$), reaching statistical significance in both cases ($p = 0.016$; $p = 0.007$, respectively). The induction of the MUCL1-encoded protein was even stronger, as evidenced by the 6.72-fold and 13.02-fold CTCF expression in vitD3-tolDC vs. iDC and mDC, respectively ($\text{MUCL1}_{\text{vitD3-tolDC}} = 1204.85 \pm 509.91 \times 10^3$; $\text{MUCL1}_{\text{mDC}} = 92.54 \pm 94.19 \times 10^3$; $\text{MUCL1}_{\text{iDC}} = 179.19 \pm 32.93 \times 10^3$). In this case, however, statistical significance was only reached in the comparison vs. mDC ($p = 0.024$), probably due to the small sample size (Figure 3B). Representative microscopy pictures of the expression of both proteins in healthy donor samples are shown in Figures 3C,D. Furthermore, the ICC analysis in 3 MS patient samples presented a similar tendency. On the one hand, as shown in Figure 3A, the study of MAP7 expression presented a 1.57-fold and a

2.14-fold higher CTCF expression in vitD3-tolDC compared to iDC and mDC, respectively ($MAP7_{vitD3-tolDC} = 132.68 \pm 91.61 \times 10^3$; $MAP7_{mDC} = 61.74 \pm 31.52 \times 10^3$; $MAP7_{iDC} = 84.32 \pm 66.48 \times 10^3$). On the other hand, and as shown in **Figure 3B**, the expression of MUCL1 was, again, even stronger, with a 2.76-fold and 4.49-fold CTCF expression in vitD3-tolDC compared to iDC and mDC, respectively ($MUCL1_{vitD3-tolDC} = 132.34 \pm 46.44 \times 10^3$; $MUCL1_{mDC} = 29.47 \pm 11.66 \times 10^3$; $MUCL1_{iDC} = 47.97 \pm 24.65 \times 10^3$). However, statistical significance for MS patient samples could only be reached for the expression of MUCL1 in vitD3-tolDC compared to mDC ($p = 0.049$), again, probably due to the small sample size. Representative microscopy pictures of the expression of both proteins in MS patient samples are shown in **Figures 3C,D**.

DISCUSSION

The identification of biomarkers is a key point for the translation of tolDC into the clinic. In this article, we have evidenced that *CYP24A1*, *MAP7* and *MUCL1* genes appear strongly induced in vitD3-tolDC, both in healthy donors and MS patients. Therefore, the differential expression of these genes in our tolerogenic cell product gives them the potential to unequivocally identify vitD3-tolDC with a simple qPCR analysis, without ambiguity, thus ensuring that they are not immunogenic (not mDC), nor susceptible of maturation (not iDC), and consequently characterizing their tolerogenic potential. Additionally, the study of the protein expression of *MAP7* and *MUCL1* further supported these results. The study of these genes in the context of the whole transcriptome of the vitD3-tolDC has also elucidated that the role of *MAP7* and *MUCL1*—but not of *CYP24A1*—seems to be closely related with important and widely described immune- and non-immune-related pathways, which correlates with many of the results that we have obtained at the phenotypical (reduction of co-stimulatory molecules and HLA-DR expression), functional (increased secretion of IL-10 and TGF- β ; non detectable production of IL-12) and transcriptomic levels (reduction of *IL1R1* gene expression). Furthermore, many of the interactions suggested by our network have also been confirmed in both healthy donor and MS patient cells. Therefore, even though the specific role of *MAP7* and *MUCL1* in the tolerogenic functionalities of vitD3-tolDC is not fully clear, our results manifested that they are at least having an effect on several relevant immune regulatory mechanisms and different metabolic pathways. On the other hand, *CYP24A1* gene, encoding the vitamin D3 24-hydroxylase, might well serve as a strong and robust biomarker of vitD3-tolDC, although it seems to have little to no influence in the actual regulatory properties of these cells. After all, this gene is directly involved in the metabolism of vitamin D3 (43, 44), and thus could constitute a robust indicator of the response of the cell product to the treatment with this compound. Even though we previously suggested the potential of *CYP24A1*, *MAP7* and *MUCL1* as candidate biomarkers of vitD3-tolDC in a previous microarray

study (33), the current report constitutes their first validation as such.

Several years ago, mucin-like 1, the protein encoded by *MUCL1* gene, was initially identified as a breast-specific gene expressed in more than 90% of breast cancer cell lines, developing an important role in the proliferation of these tumor cells (45–48). However, no other specific role has been reported for it. In fact, its name comes given by the structural analogy of MUCL1 protein with mucin proteins, characterized by regions of high tandem repeated serine and threonine content with extensive O-glycosylation of these residues (46). On the other hand, we have *MAP7*. This gene encodes the microtubule-associated protein 7, a cytoskeleton component that has been mainly related with the development of collateral axon morphogenesis and development in neurons (49, 50). For both MUCL1 and MAP7, a potential role in DC function has not been reported yet. However, interestingly, microtubule associated proteins have been described to be O-glycosylated—just like MUCL1—, although the functional significance of this modification remains to be determined (51). In the case of vitD3-tolDC, our results have demonstrated a strong induction of gene and protein expression of both *MUCL1* and *MAP7*, as already hinted in our previous microarray study, thus suggesting that they might be developing an important role in the functionality of these cells. However, further functional studies should be performed first in order to elucidate to what extent these genes might be involved in the mechanisms of immune tolerance. In any case, the fact that our results also evidence a strong induction of *MUCL1* in IL10-tolDC indicates that this gene might constitute a potential biomarker of the regulatory function of tolDC, and it would be interesting to test its expression in other protocols generating tolerance-inducing cell products. Nevertheless, on the other hand, it is also true that our above-mentioned preliminary microarray results did not show the overexpression of *MUCL1* in dexamethasone nor rapamycin-induced tolDC, although proper PCR validations should be performed in order to confirm this.

In concordance with previous reported studies by our own group (26), and with the objective to finally develop an autologous tolerogenic cell-therapy for MS, our results demonstrated that the generation of vitD3-tolDC in both healthy donors and MS patients did not significantly differ regarding their phenotypic and functional characteristics—with the exception of an increase on TGF- β production in healthy donor samples only—. In all cases, a consistent decrease in the surface expression of CD86 and HLA-DR, as well as in the induction of allogeneic proliferation, was observed, accompanied by a significant increase in the production of IL-10 and no *IL-12* detection. However, the tolerogenic potential of vitD3-tolDC generated from monocytes of MS patients, although sufficient, did not seem as robust as in healthy donors, provided that the changes in their functional and phenotypic markers were not as pronounced. Interestingly, though, the study of *CYP24A1* expression raised another concern in this regard. Even though this gene constitutes a robust biomarker of the generation of vitD3-tolDC, as discussed above, its expression also manifested important differences in iDC from healthy donors and MS patients; compared to mDC, a strong repression of *CYP24A1* was

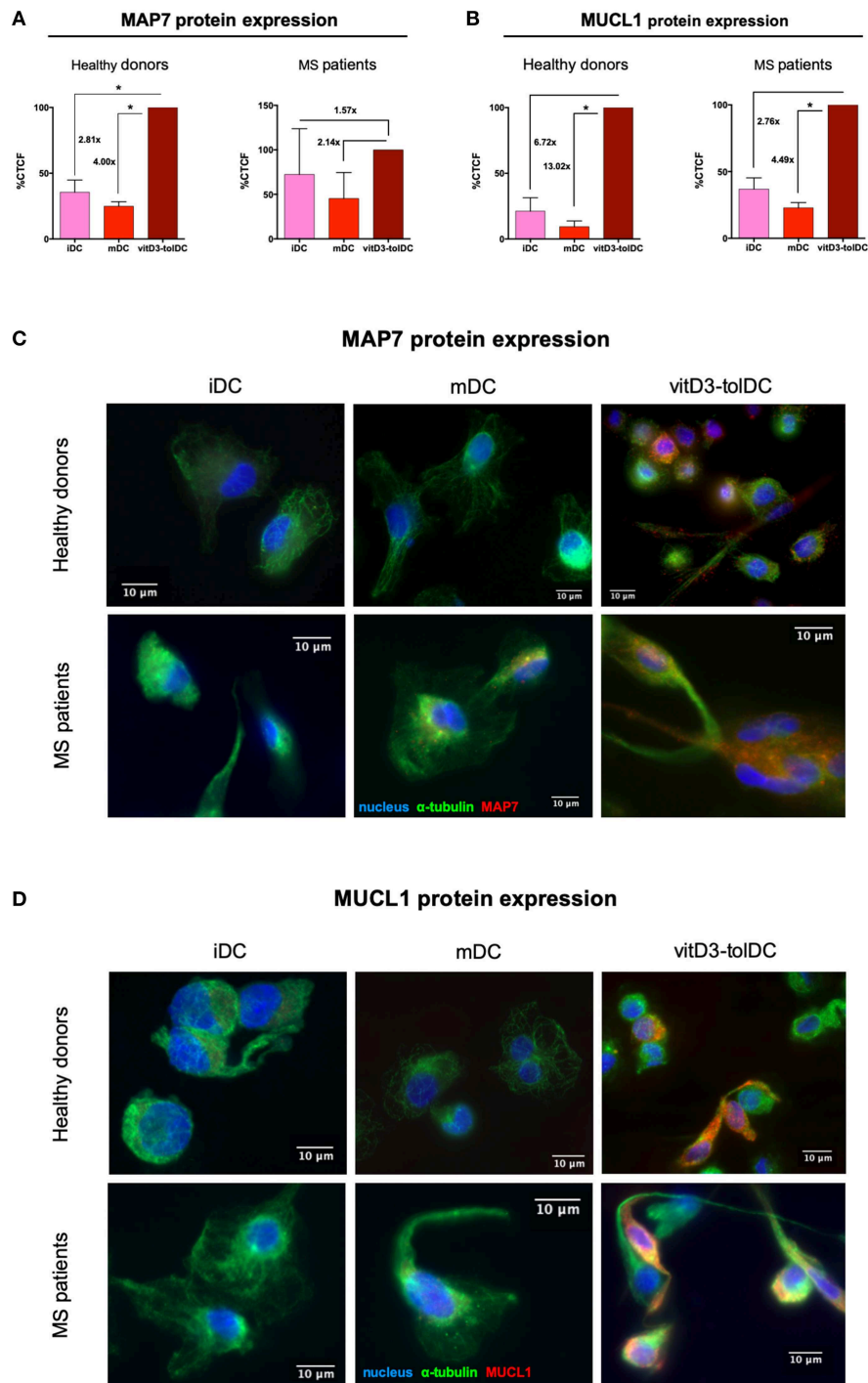


FIGURE 3 | Immunocytochemistry study of MAP7 and MUCL1 protein expression in dendritic cells. **(A)** Relative levels of expression of microtubule-associated protein 7 (MAP7) in dendritic cells differentiated from healthy donor samples ($n = 4$) and MS patient samples ($n = 3$). **(B)** Relative levels of expression of mucin-like 1 (MUCL1) in dendritic cells differentiated from healthy donor samples ($n = 4$) and MS patient samples ($n = 3$). Results are calculated as percentage (%) of corrected total cell fluorescence (CTCF) in immature dendritic cells (iDC) and mature DC (mDC) compared to vitD3-toIDC. One single immunocytochemistry experiment was performed for each sample. Error bars corresponding to SEM. $*p < 0.05$. One-way ANOVA test with Geisser-Greenhouse correction or paired t -test. Representative pictures of the expression of **(C)** MAP7 and **(D)** MUCL1 in iDC, mDC and vitD3-toIDC from healthy donor and MS patient samples. α -tubulin staining is shown in green; either MAP7 or MUCL1 staining is shown in red; nuclei staining is shown in blue. The immunocytochemistry analysis was performed on a fluorescence microscope using a 63x objective. Immunocytochemistry primary staining was performed using mouse anti-human α -tubulin and either rabbit anti-human MAP7 or rabbit anti-human MUCL1 antibodies. Secondary stainings were performed using AlexaFluor (AF) 488 goat anti-mouse IgG and AF 594 goat anti-rabbit IgG antibodies. Nuclei staining was performed using DAPI.

observed in healthy donor samples, but the expression of this gene in iDC from MS patients was similar to mDC. Therefore, this result implicates that the transcriptomic profile of DC in MS patients is already different before receiving the same treatment with vitamin D3, thus suggesting a more pro-inflammatory baseline status in these cells. However, the strong induction of *CYP24A1* observed in vitD3-tolDC still indicates that these cells are adequately responding to the effect of vitamin D3, suggesting that the problem has to be downstream.

These differences between healthy donors and MS patient samples also forced us to discard another interesting candidate transcriptomic biomarker, *DHRS9*, the gene encoding the dehydrogenase/reductase 9, involved in the metabolism of retinoic acid (52). The relevance of this gene comes given by its potential as a broad-use biomarker of immune tolerance, since it has been already described as differentially induced in vitamin D3 + dexamethasone-induced tolDC (53) and, especially, in regulatory macrophages (54). It is also worth noting that, even though *DHRS9* could not be confirmed as a biomarker of vitD3-tolDC from MS patients, this does not mean that it could not still be useful in cells generated from patients with a different autoimmune disease, such as rheumatoid arthritis or type 1 diabetes. In this regard, our results already allowed us to point out some transcriptomic differences between healthy donors and MS patients. We observed that *CLEC5A* could not be validated in MS patient samples either, and when we studied our network, we noticed that both *CLEC5A* and *DHRS9* were closely related to *MUCL1* through *GALNT2*, *GALNT7* and *GALNT11* genes. Specifically, these 3 genes have been described to be in charge of the processes of O-linked glycosylation. Consequently, this might be an indicator of a potential misfunction in the glycosylation mechanisms of MS patients, that might be indirectly affecting the expression of *CLEC5A*, *DHRS9* and probably many other genes. In fact, several glycosylation defects have already been related with the pathogenesis of MS, as reviewed by Grigorian et al. (55). However, *MUCL1* gene expression would also be expected to be affected by a defect in these genes, but our results showed that this was not the case. As a possible explanation, the glycosylation of *MUCL1* might be sufficiently mediated by *ST6GAL1* gene alone—whose expression still remains slightly induced in vitD3-tolDC from MS patient samples—. In any case, our results have raised the need to explore the mechanisms that might be different between healthy donors and MS patients. On the other hand, most of the genes closely related to *MAP7* showed a similar behavior in both healthy donor and MS patient vitD3-tolDC, such as *SPARC*, *THBS1* or, specially, *VEGFA*, which has been reported to develop a role in the recruitment, inhibition of maturation and IDO1 induction in DC (56–58).

In conclusion, our results evidenced that, despite not having an obvious involvement in the tolerogenic functionality of the cells in all cases, several genes have shown a strong differential expression in vitD3-tolDC from healthy donors, compared to both mature and immature control conditions. Among them, *CYP24A1*, *MAP7* and *MUCL1* have also been validated as robust transcriptomic biomarkers of vitD3-tolDC generated from MS patient samples. Thus, this finding opens a promising window

for the use of these genes as a reliable quality control in clinical trials with a simple qPCR analysis, before administering the cell product into the patients. Furthermore, the role of *MAP7* and *MUCL1*, but not of *CYP24A1*, seems to be strongly related to important immune-related functions. Specifically, the case of *MUCL1* is of significant relevance, since this gene has also demonstrated an interesting potential as a broad-use biomarker of tolerance, based on its validation both in vitD3-tolDC and in IL10-tolDC. Consequently, *MUCL1* sets an interesting path for future experiments with the objective to validate the role of this gene as a potential biomarker of other tolDC protocols, and thus, hopefully as a wide biomarker of tolerogenic cell products and their mechanisms of immune tolerance induction.

ETHICS STATEMENT

This study was approved by the Germans Trias i Pujol Hospital ethical committee, and all patients and healthy controls signed an informed consent.

AUTHOR CONTRIBUTIONS

EM-C, JN-B, and MM conceived the experiments. CR-T and SP-R obtained the patient samples. AtB provided cDNA samples from IL10-tolDC. AA-M, BQ-S, JN-B, and MM performed the cell cultures. AA-M, BQ-S, JN-B, and MM performed the cell characterization analyses. JN-B and MM analyzed the results. EM-C, JN-B, and MM interpreted the results. JN-B wrote the manuscript. AA-M, AtB, AT-S, BQ-S, CR-T, EM-C, JN-B, MM, and SP-R reviewed the manuscript.

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Daurinol Attenuates Autoimmune Arthritis via Stabilization of Nrp1–PTEN–Foxp3 Signaling in Regulatory T Cells

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Optimizing Treg function and improving Treg stability are attractive treatment strategies for treating autoimmune rheumatoid arthritis (RA). However, the limited number of circulating Tregs and questions about the functional stability of *in vitro*-expanded Tregs are potential limitations of Treg-based cell therapy. The aim of this study was to analyze the regulatory effect of daurinol, a catalytic inhibitor of topoisomerase II α , on Th cell differentiation and to evaluate their therapeutic potential in a preclinical experimental model of RA. We investigated the effect of daurinol on T cell differentiation by flow cytometry. Foxp3 stability and methylation were analyzed by suppression assays and bisulfite pyrosequencing. Daurinol was treated in the collagen-induced arthritis (CIA) model, and the effects *in vivo* were determined. We found that daurinol can promote Treg differentiation and reciprocally inhibit Th17 differentiation. This Treg-inducing property of daurinol was associated with decreased activity of Akt–mTOR and reciprocally increased activity of neuropilin-1 (Nrp1)–PTEN. Daurinol treatment inhibited aerobic glycolysis in Th17 conditions, indicating the metabolic changes by daurinol. We found that the daurinol increase the Treg stability was achieved by Foxp3 hypomethylation. *In vivo* daurinol treatment in CIA mice reduced the clinical arthritis severity and histological inflammation. The Treg population frequency increased and the Th17 cells decreased in the spleens of arthritis mice treated with daurinol. These results showed the anti-arthritic and immunoregulating properties of daurinol is achieved by increased differentiation and stabilization of Tregs. Our study provides first evidence for daurinol as a treatment for RA.

Keywords: daurinol, regulatory T cells, stability, FOXP3 hypomethylation, rheumatoid arthritis, neuropilin 1

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease characterized by tumor-like growth of the synovium and infiltration of immune cells through the affected joints. Interleukin 17 (IL-17)-secreting CD4⁺ T cells (Th17) have been shown to play a crucial role in the pathogenesis of autoimmune diseases (1). It has become clear that these proinflammatory cytokines including

tumor necrosis factor α (TNF- α), IL-6, and IL-1 play together with other inflammatory mediators IL-17 in an additive or synergistic way (2). Many studies have identified the pivotal roles of IL-17 and Th17 cells in the development and progression of RA (3). In contrast to Th17 cells, regulatory T cells (Tregs) comprise an indispensable mediator that sustains immune tolerance to self-antigens and helps to maintain immune homeostasis (4). The Th17-Treg paradigm is vital to understanding the pathogenesis of T cell-mediated autoimmune disorders such as RA (5, 6), and systemic lupus erythematosus (SLE) (7).

RA patients with a high number of CD4+CD25+ Tregs in whole blood respond better to the anti-TNF agent infliximab than do those with lower number of Tregs measured at baseline before the treatment (8). This finding suggests that a strategy to expand the Treg population may be helpful in controlling RA disease activity and augmenting treatment efficacy in RA patients. In addition to the lower number of Tregs in some RA patients (9), several studies provided evidence of disturbed immunoregulatory function of these cells in RA patients (10, 11). CD4+CD25+ Tregs present in RA patients do not express *FOXP3* (10). *Foxp3* acts to stabilize the immunoregulatory function of Tregs (12), and appears to be related to defects in Tregs function in RA patients. *Foxp3* is required for Treg development and function.

Daurinol is a natural aryl naphthalene lactone that can be isolated from the traditional medicinal plant *Haplophyllum dauricum* (13). Our previous studies have identified its anticancer property (14, 15). The chemical structure of daurinol is similar to that of VP-16, which is also known as etoposide, a widely used clinical anticancer drug (16). The safety profile of daurinol differs considerably for that of etoposide. Compared with etoposide, daurinol causes little loss of body weight and less bone marrow suppression (14). Previous studies showing the therapeutic potential of daurinol have focused mainly on its ability to inhibit cell proliferation and the underlying mechanisms. Until now, no one, including our research team, has studied the anti-inflammatory or anti-arthritis efficacy of daurinol.

The aim of the present study was to examine the therapeutic potential of daurinol in RA and the underlying mechanisms, especially on modulation of T cell subsets. This is the first report of the reciprocal regulation of Th17 and Tregs by daurinol treatment *in vitro* and *in vivo*. We found that daurinol induced the proliferation and differentiation of Tregs from naïve CD4+ T cells and that the increase in immunoregulatory function and Treg number induced by daurinol was achieved through *Foxp3* induction via *Foxp3* hypomethylation. We suggest that daurinol has potential as a novel compound to inhibit RA through a Treg-specific mechanism involving expansion and stabilization of this population.

MATERIALS AND METHODS

Mice

DBA/1J and C57BL/6 (B6) mice, 8–10 weeks of age, were purchased from OrientBio (Sungnam, Korea) and were maintained under specific-pathogen-free conditions in an

animal facility. The protocols used in this study were approved by the Animal Care and Use Committee of the Catholic University of Korea.

Induction of Arthritis and Daurinol Treatment

Collagen-induced arthritis (CIA) was induced in DBA1/J mice (each group: $n = 10$). Mice were immunized with 100 μ g of chicken CII (Chondrex Inc., Redmond, WA, USA) dissolved overnight in 0.1 N acetic acid (4 mg/ml) in complete Freund's adjuvant or incomplete Freund's adjuvant (Chondrex Inc.). The immunization was performed intradermally into the base of the tail. The mice were randomly assigned to three experimental groups ($n = 10$) and treated with daurinol (5 mg/kg or 25 mg/kg of body weight) or vehicle by oral gavage three times a weeks for 3 weeks since 3 weeks after 1st CII immunization.

Clinical Scoring and Histological Assessment of Arthritis

The onset and severity of arthritis were measured visually twice per week based on the appearance of arthritis in the joints, based on the previously described scoring system (17). Detailed experimental procedures are described in **Supplementary section Materials and Methods**.

Measurement of Cytokine and IgG Levels

The concentrations of IFN- γ , and IL-17 in culture supernatants and serum were measured using a sandwich enzyme-linked immunosorbent assay (ELISA DuoSet; R&D Systems, Lille, France). Serum levels of IgG, IgG1, and IgG2a antibodies were measured using a commercially available ELISA kit (Bethyl Laboratories).

Murine and Human T Cell Isolation and Differentiation

To purify mouse splenic or human CD4⁺ T cells, the splenocytes were incubated with CD4-coated magnetic beads and isolated using magnetic-activated cell sorting separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Mouse Th17 cell differentiation was induced by treatment with anti-CD3 (0.5 μ g/ml); and soluble anti-CD28 (0.5 μ g/ml), IL-6 (20 ng/ml) and TGF- β (2 ng/ml), anti-IFN- γ , and anti-IL-4 antibodies (each at a concentration of 5 μ g/ml). Human Th17 cells were stimulated with plate-bound anti-CD3 (0.5 μ g/ml); and soluble anti-CD28 (0.5 μ g/ml), anti-IFN- γ (2 μ g/ml), anti-IL-4 (2 μ g/ml), anti-IL-1 β (20 ng/ml), and anti-IL-6 (20 ng/ml) for 72 h.

Metabolic Assays

The ECAR were measured with an XF96 analyzer (Seahorse Bioscience). Cultured CD4 T cells were seeded at a density of 5×10^5 cells per well of a XF96 cell culture microplate. Before assay, cells were equilibrated for 1 h in unbuffered XF assay medium supplemented with 0.1% Insulin-Transferrin-Selenium-Sodium Pyruvate (ITSA). Compounds were injected during the assay at the following final concentrations: 2 μ M Oligomycin, 3 μ M FCCP, and 5 μ M Rotenone-Antimycin A.

Crisper Cas9 Transfection

The Alt-R CRISPR/Cas9 system was carried out as previously described (18, 19). Nrp-1 or PTEN CRISPR/Cas9 vector was transfected using an Amaxa 4D-nucleofector X unit according to the manufacturer's recommendations with program DN-100 (Lonza, Cologne, Germany).

Treg Stability

To test of Treg stability, mouse CD4⁺T cells were stimulated with Treg differentiation condition (CD3 and CD28 and rhTGF- β (5 ng/ml) for 3 days and restimulated with Th17 cell differentiation condition (anti-CD3 (0.5 μ g/ml); and soluble anti-CD28 (0.5 μ g/ml), IL-6 (20 ng/ml) and TGF- β (2 ng/ml), anti-IFN- γ , and anti-IL-4 antibodies (each at a concentration of 5 μ g/ml) with or without daurinol. After 3 days, the cell surface was stained with CD4, CD25, and FoxP3 antibodies.

Proliferation Assay

For proliferation analysis, cells were pulsed with 1 Ci 3H-thymidine (GE Healthcare) per well for the final 8 h of the 72-h culture period. Finally, 3H-thymidine incorporation was determined using a liquid beta-scintillation counter (Beckman).

CpG Methylation Analysis

CpG methylation analysis was determined by pyrosequencing of bisulphite-modified genomic DNA from non-treated CD4⁺T cells or daurinol-treated CD4⁺T cells. Pyrosequencing was performed using the PyroMark Q96 ID (Qiagen) machine, and results were analyzed with PyroMark CpG Software 1.0 (Qiagen). Methylation analysis was conducted by Genomictree, Daejeon, South Korea.

Real-Time Polymerase Chain Reaction (PCR)

Messenger RNA (mRNA) was extracted using the TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using a SuperScript Reverse Transcription system (Takara Bio Inc., Otsu, Japan). A LightCycler 2.0 instrument (software version 4.0; Roche Diagnostics, Mannheim, Germany) was used for PCR amplification. All reactions were performed using the LightCycler FastStart DNA Master SYBR Green I mix (Takara Bio Inc.), following the manufacturer's instructions. Primer sequences are described in **Tables S1, S2**. All mRNA levels were normalized to that of β -actin.

Western Blotting

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were stained with primary antibodies against phosphorylated (active) form of pSTAT3 (Tyr⁷⁰⁵, Ser⁷²⁷), STAT3, STAT5, pSTAT5, PTEN, pPTEN, Akt, pAkt (Ser⁴⁷³, Tyr³⁰⁸), pmTOR, Nrp1 (all from Cell Signaling, Danvers, MA, USA), and β -actin. A horseradish peroxidase (HRP)-conjugated secondary antibody was then added. Primary antibodies used for western blot are described in **Table S3**.

Flow Cytometry

Mononuclear cells were immunostained with various combinations of the following fluorescence-conjugated antibodies: CD25, CD4, FoxP3, IL-17, CTLA-4, and glucocorticoid-induced tumor necrosis factor receptor (GITR), ICOS, C103, and PD-1. These cells were also intracellularly stained with the following antibodies: CTLA-4 (BD Biosciences), Nrp-1(R&D), IL-17, and FoxP3 (eBioscience). Prior to intracellular staining, cells were restimulated for 4 h with phorbol myristate acetate (25 ng/ml) and ionomycin (250 ng/ml) in the presence of GolgiSTOP (BD Biosciences). Intracellular staining was conducted using a kit (eBioscience), following the manufacturer's protocol. Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences).

Labeling With 5,6-Carboxyfluorescein Succinimidyl Ester (CFSE)

Mononuclear cells isolated from mice spleens were washed once in 0.1% bovine serum albumin (BSA) in PBS and labeled with 1 μ l of 5 mM CFSE (Invitrogen) at a density of 10⁷ cells/ml in 0.1% BSA in PBS for 10 min at 37°C in the dark. CFSE-labeled cells were stimulated with Th17 differentiation condition treated with either vehicle and daurinol for 3 days. Flow cytometry was used to assess CFSE fluorescence.

Confocal Microscopy and Immunostaining

Spleen tissues were obtained 8 or 14 days after BMT, snap-frozen in liquid nitrogen, and stored at -80°C. Tissue cryosections (7 μ m thick) were fixed in 4% (v/v) paraformaldehyde and stained using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PerCP-Cy5.5-, or allophycocyanin -conjugated monoclonal antibodies to mouse CD4, CD25, pSTAT3 (Ser⁷²⁷), IL-17, Foxp3 (eBioscience), and Nrp1 (R&D). After incubation overnight at 4°C, stained sections were visualized by confocal microscopy (LSM 510 Meta; Zeiss, Göttingen, Germany).

Immunohistochemistry

Immunohistochemistry was performed using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA). Tissues were first incubated with the primary anti-IL-17, anti-Foxp3, anti-TNF- α , anti-IL-1 β , anti-IL-6, anti-RANK, and anti-RANKL antibodies overnight at 48°C. The primary antibody was detected with a biotinylated secondary linking antibody, followed by incubation with a streptavidin-peroxidase complex for 1 h. The final color product was developed using DAB chromogen (DAKO, Carpinteria, CA, USA).

Microarray Data

T cells were isolated from WT mice, and treated with Th17 condition with or without daurinol. Affymetrix microarrays HT_MG-430A were used to measure the resulting mRNA. Expression data was preprocessed using the RMA algorithm followed by quantile normalization. To identify differentially expressed genes of interest, real-time PCR (RT-PCR) was used to validate the microarray results.

Statistical Analysis

Data are presented as the mean \pm standard deviation (s.d.). The Mann–Whitney *U* test or Student *t*-test was used for comparing values between two groups. One-way analysis of variance followed by Bonferroni's *post-hoc* test was used to compare the differences between three or more groups. To assess the Gaussian distribution and the equality of variance, the Shapiro–Wilk test and Levene test were used, respectively. Differences between arthritis incidences at a given time point were analyzed by the χ^2 contingency analysis. The program used for the statistical analysis was the SPSS statistical software package, standard version 16.0 (SPSS, Chicago, IL, USA). *P*-values < 0.05 (two-tailed) were considered significant.

RESULTS

Reciprocal Regulation of Th17 and Tregs by Daurinol Treatment

First, we examined the effects of daurinol on Th17 and Treg differentiation. CD4⁺ T cells isolated from normal DBA/1J mice were cultured under the Th17-polarizing condition (as described in section Materials and Methods) in the presence or absence of daurinol (concentration ranging from 0.5 to 5 μ M) for 72 h. Flow cytometry demonstrated that daurinol treatment of murine CD4⁺ T cells inhibited Th17 differentiation and promoted Treg differentiation (Figures 1A,B). IL-17 production in culture supernatants of daurinol-treated cells was significantly inhibited in a dose-dependent manner, compared with that in culture supernatants of vehicle-treated cells (Figure 1C).

Next, we analyzed the populations of CD25⁺, Foxp3⁺, and IL-17⁺ cells among CFSE-labeled proliferating T cells cultured under the Th17-skewing condition. As expected, IL-17⁺ cells among the CD4⁺ T cells were inhibited by daurinol. Although CD25 is expressed upon activation of CD4⁺ T cells (20) and is an accepted surface marker of Tregs (21), Foxp3 is the critical master regulator of immunoregulatory function of Tregs and their development (22). Interestingly, daurinol treatment decreased the percentage of CD25⁺ cells among proliferating CD4⁺ T cells but increased that of Foxp3⁺ cells (Figure 1D).

Daurinol treatment increased the mRNA levels of Treg-related molecules (Foxp3, TGF- β , and SOCS3) in murine CD4⁺ T cells cultured under the Th17-skewing condition (Figure 1E). Hypoxia-inducible factor-1 α (HIF-1 α), a master transcription factor of hypoxia-inducible genes, plays a crucial role in the balance between Th17 and Treg cells. It directly promotes Th17 differentiation via activation of ROR γ t, the key transcription factor for Th17 cell differentiation (23), and reciprocally suppresses Treg differentiation by stimulating Foxp3 degradation (24). Daurinol treatment in murine CD4⁺ T cell decreased the mRNA expression of Th17-related molecules including IL-17, ROR γ t, and HIF-1 α (Figure 1E).

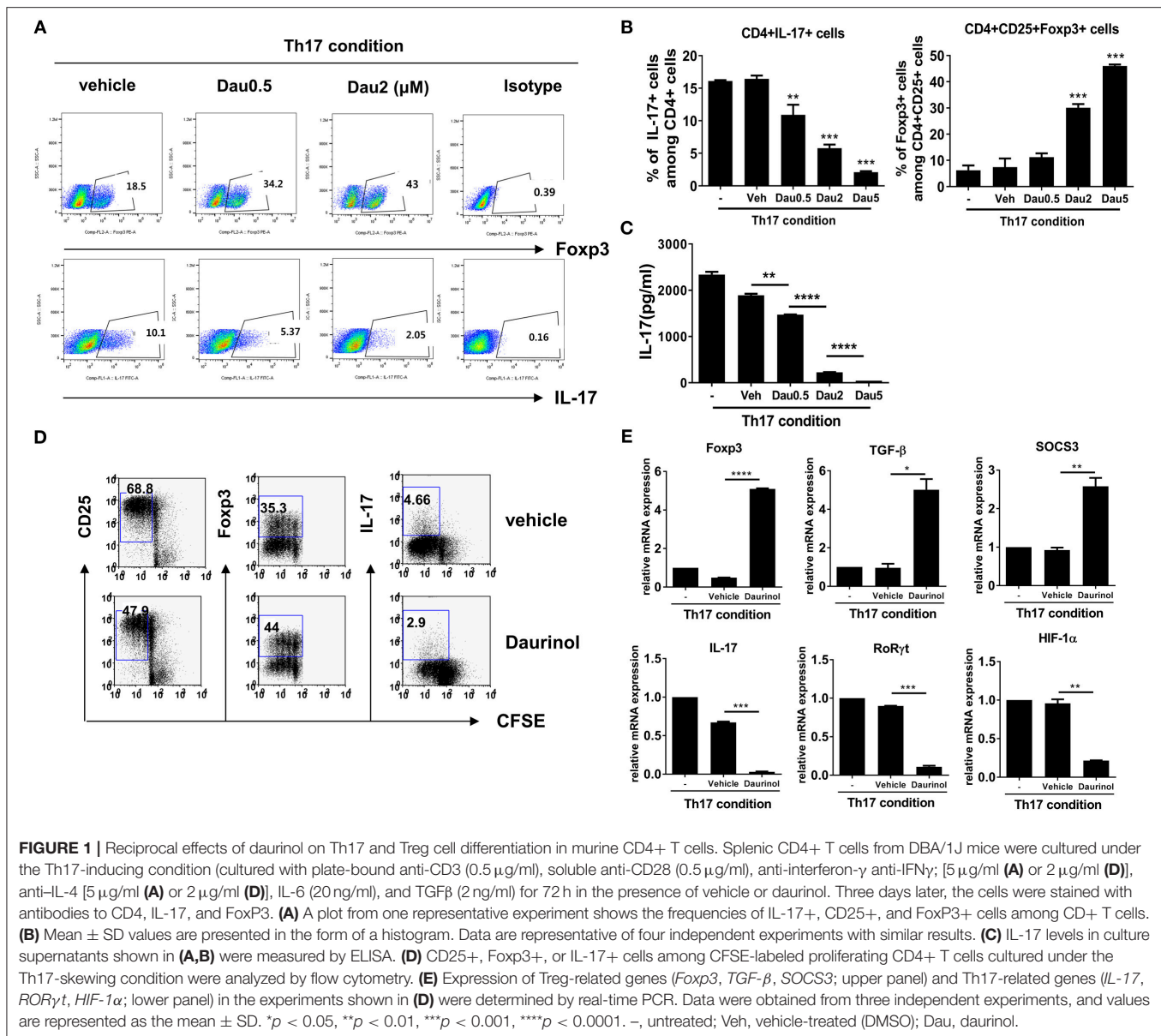
Since splenic CD4⁺ T cells also contain CD62[–] memory T cells, the differentiation from splenic total CD4⁺ T cells and CD62⁺ naïve T cells into Th17 cells are different.

Thus, Th17 differentiation experiments were conducted using only isolated CD44-CD62⁺ naïve T cells in order to more selectively identify the potential of daurinol during Th17 differentiation. We confirmed that daurinol also induced Th17–Treg reciprocal regulation in a dose-dependent manner (Figure S1A) and attenuated IL-17 production in naïve CD4⁺ T cells (Figure S1B). To elucidate the Th17–Treg-modulating mechanisms of daurinol, the mRNA expression levels of immunoregulatory mediators expressed in Tregs were analyzed by real-time PCR. Under the Th17-polarizing condition, daurinol treatment (2 μ M) of murine splenic CD4⁺ T cells significantly induced mRNA expression of *Igfbp4*, *Sell*, *Nt5e*, *IL-7R*, and neuropilin-1 (*Nrp1*) (Figure S2). Taken together, these data suggest that the immunoregulatory properties of daurinol occurs through reciprocal regulation of Th17 and Treg differentiation from naïve CD4⁺ T cells and promotion of gene expression of immunoregulatory mediators of Tregs.

Nrp1-Dependent Treg Induction by Daurinol

Nrp1 has been identified as a Treg-expressing marker at least in murine T cells (25). Nrp1-induced transcriptome augments Treg stability by promoting survival factor and inhibiting terminal differentiation (26). Nrp1 stabilizes Treg function by potentiating its downstream target, phosphatase and tensin homolog (PTEN) activity (26). PTEN is a negative regulator of the Akt–mTOR signaling axis in T cells (27). PTEN-induced suppression of Akt–mTOR activity helps to maintain Treg function, homeostasis, and stability by augmenting Foxp3 expression (27–29). Based on this rationale, we next examined whether the daurinol treatment could alter the abovementioned signaling pathways that are pivotal for Treg differentiation and stabilization in CD4⁺ T cells.

Total CD4⁺ T cells isolated from mouse spleens were cultured under the Th17-polarizing condition for 3 days. The levels of total and phosphorylated forms of STAT3 (pSTAT3, as a transcriptional factor for Th17 cells), STAT5 (as a transcriptional factor for Tregs), PTEN, and Akt, and phosphorylated mTOR, Smad3 and Nrp1 activity were evaluated by Western blotting in cells treated with or without 2 μ M daurinol. Compared with vehicle-treated cells, daurinol treatment markedly attenuated the expression levels of the phosphorylated forms of STAT3 (both Tyr705 and Ser727), Akt (both Ser473 and T308), and mTOR but reciprocally augmented pPTEN, Smad3, and Nrp1 activity (Figure 2A). Interestingly, pSTAT5 activity was inhibited by daurinol treatment (Figure 2A). Although STAT5 plays as a major driver of differentiation and homeostasis of Treg cells, STAT5-independent pathway such as mTOR have a negative impact on Treg cells (30). Previous many studies have demonstrated that specific loss of mTOR activity and mTOR inhibitor (such as rapamycin) treatment resulted in loss of Th1, Th2, and Th17 cells, while enhancing Treg differentiation, implying the selective role of mTOR during Treg differentiation (31–33). Thus, we assumed that daurinol can promote Treg differentiation



by Smad3 activity which was promoted by inhibition of mTOR activity.

We examined whether daurinol treatment increased the T cell populations expressing Treg markers such as GITR, ICOS, CTLA-4, CD103, PD-1, and Nrp1. Flow cytometry showed that the percentages of CD4⁺Foxp3⁺ T cells expressing Treg markers were increased by daurinol in CD4⁺ T cells cultured under the Th17-skewing condition; this supports the Treg-inducing property of daurinol (Figure 2B). Next, the CRISPR-Cas9 system was used to determine whether the Treg-inducing property of daurinol is dependent on Nrp1. By applying CRISPR-Cas9 system, Nrp1 activity was effectively reduced by about 50% in murine CD4⁺ T cells (data not shown). The Foxp3⁺ Treg-inducing property induced by daurinol was diminished by *Nrp1* silencing (Figure 2C). Taken together, these findings suggest that daurinol induces the differentiation of Foxp3⁺ Tregs

and that this effect depends on the Nrp1-PTEN-Akt-mTOR signaling axis.

Association Between Treg Induction by Daurinol and Decreased Aerobic Glycolysis

The activation of effector T cells, including Th1 and Th17 cells from naïve T cells, is accompanied by a metabolic switch to aerobic glycolysis to fuel the energy demands of the process (34). By contrast, Tregs have a high fatty-acid oxidation metabolic rate (35). HIF-1α is a key transcription factor that orchestrates the expression of glycolytic enzymes, thereby modulating the differentiation of Th17 and Tregs (36). To identify whether the reciprocal regulation of Th17/Treg cells by daurinol is associated with metabolic switch, we measured glycolytic activity in murine CD4⁺ T cells cultured under the Th17-polarizing condition.

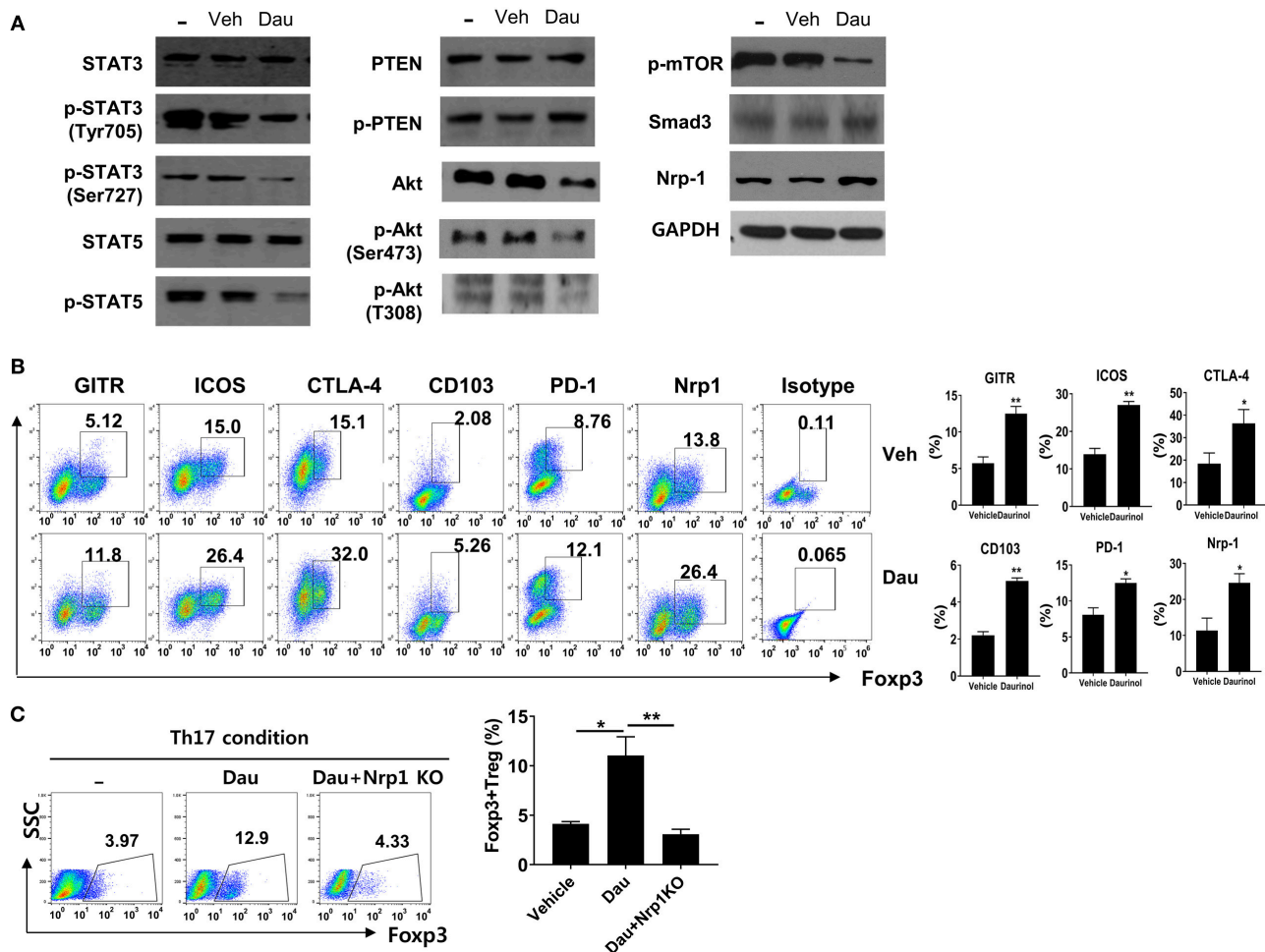


FIGURE 2 | Daurinol induce the Neuropilin-1+Foxp3 Treg cell expansion. **(A)** Mouse splenic CD4⁺ T cells were cultured under the Th17-skewing condition for 72 h in the presence or absence of daurinol (2 μ M). Cell lysates were analyzed by Western blotting to detect total and phosphorylated forms of STAT3 (pSTAT3), STAT5 (pSTAT5), PTEN (pPTEN), Akt (pAkt), and pmTOR, Nrp1, and Smad3. **(B)** The cell populations with the Treg phenotype (GITR⁺, ICOS⁺, CTLA-4⁺, CD103⁺, PD-1⁺, and Nrp1⁺ cells) after daurinol (2 μ M) treatment were analyzed by flow cytometry. **(C)** Nrp1 was deleted in CD4⁺ T cells using the CRISPR-Cas9 system. Naïve CD4⁺ T cells and Nrp1-deleted cells were cultured under the Th17-polarizing condition in the presence or absence of daurinol. The populations of Foxp3⁺ T cells were analyzed by flow cytometry. Data were obtained from three independent experiments, and values are represented as the mean \pm SD. * p < 0.05, ** p < 0.01.

To determine the influence of daurinol on glycolysis, the changes in aerobic glycolysis-associated mediators were analyzed in CD4⁺ T cells. Real-time PCR analysis of murine CD4⁺ T cells cultured under the Th17-polarizing condition revealed that daurinol treatment downregulated genes encoding various molecules involved in aerobic glycolysis, such as *Glut1* (glucose transport 1), *MCT4* (monocarboxylic acid transporter member 4), *HK2* (hexokinase 2), *GPI* (glucose-6-phosphate isomerase), *TPI* (triosephosphate isomerase), *Eno1* (enolase 1), and *PKM* (pyruvate kinase muscle), compared with those of vehicle-treated cells (Figure 3A). *Glut1* is a transporter for glucose uptake and is rapidly induced following T cell activation and plays a pivotal role in effector T cells (37). *MCT4* is a plasma membrane transporter for the lactate exporter and is involved in aerobic glycolysis (38).

Next, we measured the extracellular acidification rate (ECAR) to investigate whether daurinol could change the degree of glycolysis in CD4⁺ T cells. Daurinol decreased glycolysis

in CD4⁺ T cells cultured in the Th17-skewing condition (Figure 3B), which suggests that Treg induction by daurinol treatment may occur through the metabolic changes.

Increased Differentiation and Stabilization of Tregs by Daurinol Through Foxp3 Hypomethylation

Next, to confirm the function of daurinol in terms of Treg stabilization, murine CD4⁺ T cells were cultured under the Treg-polarizing condition for 72 h and then further cultured under the Th17-polarizing condition for 72 h. The population of Tregs induced under the Treg-polarizing condition decreased after culture under the Th17-skewing condition. Interestingly, daurinol treatment in murine CD4⁺ T cells prevented this decrease in Treg populations (Figure 4A). The concentrations of IL-17 and IFN- γ in culture supernatants were measured

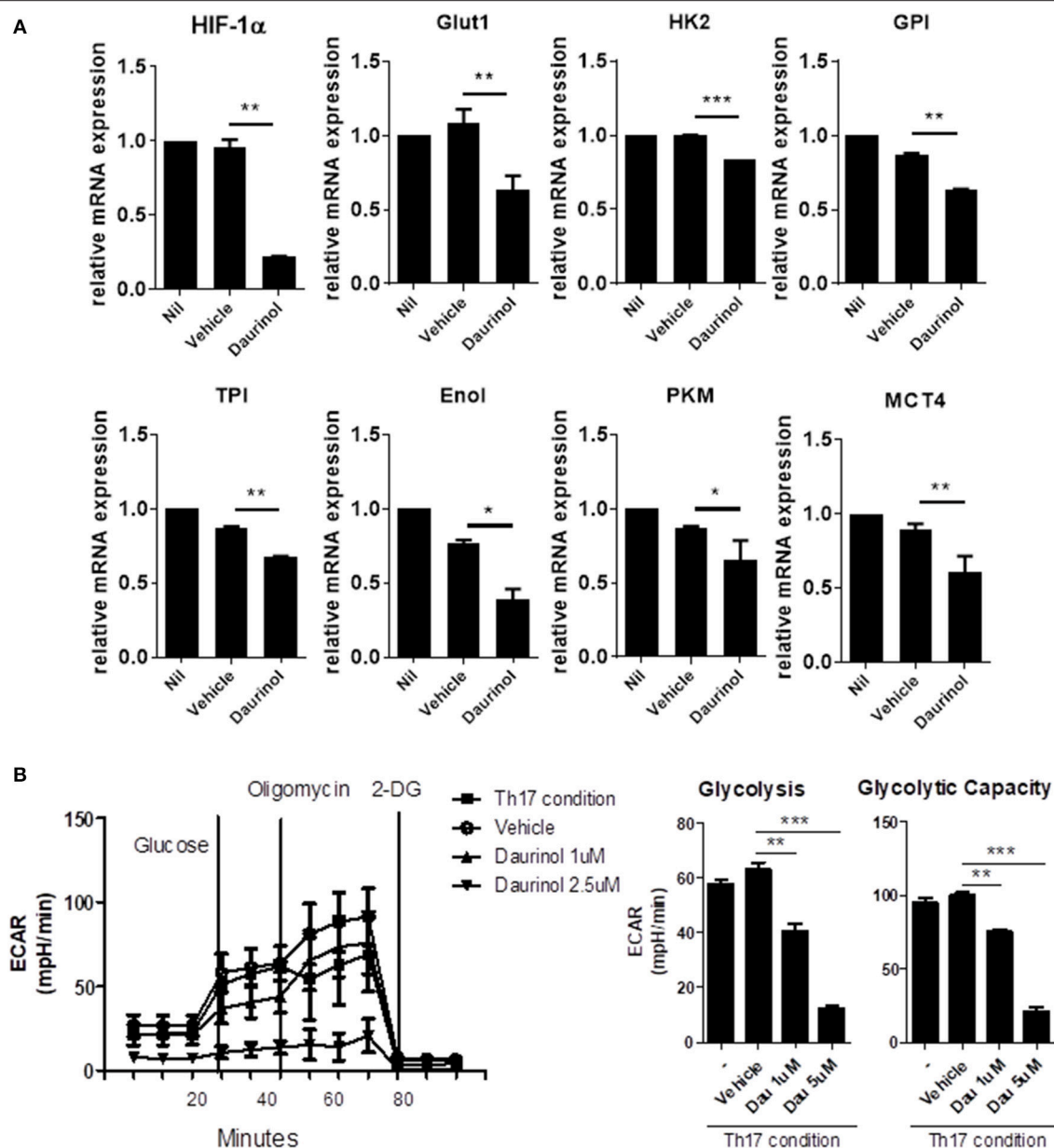
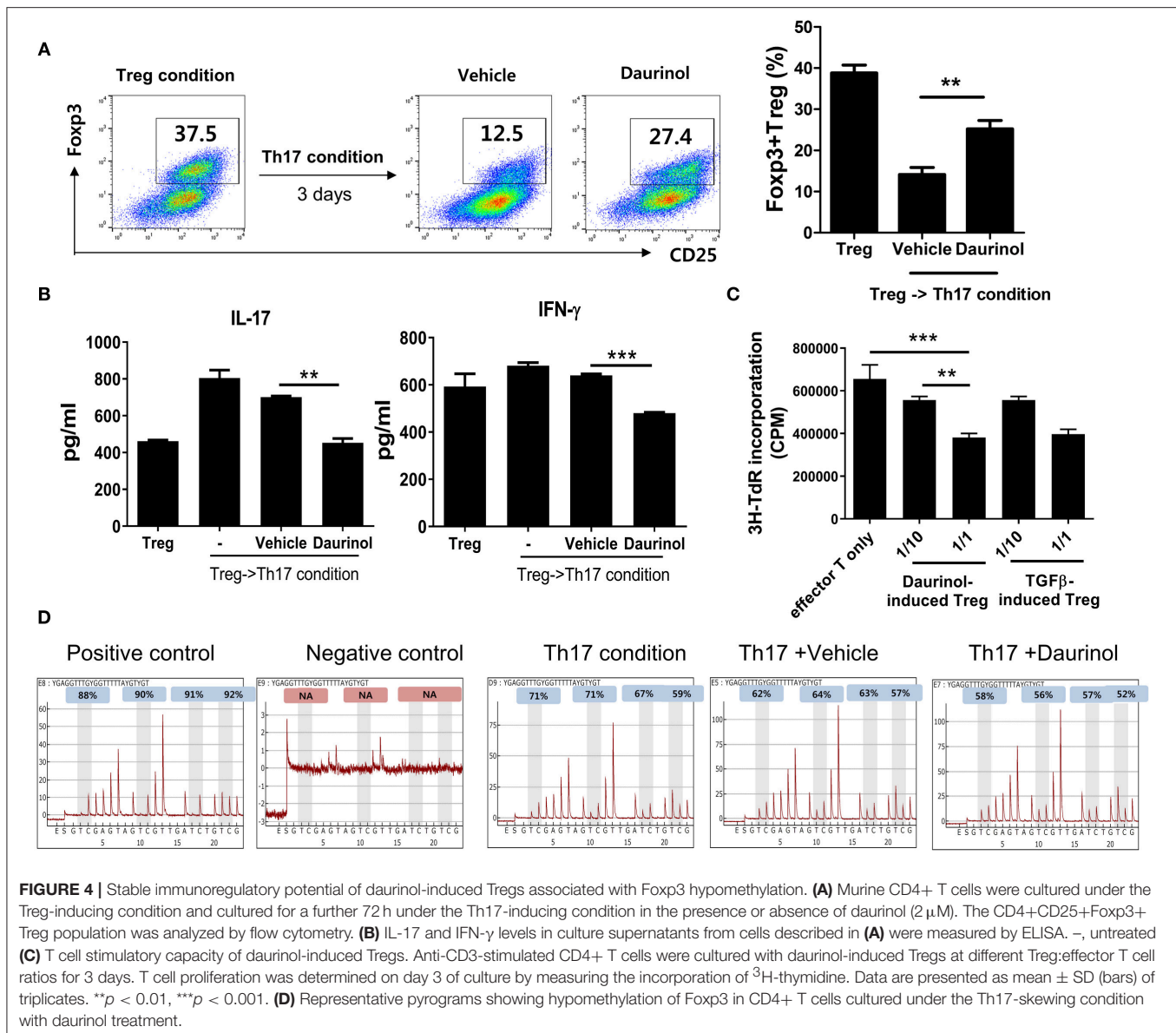


FIGURE 3 | Daurinol inhibits aerobic glycolysis in CD4⁺T cells. **(A)** The expression levels of aerobic glycolysis-associated genes were determined by real-time PCR in murine CD4⁺ T cells cultured under the Th17-polarizing condition in the presence or absence of daurinol (2 μ M). **(B)** ECAR in CD4⁺ T cells cultured under the Th17-polarizing condition in the presence or absence of daurinol. The data are representative of three independent experiments and are expressed as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

by ELISA. The production of IL-17 and IFN- γ was inhibited in daurinol-treated T cells compared with vehicle-treated T cells (Figure 4B).

Next, we studied whether daurinol-induced Tregs have an immunoregulatory function by inhibiting the proliferation of anti-CD3-stimulated CD4⁺ T cells. The proliferative response of anti-CD3-stimulated CD4⁺ T cells cultured with daurinol-induced Tregs (effector T cells alone, 1:10, 1:1) was evaluated by measuring [3 H] thymidine incorporation. Daurinol-induced

Tregs suppressed the proliferation of effector T cells in a ratio-dependent manner, and these cells showed the same suppression effect as TGF β -induced Tregs (Figure 4C), which suggests that the daurinol stimulated the differentiation of Tregs and their immunoregulatory function. Foxp3 is a major transcription factor for Treg induction and its maintenance is essential for the control of inflammation. The hypomethylated region within Foxp3 is considered to be the hallmark of stable Tregs (39, 40). Therefore, we examined whether daurinol can directly modulate

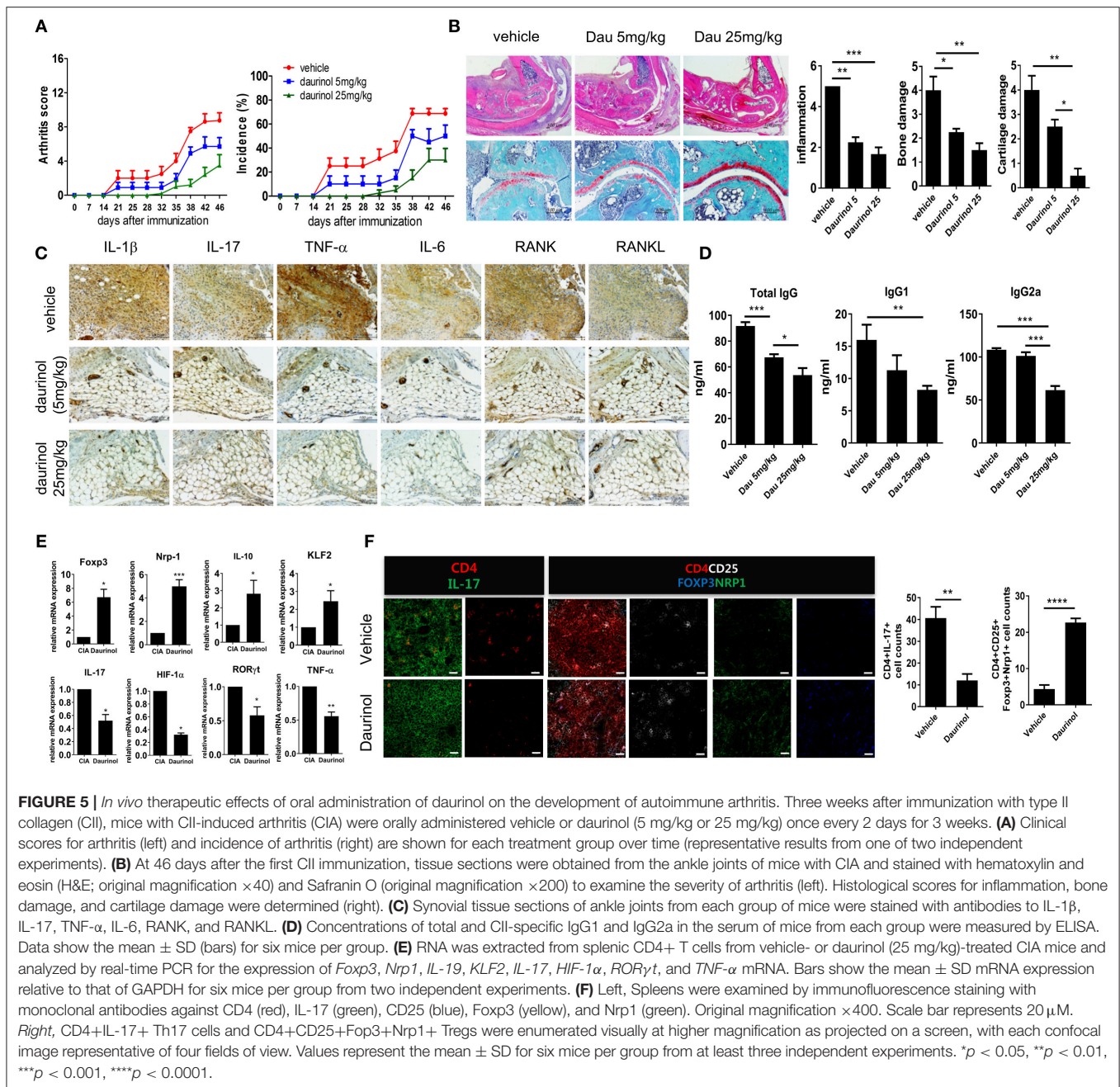


Foxp3 and, if so, the regulatory mechanism. To analyze the methylation density of the promoter region *Foxp3* gene by daurinol, we conducted pyrosequencing of bisulfite-modified genomic DNA from CpG island at promoter regions of *Foxp3*. Daurinol treatment of CD4⁺ T cells cultured under the Th17-polarizing condition induced decreased methylation of CpG sites at *Foxp3* promoter regions, which stimulated the differentiation and increased the stability of Tregs (**Figure 4D**).

Attenuation of the Development of Inflammatory Arthritis by Daurinol in a Dose-Dependent Manner

We investigated whether daurinol could suppress inflammation and joint destruction in an experimental murine model of RA (CIA). Daurinol (5 mg/kg or 25 mg/kg) was administered

orally once every 2 days for 3 weeks from day 21 after primary immunization with CII emulsified in Freund's complete adjuvant. Daurinol ameliorated arthritis severity and incidence compared with vehicle-treated CIA mice in a dose-dependent manner (**Figure 5A**). Histological sections of hind paw joints showed that daurinol treatment in CIA mice attenuated the severity of inflammation, cartilage damage, and bone erosion, as investigated by hematoxylin-eosin (H&E) and Safranin O staining (**Figure 5B**). IL-1 β , IL-17, TNF- α , and IL-6 are considered to be proinflammatory cytokines that are implicated in the pathogenesis of RA (41). The role of the receptor activator of nuclear factor κ B ligand (RANKL)/RANK system has been extensively studied in joint destruction in RA and is a major treatment target in RA. Compared with joints of vehicle-treated mice with CIA, joints of daurinol-treated mice with CIA had significantly fewer cell populations expressing IL-1 β ,

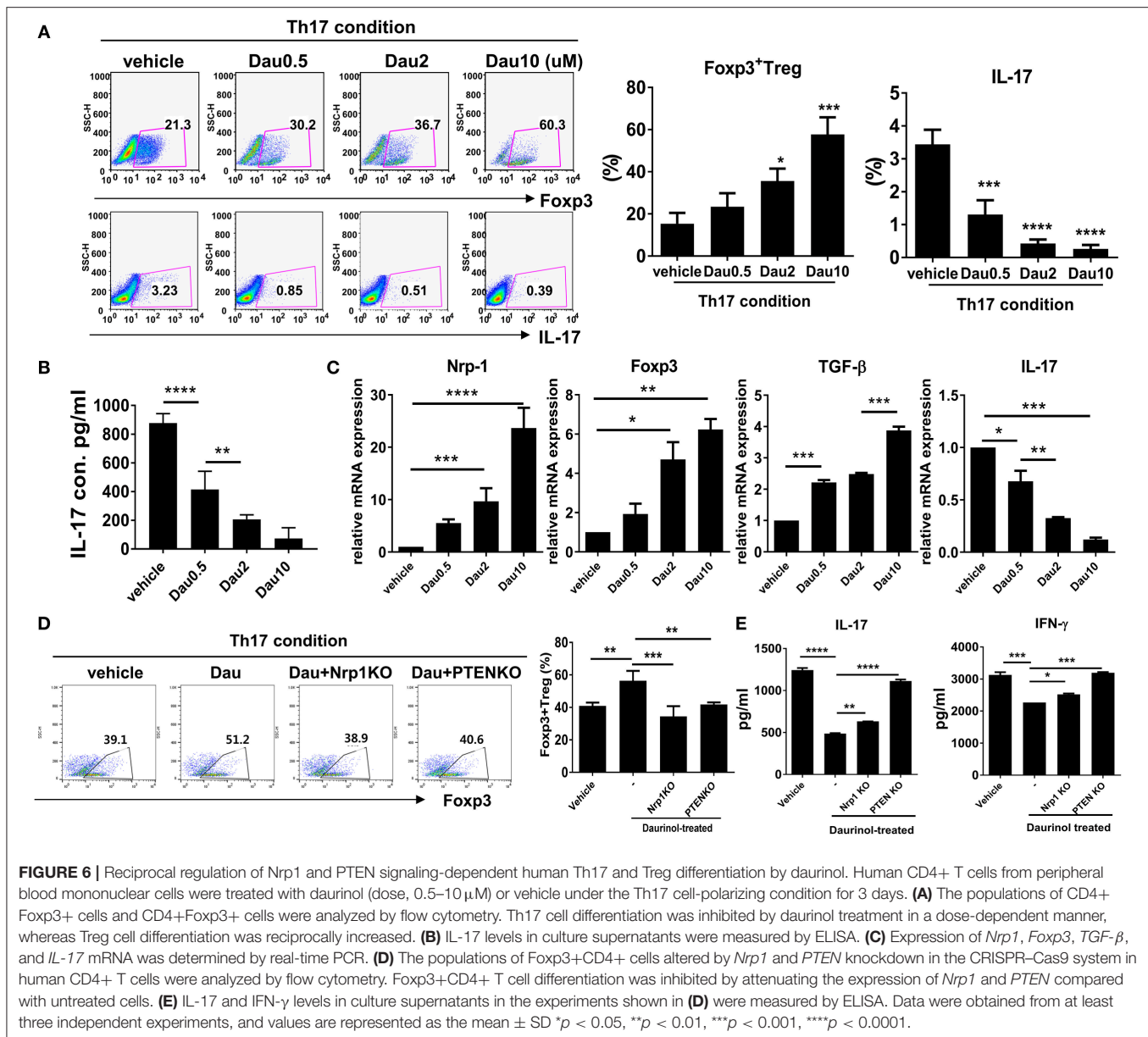


IL-17, TNF- α , IL-6, RANKL, and RANK (Figure 5C). Serum levels of CII-specific IgG, IgG1, and IgG2a antibodies were lower in daurinol-treated mice than in vehicle-treated mice, and the effects were dose dependent (Figure 5D).

IL-10, one of the anti-inflammatory cytokines, restrains the Th17-mediated inflammatory process (42). IL-10 signaling is also pivotal for maintaining the immunoregulatory function of Tregs (43). Krüppel-like factor 2 (KLF2) promotes Treg generation and function through Foxp3 induction (44). We analyzed the mRNA expression of factors associated with the differentiation and function of Th17 and Tregs in splenic CD4+

T cells isolated from each group of mice. mRNA expression levels of Treg factors in the splenic T cells were significantly higher after daurinol treatment (25 mg/kg) compared with cells from CIA mice treated with vehicle. By contrast, the mRNA expression of Th17 factors was decreased by daurinol treatment (Figure 5E). Confocal analysis showed significantly suppressed IL-17+ and reciprocally augmented CD25+Foxp3+Nrp1+ cell populations among splenic CD4+ cells after daurinol treatment in CIA mice (Figure 5F).

Next, we confirmed the anti-inflammatory effect of intraperitoneal (IP) administration of daurinol. Daurinol



(20 mg/kg) was administered intraperitoneally once every 2 days for 3 weeks from day 21 after CII immunization. IP daurinol treatment significantly reduced the arthritis severity and incidence compared with those observed in vehicle-treated CIA mice (**Figure S3A**). Serum levels of CII-specific IgG, IgG1, and IgG2a antibodies were lower in daurinol-treated mice than in vehicle-treated mice (**Figure S3B**). Histological sections of ankle joints stained with H&E, Safranin O, and toluidine blue showed less severe arthritis in IP daurinol-treated CIA mice compared with vehicle-treated mice (**Figure S3C**). Compared with joints of vehicle-treated mice, joints of IP daurinol-treated mice with CIA exhibited smaller populations of cells expressing TNF- α , IL-17, IL-6, and IL-1 β (**Figure S3D**).

To determine whether the populations of Th17, Th1, Th2, and Tregs were altered in daurinol-treated mice with CIA, we used flow cytometry to analyze IL-17-, IFN- γ -, IL-4-, and Foxp3-expressing cells among CD4⁺ cells in the spleens from mice with CIA. Spleens from daurinol-treated CIA mice showed fewer Th17, Th1, and Th2 cells and a reciprocal increase in the number of Foxp3-expressing Tregs compared with spleens from vehicle-treated mice (**Figure S4A**). IP daurinol treatment increased the populations of CD4⁺ cells expressing Treg markers, such as GITR, ICOS, CD103, CTLA-4, or PD-1 in CIA mice (**Figure S4B**). Confocal immunostaining of spleen tissue sections also showed significantly decreased populations of CD4⁺ T cells that was expressing pSTAT3Ser727, and a significantly increased population of CD4⁺CD25⁺Foxp3⁺ T cells (**Figure S5**).

Inhibition of Osteoclastogenesis by Daurinol *in vitro* and *in vivo*

Pathologically enhanced osteoclast activation is an important therapeutic target, which contribute to progressive joint damage in RA patients. Since both oral and intraperitoneal administration of daurinol showed a significant anti-arthritis effects *in vivo*, we tried to confirm whether daurinol affects not only T cell subset differentiation but also osteoclastogenesis. For this purpose, BMM cells isolated from normal DBA/1J mice were cultured with M-CSF and RANKL in the presence or absence of daurinol (2 μ M). TRAP staining showed that daurinol treatment significantly inhibited *in vitro* osteoclastogenesis in BMMs compared with vehicle-treated cells (**Figure S6A**). We next tried to identify the pro-osteoclastogenic factors affected by *in vivo* daurinol treatment. BMM cells were isolated from daurinol-treated CIA mice or vehicle-treated CIA mice and were cultured in the presence of M-CSF and RANKL. Real-time PCR was used to analyze the levels of mRNA for osteoclast markers, including *TRAP*, *MMP9*, *carbonic anhydrase II*, *calcitonin receptor*, and *Itgb3* (integrin β 3) in the cells. mRNA transcript levels of osteoclastogenesis markers were also significantly lower in daurinol-treated CIA mice than in the vehicle-treated animals (**Figure S6B**). This finding suggests that daurinol ameliorates CIA by reducing osteoclastogenesis in mice.

Daurinol Upregulates Treg Cell Through Nrp1-PTEN Signaling and Reciprocal Inhibition of Th17 Cell in Human CD4+ T Cells

We next investigated the effects of daurinol on human CD4+ T cells isolated from PBMCs obtained from normal healthy volunteers. Purified CD4+ T cells were cultured under the Th17-polarizing condition in the presence or absence of daurinol (at doses of 0.5–10 μ M). Daurinol treatment significantly increased the Foxp3+ Treg cell population in a dose-dependent manner but suppressed Th17 cell differentiation (**Figure 6A**). Daurinol also reduced the IL-17 level in the culture supernatant (**Figure 6B**). We measured the mRNA levels of Treg- and Th17-related molecules in the cells. Daurinol-induced Treg induction and Th17 suppression were associated with increased levels of mRNA encoding *Nrp1*, *Foxp3*, and *Tgfb*, and with decreased *Il17* mRNA level (**Figure 6C**). We next used the CRISPR–Cas9 system to confirm that Treg induction effect by daurinol is dependent on Nrp1 and PTEN. By applying CRISPR–Cas9 system, Nrp1, and PTEN protein activity was effectively reduced by about 50% in human CD4+ T cells (**Figure S7**). The results showed that Treg induction by daurinol in human CD4+ T cells was also dependent on Nrp1–PTEN signal (**Figure 6D**), which suggests that human T cell reactions to daurinol correspond to those observed in murine CD4+ T cells. The decreased production of IL-17 and IFN- γ induced by daurinol treatment was attenuated by knockdown of *Nrp1* and *Pten* (**Figure 6E**).

DISCUSSION

Treg-induced immune tolerance has emerged as an attractive strategy in RA treatment. However, the ability to generate

a sufficient number of Tregs with functional stability by *in vitro* expansion remains an issue that hinders the clinical exploitation of these cells in autoimmune diseases including RA. In this study, we first confirmed that daurinol diverted the differentiation of human and murine CD4+ T cells toward a Treg phenotype, even under Th17-polarizing conditions, and increased Treg stability through Nrp1–PTEN–Foxp3 signaling. Interestingly, the optimal and stable immunoregulatory function of Tregs induced by daurinol is associated with hypomethylation of the lineage-specific transcription factor Foxp3. The Tregs induced by daurinol treatment showed significantly reduced aerobic glycolysis. The change in the metabolic profile may explain the reduced populations of effector T cells including Th1, Th2, and Th17 cells, and reciprocally augmented Treg subsets in daurinol-treated CIA mice compared with vehicle-treated animals. Here, we found that systemic administration of daurinol (oral and IP administration) effectively reduced the clinical and histological scores in a murine model of RA. The Treg-inducing property by *in vivo* daurinol treatment in CIA mice was associated with significant induction of Nrp1 and Foxp3 expression. In addition, daurinol significantly inhibited osteoclast differentiation and related gene expression, which suggests that daurinol has an inhibitory effect on bone destruction. However, one of the limitation of our study is that the presentation of inherent mechanisms to explain the anti-inflammatory and immunoregulatory effects of daurinol shown *in vivo* is fairly limited.

One reasonable treatment strategy for autoimmune diseases under a new paradigm may involve optimizing the immunoregulatory function of Tregs. Some trials have targeted Tregs in autoimmune diseases including graft-vs.-host disease (45), SLE (46), and RA (47). However, few studies have targeted the epigenetic stabilization of Foxp3, a key transcription factor of Tregs. The ways by which Tregs maintain their lineage stability and immunosuppressive function include multiple epigenetic changes (48). Epigenetic changes including DNA methylation affect cell differentiation and lineage stabilization at the level of transcription (49, 50). In the research field of Treg function, several studies have been published to support the claim that demethylation of Foxp3 gene promoter sites mainly contributes to their immunoregulatory function and development of a stable suppressor cell lineage (40, 51). Through our present study, we first proved that daurinol has immunoregulatory potential via the epigenetic change (DNA demethylation) of Foxp3 promoter region, rather than the expression control of transcription factor STAT5.

We assumed that daurinol has shown Treg-induction effects through modulation of mTOR/Akt signaling activity. The sustained expression of Foxp3 is important for maintenance of Treg lineage. Foxp3 retains the immunoregulatory function of Tregs and blocks the transition of Tregs into effector T cells, such as Th1 and Th17 cells (52). Previous study demonstrated clearly that Foxp3-negative Tregs lose their suppressive function and assume an inflammatory phenotype (53). The forkhead box protein O (FoxO) is a transcription factor that induces Foxp3 gene expression by enhancing Foxp3 promoter region, thereby contributes to Tregs stabilization and maintaining their immunoregulatory function (54). Interestingly, Akt-induced

phosphorylation of FoxO protein triggers the translocation of FoxO protein from nucleus to cytoplasm, which ultimately results in the suppression of Foxp3 expression (55). Although this study could not clarify the underlying mechanisms by which mTOR/Akt axis regulation by daurinol affected the subcellular location of FoxO protein, we presumed that mTOR/Akt/FoxO axis regulation by daurinol induced Foxp3 promoter activity, which ultimately resulted in Tregs induction. Promoted Treg function driven by sustained Foxp3 expression by daurinol may be a novel treatment strategy for autoimmune diseases that involve impaired Treg function, such as RA and SLE.

Our study provides the first evidence that daurinol can regulate the differentiation of Tregs and stabilize their immunoregulatory function. Our findings suggest that daurinol can regulate Th17 differentiation by STAT3 inhibition while stimulating Treg differentiation and stabilizing Tregs through Nr1-PTEN-Foxp3 signaling. By hypomethylation of Foxp3, a Treg lineage-specific transcription factor, daurinol concurrently stimulates Treg differentiation while suppressing the Th17 population.

ETHICS STATEMENT

The protocols used in this study were approved by the Animal Care and Use Committee of the Catholic University of Korea.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. M-LC and DS had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. M-JP, S-JM, DS, and M-LC contributed to study conception, study design, data acquisition, analysis, and interpretation and drafted the manuscript. E-JL, E-KK, J-AB, S-YK, SHL, D-SK, KJ, and JC contributed to data acquisition, analysis, and interpretation. SHL, J-KM, and S-HP contributed to analysis and interpretation of data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01526/full#supplementary-material>

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Matured Tolerogenic Dendritic Cells Effectively Inhibit Autoantigen Specific CD4⁺ T Cells in a Murine Arthritis Model

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Tolerogenic dendritic cells (tolDCs) are a promising treatment modality for diseases caused by a breach in immune tolerance, such as rheumatoid arthritis. Current medication for these diseases is directed toward symptom suppression but no real cure is available yet. TolDC-based therapy aims to restore immune tolerance in an antigen-specific manner. Here we used a mouse model to address two major questions: (i) is a maturation stimulus needed for tolDC function *in vitro* and *in vivo* and is maturation required for functioning in experimental arthritis and (ii) can tolDCs modulate CD4⁺ T cell responses? To answer these questions, we compared matured and immature dexamethasone/vitamin D3-generated tolDCs *in vitro*. Subsequently, we co-transferred these tolDCs with naïve or effector CD4⁺ T cells to study the characteristics of transferred T cells after 3 days with flow cytometry and Luminex multiplex assays. In addition, we tested the suppressive capabilities of tolDCs in an experimental arthritis model. We found that tolDCs cannot only modulate naïve CD4⁺ T cell responses as shown by fewer proliferated and activated CD4⁺ T cells *in vivo*, but also effector CD4⁺ T cells. In addition, Treg (CD4⁺CD25⁺FoxP3⁺) expansions were seen in the proliferating cell population in the presence of tolDCs. Furthermore, we show that administered tolDCs are capable to inhibit arthritis in the proteoglycan-induced arthritis model. However, a maturation stimulus is needed for tolDCs to manifest this tolerizing function in an inflammatory environment. Our data will be instrumental for optimization of future tolDC therapies for autoimmune diseases.

Keywords: arthritis, tolDCs, CD4⁺ T cell, immune modulation, immune tolerance

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in the joints which causes cartilage and bone destruction (1). To date, there is no cure available and treatment is directed toward mitigating symptoms (non-steroidal anti-inflammatory drugs) or dampening the immune response (disease modifying anti-rheumatic drugs) (2, 3). These therapies

suppress the immune system non-specifically and thus are not completely effective and have side effects. RA, as other chronic inflammatory diseases, is caused by a disbalance in the immune system. Immune tolerance for self-antigens is not maintained which means that autoreactive T cells can attack the body's own cells.

Regulatory T cells (Tregs), mainly CD4⁺ T cells, are able to restore immune tolerance by suppressing effector cells in an antigen-specific manner. In patients with autoimmune disease, it is thought that a subtle change in function or presence of Tregs is involved in the pathogenesis of the disease. However, a lot of controversy exists in this area. Several studies show that the suppressive capacities of Tregs in synovial fluid of RA patients are diminished, while in peripheral blood these capacities are maintained (4–6). Other studies indicate a decrease in Treg numbers (7, 8), which could cause excessive inflammation in RA. Antigen specific Tregs are able to suppress this excessive inflammation by suppressing the immune cells that cause the pathological autoimmune response while leaving protective immunity intact.

As a tool to induce antigen specific Tregs, tolerogenic dendritic cells (tolDCs) can be used. TolDCs are dendritic cells (DCs) that are modulated to become immune tolerance-inducing. Whether a DC is immune stimulatory or immune tolerant mainly depends on the environmental cues the DCs receive determines if they become immunogenic or tolerogenic. By modulating DCs *in vitro*, they can be steered toward an immune tolerant status. Multiple approaches for inducing a tolerogenic function in antigen presenting cells *in vitro* have been described (9–12). We focus on dexamethasone and α ,25-dihydroxyvitamin D3 modulated DCs (13–15) because this type of tolDCs has recently been tested in a phase I clinical trial in inflammatory arthritis patients (16).

The advantage of tolDCs is that they can be loaded with an antigen to specifically target autoreactive T cells without affecting other immune responses. We have shown previously that loading tolDCs with disease specific-antigen enhances their efficiency compared to non-loaded tolDCs (17). Some groups show therapeutic effects with non-loaded tolDCs (18), but generating non-specific Tregs may give rise to general immune suppression thereby increasing the risk of infections. Furthermore, administering non-loaded tolDCs might not actively induce Tregs but generate T cell anergy, which in turn can suppress excessive Th17 and Th1 responses (19). Since the autoantigen that causes disease in RA is currently unknown, non-loaded tolDC treatment could be an option.

In addition to the issue of antigen loading, the stability of a tolDC is an important issue to address. Since DCs are essential for both tolerance and immunity they are the sentinels of the immune system. It is plausible that non-stimulated tolDC change their phenotype when entering an immune stimulatory environment. Therefore, partial maturation with lipopolysaccharide (LPS) (15, 20), monophosphoryl Lipid A (MPLA, lipid A portion of LPS) (21) or a cytokine cocktail (22) would be preferable. This potentially stabilizes the phenotype of the tolDC and improves antigen presentation and migration (9, 15). A more complete understanding of the working mechanism

of tolDCs will contribute to the development of tolDC treatment in the future. For that reason, we aimed to (i) determine the role of maturation in murine dexamethasone and α ,25-dihydroxyvitamin D3 induced tolDCs and function in arthritis, and (ii) study the effects of tolDCs on CD4⁺ T cell responses.

MATERIALS AND METHODS

Mice

Female Balb/cAnNCrl of 18–20 weeks old were purchased from Charles River laboratories for *in vivo* arthritis experiments. Male Balb/cAnNCrl of 10 weeks old were purchased from Charles River laboratories for co-transfer studies. hPG TCR transgenic (23) mice were bred at the central animal laboratory of Utrecht University, the Netherlands. Both sexes were used as donor mice. Animals were kept under standard conditions of the animal facility and all experiments were approved by the Animal Experiment Committee of Utrecht University (project number AVD108002016467). Mice were randomly divided in control- or treatment groups and all animals were monitored and scored three times a week during the arthritis experiments.

BMDC Culture

Bone marrow was isolated from the femur and tibia from Balb/cAnNCrl (both male and female) 10–20 weeks old mice and seeded 450.000 fresh cells/ml in 6 wells plates (Corning costar). As culture medium IMDM (Gibco) supplemented with 10% FCS (Bodinco), 100 units/ml penicillin, 100 μ g/ml streptomycin and 5×10^{-5} M β -mercaptoethanol in the presence of 20 ng/ml GM-CSF (in house produced) was used. On day 2 an equal volume of fresh culture medium containing 20 ng/mL GM-CSF was added, and on day 4/5 20 ng/mL fresh GM-CSF was supplemented to the culture. Tolerogenicity was induced by adding 10^{-6} M dexamethasone (Invivogen) and 10^{-10} M α ,25-dihydroxyvitamin D3 (Enzo Life sciences) to the BMDC culture on day 7. Simultaneously with the tolerogenic agents, 10 μ g/mL peptide (hPG: ATEGRVRVNSAYQDK) and maturation stimuli (lipopolysaccharide (LPS) 10 ng/mL; Sigma Aldrich or Monophosphoryl Lipid A (MPLA, 10 ng/mL) from Salmonella minnesota R595; Invivogen) were added. After 8 days of culture at 37°C, 5% CO₂, the BMDCs or tolDCs were harvested for further experimentation. Before co-culture, BMDCs or tolDCs were replated into 24 wells plates (Corning costar). Before injection in co-transfer experiments, BMDCs or tolDCs were thoroughly washed with medium (2x) and PBS (1x) and kept on ice.

Co-cultures

For co-culture experiments, spleens from mB29b TCR transgenic mice were pooled and CD4⁺ cells were isolated using Dynal bead isolation (Invitrogen) by negatively selecting CD4⁺ T cells with a mixture of the following in house produced antibodies: anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC-II (M5/114) and anti-CD8 (YTS169). To gain a naïve population, CD25⁺ and CD44⁺ cells were depleted by adding an anti-CD25 antibody (PC61, produced in house from hybridoma ATCC PC61 and purified from supernatants) and an anti-CD44 antibody (IM7.8,

kindly provided by Tibor Glant) in predetermined optimal concentrations. The purified naïve CD4⁺ T cells were added to BMDCs in a 10:1 ratio.

Flow Cytometry and Antibodies

Flow cytometry was performed with FACS Canto II (BD) with monoclonal antibodies CD4-V500 (RM4-5, BD Biosciences), CD25-PerCP-Cy5.5 (PC61.5, eBioscience/ThermoFisher), Thy1.1-PerCP-Cy5.5 (HIS51, eBioscience/ThermoFisher), FoxP3-eFluor450 (FJK-16s, eBioscience/ThermoFisher), CD62L-PE (MEL-14, BD biosciences), NKp46-PE-Cy7 (29A1.4, eBioscience/ThermoFisher) and CD3-APC (145-2C11, BD biosciences). Red blood cells were lysed with ACK (Ammonium-Chloride-Potassium) buffer. For BMDC phenotyping the following monoclonal antibodies were used: I-A/I-E Horizon450 (M5/114.15.2, eBioscience/ThermoFisher), CD11c APC (N418, eBioscience/ThermoFisher), CD86 FITC (GL1, BD biosciences), PD-L1 PE (10F.9G2, Biolegend), IL12p40/70 PE (C15.6, BD biosciences). To identify dead cells the Zombie NIR fixable viability kit (Biolegend) was used.

Induction of PGIA

Human proteoglycan (hPG) was isolated from human articular cartilage as described (24). To induce arthritis, Balb/c mice were injected twice intraperitoneally with a mixture of 2 mg DDA and 250 µg human proteoglycan with a 21 day interval. Subsequently, mice were randomized among experimental groups, and arthritis scores were determined in a blinded fashion using a visual scoring system based on swelling and redness of paws as described (24). tolDCs (1×10^6 cells in 200 µL PBS) were injected intravenously on day 17.

Co-transfers

Naïve CD4⁺ T Cell Co-transfer

CD4⁺CD25⁻CD44⁻ T cells were purified from spleens of hPG TCR transgenic Thy1.1⁺ mice by Dynal bead isolation (Invitrogen). A representative dot plot of the transferred cells is shown in **Supplementary Figure 1A**. The purified naïve CD4⁺ T cells (23) were labeled with 0.5 µM 5,6-carboxyfluorescein-succinimidyl-ester (CFSE, Invitrogen) and subsequently intravenously injected (max 10×10^6) in 200 µl PBS in acceptor mice (8–10 weeks old male Balb/cAnNCr) (day 0). On day one, 1×10^6 freshly cultured hPG pulsed BMDCs or tolDCs were intravenously injected in the same acceptor mice. After 3 days, spleens from acceptor mice were harvested and transferred CD4⁺ T cells were tested for proliferation, activation and phenotype. A schematic prestaton of the experiments is depicted in **Figure 1**.

Activated CD4⁺ T Cell Co-transfer

On day -3 the donor hPG TCR transgenic Thy1.1⁺ mice were intra muscularly (left quadriceps) injected with 100 µg hPG peptide in 50 µl PBS (**Figure 1**). On day 0, CD4⁺ T cells were purified from spleens from hPG TCR transgenic Thy1.1⁺ mice (23) by Dynal bead isolation (Invitrogen). Representative dot plot of the transferred cells and the expression of CD44, CD62L CD25, and FoxP3 are in **Supplementary Figure S1B**.

The purified CD4⁺ T cells were labeled with 0.5 µM 5,6-carboxyfluorescein-succinimidyl-ester (CFSE, Invitrogen) and subsequently intravenously injected in 200 µl PBS in acceptor mice (8–10 weeks old male Balb/cAnNCr) (day 0). On day one, 2×10^5 or 1×10^6 freshly cultured tolDCs (MPLA stimulated; mtolDC) or BMDCs (LPS matured; mBMDC) were intravenously injected in the same acceptor mice. Both cell types (tolDC or BMDC) were either non-loaded or pulsed with hPG. After 3 days, spleens from acceptor mice were harvested and transferred CD4⁺ T cells were tested on proliferation, activation and phenotype. A schematic prestaton of the experiments is depicted in **Figure 1**.

Cytokine Analysis

Supernatants of *ex vivo* stimulations or co-cultures were used for multiplex cytokine analysis of IL-10, IL-2, IL-6, IL-17, and IFN-γ using the Magpix (Luminex XMAP) system according to manufacturer's instructions. Briefly, supernatant together with magnetic beads coated with capture antibodies for the respective cytokines were added to polystyrene, black, 96 wells flat bottom plates (Greiner bio-one, 655096). Subsequently, biotin-conjugated detection antibodies and Streptavidin-PE (BD Bioscience) were added and incubated together. The antibody pairs used:

Cytokine	Coating	Detecting
IL-2	JES6-1A12	JES6-5H4
IL-6	MP5-20F3	MP5-32C11
IL-10	JES5-2A5	SXC-1
IL-17A	TC11-18H10	TC11-8H4.1
IFN-γ	AN-18	XMG1.2

The concentrations of cytokines in the tested samples were calculated using standard curves of recombinant proteins and the MFI data was analyzed using a 5-parameter logistic method (xPONENT software, Luminex, Austin, USA).

ELISpot

Multiscreen IP filter plate plates (Milipore) were activated with 70% Ethanol and coated with a rat anti-mouse anti-IFN-γ antibody (clone AN-18, in house produced) or anti-IL-10 antibody (JES5-2A5, in house produced) at 2 µg/ml in PBS and then blocked with IMDM medium supplemented with 5% FCS. Subsequently, single cell suspensions of spleen from co-transferred acceptor mice were cultured in 200 µl complete medium for 48 h in 96-wells flat bottom plates for ELISPOT (Millipore) at 5×10^6 cells/ml. Medium or 20 µg/ml hPG peptide were added as restimulation. After culture, the plates were washed and the IFN-γ or IL-10 producing cells were detected using the rat anti-mouse biotin-anti-IFN-γ antibody (clone XMG1.2, BD biosciences) or rat anti-mouse biotin-anti-IL-10 antibody (clone SXC-1, BD biosciences), respectively. Streptavidin-alkaline phosphatase (Sigma, S2890) and BCIP/NPT solution (Roche) were used to visualize the spots according to manufacturer's instructions. Counting of the IFN-γ or IL-10 producing cells was done by the Automated ELISpot

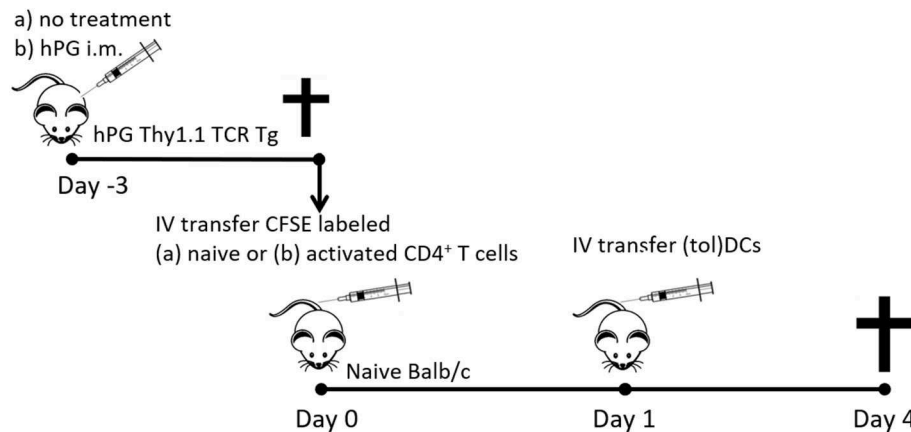


FIGURE 1 | Schematic representation of the co-transfer experiments. Activated hPG Thy1.1⁺ TCR transgenic CD4⁺ T cells were isolated either from TCR Tg mice that were immunized with hPG i.m. at day -3. Naïve CD4⁺Thy1.1⁺ T cells were isolated from untreated and depleted of CD25⁺ and CD44⁺ CD4⁺ T cells prior to transfer to naïve balb/c mice at day 0. One day after T cell transfer mice received an i.v. injection with (tol)DC as described and after 3 days spleens were isolated for further analysis.

Assay Video Analysis System (A.EL.VIS GmbH). To calculate the relative increase (stimulation index: SI), the negative (medium) control was set to 1. The stimulated conditions (hPG or α CD3) were calculated relative to the control: (hPG/ α CD3 sample \times 1)/negative control.

Statistical Analyses

The following statistical analyses were performed using Prism 7.04: repeated measures analysis of variance (ANOVA) with Dunnett or Bonferroni correction for comparisons between multiple groups, paired Student *t*-test for comparisons between two groups.

RESULTS

Peptide Pulsed tolDCs Show a Semi-mature Phenotype and Hamper CD4⁺ T Cell Activation *in vitro*

First, we investigated the change in phenotype of tolDCs after stimulation with a TLR4 agonist *in vitro*. DCs were generated from bone marrow and treated with dexamethasone and the active form of vitamin D3 to develop tolDCs. Untreated bone marrow derived dendritic cells (BMDCs) were used as controls. To compare the phenotype of BMDCs and tolDCs, both cell types were measured unstimulated (immature BMDCs; iBMDCs or immature tolDCs; itolDCs) or after stimulation with the lipid A portion of LPS: MPLA (mature BMDCs; mBMDCs or mature tolDCs; mtolDCs). TolDCs were stimulated with MPLA since TLR4 stimulation might be required for antigen-processing and migration. Considering that LPS cannot be used in humans, we used MPLA. Both itolDCs and mtolDCs exhibited a semi-mature phenotype, consisting of a lower expression of MHC-II, CD86 (Figures 2A,B) and a higher PD-L1/CD86 ratio compared to their respective control BMDCs (iBMDCs or mBMDCs). The PD-L1/CD86 ratio is considered indicative

of a tolerogenic phenotype as described in multiple studies (20, 25, 26). Furthermore, MPLA stimulation of tolDCs did not induce a proinflammatory cytokine profile as shown by the trend toward a higher IL-10 production and lower IL-12 and IL-6 (Figure 2C) which is important since cytokine signaling is one of the mechanisms of DCs to communicate. To study their function, peptide pulsed itolDCs or mtolDCs were co-cultured with naïve CD4⁺ T cells from a TCR transgenic mouse (27). After 3 days of co-culture with itolDCs or mtolDCs, fewer CD4⁺ T cells expressed the activation marker CD25. Additionally, more CD4⁺ T cells expressed CD62L compared to the controls. Next to this, there was a trend toward more CD4⁺FoxP3⁺ cells after co-culturing with tolDCs (Figure 2D, Supplementary Figure S2). To correct for inter experimental variance in FoxP3% data have been normalized and represented as ratio compared to iBMDC treated mice. CD4⁺ T cells that were in co-culture with non-peptide pulsed itolDCs or mtolDCs did not show any activation or proliferation since these CD4⁺ T cells are antigen-specific. To address CD4⁺ T cell activation and differentiation status their cytokine profile was analyzed (Figure 2E). TolDCs impair pro-inflammatory cytokine production of the CD4⁺ T cells by 2–20 fold compared to CD4⁺ T cells stimulated by control BMDC. In addition the anti-inflammatory cytokine IL-10 production was not diminished shifting the cytokine balance/profile toward a tolerogenic profile in co-culture with tolDCs, indicating that these T cells contain a more regulatory character. Thus, tolDCs modulate CD4⁺ T cell activation and drives them toward an immune tolerant state. In these experiments, maturation of the tolDCs did not influence their effect on CD4⁺ T cells.

TolDCs Restrict the Activation of Naïve Antigen Specific CD4⁺ T Cells *in vivo*

To determine if tolDCs can affect naïve CD4⁺ T cells *in vivo* we performed co-transfer experiments. First, we transferred naïve hPG-TCR Thy1.1⁺ transgenic CD4⁺ T cells and subsequently (1

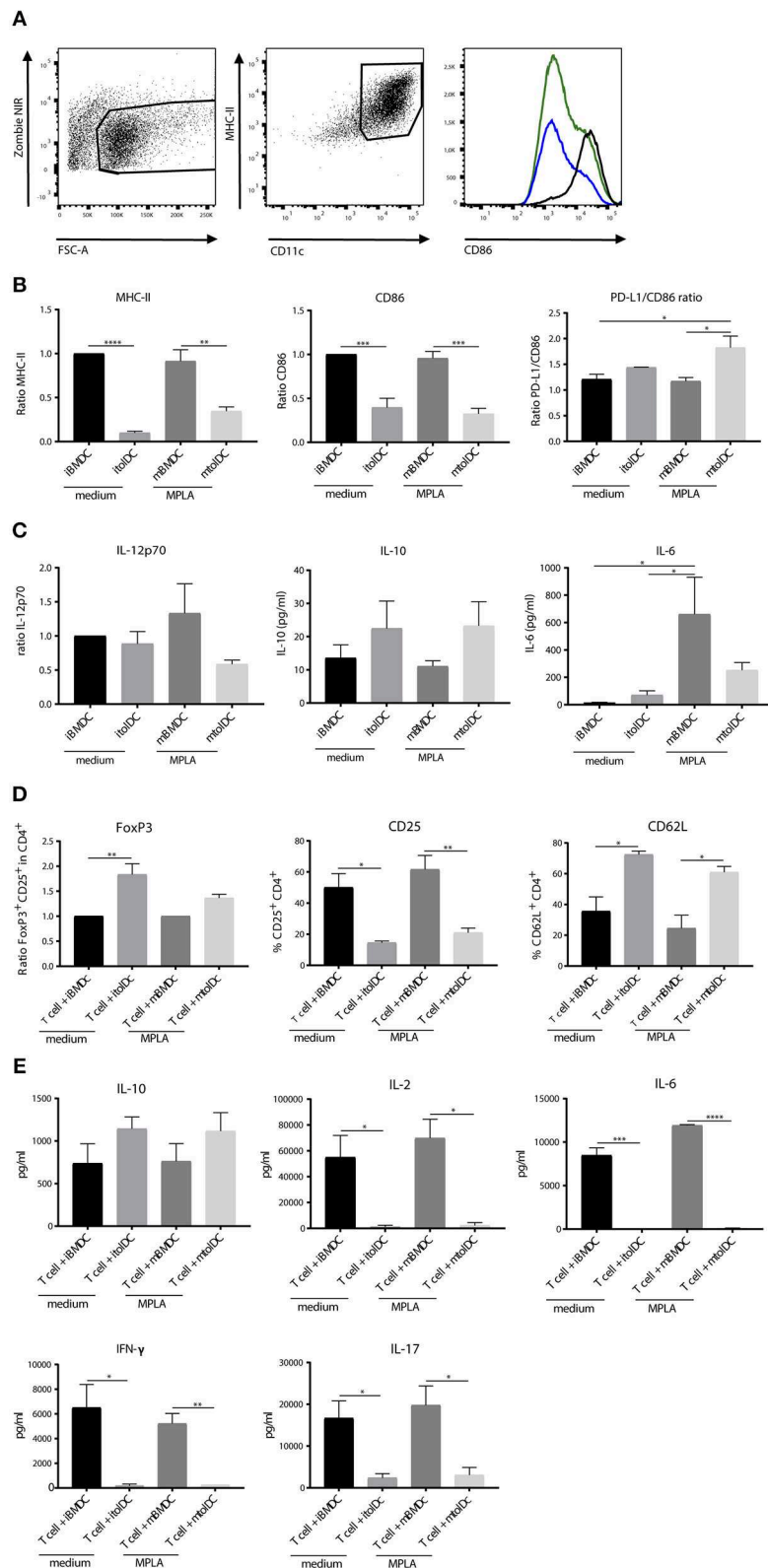


FIGURE 2 | ToDCs exhibit a semi-mature phenotype and remain stable after challenge *in vitro*. ToDCs were generated by adding dexamethasone and 1,25-dihydroxyvitamin D3 and stimulated with MPLA or medium as control. Phenotype was measured by flow cytometry. In the histogram, mBMDCs (black line), (Continued)

FIGURE 2 | itolDCs (blue) and mtolDCs (green) representatives are shown **(A)**. The ratio to immature BMDC was used to determine the difference in expression of MHC-II, CD86 and PD-L1 in the CD11c⁺ population. The ratio was used for MHC-II and CD86 because of the inter-experimental variance. iBMDCs were set to 1 and mBMDC and tolDCs were compared to these cells **(B)**. Cytokine production **(C)** was measured in the supernatant by Magpix (IL-10, IL-6) or intracellular by flow cytometry (IL-12p70). iBMDCs, mBMDCs, itolDCs or mtolDCs were pulsed with peptide and co-cultured for three days with naïve (CD25⁺ and CD44⁺ depleted) CD4⁺ T cells. On day 3, phenotype of the CD4⁺ T cells was determined by flow cytometry **(D)**. For CD25+FoxP3, the ratio (in CD4⁺ population) to CD4⁺ T cells that were in co-culture with BMDCs was used to compare the difference when co-culturing with tolDCs. The ratio was used because of the inter-experimental variance. The respective control BMDC-T cell co-cultures were set to 1 and tolDC co-cultured T cells were compared to BMDC co-cultured T cells. As markers for activation status of the CD4⁺ T cell, CD25 and CD62L were measured. Cytokine production was measured after co-culture in the supernatant by Magpix **(E)**. Data shown are mean ± SEM. Two-tailed paired student *T*-test was used. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, *****p* ≤ 0.0001. *N* = 4.

day later) itolDCs or mtolDCs pulsed with hPG peptide into a naïve acceptor mouse. By transferring CD4⁺ T cells that contain a congenic marker (Thy1.1), we were able to select only the transferred CD4⁺ T cells for analysis *ex vivo*. As a control, LPS matured BMDCs (mBMDCs) instead of tolDCs loaded with hPG peptide were transferred with naïve CD4⁺ T cells. Nearly 100% of the CD4⁺ Thy1.1⁺ T cells that were co-transferred with mBMDCs proliferated, while the CD4⁺Thy1.1⁺ T cells that were co-transferred with itolDCs or mtolDCs proliferated, respectively 45 and 75% (**Figures 3A,B**). At the same time, the CD25 expression was lower on the hPG TCR transgenic CD4⁺Thy1.1⁺ T cells that were co-transferred with itolDCs. Furthermore, the antigen specific CD4⁺ Thy1.1⁺ T cells that were in the presence of itolDCs or mtolDCs showed a lower CD62L expression compared to the control (**Figure 3B**). This indicates that next to *in vitro* modulation, both immature and mature tolDC can also modulate the CD4⁺ T cell response *in vivo*. The percentage CD4⁺FoxP3⁺ in the total CD4⁺Thy1.1⁺ transferred cells was not significantly increased when tolDCs were co-transferred. However, in the presence of itolDCs, we observed a trend toward a higher percentage CD4⁺FoxP3⁺ cells in the proliferating population (**Figure 3C** and **Supplementary Figure S3**).

To further define the phenotype of the CD4⁺ T cells *ex vivo*, IL-10 and IFN-γ secreting cells were measured by ELISpot. The amount of IL-10 producing antigen specific CD4⁺ T cells was similar when itolDCs, mtolDCs, or mBMDCs were present *in vivo*, and although not significantly visually the amount of IFN-γ producing cells tended to be lower when mice were co-transferred with itolDCs or mtolDCs (as shown by the decreasing trend in **Figure 3D**). These results confirm that CD4⁺ T cell activation is constrained after encountering tolDCs *in vivo*.

TolDCs Are Capable of Modifying Effector CD4⁺ T Cells

Since tolDCs are intended to be used as therapeutic agents under inflammatory conditions, restraining the activation of naïve CD4⁺ T cells alone may not be sufficient. In addition, tolDCs should also be able to diminish responses of activated T cells. Therefore, we performed co-transfer studies with a mixture of (both memory and effector) activated CD4⁺ T cells to investigate if tolDCs are capable to modify such proinflammatory T cells. We co-transferred itolDC, mtolDC or mBMDC pulsed with peptide and activated peptide-specific CD4⁺Thy1.1⁺ T cells into a naïve acceptor Balb/c mouse. Both itolDCs and mtolDCs inhibited further proliferation and activation of the peptide-specific CD4⁺Thy1.1⁺ T cells *in vivo* (**Figure 4A**), similar as

was seen when naïve CD4⁺Thy1.1⁺ T cells were injected. Next to this, in mice that were injected with tolDCs, not only more Tregs (CD4⁺CD25⁺FoxP3⁺) were measured (**Figure 4B**, **Supplementary Figure S4**), but these CD4⁺CD25⁺FoxP3⁺ cells were also more activated as shown by an increase in CD44 expression when compared to mice that received mBMDCs (**Figure 4B**). In addition, the spleens of mice which received tolDCs contained more naïve CD4⁺Thy1.1⁺ T cells 3 days after co-transfer as shown by an increase in CD62L positive cells and a decrease in CD25 (**Figure 4A**) and CD44 (data not shown). These results indicate that tolDCs are not only able to modulate naïve CD4⁺ T cells but also previously activated CD4⁺ T cells *in vivo*. Mice in which activated CD4⁺ T cells were co-transferred with itolDC, mtolDC or mBMDC pulsed with an irrelevant peptide did not show any proliferation or activation of the peptide-specific CD4⁺ T cells, showing that these are antigen dependent (data not shown).

TolDCs Ameliorate Proteoglycan Induced Arthritis

To study if tolDCs are able to affect arthritic symptoms, we performed *in vivo* arthritis studies in the proteoglycan induced arthritis (PGIA) model. Female Balb/c mice were injected with hPG protein and dimethyl-diocetadecylammonium (DDA) two times intraperitoneal with a 3-week interval to induce arthritis. Non-loaded tolDCs or tolDCs loaded with hPG peptide were injected intravenously on day 17 in the pre-clinical phase of disease. As pre-clinical phase is considered the stage in which the mice received the first immunization with hPG protein and DDA. In these 2 weeks before the second hPG/DDA injection, the mice develop antibodies against hPG but do not experience symptoms yet (28). This is comparable to humans in whom autoantibodies can be detected years before symptoms occur. Mice treated with itolDCs showed equal arthritis scores compared to the PBS mice (**Figure 5A**). However, treatment with non-loaded mtolDCs as well as mtolDCs loaded with hPG resulted in a reduced and delayed development of arthritis (**Figure 5B**). No significant difference was observed between mice that were injected with non-loaded mtolDCs or mtolDCs loaded with hPG peptide. These results indicate that a maturation stimulus is needed for tolDCs to be effective *in vivo*. Taking the day of onset and maximum arthritis score into account (**Table 1**, data combined from two independent *in vivo* studies), the mice that were treated with mtolDCs not only show less arthritis but develop symptoms at a later time point than PBS mice.

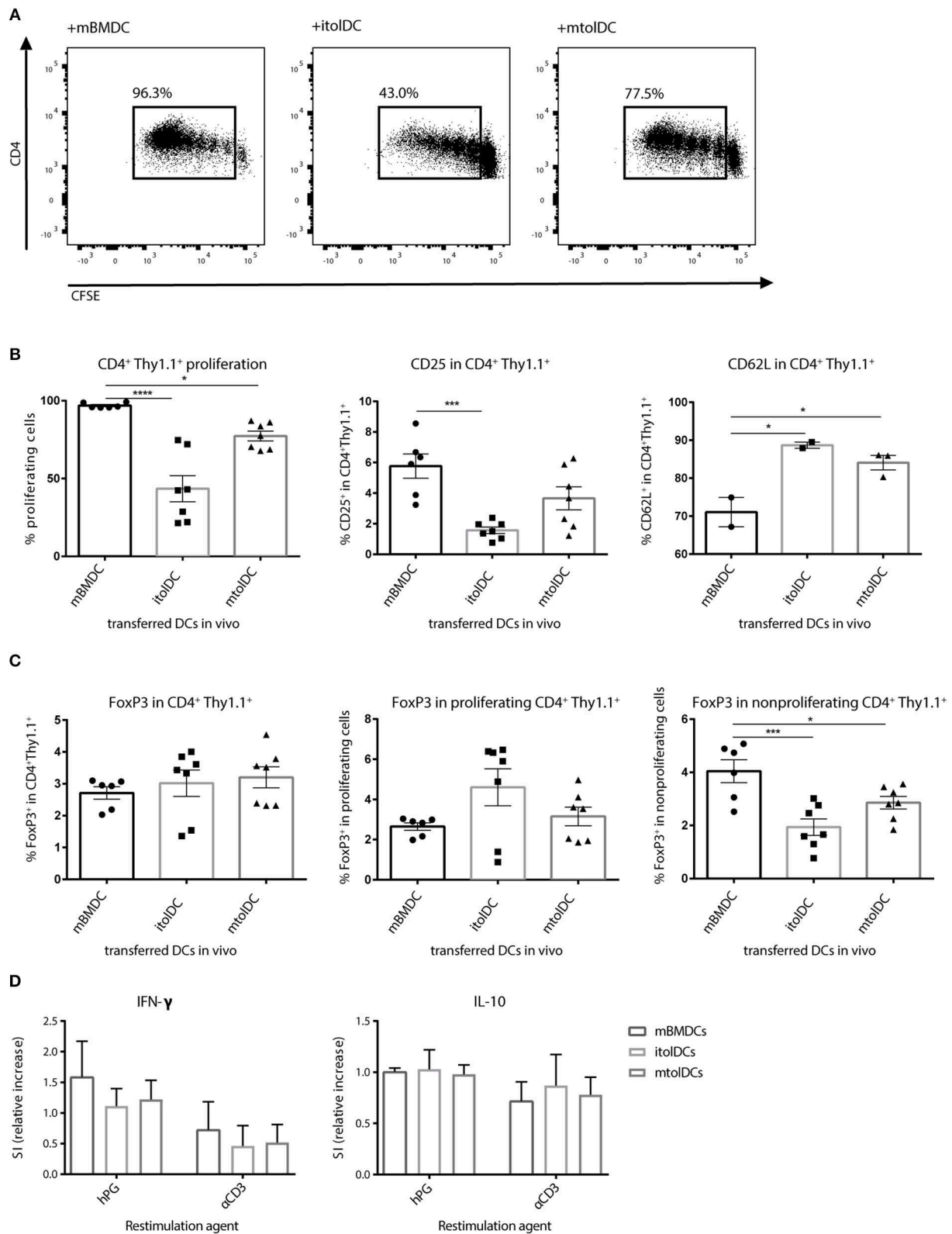
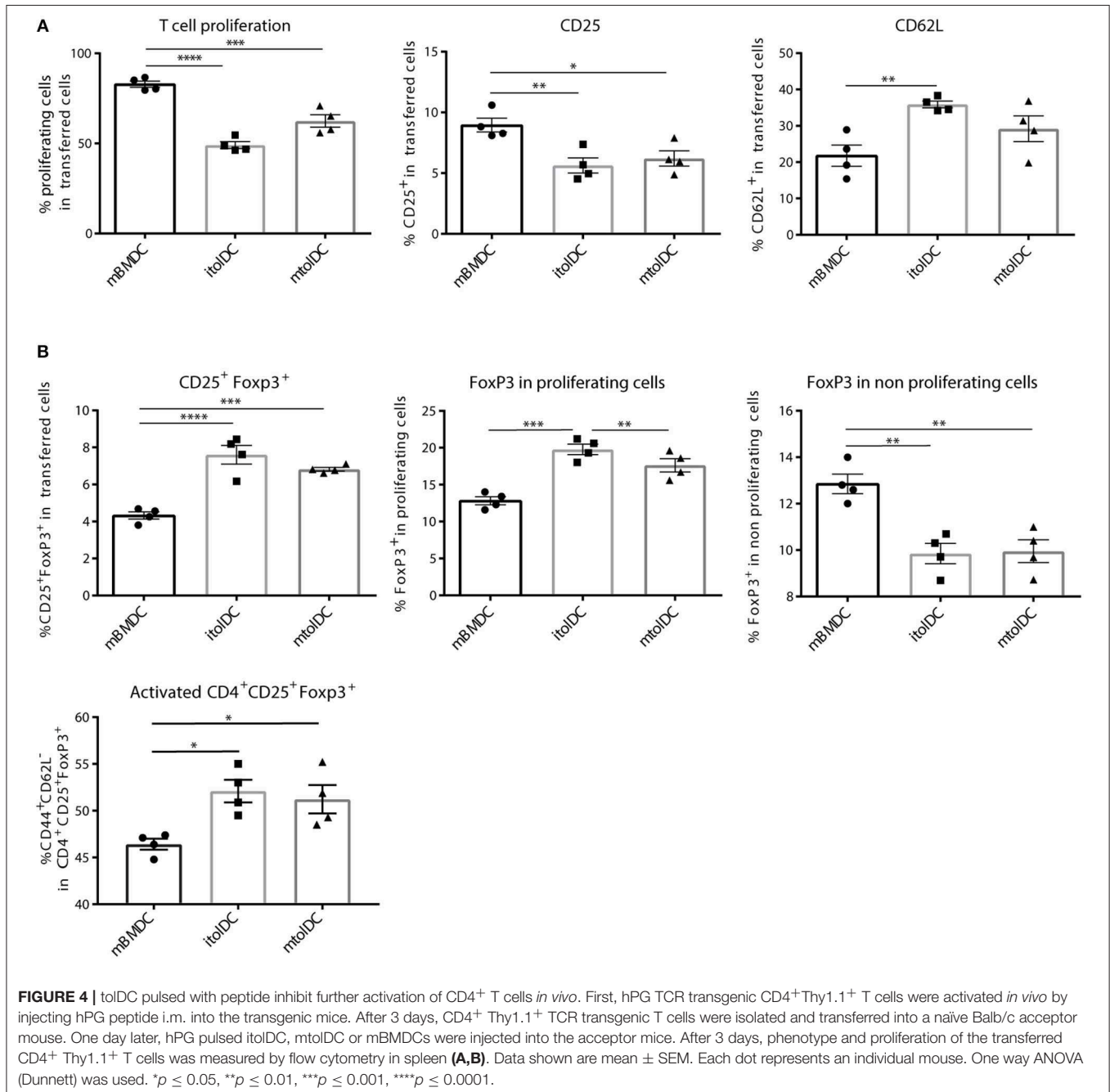


FIGURE 3 | tolDCs restrict the activation of naïve antigen specific CD4⁺ T cell *in vivo*. (Im)mature tolDCs or mature BMDCs were pulsed with hPG peptide and transferred (1×10^6 cells/ injection) 1 day after the naïve CFSE labeled hPG TCR transgenic CD4⁺ T cells. Proliferation (A,B) and phenotype (B,C) from the

(Continued)

FIGURE 3 | transferred $CD4^{+}Thy1.1^{+}$ T cells was measured by flow cytometry. To further examine the activation status of the transferred $CD4^{+}Thy1.1^{+}$ T cells, splenocytes were stimulated with hPG (antigen specific) or soluble $\alpha CD3$ (general). After 3 days, $IFN\gamma$ and IL-10 producing cells were measured by ELISpot (**D**). Data shown are mean \pm SEM. Each dot represents an individual mouse. Data are from three independent experiments. One way ANOVA (Dunnett) was used. $*p \leq 0.05$, $***p \leq 0.001$, $****p \leq 0.0001$.



Ex vivo analyses of spleen and draining lymph nodes (popliteal lymph nodes) show that Tregs (defined as $CD4^{+}CD25^{+}FoxP3^{+}$ cells) are present in lower quantities in the spleen (**Figure 5C**) in mtolDC treated mice as compared to PBS mice (controls). However, in the popliteal lymph node there are more

$CD4^{+}CD25^{+}FoxP3^{+}$ cells present only in mice that received antigen-pulsed mtolDCs compared to PBS mice (**Figure 5C**, **Supplementary Figure S5**). The arthritis experiments described show that tolDC treatment can be effective if tolDCs are stimulated with MPLA prior to infusion.

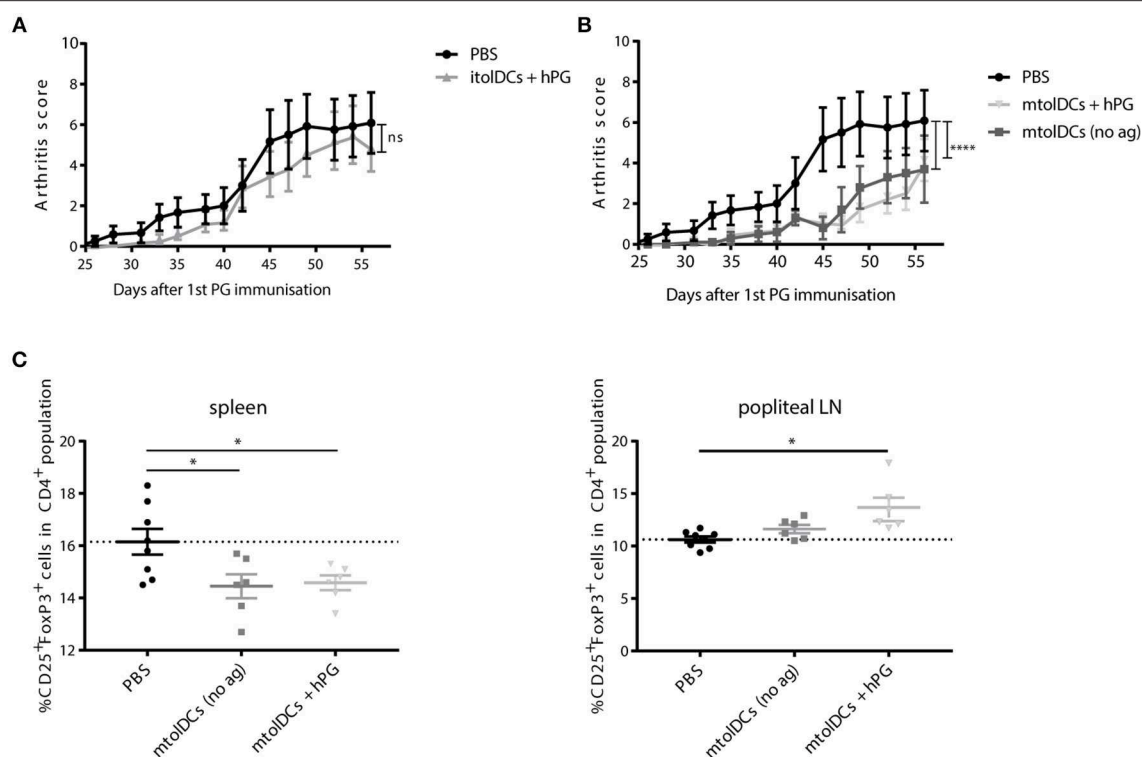


FIGURE 5 | hPG loaded or non-loaded tolDCs matured with MPLA ameliorate PGIA. Arthritis was induced by injecting the mice two times (day 0 and day 21) with hPG protein and DDA. TolDCs (1×10^6 cells in 200 μ L PBS) were administered intravenously on day 17, before the second hPG/DDA injection. Mice were scored three times a week for arthritis. The arthritis scores are based on a visual scoring system. Mice received either PBS, immature tolDCs (itolDC) (A) or mtolDCs (B) non-loaded or pulsed with disease specific antigen (hPG). Data shown in A and B are from one *in vivo* experiment. PBS: $n = 6$, mtolDCs no ag: $n = 5$, mtolDCs + hPG: $n = 7$ (C) The percentage CD4⁺CD25⁺FoxP3⁺ (Treg) was analyzed *ex vivo* in the spleen and popliteal lymph nodes by flow cytometry. Data shown are mean \pm SEM. Each dot represents an individual mouse. Two way ANOVA (Dunnett) was used to compare all three groups in an unbiased fashion. * $p \leq 0.05$, **** $p \leq 0.0001$.

TABLE 1 | MtolDCs ameliorate PGIA and delay the onset of disease.

Group	Day of onset	Maximum arthritis score	Total number of mice (n)
PBS	35.36 \pm 8.0	5.82 \pm 3.52	14
mtolDCs (no ag)	41.27 \pm 7.45	3.36 \pm 3.44	11
mtolDCs + hPG	41.85 \pm 7.49	3.269 \pm 2.48	13

MtolDCs reduce arthritis scores (maximum arthritis scores) and delay the onset of PGIA (day of onset) when compared to the PBS mice. As day of onset is considered the first day that a mouse has a score of 1. Two-tailed paired student T-test was used, no significant differences.

Matured tolDCs Are Able to Take Up Antigen *in vivo*

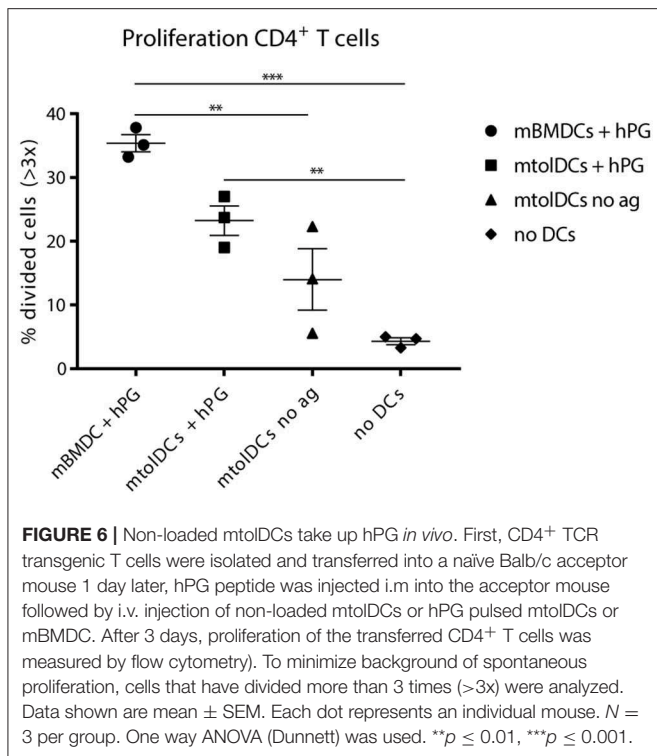
We observed in the arthritis experiments (Figure 5) that non-loaded mtolDCs are also able to lower arthritic symptoms. To investigate if this was due to general immunosuppressive factors (e.g. cytokines) or antigen dependent, we performed a co-transfer experiment in which we tested if mtolDCs are able to take up hPG *in vivo*. Therefore, hPG TCR transgenic CD4⁺Thy1.1⁺ T cells were injected into an acceptor mice followed 1 day later by an intramuscular injection with hPG peptide and an intravenous

injection with mtolDCs or mBMDCs. To study if non-pulsed mtolDCs could internalize hPG *in vivo*, we included a condition in which the acceptor mice received no DC injection but only the hPG TCR transgenic CD4⁺Thy1.1⁺ T cells.

The mice that received no intravenous (tol)DC injection show 3–5% CD4⁺ T cells that proliferated adequately (Figure 6). This is most likely proliferation induced by APCs from the recipient mouse presenting hPG. The mice that were injected with non-loaded mtolDCs (“mtolDCs no ag” in Figure 6) show 5–22% proliferating CD4⁺ T cells. This indicates that the non-pulsed mtolDCs indeed are able to take up hPG *in vivo* and actively presented it to CD4⁺ T cells. The mice treated with hPG loaded mBMDCs and mtolDCs show similar responses as in Figures 2, 3.

DISCUSSION

TolDCs are potentially useful for inducing immune tolerance in RA. In this study we addressed two questions: (i) is tolDC maturation required for their function *in vitro* and *in vivo*, and (ii) can tolDCs modulate CD4⁺ T cell responses. TolDCs are able to hamper activation and proliferation of naïve and effector CD4⁺ T cells *in vitro*



or under immune homeostatic circumstances. However, tolDC maturation is required under inflammatory conditions to inhibit arthritis.

Since maturation of tolDCs induces metabolic changes in the DC which might be necessary for stabilization, cell survival and function of tolDCs (9, 15, 29), we tested both non-stimulated and MPLA stimulated tolDCs. Furthermore, we also tested LPS stimulated tolDCs on phenotype and functionality which gave similar results to MPLA stimulated tolDC (Supplementary Figure S6). In addition, mtolDC phenotype remained stable even in the presence of a pro-inflammatory cytokine mix consisting of IL-1 β , GM-CSF, IL-6, TNF, and IFN γ *in vitro* (data not shown).

As shown in Figure 5, tolDCs need stimulation with MPLA to exert their function in the arthritis model. However, itolDCs were also efficient in modulating the CD4⁺ T cell response in the co-transfer studies. We hypothesize that this difference is caused by the proinflammatory milieu in the arthritis experiments, which is not present in the *in vivo* co-transfer experiments. itolDCs are able to modulate CD4⁺ T cells in these “neutral” environments, but when a proinflammatory milieu is present they are not potent enough to induce tolerance and/or disease suppressing responses or cannot reach the inflammatory sites due to a lack of migration.

To answer the second question, if tolDCs can modulate CD4⁺ T cells, we investigated how tolDCs influence CD4⁺ T cells to adopt a tolerant phenotype. As shown in the co-transfer studies, tolDCs abrogated not only the activation and proliferation of naïve CD4⁺ T cells (Figure 3) but also inhibited

further activation of pre-activated CD4⁺ T cells (Figure 4). Next to effects on CD4⁺ T cell activation, we have shown that tolDCs also affect CD4⁺CD25⁺FoxP3⁺ cells (Tregs). The observation locally enhanced numbers of CD25⁺FoxP3⁺ are present (Figure 5C) can imply that Tregs migrate to the site of inflammation under the influence of mtolDCs, however intrinsic differences in Treg homeostasis in the different lymphoid organs cannot be excluded (30). Furthermore, the co-transfer studies indicate that tolDCs have the potential to activate Tregs (Figure 4).

Currently, there is still a lot of debate about antigen loading of tolDCs. In our experiments antigen loading did not significantly enhance the efficacy of treatment. Both the non-loaded mtolDC as the hPG loaded mtolDC injections caused reduced and delayed arthritis symptoms in the treated mice (31). However, this antigen-independence might be evoked by the fact that the mtolDCs were administered before the second injection with hPG/DDA. A possible explanation that the non-loaded mtolDCs reduced arthritis, is that they took up hPG *in vivo* since by injecting hPG/DDA a depot is formed thereby inhibiting arthritis in an antigen-specific manner. This hypothesis is supported by the data shown in figure 6. In addition, preliminary studies have shown that if we add supernatant from cultured tolDCs to antigen presenting cells in the presence of CD4⁺ T cells *in vitro*, the proliferation of the CD4⁺ T cells is abrogated (unpublished observations), indicating that the tolDCs exert their function, at least partly, via soluble mediators (e.g., cytokines).

In conclusion, tolDCs abrogate activation and proliferation of both naïve as pre-activated CD4⁺ T cells and potentially activate Tregs. Although itolDCs are effective under immune homeostasis, maturation of the tolDCs is needed to inhibit experimental arthritis.

ETHICS STATEMENT

All experiments were approved by the Animal Experiments Committee of Utrecht University (project number AVD108002016467).

AUTHOR CONTRIBUTIONS

MJ designed and performed experiments, analyzed data, wrote the paper, and approved the submitted version. RS and CH analyzed data, commented on the manuscript at all stages, and approved the submitted version. IL performed experiments, commented on the manuscript at all stages, and approved the submitted version. WvE commented on the manuscript at all stages and approved the submitted version. FB designed experiments, analyzed data, commented on the manuscript at all stages, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02068/full#supplementary-material>

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Conflict of Interest Statement: WvE has shares in Trajectum Pharma, Inc., a SME that develops HSP peptides for immunotherapy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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