



Regulatory T cell-derived exosomes: possible therapeutic and diagnostic tools in transplantation

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Exosomes are extracellular vesicles released by many cells of the body. These small vesicles play an important part in intercellular communication both in the local environment and systemically, facilitating in the transfer of proteins, cytokines as well as miRNA between cells. The observation that exosomes isolated from immune cells such as dendritic cells (DCs) modulate the immune response has paved the way for these structures to be considered as potential immunotherapeutic reagents. Indeed, clinical trials using DC derived exosomes to facilitate immune responses to specific cancer antigens are now underway. Exosomes can also have a negative effect on the immune response and exosomes isolated from regulatory T cells (Tregs) and other subsets of T cells have been shown to have immune suppressive capacities. Here, we review what is currently known about Treg derived exosomes and their contribution to immune regulation, as well as highlighting their possible therapeutic potential for preventing graft rejection, and use as diagnostic tools to assess transplant outcome.

Keywords: regulatory T cells, exosomes and immune modulation

TREG EXOSOMES – IMMUNE MODULATORS

Exosomes are small, cup-shaped, secreted membrane vesicles (approximately 50–100 nm in diameter) that are formed by the inward budding of endosomal membranes (1–6). Exosomes are released into the extracellular environment following the fusion of multivesicular endosomes with the plasma membrane (7). Several proteins involved in their biogenesis and release have been described and have recently been reviewed by Colombo et al. (7). Exosomes released by many immune and non-immune cells have been shown to have a range of physiological properties within the immune system. These include antigen presentation, immune regulation, and programmed cell death, each of which is linked to the cell from which they are released (6, 7). They play an important role in intercellular communication and can act as shuttles for transferring proteins, miRNA, mRNA, and cytokines from one cell to another (8).

Many cells of the body produce these extracellular vesicles (EVs) including those of the immune system such as CD4⁺ and CD8⁺ T cells, B cells, and dendritic cells (DCs). Exosomes from these cells have been shown to mediate either immune stimulation (DCs) or immune modulation (T cells) (9–14). Recently, the release of exosomes by murine CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), following TCR activation, was shown, initially by Smyth et al. (15) and later by Okoye et al. (16). In addition to CD4⁺CD25⁺Foxp3⁺ cells, other murine T cells with regulatory capacities were found to also release exosomes following activation. Bryniarski et al. observed that “exosome like” particles were present in the supernatants of cultured CD8⁺ T cells with suppressive capacity (17), whilst Xie et al. observed that CD8⁺CD25⁺Foxp3⁺ T cells secreted exosomes capable of inhibiting DC induced CD8⁺ CTL responses (18).

Exosome production by murine CD4⁺CD25⁺Foxp3⁺ Tregs appears to be quantitatively greater than other murine T cells, including naïve CD4⁺ and CD8⁺ T cells, T helper 1 (Th1), and Th17 cells, and is regulated by changes in intracellular calcium, hypoxia, and sphingolipids ceramide synthesis, as well as in the presence of IL-2 (16). Exosomes contribute significantly to the function of murine CD4⁺CD25⁺FoxP3⁺ Tregs, inhibiting the release of exosomes reversed these cells suppressive capabilities (16). In parallel, murine Tregs exosomes were found to be immune modulatory. Reduced CD4⁺ T cell proliferation and cytokine (IL-2 and IFN γ) release was observed in their presence *in vitro* (15). The suppressive nature of Treg exosomes, in one study, has been attributed to the ectoenzyme CD73 (15). The loss of CD73 on Treg exosomes reversed their suppressive nature. Expression of both CD39 and CD73 on Tregs contributes to immune suppression through the production of the anti-inflammatory mediator adenosine (19–21). Binding of this molecule to adenosine receptors A2aR, expressed by activated T effector cells (Teffs) triggers intracellular cAMP leading to the inhibition of cytokine production, thereby limiting T cell responses (22). Given that adenosine was produced following incubation of CD73 expressing Treg exosomes with exogenous 5'AMP it is feasible that the release of exosomes expressing CD73 within the local environment increases the surface area by which this membrane-associated enzyme, and ultimately Treg suppression, can function (15).

Several molecules associated with immune modulation including CD25 and CTLA-4, were also found on CD4⁺CD25⁺Foxp3⁺ Treg exosomes (15). Nolte-t Hoen et al. have previously shown that exosomes, derived from anergic rat T cells, inhibited Teffs responses following co-culture with B cells and DCs *in vitro* (23).

These T cell-derived exosomes expressed high levels of CD25 and the authors suggested that CD25 expressing exosomes, binding to the surface of an antigen presenting cells (APC), bestows that cell with the ability to bind free IL-2 in the local environment leading to depletion of available cytokines and apoptosis of Tregs (23). Although CD25 expression was observed on Treg exosomes, this molecule may not play a role in their suppressive function given the observation that exosomes isolated from a T cell line, incapable of suppressing proliferation or cytokine production of CD4⁺ T cells, in the presence of B cells, expressed similar levels of CD25 to Treg exosomes with regulatory function (15). A redundant role for CTLA-4 molecules has also been reported. Although present on Treg exosomes, blocking CTLA-4 did not modulate their suppressive function (15). So far, no molecules have been associated with the regulatory capacity of CD8⁺25⁺FoxP3⁺ exosomes (18).

Recently, the transfer of miRNAs contained in T cell exosomes has been shown to affect the function of recipient APCs by inhibiting translation of target mRNA molecules (14, 24). Likewise, the transfer of miRNAs, including Let-7d, miR-155, and Let-7b, to Tregs through the acquisition of CD4⁺CD25⁺Foxp3⁺ Treg exosomes has been shown (16). Inhibiting Let-7d expression in Treg exosomes reversed the suppressive nature of these vesicles suggesting that miRNAs present in Treg exosomes may also play a role in their suppressive capacity (16). These findings confirm those of Bryniarski et al. (17) who observed the targeted delivery of an inhibitory miRNA, miR-150, to Tregs using exosomes isolated from CD8⁺ T cells with suppressive capacity.

Several molecules present on exosomes isolated from Tregs, DCs, and B cells have been shown to have immune modulatory properties. Whether they also contribute to the suppressive nature of Treg exosomes has yet to be validated. For example, expression of FasL on murine CD8⁺ T cell exosomes induced death of APCs (12, 25), in addition, FasL-expressing exosomes isolated from DCs, genetically modified to express FasL, suppressed antigen-specific immune responses *in vivo* (26) and lastly, MHCII⁺FasL⁺ exosomes constitutively produced by a human B cell-derived lymphoblastoid cell lines induced apoptosis in CD4⁺ T cells (27). Murine and human CD4⁺25⁺ Tregs express FasL (28). Whether FasL is expressed on Treg exosomes and contributes to the death of Tregs is yet to be tested. Other molecules, present on Tregs such as the inhibitory cell surface ligand programmed cell death 1 ligand 1 (PDL-1) and Galectin-1 (29–31) may also be present on Treg exosomes. PDL-1 was found on mesenchymal stem cell EVs (32) and exosomes have been identified as transport vehicles for the secretion of molecules that lack a signal sequence such as Galectin-1 (33). Not only is this molecule highly expressed on Tregs it is essential for their function (34).

Regulatory T cells produce immune modulating cytokines such as IL-10, IL-35, and TGFβ (35). Presently, it is unknown whether these cytokines are contained in Treg exosomes however, expression of IL-10 and TGFβ in exosomes isolated from DCs, transduced to express these cytokines, has been shown (36, 37) as has surface TGFβ on MSC derived EVs (32). Given the aforementioned it is a theoretical possibility, that Treg exosomes may contain one or more of these cytokines.

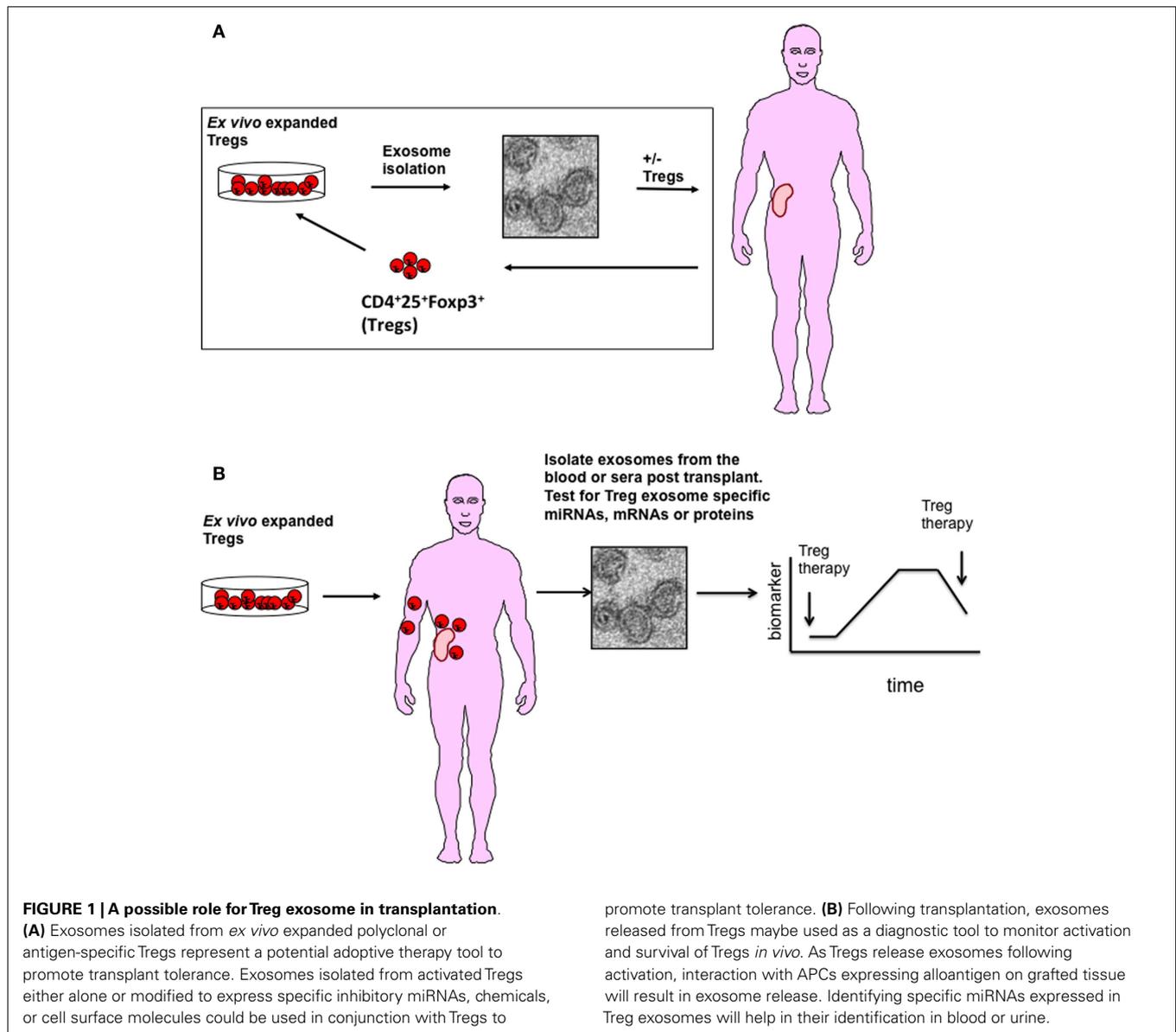
ROLE OF Treg EXOSOMES IN TRANSPLANTATION POSSIBLE THERAPY?

In 1990, Hall et al. observed that the adoptive transfer of CD4⁺CD25⁺ T cells resulted in long-term cardiac allograft survival in cyclosporine-treated rats (38). Since then this field of immunotherapy has been intensely studied in mouse (39–41), and recently in preclinical humanized mouse models (mice reconstituted with a human immune system and transplanted with human skin or human pancreatic islets of Langerhans) (42, 43). In the latter, human CD4⁺25⁺Tregs, expanded with anti-CD3/28 antibody coated beads, have been found to prolong islet transplant survival and function (42, 44). These positive outcomes have led to the application of human Tregs for the prevention of graft versus host disease (GvHD) and to promote transplant tolerance (45–48). Currently, several organizations around the world are investigating the use of CD4⁺CD25⁺ Tregs to promote “tolerance” to transplanted organs. At King’s College London, UK, phase I/II clinical trials are currently under way to test the safety and efficacy of using these cells in human kidney (One Study) and liver (ThRIL) transplant patients. Other clinical trials using human Tregs are also underway and are described elsewhere (49). Presently, we do not know the efficacy and efficiency of Tregs in these trials. Although Tregs are now being used in patients how they function *in vivo* is still unknown.

Given their immune modulatory capacity, the question arises, what is the contribution of Treg exosomes to transplant tolerance seen in the preclinical mouse models and can Treg exosomes be used *in vivo* as an alternative/or complementary therapy? At present, we are a long way away from using Treg exosomes in man given that the optimal Treg subset required to induce transplant tolerance is as yet unknown, as is whether they prolong graft survival in a patient setting. So why should we consider these EVs as a therapy? Several studies have suggested that inflammatory environments can subvert human Foxp3⁺Treg cell function by converting them to Tregs *in vivo* (50, 51). However, unlike Foxp3⁺ Tregs, adoptive transfer of human Treg exosomes are unlikely to be modified during inflammatory conditions *in vivo* (1) making them an ideal immune modulatory reagent (Figure 1A).

Several lines of evidence exist, some preliminary, some not, suggesting that studying these vesicles for this purpose is worthwhile, albeit challenging. So far, Yu et al. are the only group that have investigated the use of Treg exosomes as a therapy in a transplantation setting (52). These authors observed that the adoptive transfer of autologous rat Treg exosomes, post transplant, prolonged both survival and function of kidney allografts (52). Suggesting that Treg exosomes may represent an exciting new therapy for the induction of transplant tolerance.

Can this observation be translated into a human setting? Using preclinical methods to isolate and expand human Tregs, from peripheral blood of health individuals (53), we have successfully identified the release of CD63 and CD81 expressing exosomes from CD4⁺CD25^{hi}Foxp3⁺ suppressive human Tregs, following TCR activation (Agarwal et al., personal communication). Whether human Treg exosomes display molecules that can modulate the immune response *in vivo* is still being assessed. However, given that Jurkat CD4⁺ T cells (a human T cell line) as well as human



CD3⁺ T cells, isolated from PBMCs, produce exosomes (54–56) containing molecules with potential immune regulatory effects, such as TCRs (54) and CTLA-4 (56) the possibility that human Treg exosomes contain immune regulatory molecules is very high.

Two phase I clinical trials using exosomes isolated from immature DCs have been conducted in advanced stage melanoma and MAGE-expressing non-small cell lung cancer patients (57–59). Despite a lack of antigen-specific T cell responses, stable disease was observed in some patients with tumor regression reported in one patient following treatment (60–62). These positive outcomes have paved the way for Phase II clinical trials using exosomes isolated from LPS or IFN γ activated DCs in non-small cell lung cancers. These studies have validated the efficacy and safety of exosomes as a therapy in man. In spite of these encouraging findings, several key limitations pertaining to the use of exosomes cannot be ignored. Firstly, at present there is no standardized protocol

for isolating and analyzing “pure” exosomes (7). Contamination from other EVs as well as membrane free aggregates may be an issue depending on the isolation method used. Therefore, careful analyses of the purified exosomes will be required before administration. This will require the use of expensive equipment such as EM and Nanosight, which are not always readily available (7, 63). Secondly, given that exosome release by Tregs is not constitutive and requires activation using anti-CD3/CD28 antibodies, the possibility that these antibodies contaminate Treg exosome preparations is as yet untested. Additionally contaminating molecules, for example, proteins/cytokines present in media, may pose a potential problem especially as exosomes will be isolated from culture supernatants. Thirdly, the quantity of exosomes isolated and the amount required for therapy purposes are at present unknown, as is whether large-scale production of Treg exosomes is actually possible. Lamparski et al. published that 1.8–5.8 mg

of exosomes could be isolated from human monocyte derived DCs, expanded from peripheral blood leukopacks (originally containing $12\text{--}25 \times 10^9$ cells) highlighting the feasibility of large-scale production of DC exosomes (64). However, DCs produce these EVs vesicles constitutively making their production easier than those from Tregs, which are isolated only after activation (65). Yu et al. obtained $117 \mu\text{g}$ of exosomes from 4×10^9 freshly isolated rat Tregs, following activation, and the administration of $33 \mu\text{g}$ of exosomes, given over 3 time points, was sufficient to prolong the lifespan of a kidney transplant (52). Whether large quantities of pure exosomes can be isolated from human Tregs grown under GMP conditions is as yet unknown. Lastly, what happens to Treg exosomes *in vivo*, which cells acquire them and whether is it receptor driven is poorly understood. Recently, Teffs were shown to acquire Treg exosomes (16) whilst exosomes from EL4, a T cell lymphoma, have been shown to be preferential acquired by macrophages (66), perhaps via the CD169 pathway (67). Therefore, *in vivo* analysis of Treg exosomes is essential before they can be used in a clinical setting. Until all of these factors are addressed, using Treg exosomes in a transplant setting remains challenging and potential advantages remain at present theoretical.

DIAGNOSTIC TOOL?

Biomarkers are quantitatively, measurable biological parameters that help indicate health and disease. The use of exosomes as biomarkers is a relatively new concept. Although it has not yet reached clinical practice, it is one area of exosome research that is rapidly expanding, with many clinical trials focusing on their use as a diagnostic tool, particularly for cancer (Table 1). Several factors make exosomes suitable for this purpose, firstly, they travel through the bloodstream and can be isolated from plasma, serum, and urine (68, 69). Secondly they receive surface markers from the cell from which they are derived, such that they can be identified and isolated. Lastly, they express unique miRNA and mRNA (Table 1).

Valadi et al. were the first group to publish that exosomes contained RNA (8). Exosome RNA is small, typically of about 200

bases in length and lacks the 18S and 28S RNA found in cells (74). Different RNA species including small ribosomal RNA, specific tRNA fragments, long interspersed elements, and long terminal repeats, have all been found in exosomes (75). Additionally, and as discussed earlier, there is also a selective enrichment of specific miRNAs into exosomes (24, 76). The miRNA repertoire of an exosome is generally different to that of the parent cell, suggesting that exosome packaging is an active process (14). In T cells, for example, Rossi et al. identified a set of 20 miRNAs of which only 2 were differentially expressed in T_H cell-derived exosomes (77). Upon activation primary $CD4^+$ T cells down-regulate their miRNA content. Some of these miRNAs accumulate in exosomes, for example, miR-150, suggesting that the cell may be shedding miRNA as part of a regulation step (70). de Candia et al. quantified the amount of miR-150 present in sera isolated from mice immunized with OVA plus an adjuvant, and reported an increased level of this miRNA in immunized mice as compared to non-immunized mice (70, 78). When they removed $CD4^+$ T cells no elevated miR-150 levels were observed. They next validated this observation using sera collected from adults and children vaccinated with the 2009 pandemic flu (H1N1) vaccine. Similar to the mouse model, they observed that miR-150 was evident in the sera following vaccination, and that this miRNA was associated with lymphocyte derived exosomes. In addition, increased levels of miR-150 correlated with high antibody levels post vaccine, suggesting a link between activation of the adaptive immune responses and expression of a specific miRNAs in exosomes (70, 78). From the adoptive cellular therapy point of view, this data is very exciting as it highlights the possibility of using exosomes to monitor cellular therapies such as Tregs *in vivo*. Given that Tregs produce exosomes only following activation, and in the case of transplantation this will be following recognition of alloantigen presented by donor and recipient DCs, it may be possible to assess Treg viability and function *in vivo* by monitoring Treg exosomes in the blood of transplant recipients. If this is possible Treg exosomes may be unique biomarkers for immune suppression (Figure 1B).

As mentioned earlier in addition to miRNA, mRNA, and proteins associated with exosomes can also act as diagnostic tools. For example, in patients with kidney disease CD2AP mRNA was associated with urinary exosomes (79). Several specific proteins have been identified in exosomes isolated from: (1) the urine of healthy individuals (CD24 and Aquaporin 2) (80), (2) sera from cancer patients (MUC1, LRG1, Hsp90a, and RAD21) (81), (3) the placenta (syncytin-1) (82), and 4) from patients with multiple sclerosis (IB4) (83). Taken together, these studies suggest the importance of validating the expression of mRNA and proteins, in addition to miRNAs, in Treg exosomes if unique biomarkers are to be identified.

In conclusion, at present Treg exosomes are still in their infancy with regard to transplantation, either as a therapy or a diagnostic tool. As outlined in this review, several key questions regarding their composition and function need to be addressed. In addition, better isolation and analysis protocols, as well as preclinical models are required before Treg exosomes can make the transition from the lab to the clinic, even for diagnostic purposes. Although some of the ideas presented here are speculative, pursuing the use of Treg exosomes for immune modulation and diagnostic purposes

Table 1 | miRNAs present in exosomes isolated from the sera of patients with specific cancers or following immunization are being used as diagnostic biomarkers.

miRNA identified in exosomes	Cells origin	Reference
miR-150	$CD4^+$ T cells	(70)
miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214	Ovarian cancer	(71)
miR-205, miR-19a, miR-19b, miR-30b, and miR-20a	Lung squamous cell carcinoma	(72)
let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a	Colon cancer	(73)
hsa-miR-31, miR-185, and miR-34b	Melanoma	(44)

within a transplantation setting is timely given that clinical trials are now underway using Treg cells themselves.

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