THE ROLE OF IMMUNOMETABOLISM IN AUTOIMMUNE MEDIATED AND AUTOINFLAMMATORY DISORDERS

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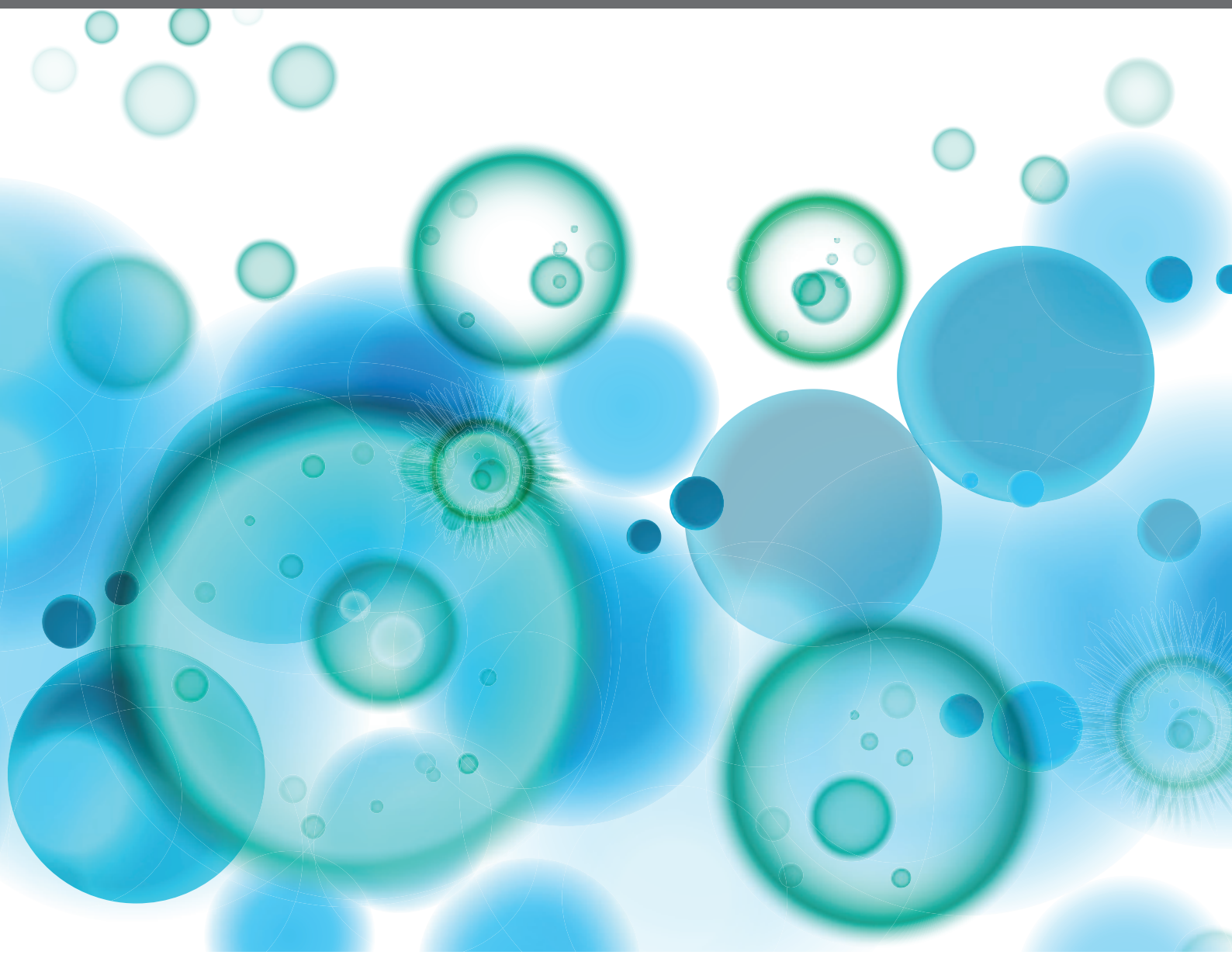

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THE ROLE OF IMMUNOMETABOLISM IN AUTOIMMUNE MEDIATED AND AUTOINFLAMMATORY DISORDERS

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Editorial: The role of immunometabolism in autoimmune mediated and autoinflammatory disorders

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Editorial on the Research Topic
The Role of Immunometabolism in Autoimmune Mediated and Autoinflammatory Disorders

Over the last years, cell metabolism has become one of the most exciting areas of investigation in the field of immuno-rheumatology. Convincing evidence has revealed that metabolic pathways closely regulate cell activities and immune as well as stromal cells adopt distinct metabolic programs to sustain their function in order to cope with environmental demands. It has been shown that dysregulated cell metabolism contributes to the development of several autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS) where current treatment options are effective only in some patients. Predictive biomarkers of prognosis and therapeutic response remain inadequate from a clinical perspective. Thus, new therapeutic options as well as disease predictors are needed. Our aim in assembling this Research Topic is to highlight the current understanding of the role of cell metabolism in regulating immune responses in health and in autoimmunity with the purpose to find opportunities for clinical translation.

Qiu et al., focus on metabolic abnormalities of T cells from RA patients. They discuss how metabolic dysregulation is present in the naïve population and sustained in tissue-residing memory T cells, placing metabolic dysregulation upstream of the joint. Similar to RA T cells, type 1 diabetes (T1D) CD4+ T cells exhibit a pro-inflammatory phenotype which is accompanied by an enhanced glycolytic metabolism (Martins et al.) Martins et al., showed that targeting glycolysis with the use of the small molecule PFK15, a competitive inhibitor of the rate limiting glycolysis enzyme 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3 (PFKFB3) was able to dampen inflammation *in vitro* and *in vivo*, in an adoptive transfer model of T1D.

Similar to T cells, synovial like fibroblasts (FLS) also show metabolic dysregulation in autoimmune arthritis. O'Brien et al. show that janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling mediate a complex interplay between inflammation and cellular metabolism in psoriatic arthritis (PsA). The inhibition of this pathway with JAK inhibitor shows effective suppression of inflammatory mechanisms that drive pathogenic functions of PsA FLS. Similarly Falconer et al. found that RA FLS display an impairment of mitochondrial function which alter their capability to resolve inflammation. The role of mitochondria in the pathogenesis of RA is further discussed by Clayton et al. who illustrate the role of mitochondria in the pathogenesis of RA and how current and future therapeutic strategies can function through modulation of mitochondrial activity.

Targeting glygolytic enzymes has also revealed to be a powerful tool to reduce inflammation in RA and in a collagen induced arthrtits mouse model as outlined by Zuo et al. and Wang et al. respectively. Continuing on the field of arthritis, Tripolino et al. discuss the cause-effect relationship between arthritis and metabolic abnormalities with a focus on insulin signaling. They also offer their view on the effect of glucose-lowering agents on arthritis. On the same topic, Jutley et al., assess the relationship between an objective measure of systemic inflammation [C-reactive protein (CRP)] and both the serum and urinary metabolome in patients with newly presenting RA. These findings suggest that NMR spectroscopy is a valid tool for the identification of metabolic biomarkers in disease. New metabolic pathways are emerging as novel potential driver of inflammation in RA. In this vein, Zhao et al. speculate the possible role of ferroptosis in the pathogenesis of RA. Mormile et al. illustrate the multifaceted roles of formyl peptide receptors in promoting resolution or inflammation in RA. Moving toward other autoimmune diseases, De Luca et al. illustrate the link between IL1, metabolism, inflammasome activation and cardiovascular complications in systemic sclerosis. Nardone et al. discuss the relationship between gut microbiota and sarcopenia in inflammatory bowel disease. Robinson et al. highlight the latest understanding of the role of immunometabolism in SLE with particular focus on the role of abnormal mitochondrial function, lipid metabolism, and mammalian target of rapamycin (mTOR) signaling. Hwang et al. examined the impacts of changes associated with aging or metabolic abnormalities on populations of T and B cells and Sjogren's disease severity. Peruzzotti-Jametti discuss how cell metabolism and mitochondrial function govern the function of chronic active microglia and macrophages in neuroinflammatory conditions. Finally, Lin et al. in their review discuss the role of reactive oxygen species (ROS) in regulating interactions between innate and adaptive immunity in autoimmunity. Raza and Clarke offer an overview of B cell metabolism and autophagy in health and disease.

In conclusion, these are exciting times for those who are investigating cell metabolism during homeostasis and in autoimmunity. Gaining deeper understanding of how cell metabolism and immune responses regulate each other will lead to new insights on disease mechanisms and, eventually, to the development of novel therapeutic options.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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REVIEW
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Reactive Oxygen Species in Autoimmune Cells: Function, Differentiation, and Metabolism

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Accumulated reactive oxygen species (ROS) directly contribute to biomacromolecule damage and influence various inflammatory responses. Reactive oxygen species act as mediator between innate and adaptive immune cells, thereby influencing the antigen-presenting process that results in T cell activation. Evidence from patients with chronic granulomatous disease and mouse models support the function of ROS in preventing abnormal autoimmunity; for example, by supporting maintenance of macrophage efferocytosis and T helper 1/T helper 2 and T helper 17/ regulatory T cell balance. The failure of many anti-oxidation treatments indicates that ROS cannot be considered entirely harmful. Indeed, enhancement of ROS may sometimes be required. In a mouse model of rheumatoid arthritis (RA), absence of NOX2-derived ROS led to higher prevalence and more severe symptoms. In patients with RA, naïve $CD4^+$ T cells exhibit inhibited glycolysis and enhanced pentose phosphate pathway (PPP) activity, leading to ROS exhaustion. In this "reductive" state, $CD4^+$ T cell immune homeostasis is disrupted, triggering joint destruction, together with oxidative stress in the synovium.

Keywords: reactive oxygen species, autoimmunity, macrophage, T cell, rheumatoid arthritis, metabolism

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INTRODUCTION

Oxidative stress represents an imbalance between pro- and anti-oxidants, in favor of the former, and has generally been considered as potentially harmful, since it leads to phenomena including DNA damage, protein oxidation, and lipid peroxidation (1). Based on this dogma, items, such as antioxidant skin care products, natural foods, herbal medicine, and even vitamins have been in demand in recent decades. A typical example of the effects of oxidative stress is ROS-related cell aging. Strikingly, a recent study demonstrated that a modified oxidized form of cysteine residues in proteins is not elevated in old (80 weeks) compared with young (16 weeks) mice, providing strong evidence against the theory of oxidative aging, which involves accumulation of indiscriminate oxidation of biological macromolecules. This study found that protein oxidative state is tissue- and age-specific, and can influence various physiological networks. For example, reversible cysteine oxidation modification of hexokinase controls the flux of glycolysis and the PPP, and polymerization and dissociation of some protein complexes are also regulated by redox state. Researchers have proposed that oxidative modification site, rather than oxidative modification level, is the main target of anti-aging (2).

Numerous studies have demonstrated the role of oxidative stress in the pathogenesis of autoimmune disease, varying from biomacromolecule damage to pro-inflammatory responses (3); however, antioxidant supplements may be not beneficial for primary or secondary prevention (4, 5); indeed, beta carotene, vitamin A, and vitamin E supplements may increase mortality (6). In recent years, ROS has become widely regarded as a signaling molecule, involved in many immune cell relationships and functions (**Figure 1**). In this review, we discuss the importance of ROS in adaptive immune responses, and the damage to immune tolerance caused by excessive ROS elimination. Further, we provide a detailed review of the roles of redox regulation in the glycolytic/PPP equilibrium in $CD4^+$ T cells, focusing on rheumatoid arthritis (RA) as a model autoimmune disease.

ROS AND IMMUNE CELLS

ROS Production and Disease

There are two main physiological sources of ROS: the NADPH oxidase complex, NOX2, and mitochondria. NOX2 is a multi-component enzyme system, composed of three cytoplasmic protein subunits (p47phox, encoded by *NCF1*; p67phox, encoded by *NCF2*; and p40phox, encoded by *NCF4*), two transmembrane protein subunits (p22phox, encoded by *CYBA* and gp91phox encoded by *CYBB*), and a small GTP-binding protein (Rac) (7, 8). In mitochondria, ROS production occurs when O_2 receives an electron from the mitochondrial complex, which is a complex process influenced by the concentration of electron donors and O_2, and the reactions rate constants between them (9). Chronic granulomatous disease (CGD) is an inherited disease, characterized by non-functional NOX2. Patients with CGD always suffer from recurrent life-threatening infections, due to deficient neutrophil- and macrophage-mediated innate immune responses. Interestingly, patients with CGD patients also have an increased risk of developing autoimmune diseases, resulting from their adaptive immune response disorder (8). In contrast, mitochondrial disease pathology is invariably considered to involve elevation of ROS (10); however, a study found that double mutants of alternative oxidases and severe myopathic skeletal muscle-specific *COX15* gene mutation led to decreased ROS production, and consequent impairment of PAX7/MYOD-dependent muscle regeneration. This study indicated the benefits of mitochondrial ROS (mtROS) signaling and the potential hazards arising from ROS elimination (11).

ROS in Antigen Presentation

Mononuclear phagocytes and dendritic cell are the main professional antigen-presenting cells (APCs), in which exogenous antigens are proteolytically processed, then complexed, generally with MHC class II, or with MHC class I by a special process referred to as cross-presentation. NOX2-derived ROS in phagosomes can kill ingested pathogenic microorganisms and prevent excessive reduction of proteolysis and disulfide bond formation, by modulating the redox microenvironment, including the pH and oxidative modification of cysteine residues (12–14). In this way, the stability of effective epitopes of antigenic peptides and efficiency of their presentation are enhanced, so that APCs can better activate T cells. For example, activation of $CD4^+$ T cell clones is regulated by NOX2-derived ROS through alteration of phagosome cysteine cathepsin activity, based on the immunodominant peptide epitope presented in the context of MHC Class II (14). In contrast, dendritic cells from p47phox-null mutant NOD mice (a spontaneous mouse model of autoimmune diabetes) and patients with CGD showed reduced ability to activate $CD8^+$ T cells, due to antigen degradation and deficient antigenic peptide loading on MHC Class I (15). In addition, discovery of many oxidation autoantigens in APCs from individuals with autoimmune diseases indicated that ROS can change antigen structure directly, thus affecting T cell behavior (16, 17).

The other main source of ROS, MtROS may also influence the antigen presentation process in more complex ways. One study found that increased mtROS in aged murine dendritic cells (DCs) hampered the cross-presentation process, which could be restored by scavenging of ROS *in vitro*. This change is not influenced by phagocytosis function and pH (18); however, in plasmacytoid dendritic cells (pDCs), mtROS-dependent pH alkalization and antigen protection are key factors in induction of cross-presentation. This obvious difference may result from specialized toll-like receptor (TLR) activation and NOX2 independence of pDCs (19, 20). In pDCs, ROS also participates in responses to damage-associated molecular pattern (DAMP) molecules (such as mitochondrial DNA), and influences their capacity to stimulate pDCs (21). NOX2-derived ROS and mtROS may act synergistically, since one study found that, in macrophages that had already engulfed bacteria, mitochondria translocated and juxtaposed to the phagosome (22), and mtROS can be packaged by Parkin-based mitochondrial vesicles and transferred to bacteria-containing phagosomes (23).

Costimulatory molecules on the surface of APCs also influence the activation of adaptive immune cell. NOX2-derived ROS in dendritic cells endocytosing tumor cell-derived microparticles can upregulate the costimulatory molecules, CD80 and CD86, thereby activating $CD8^+$ T cells. The underlying mechanism involves generation of the ROS-activated calcium channel, Mcoln2, in the lysosomal membrane, leading to Ca^{2+} release and activation of the transcription factor EB (TFEB), which can bind to the promoters of the genes encoding CD80 and CD86 (24, 25); however, in human primary monocytes infected by Epstein-Barr virus, TLR signaling activation increases ROS production. Further, ROS is an important contributor to marked up-regulation of the inhibitory costimulatory molecule, PD-L1, leading to immune escape (25); interestingly, the antioxidants, N-acetyl cysteine (NAC) and apigenin, can offset this change (26). In addition, alloantibody-FcγR I/FcγR III-dependent ROS production in macrophages is an important mediator of humoral immune damage during liver graft rejection (27). Opsonization of IgG on IFN-γ-activated macrophages led to diminished phagosomal processing of proteins in a PKC/Syk- NOX2-dependent manner, which occurs at the level of the individual phagosome (28). Altered IgG subtype distribution and the resulting increase in IFN-γ production are observed in both

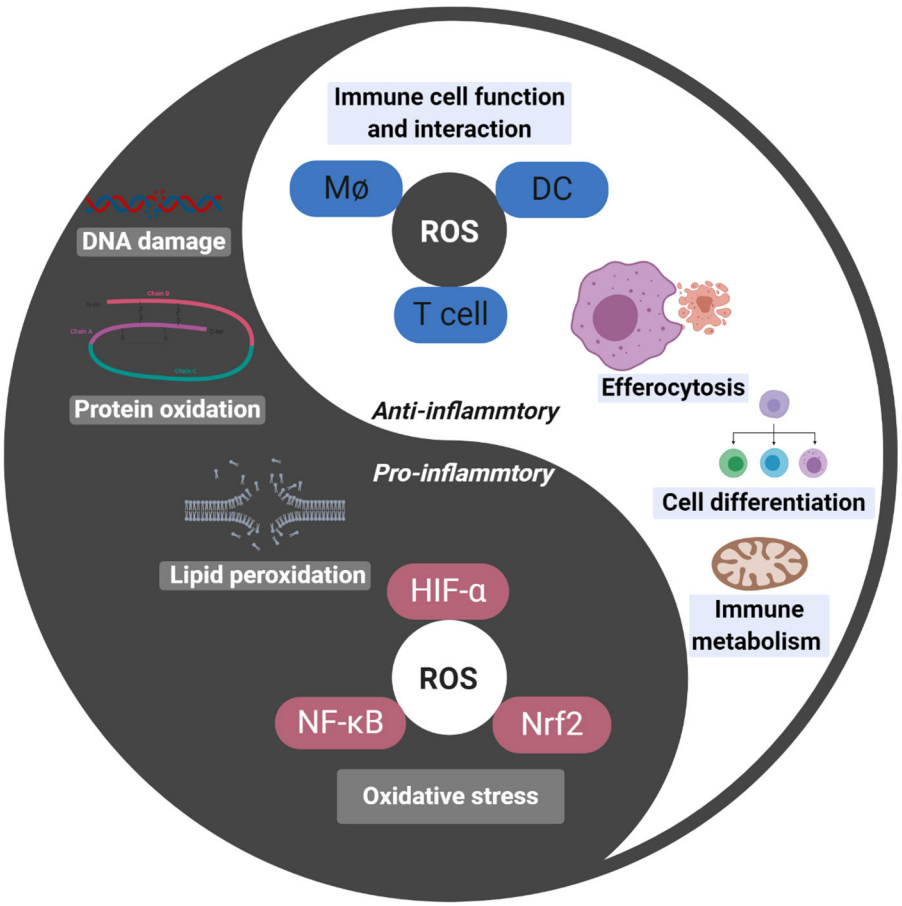

FIGURE 1 | Darkness (oxidative stress) and light (signal molecule) of ROS. Oxidative stress induced by ROS lead to DNA damage, protein oxidation and lipid peroxidation, thus injuring cells. ROS also are involved in HIF-α, NF-κB, and Nrf2 mediated pro-inflammatory response. ROS act as important signal molecular in cell, which connect innate immunity and adaptive immunity, as well as participate in cell biological behaviors like metabolism, differentiation, and apoptosis.

patients with CGD and a CGD mouse model (29), indicating a possible feedback loop involving IFN-γ, IgG, FcγRs, and NOX2.

ROS and Macrophage Efferocytosis

Macrophages are responsible for anti-pathogen immunity and ROS is a powerful weapon in this context. Reactive oxygen species promotes both M1 and M2 macrophage polarization (30), which appears to be contradictory, as the M1 phenotype is pro-inflammatory while M2 polarization is anti-inflammatory; however, the mechanism occurs in the context of mixed M1/M2 populations present under physiological conditions, defective innate immunity, and the tendency toward autoimmunity in patients with CGD. Normally, macrophages do not require antioxidants to protect themselves from ROS-related oxidative stress, because they possess defensive measures against ROS-mediated damage, such as the Mst-Nrf2 axis (31). In contrast, the addition of anti-oxidants may disturb macrophage-mediated autoimmunity, by altering macrophage polarization and homeostasis (30).

Efferocytosis is the process by which macrophages engulf and clear billions of apoptotic cells. Impaired efferocytosis plays a vital role in inflammation in CGD patients. Peritoneal, bone marrow-derived, and alveolar macrophages from NOX2-deficient mice and primary macrophages from CGD patients showed diminished efferocytosis of apoptotic Jurkat T cells and human neutrophils both *in vivo* and *in vitro* (32, 33). The mechanism involved has several aspects: "find me" signals in the prepare phase, "eat me" signals in the implementation phase and "digest me" in the rehabilitation phase.

"Find me" signals are some soluble substances released by apoptotic cells themselves, which recruit macrophages and reshape their scavenging potential. Among these signals phingosine-1-phosphate and some metabolites (AMP, GMP, creatine, spermidine, and glycerol 3-phosphate) are reported as phagocyte gene expression modulators (34, 35). Interestingly, this characteristic of apoptotic cells seems to be changed in CGD mice. In zymosan A induced self-limited peritonitis CGD mice, researchers observed reduced macrophages/monocyte infiltration and delayed neutrophils clearance as well as

diminished macrophage efferocytosis. The mechanism lays in defective respiratory burst in CGD neutrophils, thus failed to deplete local O_2 and produce enough ROS to maintain HIF-1α protein stability that is essential to upregulate macrophage efferocytosis enhancer erythropoietin- PPARγ signals (36).

As for "eat me" signals exposed on the surface of apoptotic cells, phosphatidylserine (PS) is the strongest (37). Apoptotic neutrophils in patients with CGD are prevented from PS externalization, as this process requires the participation of NOX2-derived ROS (33, 38, 39), which is verified by treatment of normal neutrophils with NOX2 inhibitor diphenyleneiodonium (33). And peroxidized PS species (PSox) are even stronger "eat-me" signals than PS alone (40). Further, PS exposure seems to modulate macrophage program such as classical and alternative activation in M1/M2 balance, above and beyond its effect on phagocytosis (32). M2, rather M1, macrophages are the protagonists of efferocytosis; and CGD patients and NOX2-deficient mice have macrophages with an M1 phenotype that tend to promote inflammation (32, 41, 42). Finally, the difference in efferocytosis ability between M1 and M2 macrophages is primarily attributable to the central role of interleukin 4 (IL-4) signaling through peroxisome-proliferator activated receptor γ (PPARγ). *Ex vivo* treatment of macrophages from patients with CGD and NOX2-deficient mice with IL-4 or IL-13 leads to re-establishment of normal efferocytosis, as do monocytes treated with the PPARγ agonist, pioglitazone (a drug for treatment of type 2 diabetes) (32, 43). PPARγ agonist treatment can not only reverse impaired efferocytosis in CGD monocytes, but also enhances mtROS production (43, 44). Interestingly, mtROS production can promote M2 macrophage polarization in the intestine (45).

Works go on in macrophage after ingesting apoptotic cells. In contrast to function of preventing excessive antigen reduction characterized in previous section, NOX2-derived ROS in the degradation of apoptotic cells seems to be a positive correlation: efferosomes maturation (acquisition of LC3 and LAMP-1), enhanced acidic environment mediated by V-ATPases, competent proteolytic activity, and these are obviously delayed in macrophage of CGD patients. The key element of this difference lies on the nature of phagosomal protein cargo. Apoptotic neutrophils cargo contributes to activation of macrophage NOX2 in a CD11b-TLR2/TLR4-myeloid differentiation primary response 88 (MyD88)-dependent manner and the subsequent ROS production, which is significantly delayed in macrophages from NOX2-deficient mice (46). While IgG-opsonized antigen cargo activates NOX2 dependent on FcγR-PKC/Syk pathway rather than V-ATPase (13, 28, 46).

NOX2 deficiency has been identified as associated factor in autoimmune disease. For example, Ncf1 polymorphism is a stronger genetic factor of systemic lupus erythematosus (SLE) (47). It is well-known that autoantigen triggered autoantibody plays a vital role in pathology of SLE, and this process is dramatically enhanced in experimental lupus in NOX2 deficient mice (48, 49). Failure in timely clearance of apoptotic cells that is founded in NOX2 deficient lupus mice contributes to accumulation of secondary necrotic cells leading to increased secretion of inflammatory cytokines and chemokines (50).

The Effects of ROS on T Helper 1/T Helper 2 and T Helper 17/Regulatory T Cell Balance

It is widely accepted that ROS is essential in adaptive immunity. T cell receptor (TCR) activation is accompanied by production of large amounts of ROS over a few minutes (51), which is associated with mTOR/AMPK axis-mediated metabolic reprogramming (52, 53). Reactive oxygen species is a critical link in the signaling events mediating T cell activation, proliferation, and differentiation (54, 55); however, number of studies have contradicted these findings (56). Here, we review understanding of the function of ROS in T helper 1 (Th1)/T helper 2 (Th2) cell, and T helper 17 (Th17) cell/regulatory T cell (Treg) balance, the importance of which in autoimmunity is universally acknowledged.

Th1/Th2 were the first $CD4^+$ T helper cell subsets determined to contribute to autoimmune diseases (57). Characterized by IFN-γ and IL-2, Th1 cells mainly function in cellular immunity, while Th2 cells are focused on humoral immunity. The Th1/Th2 equilibrium manifests in both directions during autoimmune disease; for example, the Th1 predominance in RA (58) and Th2 advantage in SLE (59). Further the Th17/Treg equilibrium has a major role in inflammatory and autoimmune diseases (60). Interconnected developmental pathways facilitate the plasticity between Th17 and Treg phenotypes in various inflammatory (61) and oxidative (56) microenvironments.

Evidence from patients with CGD and NOX2-deficient mice includes experimental data on the "third signal" function of ROS in Th1/Th2 and Th17/Treg balance (**Table 1**). In NOX2-deficient mice, the T cell phenotype is skewed toward the Th1 and Th17 lineages (62, 63, 67), while macrophage-restricted restoration of ROS production improved resistance to collagen-induced arthritis in NOX2-deficient mice (68). This effect may depend on Treg induction by macrophage-derived ROS, and was confirmed in experiments using macrophages from patients with CGD (64). Further, compared with wild-type mice, Tregs from NOX2-deficient mice exert much weaker inhibition of $CD4^+$ effector T cells (65), and antioxidant NAC or NOX inhibitors also induce changes in the Th1/Th2 and Th17/Treg balance (**Table 2**).

However, it is noteworthy that NOX2 deficiency in combination with transgenic mice shows different change of T cell subsets. For example, NOX2 deficiency in OT-II mice (a transgenic mouse model with antigen-specificity for chicken ovalbumin 323-339 in $CD4^+$ T cell) lead to both decreased Th1 and Th17 lineages in contrast with NOX2-deficient mice (66). Further, NOX2 deficiency in NOD mice serves as protector reflected in significant reduction and delay in autoimmune diabetes development (67, 70, 71). And macrophage, $CD4^+$ T cell, $CD8^+$ T cell are involved in the protection (**Table 3**). Weakened Th1 lineage proficiency resulted from absence of Th1 transcription factors (T-bet, STAT4, and STAT1α) and Th17 proneness by STAT3 activation are observed in NOX2-deficient NOD mice (67). Followed research revealed that the protection afforded by NOX2 deficiency is rely on ROS lack in macrophages and DCs leading to reduced $CD4^+$ T-cell autoreactivity [one possible mechanism is reduced MHC-II

TABLE 1 | Th1/Th2 and Th17/Treg related change in NOX2-deficient mice or CGD patients.

Cell type	NOX2 mutation	Th1 change	Th2 change	Th17 change	Treg change	Other
$CD4^+$ T cell of C57BL/6 (62)	$gp91^{phox}$-	IL-2 ↑	IL-4 ↓ IL-5 ↓	NA	NA	TNF ↑
Total splenocytes of C57BL/6 (63)	$gp91^{phox}$-	IFN-γ ↑	NA	IL-17↑	NA	NA
Naïve $CD4^+$ T cells of C57BL/6 (63)	$gp91^{phox}$-	IFN-γ ↑ T-bet ↑	IL-4 ↓ IL-4Rα↓ IL-5 ↓ GATA-3↓	IL-17↑	NA	STAT5 Phosphorylation↓ NA
PBMC from CGD patients (64)	$p47^{phox}$-	NA	NA	NA	No change of $CD4^+$CD25+ Foxp3+ cells	NA
Total spleen cell from C57BL/6 (65)	$p47^{phox}$-	NA	NA	NA	No change of $CD4^+$ Foxp3+ cells	NA
Spleen CD4 T cells of OT-II mouse (66)	$p47^{phox}$-	IL-2 ↓ IFN-γ↓ $CD4^+$T-bet+ cell↓		IL-17A↓	IL-10↓	TNF-α↓ TGF-β↓ IL-5↓ Il-12p70↓

Frequencies of Th1, Th2, Th17, Treg cells, or levels of related cytokines are compared between immune cells from NOX2 mutation mice/CGD patients and wild type mice/healthy people. ↑: levels of cytokines or frequencies of cells are higher than wild type mice/healthy people. ↓: levels of cytokines or frequencies of cells are lower than wild type mice/healthy people.

TABLE 2 | Th1/Th2 and Th17/Treg related change in different treatments.

Cell type	Treatment	Th1 change	Th2 change	Th17 change	Treg change	Other
$CD4^+$ T cell from BALB/c mice (63)	10 mM NAC (ROS scavenger)	IFN-γ ↑	IL-4 ↓ IL-5 ↓	NA	NA	STAT5Phosphorylation↓
Human CD4+CD25– T cells (64)	Primed with macrophage from $p47^{phox}$-CGD patients	IFN-γ ↑	NA	IL-17↑	CD4+CD25+Foxp3+ cell ↓	NA
CD4+ CD45RO– T-cells under Th1- and Th17-skewing conditions (69)	Tempol (ROS scavenger)	IFN-γ+ cells ↑	NA	IL-17+ cells ↑	NA	NA

Thayer et al. (70) immune cells whether receiving ROS clearing treatment, or primed with macrophage from CGD patients and healthy people. ↑: levels of cytokines or frequencies of cells are higher than control group. ↓: levels of cytokines or frequencies of cells are lower than control group.

complex by macrophage (72)] and $CD8^+$ T cell effector function [one possible mechanism is defective cross presentation by DC (15)], rather than isletβ-cell and neutrophils (70). Surprisingly, NOX2 deficiency in NOD.BDC-2.5 mice (a TCR transgenic mice whose TCR specifically recognizes islet antigen) that was meant to prevent from autoimmune diabetes has instead resulted in spontaneous type 1 diabetes. In followed adoptive transfer experiment, $CD4^+$ T cells of NOX2-deficent NOD.BDC-2.5 mice were more diabetogenic upon adoptive transfer into NOD due to less suppressive Tregs (73). These studies implied the complication of ROS in immune cell response to heterogeneous microenvironment.

A key factor influencing Th1/Th2 differentiation from Th0 cells is the cytokine microenvironment, where APC represent a cytokine source. If glutathione (GSH; a major cellular antioxidant) is depleted in APCs over a short time period, production of Th1-associated cytokines will be inhibited and Th2-associated cytokine generation favored (74). Similarly, low doses of H_2O_2 can prevent activated Th1 clones from producing INF-γ and potentiate IL-4 secretion by activated Th2 clones (75). T cell activation-triggered IL-2 and IL-4 expression is partly dependent on ROS generation and follows oxidative signaling via mitochondrial respiration chain complex I. Inhibition of complex I function will decrease mtROS generation, thus blocking activation-induced secretion of IL-4 in $CD4^+$ T cells from patients with atopic dermatitis, a disease characterized by elevated IL-4 and IgE. Prolonged ciprofloxacin treatment has the same effect on $CD4^+$ T cells as the complex I inhibitor, rotenone, which may explain the immune regulation function of ciprofloxacin (76). The transcription factor, Bach2, plays a vital role in shaping the balance between $CD4^+$ T cell subsets (77). TCR signaling induced by ROS specifically limits the degradation of the SUMO-specific protease, SENP3, leading to its rapid accumulation in Tregs. SENP3 promotes Bach2 deSUMOylation and prevents its nuclear export, which inhibits the expression of IFN-g, IL-17, and other effector cytokines, and maintains Treg-specific gene signatures (78). Reactive oxygen species promotes PAC1/DUSP2 expression by activating the

TABLE 3 | Th1/Th2 and Th17/Treg related change in NOX2-deficient (*NCF1* mutation) NOD mice.

Cell type and mice	Type 1 diabetes	Macrophage related change	CD4/CD8+ T cell related change	Other
$CD4^+$ T cells of NOD mice (67)	Remission	NA	IFN-γ ↓, IL-2 ↓, T-bet ↓ IL-4 ↓, IL-17↑, IL-10↑ CD4+/IL-17A+ cell↑ No change of CD4+CD25+ Foxp3+ cells	TNF-α↓ TGF-β↑
Spleen $CD8^+$ T cells (70) of NOD mice	Remission	NA	CD8+/IFN-γ+ cells↓ CD8+/GzmB+ cells↓	NA
BM-Mφs and $CD4^+$ T cell of NOD mice (72)	Remission	MHC-II↑, TNF-α↓, IFN-β↓, TLR3↓, NF-κB↓	No change of CD4+ Foxp3+ cells	NA
Islets and BM-Mφs with M1/M2 Polarization of NOD mice; (71)	Remission	M1 Marker↓ (cxcl10, ccl5, iNOS, TNF-α, IFN-γ,STAT1) M2 Marker↑ (ccl17, Arg1, Retnla, CD206, STAT6)	NA	NA
Splenocytes of NOD.BDC-2.5 mice (73)	Exacerbation	NA	IFN-γ↑, IL-17↑ IL-2↑, IL-12Rβ2↑ Activation Markers↑ (CD25, CD44, CD69)	TNF-α↑ IL-1β↑

Frequencies of Th1, Th2, Th17, Treg cells, or levels of related cytokines are compared between immune cells from NOX2 mutation NOD mice and NOD mice. ↑: levels of cytokines or frequencies of cells are higher than NOD mice. ↓: levels of cytokines or frequencies of cells are lower than NOD mice. BM-Mφs, Bone marrow macrophages.

transcription factor, EGR1, while PAC1 suppress the STAT3 signaling crucial for Th17 lineage differentiation (79, 80). In addition, we have described above that the post-translational oxidative modification is the main change in aging (2). Protein oxidative modification state is regulated by balance between ROS and methionine sulfoxide reductase (Mrs). Mrsb1 deficiency in DC delays its maturation and decreases DC-induced Th1 differentiation, and is associated with defective differentiation of follicular helper T cell cells *in vivo* (81).

Transforming growth factor β (TGF-β) is a cytokine with broad regulatory functions in T cell development and differentiation. The complexity of TGF-β-related signaling is partly reflected in its contradictory functions and mechanisms in Th17/Treg differentiation. TGF-β is normally synthesized as a precursor, whose C-terminal portion is referred to as latency-associated peptide (LAP). LAP is cleaved by Furin, to generate latent TGF-β, which can occur both intracellularly and extracellularly (82). Latent TGF-β can be activated by various molecules, including integrin αvβ8 and ROS in T cells (83, 84). Human $CD4^+$ $CD25^-$ naïve T cells can be induced to express Foxp3 by stimulation with anti-TCR and anti-CD28 antibodies plus ROS (85). This effect relies on production of latent TGF-β by TCR and CD28 engagement, as well as subsequent activation of latent TGF-β by ROS on TCR stimulation (84). Promotion of Th17 generation by high glucose and Treg generation by D-mannose are dependent on addition of exogenous latent LAP-TGF-β, rather than active TGF-β, indicating that activation related signals contribute to this effect, but not active TGF-β itself (86, 87) (**Figure 2**). Why active TGF-β does not function in this context and how the "activation-related signals" promote TGF-β signal transduction remain unclear; we speculate that intercellular communication, controlled by receptor-mediated TGF-β activation, may be a contributing factor, while "activation-related signals" may trigger some change that influences TGF-β signal transduction. Reactive oxygen species are key intermediary molecules for activation of latent TGF-β, and NAC abolishes the induction of Th17 cells and Tregs (86, 87). Reactive oxygen species not only acts as an upstream molecule to activate latent TGF-β, but also participates in TGF-β-Smad signaling. Misshapen (Msn)/NIK-related kinase 1 (MINK1) is a serine-threonine kinase that can induce Th17 differentiation by directly phosphorylating the T324 site at the α1 helix region of the Smad2 protein (88). Reactive oxygen species are involved in activation of MINK1 (89), and NAC treatment profoundly reduced MINK1 activity and increased the frequency of IL-$17A^+$ cells, which did not occur in MINK1-deficient T cells. The processes described above are shown in **Figure 2**. Further, in Th17 cells differentiated from MOG35–55-immunized $CD4^+$ T cells, NAC treatment resulted in more severe experimental autoimmune encephalomyelitis (EAE) disease after transfer into $Rag1^{-/-}$ mice (88), while knockout of GSH in T cells led to EAE resistance in mice. IsoalloLCA is a bile acid metabolite, which can enhance mtROS production in $CD4^+$ T cells. Elevated mtROS increased H3K27 acetylation at the Foxp3 promoter region in a TGF-β-Smad3 signal dependent manner, thereby promoting Treg differentiation (90). Moreover, ROS also contributes to suppression of $CD4^+$ effector T cells mediated by Tregs, which is partly dependent on TGF-β and can be blocked by thiol-containing antioxidants (65).

Abnormal redox related Th subsets change has been identified in autoimmune diseases, such as RA. Naïve $CD4^+$ T cells from RA patients possess a distinct carbohydrate metabolic signature,

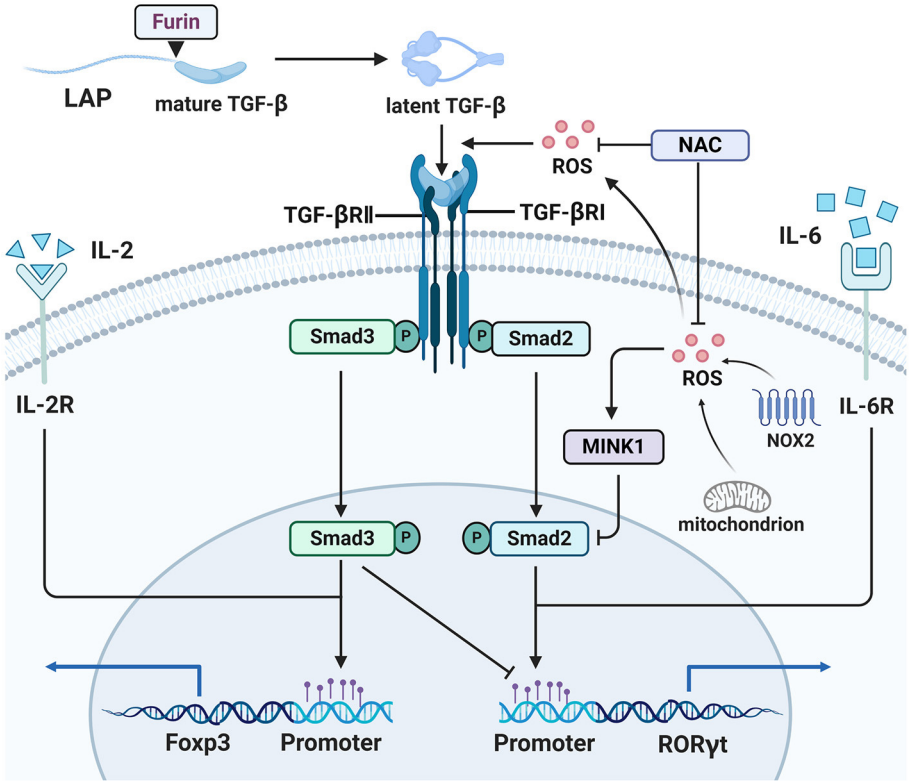

FIGURE 2 | ROS participate in Th17/Treg balance. ROS are involved in TGF-β mediated Th17/Treg balance. Latent TGF-β formed after LAP in the TGF-β precursor was cut by Furin, and then ROS activated TGF-β from its latent form. NOX2 or mitochondrion derived ROS activated MINK1 that inhibits the phosphorylation of Smad2 in T324 residue, so that expression of Th17-associted genes is blocked, and ROS scavenger NAC will reverse this function. ROS also participate in expression of Treg-associated genes.

which is manifested as an excessive shunt of glucose into the PPP, resulting in high levels of reduction mediated by GSH and NADPH, and exhaustion of ROS (69, 91, 92). Low levels of ROS lead to ataxia telangiectasia mutated (ATM) deficiency, causing rapid T cell proliferation and differentiation toward Th1 and Th17 lineages (69, 91); however, in a study of inflammasomes in RA, naïve $CD4^+$T cells from RA patients produced more ROS compared with those from healthy controls (93); this contradictory result may be attributable to measurement being conducted as early as 8 h after anti-CD3/CD28 stimulation, which is much earlier than the 3–6 day time points used in two other studies (69, 92). In those reports, treatment of $CD4^+$ T cells from RA patients with the pro-oxidant, plumbagin, reduced the frequencies of Th1 and Th17 cells, as well as decreasing production of the inflammatory cytokines, TNF-α and IL-6. The antioxidant, NAC, completely reversed the regulatory effects of plumbagin (94). These studies demonstrate the potential pro-inflammatory function of ROS exhaustion in RA $CD4^+$ T cells, and anti-inflammatory benefits of supplementation with oxidants.

Based on the evidence reviewed above, it remains unclear how strong the effect of ROS is in autoimmunity-related Th1/Th2 and Th17/Treg balance. Regarding patients with CGD, it is difficult to determine a clear role of $CD4^+$ T cells in CGD-related autoimmunity risk. For example, one study reported that only gp91phox-deficient CGD is associated with diminished Tregs (95); however, a review demonstrated that oxidative stress leads to T-cell dysfunction in SLE by altering Th cell lineages and gene transcription (96).

ROS and Inflammatory Metabolism: Kyn-IDO1

Indoleamine 2,3-dioxygenase (IDO) has maintained a central role in tryptophan metabolism over hundreds of millions of years of evolution (97, 98). It is well-established that IDO has suppression and feedback roles in immune regulation (99), and clinical trials of IDO inhibitors for treatment of cancers were very successful. The normal dioxygenase activity of IDO is post-translationally activated by biological reduction of Fe^{3+} to Fe^{2+} in heme. Of these reducing agents, ROS, and particularly superoxide anion (O2?), are most widely studied in mice and rabbit (100, 101). Specifically, hyperbaric oxygen can increase kynurenine concentration in rat brains by 60% compared with air (102). In murine atopic dermatitis and psoriasis lesions, hyperbaric oxygen therapy can elevate ROS levels to attenuate disease, which may be mediated by enhanced IDO expression and Treg function (103, 104). Precisely because of the immunosuppressive effect of IDO and its ROS dependency,

defective IDO activation and tryptophan metabolism were once considered to be important factors contributing to hyper inflammatory responses in murine CGD (101, 105). Interestingly, long-term evolution appears to have freed human IDO activity from ROS restriction. The IDO metabolic activity of leukocytes and monocyte-derived dendritic cells is fully maintained and intact in human patients with CGD (106–108), indicating that ROS is not indispensable for human IDO activity. Cytochrome b5, rather than ROS, has a major role in IDO reduction in human cells (100, 109); however, it is not clear whether the entire tryptophan metabolism process is independent of ROS in humans. LPS-induced ROS promotes DC maturation mediated by IDO and NF-κB activation, leading to expansion of $CD4^+$ $CD25^{high}$ Tregs (110). A tryptophan catabolite of IDO, L-kynurenine (Kyn), can induce apoptosis of NK cells in a ROS-dependent manner. This pro-apoptotic effect can be entirely prevented by the antioxidant, NAC (111). In turn, Kyn also can elevate ROS levels in activated T cells and promote their proliferation, which relies on inhibition of sepiapterin reductase, the terminal enzyme in the *de novo* tetrahydrobiopterin synthesis pathway (112). Further, IDO1 can even produce single molecular oxygen in the presence of hydrogen peroxide. The generated $^1O_2$ oxidizes L-tryptophan to a tricyclic hydroperoxide, thus regulating vascular tone and blood pressure under inflammatory conditions (112).

ROS AND RHEUMATOID ARTHRITIS

Due to the physiological function of ROS, high levels of reducing equivalents and excessive ROS scavenging may lead to damage of the opposite type to oxidative stress, sometimes referred to as reductive (113) or antioxidative (114) stress.

Redox regulation treatments should be disease-specific as the different redox characteristics among diseases. For example, unlike the central role of oxidative stress in lupus pathogenesis, $CD4^+$ T cells of RA patients experienced reductive stress (115). In addition, variations in redox state exist among types of lesions and cells in RA. In the next section, we discuss the metabolic origin of these variations and their influences on pathology.

ROS in RA: Every Coin Has Two Sides

RA pathogenesis is not well-understood, but can be summarized by loss of peripheral immune tolerance to autoantigens, followed by excessive activation of T and B cells, leading to increased levels of cytokines and autoantibodies (rheumatoid factor, anti-cyclic citrullinated peptide antibodies, etc.). The homeostasis between pro- and anti-inflammatory states is destroyed, eventually leading to damage of multiple joints and other organs throughout the body.

Numerous studies have confirmed the vital role of ROS-related oxidative stress in joint pathology, including in angiogenesis, synovial proliferation, and inflammatory infiltration (116, 117). Further, functional genetic analysis showed that the rs201802880 polymorphism in the *NCF1* coding region is associated with genetic susceptibility to RA (47). Patients with CGD also have increased susceptibility to RA (47, 118). Moreover, a study of NOX2-deficient mice demonstrated that the absence of ROS prevents resistance to autoimmune arthritis. A collagen-induced arthritis model generated by Ncf1 mutation in mice has more severe symptoms, higher anti-CII IgG levels, and stronger Th1 responses than wild-type mice, which can be reversed by restoration of functional Ncf1 solely in macrophages. Interestingly, this research also found that T cells from Ncf1-mutated mice responded more vigorously to APCs (68). Mice with mutated mouse collagen (MMC) have higher resistance to arthritis mediated by a mutated immunodominant epitope in collagen type II that binds to the MHC class II molecule. When these MMC mice are bred with NOX2-deficient mice, their immune tolerance to arthritis disappears, and they exhibit enhanced autoimmune T cell responses and higher anti-CII IgG levels (119).

Poly-N-isopropylacrylamide (PNiPAAm)-based polymers are new synthetic substances that can serve as adjuvants in inducing experimental arthritis. Mixture of these new "adjuvants" with natural CII triggers more severe arthritis and stronger autoantibody responses in Ncf1-mutated mice, in which macrophage ROS also plays a central role (120). DC function is also influenced by redox state. *Mycobacterium tuberculosis*-activated DC with ROS exhaustion (through incubation with SOD and catalase, or derived from NOX2-deficient mice) produced more IL-1β, TNF-α, TGF-β, and IL-6. NOX2 mutation breaks the resistance to arthritis of wild-type C57BL/6 mice, and this is CII specific, as Freund's complete adjuvant alone cannot induce arthritis. In addition, when DC from NOX2-deficient mice serve as APC, T cells produce more IL-17 after activation by toxic shock syndrome toxin-1 (TSST-1) (121); however, research into SKG mice (a spontaneous arthritis animal model caused by ZAP70 mutation) indicates that Ncf1 mutation-related ROS deficiency does not exhibit further alteration of T-cell activation or differentiation profile because of ZAP70 mutation, while transgenic restoration of functional Ncf1 in macrophages modified the arthritis in Ncf1-mutated SKG mice to the state observed in ROS-sufficient SKG mice. This research indicates that innate, but not adaptive, inflammation contributes to more severe arthritis related to ROS deficiency (122). Therapeutic strategy that increasing NOX2-derived ROS has been tested, in which phytol, an oxidative burst-inducing substance, ameliorated pristane induced rat arthritis in T cell and IFN-βdependent pathway (123, 124). Other free radicals, namely reactive nitrogen species, also contribute to arthritis pathogenesis in Ncf1-mutated mice, and may counteract the effects of ROS. Treatment with the NOS inhibitor, L-NAME, in the priming, rather than the effector, phase prevents Ncf1-mutated mice from developing CII-induced arthritis (125).

The Reductive State in RA Naïve $CD4^+$ T Cells

Subsets of $CD4^+$ T cells, including Th17 cells and Tregs, are recognized as important targets for the treatment of autoimmune disease; however, few studies have concentrated on the role of naïve $CD4^+$ T cells. After recognizing the MHC complex, naïve $CD4^+$ T cells initiate rapid clonal expansion, with a consequent explosive increase in energy and biosynthesis

demands. The energy demands are dependent on transformation from oxidative phosphorylation to glycolysis, referred to as metabolic reprogramming (52). This may appear to be a retrogression, where an efficient method of ATP generation is abandoned in favor of a wasteful method; however, glycolysis actually is the more logical choice for cells undergoing rapid proliferation. Oxidative phosphorylation has a much higher proteome costs than glycolysis, because of the prerequisite complicated mitochondrial infrastructure, that requires huge energy expenditure (126). The enhanced PPP and glutamine decomposition meet the NADPH and biosynthetic precursor needs during T cell growth and proliferation (127), and both participate in GSH generation. The balance of energy generation and biosynthesis is also very important. In contrast, deficiency of any of these processes will destroy the basic support for normal physiological activities after T cell activation. In contrast, excessive glycolysis is associated with pro-inflammatory T cell subsets (128) and excessive biosynthesis indicates a worse outcome (i.e., a tumor). In normal naïve $CD4^+$ T cells, glycolysis activation occurs in response to upregulation of the glucose transporter, GLUT, which increases glucose uptake (129), and activities of several rate-limiting enzymes, including 6-phosphofructo-1-kinase (PFK-1) (130). PFK-1 activity is mainly dependent on allosteric activation by fructose-2,6-bisphosphate (F2,6P2), and production of F2,6P2 is primarily controlled by four fructose-2,6-bisphosphatase (PFKFB) isoenzymes (131), among which PFKFB3 is the strongest. During the activation of $CD3^+$ T cells, PFKFB3 expression is increased in response to engagement of the TCR and the co-stimulatory receptor, CD28 (132). Two studies found that, compared with those from healthy people, naïve $CD4^+$ T cells from patients with RA failed to upregulate PFKFB3 expression during the activation process, leading to reduced glycolytic flux and diminished ATP generation. As a rate-limiting enzyme of the PPP, glucose-6-phosphate dehydrogenase (G6PD) initiates the PPP by dehydrogenation of glucose 6-phosphate. Unlike PFKFB3, these naïve $CD4^+$ T cells successfully upregulated G6PD expression, which controls the fate of residual glucose, namely, influx to the PPP. Hence, there is an imbalance of energy generation and biosynthesis in naïve $CD4^+$ T cells from patients with RA, resulting in accumulation of GSH and NADPH, reduced ATP generation, and ROS exhaustion (**Figure 2**) (60, 79).

Altered Metabolism Influences T Cell Differentiation and Proliferation

Glutaminolysis is a basic and widespread metabolic process that links oxidative phosphorylation (OXPHOS), biosynthesis, and redox regulation. The main branch point hinges on glutamate, the first product of glutamine decomposition, which can serve as material for *de novo* synthesis of GSH to regulate oxidation, or transform into α-KG and enter the TCA cycle to generate ATP, mtROS, and biosynthetic precursors. Hence, the different destinies of glutamate generate counteracting metabolites (GSH and ROS), facilitating precise coordination of metabolic flux by altering enzyme activity (133). Activation of primary T cells requires rapid glutamine uptake mediated by the amino acid transporter, ASCT2 (127). Glutaminolysis inhibition of $CD4^+$ T cells has an anti-inflammatory function in autoimmunity, promotes high levels of Foxp3 expression (134) and decreases Th17 differentiation in SLE and EAE (135, 136). In RA, fibroblast-like synoviocytes (FLS) express increased levels of glutaminase 1, and inhibition of glutaminase 1 reduces RA-FLS proliferation (137); however, there has been no study of the glutaminolysis phenotype of RA naïve $CD4^+$ T cells. Given the dysregulated redox state in RA T cells, whether or not glutaminolysis contributes to this phenomenon warrants discussion and study.

Other than sharing the same substrates, connections between PPP and glycolysis also include their interactions in metabolic signaling. Although excessive PPP and accumulated GSH lead to reductive stress in RA primary $CD4^+$ T cells, T cells lacking GSH also appear to be incapable of initiating metabolic reprogramming, because of impaired Myc expression, NFAT activation, and mTOR activation. Higher ROS levels appear to be the protagonist, as ROS scavengers reverse the influence of lack of GSH (138). Briefly, both high levels of ROS and exhaustion of ROS harm normal transfer into glycolysis during metabolic reprogramming following T cell activation; recalling the old Chinese idiom, "Beyond is as wrong as falling short."

Similar with the higher risk of fetal deformity occurring during the first 3 months of pregnancy, naïve $CD4^+$ T cell may represent a stage at which pathogenic factors can readily influence T cells, with pathological changes at this stage having profound and lasting influences. Reactive oxygen species exhaustion disturbs normal DNA repair capabilities in naïve $CD4^+$ T cells from patients with RA, and ATM insufficiency is a key factor influencing this phenomenon (69, 139). The resulting accumulation of DNA damage, ATP deficiency because of reduced glycolysis, and impaired autophagy induction (92) mean that naïve $CD4^+$ T cells are more sensitive to apoptosis, leading to an abnormal loss of T cells. Since patients generally present with RA in middle-age, it is T cell auto proliferation, rather than newborn T cells, that maintain homeostasis of the T cell compartment. Remaining T cells are confronted with replicative stress, under the influence of lymphopenia, excessive biosynthesis, and pro-inflammatory cytokines. Consequently, the T cell immune aging process begins (140, 141). In addition, this imbalance supports pro-inflammatory functions, such as Th17 lineage expansion and enhanced synovial invasiveness (69, 142).

Division of Glycolysis Between Naïve $CD4^+$ T Cell and Synovium in RA: Hypoxia, Lactic Acid, and ROS

Hypoxia has been identified as a constant feature of RA synovial tissue, which occurs in the pre-arthritic phase because of increased cell proliferation, capillary network collapse, and maintenance of the inflammatory phase, due to invasive synovial proliferation, dysregulated architecture of the microvasculature, and pro-inflammatory signals, such as HIF-1α and JAK–STAT signaling (143). In energy metabolism, hypoxia is always accompanied by aerobic glycolysis and mitochondrial dysfunction, leading to accumulation of lactic acid and ROS

(9). Indeed, accumulated lactic acid supports pro-inflammatory T cells to remain at the double-positive stage and produce more IL-17 (144, 145), while ROS causes oxidation with broad impacts (116, 117); however, as discussed above, in $CD4^+$ T cells at the preliminary stages of RA, glycolysis is decreased and ROS is exhausted, resulting in a reductive metabolic microenvironment (low pyruvate and high NADPH) and triggering aberrant lipogenesis. This induces up-regulation of the podosome scaffolding protein, TKS5, and formation of cell membrane structures beneficial to T cell synovial invasion (142). Interestingly, this division of glycolysis between RA T cells and the synovium may combine to induce joint destruction: pro-inflammatory T cells invade synovial tissue quickly and easily, while departure is more difficult (**Figure 3**). In addition, lactate, which was once considered a metabolic waste product, is now thought to act as a homeostatic regulatory substance capable of counteracting the inflammatory responses caused by HIF1α and glycolysis metabolites, such as macrophage polarization, tumor immunity, and antiviral responses (146), and the newly discovered histone lysine lactate modification, lactylation, may be an important mechanism underlying these processes (147). Therefore, the decreased lactate levels in RA naïve $CD4^+$ T cells, due to deficient glycolysis, may also contribute to the immune pathology of RA.

PROGRESSIVE OPINION IN REDOX MODULATION

Studies indicate that oxidatively modified lipids, proteins, and nucleic acids, may be typical of atherosclerosis. Oxidation of low-density lipoprotein has been clearly identified as an important initial event for the onset of atherosclerosis (148). Further, regarding oxidative stress in immune-related disease, oxidatively modified autoantigens are a major topic of interest, because of their induction of loss of immune tolerance. Nevertheless, cardiovascular patients do not benefit from antioxidant supplements (5), and their effects in autoimmune diseases, such as RA, are highly contentious (149–151).

Several points may explain the failure of "one-size-fits-all" antioxidant supplements in human studies. First, the reactivity of antioxidants is dependent on the oxidants they encounter, and rate constants are highest in specific pairs: α-tocopherol and peroxyl radical, glutathione and peroxynitrous acid, ascorbic acid and carbonate anion radical, glutathione and hypochlorous acid, and β-carotene paired with singlet oxygen (152). In view of different types of oxidative modification of biological macromolecules in various degenerative and aging related diseases, as well as selectively or indiscriminately produced oxidation products, application of bulk antioxidants are expected to be more precise and targeted. Second, bioavailability in target organs is a key factor. Take ischemic stroke for example, edaravone works by eliminating free radicals and suppressing oxidative stress. However, low bioavailability and inefficient penetration across the blood-brain barrier limits the curative. Treatments based on drug nano-systems loading with edaravone possess better scavenging efficiency of free radicals (153, 154). Third, different roles of antioxidants between target organ and the others may be an important reason for side effects. In view of ROS as the key beneficial messenger in the barrier ecosystem, oral administration of antioxidants which is the main application way by people, may starts its disturbance on the body upon first barrier—gastrointestinal tract (155).

Inspired by these findings in exogenous antioxidants, new strategies may combat oxidative stress shifts by promoting innate redox modulation systems; For example, by increasing the level of endogenous NADPH (156). Another meaningful progress is the use of the SOD mimics. Superoxide dismutase 2 (MnSOD) is a part of innate redox modulation systems, which plays an important role in regulating the ROS level in mitochondria. Based on therapeutic potential of MnSOD in human diseases, SOD mimics have been developed and are currently in several clinical trials (157). Interestingly, due to the redox potential falling in between the potentials for the oxidation and reduction of $O_2\bullet$-, these MnSOD mimics are capable of role transformation from reductants to oxidants decided by chemical properties of the reaction and the cellular environment (157). Except for drug therapy, there are some special treatments promoting redox balance and deserve attention. A recent study found static magnetic and electric fields rapidly ameliorate insulin resistance and glucose intolerance in type 2 diabetes dependent on mitROS induction, and SOD2 application fully abolishes these positive effects (158). Further, exercise especially acute exercise is widely believed a process producing large numbers of ROS which causes skeletal muscle damage, fatigue and impair recovery. As a result, antioxidants supplement has become common practice. However, several reviews have well-characterized that increase of free radicals explain the health promotion effect of exercise, and antioxidants supplement reduce the positive effects of exercise (159–161). These discoveries imply the potential bidirectional therapy, which fits the point we mentioned in this review: enhancement of ROS may sometimes be required in diseases.

CONCLUSION

Unlike their past reputation as harmful factors, a focus on ROS as important signaling molecule has developed in recent years. A typical example of this change in research direction is illustrated by the fact that tissue-specific redox modification of proteins has replaced biomacromolecule damage as the main agent involved in the process of aging. Failures of clinical trials into antioxidant supplements led scientists to deeply consider the problems of whether and how antioxidation strategies should be implemented.

This review has discussed the important roles of ROS in various autoimmune functions. For example, ROS influences interactions between innate and adaptive immunity by controlling the antigen presentation and apoptotic cell clearance. Evidence from NOX2-deficient mice and patients with CGD support the functions of ROS in regulating Th1/Th2 and Th17/Treg balance. Immunometabolism is an important process to which ROS contributes. Tryptophan metabolism

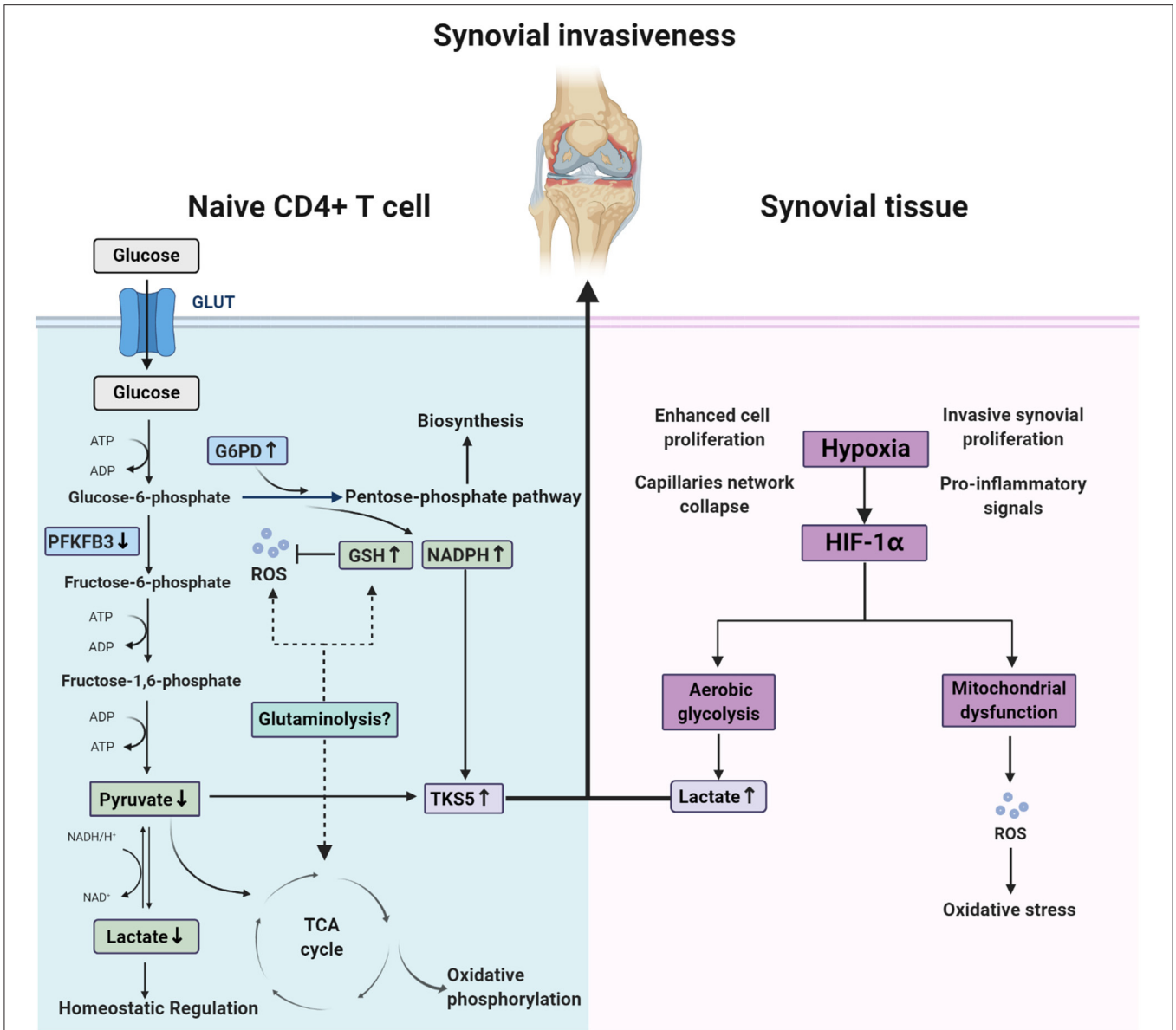

FIGURE 3 | Different redox state between naïve CD4$^+$ T cell and synovium in rheumatoid arthritis. In naïve CD4$^+$ T cell of rheumatoid arthritis patients, glucose shunting into pentose phosphate pathway due to the change of metabolic key enzymes (PFKFB3 and G6PD) activity leads to a special microenvironment characterized by low ATP, high reducing equivalences (GSH and NADPH) and exhausted ROS. Whether glutaminolysis is associated with this remains to unclear. In contrast, synovium of patients experienced oxidative stress and abnormal aerobic glycolysis that result from hypoxia in the joint. These two split situation destruct joints together by the integration of up-regulated TKS5 in naïve CD4$^+$ T cell and high lactates in synovium.

deficiency contributes to the stronger, harmful inflammatory response in CGD mice. Regarding autoimmune diseases, such as RA, alterations in glucose metabolism-related redox imbalance have broad impacts. Due to glucose shunting into the PPP from glycolysis, naïve CD4$^+$ T cells from RA patients are a good cellular level model system to explore T cell immune responses in a naturally ROS deficient environment. This special change in metabolism and redox balance leads to DNA repair deficiency, susceptibility to apoptosis, and differentiation into inflammatory subsets of RA naïve CD4$^+$T cells. In addition, although the metabolism and redox state in RA synovial tissue are completely contrary to that of naïve CD4$^+$T cells, we speculate that they act in combination to mediate joint destruction; however, the origin of this specific type of metabolic reprogramming remains unclear, and whether ROS contributes to triggering this process also awaits further investigation.

Overall, ROS clearance may be beneficial in specific situations, but harmful in others. Under no circumstances should we regard antioxidant supplements as completely

safe treatments, particularly for immune disease. Moreover, antioxidant supplements are not equivalent to ROS clearance.

AUTHOR CONTRIBUTIONS

WL: writing, original draft, and figures. PS: review and editing. YS: resources. YH: resources. ST: conceptualization. All authors contributed to the article and approved the submitted version.

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Interleukin-1 and Systemic Sclerosis: Getting to the Heart of Cardiac Involvement

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Systemic sclerosis (SSc) is rare, severe connective tissue disease characterized by endothelial and vascular damage, immune activation, and resulting in inflammation and fibrosis of skin and internal organs, including the heart. SSc is associated with high morbidity and mortality. Cardiac involvement is frequent in SSc patients, even though often asymptomatic at early stages, and represents one of the major causes of SSc-related mortality. Heart involvement has a variable clinical presentation, and its pathogenesis is not completely understood. Myocardial fibrosis is traditionally considered the immunopathologic hallmark of heart involvement in SSc. This unique histological feature is paralleled by distinctive clinical and prognostic features. The so-called "vascular hypothesis" represents the most credited hypothesis to explain myocardial fibrosis. More recently, the prominent role of an inflammatory myocardial process has been identified as a cardinal event in the evolution to fibrosis, thus also delineating an "inflammation-driven pathway to fibrosis". The pro-inflammatory cytokine interleukin (IL)-1 has an apical and cardinal role in the myocardial inflammatory cascade and in cardiac dysfunction. The primary aim of this perspective article is: to present the emerging evidence on the role of IL-1 and inflammasome in both SSc and heart inflammation, to review the complex interplay between cellular metabolism and inflammasome activation, and to discuss the rationale for targeted inhibition of IL-1 for the treatment of SSc-heart involvement, providing preliminary experimental and clinical data to support this hypothesis.

Keywords: systemic sclerosis (scleroderma), heart inflammation, interleukin-1, inflammasome, cellular metabolism

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INTRODUCTION

Heart involvement is frequent and is a major cause of mortality in systemic sclerosis (SSc), being responsible for up to 30% of disease-related deaths (1–3). Heart involvement has a variable clinical presentation: at early stages most patients are asymptomatic, but some go on to develop arrhythmias, dyspnea, chest pain, and heart failure (HF) (1, 4–9). In comparison to other

inflammatory myocardial disease, myocardial fibrosis is usually considered the immunopathologic hallmark of SSc heart disease. This unique histological feature is paralleled by distinctive clinical and prognostic features (10, 11).

The most credited hypothesis to explain myocardial fibrosis is the one attributed to "vascular" (12): intermittent vascular spasm, ischemic necrosis, and reperfusion injury are considered pivotal mechanisms in fibrogenesis. More recently, the prominent role of an inflammatory myocardial process, clinically identified as a myocarditis, has been also identified as a crucial event in the evolution to fibrosis, thus also delineating an "inflammation-driven pathway to fibrosis" (1, 4, 13–18). In this "bimodal" ischemic-inflammatory pathogenic model, reperfusion products and pro-inflammatory cytokines may jointly orchestrate SSc-related heart involvement (SSc-HI). Therefore, the fact that only the inflammatory pathway to fibrosis is similar to other inflammatory cardiomyopathies (HF, dilated cardiomyopathy [DCM], virus-negative myocarditis) makes myocardial involvement in SSc a really peculiar and complex multifaceted event (19–24).

The pro-inflammatory cytokine interleukin (IL)-1 has an apical and cardinal role in the myocardial inflammatory cascade and in cardiac dysfunction (24). In this article, we provide an expert perspective on the emerging evidence on the role of IL-1 in both SSc and heart inflammation, and discuss the rationale for targeted inhibition of this cytokine for the treatment of SSc-HI.

IL-1 FAMILY AND IL-1 BIOLOGY

The IL-1 cytokines family includes seven members with agonistic activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) and four members with antagonistic functions (IL-1Ra, IL-36Rα, IL-37, and IL-38) (25–28).

IL-1 is an archetypal pro-inflammatory cytokine. The term IL-1 hints at two different molecules, IL-1α and IL-1β, which share a significant sequence homology and bind the same IL-1 type-I receptor (IL-1RI), which then transduces pro-inflammatory signals and leads to the synthesis and expression of myriad secondary inflammatory mediators (29). IL-1α is constitutively present in epithelial cells as a fully active pro-inflammatory mediator, and is released upon cell death thus acting as an "alarmin". Alarmins are a group of intracellular mediators, of which High Mobility Group Box 1 (HMGB1) likely represent the best characterized member, signaling tissue damage and activating inflammatory patrolling when found in the extracellular space. In scleroderma, mechanisms leading to alarmin release include ischemic cell death or inflammation-mediated tissue damage (30). Conversely, IL-1β is primarily produced by myeloid cells as an inactive precursor. Production of the mature pro-inflammatory cytokine follows activating cleavage of the precursor by an intracellular molecular complexes termed "inflammasomes" (31). To dampen excessive inflammation, the same cells that produce IL-1α or IL-1β also synthesize diverse regulatory molecules, including the IL-1 receptor antagonist (IL-1Ra). IL-1 signaling and IL-1-mediated inflammation are prevented by competitive binding of IL-1Ra to IL-1RI (30–33), thus curbing IL-1-mediated inflammation.

IL-1 AND THE INFLAMMASOME IN SSC

Expression or biologic activity of most IL-1 family cytokines can be abnormal in many autoimmune diseases, including SSc (34). IL-1α regulates differentiation of fibroblast into myofibroblast, as well as myofibroblast longevity, which are considered central events in SSc (34). Indeed, dermal fibroblasts from SSc patients cultured *ex vivo* express higher levels of intracellular IL-1α than healthy counterparts (35). Immunohistochemical studies indicated that intracellular IL-1α is also markedly expressed in fibroblasts isolated from skin lesions of SSc patients; in addition, endogenous IL-1α induces fibroblast proliferation and production of collagen by inducing IL-6 and platelet-derived growth factor (PDGF) (36). Consistently, the production of IL-6, suppression of IL-1α through IL-1α siRNA results in decreased PDGF and procollagen production in SSc-affected fibroblasts (37), whereas overexpression of IL-1α through transfection in healthy fibroblasts promotes differentiation into a SSc-related phenotype (34). In SSc fibroblasts, the NLRP3 inflammasome is over-expressed and caspase-1 activity is up-regulated with consequent increased production of IL-1β and IL-18, whereas inhibition caspase-1 and inflammasome activity abrogated the myofibroblast phenotype in SSc dermal and lung fibroblasts (38–40). A separate study revealed that SSc fibroblasts exhibit increased synthesis of micro-RNA (miR)-155, which can also be induced by IL-1β (22). To date, miR-155 was implicated in various biological processes, including inflammation, immunity, and fibrosis (41). MiR-155, moreover, has been involved in cardiac remodeling, and miR155 deletion or inhibition reduced inflammatory and fibrotic responses in animal models of cardiac fibrosis induced by angiotensin-II (Ang-II) or diabetes (42, 43). In addition, miR-155 is required for the synthesis of collagen induced by activation of the inflammasome. Indeed, inhibition of caspase-1 activity abrogated miR-155 expression and significantly dampened collagen synthesis in a bleomycin-induced SSc mouse model (22).

In SSc patients, high levels of IL-1βcan be observed both in the bronchoalveolar lavage fluid (BAL) and in the serum (44). In the affected skin of SSc patients, IL-1β and IL-18 were significantly over-expressed, a finding correlating with the area of skin fibrosis assessed by the modified Rodnan skin score (mRSS) (38). This finding is not surprising, since IL-1β also induces myofibroblast activation, endothelial to mesenchymal transition, and fibrosis through IL-6 and TGF-1β (45).

IL-1α is an intracellular cytokine which is rarely if ever detectable in the circulation, including in SSc patients (35). Similarly, even though associations between genes encoding IL-1 family cytokines and SSc susceptibility were revealed by genome-wide association studies, the results are not conclusive (46–51).

INFLAMMASOME, IL-1, AND METABOLISM

Recent studies have identified a strong interplay between cellular metabolism and inflammasome activation (52). Specifically, NLRP3 inflammasome is regulated by cellular metabolism, and growing evidences suggest that cellular metabolism is a crucial driver for macrophage polarization and inflammation, as well as myofibroblast differentiation and fibrosis (53, 54).

There are several molecular pathways involved in the metabolic regulation of the inflammasome: glycolysis, tricarboxylil acid (TCA) cycle, amino-acid metabolism, and fatty acid metabolism, and and most of them have been found to be dysregulated in SSc, providing a potential mechanism involved in inflammasome activation, and thus IL-1β release (52) (**Figure 1**).

Enhanced glycolisis is a hallmark of activated macrophages (53–57). Recents studies of SSc patients undergoing positron emission tomography using the glucose analogue tracer ^{18}fluorodeoxyglucose revealed both increased glucose uptake (58). Glycolysis is critical in fibroblast differentiation and has been associated with the development of pulmonary fibrosis in bleomycin-induced experimental models (52). A recent study indicated that TGF-β1, a key cytokine in scleroderma, up-regulates glycolysis in dermal fibroblasts derived from SSc patients, and inhibition of glycolysis attenuates its pro-fibrotic effects (59).

Glutaminolysis through the TCA cycle and its intermediate metabolites was also evaluated in fibrotic conditions. TCA intermediate succinate binds the G-protein-coupled receptor-91 (GPR91) and increases GPR91, type-I collagen, α-SMA, and TGF-β levels. Levels of succinate are up-regulated in lung myofibroblasts of patients with idiopathic pulmonary fibrosis, where they induce TGF-β1, hypoxia-inducible factor-1alpha (HIF-1a), and fibroblast differentiation (58). Of note, succinate levels stabilize HIF-1a and promote IL-1β expression (53); this process is inhibited by itaconate, an anti-inflammatory metabolite required for the activation of the anti-inflammatory transcription factor Nrf2 by lipopolysaccharide in mouse and human macrophages, thus enabling *Nrf2* to increase the expression of downstream anti-oxidant genes as NAD(P)H Quinone Dehydrogenase 1 (60, 61). Interestingly though, the *Nrf2* pathway is highly down-regulated in human and SSc mice with detrimental consequences on inflammation and fibrosis. The $nrf2^{-/-}$ mice, indeed, develop a more severe SSc with enhanced fibrosis and inflammation compared to wild-type mice (62).

Results from the aformentioned study about the role of metabolic reprogramming in SSc pathogenesis (59), demonstrated that TGF-β1 is able to enhance succinate production, which determines an increase of collagen expression, thus providing a link between the pro-fibrotic milieu

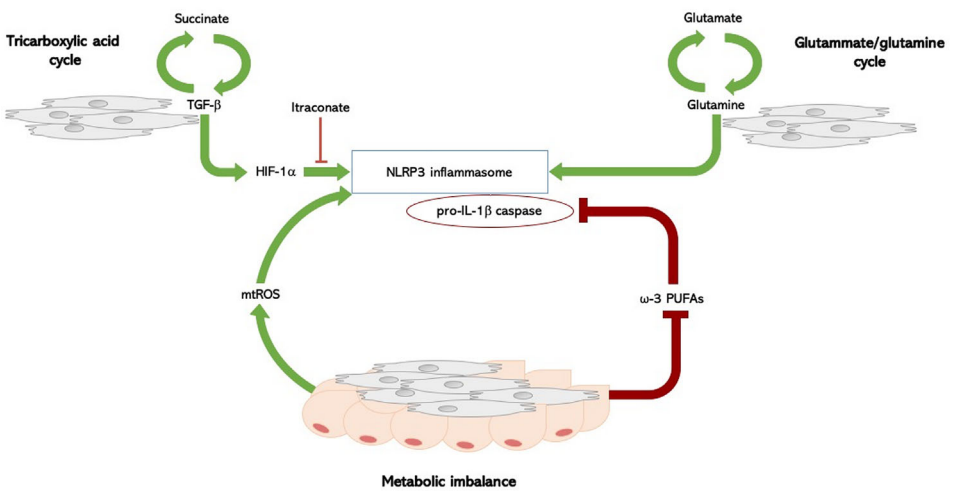

FIGURE 1 | Inflammasome and metabolism in systemic sclerosis. Molecular pathways involved in the metabolic regulation of the NLRP3 inflammasome in SSc: TCA cycle, fatty acid imbalance, and amino-acid metabolism. In SSc, a complex biological loop in which the TGF-β1 rich microenvironment, the upregulated glutamine metabolism, and the fatty acid dysregulation, could lead to both inflammasome activation with IL-1β release and myofibroblasts differentiation, thus foraging the inflammation-driven fibrosis. Succinate is formed in the TCA cycle; its levels increase the TGF-β1-induced HIF-1α expression, promoting fibroblast differentiation. High levels of succinate can support IL-1β expression by stabilizing HIF-1α for IL-1β transcript expression to occur. This process is inhibited by itaconate. TGF-β1 itself is able to enhance succinate production, thus foraging this biological loop. SSc fibroblasts have an increased glutaminase expression, and an altered glutamine metabolism is an ubiquitous trait in SSc. The glutammate-glutamine pathway activates the NLPR3 inflammasome. Fatty acid metabolism has been implicated in the regulation of NLRP3 inflammasome: metabolic imbalance itself act as a cue to activate an inflammatory response, though the production of mitochondrial reactive oxygen species (mtROS), which directly activate the NLRP3 inflammasome. PUFAs, particularly ω-3 PUFA, regulate NLRP3 inflammasome activation, acting as potent inhibitors of both caspase-1 activation and IL-1β release. Fatty acid metabolism is dysregulated in SSc, and intradermal adipose tissue is atrophied and replaced by collagen-rich fibrous tissue in SSc. SSc, systemic sclerosis; TGF-β1, transforming-growth factor beta-1; HIF-1α, hypoxia-inducible factor-1alpha; TCA, tricarboxylil acid; IL-1β, interleukin-1 beta; PUFAs, long-chain polyunsaturated fatty acids.

of the disease and the metabolic activation of the inflammasome. Consistently, SSc fibroblasts incubated with itaconate exhibited reduced expression of collagen (59).

The same study showed that inhibition of glutamine metabolism, another pivotal metabolic pathway fuelling cellular growth, inflammation, and myofibroblast differentiation, antagonises TGF-β1-induced glycolysis and fibrosis in normal human dermal fibroblasts. Furthermore, SSc fibroblasts showed an increase in glutaminase expression, suggesting that an altered glutamine metabolism may be a hallmark metabolic feature in SSc (59). Also of note, the same glutammate-glutamine pathway has been shown to activate the NLPR3 inflammasome (63).

Finally, fatty acid metabolism might also be implicated in the regulation of NLRP3 inflammasome. However, current evidence is conflicting and synthesis and degradation of fatty acids were linked to inflammasome activation in different studies, perhaps indicating that imbalance itself may activate an inflammatory response. It is also possible that these metabolic pathways activate a common intermediate mediator able to directly activate the NLRP3 inflammasome, the main candidate being mitochondrial reactive oxygen species (mtROS) (53). The ω-3 PUFA, docosahexaenoic acid (DHA), inhibits the activation of caspase-1, thus lowering the production of active IL-1β (64, 65). Apart from DHA, other ω-3 PUFAs, such as eicosapentaenoic acid and α-linolenic acid, can inhibit the activation of the inflammasome (64, 65).

The notion the fatty acid metabolism is dysregulated in SSc dates back to the 1970s, by studies showing that intradermal adipose tissue is progressively replaced by fibrotic tissue in SSc (52, 66).

Taken together, these findings support the existence of a complex biologic loop in SSc, in which the TGF-β1 rich microenvironment, the up-regulated glutamine metabolism, the ehnahced glycolysis, and the fatty acid dysregulation, could all contribute to both inflammasome activation with IL-1β release and myofibroblasts differentiation, thus possibly foraging the occurrence of inflammation-driven fibrosis.

IL-1 AND HEART INFLAMMATION

Recent clinical and experimental data support the relevance of IL-1 in heart inflammation and cardiac dysfunction in several heart diseases. The heart exhibits a highly conserved response to tissue damage, characterized by a stereotyped inflammatory reaction that is centrally mediated by the pro-inflammatory cytokine IL-1 (24). Specifically, IL-1α is released from the dying myocardial cells together with and other intracellular contents, which act as mediators activating the inflammasomes in bystander cells (29, 32, 33, 67–69). IL-1-mediated inflammation ensues; if protracted, this leads to the apoptosis of cardiomyocytes and to the loss of contractile tissue progressively replaced by fibrosis, clinically manifested with cardiomyopathy, HF, and arrhythmic outburst (24).

Previous studies evaluating endomyocardial biopsy (EMB) samples from patients with acute lymphocytic myocarditis indicated that intracellular aggregates of either Apoptosis-associated speck-like protein containing CARD (ASC) or caspase-1, both indicative of inflammasome activation, can cardiomyocytes and infiltrating immune cells. Notably, the number of inflammasome-containing leukocytes correlated with the clinical severity of HF (67).

Moreover, IL-1 causes impaired contractile function by inducing multiple downstream events, including uncoupling of the β-adrenergic receptor from the adenylyl cyclase, inhibition of L-type calcium channels (24, 28, 67–74), transcriptional and post-translational changes in phospholamban and sarcoplasmic/endoplasmic reticulum calcium ATPase (24, 75), mytochondrial dysfunction, and nitric oxide (NO) synthesis (24, 76–78). Animal studies also confirm a role of IL-1-mediated inflammation in HF: injection of plasma from HF patients induced contractile dysfunction in mice, suggesting the existence of cardiodepressant factors in the circulation (79–82). Notably, administration of IL-1β to mice had similar effects, whereas pre-administration of IL-1 inhibitors prevented contractile dysfunction induced by HF serum: collectively considered, these findings indicate that cardiodepressant effects are centrally mediated by IL-1 (75–77), as also in sepsis (82).

Robust evidence also indicates that IL-1 signaling is central to the development of inflammation in both viral and autoimmune acute myocarditis (AMy). Mouse models of coxsackievirus-induced myocarditis exhibit heart infiltration with myeloid cells secreting IL-1 and TNF-α (83). Increased IL-1β expression is also a feature of chronic heart inflammation in experimental models of post-myocarditis DCM, induced by infection with encephalomyocarditis virus (84). Mice lacking IL-1RI did not develop AMy (85), and administration or over-expression of IL-1Ra reduced disease severity in experimental models of cardiomyopathy (86–89). These pre-clinical findings were paralleled by clinical observations in humans: EMBs from patients with viral myocarditis (85) and idiopathic DCM (86) revealed increased IL-1β mRNA levels.

HEART INFLAMMATION DOWNSTREAM IL-1

Once induced, inflammation escalates into a redundant process: hence, other pro-inflammatory cytokines may also play a key role in heart inflammation and inflammation-driven fibrosis. The IL-1 biological activity sustains an inflammatory process which involves IL-1 itself as well as downstream mediators. IL-6 is induced by IL-1, and acts as a downstream mediator of several inflammatory effects (**Figure 2**). It is thereby not surprising that IL-6 concentrations are elevated in the serum and myocardium of patients with HF and myocarditis, while also being predictive of adverse outcomes (90). In myocarditis, the primary sources of IL-6 are likely cardiomyocytes and cardiac fibroblasts (91, 92). Overexpression of IL-6 in experimental animals subjected to viral myocarditis results in extensive myocardial inflammation, whereas IL-6 inhibition with tocilizumab reduced heart inflammation and infiltration with CD3+T-cells and CD68+ macrophages (20).

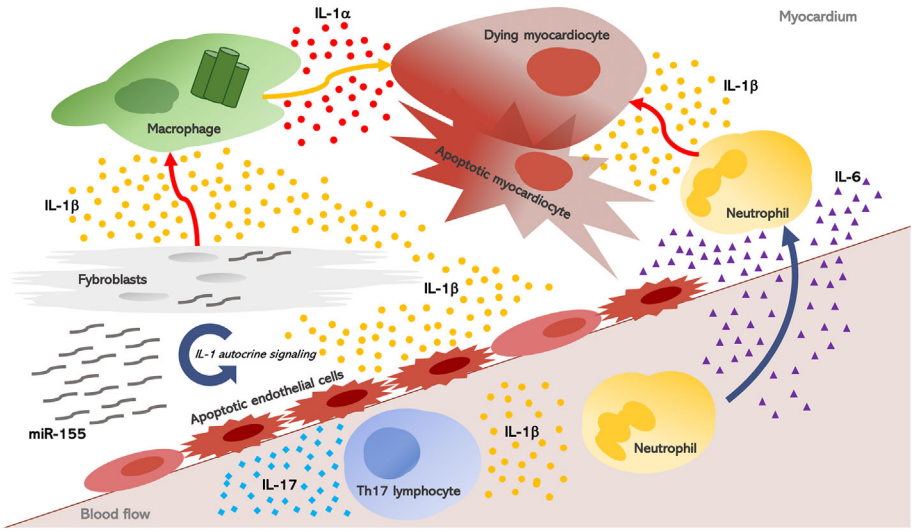

FIGURE 2 | Interleukin-1, myocardial inflammation, and heart fibrosis in systemic sclerosis. Heart inflammation results in myocardial injury. As a consequence, IL-1α is released from dying myocardiocytes, together with intracellular debris and inflammatory mediators; these in turn activate a molecular complex known as the "inflammasome" inside macrophages which processes and releases active IL-1β. Once induced, inflammation escalates into a redundant process: hence, other pro-inflammatory cytokines, mainly IL-6, are produced and they perpetuate heart inflammation and inflammation-driven fibrosis. IL-1 and IL-6 also promote Th17 differentiation, and in post-myocarditis, the role of IL-17A emerged in myocardiac remodeling, thus contributing to both myocardial fibrosis and progression to dilated cardiomyopathy. Finally, in SSc fibroblasts, the NLRP3 inflammasome is over-expressed with consequent increased production of IL-1b. IL-1 also stimulate SSc fibroblasts and induce the synthesis of micro-RNA (miR)-155 which establishes an autocrine loop further increasing IL-1 signaling.

Recently, tocilizumab was used to effectively treat SSc-related myocarditis, and improvement of myocardial inflammation was revealed as a reduction in myocardial edema at cardiac magnetic resonance (CMR), and by the improvement of cardiac function, clinical status and cardiac enzymes (20). Moreover, IL-6 plays a major role in heart fibrosis induced by Ang-II, through TGF-β/Smad activation. Consistently, IL-6 deficiency reduces cardiac inflammation, as well as contractile dysfunction and interstitial fibrosis, without affecting blood pressure in Ang-II-high salt-induced hypertension in IL-6 knockout (IL-6−/−) mice (21). Furthermore, deletion of IL-6 alleviates interstitial fibrosis also in experimental diabetic cardiomyopathy in IL-6−/− mice (81). The deletion or inhibition of miR155 yielded the same protective effects (42, 43), and recent studies revealed that the soluble IL-6 receptor (IL6R) is a target of miR155 (93). In summary, these studies delineate a miR-155/IL-1/IL-6 loop sustaining inflammation-driven fibrosis: overexpression of miR-155 in SSc fibroblasts induces inflammasome-mediated release of IL-1β, which in turn stimulates IL-6 production and collagen synthesis during fibrosis.

Another important signaling axis which potentially contributes to fibrosis and inflammation in SSc is the IL-1/IL-17 axis. Many of inflammatory cytokines that are involved in the SSc pathogenesis, (i.e., IL-1, IL-6, TGF-β), also promote Th17 differentiation. This strongly suggests their potential role in skewing CD4+ T cells toward Th17 differentiation in SSc. A recent *in vivo* study showed that IL-17 is involved in fibrosis and inflammation in bleomycin (BLM)-induced SSc. The authors also used another murine model of SSc, chronic graft-*versus*-host disease(cGVHD), to show that blocking IL-17 activity was able to attenuate disease severity. IL-1 and IL-17 synergically induce the expression of profibrotic and inflammatory mediators, both in human and murine dermal fibroblasts. Subsequent animal studies *in vivo* confirmed the antifibrotic and anti-inflammatory potential of IL-1Ra (94). Hence, IL-17 inhibition, either directly or by blocking IL-1, has therapeutic rationale for tissue fibrosis in SSc. In post-myocarditis, the role of IL-17A emerged either in myocardiac remodeling and the progression to DCM, thus contributing to myocardial fibrosis following experimental AMy by a protein-kinase-C(PKC)b/Erk1/2 Nuclear Factor (NF)-kB signaling (95, 96).

THERAPEUTIC APPLICABILITY OF IL-1 INHIBITION AND FUTURE PERSPECTIVES

Despite extensive experimental evidence pointing at a central role for IL-1 in the pathogenesis of heart inflammation, systolic dysfunction, and fibrosis, and despite a possible role of this cytokine in SSc skin and lung inflammation, the use of available IL-1 blocking agents in SSc was only anedoctally reported.

Rilonacept, a fusion protein consisting of the human IL-1 receptor (IL-1R1) and IL-1 receptor accessory protein (IL-1RAcP) which binds and neutralizes both IL-1α and -β, was evaluated in single phase I/II randomized, double-blind, placebo-controlled trial on SSc patients. The primary endpoint was the

level of skin expression of the 2G SSc gene biomarkers, which functions as a proxy for the mRSS, while the secondary endpoint was the change in mRSS. Nineteen patients were randomized 2:1 rilonacept 320 mg loading dose at day 0 and then 160 mg weekly *versus* placebo. Skin biopsies were obtained before rilonacept treatment initiation and at week 7. Both the primary and the secondary endpoints were not met, as no changes in gene expression or in the mRSS between treated and placebo patients were observed after 6 weeks (97). However, this trial had several limitations, including the small sample size and the short duration of therapy, even more important in the context of a chronic fibrotic disease. Moreover, no exploratory secondary endpoints to evaluate SSc-HI were considered.

Data from animal models are scarce and conflicting. Treatment with anakinra, a recombinant IL-1 receptor antagonist, improved BLM-induced pulmonary fibrosis in mice and of pulmonary silicosis in humans (98, 99), as well as pulmonary function in patients with COVID-19 and systemic hyperinflammation (100). However, anakinra aggravated pulmonary fibrosis due to Th2 skewing in the fos-related antigen-2 (Fra-2) mouse model of SSc, and was not associated to changes in lung inflammation profile in wild type mice (101). To date, the Fra-2 transgenic mice spontaneously develop pulmonary inflammation. These findings on animal models suggest that the net effect of IL-1 (or of its inhibition) is context-dependent. For this reason, it is still premature to transfer these limited data, obtained on animal models, into clinical practice.

It is important to note that recent evidence supports IL-1 therapeutic blockade in HF and myocarditis. In fact, given the role of IL-1 in both heart inflammation and contractile dysfunction, and the potential role in mechanisms of inflammation-driven heart fibrosis through different pathways, IL-1 inhibition might fit in a proof of concept rationale for an anti-inflammatory therapeutic strategy in SSc myocarditis. In patients with myocardial inflammation and HF, the short-term treatment regimen (14 days) with anakinra improved exercise capability, as determined by oxygen consumption (VO_2) (79, 102). Prolonged administration (12 weeks) reduced hospitalizations and further improved VO_2, NTproBNP, and the quality of life in a randomized clinical trial (RCT) (103). Similarly, treatment with the anti–IL-1β monoclonal-antibody canakinumab significantly reduced cardiovascular events in 10,061 patients with previous myocardial infarction and C-reacrive protein >2 mg/L in the CANTOS-trial (104). Recently, our group firstly described the dramatic dampening of heart inflammation and an unprecedented clinical improvement after IL-1 suppression with anakinra in a patient with DCM. Improvement began soon after anakinra administration with improvement of the arrhythmic outburst, decrease of cardiac biomarkers, and normalized echocardiography and CMR imaging—including those that measured LVEF (105). Anakinra treatment was also beneficial in patients with fulminant myocarditis (106–108). Taken together, these observations suggest that IL-1 inhibition could curb heart inflammation while also ameliorating myocardial contractility. Thus, IL-1 inhibition may dampen disease progression and fibrotic damage in patients with myocarditis and other inflammatory cardiomyopathies.

A significant bulk of data also demonstrates the efficacy of IL-1 blockade in pericardial inflammation and recurrent pericarditis (109–113), which usually requires steroids and immunosuppressive treatment when clinically evident (1), including a RCT of anakinra in 21 patients (96), and more recently a positive trial with rilonacept (114). This is of great importance since pericarditis is common in SSc: symptomatic pericarditis occurs in 5–16% patients, whereas autopsy-demonstrated pericardial involvement occurs in 33–72% patients (1).

Beside the aforementioned RCTs in HF (79, 102, 103), double blind, phase IIb, randomized, placebo-controlled clinical trials of anakinra are ongoing to evaluate this treatment in acute myocarditis [ARAMIS-trial, ClinicalTrials.gov: Identifier: NCT03018834] and EMB-proven virus-negative myocarditis [MYTH-1 trial; Eudract: 2018-003472-13]. The monoclonal antibody canakinumab is also clinically available, which blocks IL-1β111). More recently, oral NLRP3 inflammasome inhibitors have been proposed to treat a wide spectrum of inflammatory cardiovascular diseases, and RCTs are ongoing (115).

Results from these trials, together with the robust biologic proof of concept, could potentially pave the way to the use of IL-1 therapeutic blockade to treat SSc-related inflammatory heart involvement.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GDL: conceived the hypothesis, contributed to the understanding of pathogenic mechanisms of both systemic sclerosis heart involvement and IL-1 mediated heart inflammation, generated original data to support the hypothesis, and drafted the manuscript. GC: contributed to the understanding of biological effects of IL-1 and IL-1 therapeutic blockade in a broad spectrum of rheumatic and inflammatory diseases, generated original data to support the hypothesis, and critically revised the manuscript. CC: contributed to the understanding of pathogenic mechanisms of both systemic sclerosis heart involvement and IL-1 mediated heart inflammation, and critically revised the manuscript. CB: contributed to the understanding of pathogenic mechanisms, clinical presentation, and prognostic meaning of systemic sclerosis, particularly heart involvement, and critically revised the manuscript. AT: contributed to the understanding of biological effects of IL-1 and IL-1 therapeutic blockade in a broad spectrum of rheumatic and inflammatory diseases, created

the figures, and critically revised the manuscript. LD: conceived the hypothesis, critically revised the manuscript, and gave the approval of the final version. MM-C: conceived the hypothesis, critically revised the manuscript, and gave the approval of the final version. All authors contributed to the article and approved the submitted version.

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REVIEW
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Metabolic Control of Autoimmunity and Tissue Inflammation in Rheumatoid Arthritis

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Like other autoimmune diseases, rheumatoid arthritis (RA) develops in distinct stages, with each phase of disease linked to immune cell dysfunction. HLA class II genes confer the strongest genetic risk to develop RA. They encode for molecules essential in the activation and differentiation of T cells, placing T cells upstream in the immunopathology. In Phase 1 of the RA disease process, T cells lose a fundamental function, their ability to be self-tolerant, and provide help for autoantibody-producing B cells. Phase 2 begins many years later, when mis-differentiated T cells gain tissue-invasive effector functions, enter the joint, promote non-resolving inflammation, and give rise to clinically relevant arthritis. In Phase 3 of the RA disease process, abnormal innate immune functions are added to adaptive autoimmunity, converting synovial inflammation into a tissue-destructive process that erodes cartilage and bone. Emerging data have implicated metabolic mis-regulation as a fundamental pathogenic pathway in all phases of RA. Early in their life cycle, RA T cells fail to repair mitochondrial DNA, resulting in a malfunctioning metabolic machinery. Mitochondrial insufficiency is aggravated by the mis-trafficking of the energy sensor AMPK away from the lysosomal surface. The metabolic signature of RA T cells is characterized by the shunting of glucose toward the pentose phosphate pathway and toward biosynthetic activity. During the intermediate and terminal phase of RA-imposed tissue inflammation, tissue-residing macrophages, T cells, B cells and stromal cells are chronically activated and under high metabolic stress, creating a microenvironment poor in oxygen and glucose, but rich in metabolic intermediates, such as lactate. By sensing tissue lactate, synovial T cells lose their mobility and are trapped in the tissue niche. The linkage of defective DNA repair, misbalanced metabolic pathways, autoimmunity, and tissue inflammation in RA encourages metabolic interference as a novel treatment strategy during both the early stages of tolerance breakdown and the late stages of tissue inflammation. Defining and targeting metabolic abnormalities provides a new paradigm to treat, or even prevent, the cellular defects underlying autoimmune disease.

Keywords: T cell, metabolism, autoimmunity, rheumatoid arthritis, mitochondria, glycolysis, glutaminolysis, fatty acid

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease characterized by inflammation of the synovial tissue and autoantibody production (1, 2). Typically, the synovial membrane is infiltrated with T cells, B cells and macrophages, eliciting a maladaptive response-to-injury in fibroblast-like synoviocytes (FLS) that hyper-proliferate and destroy cartilage and bone (1, 3). Over the decade-long course of RA, a combination of genetic, epigenetic, and environmental factors contributes to rendering the host susceptible to autoimmunity and the eventual appearance of joint inflammation. An array of immune cells, including macrophages, dendritic cells, mast cells, neutrophils, T cells and B cells, have all been implicated in the disease process and make a pathogenic contribution during early loss of self-tolerance, the subsequent progression to joint inflammation when joint-specific protection factors fail and the final "non-healing wound" when the collective action of immune cells and stromal cells demolishes the joint (1, 4).

Patients diagnosed with RA produce a specific set of autoantibodies, typically reactive against post-translational modifications of proteins (5–7). Such autoantibodies appear decades before clinically apparent disease (8). Reactivity against a broad spectrum of citrullinated proteins, instead of a single autoantigen, has questioned the relevance of classical models of autoimmunity in RA and instead has emphasized the potential role of antigen-nonspecific factors, such as the metabolic control of immune cell function (9, 10). The genetic risk conferred by HLA class II molecules, the accumulation of chronically activated T cells in the diseased joint and the T-cell dependence of autoantibody production all support a critical role of dysfunctional memory T cells as a salient feature of RA. Differentiation and functional commitment of T cells is critically dependent on metabolic adaptations that co-ordinate the biosynthetic and energy demands imposed by chronic activation and the massive generation of cellular offspring (11). It is now clear that T cells not only differentiate into memory T cells, they also can become aberrantly activated, phenotypically unstable, exhausted, or senescent (12, 13). All these different functional states are ultimately interlinked with a specific metabolic program. RA T cells are characterized by a metabolic signature and progress has been made in defining molecular mechanisms underlying the metabolic deviations. Here, we will review how T cell metabolism influences the development of inflammation in the synovial tissue, focusing on three major metabolic pathways: glucose metabolism, glutamine metabolism and lipid metabolism. Ultimately, understanding how energy utilization and metabolite production dictates the cells' fate outside and inside of the joint will set the stage for the design of novel immunotherapeutic strategies.

GLUCOSE METABOLISM AS AN ARTHRITOGENIC RISK FACTOR

Glucose metabolism, one of the primary metabolic pathways in the body, primarily refers to the process of breaking down glucose into ATP and intermediate metabolites, including glycolysis, aerobic oxidation, and processing in the pentose phosphate pathway (PPP). Glucose metabolism not only provides energy for physical activity but also mediates a variety of physiological processes through the formation of complex signaling networks with metabolic substrates. Glycolysis involves the catalytic conversion of one molecule of glucose into lactic acid, producing two adenosine triphosphate (ATP) molecules, whereas aerobic oxidation catalyzes one molecule of glucose into CO_2 and H_2O and generates 38 or 36 ATP molecules. Both glycolysis and aerobic oxidation are important ways to generate ATP, but the choice is typically adaptive to the physiological and pathological needs occurring in cells, tissues, and organs. In recent years, data have accumulated identifying glucose metabolism as a key component in the pathogenesis of RA.

Glycolytic Breakdown in Inflammatory Effector Cells

In normal synovial tissues, glycolysis is the primary pathway promoting mitochondrial substrate oxidation of pyruvate. The activity levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH), the major enzymes of the glycolytic pathway, are increased in RA synovial cells (14). Accordingly, lactate concentrations are high and glucose concentrations are distinctly low in the inflamed joint (15). Hexokinase, which catalyzes the first step in glucose metabolism, enhances the ability of FLS to migrate and invade (16, 17). In RA FLS, the balance between glycolysis and oxidative phosphorylation is shifted toward glycolysis (18). Synovial fibroblasts are key effector cells in the final stages of RA (19), are under considerable metabolic stress and produce competition for energy sources. Synovial tissue with its increased glycolytic activity represents only one "battle ground" of the disease. Disease-relevant cells live and function in other tissue environments, particularly the lymphoid tissues from which they originate (**Figure 1**).

Accordingly, naïve $CD4^+$ T cells isolated from RA patient exhibit diminished glycolytic activity (20). Before such naïve $CD4^+$ T cells become pathogenic memory and effector T cells, they utilize glucose in a distinctly different manner than naïve $CD4^+$ T cells from healthy individuals: they avoid glycolytic breakdown into lactate and instead divert glucose into the PPP, driving the accumulation of NADPH and consumption of cellular reactive oxygen species (ROS). With an excess of reducing equivalents, T cells are unable to activate the relevant redox kinases, which allows them to bypass the regulatory checkpoints of the G2/M cell cycle and enter unrestrained proliferation (21). The lack of cellular ROS appears to be a T-cell-specific feature.

Macrophages from RA patients have high mitochondrial activity and can efficiently generate ROS (22–24). In patient-derived macrophages, suppression of GSK-3β fuels mitochondrial activity, enhances ATP synthesis and ROS release. This metabolic constellation causes the cytoplasm-to-nucleus translocation of the glycolytic enzyme pyruvate kinase M2 (PKM2). Functional outcomes include the PKM2-dependent

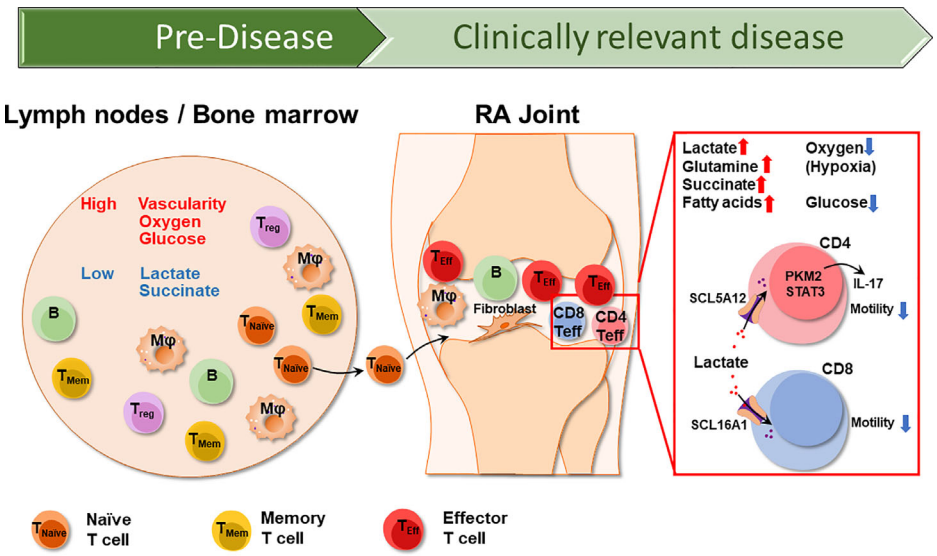

FIGURE 1 | The different Tissue Environments of Rheumatoid Arthritis. Early events in the RA disease process, e.g., the loss of self-tolerance of RA T cells occurs in lymphoid tissues, such as lymph nodes and the bone marrow. Over lifetime, lymphoid tissues remain the home of naïve and memory T cells, which circulate through the peripheral blood where they can be sampled. Lymph nodes are highly vascularized, therefore have access to oxygen and glucose. As RA naïve T cells differentiate into short lived effector T cells (SLEC), they acquire the ability to leave the circulation and invade into the synovial tissue. Synovial effector T cells encounter a different metabolic environment which is hypoxic, low in glucose and high in lactate due to the chronic activation of macrophages and stromal cells. $CD4^+$ effector T cells and $CD8^+$ effector T cells uptake lactate through SCL5A12 and SCL16A1, respectively. Imported lactate promotes IL-17 secretion in $CD4^+$ T cells and inhibits both $CD4^+$ and $CD8^+$ T cell motility, essentially arresting T cells in the lactate^{hi} synovium. $T_{naïve}$, naïve T cell; T_{Mem}, memory T cell; T_{reg}, regulatory T cell; Mφ, macrophage; B, B cell.

activation of STAT3, boosting the production and secretion of pro-inflammatory cytokines (25), such as IL-6 and IL-1 (**Figure 2**). Such pro-inflammatory macrophages accompany T cells in the inflamed joint, where both cell types compete for the access to glucose. Naïve $CD4^+$ T cells live in lymphoid organs and it is unlikely that inflammatory macrophages regulate the fate decisions of such T cells prior to their differentiation into memory/effector cells. In the inflamed joint, multiple, functionally diverse macrophage subsets are now recognized (26). In general, inflammatory macrophages are considered to mainly rely on glucose as an energy carrier and resolving/anti-inflammatory macrophages are known to require less glucose and supply their bioenergetic and biosynthetic needs through mitochondrial oxidative phosphorylation (27). Whether synovial macrophage subsets differ in their energy generation and utilization and the metabolic environment they build in their surroundings remains unexplored.

Oxidative Phosphorylation in Inflammatory Effector Cells

The major metabolic abnormality of RA $CD4^+$ T cells lies in the mitochondria (**Figure 3**). Mitochondrial failure presents as low ATP generation and reduced release of ROS (28). Production of the mitochondrial intermediate succinate is impaired, and the mitochondrial tricarboxylic acid cycle (TCA) cycle changes direction (29). Unable to convert acetyl-CoA into ATP, RA $CD4^+$ T cells produce excess citrate and transport it out of the mitochondria into the cytosol (29). Here, acetyl-CoA participates in post-translational modification of proteins, shifting the functional commitment of the cells toward mobility and cytokine release (29). Mitochondrial failure in RA T cells results from insufficient repair of mitochondrial DNA, due to loss-of-function of the DNA repair nuclease MRE11A (30). A second dimension in maintaining mitochondrial fitness derives from the misplacement of the energy sensor AMP-activated protein kinase (AMPK) (31). Under physiologic conditions, a decline in ATP would trigger mitochondrial biogenesis *via* activation of AMPK (32). In RA T cells, this mechanism is paralyzed, forcing the cell to function with damaged mitochondria. The ATP^{lo} status of RA T cells is aggravated by suppressed glycolysis; attributable to the transcriptional repression and functional impairment of the glycolytic enzyme phosphofructokinase (20). Instead, glucose is shunted into the PPP, supporting anabolic metabolism and laying the groundwork for biosynthesis, biomass production and the generation of new cells (21). Together, these data support the concept that irregularities in glucose metabolism are evident during an early stage of RA, when naïve $CD4^+$ T cells deviate from a normal differentiation pattern and make a commitment to become a pro-inflammatory effector cell.

Immunoregulation Through Glucose Uptake

As part of their activation program, T cells switch to high glycolytic flux (33, 34), securing rapid access to ATP, but also to biosynthetic precursor molecules required for cellular

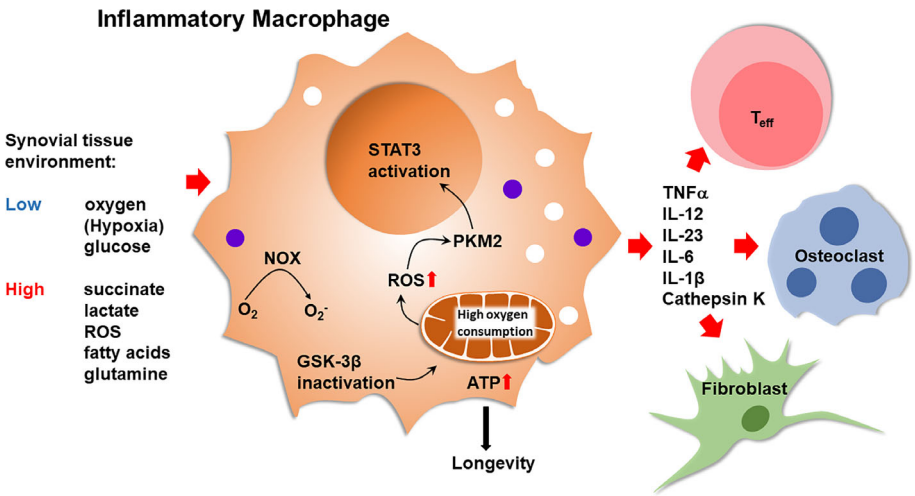

FIGURE 2 | Inflammatory Macrophages in Rheumatoid Synovitis. Bone marrow-derived macrophages that infiltrate into the arthritic joint enter an oxygen^{lo}, glucose^{lo}, ROS^{hi}, succinate^{hi}, fatty acid^{hi}, lactate^{hi} tissue environment. Inactivation of glycogen synthase kinase 3β (GSK-3β) in macrophages optimizes pyruvate import, enhancing mitochondrial activity. High oxidative phosphorylation increases ATP production and ROS release. ATP abundance promotes macrophage longevity. ROS facilitate the dimerization of the cytosolic enzyme pyruvate kinase M2 (PKM2), enabling nuclear translocation and STAT3 activation by the kinase. High mitochondrial activity supports the production of TNFα, IL-12, IL-23, IL-6 and IL-1β. Inflammatory macrophages trigger and sustain synovitis by modulating the function of neighboring T effector cells, endothelial cells, osteoclasts and synovial fibroblasts. NOX, NADPH oxidase; T_{eff}, T effector cell.

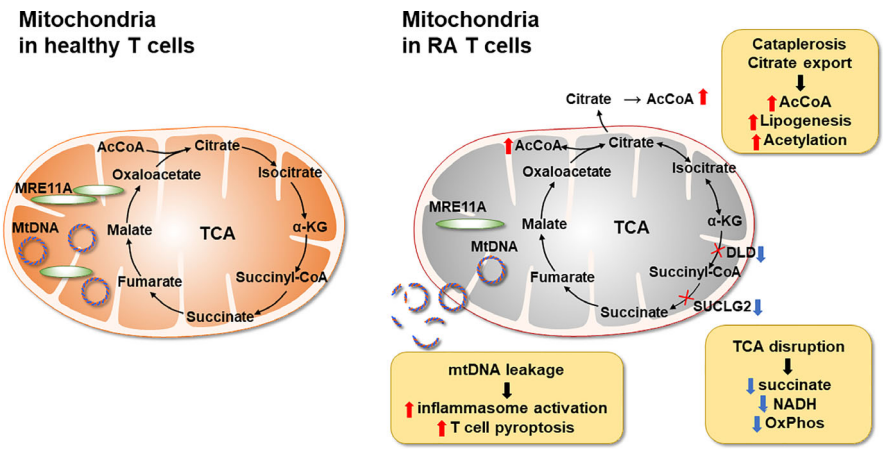

FIGURE 3 | Mitochondrial Defects in T cells in rheumatoid arthritis. RA CD4$^+$ T cells fail to maintain mitochondrial DNA due to the transcriptional repression of the nuclease MRE11A, a limiting factor in DNA double-strand break repair and replication fork protection. Instability of mtDNA causes leakage of mtDNA fragments into the cytosol, triggering activation of the inflammasome and eventually T cell pyroptotic death. Mitochondrial oxidative phosphorylation is further impaired by the disruption of the TCA cycle. Transcriptional repression of succinyl-CoA ligase (SUCLG2) prevents the conversion of a-ketoglutarate into succinate and forces the TCA cycle to change direction. In a cataploretic reaction, mitochondria of RA T cells produce and export excess citrate, creating an acetyl-CoA-rich environment in the cytoplasm. Together with NADPH generated in the hyperactive pentose phosphate pathway surplus acetyl-CoA enters biosynthesis, specifically lipogenesis. Also, excess acetyl-CoA drives protein acetylation, including the acetylation of microtubules. α-KG, α-ketoglutarate; mtDNA, mitochondrial DNA; SUCLG2, Succinate-CoA Ligase GDP-Forming Subunit Beta.

expansion. The swift upregulation of glycolysis is linked to the increased expression of the glucose transporter 1 (GLUT1) maximizing glucose uptake by the cell (35). Glucose uptake in T cells undergoing activation is strictly correlated to GLUT1, identifying the transporter as a key determinant in glucose utilization (36, 37). Notably, GLUT1 expression differs among different T cell lineages and is highest in Th17, Th2, and follicular helper T cells (TFH) cells, followed by Th1 cells (38, 39). This difference in expression correlates with differential glycolytic rates in these T cell subsets (38) and identifies GLUT1 as a potential target for immunomodulation. Induction of *GLUT1* transcripts is an early event during T cell receptor stimulation

and is well known to depend on co-stimulatory signals. Signaling through CD28 and the downstream PI3K/Akt activation determines glucose uptake though GLUT1 upregulation (40). Cross-linking of the T cell receptor alone or in combination with IL-2 appears to have no effect on the level of GLUT1 expression (41, 42). Like T cells, B cells require GLUT1 upregulation for optimal activation responses and similarly, rely on PI3K-dependent signaling (43).

Experimental data suggest that GLUT1-mediated glucose transport may have differential effects in $CD4^+$ and $CD8^+$ T cells. GLUT1 knockout (KO) T cells fail to proliferate in response to immunization with an experimental antigen or *in vitro* stimulation (44). Similarly, treatment with CG-5, a nonselective small molecule glucose transport inhibitor inhibited T cell proliferation (45). CG-5 blocked Th1 and Th17 polarization and reduced T cell expansion in a mixed lymphocyte reaction. Interestingly, $GLUT1^{neg}$ $CD8^+$ T cells proliferated to a similar extent and expressed equal levels of granzyme B as their wild type counterparts following stimulation. Thus, the cytotoxic potential of $CD8^+$ T cells may not depend on GLUT1. Increased expression of GLUT3 and GLUT6 in GLUT1 KO $CD8^+$ T cells could possibly compensate for the deficiency of glucose uptake by GLUT1 (44). More importantly, immunosuppressive T regulatory cells were not affected by GLUT1 deletion as they rely on fatty acid oxidation rather than glycolysis for their activity (38). GLUT1 expression had major consequences not only for proliferation but also for differentiation of T cells, as exemplified by the deficient induction of IFN-γ and IL-17 in $GLUT1^{lo}$ T cells (45).

The function of GLUT family member in T cells is well studied, but not clear in RA. There are only a few reports on GLUT1, GLUT3 and GLUT4 in RA. Heterozygous deletion of *GLUT3* correlates directly with expression levels of GLUT3 and influences glycolysis rates in the human immune system (46), but the frequency of the GLUT3 copy number variant is not different among RA, multiple sclerosis and heathy control, providing no evidence for RA protection in deletion of GLUT3 (46), the study of which demonstrated GLUT3 is not necessary for glucose transfer in patients with RA. However, another study showed the mutual activation between $CD4^+$ T cells and FLS, which resulted in increased proliferation and expression of glucose transporters GLUT1 and GLUT3 in FLS (47). In rat Arthritis model, arthritic rats showed cachexia, reduced adipocyte size, and downregulated GLUT4 in adipocyte membranes (48). Although the function of GLUT4 in pro-inflammatory T cells is unknown, the downregulated membrane GLUT4 confirms its role in autoimmunity.

Taken together, the intensity of glycolysis is highly dependent on active glucose transport, making GLUT1 an excellent marker of glycolytically active T cell populations. Glucose utilization determines T cell proliferation and differentiation, identifying this metabolite as an excellent candidate for metabolic interference. Immunoinhibitory Treg cells are less dependent on glucose uptake and should therefore be less vulnerable to glucose withdrawal. Controlling glucose import may represent an excellent approach to immunomodulation.

Tissue Lactate as an Immunoregulatory Mediator

Glycolysis has a low efficiency in generating energy. The terminal product of the anaerobic oxidation of glucose is lactate. Lactate has long been considered a "waste" by-product of cell metabolism, and it accumulates at sites of high glycolytic activity, such as tumor surroundings or inflamed tissue areas. Clinically, elevated serum lactate levels are a sensitive indicator of stress-induced glycolysis during sepsis (49). Since lactate production acidifies the environment, physiologic lactate concentrations are kept in a narrow range (1.5-3 mM) in blood and tissues of healthy individuals. Lactate can serve as an energy carrier and can be transported across cell membranes by mono-carboxylate transporters and can be reabsorbed by the kidneys to prevent energy loss. However, fueled by extensive glycolysis, lactate can increase up to 10 mM in inflammatory lesions and even higher (30-40 mM) in cancerous tissues (50, 51). Lactate accumulates in the synovial fluid of RA patients (52), reflecting high glucose turnover by cells trapped in the synovial membranes. It has been proposed that synovial lactate measurement may function as a reliable indicator to differentiated subtypes of inflammatory arthritis (53) (**Figure 1**).

Elegant work has examined the immunomodulatory impact of synovial tissue lactate in RA patients. T cells sense lactate *via* the expression of specific transporters and RA T cells may even be more reliant on the uptake of energy carriers, given their inherent difficulties of generating ATP in their mitochondria. Lactate uptake by $CD4^+$ T cells has been implicated in inhibiting migratory capability, essentially arresting T cells in the $lactate^{hi}$ microenvironment (**Figure 1**). Also, lactate availability fostered differentiation of a Th17 subset, further supporting the chronic inflammatory process (49, 54). As reported by Pucino et al., synovial T cells adapt to their milieu by upregulating the lactate transporter SLC5A12. In line with the fundamental difficulties of RA T cells to secure energy production through mitochondrial activity (29, 30), SLC5A12 high-expressing tissue $CD4^+$ T cells appear to be particularly dependent on utilizing alternative energy resources. Nurtured by metabolically highly active macrophages and synovial cells (18, 55) lactate-depending $CD4^+$ T cells will be functionally biased toward IL-17 production, mediated by nuclear PKM2/STAT3 and enhanced fatty acid synthesis (56). Here, tissue-resident T cells display a metabolic signature previously described for pro-inflammatory macrophages that use nuclear PKM2/STAT3 to sustain IL-1 and IL-6 production (57).

Furthermore, tissue lactate not only shapes the functional commitment of $CD4^+$ T cells, but also affects $CD8^+$ effector T cells. Extracellular sodium lactate and lactic acid inhibit the motility of $CD4^+$ and $CD8^+$ T cells, respectively. The selective regulation of T cell mobility is mediated *via* subtype-specific transporters, SLC5A12 and SLC16A1, selectively expressed on $CD4^+$ and $CD8^+$ T cells, respectively (50). Underlying mechanisms have been defined and show that the lactate-induced inhibition of $CD4^+$ T cell movement results from an interference with glycolysis that is activated when the chemokine CXCL10 engages the chemokine receptor CXCR3. CXCR3 is

typically expressed on effector T cells, both $CD4^+$ and $CD8^+$ subtypes (58), emphasizing that the lactate-rich environment modifies the function of differentiated effector T cells as opposed to lymph node-residing naïve T cells that are upstream of synovitis induction (**Figure 1**). Interestingly, lactate exposure appears to result in a very different outcome for $CD4^+$ and $CD8^+$ effector T cells, stimulating the prior and inhibiting the latter (50). Single cell technology may be helpful in further breaking down the heterogeneous nature of tissue-residing effector T cells (59, 60), enabling the assignment of functional subsets to particularly patterns of nutrient uptake and utilization.

Lactate has drawn broad attention as a metabolite shaping the tissue milieu within and around tumors. A positive correlation between lactate dehydrogenase A (LDH-A), high local lactate levels, and tumor progression has been documented in various tumors (61). Autoimmunity-related tissue inflammation causes less of an acidification, but infiltrating cells may be exposed to similar cues. LDH isoenzymes have been reported to be higher in serum and synovial fluid of RA compared to osteoarthritis patients (62). And, it has long been known that LDH activity is increased in RA synovial tissues compared to healthy controls, emphasizing the dependence of the lesion on glycolytic metabolism (63). In healthy T cells, LDH-A is important in regulating differentiation and lineage assignment. By increasing availability of substrate for acetylation and shifting the T cell epigenome, LDH promotes Th1 commitment and IFN-γ production (64). A recent study has identified LDHA high expression as a feature of all $CD8^+$ T cell subsets in RA patients (65). Inhibition of LDHA with FX11 (LDHA inhibitor) led to reduction in lipogenesis, migration, and proliferation of $CD8^+$ T cells, and lowered $CD8^+$ T cell effector functions (65). LDHA inhibition successfully abrogated the ability of RA $CD8^+$ T cells to sway healthy B cells toward a pro-inflammatory phenotype. The $LDHA^{hi}$ phenotype of peripheral CD8 T cells was maintained in $CD8^+$ T cells from the synovial membrane (65).

In summary, upregulation of glycolysis, and with it the production of lactate, is a marker of cellular activation and growth. The dependence of highly proliferative cells on glucose as an energy carrier leads to localized lactate accumulation and to acidification of the tissue site. During the late stages of the RA disease process, after T cells have arrived in the tissue environment, they are exposed to a lactate-rich milieu. Lactate providing a cellular arrest signal fits well into the concept that T cells build an extra-lymphoid site where they persist, commit to pro-inflammatory effector functions, and promote the building of self-sustained lymphoid architecture (66–68).

Immunoregulation Through the Mitochondrial Intermediate Succinate

By producing NADH, the tricarboxylic acid (TCA) cycle is a central route for oxidative phosphorylation, fueling complex I of the electron transport chain and eventually ATP generation. The TCA cycle fulfills other bio-energetic, biosynthetic, and redox balance requirements and functions as a metabolic hub. One major TCA function is the production of metabolic intermediates, that can be transported out of the mitochondria and participate in cytosol, nuclear and extracellular processes. An abundant TCA metabolite is succinate, which, like lactate, appears in the extracellular milieu, where it can be taken up by surrounding cells. Succinate is a product of myeloid and lymphoid cells, but the succinate receptor GPR91 is selectively expressed on monocytes/macrophages, granulocytes, and dendritic cells (69–71). GPR91 plays a critical role in the development of immune-mediated arthritis, supports the expansion of the Th17 cell population and acts as an overall amplifier of experimental antigen-induced arthritis. *GPR91-/-* mice show reduced articular hyperalgesia, neutrophil infiltration and inflammatory cytokines in the joint, and reduced frequency of Th17 cells in the draining lymph nodes (71).

Evidence has been provided describing succinate as a signaling molecule in the arthritic joint, with macrophages as the recipients of the stimulatory signals (70) (**Figure 2**). However, the cellular source of the succinate has not been determined. In general, succinate is recognized as a strong pro-inflammatory stimulator (72). LPS stimulation of macrophages results in abundant succinate production, which stabilizes Hypoxia-inducible factor 1α (HIF-1α) and IL-1β an important downstream target (73). In innate immunity, succinate may be one of the major signaling molecules (72), identifying metabolic activity as a key determinant of regulating the intensity of inflammation. Conversely, in the adaptive immune system, lack of succinate appears to be of particularly relevance in breaking tissue tolerance and causing inflammation (**Figure 3**). T cells isolated from RA patients are distinctly low in succinate, due to repression of the *SUCLG2* gene, which brings the TCA to a halt and shifts from the oxidative to the reductive direction. One outcome is the accumulation of the oncometabolite α-ketoglutarate, feeding the production of citrate and acetyl-CoA. Excess acetyl-CoA imposes a pro-inflammatory effector phenotype through acetylation of the microtubular cytoskeleton (**Figure 3**). $Succinate^{low}$ $acetylCoA^{hi}$ T cells rapidly reshape their cellular body, form a rear uropod, become hypermobile and invade into the tissue site (29). In that scenario, succinate supplement could be beneficial, reestablishing directionality of the TCA cycle, supporting the electron transport chain and forcing the T cell into a more tolerant state. Diametrical metabolic states in innate and adaptive immune cells seems to be a consistent finding in RA (55), beyond the role of succinate. Also, ATP and reactive oxygen species are abundant in monocytes/macrophages but scares in T cells. It is currently unknown how hypermetabolic macrophages communicate with hypometabolic T cells and vice versa and what the functional consequences are. But succinate appears to have a context-specific and cell-type specific role extending beyond its function as an energy carrier.

HYPOXIA AS AN AMPLIFIER OF TISSUE INFLAMMATION

Mitochondrial metabolism ultimately depends on the availability of oxygen, which functions as the final electron acceptor in the

electron transport chain. Oxygen pressures are highest in the lung, considerably lower in the blood and reach hypoxic levels in the tissue. Immune cells and tissue stromal cells need to adapt to hypoxic conditions and hypoxia is now recognized as a critical modulator of RA tissue inflammation (74). In line with the concept that reduced mitochondrial fitness is a risk factor for pro-inflammatory behavior, levels of synovial oxygen have been reported to negatively correlate with disease activity. Hypoxia induces a wide spectrum of alterations in mitochondrial structure, dynamics, and mitochondrial DNA (mtDNA) stability, resulting in impaired mitochondrial respiration, excessive production of reactive oxygen species (ROS), loss of ATP, increased oxidative damage and the accumulation of mtDNA mutations (75). One consequence of hypoxia is the activation of the transcription factor HIF-1α, which in turn promotes a gene program designed to enhance the production of glycolytic energy, including glucose transporters and glycolytic enzymes (76, 77). Hypoxic conditions in the rheumatoid joint should favor the survival of T cells that are less dependent on glycolytic breakdown, e.g., T cells with repression of the glycolytic enzyme PFKFB3 (78). PFKFB3^{lo} RA T cells shift glucose toward the PPP (20) and produce biosynthetic precursors even under low oxygen conditions. Cells less dependent on proliferative expansion, such as synovial fibroblasts and macrophages, switch toward glycolysis as an oxygen-independent way of generating ATP.

By sustaining chronic upregulation of HIF-1α, synovial hypoxia provides a feed-forward mechanism amplifying synovitis. HIF-1α increases the activity of lactate dehydrogenase A (LDHA), which converts pyruvate to lactate. The resultant acidic environment promotes fibroblast and immune cell proliferation and persistence. High lactate and low O_2 enable the survival of T cells by stabilizing HIF-1α (79, 80). HIF-1α has been reported to be highly expressed in RA synovium (81), identifying this transcription factor as a hallmark of synovial tissue inflammation. Hypoxia further enhances the stabilization of HIF-1α induced by T-cell-receptor-mediated activation of the PI3K-mTOR pathway (82), functioning as a stabilizer of autoimmune tissue inflammation. HIF-1α$^+$ T cells may be particularly adapted to serve as pro-inflammatory effector cells (74). Driven by the hypoxic microenvironment of the joint, FOXP3$^+$ regulatory T cells promptly convert to pathogenic Th17 cells (83), further weakening anti-inflammatory mechanisms. HIF-1α-dependent induction of retinoic acid-receptor-related orphan receptor-γt (RORγt) combined with targeting FOXP3 for degradation is detrimental to Treg cells and fosters Th17 cell generation (84), identifying hypoxia as a potent risk factor for unrelenting inflammation.

Low availability of oxygen equally encourages pathogenic traits of stromal cells. Hypoxia and IL-17 synergize to drive migration and invasion of synovial fibroblasts through MMP2 and MMP9 expression (85). HIF-1α also controls fibroblast IL-33 production, which in turn enhances HIF-1α expression and generates a regulatory cycle that perpetuates RA inflammation (86). Also, cooperation of HIF-1α with intracellular signaling cascades may accelerate pro-inflammatory pathways. Under hypoxic conditions, HIF-1α interacts with Notch-3 and STAT-1 in RA synoviocytes to stabilize and enhance stromal cell inflammation (87).

GLUTAMINE AS A FUEL SOURCE FOR JOINT INFLAMMATION

While much of the focus of immunometabolism has been directed to central carbon metabolism, e.g., glycolysis and the TCA cycle, amino acids are now emerging as critical regulators of immunocompetence. Among the 20 amino acids edited by gene codons, glutamine appears particularly important, contributing to bioenergetic as well as biosynthetic processes, while also helping to maintain redox balance. Glutamine is the most abundant and widely used amino acid in the human body. Glutamine is largely anaplerotic and relinquishes both of its amino groups to fuel the TCA cycle. It participates in the inter-organ nitrogen exchange through ammonia (NH3) transport between tissues and is critically involved in maintaining pH stability. Glutamine serves as a carbon and nitrogen donor for nucleotide biosynthesis and is a requirement for nicotinamide adenine dinucleotide phosphate (NADPH) generation. Thus, glutamine is an alternative fuel source, serves as a biosynthetic material, contributes to epigenetic and posttranslational modifications and determines the redox status, identifying this amino acid as a central regulator of immune cell fitness (88, 89).

Glutamine to the Rescue: Maintaining Energy Production in Unfit Mitochondria

Glutamine, and its breakdown product glutamate, have both been identified as critical energy carriers in tumors, and the glutaminolytic pathway is considered a potential therapeutic target to suppress tumor growth (90). Both glucose and glutamine can provide carbons to feed the mitochondrial TCA chain and preference for one over the other may depend on local availability but may also affect the outcome of pathogenic immune responses. Glucose is primarily supplied by the liver, glutamine is synthesized by the muscle (91, 92), implicating these two organ systems in regulating protective and pro-inflammatory immunity. To make glutamine available for ATP generation, T cells utilize the Glutaminase isoenzymes (GLS1 and GLS2). Transcriptomic studies combined with metabolomic studies have defined GLS1 as a marker enzyme of fibroblast-like synoviocytes (FLS) isolated from RA patients (93). Notably, withdrawal of glutamine, but not of glucose, reduced RA-FLS proliferation, suggesting that the amino acid is critical in supporting pannus formation in the arthritic joint. Glucose may be a limiting factor, required to support multiple cell types in the inflamed synovial membrane, while glutamine may be freely accessible, excluding the proliferative FLS from glucose competition. Takahashi et al. have examined which factors can upregulate GLS1 in RA-FLS and found that both, IL-17 and platelet-derived growth factor acted as GLS1 inducers (93). The emerging model suggests that the T cell cytokine IL-17

directs synovial stromal cells to utilize glutamine instead of glucose, implicating metabolic regulation in the coordination of innate and adaptive immune responses in RA.

If the rheumatoid joint is a glutamine rich environment, then breakdown products derived from glutamine could potentially have pro-inflammatory functions. Through a series of enzymes, T cells convert glutamine into glutamate, which then is transformed into α-Ketoglutarate (α-KG) (94). The oncometabolite α-KG has versatility and has access to multiple cellular compartments, including the mitochondria, the cytosol, and the nucleus (95–97). α-KG can directly enter the TCA cycle, where it can be metabolized through oxidation and reduction. Conversion into succinate is a rate-limiting step in oxidative phosphorylation. Transformation into citrate feeds carbons into lipid metabolism. By functioning as a precursor for glutamine, α-KG also takes a center position in maintaining the redox balance of the cell. Given the enrichment in glutamate, discussions have focused on whether glutamatergic signaling has a role in the pathophysiology of RA (98). Measurements in collagen-induced arthritis (CIA) have shown a marked increase of glutamate in the synovial fluid. Glutamate functioned as an arthritogenic effector molecule by driving proliferation of synovial fibroblasts. Further support for a direct contribution of glutamate in the disease process came from blocking studies, applying Memantine, an N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor antagonist. This receptor antagonist had anti-inflammatory efficacy by upregulating $CD4^+CD25^+$ regulatory T cells in the spleen (99). These data suggest that glutamate is not only a stimulatory neurotransmitter, but it also has immunoregulatory functions. To better understand how glutamate promotes disease, it would be important to know the cellular origin and the glutamate receptor expression profile of innate, adaptive, and stromal cells participating in synovitis.

A particularly appealing concept is the idea that the tolerance-breaking effect of glutamate is a consequence of its function as a neurotransmitter. Support for this concept has come from studies applying the non-competitive NMDA ionotropic glutamate receptors antagonist ketamine intrathecally (100). When injected intrathecally into animals with antigen-induced arthritis, arthritis severity is reduced, the density of inflammatory cells in the joints is lowered and joint destruction is halted (100). Therapeutic application would require a more precise understanding of which cells are signal-sending and which cells are signal-receiving.

Pumping in Amino Acids: Glutamine Transporters as Disease-Promoting Molecules

Mechanistic studies have attempted to define upstream mediators that trigger the enhancement of glutaminolysis in T cells. During T cell activation, glutamine transporters and the components of the glutaminolysis machinery are upregulated through a MYC-dependent pathway (101). Compared to the resting state, activated T cells increase their glutamine uptake 5-10-fold. Several amino acid transporters facilitate glutamine uptake in T cells; e.g., the sodium-coupled neutral amino acid transporters of the SLC38 gene family (102). Upon T cell activation, transcription of SLC38A1 and SLC38A2 is activated in a coordinated fashion. Interestingly, SLC38A1 and SLC38A2 protein is stored in intracellular vesicles from where it is rapidly transferred to the cell surface (103). Early events after T cell receptor triggering, such as membrane-proximal signaling and induction of T cell activation markers are unaffected by the withdrawal of glutamine. Conversely, late processes, such as T cell clonal expansion and cytokine release are highly sensitive to the lack of glutamine, predicting that glutamine is partially important for pro-inflammatory effector functions of T cells.

Several transporters ensure sufficient glutamine uptake in T cells undergoing activation, including SLC1A5 (104). Naïve $CD4^+$ T cells rely upon SLC1A5 to fulfill their need for glutamine. In line with the concept that T cell activation and differentiation require metabolic adaptation, SLC1A5 couples TCR and CD28 signals to ultimately activate the mTORC1 pathway and enable the cell to undergo clonal expansion (105). SLC1A5 expression seems to be a prerequisite for the lineage commitment of both Th1 and Th17 cells (105), placing it high on the list of potential upstream regulators of the pathogenic differentiation of short-lived effector T cells encountered in RA patients (31). No information is currently available how a specific tissue microenvironment affects metabolically regulated aspects of T cell differentiation. Considering that T cell pathology in RA occurs both in lymphoid tissues as well as the inflamed joint, it would be important to know whether differences exist for glutamine availability, to which degree glutamine substitutes as an energy carrier under conditions of glucose deprivation and how glutamine interferes with the pathogenic mTORC activation driving disease-relevant effector functions.

LIPIDS AS A CATALYST FOR AUTOIMMUNE JOINT INFLAMMATION

Like glucose and amino acids, lipids are an essential nutrient resource for protective and pathogenic immune cells. Lipids are a high-impact energy source, affecting multiple biological processes, including the production and the storage of energy (106), the assembly and functionality of cellular membranes (107), gene regulation of metabolic pathways (108) and post-translational modifications in intracellular signaling pathways (109). Intracellular lipid concentrations are regulated both by nutritional uptake and by ongoing biosynthesis. Fatty acid synthesis involves the creation of fatty acids from acetyl-CoA and NADPH through the action of enzymes called fatty acid synthases (FAS), through which simple lipids are bio-synthesized and sequentially elongated through addition of acetyl-CoA (110).

Disordered Lipid Profiles in Inflammatory Disease

Numerous autoimmune diseases, including RA, psoriasis, and systemic lupus erythematosus, in which autoreactive $CD4^+$ T cells participate in inflammatory tissue lesions share the accumulation of intracellular lipid droplets in such T cells

(111–113). These autoimmune diseases are associated with elevation of serum triglycerides and cholesterol (113–115). Whether this dyslipidemia is causative for inflammation or a consequence of persistent inflammatory activity remains unresolved. Evidence for a disease-relevant role of lipids has come from reports that lowering blood lipid levels by diet or drug treatment may improve symptoms, including T cell-dependent autoantibody responses (116–118). Nevertheless, it remains speculative whether modification of lipid profiles could reestablish tissue tolerance and revert pathogenic immunity.

Lipids accumulating in the synovial fluid of RA patients have been analyzed through liquid chromatography mass spectrometry, and more than 70 different components from different lipid classes have been detected (119). Among the broad spectrum of lipids found in synovial fluid, maresin 1, lipoxin A4 and resolvin D5 were associated with RA and 5S,12S-6E,8Z,10E,14Z-dihydroxyeicosatetraenoic acid was identified as a marker of lipoxygenase activity. Nevertheless, lipidomic studies have not yielded any unexpected insights. In early studies more than 50 years ago, increased amounts of phospholipid, cholesterol, and neutral lipids were described, but synovial fluid lipid levels were not predictive of severity of synovitis, questioning a direct involvement of lipids in driving arthritis (120, 121).

The abnormal cholesterol profile in patients with preclinical RA and early RA is typical of metabolic syndrome: normal or mildly elevated total cholesterol, LDL cholesterol and triglycerides, associated with decreased HDL cholesterol levels (122, 123). The cholesterol biosynthesis pathway appears to be a key regulator of controlling $CD4^+$ T cell commitment to inflammatory versus anti-inflammatory effector status. Inhibition of the cholesterol biosynthesis pathway produces a specific block in immune resolution, defined as a significant decrease of T cell IL-10 production (124). Thus, anti-inflammatory response in $CD4^+$ T cells may be particularly dependent on sterol metabolism.

Cytoplasmic Lipid Droplets As Immunoregulatory Organelles

Intracellular lipids, however, play a quintessential role in how immune cells differentiate into effector cells. Indeed, the activation of the T cell receptor is coordinated with up-regulation of genes involved in the biosynthesis of cholesterol and fatty acids (125). This anabolic program is regulated by specialized transcription factors, the sterol regulatory element-binding proteins (SREBP) (125). Without SREBP signaling, T effector cells could not blast and could not undergo clonal expansion (125). T cell activation is associated with massive cellular proliferation, imposing high biosynthetic demands, particularly when it comes to the generation of bio-membranes. Besides their essential role as biosynthetic precursor, lipids also serve a non-replaceable role a bioenergetic materials, specifically in the induction of T memory cells, which are known to dependent on mitochondrial fatty acid β-oxidation (126). Notably, T memory cells, unlike T effector cells, do not uptake extracellular palmitate (126), delineating a stringent association between T cell subtype and preferences for fatty acid substrates. How different T cell subpopulations acquire and mediate their taste for select lipids is not entirely clear. Different fatty acid transporters are expressed on the membrane of distinct T cell subsets. In the case of memory T cells, survival is impaired in the presence of the FAS inhibitor C75 (127), indicating that the fitness of T memory cells depends on *de novo* fatty acid biosynthesis.

Data that have accumulated over the last 5 years support the concept that lipid metabolism in RA T cells is fundamentally abnormal. Due to the mitochondrial defects (**Figure 3**), RA T cells lack the flexibility to use lipids as an energy carrier. Instead, RA T cells commit fatty acids to the lipogenesis program to support the biomass building program. Excess lipids that cannot be metabolized are stored as cytoplasmic lipid droplets (29, 128). Fatty acids that are not committed to the building of cellular offspring need to be safely packed and stored. Notably, tissue-residing T cells in rheumatoid synovitis accumulate cytoplasmic lipid droplets (128). Currently, there is no evidence that the lipogenesis>>lipolysis status of RA T cells is a primary defect, but rather appears to be a consequence of abnormal mitochondrial function and rerouting of glucose into the biosynthetic PPP. Reversal of the TCA cycle and the export of citrate out of the mitochondria leads to the accumulation of acetyl-CoA in the cytoplasm (129, 130). Fatty acid synthesis is further facilitated by the availability of NADPH, a cofactor required for lipogenesis and available due to the commitment to the PPP instead of glycolysis (131). Surplus in fatty acids has direct impact on the pathogenic effector functions of RA T cells, as fatty acids can be integrated into phospholipids, creating building blocks for cell membranes. Data on the composition of cellular and subcellular membranes in RA T cells are currently not available, but structural analysis has yielded insights on how these autoimmunity-prone cells adapt to their metabolic programming (132). Colocalization studies of the cytoskeletal marker F-actin and the membrane marker cortactin have demonstrated membrane extrusions indicative of rapid membrane turnover and structural flexibility (133, 134). Membrane ruffling occurs in cells loaded with cytoplasmic lipid droplets and translates into high mobility in 3-dimensional matrix and rapid transition from the blood to the tissue (128). Acetyl-rich RA T cells easily change their cellular shape, building a uropod known to promote cellular motion (29). Studies applying metabolic inhibitors have placed the redirection of glucose and lipids upstream of membrane ruffling and podosome formation (**Figure 4**) (128). Nevertheless, the question arises whether the loss of a circular cellular shape has metabolic implications by itself. Analysis of the content of the uropod in RA T cells has revealed the displacement of mitochondria into a perinuclear position (29). The mitochondrial rearrangement is rooted in the hyperacetylation of cytoskeletal proteins, specifically tubulin, which propels mitochondria away from the periphery close to the nuclear membrane. The proximity of ROS-producing mitochondria to the nucleus and to chromosomes placed near the nuclear membrane enables mitochondria to impose gene expression changes that by itself can mediate information from the metabolic hub of the cell to the "cockpit". Essentially, metabolic rewiring may function as a feed forward mechanism, profoundly altering programs that oversee cellular behavior and adaptive immunity.

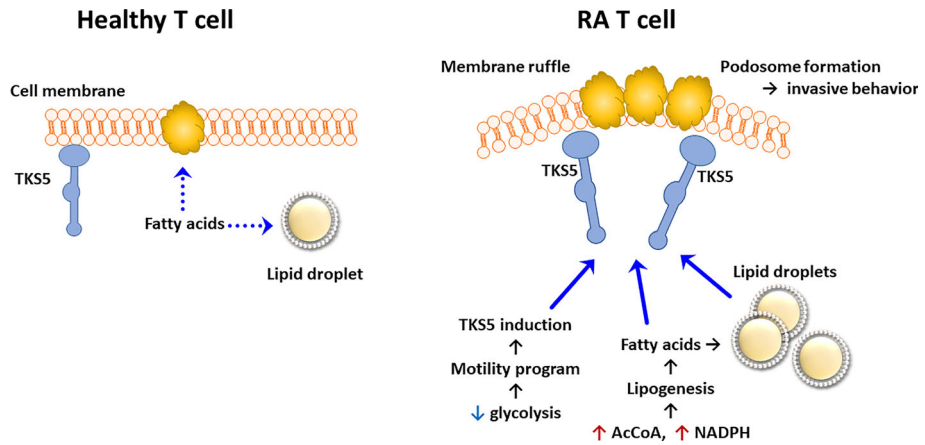

FIGURE 4 | Pathogenic Lipogenesis in RA T cells. Metabolic rewiring in RA T cells, including the shunting of glucose to the pentose phosphate pathway, the export of mitochondrial citrate and the suppression of mitochondrial lipid oxidation, all favor lipogenesis over lipolysis. As a result, RA T cells deposit lipid droplets in the cytoplasm and are rich in biosynthetic precursors for membrane lipids. Lowered glycolytic activity upregulates a module of motility genes, including the scaffolding protein TKS5. As part of their pro-inflammatory phenotype, lipogenesis-biased TKS5^{hi} RA T cells spontaneously form actin- and cortactin-rich membrane ruffles, equipping them tissue invasion and mobility in extracellular matrix. AcCoA, acetyl-CoA.

METABOLIC NETWORKS AS THERAPEUTIC TARGETS

Immunomodulatory therapies currently applied in RA have metabolic "side effects" (**Table 1**). It is difficult to say whether the metabolic consequences induced by these therapeutics are primary or secondary. Obviously, effective control of systemic inflammation may leave a metabolic footprint, even if the pathways of intracellular metabolic control are ignored by the therapeutic. Some of these immunosuppressive medications may directly interfere with glycolysis, mitochondrial function, lipogenesis, and amino acid metabolism, but mechanistic understanding of such effects remains scarce. The gold standard of RA management, Methotrexate (MTX), is a potent antimetabolite drug that targets the processing of purine (155) and folic acid (156) by inhibiting dihydrofolate reductase. MTX, corticosteroids, sulfasalazine, leflunomide and cyclosporine A function as immunomodulators by inhibiting pro-inflammatory Th1 and Th2-dependet immunity (157). However, their metabolic impact is not always protective. Corticosteroids as well as nonsteroidal anti-inflammatory drugs have metabolic consequences that enhance cardiovascular risk (158).

Anti-TNF-α drugs have become a cornerstone in managing RA, particularly in patients resistant to traditional therapeutic approaches (159, 160). Anti-TNF-α treatment decreases glycolytic activity in RA synovium, likely through its potent anti-inflammatory effect (161). Blockade of IL-6 appears to have primarily a systemic are metabolic impact. Anti-IL-6 receptor treatment leads to weight gain and modified fat distribution and gain in muscle mass suggests that blocking IL-6 might be efficient in treating sarcopenia associated with RA (151). Anti-IL-6 receptor treatment has been reported to decreases oxidative stress in RA leucocytes (152). Inhibitors of the JAK-STAT pathway (e.g. Tofacitinib) display powerful anti-inflammatory effects and significantly decrease mitochondrial membrane potential, mitochondrial mass and ROS production in RA synovial fibroblasts (162). In an elegant study, McGarry et al. have provided evidence that tofacitinib directly modulates mitochondrial function, such as regulating key mitochondrial genes, increasing oxidative phosphorylation, enhancing ATP production while diminishing glycolytic flow and key glycolytic genes (162).

Capitalizing on the understanding of how fuel selection guides cellular behavior and on the concept that fuel determines function, multiple novel therapeutic targets emerge. Besides the central role of glucose itself (163), metabolic intermediates such as lactate (53) and succinate (70) appear to be critically important in regulating tissue inflammation. Much will be learned from therapeutic approaches in cancer therapy that rely on disrupting bioenergetic needs to the tumor and metabolic vulnerabilities in anti-tumor immune

TABLE 1 | Metabolic effects of currently used therapies in rheumatoid arthritis.

Therapies for RA	Metabolic effects	References
Methotrexate	Accumulation of polyamines, inhibition of purine and pyrimidine synthesis, promotion of adenosine release	(135–138)
Anti-TNF	Decreased insulin serum levels, insulin resistance, increased HDL cholesterol serum levels, hypertriglyceridemia	(139–145)
Anti-CD20	Decreased succinate, taurine, lactate, pyruvate and aspartate in serum	(146)
Hydroxychloroquine	Decreased total cholesterol, decreased LDL, increased HDL and decreased triglycerides	(147–150)
Anti-IL-6	Altered body composition, increased lean mass and skeletal muscle mass, decreases oxidative stress in leucocytes	(151, 152)
Leflunomide	Decreased synthesis of pyrimidine, decreased uric acid	(153, 154)

responses (164). Unselected approaches, such as shutting off glucose consumption, amino acid utilization or lipid import are likely going to fail, as all organ systems are dependent on energy supply from these energy carriers (165) and more sophisticated strategies are needed to manipulate cellular metabolism.

CONCLUSION

Autoimmune diseases, such as RA, begin with T cells losing self-tolerance and providing help to auto-antibody producing B cells. The root cause is believed to be the recognition of autoantigen and the failure of mechanisms that sort out autoreactive T cells. Recent data have questioned this simplified model and have added metabolic signals as decisive determinants in T cell fate and behavior. Based on the concept that "fuel feeds function", the metabolic networks of disease-inducing T cells have been defined and are being explored as liabilities that may be targeted to re-engineer "bad" T cells into "good" T cells. An additional layer of metabolic control is introduced by the metabolic cues provided by the tissue environment in which cells live, die, proliferate, and communicate. RA $CD4^+$ T cells lose tolerance decades before joint inflammation begins, while living in lymphoid organs, such as lymph nodes and the bone marrow. Once such T cells differentiate into short-lived effector cells (SLEC) instead of long-lived memory cells, they function as tissue-infiltrating and pro-inflammatory effector cells.

Here are the hallmarks of altered immunometabolic regulation in RA:

- RA T cells are characterized by abnormal glycolysis and inappropriate lipogenesis (**Figure 5**).
- The underlying defect is a malfunctioning mitochondrion. Lacking DNA repair of the mitochondrial genome leads to DNA instability and cytosolic leakage. Triggering of the inflammasome gives rise to T cell pyroptosis, a process that by itself functions as a strong instigator of tissue inflammation.
- Insufficient mtDNA damage repair undermines OxPhos and ATP generation. The mitochondrial TCA cycle is disrupted due to transcriptional repression of *SUCLG2*, impairing the conversion of α-KG into succinate. Unprocessed α-KG leads to reversal of the TCA cycle and results in the accumulation and the export of citrate.
- This cataploretic step promotes acetyl-CoA accumulation in the cytoplasm and favors acetylation of cytoplasmic proteins. Hyperacetylation of microtubules stiffens the cytoskeleton, fundamentally changing the cellular shape and the distribution of subcellular organelles. The most significant consequence of the hyperacetylate state is transfiguration of the cell away from a circular shape toward rear-front polarization and uropod formation. The resulting cell is hypermobile, swiftly transmigrating from the blood to the tissue.
- Besides the change in migratory behavior and invasiveness, RA T cells differentiate into pro-inflammatory cytokine producers, favoring IFN-γ and IL-17 as effector cytokines. The commitment to Th1 and Th17 differentiation results from the persistent activation of mTORC1, a consequence of the misplacement of the energy sensor AMPK. Lacking a myristoylation tail, AMPK

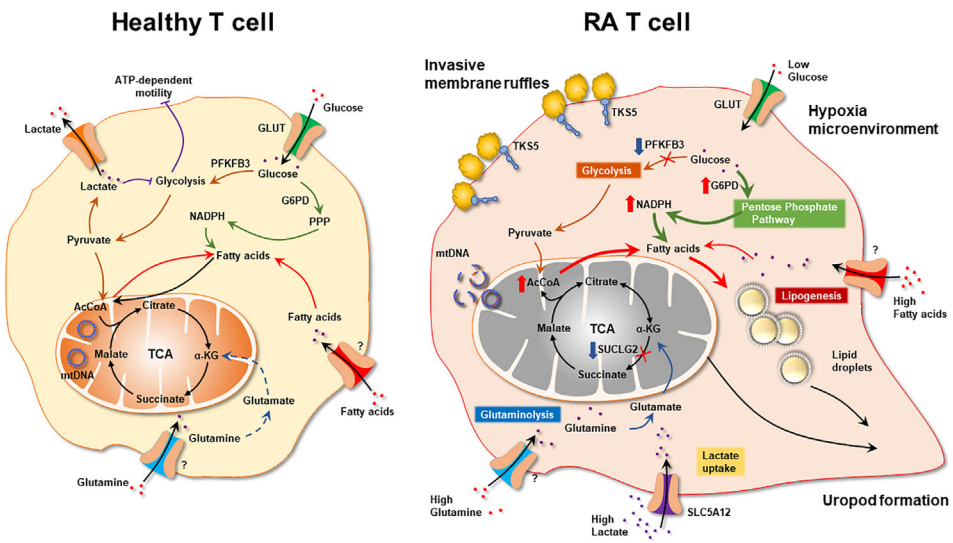

FIGURE 5 | The Metabolic Signature of Autoreactive T cells in Rheumatoid Arthritis. Schematic diagram of metabolic pathways in health and RA T cells. Healthy T cell: Low-proliferating healthy T cells (naïve, memory) rely on mitochondrial energy production. When differentiating into highly replicating effector T cells, bioenergetic and biosynthetic needs are fulfilled by engaging glycolysis. RA T cells: Biased toward biomass generation, RA T cells shunt glucose into the pentose phosphate pathway and produce NADPH. Mitochondria with unrepaired mtDNA utilize relatively low amounts of oxygen and produce low concentrations of ROS. Mitochondrial succinate production is suppressed due to low activity of Succinate-CoA ligase and a-ketoglutarate is converted into citrate. Exported citrate supplies cytoplasmic acetyl-CoA pools and promotes lipogenesis. Surplus lipids sustain the generation of invasive membrane ruffles and of cellular uropods. With low ATP production, RA T cells require external energy sources, such as glutamine and lactate to meet basic bioenergetic needs. By diverting energy away from ATP production toward assembly of biosynthetic precursor molecules, RA T nourish the emergence and the survival of replicating effector T cells that are tissue-invasive and pro-inflammatory. α-KG, α-ketoglutarate; mtDNA, mitochondrial DNA.

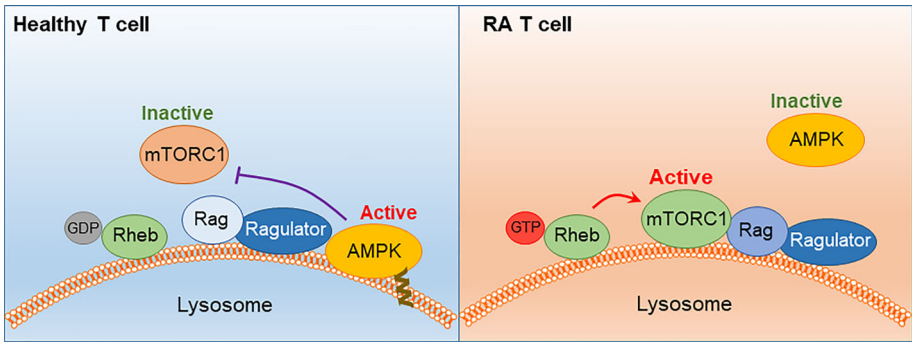

FIGURE 6 | AMPK signaling failure and unopposed mTORC1 activation in RA T cells. In healthy T cells, AMPK senses the cell's energy state by trafficking to the lysosomal surface, where it is anchored in the lysosomal membrane through a myristoylated tail. AMPK interacts with the Ragulator-Rag complex, resulting in dissociation and inactivation of mTORC1. In RA T cells, a defect in protein myristoylation leads to mistrafficking and cytosolic retention of AMPK. mTORC1 is retained at the lysosomal surface, where Rheb-GDP converts into Rheb-GTP and switches on the kinase activity of mTORC1. mTORC1 activation persists in RA T cells despite low ATP availability.

in RA T cells fails to anchor on the lysosomal surface and thus forgoes the suppression of mTORC1 (**Figure 6**). Unopposed mTORC1 activation encourages RA T cells to proliferate and build daughter cells despite the low ATP state. The proliferation program of SLECs, however, is supported by the shunting of glucose from bioenergetic to biosynthetic utilization. Instead of breaking glucose down into ATP and pyruvate, RA T cells shift glucose into the PPP and generate NADPH plus biosynthetic precursors. Ongoing lipogenesis provides building blocks for membranes and organelles and the cells' reductive state promotes fatty acid synthesis.

- Once transitioned into the inflamed joint, RA T cells encounter a hypoxic, lactate-rich, glucose- low environment. The chronic activation of stromal cells and invading immune cells depletes the tissue of glucose and acidifies the environment with lactate. Synovial T cells have been shown to uptake lactate, supplementing their energy supply but also signaling the cells to undergo arrest and persist in the tissue niche.
- Most metabolic abnormalities in RA T cells are present in the naïve population and sustained in tissue-residing memory T cells, placing metabolic dysregulation upstream of the joint. These data classify the metabolic malfunction, at least in part, as an "original sin", leading to autoimmunity.

Based on the novel paradigm that metabolic programming determines the risk for inappropriate immune function, new therapeutic interventions can be developed. Ideally, a dietary approach can be designed to direct the flow of energy carriers and that of intracellular and extracellular metabolites. Alternatives include small molecules designed to finetune glycolysis, glutaminolysis and lipogenesis. Metabolic intervention may eventually be the most elegant way to manipulate the energy sensor mTORC1, which is critically involved in misleading T cells and turning them into multiplying and self-aggressive effector cells.

AUTHOR CONTRIBUTIONS

All authors contributed to concept development. JQ, BW, GB, SG, JG and CW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mitochondria as Key Players in the Pathogenesis and Treatment of Rheumatoid Arthritis

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Mitochondria are major energy-producing organelles that have central roles in cellular metabolism. They also act as important signalling hubs, and their dynamic regulation in response to stress signals helps to dictate the stress response of the cell. Rheumatoid arthritis is an inflammatory and autoimmune disease with high prevalence and complex aetiology. Mitochondrial activity affects differentiation, activation and survival of immune and non-immune cells that contribute to the pathogenesis of this disease. This review outlines what is known about the role of mitochondria in rheumatoid arthritis pathogenesis, and how current and future therapeutic strategies can function through modulation of mitochondrial activity. We also highlight areas of this topic that warrant further study. As producers of energy and of metabolites such as succinate and citrate, mitochondria help to shape the inflammatory phenotype of leukocytes during disease. Mitochondrial components can directly stimulate immune receptors by acting as damage-associated molecular patterns, which could represent an initiating factor for the development of sterile inflammation. Mitochondria are also an important source of intracellular reactive oxygen species, and facilitate the activation of the NLRP3 inflammasome, which produces cytokines linked to disease symptoms in rheumatoid arthritis. The fact that mitochondria contain their own genetic material renders them susceptible to mutation, which can propagate their dysfunction and immunostimulatory potential. Several drugs currently used for the treatment of rheumatoid arthritis regulate mitochondrial function either directly or indirectly. These actions contribute to their immunomodulatory functions, but can also lead to adverse effects. Metabolic and mitochondrial pathways are attractive targets for future anti-rheumatic drugs, however many questions still remain about the precise role of mitochondrial activity in different cell types in rheumatoid arthritis.

Keywords: mitochondria, rheumatoid arthritis, metabolism, oxidative phosphorylation, NLRP3, DAMP, DMARD (disease modifying anti-rheumatic drug)

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INTRODUCTION

Mitochondria have long been described as central energy-producing organelles and regulators of cellular metabolism, but throughout the years many additional cellular functions of mitochondria have become apparent. It is now appreciated that these complex organelles also contribute to intra- and inter-cellular signalling through the actions of proteins, DNA, lipids, metabolites, and reactive oxygen species, and mitochondrial components are capable of directly activating the immune system.

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune condition driven by a complex interplay of different immune and non-immune cell types. Dysregulation of immune signalling pathways causes local inflammation within the synovial joint, as well as a host of systemic complications such as an increased risk of cardiovascular diseases, all of which pose a significant risk to the affected individual's quality of life (1). Known risk factors for the development of RA are both genetic and environmental, with the most important genetic link being the "shared epitope" of the MHC class II HLA-DR allele (1). Auto-antibodies such as rheumatoid factor or anti-citrullinated protein antibodies (ACPA) are found in a high percentage, though not all, of RA patients, and are associated with a more severe disease, but the presence of these antibodies generally pre-dates the development of clinical symptoms of arthritis by several years (2). It is considered that an assortment of cumulative triggering events, which are variable and still incompletely understood, then lead to the progression of a pre-clinical arthritis into established RA (1). Active RA disease involves expansion of the synovial membrane of the joint to form an invasive pannus, leading to joint damage and dysfunction. The identification of different subtypes of RA, which show varying degrees of contribution from infiltrating leukocytes to synovial pathology, highlights the heterogeneity of this disease and indicates that a range of diverse mechanisms likely drive disease pathogenesis in different individuals (1, 3, 4).

Metabolic dysregulation is a key contributing factor to the initiation and development of disease in autoimmunity, and much recent work has focused on the study of metabolic processes in RA and other inflammatory diseases in order to better understand and treat these complex conditions. Due to their pleiotropic effects on the cell, mitochondria contribute to disease pathogenesis *via* metabolic actions as well as through direct effects on signalling pathways.

In this review we address the contribution of mitochondria to pathological processes in RA, as well as how mitochondrial function can be altered by therapeutics in the context of RA treatment. We focus on the metabolic function of mitochondria in different cell types in RA, as well as how this organelle facilitates immune cell activation and production of inflammatory mediators. This has mostly been studied in T cells, macrophages, and fibroblasts, but there is also some evidence for mitochondrial regulation of endothelial cells, osteoclasts, neutrophils and chondrocytes in the context of arthritis. Mitochondria have a well-established role in the process of apoptosis, however this topic is outside the scope of the current review except where it directly relates to metabolic processes, and we direct the readers to other reviews on the topic (5), including in the context of rheumatoid arthritis (6, 7).

MITOCHONDRIAL DYNAMICS

Mitochondrial Fusion Versus Fission

The opposing processes of fusion and fission regulate the gross structure and overall organisation of mitochondria, and impact upon many aspects of mitochondrial function, including DNA segregation, oxidative phosphorylation efficiency, reactive oxygen species (ROS) production, and apoptosis. These mitochondrial activities can in turn impact cellular function in a multitude of ways, therefore mitochondrial dynamics plays a key role in cellular homeostasis and signalling (8). The balance of fusion and fission regulates the activity and survival of immune cells including T cells and macrophages. A fragmented mitochondrial state is observed in effector T cells, as well as macrophages treated with lipopolysaccharide (LPS) or infected with *Mycobacterium tuberculosis in vitro*, and this fission is linked to inflammatory cell function (8–11). In contrast, LPS treatment of human monocytes has been linked to a large, fused mitochondrial state (12, 13). Hyperfused mitochondria with tight cristae are observed in memory T cells, which retain high oxidative capacity and generate ATP through fatty acid oxidation and oxidative phosphorylation (9).

Due to their central role in mitochondrial regulation and cellular homeostasis, the fusion and fission processes are tightly controlled by a variety of interconnected mechanisms. In monocytes, the microRNA miR-125b regulates mitochondrial dynamics and apoptosis in several ways, including by downregulation of the mitochondrial fission protein MTP18 (12). Expression of miR-125b was found to be reduced in peripheral monocytes from RA patients compared with healthy individuals, although the precise contribution of this finding to disease processes has not been determined (12). T cells in systemic lupus erythematosus (SLE) contain large, fused mitochondria characterised by high membrane potential and excessive ROS production (14), however dysregulated fusion/fission processes have not been described in T cells from RA patients to our knowledge.

In addition to the regulation of immune cell function, mitochondrial dynamics also play a role in stromal cell activity. Fibroblasts are a major cell type present in the healthy synovial membrane, and these cells significantly contribute to disease processes in RA (15). Proliferation of fibroblasts leads to formation of the expanded synovial pannus, and these cells mediate extracellular matrix and cartilage degradation, contribute to immune cell recruitment and activation, and activate endothelial cells (15). Synovial tissue and *ex vivo*-cultured fibroblast-like synoviocytes (FLS) from RA patients show shortened mitochondria and elevated expression of the mitochondrial fission GTPase dynamin 1-like protein (DNM1L, also known as dynamin-related protein 1: Drp1) (16, 17). Inhibition of mitochondrial fission with the GTPase inhibitor m-divi in a collagen-induced arthritis (CIA) mouse model reduced disease severity, decreased synovial tissue ROS levels,

and inhibited expression of inflammatory and destructive mediators (17).

Determining the precise contributions of fusion and fission processes to cellular function and disease is complicated by the fact that the machinery responsible for these processes also serve additional roles (8). For example, the fusion GTPase Opa1 also regulates mitochondrial cristae structure, which in turn modulates supercomplex formation and apoptosis (18); and the fusion-related protein mitofusin 2 has been linked to inflammasome activation and interleukin-1β (IL-1β) production, which shall be discussed in more detail in a later section (19). The specificity of tools that are commonly used to inhibit such machinery has also been brought into question, for example m-divi was shown to inhibit mitochondrial complex I activity independently of any effects on fusion/fission (20).

Mitochondrial Biogenesis Versus Mitophagy

As well as showing dynamic regulation in the form of fusion and fission, cellular mitochondrial content is regulated by the balance of mitochondrial biogenesis and the degradation and recycling of mitochondrial components through autophagy – termed mitophagy. During homeostasis, this balance helps to maintain a healthy and functional population of mitochondria and may be indicative of overall respiratory capacity. The balance of fusion/fission and biogenesis/mitophagy are exquisitely sensitive to metabolic cues, such as nutrient availability and mitochondrial membrane potential (21). Cellular stress can be associated with an imbalance of these processes, and accumulation of damaged mitochondria can drive cellular dysfunction and/or immune cell activation. Disruptions to these pathways have been linked to a wide range of diseases, including cancers and several neurodegenerative diseases (21, 22).

The precise impact of mitophagy or its disruption on RA pathogenesis is poorly understood. Dysregulation of autophagy, whether increased or decreased, has been demonstrated in several different cell types in RA and has been linked to pathogenic processes (16, 23, 24), however the distinction between mitophagy and general autophagy in these studies has not been made. Clearance of defective mitochondria by mitophagy is important for the survival of chondrocytes from osteoarthritis (OA) patients, therefore this process likely has a role in protecting against cartilage loss in arthritis, but a specific link to rheumatoid arthritis was not shown (25).

Total mitochondrial mass is maintained in RA T cells, despite a decrease in respiratory activity in these cells compared with healthy controls (26). In contrast, T cells in SLE show higher mitochondrial load, attributable to increased biogenesis and decreased mitophagy (14). It is plausible that both mitochondrial biogenesis and mitophagy are disrupted in RA T cells, as it has been shown that activation of the energy sensing kinase AMPK is dysfunctional in these cells (27). AMPK serves as a crucial regulator of metabolic processes and acts to maintain mitochondrial homeostasis (28). The fact that AMPK is responsible for stimulating both mitophagy and mitochondrial biogenesis could explain the lack of changes in total mitochondrial mass in RA T cells. Activation of AMPK is also required to initiate mitochondrial fission processes in response to mitochondrial damage (28). The disrupted activity of AMPK in RA T cells results in uncontrolled activity of the mTOR complex mTORC1, which promotes T cell proliferation and inflammatory Th1 and Th17 differentiation that are associated with synovitis (27). mTOR is also a known regulator of mitochondrial dynamics through the control of mitochondrial protein synthesis (29). Whether the disruption of these energy sensing pathways in RA gives rise to a pool of dysfunctional mitochondria and contributes to oxidative stress needs to be determined.

MITOCHONDRIAL METABOLIC ACTIVITY AND ATP PRODUCTION

One of the central functions of the mitochondrion is the generation of adenosine triphosphate (ATP), which acts as the cell's energy currency. ATP is produced in high quantities by the process of oxidative phosphorylation, carried out by the mitochondrial respiratory chain (aka electron transport chain/ETC) within the inner mitochondrial membrane. Coupling of electron transport and proton transfer produces an electrochemical gradient across the inner mitochondrial membrane (known as the mitochondrial membrane potential), allowing proton-driven phosphorylation of ADP by the enzyme ATP synthase (30). Mitochondrial respiration consumes oxygen, therefore cellular consumption of oxygen is commonly used as a readout of mitochondrial respiratory chain activity, with the ATP synthase inhibitor oligomycin being used to distinguish ATP-coupled oxygen consumption from other oxygen-consuming processes (30). The process of glycolysis involves the metabolism of glucose in the cytoplasm to generate pyruvate or lactate, allowing ATP production without oxygen consumption. Akin to the Warburg effect that is critical for cancer cell survival and proliferation, an increased rate of aerobic glycolysis is strongly linked to inflammatory activity in a number of different immune and stromal cell types (31, 32). Mitochondrial activity in inflammatory contexts, particularly in the context of inflammatory disease, is more varied and is still incompletely understood, however important advances in our understanding have been made in recent years (**Figure 1**).

Monocytes and Synovial Tissue Macrophages

Inflammatory activation of macrophages, dendritic cells and monocytes results in upregulation of glycolytic metabolism, which is necessary for many of the pro-inflammatory functions of these cells (13, 32, 33). Numerous *in vitro* studies, which have predominantly focused on cells of mouse origin, show that this increase in glycolysis is accompanied by a robust downregulation of oxygen consumption and mitochondrial ATP production. This is thanks to the repurposing of the electron transport chain (ETC) and the tricarboxylic acid (TCA) cycle for ROS generation and the provision of specific metabolites for signalling and biosynthetic functions. These processes are reviewed extensively elsewhere (32–35), and are further discussed below. However, recent evidence has shown that metabolic responses to

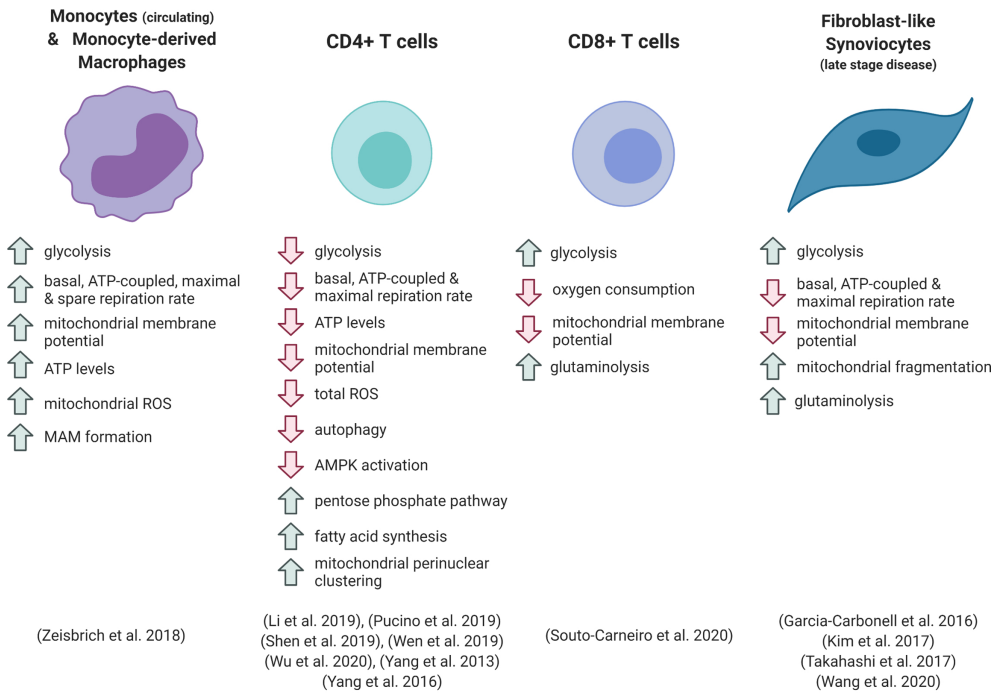

FIGURE 1 | Metabolic phenotype of different cell types in rheumatoid arthritis. ROS, reactive oxygen species; MAM, mitochondria-associated membrane.

toll-like receptor 4 activation with LPS differ in human myeloid cells, and that different forms of inflammatory stimulus result in distinct metabolic phenotypes (13, 36). Therefore, the study of these pathways in the context of inflammation in human disease is required to fully appreciate the immunometabolic landscape of disease.

Circulating monocytes and monocyte-derived macrophages from the peripheral blood of RA patients are hyper-metabolic, displaying enhanced rates of both glycolysis and oxidative phosphorylation (37). Patient-derived cells showed elevated basal, ATP-linked and maximal oxygen consumption under *ex vivo* analysis compared with cells from healthy individuals (37). RA patient-derived macrophages had increased numbers of mitochondrial-ER contacts, forming structures known as mitochondria-associated membranes (MAMs). These structures facilitate calcium transfer between organelles, which can increase mitochondrial enzyme efficiency. The increased mitochondrial-ER associations were linked to deactivation of glycogen synthase kinase 3b (GSK3b), a kinase implicated in the regulation of mitochondrial respiratory activity. Increased levels of the inactive, phosphorylated form of GSK3b were detected in RA patient blood monocytes and synovial CD68+ macrophages. From a functional perspective, these metabolic adaptations were linked to increased macrophage production of the collagenase cathepsin K (37). This enzyme is involved in bone resorption and contributes to joint destruction in arthritis (38). Zeisbrich et al. demonstrated that the activity of cathepsin K correlated with RA disease activity (37). Cathepsin K is also associated with atherosclerotic lesions, and the authors suggest that the hypermetabolic and destructive phenotype of the RA patient macrophages, which mechanistically mirror that of coronary artery disease macrophages, may increase the risk of systemic complications associated with disease, such as cardiovascular complications (37). Whether this metabolic phenotype in the periphery is a cause or a consequence of joint inflammation is difficult to determine, and further investigation is required to understand whether macrophages in the synovium share this hypermetabolic signature.

Recent studies identified distinct types of synovial tissue macrophages with either protective or inflammatory functions in mouse and humans (39–41), and showed that these synovial tissue macrophage subpopulations appear to have opposing preferences for mitochondrial *versus* glycolytic metabolism (39). In the human synovium, macrophage subsets that express the TAM receptor MerTK predominantly display a pro-resolution gene expression signature and are associated with the healthy joint and RA disease remission (39). These subsets show elevated expression of genes linked to oxidative phosphorylation, including eleven out of the thirteen mitochondrially-encoded ETC subunit genes (**Figure 2**). In contrast, subsets lacking MerTK, which express high levels of pro-inflammatory mediators and induce an inflammatory and destructive phenotype in co-cultured fibroblasts, display elevated expression of genes of glycolysis, the pentose phosphate pathway (PPP) and transporters for glucose and lactate (**Figure 2**) (39). These expression profiles support our current understanding of general concepts of macrophage immunometabolism, that inflammatory macrophage function is driven by a skewing of metabolic activity towards glycolysis and the PPP, and away from mitochondrial

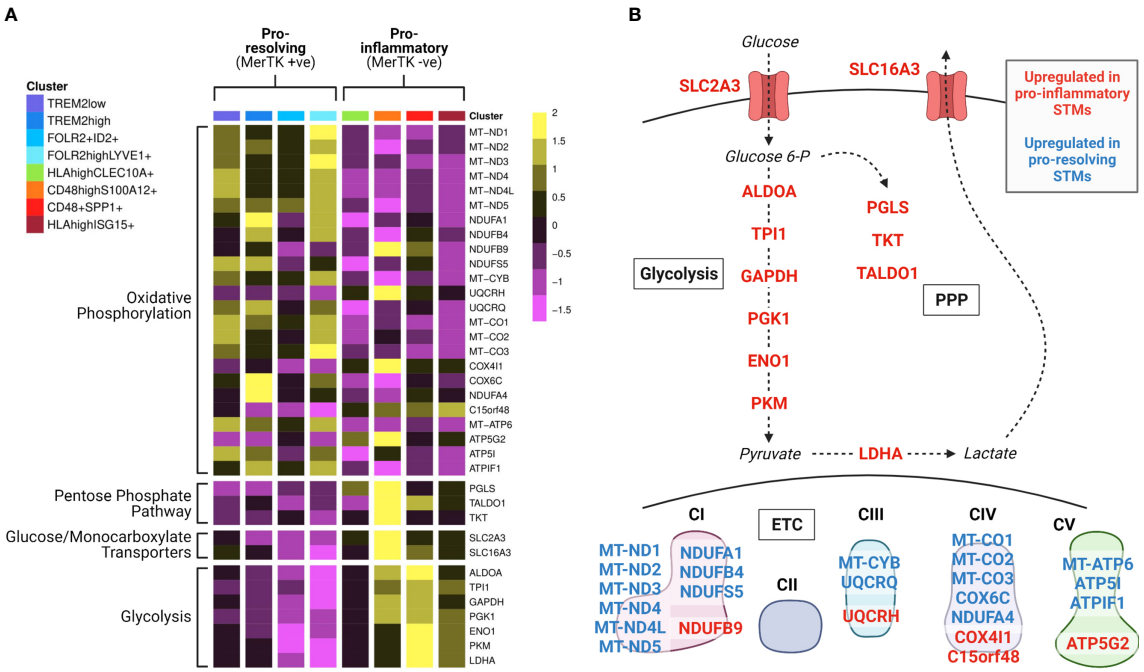

FIGURE 2 | Synovial tissue macrophage subpopulations display distinct metabolic gene expression signatures. Data from Alivernini et al. (39). **(A)** Heatmap of synovial tissue macrophage single cell RNA sequencing data displaying differential expression of metabolism-associated genes across macrophage subpopulations (clusters). Detailed information on the characterisation and function of different clusters and their contribution to different disease outcome groups can be found in Alivernini et al. (39). **(B)** Pathway schematic of the data from **(A)**. Genes upregulated in at least one cluster of pro-inflammatory macrophages are displayed in red, and genes upregulated in at least one cluster of pro-resolving macrophages are displayed in blue. STM, synovial tissue macrophage; PPP, pentose phosphate pathway; ETC, electron transport chain.

ATP production (42). However, it remains to be seen precisely how the activity of different metabolic pathways varies amongst subpopulations of cells in RA, and to what extent these metabolic phenotypes dictate cellular function *in vivo*.

T Cells

The metabolic regulation of T cells is crucial to allow their proliferation and effector functions following activation. Upon antigen recognition, quiescent naïve T cells transition into rapidly expanding effector T cells. These effector cells are driven by high rates of glycolysis, which allows fast energy production and provides substrates for DNA, lipid and protein synthesis. In contrast, long-lived memory T cells upregulate catabolic processes and depend upon mitochondrial oxidative metabolism (8). Inflammatory microenvironments such as the RA synovium induce metabolic reprogramming of T cells, which contributes to pathogenic processes. Retention of CD4+ T cells within the inflamed tissue is brought about by uptake of lactate, which drives increased fatty acid synthesis and elevated production of IL-17 (43).

In contrast to the hypermetabolic RA monocytes, circulating CD4+ T cells from RA patients show reductions in both oxygen consumption and lactate production in comparison with healthy controls. Consequently, ATP production rate and intracellular ATP levels are reduced in patient cells (24, 26, 44, 45). Several mechanisms have been linked to these phenomena.

One such mechanism is the diversion of glycolytic flux away from the mitochondria and lactate production, with carbon instead being rerouted into the PPP for production of NADPH and nucleotides. This was found to be achieved through the balance of two key rate-limiting enzymes, PFKFB3 and G6PD (44). PFKFB3 produces fructose 2,6-bisphosphate, the allosteric activator of the glycolytic enzyme PFK-1, and its upregulation in response to T cell activation was attenuated in RA T cells (24). In contrast, glucose-6-phosphate dehydrogenase (G6PD), which catalyses the first and rate-limiting step of the PPP, was over-expressed in RA T cells. This resulted in enhanced NADPH production and elevated levels of reduced glutathione in the patient cells. The increased antioxidant capacity was found to hinder the signalling function of intracellular ROS that are produced upon TCR activation, preventing activation of the cell cycle checkpoint kinase ATM, and giving rise to hyperproliferative T cells skewed towards inflammatory Th1 and Th17 differentiation (24, 44). Intracellular signalling by reactive oxygen species is also an important factor in other aspects of inflammatory cell function, which shall be discussed later.

In addition to reduced pyruvate entry into the mitochondria, RA CD4+ T cells possess a disrupted TCA cycle, which contributes to the loss of mitochondrial activity and reduced mitochondrial membrane potential in these cells (45). Specifically, T cells from RA patients have low activity of the mitochondrial enzyme succinyl-CoA ligase due to suppressed

expression of the *SUCLG2* subunit gene. This was shown to result in elevated levels of upstream metabolites α-ketoglutarate, citrate and acetyl-CoA. From a functional perspective, this metabolic disruption increased acetylation of tubulin and promoted T cell motility and migration, resulting in greater pro-inflammatory potential and synovial invasiveness *in vivo* (45). Mitochondria were shown to cluster to a perinuclear region within RA T cells, which was also dependent upon tubulin acetylation, and which likely contributes to the regulation of mitochondrial signalling in these cells (45).

These findings demonstrate the intimate and inter-connected relationships between different metabolic pathways and their interplay with signalling pathways and multiple aspects of cellular functionality, as well as the relevance of these relationships to inflammatory disease.

Peripheral blood CD8+ T cells from RA patients demonstrate enhanced glycolysis and lactate production compared with healthy controls or other types of inflammatory arthritis (46). This glycolytic phenotype was accompanied by decreased mitochondrial membrane potential and oxygen consumption in RA patient cells. A glycolytic gene expression signature was also evident in CD8+ T cells from the RA synovium, which are predominantly present in ectopic lymphoid follicles. The reduced dependence on oxidative metabolism in the RA patient cells is thought to underlie their enhanced ability to proliferate in hypoxic conditions, and the RA T cells were shown to increase uptake of glutamine and its conversion to lactate when subjected to low oxygen and glucose (46). The increased lactate production from RA CD8+ T cells indicates that these cells are not subject to the same glycolytic checkpoints as seen in RA CD4+ T cells, however both metabolic phenotypes are associated with increased production of inflammatory mediators and are linked to disease processes.

Fibroblast-Like Synoviocytes

Stromal cell metabolic function is also associated with RA pathogenesis. Fibroblasts consume glucose at high rates under inflammatory conditions, including in animal models of arthritis (47, 48). Fibroblast-like synoviocytes (FLS) from RA patients demonstrate a Warburg-like metabolic shift in comparison to OA FLS, favouring glycolysis over oxidative metabolism, and silencing of the glycolytic enzyme hexokinase-2 was able to reduce FLS migration and ameliorate disease in a serum-transfer arthritis model (47, 49). Kim et al. found that RA FLS show decreased basal respiratory rate and respiratory capacity compared with OA FLS, which was associated with mitochondrial depolarisation and abnormal morphology of cristae (16). These traits could be recapitulated *in vitro* by treatment of FLS with IL-17 or co-culture with Th17 cells, implicating inflammatory T cell activity in RA fibroblast dysregulation. Expression of several components of the mitochondrial ETC was decreased by IL-17 treatment (16). A hallmark of FLS in RA is their resistance to apoptotic signals, which contributes to FLS activation, synovial hyperplasia and formation of the invasive pannus (6). One mechanism that has been implicated in this apoptosis resistance is increased autophagy, which allows the cell to withstand nutrient deprivation and inhibits the ER stress response (50, 51). In the study by Kim et al., IL-17 treatment reduced apoptosis of RA FLS, and inhibition of autophagosome formation could reverse this effect. As mitochondrial dysfunction can be a trigger of autophagic processes, these results suggest a link between IL-17-mediated mitochondrial stress, autophagy and FLS survival, which could be an important pathway in disease progression (16).

Similarly to macrophages, recent studies have highlighted important distinctions between the roles of different subpopulations of T cells and fibroblasts in RA (52, 53). It will therefore be interesting to determine whether these different subsets also show distinct metabolic phenotypes that determine their specific functions.

Other Cell Types

There is little study of mitochondrial activity in other immune cell types in RA, with the majority of the focus in this context being on macrophages and T cells. B cells contribute to various stages of RA pathogenesis through the production of autoantibodies and by contributing to cytokine production and antigen presentation (1). Activated B cells upregulate both glycolytic and oxidative metabolism to meet the high energetic demands of proliferation and antibody production, and mitochondrial mass increases upon B cell stimulation *in vitro* (54). Mitochondrial signalling, in particular mitochondrial ROS generation, is important for dictating specific B cell differentiation paths (55). The precise metabolic state of B cells in RA has not been described, however deletion of one allele of the *PPARγ* gene resulted in hyperproliferative and hyperresponsive B cells and exacerbated disease in an antigen-induced arthritis model. This suggests that lipid metabolism may be important in regulating B cell responses in RA (56).

Neutrophils also play a role in RA disease, contributing to inflammatory and destructive processes and acting as sources of autoantigens (57). Neutrophils rely heavily on glycolysis for energy production (58), and neutrophils from RA synovial fluid displayed an enhanced glycolytic gene signature compared with peripheral blood neutrophils from the same patients (57). Changes in mitochondrial activity have been shown to impact upon neutrophil functions such as chemotaxis (58), however this has not yet been demonstrated in the context of RA. Neutrophils are thought to be a source of immunostimulatory extracellular mitochondrial DNA in autoimmune diseases (59–61), which shall be discussed in a later section, but the metabolic regulation of neutrophils in RA warrants further study.

Hypoxia

The precise contribution of mitochondrial activity to ATP production within the synovial environment is yet to be fully understood. One important factor to take into consideration is the availability of oxygen within this microenvironment, as the inflamed synovium has been demonstrated to be profoundly hypoxic. Synovial tissue oxygen tension (pO_2) varies considerably between patients, but average measurements of around 20mmHg have been reported (equivalent to roughly 3% ambient concentration), and values as low as 3.2mmHg (0.45%) have been detected (62–64). *In vivo* pO_2 was shown to negatively correlate with macroscopic synovitis, as well as with the numbers of CD3+ and CD68+ cells within the synovial sublining layer (64). The hypoxic microenvironment can exacerbate disease *via* many

different mechanisms, which are reviewed in detail by McGarry et al. and Deng et al. (65, 66). These include the induction of oxidative stress, as well as the stabilisation of HIF-1α, which directly stimulates the production of inflammatory cytokines such as IL-1β and promotes glycolytic metabolism (65, 66).

A number of regulatory mechanisms (in the most part coordinated by HIF-1α) act to reduce mitochondrial activity during low oxygen conditions. This serves to prevent excessive ROS production at complexes I and III of the ETC (67). These mechanisms include decreasing expression and promoting subunit remodelling of ETC complexes, as well as reducing substrate entry into the mitochondrial TCA cycle through inhibition of pyruvate dehydrogenase (67–69). Hypoxia has also more recently been shown to cause reduced expression of the mitochondrial pyruvate carrier (MPC), which is critical for transport of glycolysis-derived pyruvate into the mitochondria (70). Chronic hypoxia may result in an additional suite of adaptations over those of an acute hypoxia insult. Exposure of the monocyte cell line THP1 to chronic hypoxia (72h) substantially reduced oxygen consumption compared with normoxia or acute hypoxia, however a low level of oxidative respiration was maintained through increased electron-transferring flavoprotein expression and oxidation of fatty acids and glutamine (71).

Hypoxia can also have cell type-specific effects. In contrast to other cell types, exposure of osteoclasts to hypoxia did not cause inhibition of pyruvate dehydrogenase activity, and these cells actually increased their mitochondrial activity in hypoxia compared with normoxia (72). This enabled increased ATP production, which is necessary for the increase in bone resorptive activity demonstrated by osteoclasts in hypoxia, suggesting that increased mitochondrial activity in osteoclasts may contribute to bone loss in severe RA (72).

The activity of mitochondria within synovial cell populations is therefore dependent upon the extent to which these regulatory mechanisms are deployed, and this highlights the importance of studying these cells within physiologically relevant conditions.

Nitric Oxide

Another important factor affecting the activity of mitochondrial metabolism in immune cells is the production of nitric oxide (NO). This short-lived signalling molecule is produced from arginine by the nitric oxide synthase (NOS) enzymes, and inhibits mitochondrial metabolism through a variety of mechanisms. NO reversibly inhibits cytochrome *c* oxidase activity through competition for the oxygen binding site (73). In addition, NO irreversibly inhibits several mitochondrial enzymes through direct modification of cysteine residues by the process of nitrosylation. This particularly affects iron-sulfur cluster-containing enzymes, including the TCA cycle enzyme aconitase and complexes I and II of the ETC (73–75). NO signalling has also been linked to reduced abundance of complex I subunits, including key catalytic subunits (76). These mechanisms cooperatively lead to decreased mitochondrial activity and oxygen consumption.

The expression of inducible nitric oxide synthase (iNOS, encoded by the *Nos2* gene) and production of NO is a hallmark of mouse "M1"-type pro-inflammatory macrophages and LPS-treated dendritic cells. In these cells NO is implicated in the strong inhibition of oxidative metabolism following toll-like receptor activation, and contributes to cellular commitment to glycolysis (75–78). Despite this striking effect in mouse cells, human macrophages show little or no synthesis of NO following the same activation signals *in vitro*, which has been explained by the detection of extensive CpG methylation of the *Nos2* promoter in human cells (79–81). This likely underlies the observation that cultured human monocyte-derived macrophages do not downregulate mitochondrial oxygen consumption in response to LPS treatment (36). However, it has been shown that PBMCs from RA patients demonstrate detectable iNOS expression and produce NO (82), suggesting that this signalling molecule may be relevant to mitochondrial regulation in the inflammatory environment *in vivo*. These findings further stress the importance of studying metabolic processes under disease-relevant conditions, as well as the vital consideration of the differences between human disease and animal models (81).

Nitric oxide is also produced by T cells, and increased NO production by these cells in SLE has been linked to increased mitochondrial mass and mitochondrial hyperpolarisation (83). Elevated NO production was also detected in circulating T cells from RA patients compared with controls, although no differences in mitochondrial mass were detected between these groups (84). NO has also been suggested to influence T cell differentiation, however opposing effects have been reported on Th17 differentiation in different studies (79). Differing consequences of T cell-intrinsic production *versus* exogenous exposure, as well as opposing effects of large *versus* small quantities of NO on T cell survival, also complicate the formation of conclusions on the contribution of this signalling molecule to RA disease (79). However, the striking impact of NO on a variety of different metabolic processes means that this small molecule deserves further study in disease-relevant situations.

FATTY ACID OXIDATION

Oxidative phosphorylation is fuelled from several different sources. In addition to pyruvate derived from glycolysis and glutamate produced by the metabolism of glutamine, the TCA cycle can receive carbon from fatty acids through acetyl-CoA generation by the process of fatty acid β-oxidation (FAO). This process also generates NADH and $FADH_2$, which can directly drive the mitochondrial respiratory chain. β-oxidation occurs within the mitochondria through a series of enzymatic reactions. The rate limiting step is entry of fatty acid acyl-CoA into the mitochondria, which involves conjugation to carnitine by the enzyme carnitine palmitoyltransferase I (CPT1) (32). FAO is an important mechanism of energy production in certain immune cell types, including regulatory T cells and "M2"-type macrophages (32). While FAO was previously described as essential for macrophage differentiation in response to IL-4 (M2 differentiation), as well as for memory T cell differentiation, the dependence of these cells on FAO has more recently been brought into question. This was thanks to evidence of non-specific

effects of the inhibitor etomoxir, which is used to inhibit CPT1 activity (85, 86), as well as the finding that inhibition of FAO in human monocyte-derived macrophages did not prevent M2 gene expression or anti-inflammatory function (87). Despite these question marks, fatty acid metabolism has been shown to be disrupted in RA, although more work is required to fully understand the dysregulation of FAO processes in different cell types.

Rodgers et al. described a link between carnitine shuttling of fatty acids and the production of the chemokine CCL20, which has roles in lymphocyte recruitment and osteoclast activity (88). Treatment of human monocytes with exogenous carnitine enhanced LPS-induced CCL20 production, and culture of monocytes in RA synovial fluid led to an increase in intracellular carnitine metabolites under hypoxic conditions. It was proposed that entry of monocytes into the hypoxic and inflamed synovial joint brings about alterations in fatty acid dynamics, supporting the production of CCL20 and promoting further inflammation and joint damage (88). A different study found that carnitine was elevated in synovial fluid samples from RA patients compared with healthy subjects (89). However, this study also showed that enzymes involved in FAO, including HADHA and ACADVL, were significantly downregulated in RA FLS compared with healthy subjects (89). These results suggest that modulation of fatty acid metabolism may vary between different cell types in RA. Hypoxia may be a key regulator of these processes in the synovium, as silencing of HIF-1α in FLS could increase expression of FAO enzymes (89).

Fatty acid metabolism is also disrupted in T cells during RA disease. RA patient T cells accumulate lipid droplets within their cytoplasm through high rates of fatty acid synthesis, which facilitates T cell hypermotility and tissue invasion (90). Enzymes of the FAO pathway were found to be elevated in RA T cells, but there were no differences in expression of *Cpt1*. Therefore it remains to be seen how delivery of fatty acids into the mitochondria and their degradation are regulated in this context, and whether insufficient FAO contributes to the lipid accumulation and low levels of ATP seen in the patient T cells (90). It was subsequently shown that AMPK activation is dysfunctional in RA T cells (27), and AMPK is known to inhibit fatty acid synthesis and to activate mitochondrial fatty acid uptake (28). Therefore dysregulated energy sensing in these cells may result in an imbalance between β-oxidation and fatty acid synthesis, contributing to pathogenic activity of inflammatory T cells.

Elevated levels of free fatty acids of multiple types have been found in the synovial fluid of RA patients relative to healthy individuals (91), however fatty acids were lower in RA synovial fluid compared with other inflammatory arthritis types (92). Different types of fatty acid can have a range of pro- or anti-inflammatory functions *via* multiple mechanisms, including by directly stimulating immune cell receptors, or by influencing membrane synthesis and composition (93–95). Therefore, it is difficult to separate the energy-generating functions of fatty acids in the mitochondria from other roles in RA based solely on abundance measurements. Changes in fatty acid metabolism may also perpetuate extra-articular symptoms of RA, for example differential expression of enzymes involved in fatty acid metabolism were observed in skeletal muscle tissue in an arthritis model and RA patients (96).

GLUTAMINOLYSIS

The metabolism of amino acids has emerged as a vital process that drives both the proliferation of cancer cells and the function of immune cells, and which exceeds the requirement for protein synthesis (97, 98). The amino acid glutamine acts as the principal nitrogen donor for production of nucleic acids and non-essential amino acids, as well as contributing carbons to the mitochondrial TCA cycle through its conversion first to glutamate and subsequently to α-ketoglutarate *via* a process known as glutaminolysis (98). In this way glutamine metabolism is an important anaplerotic mechanism that helps to maintain TCA cycle flux (97–99). Glutaminolysis also contributes to epigenetic regulation through the generation of cofactors or inhibitors of chromatin remodelling enzymes (100–103), and affects protein modification through hexosamine biosynthesis (104).

The mitochondrial enzyme glutaminase 1 (GLS1) catalyses the first step of glutaminolysis: the deamidation of glutamine to glutamate. Takahashi et al. showed that expression of GLS1 was higher in RA FLS compared with those from OA patients, and glutamine starvation or knock-down of GLS1 significantly inhibited RA FLS proliferation. Pharmacological inhibition of glutaminase also reduced RA FLS proliferation both *in vitro* and *in vivo*, and significantly ameliorated disease in an SKG mouse model of arthritis (105). These results suggest that RA FLS exhibit a "glutamine addiction" similar to certain cancer cells (98, 105). However, this reliance on glutamine may relate only to specific pathological properties of FLS, such as proliferation, as inhibition of GLS1 did not alter FLS production of either IL-6 or matrix metalloproteinase-3 (105).

Activated T cells strongly upregulate both glucose and glutamine uptake, and through its numerous functional roles within the cell glutamine metabolism differentially impacts effector activity of different T cell subsets (101). The presence of glutamine has been shown to control the balance of differentiation between Treg cells and Th1 or Th17 cells (103, 106). Recent work reported that Th17 cells are more reliant upon glutaminolysis than are other T helper subsets, and GLS1 is preferentially upregulated in Th17 cells (101, 103, 107). GLS1 inhibition reduced Th17 proliferation and IL-17 production *in vitro*, and ameliorated Th17-driven inflammation *in vivo* in models of inflammatory bowel disease, allergic airway disease and experimental autoimmune encephalomyelitis (101, 107). Despite these reports of a dependence on glutaminolysis for Th17 cell differentiation and proliferation, Takahashi et al. found no difference in Th17 numbers in the spleen of SKG mice following administration of a GLS1 inhibitor (105). However in a different study, the glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON) reduced the proportion of splenic Th17 cells, and showed an additive beneficial effect on arthritis severity

when used along with the mTOR inhibitor rapamycin in the SKG mouse model (108). The precise role of glutaminolysis in T helper cell function in RA therefore requires further study.

As mentioned above, CD8+ T cells from RA patients were able to metabolise glutamine to lactate when subjected to low glucose conditions (46). Therefore, glutamine may be particularly important in the inflamed synovial environment, where competition for glucose is fierce due to the abundance of activated immune and stromal cells. RA CD8+ T cells strongly upregulated expression of the glutamine transporter *SLC5A1* upon stimulation, resulting in significantly elevated expression compared with healthy control cells (46).

Glutamine metabolism has also been shown to be important for monocyte/macrophage function. Glutamine feeds the TCA cycle in both LPS- and IL-4-treated macrophages (97, 99, 102, 104), and the abundance of glutamine-derived metabolites α-ketoglutarate, succinate and fumarate influences macrophage polarisation and innate immune memory through epigenetic mechanisms and prolyl hydroxylase regulation (99, 100, 102). How these mechanisms influence cellular activity in RA is thus far unknown.

A metabolomic study comparing different inflammatory arthropathies found that synovial fluid glutamine levels were highly elevated in RA patients in comparison with ankylosing spondylitis, Behçet's disease or gout (92). Baseline levels of glutamine in the urine or serum of RA patients also contributed to two independent metabolite profiles that could distinguish clinical responders *versus* non-responders to anti-TNFα therapy, with elevated glutamine levels associating with a favourable response (109, 110). Priori et al. showed that serum glutamine levels significantly increased following six months of etanercept treatment in good responders (110). While it is difficult to directly infer mechanistic changes based on these metabolomic studies, the differences in glutamine levels may help to stratify patients for effective treatment strategies.

TCA CYCLE METABOLITES

The tricarboxylic acid (TCA) cycle is a series of enzymatic reactions that occur in the mitochondrial matrix to produce reducing equivalents in the form of NADH and $FADH_2$ to fuel the ETC. TCA cycle metabolites also participate in branching pathways for the biosynthesis of alternative small molecules and macromolecules, or can have signalling roles in their own rights (30). All of these functions have the potential to contribute to disease processes in inflammatory conditions such as RA. Measurement of metabolite concentrations in different biofluid samples from patients can give insights into mechanisms of disease, as well as identifying potential disease biomarkers that will assist in accurate diagnosis or prediction of response to therapy.

Succinate

Succinate is produced from succinyl-CoA in the TCA cycle, and is metabolised to fumarate by the enzyme succinate dehydrogenase, which forms complex II of the ETC. Extracellular succinate was found to be elevated in the synovial fluid in an antigen-induced arthritis model compared with naïve mice, and the level of paw swelling correlated with the level of succinate in the synovial fluid (111). Succinate can also be found in the synovial fluid of patients with RA (111). Similarly to glutamine, succinate was strongly elevated in synovial fluid from RA patients compared with other forms of inflammatory arthritis (92). However, while succinate was detected in the synovial fluid in a separate comparison of several inflammatory and non-inflammatory arthritis types, no metabolic distinction was found between patient groups in this study (112). Metabolomic studies of plasma and serum found no significant differences in succinate levels between RA patients and controls (91, 113–115), or between RA patient groups with differing disease activity (114), meaning that succinate levels are unlikely to be a useful clinical biomarker of disease. It is unclear whether these different results represent mechanistic differences in disease processes between the circulation and synovium, or if the power of the studies was insufficient to detect differences in this metabolite in serum samples.

Macrophages are known to accumulate succinate following an inflammatory challenge, due to a break in the TCA cycle caused by inhibition of succinate dehydrogenase, as well as through increased anaplerosis of TCA cycle metabolites from glutamine and the GABA shunt (99, 116, 117). In addition to intracellular accumulation, macrophages have been shown to release succinate into the extracellular environment following LPS treatment *in vitro* (111). LPS treatment of endothelial cells also induces succinate accumulation, as does hypoxia exposure of both endothelial cells and synovial fibroblasts, indicating that several cell populations could contribute to elevated succinate levels within the synovial environment (118).

From a mechanistic perspective, succinate can have inflammatory effects in both the intra- and extra-cellular compartments. Accumulation of intracellular succinate in macrophages inhibits prolyl hydroxylase (PHD) enzymes either directly or through increased ROS generation (117). This leads to stabilisation of HIF-1α, promoting glycolytic metabolism and IL-1β production, amongst other effects (99). Inhibition of PHDs can also induce NF-κB-mediated inflammatory gene expression through increased activity of the kinase IKKβ, which promotes the degradation of IκBα, the negative regulator of NF-κB (102, 119).

Extracellular succinate can act as an alarmin or danger signal, enhancing immune cell activation by autocrine and paracrine signalling through the plasma membrane succinate receptor SUCNR1/GPR91 (120). This G protein-coupled receptor is expressed by several different cell types, including dendritic cells (DCs) and macrophages, where its expression is further enhanced following inflammatory stimulation (111, 120). Activation of SUCNR1 on macrophages enhances HIF-1α protein expression and augments production of IL-1β in response to inflammatory challenge. Deletion of the *Sucnr1* gene led to a reduction in synovial IL-1β levels and significantly reduced knee swelling in an antigen-induced arthritis model (111). Activation of SUCNR1 on DCs promotes their migration to lymph nodes and enhances costimulatory capacity of DCs

towards T cells (120). This was linked to exacerbation of disease in antigen-induced arthritis through increased expansion of Th17 cells (121).

Succinate also promotes angiogenesis within the synovial membrane by stimulating the production of vascular endothelial growth factor (VEGF) by endothelial cells. This has been linked to intracellular accumulation of succinate in these cells leading to stabilisation of HIF-1α, as well as activation of endothelial cell SUCNR1 by extracellular succinate, both of which promote VEGF production (118). Endothelial cell activation and increased angiogenesis are known to contribute to pathogenic processes in RA, for example by enabling and promoting leukocyte recruitment and migration, as well as supporting hyperplasia of the synovial pannus (122). Treatment of RA synovial fibroblasts with succinate *in vitro* increased production of basic fibroblast growth factor (bFGF) and cellular invasion, which could also contribute to the invasive pannus during disease (123).

Despite these numerous reports of an inflammatory role for succinate signalling, several groups have instead described an anti-inflammatory function of this metabolite, including in isolated bone marrow-derived or peritoneal macrophages (124, 125), and in adipose tissue under steady state and in the context of obesity (125). These anti-inflammatory effects are suggested to occur through both SUCNR1-dependent and -independent mechanisms (124). Keiran et al. also reported that macrophage expression of *Sucnr1* is reduced by LPS treatment and increased by the type 2 cytokine IL-4 (125), which is in opposition to previously reported findings (111). In the context of cancer, tumour-derived succinate induced migration and IL-6 production in tumour-associated macrophages, but also increased *Arg1* expression, a marker of both "M2"-type and tumour-associated macrophages (126). Collectively these results suggest that cell type and context are key for the precise role of succinate in immune cell regulation.

The mechanism of export of succinate from cells of the synovium is also not fully understood. Release of succinate into the extracellular environment upon cell death and rupture is well documented and is consistent with the action of this metabolite as an alarmin (120). Membrane transport of succinate in viable cells is less well understood, although cancer cells have been shown to take up succinate in order to fuel mitochondrial metabolism *via* sodium-coupled dicarboxylic acid transporters such as NaDC3 (*SLC13A3*) (127). Reddy et al. also showed that the monocarboxylate transporter MCT1 (*SLC16A1*) can export succinate from active muscle cells, with important paracrine effects (128). Both of these transport activities required a low pH environment (127, 128). It remains to be seen whether these mechanisms are responsible for succinate transport in the synovial environment, which, similarly to active muscle and the tumour microenvironment, can exhibit localised acidic pH due to high glycolytic rates and enhanced lactic acid production (129, 130). MCT1 has also been shown to be expressed in the arthritic synovial joint (131).

Citrate and Itaconate

Citrate is a TCA cycle metabolite produced from acetyl-CoA and oxaloacetate by the enzyme citrate synthase. Similarly to succinate, citrate accumulates in inflammatory macrophages due to interruption of the TCA cycle. Mechanisms implicated in this accumulation are downregulation of the downstream enzyme isocitrate dehydrogenase (IDH) (104), as well as NO-mediated inhibition of aconitase, the enzyme that converts citrate to isocitrate (75). Citrate also accumulates in RA T cells due to succinyl-CoA ligase deficiency and reversal of the TCA cycle, as well as through uptake and metabolism of lactate from the inflamed microenvironment (43, 45). Citrate is a key biosynthetic metabolite that supports inflammatory macrophage and T cell function. It is used to generate acetyl-CoA, which itself is utilised for the synthesis of fatty acids and lipids, including prostaglandins, and for protein modification. Citrate is also used for the production of NADPH, which is required for NO and ROS generation and to support antioxidant processes (42).

Another fate of intracellular citrate is its conversion into the immune-related metabolite itaconate by the enzyme aconitate decarboxylase 1 (ACOD1), also known as immune-responsive gene 1 protein (IRG1). *Irg1* is one of the most strongly induced genes upon LPS stimulation of macrophages, and intracellular itaconate accumulates to high levels (132). The original described function of itaconate was its direct anti-microbial action through inhibition of the glyoxylate shunt, but it has since been shown to have a number of immunomodulatory functions. These include but are not limited to: anti-inflammatory action through the inhibition of succinate dehydrogenase activity; antioxidant roles through indirect activation of the transcription factor Nrf2; and contribution to innate immune tolerance (116, 132–135).

Despite the accumulation of citrate in inflammatory macrophages *in vitro* and peripheral blood T cells from RA patients, levels of citrate were found to be significantly reduced in synovial fluid, serum and urine samples from RA patients in comparison with healthy individuals (89, 136). The decrease in synovial fluid citrate was linked to decreased expression of citrate synthase in RA patient synovial tissue. Other enzymes of the TCA cycle were also found to be reduced in RA synovial tissue in this study, including malate dehydrogenase and a component of the α-ketoglutarate dehydrogenase complex (DLST), suggesting an overall decrease in TCA cycle activity during disease (89).

An alternative explanation for the decrease in citrate levels in RA could be increased consumption of this metabolite in alternative pathways, for example for the production of itaconate, which has been detected in the plasma of patients with early RA (137). In these patients the change in itaconate levels showed highly significant negative correlation with changes in disease activity following the initial 3 months of conventional disease-modifying anti-rheumatic drug (DMARD) therapy, with a decrease in overall disease activity (DAS44) or measures of inflammation (CRP and ESR) being associated with an increase in plasma itaconate (137). While itaconate production is strongly induced by inflammatory stimuli, its anti-inflammatory and antioxidant properties could account for the negative correlation found here, and the role of this metabolite in the resolution of disease warrants further investigation.

In contrast with these findings in patients, a metabolomic analysis of the transgenic human TNFα mouse model of polyarthritis (Tg197) found that itaconate was high in samples from these transgenic mice following spontaneous disease development, whereas no itaconate was detected in wild-type mice and transgenic animals following treatment with infliximab (anti-hTNFα). The expression of the *Irg1* gene was accordingly higher in the hind limb tissue from transgenic animals relative to wild-type (138). This study also found higher levels of citrate in synovial fibroblasts of the transgenic mice compared with wild-type, in opposition to the decreased citrate seen in patient studies (136, 138). The differences in these studies may represent different disease mechanisms, as well as distinct mechanisms of action of conventional DMARDs and the anti-TNF biologic. These results also highlight the difficulties in interpretation of correlation data when it comes to deciphering mechanisms of disease or therapeutic activity, especially when it comes to feedback mechanisms such as anti-inflammatory mediators that are regulated by inflammatory signals.

The intracellular functions of itaconate are orchestrated both inside the mitochondrion (e.g. inhibition of succinate dehydrogenase) and in the cytosol (e.g. alkylation of KEAP1 to activate Nrf2), and itaconate can be transported across the mitochondrial membrane by the human citrate carrier and 2-oxoglutarate/malate carrier (135). Transporters of itaconate have been identified in certain fungal species that are utilised for industrial synthesis of itaconate (139), however the mechanism by which mammalian cells may excrete or take up itaconate is not known. As a dicarboxylic acid salt, itaconate is unable to freely cross the plasma membrane, and this has resulted in the use of various modified membrane-permeable forms of the metabolite to investigate its role in immune regulation (140). Puchalska et al. showed that exogenous unmodified itaconate could be taken up by bone marrow-derived macrophages, resulting in an altered metabolic fate of glucose. This occurred to a greater extent in unstimulated and IL-4-treated macrophages compared with LPS-treated, suggesting that uptake may be dependent upon an itaconate concentration gradient (141).

The extent to which itaconate is taken up from the extracellular environment in RA and what metabolic or inflammatory consequences this may have are yet to be determined. It is also unclear whether itaconate may have additional effects by acting at the cell surface. For example, the inhibition of succinate dehydrogenase has been attributed to the structural similarity of itaconate to succinate, allowing it to act as a competitive inhibitor of the succinate-consuming enzyme (132). This begs the question of whether extracellular itaconate can act as an antagonist of the succinate receptor SUCNR1 in a similar way.

THE NLRP3 INFLAMMASOME

The term inflammasome describes a family of multi-protein complexes that have roles in cell survival and inflammation. These complexes are characterised by pattern recognition receptors that undergo oligomerisation and act as signalling hubs for the recruitment of the caspase-1 effector protein (142). One of the most well-studied of the inflammasomes, which we shall focus on here, is NLRP3. In addition to its roles in infection, cardiovascular disease, cancer and Alzheimer's disease to name but a few, the NLRP3 inflammasome is widely studied in the context of inflammatory and autoimmune diseases (142–144). Gain-of-function mutations within NLRP3 are associated with the autoinflammatory disease cryopyrin-associated periodic syndrome (CAPS), and aberrant or excessive activation of NLRP3 has been linked to pathogenesis in SLE and various forms of arthritis (142, 144, 145). The NLRP3 inflammasome is also considered to be an important sensor of systemic metabolic disturbance, and is linked to the low-grade inflammation that plays a pathological role in metabolic disorders such as obesity and type 2 diabetes (146).

NLRP3 is a nucleotide-binding leucine-rich repeat (NLR) receptor protein that is activated in response to a wide variety of stress signals, including bacterial, viral and fungal infections; endogenous damage-associated molecular patterns (DAMPs); cytokines; and lipid metabolites and other markers of metabolic stress (145, 146). Oligomerised NLRP3 recruits the adaptor protein ASC, and this in turn recruits procaspase-1, which undergoes self-cleavage to generate the active caspase-1 effector (**Figure 3**) (145). One outcome of NLRP3 inflammasome activation that underlies its link to inflammation, is the release of two members of the interleukin-1 family: IL-1β and IL-18. These cytokines are initially produced as extended precursor forms in response to an initial priming signal, for example stimulation of toll-like receptors and activation of classical inflammatory signalling pathways such as NF-κB. Subsequent release of the active cytokines requires their cleavage by activated caspase-1 (145). Another outcome of NLRP3 activation is a form of cell death termed pyroptosis, which also plays a role in propagating inflammation. Pyroptosis involves cleavage of the protein gasdermin D (GSDMD), which subsequently forms oligomeric pore structures within the plasma membrane (**Figure 3**). This allows release of inflammatory cytokines into the extracellular space, including IL-1β and IL-18, as well as cytoplasmic material that can further act as DAMPs and activate neighbouring immune cells (147).

Methods of activation and regulation of the NLRP3 inflammasome are extensively reviewed elsewhere (142, 145, 148). Here we shall focus solely on the mitochondria-linked mechanisms of regulation, and the relevance of NLRP3 activity to rheumatoid arthritis.

Mitochondria act as important stress-signalling organelles, and mitochondrial disruption results in NLRP3 activation *via* several mechanisms. Reactive oxygen species were identified as important mediators of NLRP3 activation, however the phagosome-located NADPH oxidase enzymes, which are major producers of ROS in myeloid cells, were shown not to be the source of the activating signal for NLRP3 (149–151). Dysfunctional mitochondria produce high levels of ROS, termed mtROS, through the transfer of electrons from the ETC to molecular oxygen (152). Artificial induction of high mtROS levels *in vitro* using ETC inhibitors resulted in production of active IL-1β, indicative of NLRP3 activation (151, 153, 154).

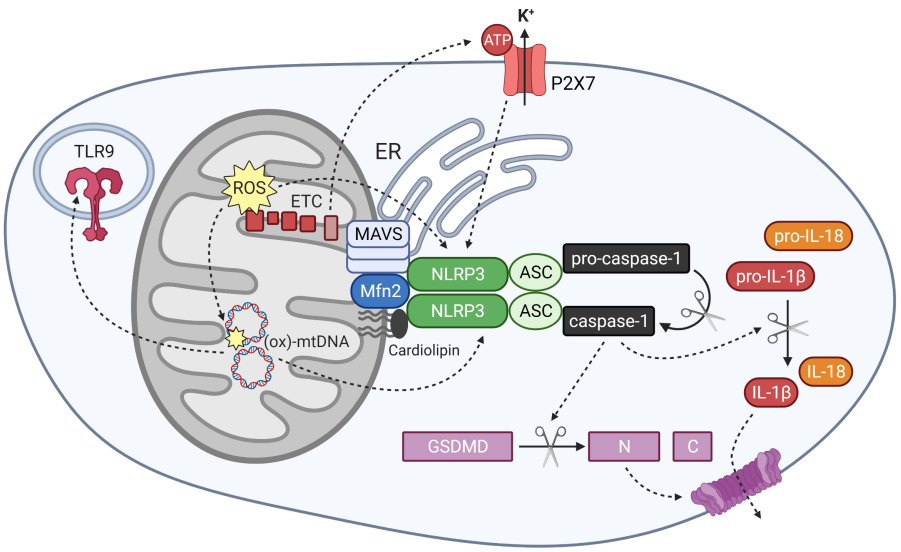

FIGURE 3 | Mitochondrial regulation of NLRP3 inflammasome activation and mitochondrial DAMP activity. The NLRP3 inflammasome is activated by signals including mitochondrial reactive oxygen species (ROS), oxidised mitochondrial DNA (ox-mtDNA) and potassium efflux through the ATP-gated channel P2X7. Components of the inflammasome localise to mitochondrial and ER membranes upon activation, where they associate with MAVS, Mfn 2 and cardiolipin in the outer mitochondria membrane. Oligomerised NLRP3 and its adapter protein ASC recruit and activate caspase-1, which cleaves gasdermin D and interleukin-1 family cytokines into their active forms. Ox-mtDNA also exhibits DAMP activity by stimulating other pattern recognition receptors including the endosomal toll-like receptor TLR9.

Conversely, inhibition of ROS formation, or ROS scavenging by either endogenous or exogenous antioxidants, strongly impaired inflammasome activation and IL-1β release (151, 153, 154). Autophagic degradation and recycling of mitochondrial components by mitophagy is an important process for the removal of damaged or defective mitochondria. Inhibition of autophagy/mitophagy strongly elevated the cellular mtROS levels and was accompanied by an increase in IL-1β release, identifying mitophagy as an important process in preventing excessive inflammatory responses through intracellular mitochondrial stress (151, 154). Autophagy can also play another role in dampening the inflammatory response through direct destruction and recycling of inflammasome components, preventing cleavage and release of IL-1β and IL-18 (155).

In addition to ROS-dependent inflammasome activation, several groups have reported ROS-independent activation, and a requirement for functional mitochondria and an intact mitochondrial membrane potential has been described. It is likely that the precise activating signal dictates the method of inflammasome activation (19, 156, 157). Consistent with the role of the inflammasome as a metabolic sensor, fatty acid metabolism is linked to regulation of NLRP3 activation, and mitochondrial processes contribute to this regulation. The mitochondrial uncoupling protein UCP2 enhances NLRP3 and IL-1β expression by promoting fatty acid synthesis, which is achieved through increased expression of the enzyme fatty acid synthase. Increased fatty acid synthesis promotes activation of Akt and p38 MAPK signalling pathways, which drive NLRP3 and cytokine gene expression (158). Somewhat contradictory to these findings, fatty acid oxidation was also shown to promote NLRP3 activation, regulated by the mitochondrial fatty acid transport enzyme CPT1A and ROS generation by the enzyme NADPH oxidase 4, which also localises to mitochondria (159).

Un-activated NLRP3 protein resides in the cytoplasm, where it associates with the endoplasmic reticulum (ER). Upon priming and activation, the components of the inflammasome relocate to the mitochondria, where they co-localise with both mitochondria and ER at mitochondria-associated membrane (MAM) structures (151, 160). Several different mitochondrial components have been identified that interact with NLRP3 and facilitate the mitochondrial association and activation of the inflammasome. The mitochondrial-specific phospholipid cardiolipin associates directly with both NLRP3 and caspase-1, and was shown to be crucial for inflammasome activation in response to various stimuli (161, 162). NLRP3 also interacts with MAVS, an anti-viral protein that forms large aggregates within the outer mitochondrial membrane upon sensing of viral RNA. MAVS was found to recruit NLRP3 to the mitochondria and facilitate its activation in response to viral infection (160, 163). Mitofusin 2 (Mfn2), a protein involved in mitochondrial fusion, also interacts with NLRP3 and facilitates its activation in response to RNA viruses (19). Mfn2 is also known to associate with MAVS, suggesting that a large protein complex assembles at the mitochondrial surface and regulates inflammasome localisation and function (**Figure 3**) (19).

ATP is a well-recognised activator of NLRP3, and mitochondria represent an important source of ATP within the cell. Mitochondrial DNA (mtDNA) has also been implicated in inflammasome activation (164). These shall be discussed in more detail in a later section. Mitochondria therefore act as important signal generators, as well as signalling platforms, for the activation of the NLRP3 inflammasome.

Activity of the inflammasome has been shown to contribute to pathogenesis in RA. IL-1β has long been known to promote inflammatory and destructive processes within the RA synovium. This occurs through its actions on both immune cells and stromal cells, inducing the production of inflammatory cytokines, chemokines and adhesion molecules, as well as matrix-degrading enzymes and activators of osteoclast-mediated bone resorption (165). IL-1β also potently inhibits the tissue repair process, thereby exacerbating and prolonging joint damage (165). Infiltrating monocytes/macrophages are considered to be the major producers of IL-1β within the synovium, and NLRP3 was found to be activated within this cell population in RA synovial tissue samples (165, 166). Pharmacological inhibition of NLRP3 in the mouse CIA model significantly reduced disease severity and diminished both synovial inflammation and cartilage erosion (166). The ubiquitin-editing enzyme A20 (aka TNFAIP3) counteracts inflammatory signals and is important in the prevention of arthritis, as reviewed by Wu et al. (167). One mechanism by which A20 inhibits inflammation is through negative regulation of NLRP3 and caspase-1 activation, suppressing interleukin production and pyroptosis (167, 168). Myeloid-specific deletion of A20 results in a spontaneous polyarthritis with characteristics of RA, and deletion of NLRP3 could protect these mice from disease (168).

Due to the apparent importance of IL-1β action in RA, a recombinant IL-1 receptor antagonist, therapeutically named anakinra, was investigated in clinical trials. IL-1R antagonism showed significant clinical benefit in RA patients compared with placebo, and anakinra was approved for the treatment of conventional DMARD-resistant RA (169, 170). However, the results of these trails were less striking than was anticipated based on pre-clinical studies, and anakinra treatment showed efficacy in a lower proportion of patients in comparison to trials of other biologic DMARDs, such as anti-TNFα therapies (166, 170).

Interleukin-18, the second cytokine dependent upon inflammasome activation and cleavage by caspase-1, has also been implicated in driving pathogenic mechanisms in RA. Synovial tissue expression of IL-18 protein was found to correlate with CRP, and macrophages were also implicated in the production of this cytokine (171, 172). Deletion or therapeutic neutralisation of IL-18 reduced incidence and/or severity of disease in CIA mouse models, and the pathogenic roles of this cytokine include promoting polarisation and activation of Th1 cells and macrophages (171, 173, 174). Therefore, the direct targeting of the NLRP3 inflammasome may prove more effective in RA treatment than blocking IL-1 activity alone, due to inhibition of both IL-1β and IL-18 signalling, as well as preventing the release of alarmins *via* pyroptosis (166).

MITOCHONDRIAL DAMPS

Mitochondria act as an important source of damage-associated molecular patterns (DAMPs). These endogenously derived molecules signal the occurrence of tissue injury, cellular destruction, or cellular stress, and can activate immune cells in a similar way to microbial-derived pathogen-associated molecular patterns (PAMPs). Due to the bacterial ancestry of mitochondria as described by the endosymbiont theory, mitochondrial components are able to activate the same pattern recognition receptors (PRRs) as exogenous PAMPs (175). DAMPs signal through several different receptors, including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and purinergic receptors (175). A large number of studies have focused on the role of the mitochondrion as a source of DAMPs and the potential involvement in the pathogenesis of RA.

ATP

Mitochondria are the major cellular producers of ATP, with oxidative phosphorylation producing 18 times more ATP per glucose molecule than glycolysis. Release of ATP into the extracellular environment can occur as a result of cell death, and thus this small molecule acts as an alarmin, stimulating an immune response by acting on a variety of cell surface receptors (176). Extracellular ATP and UTP released from apoptotic cells act as chemoattractants for phagocytes such as monocytes, facilitating the removal of apoptotic cell debris. This is achieved through the activation of the purinergic G-protein coupled receptor P2Y2 on the cell surface of monocytes and macrophages (177). Early work in this field showed that *in vitro* treatment of human RA synovial fibroblasts with ATP or UTP was able to mobilise intracellular calcium, consistent with the activation of G-protein coupled receptors (GPCRs) such as P2Y2. Extracellular nucleotides and IL-1α synergised to stimulate synovial fibroblasts to secrete prostaglandins E_2, an important lipid mediator of inflammatory signalling (178).

The most extensively studied link of extracellular ATP to inflammation is through its function as an activator of the NLRP3 inflammasome. ATP is commonly used *in vitro* as the second signal of inflammasome activation, as it promotes NLRP3 oligomerisation and cleavage of pro-IL-1β and pro-IL-18 (145). One mechanism by which ATP achieves activation of NLRP3 is through stimulation of the alternative purinergic receptor P2X7 (**Figure 3**). This is a ligand-gated ion channel that is broadly expressed, but which shows highest expression in the monocyte-macrophage lineage and has been extensively linked to regulation of the innate and adaptive immune systems (179, 180). Activation of the P2X7 receptor by ATP results in efflux of potassium ions, which is a common mechanism of NLRP3 activation that is also employed by the ionophore nigericin (145).

As discussed above, inflammasome activation and the upregulation of IL-1β and IL-18 production has been linked to RA disease processes, and the specific involvement of the P2X7 receptor has also been demonstrated. Elevated expression of the P2X7 receptor has been shown on total PBMCs and circulating

monocytes from RA patients compared with control subjects, and RA patient blood cells produced significantly higher levels of IL-1β in response to LPS+ATP stimulation compared with healthy control cells (181–183). A recent study found that significantly elevated proportions of circulating Th17 and Th1 cells stained positive for P2X7 in samples from either SLE or RA patients compared with healthy controls. The percentage of P2X7-expressing Th17 cells correlated with both DAS28 and serum concentrations of IL-1β (184). Activation of the P2X7 receptor and NLRP3-dependent IL-1β production by dendritic cells is important for priming of T cells, and this pathway was shown to regulate CD8+ T cell activity in the anti-cancer immune response (185) and Th17 differentiation in arthritis models (181).

Cytochrome c

Permeabilization of the outer mitochondrial membrane and release of cytochrome *c* from the intermembrane space into the cytosol is an important initiating signal for the intrinsic pathway of apoptosis. Cytosolic cytochrome *c* activates a caspase cleavage cascade that culminates in apoptotic cell death, which unlike other forms of cell death, is generally not inflammation-inducing (5, 186).

In contrast to its role in the cytosol, cytochrome *c* released into the extracellular environment may be able to act as a DAMP and stimulate immune cell activation (175, 186). Intra-articular injection of cytochrome *c* into mice resulted in a short-lasting inflammatory arthritis characterised by pronounced myeloid cell infiltration, in which neutrophils were the key drivers of pathology (187). Despite this link to arthritis symptoms, lower levels of cytochrome *c* were found in the serum of RA patients compared with healthy controls, and synovial levels were lower than matched serum samples. While the authors suggest that this may be due to increased consumption of cytochrome *c* in the inflammatory synovial environment, it remains to be seen whether there is a physiological role for the inflammation-promoting activity of this protein in RA (187). Extracellular cytochrome *c* may have relevance to autoimmunity in SLE, as autoantibodies to cytochrome *c* were detected in a small proportion of SLE patients in an early study (188).

mtDNA

Mitochondrial DNA (mtDNA) consists of small circular DNA structures resembling plasmids that encode a limited number of genes, including ribosomal components, tRNAs, and key subunits of the ETC (189). These DNA molecules contain unmethylated CpG motifs similar to bacterial DNA, allowing them to act as agonists for PRRs including TLR9, cGAS and inflammasomes (189). MtDNA can be readily oxidised due to close proximity to the ETC machinery that acts as the site of mtROS production, and the oxidation state of mtDNA is an important factor in modulating its ability to activate PRRs (189, 190). The inflammation-promoting activity of mtDNA can be a cell intrinsic process through its release into the cytoplasm; or alternatively extracellular mtDNA can stimulate neighbouring cells or have wide-spread stimulatory effects through release into the circulation. The precise mechanisms of mtDNA DAMP activity, including proposed methods of release from the mitochondrion and receptor signalling pathways, are reviewed elsewhere (189, 191).

As a demonstration of the inflammatory properties of mtDNA, Collins et al. showed that intra-articular injection of mtDNA promoted inflammatory arthritis in mice, whereas injection of nuclear DNA had no effect. Pathology was driven by myeloid cells and could be ameliorated by inhibition of NF-κB activity (192). A synthetic oligodeoxynucleotide containing an oxidised residue was also able to induce inflammatory pathology, whereas the non-oxidised form of the same sequence had no inflammatory effect, demonstrating the greater immunostimulatory properties of oxidised DNA (192).

In the context of RA, extracellular mtDNA can be detected in synovial fluid and blood plasma (60, 190, 192). Extracellular mtDNA levels were found to be significantly higher in RA than in OA or healthy controls, and mtDNA copy number positively correlated with CRP (60). Levels of mtDNA bound to the surface of circulating blood cells were also elevated in RA, although there was no significant correlation with either disease activity or treatment response in this study (193). Hajizadeh et al. found that RA patients who tested positive for synovial fluid mtDNA were more likely also to be positive for rheumatoid factor, and that levels of 8-hydroxy-2'-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage, were higher in RA synovial fluid than controls (190). These results suggest that oxidised mtDNA (ox-mtDNA), a potent PRR agonist, may contribute to immune cell activation in RA.

As discussed earlier, the synovial environment can be profoundly hypoxic, particularly in the presence of high levels of inflammation and dysregulated angiogenesis that can occur in RA and other forms of inflammatory arthritis (63, 64, 194). Low oxygen conditions cause increased production of mtROS, which can exacerbate oxidative damage of mtDNA and contribute to PRR activation (194). Biniecka et al. demonstrated that *in vitro* hypoxia exposure led to increased levels of 8-oxodG in a human synovial fibroblast cell line (63).

Neutrophils have been implicated as a source of extracellular mtDNA that contributes to inflammatory disease. Neutrophils from RA patients demonstrate an oxidative stress gene signature, and mtDNA levels have been shown to correlate with synovial neutrophil numbers (60). Direct stimulation of isolated neutrophils with mtDNA induced expression of RANKL, suggesting that this DAMP could drive osteoclast-mediated bone erosion in the RA joint (60). NETosis is a form of inducible cell death that occurs in neutrophils involving the release of complexes of decondensed chromatin and anti-microbial proteins, termed neutrophil extracellular traps (NETs). NETs function to trap and neutralise infectious agents, but are also implicated in the pathogenesis of autoimmune diseases including SLE and RA through stimulation of cytokine production and the release or extracellular generation of citrullinated proteins that act as autoantigens (57, 61, 195–197). Hypopolarised mitochondria have been shown to be released during NETosis, and ox-mtDNA contributes to NET

formation in SLE, promoting interferon production and interferon stimulated gene signatures (61). In addition to promoting oxidation of mtDNA with immunostimulatory consequences, mitochondria-derived ROS are pivotal for the initiation of NETosis in response to certain triggers, as well as the spontaneous NETosis of pro-inflammatory low-density granulocytes from SLE patients (61, 198). Vorobjeva et al. also showed that the mitochondrial permeability transition pore is required for ROS production and NETosis in response to a calcium ionophore (198). NETosis-independent release of ox-mtDNA from neutrophils has also been demonstrated, which potently stimulates type I interferon production by plasmacytoid DCs, and this study found that some individuals with SLE develop antibodies against ox-mtDNA (59).

Cytosolic mtDNA has also been linked to the activation of the NLRP3 and AIM2 inflammasomes, resulting in caspase-1 activation and release of IL-1β and IL-18 (154, 164). The induction of apoptosis by inflammatory stimuli is associated with the release of mtDNA into the cytosol, and ox-mtDNA can interact with and activate NLRP3 in cells undergoing apoptosis (164). Inhibition of apoptosis, for example through the overexpression of the anti-apoptotic protein Bcl-2, could prevent NLRP3 activation and IL-1β release (164). Elevated levels of cytoplasmic ox-mtDNA have been detected in CD4+ T cells from RA patients. This was linked to reduced expression of the nuclease MRE11A, which forms part of the MRN DNA repair complex. Inhibition of this nuclease caused mitochondrial dysfunction, indicated by reduced oxidative metabolism and increased mtROS production. This led to enhanced oxidative damage of mtDNA and its release into the cytoplasm, activating the inflammasome and resulting in IL-1β release and pyroptotic cell death. Restoring MRE11A function in a chimeric mouse model of arthritis reduced ox-mtDNA levels, caspase-1 activation and synovial tissue inflammation (26). Reduced mtDNA repair capacity and mitochondrial damage has also been linked to chondrocyte apoptosis in osteoarthritic cartilage (199, 200).

The ability of other mitochondrial components to act as DAMPs has also been demonstrated. Cardiolipin, which is present in mitochondrial and bacterial membranes, has been shown to bind to TLR4 and either inhibit or activate signalling from this receptor, depending on the level of saturation or oxidation of the cardiolipin acyl chains (201). The relevance of these observations to autoimmune diseases has not yet been determined. Exposure of cardiolipin on the outer face of mitochondria also contributes to NLRP3 inflammasome activation, as previously discussed (161, 162, 201). Anti-cardiolipin antibodies can be detected in samples from RA patients, although these are more frequently associated with primary antiphospholipid syndrome or SLE (202), and this autoimmunity does not constitute true DAMP activity.

N-formyl peptides are also well-established mitochondria-derived DAMPs, which are a product of mitochondrial protein synthesis and act as strong chemoattractants for neutrophils (175). While an agonist of a formyl peptide receptor was shown to ameliorate disease in a serum transfer model of arthritis (203), this may be related to mimicking of endogenous anti-inflammatory agonists of this receptor rather than recapitulating mitochondrial N-formyl peptide detection, and the direct relevance of these mitochondrial-derived peptides to RA has not been investigated to our knowledge.

mtDNA MUTATION

In addition to its role as a DAMP, mitochondrial DNA is highly prone to mutation, which can promote inflammatory and autoimmune processes in a number of ways. The vulnerability of mtDNA to mutation is caused by the lack of extensive high fidelity repair processes, the high copy number of mtDNA within each cell, and the close proximity to ROS generating machinery (189, 204, 205). Mutations within the mitochondrial protein-coding genes can result in changes to peptide sequences that bring about mitochondrial dysfunction. Mutation of a mitochondrially-encoded subunit of the ETC may lead to increased ROS generation, which could subsequently exacerbate DNA mutation, as well as having other inflammatory consequences as discussed above (205). Mitochondrial peptides can activate the adaptive immune system through their presentation *via* MHC molecules. A change in the sequence of these peptides brought about by mtDNA mutation can result in breach of self-tolerance, leading to immune cell activation and the generation of autoantibodies (204–206). MtDNA mutation may also alter the ability of mtDNA to activate PRRs (189).

The frequency of mtDNA mutations was found to be significantly higher in the synovial tissue of patients with rheumatoid or psoriatic arthritis compared with OA and/or healthy individuals, and many of these mutations created amino acid changes (205, 207). MtDNA mutation frequency positively correlated with macroscopic synovitits and synovial levels of TNFα or IFNγ (207). *In vitro* exposure of immortalised RA fibroblasts to hypoxia (1% O_2) increased the number mtDNA mutations, which could be prevented by treatment with antioxidants. This is consistent with hypoxia leading to increased oxidative stress and DNA oxidation. An increase in random mtDNA mutations showed negative correlation with synovial oxygen tension in patients with rheumatoid or psoriatic arthritis, suggesting that hypoxia-induced oxidative stress is likely a key driver of the elevated mutational burden in the hypoxic synovial environment (63). *In vitro* treatment of RA FLS with TNFα also led to increased mtDNA mutation frequency, indicating that in addition to mtDNA mutation promoting inflammation, an inflammatory environment also promotes mtDNA mutation (207). This damaging positive feedback loop may contribute to the persistent inflammatory response in RA.

As well as novel mutations arising as a result of inflammation or hypoxia, specific mitochondrial haplotypes have been linked to RA. The main variations found to be associated with the disease were within genes encoding components of the ETC (208). Rare single nucleotide variants of genomically-encoded ETC subunit or assembly factor genes were also found to associate with severe erosive RA (209).

TARGETING MITOCHONDRIA IN RA TREATMENT

The therapeutic targeting of metabolic pathways initially emerged as a prospect for cancer treatment due to the relatively early discovery that metabolic reprogramming is essential to sustain cancer cell survival and proliferation. It is now apparent that targeting of metabolic processes, including mitochondrial modulation, could be therapeutically beneficial for a wide range of diseases including cardiovascular, neurodegenerative and autoimmune diseases (210, 211). As the importance of mitochondrial biology in inflammatory disease emerges, it is also becoming evident that many therapeutics currently in use for the treatment of RA have effects on mitochondrial activity, whether directly or indirectly. In some cases, these effects are known to contribute to the beneficial anti-inflammatory actions of the drugs, but can also be responsible for adverse effects of therapy. In other cases it remains to be seen whether the impact on mitochondrial function underlies the therapeutic efficacy.

Methotrexate

Methotrexate (MTX) is a conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) that is one of the first line therapeutics used for the treatment of RA and other forms of inflammatory arthritis, and which is also used in cancer treatment. MTX inhibits the mitochondrial folate pathway by acting as a competitive inhibitor of folate-dependent enzymes such as dihydrofolate reductase (DHFR), due to the structural similarity of MTX to folic acid. This results in reduced synthesis of purines and pyrimidines, thus bringing about anti-proliferative effects (212). Whilst this was the main intended target pathway of MTX, it is now thought that additional mechanisms are responsible for many of the anti-inflammatory effects of this drug. This is reviewed by Bedoui et al. (212).

Elevated ROS production is an important mechanism by which MTX affects cellular function and survival, and mitochondria have been shown to contribute to this ROS generation (213, 214). Elevated ROS can propagate cellular oxidative stress, which contributes to the anti-inflammatory or anti-cancer actions of the drug, for example through induction of T cell apoptosis (213, 215). Lee et al. found that responsiveness to MTX therapy in RA patients was linked to susceptibility of the patient's FLS to mitochondrial depolarisation and apoptosis in response to the drug (216).

However, oxidative stress and mitochondrial dysfunction have also been linked to common adverse effects of MTX therapy. Various combinations of lipid peroxidation, mitochondrial depolarisation, respiratory chain inhibition, reduced ATP levels, cytochrome *c* release and mitochondrial swelling have been reported in liver, kidney, small intestine and platelets following either *in vivo* or *in vitro* MTX treatment (214, 217–219). Hepatoxicity is one of the most common adverse effects of MTX therapy, however severe liver injury is more commonly associated with high dose therapy used in cancer treatment rather than the low doses used in the treatment of inflammatory diseases (212). Thrombocytopenia can be experienced by cancer or RA patients after long-term MTX therapy, which may be due to oxidative stress and apoptosis of platelets (214).

In contrast, *in vitro* MTX treatment was found to increase respiratory rate and respiratory capacity in the contexts of B cell lymphoma and breast cancer. This was due to AMPK activation through the accumulation of the purine synthesis intermediate AICAR, which was shown to contribute to the therapeutic effects of MTX in cancer (220). AMPK activation also has therapeutic potential for inflammatory diseases, which shall be discussed below.

Leflunomide

Leflunomide is also an approved csDMARD that is indicated for the treatment of adults with RA, although its use is not as common as other csDMARDs due to the risk of hepatic toxicity (221). Mitochondrial disruption has been implicated in this hepatotoxicity, as the drug caused ATP depletion and mitochondrial depolarisation in a hepatocellular carcinoma cell line. These effects were linked to ER stress and inhibition of ATP synthase activity, resulting in cytotoxicity (222).

Leflunomide inhibits the mitochondrial inner membrane protein dihydroorotate dehydrogenase (DHODH), reducing the *de novo* synthesis of pyrimidines and thus inhibiting proliferation. Pyrimidine depletion by leflunomide was recently shown to promote enhanced expression of mitofusins 1 and 2 (Mfn1/2), resulting in increased mitochondrial fusion (223, 224). Leflunomide and its active metabolite teriflunomide also inhibit the activity of ETC complex III, which may be explained by coupling of the pyrimidine synthesis pathway to ETC activity through DHODH-mediated reduction of ubiquinol (223, 225). DHODH inhibition in T cells preferentially reduced proliferation of high affinity antigen-specific T cells, due to their high demand for oxidative metabolism in the early stages of proliferation. In this way the drug is thought to inhibit autoreactive T cell expansion in autoimmune diseases such as multiple sclerosis and RA (225). As complex III inhibition is known to result in mtROS production, and DHODH silencing has been shown to increase ROS (226), it is possible that leflunomide brings about apoptosis induction by oxidative stress, similarly to MTX.

Sulfasalazine

Sulfasalazine is another csDMARD that has been used by rheumatologists for decades, but a full understanding of its anti-inflammatory mechanisms of action is lacking. It has been reported that sulfasalazine inhibits *de novo* purine synthesis, resulting in adenosine release that contributes to the anti-inflammatory activity of this drug, similarly to MTX (227). Sulfasalazine has anti-proliferative and cytotoxic effects, making it an effective therapeutic for cancer and inflammatory disease treatment, and it induces apoptosis of T cells through mitochondrial permeabilization (228). Ferroptosis is a form of cell death characterised by increased mitochondrial membrane densities and outer membrane rupture, which is associated with excessive ROS production through iron metabolism (229). These characteristics were demonstrated in response to sulfasalazine treatment in lymphoma and breast cancer cells *in vitro*, and inhibition of plasma membrane cystine/glutamate antiporters was implicated in sulfasalazine-induced ferroptosis (229–231).

Mitochondrial disruption has also been implicated in renal injury caused by sulfasalazine. High doses of the drug caused oxidative stress in rat kidneys, demonstrated by increased ROS, lipid peroxidation and decreased levels of reduced glutathione, which were associated with mitochondrial depolarisation and swelling (232).

Biologics

The development of targeted biologic therapies for rheumatoid diseases has revolutionised RA treatment and significantly improved quality of life for many people living with this disease. Whilst none of these therapeutics are designed to target the mitochondrion directly, many biologics block cytokine signalling, and it has been widely demonstrated that inflammatory cytokine signalling has robust effects on mitochondrial biology. For example, treatment with TNFα blocking therapy resulted in reduced frequency of mitochondrial mutations and decreased markers of oxidative stress in the synovia of patients who clinically responded to the therapy (233). Several other studies have shown reductions in reactive oxygen and nitrogen species and increases in antioxidants in response to anti-TNFα therapy (234). Reduced oxidative stress has also been demonstrated in patients treated with tocilizumab, an antibody directed against the IL-6 receptor, suggesting that combating cytokine-induced ROS production may be an important therapeutic mechanism (234).

Gene expression analyses have been used to interrogate the effects of RA therapies on immune cells. Meugnier et al. profiled gene expression changes in PBMCs from RA patients following clinical anti-TNFα treatment and found enrichment of genes related to oxido-reduction and electron transfer. This included upregulation of several ETC subunit genes and mitochondrial ribosomal components (235). Derambure et al. analysed whole blood RNA of patients treated with abatacept. This biologic is a fusion of the extracellular domain of CTLA-4 and the Fc portion of IgG1, which disrupts T cell co-stimulation through CD28 and affects antigen presenting cell signalling. Pre-therapy expression of several ETC subunit genes was significantly lower in patients who responded to abatacept plus MTX therapy compared with non-responders, and five of these genes were significantly upregulated following six months of therapy in the responder group (236). These findings suggest that an increase in respiratory chain activity may play a role in the therapeutic action of diverse biologic drugs.

JAK/STAT Inhibitors

The JAK/STAT inhibitors are a class of small molecule targeted synthetic DMARDs that are relatively new to the arsenal of therapeutics used by rheumatologists, offering a useful alternative to biologic therapies. The JAK family of tyrosine kinases are activated downstream of several different cytokine receptors and signal through activation of the STAT family of transcription factors [reviewed in (237)]. Tofacitinib, a pan-JAK inhibitor that is approved for the treatment of rheumatoid and psoriatic arthritis, increased expression of several mitochondrial genes and led to increased oxidative respiration rate and ATP synthesis in RA FLS, while also decreasing ROS levels and glycolytic enzyme expression (238). STAT3 activation has previously been linked to hypoxia and promotes HIF-1α activity, which may be an important mechanism by which JAK/STAT inhibition can regulate metabolic processes during inflammation (66). Interestingly, tofacitinib also increased lipolysis, mitochondrial activity and uncoupling protein 1 (UCP1) expression in adipocytes, which was suggested to be of therapeutic interest in obesity and may help to combat the systemic metabolic complications of inflammatory diseases (239).

Future Therapeutic Opportunities

Despite the array of therapeutics that are already approved for the treatment of RA, some patients fail to respond to any of these treatments, and sustained clinical remission is achieved in fewer than half of patients (240, 241). These facts highlight the continued need for additional therapies that are tailored to an individual patient's disease. Due to the mounting evidence of metabolic involvement in disease, metabolic targets are coming to the forefront in the search for novel therapeutic strategies. The wide array of potential metabolic targets for treatment of autoimmune disease are reviewed by Piranavan et al. (211).

A pathway that has gained much attention in this area is the energy and metabolite-sensing system, coordinated by the opposing actions of AMPK and mTOR. This system plays a number of roles in the regulation of immune cell function, including cell fate and survival decisions in lymphocytes (242). Complex crosstalk exists between these two sensors and the mitochondria, with each influencing the activity of the others. Inhibition of mTOR activity with compounds such as rapamycin or everolimus, which are used to prevent transplant rejection and in cancer therapy, has shown therapeutic benefit in clinical trials to treat multiple different autoimmune conditions (243).

An alternative approach is the activation of AMPK, which itself inhibits mTOR. This can be achieved using metformin, a drug used in the management of type 2 diabetes, which inhibits complex I of the ETC, thereby reducing mitochondrial activity and ATP generation. The resultant increase in AMP/ATP ratio causes AMPK activation, bringing about inhibition of anabolic metabolism and cellular proliferation. AMPK activation also downregulates several inflammatory signalling pathways such as NF-κB and JAK-STAT pathways, and as a result has broad anti-inflammatory actions (244). Efficacy of metformin has been shown in several pre-clinical models of arthritis, associated with decreased inflammatory cytokine production, reduced Th17 numbers and increased Tregs, and inhibition of osteoclastogenesis, amongst other effects (244–247). The examination of metformin use specifically for RA in clinical trials is currently lacking, but this drug is generally well-tolerated and could have the added benefit of reducing the systemic metabolic complications that can be associated with RA, such as obesity and diabetes, due to its effects on glucose homeostasis (244). However, Wen et al. reported that *ex vivo* treatment of RA patient T cells with metformin was unable to oppose inflammatory T cell differentiation due to impairment of the AMPK activation

pathway in these cells. This suggests that direct targeting of mTOR activity may be a better therapeutic option than AMPK activation for human RA disease (27).

Another possible therapeutic strategy is the targeting of ROS production, as ROS and oxidative stress contribute to inflammatory processes and RA pathogenesis in multiple ways, as discussed throughout this review. Metformin inhibits oxidative stress through upregulation of antioxidant enzymes and downregulation of the ROS producer NADPH oxidase, and these effects are thought to contribute to the anti-inflammatory and anti-fibrotic effects of this drug (244, 248, 249). A mitochondrially-targeted antioxidant showed modest therapeutic benefit in a rat CIA model, suggesting that mitochondrial ROS production could be a viable target (250). The targeting of ROS systems must be approached with caution, however. In contrast to pathological roles of ROS, reduced ROS signalling downstream of TCR activation is implicated in hyperproliferation and inflammatory differentiation of RA T cells, as discussed earlier (44). The induction of ROS generation and ROS-induced apoptosis appears to be a mechanism by which current csDMARDs such as MTX mediate their therapeutic effects, although this also contributes to adverse effects through apoptosis of other cell types. It has been suggested that antioxidant treatment could be used in combination with other therapeutics, either to enhance efficacy or reduce side effects, but this approach requires further validation and will depend on the specific mechanism of action of the drug in question (251, 252).

As discussed above, the NLRP3 inflammasome, which is regulated by mitochondria and acts as an important sensor and signal disseminator for both metabolic and infectious disturbances, is also a potentially viable future target that is being investigated for multiple conditions (148, 166). Preventing NLRP3 activation by inhibiting activation of ATP-gated ion channels such as P2X7 has been explored for its potential in RA treatment (179, 253). P2X7-KO mice showed significantly reduced incidence and severity of collagen antibody-induced arthritis (180). *In vivo* treatment with P2X7 antagonists suppressed inflammation and reduced disease severity in both a mouse collagen antigen-induced arthritis model (181) and a rat streptococcal cell wall arthritis model (253). However, in two clinical trials of small molecule P2X7 antagonists, targeting of this receptor showed no clinical benefit over placebo in RA patients who had shown no response to first line conventional DMARDs (254, 255). This therapy therefore showed less efficacy than directly targeting IL-1β itself with anakinra (170), suggesting that mechanisms in addition to ATP signalling through this receptor contribute to NLRP3 activation and IL-1β production in RA disease. It remains to be seen whether targeting of extracellular ATP signalling can prove beneficial in other autoimmune conditions (179).

Overall, there is plenty of scope for the targeting of metabolic and mitochondrial pathways for the treatment of autoimmune and inflammatory diseases such as RA, particularly as more is learnt about the contribution of these processes to the immune response and disease pathogenesis.

CONCLUSIONS AND OUTSTANDING QUESTIONS

We have made great strides in recent years in our understanding of the contributions made by metabolic regulation to immune cell function and disease processes in autoimmunity. Through their central role in metabolism and their ability to act as signalling hubs, mitochondria influence numerous cellular functions and can module immune cell activity. The capacity of mitochondrial components to directly activate immune receptors can also have potentially damaging consequences, however whether these signals act as initiating stimuli in RA disease is yet to be fully established.

Despite the progress already made, there remain many unanswered questions regarding the precise roles of mitochondria in driving disease in RA. Several of these have been highlighted in the body of this review, including the impacts of hypoxia and nitric oxide on mitochondrial ATP production in the synovium, the precise signalling roles of mitochondria-derived metabolites, and the differences in metabolic commitments between different subpopulations of synovial cells.

One of the major gaps in our understanding of metabolic regulation is the lack of data from the site of disease manifestation – the synovial joint itself. This has been due to the technical complexities involved in accessing and studying the very small numbers of cells from this environment, but this analysis will be crucial in determining whether direct targeting of metabolic and mitochondrial pathways will have real therapeutic benefit. Advances in single cell and *in situ* imaging technologies will be very helpful in answering these questions and will add to existing data from circulating immune cells. In addition, many studies that have looked at fibroblast-like synoviocyte biology have done so using tissue from joint replacements, which represents very late-stage disease. These cells may not bear much resemblance to FLS at the initiation of disease, with the latter likely to give more useful information about disease causes and potential opportunities for early intervention to prevent disease progression.

Another gap in our knowledge is the understanding of mitochondrial function in other cell types that contribute to RA disease, such as B cells and neutrophils, as the majority of the work in this field has focused on T cells and macrophages. This mirrors the landscape of the immunometabolism field in general, however the availability of large single cell gene expression datasets that cover multiple synovial cell populations should aid in the more rapid expansion of these areas in a disease-relevant manner (256). The metabolic communication between these cell populations is also an intriguing and important consideration, as no cell exists in isolation. This communication may constitute competition for, or exchange of, metabolites in the synovial environment. A more extreme form of metabolic communication is the exchange of whole mitochondria between cells, which has been demonstrated in other contexts including tumour models, lung inflammation and wound healing (257, 258). Whether this occurs in RA is currently unknown.

The precise regulation of mitochondrial architecture, including fusion/fission, supercomplex organisation and respiratory chain

composition, also requires further investigation in the context of inflammatory disease. These aspects help to control ATP production, oxidative stress and optimisation of substrate usage, factors that can all impact upon immune cell function. Mitochondrial architecture is known to be regulated by hypoxia and inflammatory signals in other contexts (259–261).

The impact of therapy on mitochondrial function is apparent for several currently used therapeutics, and it will be interesting to see whether other anti-rheumatic drugs in use or development similarly affect this organelle. The targeting of metabolic processes has become an attractive prospect for novel therapeutic development, as well as for the re-purposing of treatments from other diseases for use in inflammatory and autoimmune conditions, for example mTOR and AMPK modulators. However, a more complete understanding of the nuances of mitochondrial function in different cell types in RA is required before the opportunities for targeting mitochondrial pathways can be fully realised. Since metabolic and mitochondrial activities are essential for all cell and tissue types, an important consideration will be to achieve therapeutic benefit without unacceptable adverse effects.

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SC and AC devised, and SC wrote the manuscript. LM contributed to figure preparation. MK-S and AC reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Insulin Signaling in Arthritis

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Inflammatory arthritis is burdened by an increased risk of metabolic disorders. Cytokines and other mediators in inflammatory diseases lead to insulin resistance, diabetes and hyperlipidemia. Accumulating evidence in the field of immunometabolism suggests that the cause-effect relationship between arthritis and metabolic abnormalities might be bidirectional. Indeed, the immune response can be modulated by various factors such as environmental agents, bacterial products and hormones. Insulin is produced by pancreatic cells and regulates glucose, fat metabolism and cell growth. The action of insulin is mediated through the insulin receptor (IR), localized on the cellular membrane of hepatocytes, myocytes and adipocytes but also on the surface of T cells, macrophages, and dendritic cells. In murine models, the absence of IR in T-cells coincided with reduced cytokine production, proliferation, and migration. In macrophages, defective insulin signaling resulted in enhanced glycolysis affecting the responses to pathogens. In this review, we focalize on the bidirectional cause-effect relationship between impaired insulin signaling and arthritis analyzing how insulin signaling may be involved in the aberrant immune response implicated in arthritis and how inflammatory mediators affect insulin signaling. Finally, the effect of glucose-lowering agents on arthritis was summarized.

Keywords: rheumatoid arthritis, insulin, insulin receptor, metabolism, T cell, macrophage, synoviocyte

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INTRODUCTION

Insulin, the main actor of glucose homeostasis, exerts its action through the transmembrane insulin receptor (IR), expressed on target cells such as hepatocytes, adipocytes, synoviocytes, or muscle cells (1). However, IR can also be found on the membrane of T cells, macrophages or dendritic cells, and an immunoregulatory function of insulin has been suggested. Indeed, glucose is necessary for immune cells to produce energy and to maintain normal activity (2). For this reason, insulin plays a pivotal role in maintaining physiological immune response. In diabetic patients, administration of insulin may decrease levels of C-reactive protein (CRP), reduce the ability of neutrophils to generate reactive oxygen species (ROS) and suppress transcription of different Toll-like receptors (TLRs) on circulating mononuclear cells (3, 4). The interplay between inflammation, immunity and

metabolism has been outlined (5) and, in this context, insulin signaling, similarly to what is observed in type 2 diabetes (T2D) or in metabolic syndrome, may be involved in the dysregulation of immune response in inflammatory diseases. Epidemiological and laboratory studies reported a possible correlation between insulin resistance and osteoarthritis (OA), rheumatoid arthritis (RA), spondyloarthritides or systemic lupus erythematosus (6–13).

In this review, we summarize the available literature about the bidirectional cause-effect relationship between impaired insulin signaling in inflammatory and degenerative arthritis, analyzing how insulin signaling may contribute to the aberrant immune response found in arthritis and how inflammatory mediators impair insulin signaling. Finally, the effect of glucose-lowering agents on arthritis was reviewed.

PHYSIOLOGY OF INSULIN RECEPTOR AND INSULIN SIGNALING CASCADE

The IR is a dimer located on cellular membrane and composed of four domains linked by disulfide bonds: two extracellular (α) and two intracellular (β). The α domains host the binding site of insulin, while the β domains have tyrosine kinase activity (14–17). Once circulating insulin binds the α subunits, IR undergoes autophosphorylation, with consequent phosphorylation of intracellular substrates. In the first step, IR substrates 1 and 2 (IRS-1 and IRS-2) and the docking protein Src-homology collagen (Shc) are phosphorylated, leading to the activation of two main pathways, metabolic and mitogenic (**Figure 1**). The former involves phosphoinositide3-kinase (PI3K), while the latter is mediated by mitogen activated protein kinase (MAPK) (17).

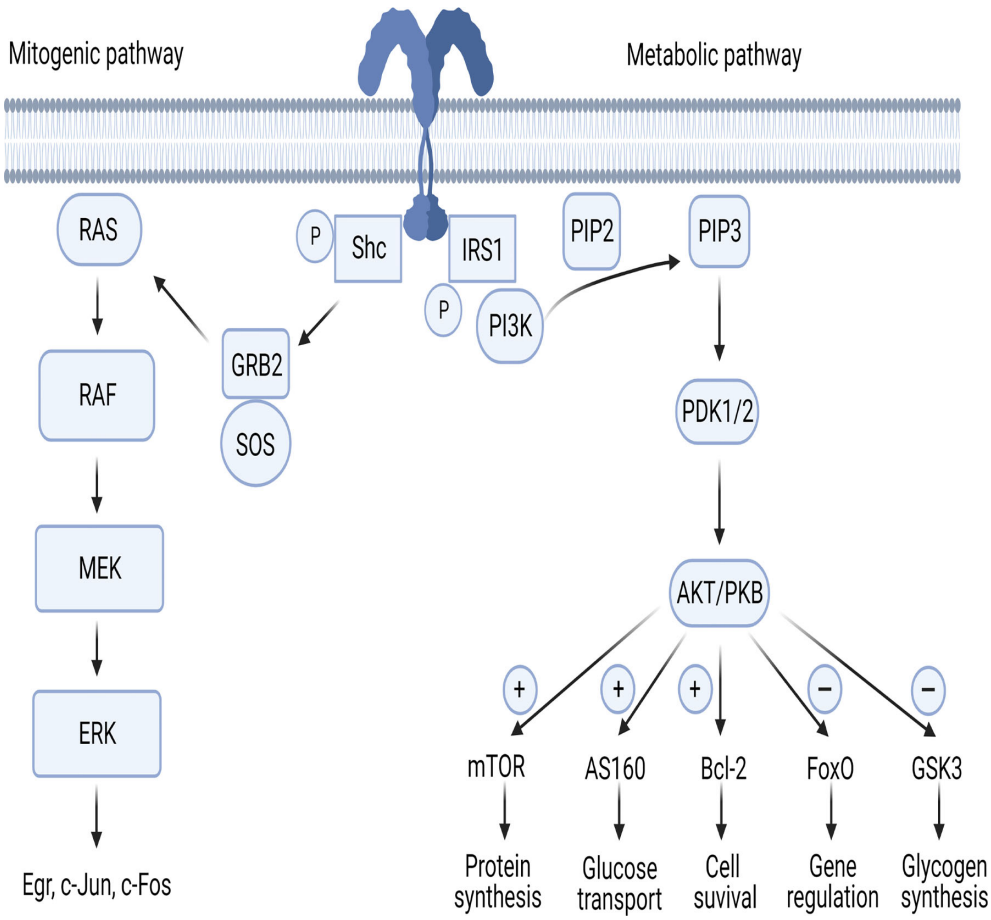

FIGURE 1 | The mitogenic and metabolic pathways of insulin signaling. AKT/PKB, protein-kinase B; AS160, AKT substrate of 160kDa; Bcl-2, B-cell lymphoma 2; ERK, extracellular signal-regulated kinases; FoxO, forkhead box-containing protein O subfamily; GRB2, growth factor receptor-bound protein 2; GSK3, glycogen synthase kinase 3; IRS, insulin receptor substrate; MEK, MAPK/ERK Kinase; mTOR, mammalian target of rapamycin; PDK1/PDK2, phosphoinositide-dependent protein kinase; PI3K, phosphoinositide-3 kinase; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; RAF, rapidly accelerated fibrosarcoma; RAS, Rouss avian sarcoma; Shc, Src-homology collagen; SOS, son of sevenless. **Figure 1** has been created using BioRender (www.biorender.com).

The Metabolic Pathway

In the first step of the metabolic pathway, the PI3K regulatory subunit p85 or p55 binds to IRS-1 and IRS-2 activating the PI3K cascade. Next, the p110 catalytic subunit activation results in phosphatidylinositol-3,4,5-triphosphate (PIP3) generation leading phosphoinositide-dependent protein kinase (PDK) 1 and 2 to activate the three isoforms of AKT/PKB (**Figure 1**). PDKs become then activated after binding to PIP3 in the cell membrane (18, 19). AKT/PKB regulates five main substrates: 1- activates mammalian target of rapamycin (mTOR), responsible of protein synthesis; 2- inhibits glycogen synthase kinase 3 (GSK3), responsible of glycogen synthesis; 3- inhibits forkhead box-containing protein O subfamily (FoxO), involved in the regulation of gluconeogenic and adipogenic genes; 4- increases the AKT substrate of 160kDa (AS160), responsible of glucose transport (20, 21); 5- upregulates Bcl-2 expression, involved in cell survival (22).

The Mitogenic Pathway

In the first step of the mitogenic pathway, growth factor receptor-bound protein 2 (GRB2) is activated by phosphorylated Shc protein (**Figure 1**). GRB2 acts as a bond that links IRS-1 to son-of-sevenless (SOS), which is a guanine nucleotide exchange factor. GRB2/SOS promotes exchange of GDP with GTP on Rouss avian sarcoma (Ras), thus activating it (23). Activated Ras recruits Raf serine/threonine protein kinase and then the MAPK pathway transcription factors MEK, ERK and p90 inducing the activation of the Egr genes c-Jun and c-Fos (24, 25).

INSULIN, IMMUNE CELLS AND ARTHRITIS

Immune cells need glucose to produce energy (14) and, similar to adipose, muscle and liver cells, also immune cells express IR on their surface (26, 27). Through IR, insulin acts as a glucose-regulating hormone and behaves as a growth-like factor and cytokine regulator (28–30), exerting its immunomodulatory effects (2, 31).

Insulin modulates the immune response either indirectly through the glucose-lowering effect or directly by acting on immune cells and influencing their proliferative responses and signal transduction (32) (**Figure 2**). Regarding the first point, hyperglycemia has negative effects on the immune system since it induces cell stress and leads to the generation of advanced glycation end products (AGEs) and ROS, which stimulate release of various pro-inflammatory mediators. It can therefore be hypothesized that insulin, through its glucose-lowering role, reduces "glucose toxicity" and cell stress, exerting an anti-inflammatory effect (33).

In addition to these actions on glucose metabolism, insulin exerts anti-inflammatory effects *via* the stimulation of various intercellular pathways. Activation of PI3K/Akt pathway reduces the transcriptional activity of FoxO proteins that in turn suppress TLR4 signaling in response to lipopolysaccharides (LPS) in leukocytes (34) leading to a downregulation of the immune system. Furthermore, insulin antagonizes the pro-inflammatory transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activates the mTOR complex (mTORC), enhancing p62 phosphorylation and then p62-mediated degradation of the Kelch-like ECH-associated protein 1 (Keap1), thus allowing antagonism of pro-inflammatory processes mediated by nuclear factor erythroid 2-related factor 2 (Nrf2). Lastly, insulin inhibits the transcription of various TLRs on circulating mononuclear cells, including TLR1, 2, 4, 7 and 9 (35), causing a decreased immune response.

On the other hand, several investigations suggested a role of insulin as pro-inflammatory hormone. Studies in healthy, nondiabetic subjects explored the effects of insulin on polymorphonuclear (PMN) leukocytes functions. In vivo experiments with hyperinsulinemic clamp demonstrated that insulin stimulated PMN chemotaxis ability and phagocytosis but it didn't affect ROS production or density of surface receptors such as IR, CD11b, CD15, CD62L and CD89 (36). Similar experiments in monocytes demonstrated a suppressive action of insulin on formyl-methionyl-leucyl-phenylalanine-induced ROS production. Furthermore, again in monocytes, insulin inhibits in a dose-dependent manner the upregulation of tissue factor procoagulant activity. The inhibition is caused by a mechanism that interferes with the regulation of cyclic AMP and intracellular calcium, independently of the PI3K–PKB pathway (37).

RA is an autoimmune inflammatory joint disease often characterized by the presence of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs). Epidemiological studies suggested a direct relationship between diabetes and rheumatoid arthritis especially in female patient (38).

Treg cells mediate tolerance to self-antigens, whereas Th17 cells are involved in the pro-inflammatory reaction to pathogens. In RA and in other autoimmune diseases, the Treg-Th17 equilibrium is altered. Glycolytic pathways and increased glucose consumption lead to a metabolic switch from low-energy to highly active state in RA (39). Insulin and insulin-like growth factors (IGFs) are similar polypeptide hormones. Insulin regulates the use of carbohydrates, while IGF-1 is involved in cell growth, differentiation, and survival. Insulin and IGFs use signaling pathways involving PI3K and Akt or Ras and MAPK, which are also involved in other cellular stimuli (40). RA is characterized by an alteration of IGF-1 axis and its receptor (IGF-1R), which is expressed on chondrocytes, synovial fibroblasts and leukocytes (41–43). In RA patients there is upregulation of the IGF-1R expression on CD4+ T cells compared to healthy controls and in RA patients the IGF-1 levels are lower (44).

Moreover, the proinflammatory adipokine resistin, involved in obesity and diabetes, has a relevant role in the pathogenesis of RA and in the induction of the inflammatory response (45). When resistin is suppressed, the result is reduced expression of IGF-1R and decreased phosphorylation of Akt (42). Resistin modulates Akt-dependent processes and IGF-1R expression in human synovial tissue interfering with IR/IGF-1R signaling.

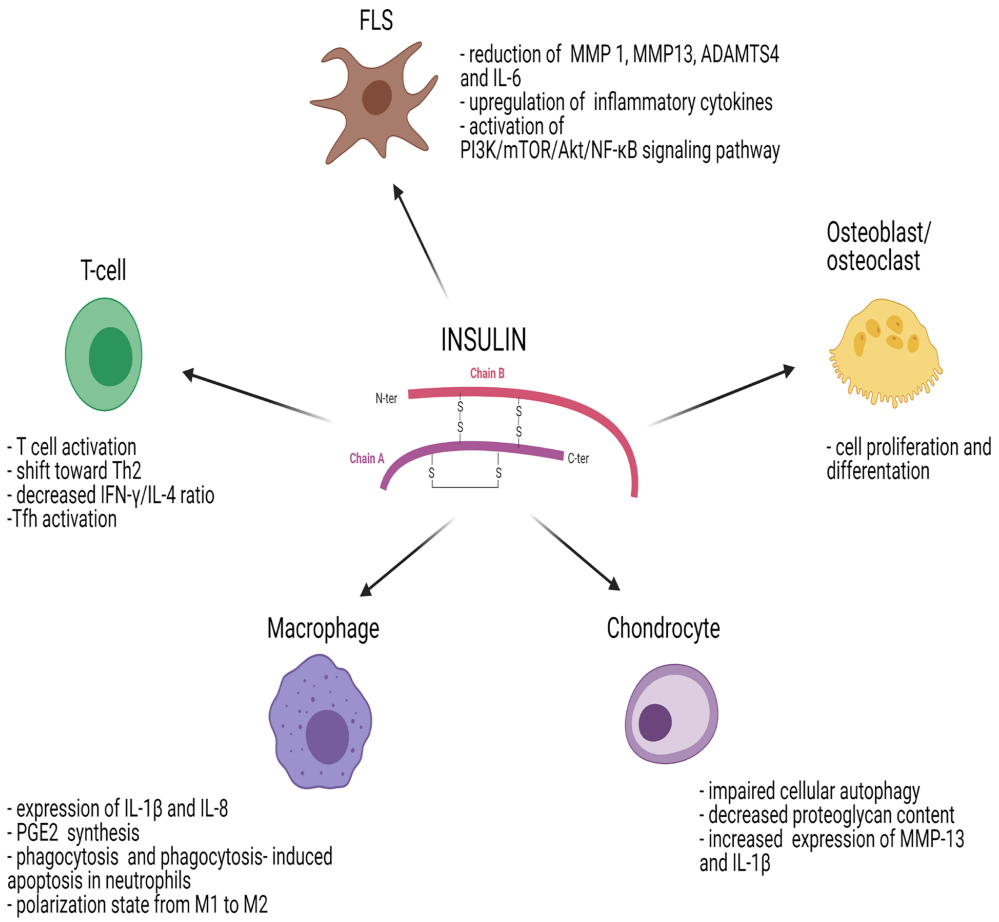

FIGURE 2 | Effects of insulin on immune cells and joint cells. ADAMTS4, metalloproteinase with Thrombospondin Type 1 Motif 4; AKT/PKB, protein-kinase B; FLS, fibroblast-like synoviocyte; IL, interleukin; IFN-γ, interferon gamma; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2, prostaglandin E2; PI3K, phosphoinositide-3-kinase; Tfh, follicular helper T cells; Th2, T helper 2. **Figure 2** has been created using BioRender (www.biorender.com).

Similar to insulin, IGF-1R signaling plays a role in inflammation mediated by T cells in arthritis. IGF-1R has an inhibitory effect on the level of IR substrates, reducing IL6-dependent formation of Th17 cells. The effects of insulin on different immune cells and joint cells are summarized in **Figure 2**.

Insulin Signaling in T Cells

While IR can be detected on the surface of B cells, monocytes and resting neutrophils, it is not expressed on resting T cells (36, 46). However, IR is significantly upregulated on activated T cells (47, 48) and it is essential to meet the large glucose demand that T cells need to acquire full effector functions. Insulin signaling in T cells promotes T cell activation by increasing protein synthesis, glucose uptake and amino acid transport (49). Viardot et al. demonstrated *in vitro* that insulin induced a shift toward T helper type 2 (Th2) response, reducing the T helper type 1 (Th1) to Th2 ratio (**Figure 2**). This resulted in a change of cytokine secretion with decreased interferon-gamma to IL-4 ratio with enhanced phosphorylation of extracellular signal-regulated kinase (ERK) (48), one of the four MAPK signaling pathways.

Experiments on IR knockout mice demonstrated that there was an impairment of polyclonal activation of CD4+ T cells and of cytokine production, migration and proliferation (50). Similar results were observed also in CD8+ T cells showing impaired cytotoxicity in response to alloantigens. Studies on obese patients have outlined that insulin resistance and related disorders are characterized by a cytokine imbalance, with high levels of TNFα, IL-6, IL-1β, CRP, and NF-κB (51). In this regard, Tao et al. found an imbalance between Th17 and regulatory T cells (Treg) in insulin resistance state (52). Th17 and Treg represent two CD4+ T cell subsets that share some developmental elements but express different phenotypes with opposite actions. The former have pro-inflammatory activity and the latter show anti-inflammatory activity (53). An altered balance between Treg and Th17 cells is involved in RA and other immune-mediated conditions (54).

Stimulation of the T cell receptor (TCR) complex and ligation of the co-receptor CD28 by co-stimulatory molecules activate quiescent T cells (55). Engagement of the TCR stimulates intracellular signaling through the ERK/MAPK pathways, while the PI3K-Akt-mTOR axis is activated by CD28 signaling (56, 57).

PI3K-Akt signaling activates glycolysis and increases glucose transporter 1 (Glut1) expression and thus glucose uptake. Overexpression of Glut1 enhances T follicular helper cells (Tfh) differentiation, a T cell subset involved in B cell regulation (58), potentially favoring autoimmunity in both type 1 diabetes and RA (59). Downstream, PI3K-Akt activates mTOR, which promotes the differentiation of Th1, Th17 and Tfh cells (60). Furthermore, mTOR can inhibit the formation of long-lived Tregs favoring effector Tregs (61). Treg knockout for mTOR reduced their frequency, leading to spontaneous effector T cell activation and inflammation (62).

AMP-activated protein kinase (AMPK) can inhibit cellular growth *via* suppression of the mTORC1 pathway (63). AMPK activation and interruption of mTOR signaling mitigated the inflammation in experimental arthritis. AMPK-dependent control of fatty acid metabolism may also impact cell fate decisions in CD4+ T cells, particularly the balance between Th17 and Treg lineages (64).

In addition, growth factors such as insulin, IGF-1 or IL-2 can stimulate PI3K-Akt-mTOR signaling. The roles played by insulin and IGFs are different, but they share the PI3K-AKTmTOR and RAS-RAF-MEK-ERK signaling pathways. Signaling through IGF-1 receptor (IGF1R) activates the Akt-mTOR pathway, increases aerobic glycolysis and ultimately favors Th17 cell differentiation over Treg cells. In experimental models, the inhibition of IGF-1R signaling may improve arthritis by decreasing IL6 production and modifying the balance between Th17 and Treg generation dependent on IL6 (44).

Insulin Signaling in Synoviocytes

Insulin signaling plays an important role in synoviocytes, which express a large number of IRs. In synoviocytes, insulin promotes the inflammatory phenotype of fibroblast-like synoviocytes (FLSs), increases cell viability, promotes production of inflammatory cytokines and chemokines and facilitates chemotaxis of macrophages, leading to synovial membrane inflammation (**Figure 2**). In synovial tissue isolated from patients with OA, a condition with persistent low-grade inflammation, and T2D, there was phosphorylation of Akt and reduced autophosphorylation of the IR induced by insulin. In OA patients with T2D, insulin resistance may develop not only in insulin-sensitive tissues such as muscle, liver or fat, but also in the synovial membrane. Furthermore, Hamada et al. demonstrated that insulin markedly reduced TNFα-stimulated production of matrix metalloproteinase (MMP) 1, MMP13, ADAMTS4, BMP2 and IL-6 in nondiabetic human FLSs without reducing TNFα itself, implicating a pivotal role of insulin in the inhibition of synovial inflammation (6, 65).

Qiao et al. showed that, in FLSs, insulin activates the PI3K/mTOR/Akt/NF-κB signaling pathway and inhibits autophagy. Insulin can also upregulate inflammatory cytokine receptors whereas PI3K/mTOR/Akt/NF-κB signaling inhibitors can reverse this process in FLSs (66).

Insulin Signaling in Osteoblasts and Osteoclasts

Previous studies supported the role of insulin signaling in the biology and pathology of the joint (67), mainly through its capability to control bone architecture acting on osteoblasts and osteoclasts (68–73) (**Figure 2**). In vitro experiments demonstrated that insulin upregulated IR expression. Moreover, through MAPK and PI3K pathway, insulin stimulated cell proliferation and differentiation by increasing alkaline phosphatase activity, secretion of type I collagen and expression of osteocalcin in MG-63 cells (69). In IR knockout mice, increased expression of osteoprotegerin in osteoblasts and inhibited osteoclastogenesis and osteoclastic activity were observed (74, 75). The activation of mTORC1 by IGF-1, which is released in the bone resorption phase, stimulates osteoblast differentiation of mouse bone marrow stromal cells (BMSC) (76). mTORC1 is in fact required for the transition of pre-osteoblasts to mature osteoblasts (77).

However, insulin acts also on osteoclasts. Through the ERK1/2 pathway, insulin induces the upregulation of receptor activator of nuclear factor-κB (RANK) contributing to the enhancement of osteoclast differentiation by RANKL (78). The effects of mTORC1 on osteoclasts have not been completely elucidated. In osteoclast precursors, the deletion of *raptor* leading to inactivation of mTORC1, or the activation of mTORC1 by deletion of tuberous sclerosis complex 1 (Tsc1), could respectively increase or reduce osteoclastogenesis. Mechanistically, this was due to mTORC1 inhibition of NF-kB and nuclear factor of activated T Cells 1 (NFATc1), both critical transcription factors of osteoclastogenesis (79). Another study shows how RANK ligand (RANKL)-dependent osteoclastogenesis is impaired in Tsc1-deficient bone marrow macrophages, where TSC1 is a negative regulator of mTORC1 (80).

Dai et al. suggested that in bone marrow macrophages, inhibition of mTORC1 by treatment with rapamycin or by genetic deletion, suppressed *in vitro* osteoclast differentiation rescued by upregulation of mTOR downstream target S6K159 (81). Collectively, these studies outline how the insulin/mTOR pathway plays a role in bone biology, however further investigation is needed to properly dissect its anabolic and catabolic role.

Insulin Signaling in Chondrocytes

Insulin resistance and hyperinsulinemia were shown to be involved in the pathogenesis of OA and metabolic syndrome (82, 83) (**Figure 2**). In human chondrocytes, insulin increases the mTOR signaling pathway and Akt phosphorylation in a dose-dependent manner, leading to impaired cellular autophagy, an important mechanism regulating the removal and degradation of damaged intracellular products (84). Treatment with rapamycin, an mTOR inhibitor, reversed the effects of insulin on autophagy activity and beneficial effects on cartilage integrity were observed (85). Furthermore, insulin reduced the content of proteoglycans and upregulated MMP-13 and IL-1β, which have a significant role in chondrocytes and in cartilage degradation (84, 86).

Insulin Signaling in Macrophages

During insulin resistance state, Akt signaling is impaired leading to hyperactivation of mTORC1 and increased glycolysis. In macrophages, increased glycolysis affects responses to pathogens and danger signals (87). Insulin significantly

enhances the LPS-dependent expression of IL-1β and IL-8 and the induction of enzymes involved in the prostaglandin E2 (PGE2) synthesis by macrophages (88) (**Figure 2**). In vivo and *in vitro* studies suggested that insulin re-established phagocytosis and fostered phagocytosis-induced apoptosis in neutrophils. Furthermore, insulin treatment induced macrophages to change their polarization state from M1 to M2 (87).

Insulin Resistance and Arthritis

Prevalence of insulin resistance is increased in RA patients (89–92) and it is correlated with disease activity and disease-specific factors such as chronic systemic inflammation and use of glucocorticoids which may cause dysfunction of pancreatic β cells (90, 93, 94). Indeed, it has been hypothesized that the glucose intolerance observed in RA is contributed by the inefficacy of β cells to compensate for insulin resistance (93) and that, in β cells of non-diabetic RA patients not receiving glucocorticoids, there is an impairment of proinsulin to insulin processing possibly explained by the sustained pro-inflammatory state (95).

Furthermore, in OA, it has been suggested that pathophysiological mechanisms similar to those observed in T2D are present and insulin resistance-related traits might have a role in the development of the disease (96).

GLUCOSE-LOWERING AGENTS AND ARTHRITIS

Increasing evidence suggests that glucose-lowering agents exert a number of anti-inflammatory activities (97). The anti-arthritis effects of metformin, thiazolidinediones (TZDs), dipeptidyl peptidase-4 (DPP-4) inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists were investigated in several studies and are summarized in **Table 1**. Currently, no data exist about the sodium-glucose cotransporter type 2 (SGLT2) inhibitors.

Metformin

Metformin represents the first line treatment of T2D and insulin resistance states. Besides its anti-hyperglycemic effects, metformin has antiproliferative, antifibrotic, and antioxidant potential (98, 99) (**Table 1**). The main mediator of the anti-inflammatory properties of metformin is AMPK activation, which controls inflammation and immunity through a variety of mechanisms (100). The anti-inflammatory effects of metformin are also independent from AMPK. Indeed, metformin is a potent inhibitor of mitochondrial respiratory chain complex I (NADH: ubiquinone oxidoreductase) (101, 102) which is implicated in the production of ROS (103).

TABLE 1 | Effects of glucose-lowering agents on arthritis.

Metformin
- antiproliferative, antifibrotic, and antioxidant potential
- ↓ Th17 cells and ↓ proinflammatory cytokines
- inhibition of mitochondrial respiratory chain complex I and ↓ ROS
- ↓ STAT3 phosphorylation *via* AMPK/mTOR pathway and ↓ Th17 differentiation
- phosphorylation of AMPK
- in synovial fibroblasts ↑ glycolytic activity and ↓ IL 6, IL 8 and monocyte chemotactic protein 1
- in osteoclasts ↓ osteoclastogenesis by the AMPK-mediated inhibition of mTOR
- in macrophages ↓ of TNFα, IL-6, and MCP-1; ↑ release of IL-10
- ↓ inflammatory cytokines by suppressing NF-κB pathway

Thiazolidinediones
- anti-inflammatory activity, immuno-modulation, antioxidant effect
- in macrophages ↓ proliferation
- in T-cells ↑ immunosuppressive effects
- ↓ production of IL-17; ↓ mRNA expression levels of inflammatory mediators; ↓ levels of MMPs
- in synoviocytes/synovial fibroblast ↓ growth and ↓ IL 1β induced PGE2 synthesis
- maintained expression of aggrecan and type II collagen
- ↓ inflammatory cell infiltration, ↓ pannus formation, ↓ cartilage/bone damage
- ↓ TNFα, IL-1β, MCP-1 and RANKL mRNA, ↓ osteoclasts differentiation
- in chondrocytes ↓ NO synthase expression, ↓ IL-1β and MMP-13
- in chondrocytes/synovial fibroblasts ↓ COX-2 expression and PGE2 production
- ↓ NF-κB pathway

Dipeptidyl peptidase-4 inhibitors
- ↓ proliferation of T cells
- ↓ anti-CCP, RANKL, TNFα and IL-6 by ↓TLR/NF-κB pathway
- action on cytokine secretion, T cell-dependent antibody production and immunoglobulin isotype switching of B cells
- in chondrocytes ↓ degradation of type II collagen by MMP-1, MMP-3, and MMP-13
- ↓ oxidative stress
- ↓ ADAMTS-4 and ADAMTS-5 leading to ↓ degradation of aggrecan
- ↓ p38 MAPK signaling pathway and ↓ NF-κB

Glucagon-like peptide 1 analogues
- in FLSs ↓ TNFα, IL-6, IL-8, IL-1β, MMP-3, MMP-13, HMGB-1, MCP-1, p38/MAPK and NF-κB pathways
- in FLSs improved oxidative stress and prevented cell death
- in chondrocytes ↑ anti-apoptotic marker Bcl-2 and ↓ apoptotic proteins active caspase 3 and Bax
- in chondrocytes ↑ deterioration of type II collagen and aggrecan

Metformin was found to decrease IL-1β and to boost IL-10 as well as to inhibit ROS production in LPS-activated murine macrophages in an AMPK independent manner (103).

In a mouse model of autoimmune arthritis, metformin downregulated Th17 cells decreasing proinflammatory cytokines and inhibiting the differentiation of Th17 differentiation through inhibition of STAT3 phosphorylation (104).

The phosphorylation of AMPK induced by metformin may partially improve synovial inflammation in RA. Under physiologic conditions, phosphorylation of AMPK reconstitutes cell stores of ATP, generating new ATP and inhibiting the inflammatory pathways, which are energy-expensive (105, 106). In RA synovial fibroblasts, metformin increases glycolytic activity and decreases oxidative phosphorylation and generation of IL-6, IL-8 and monocyte chemotactic protein 1. Metformin can also suppress the differentiation of osteoclasts and the AMPK-mediated inhibition of mTOR negatively regulates osteoclastogenesis (107). In vitro, metformin can act on macrophages to inhibit the release of TNFα, IL-6, and MCP-1 while enhancing IL-10. In vivo, metformin can reduce inflammatory cytokine production leading to clinical improvement of arthritis. These effects were exerted by correcting the impaired autophagic flux and selectively degrading IκB kinase causing suppression of NF-κB-mediated signaling (108).

Thiazolidinediones

TZDs act as insulin sensitizing agents in liver, fat and skeletal muscle cells, through the activation of the nuclear peroxisome proliferator-activated receptor γ (PPARγ). When PPAR-γ is activated, insulin-responsive genes controlling glucose and lipid metabolism are transcribed. Similar to metformin, also TZDs have pleiotropic properties including anti-inflammatory activity, immuno-modulation and antioxidant effects (109–114) (**Table 1**). From a molecular point of view, the anti-inflammatory action is exerted through the inhibition of NF-κB signal pathway (115). PPARγ acts as an E3 ubiquitin ligase, physically interacting with NF-κB p65 subunit to induce its ubiquitination and degradation thus limiting pro-inflammatory cytokine production (116).

Interestingly, PPAR-γ ligands seem to have an immunomodulatory role on monocytes and macrophages. Pioglitazone suppressed macrophage proliferation without inducing apoptosis (117, 118). Furthermore, PPARγ ligation induces T-cell immunosuppressive effects (118, 119).

The role of pioglitazone has been investigated in models of IL-17-induced human intervertebral disc degeneration. Its administration reduced the levels of inflammatory cytokines such as IL-17, and downregulated mRNA expression of inflammatory mediators. Furthermore, pioglitazone suppressed MMPs and preserved the expression of the extracellular matrix molecules aggrecan and type II collagen (120).

Anti-inflammatory effects of TZDs were demonstrated also in models of RA. In vitro, the growth of RA synoviocytes is inhibited by PPAR-γ ligands which also downregulate, in RA synovial fibroblasts, the synthesis of PGE2 mediated by IL-1β (121). Indeed, available evidence suggests that synthetic PPARγ agonists such as rosiglitazone and troglitazone, but also the natural PPARγ ligand 15-deoxy-Delta (12,14)-prostaglandin J2, can improve arthritis in murine models (122, 123).

In another set of experiments, it has been suggested that using methotrexate in combination with pioglitazone may have a synergistic effect in RA combining inhibition of inflammatory cytokines TNFα, IL-1β and prevention of the activation of ROS (124, 125).

Tomita et al. explored the effects of THR0921, a PPAR-γ ligand, in mice models of collagen-induced arthritis. Compared with normal mice, those treated with THR0921 had milder synovial hyperplasia, with no pannus formation, lower degree of inflammatory cell infiltration and modest damage to cartilage and bone (126). Furthermore, where joint damage was observed, the expression levels of TNFα, IL-1β, MCP-1 and RANKL mRNA were reduced. Koufany et al. explored the role of oral treatment with pioglitazone and rosiglitazone in murine models with adjuvant-induced arthritis, observing decreased expression of IL-1β and TNFα in inflamed synovial tissue (127). Treatment with PPAR-γ ligands inhibits nitric oxide synthase expression induced by IL-1β in OA patients' chondrocytes. PPAR-γ ligands reduced IL-1β and MMP-13 production in a dose-dependent manner. The inhibitory effect of PPAR-γ activation was also demonstrated on the generation of nitric oxide and MMP-13 induced by TNFα and IL-17 (128). Finally, in human chondrocytes and synovial fibroblasts, the activation of PPAR-γ decreases IL-1β induced COX-2 expression and production of PGE2 (129). Since high levels of COX-2-induced prostaglandins are associated with the generation of free radicals and lipid peroxide (130), pioglitazone exerts antioxidative and anti-inflammatory effects also through modulation of COX-2. Moreover, PPAR-γ agonists can inhibit the NF-κB pathway, having an anti-arthritic role (109).

Dipeptidyl Peptidase-4 Inhibitors

DPP-4 inhibitors have been widely used in T2D. They act by reducing the degradation of the incretin hormones inhibiting the DPP-4 enzymes (**Table 1**). Incretins lead to improved glycemic control by delaying satiety, favoring the release of insulin, inhibiting the production of glucagon and preserving β-cell mass. DPP-4 can modify the functioning of immune system and also disease pathogenesis interfering with mechanisms of T cells development and migration but also with cytokine secretion, T cell-dependent antibody production and immunoglobulin isotype switching (131, 132). In large, prospective, population-based cohort studies, the risk of developing an autoimmune disease was lower in patients receiving antidiabetic therapy with DPP-4 inhibitors compared to T2D patients not treated with DPP-4 inhibitors (133, 134). In a series of pioneering studies conducted *in vivo*, Tanaka et al. demonstrated that DPP-4 inhibitors suppressed inflammation in two murine models of collagen-induced and alkyldiamine-induced arthritis, with pathological characteristics similar to RA. In particular, the inhibition of DPP-4 reduced mitogen-induced and antigen-induced proliferation of T cells (135, 136).

Ibrahim et al. (137) evaluated the effects of a combination therapy with sitagliptin and tofacitinib on JAK/STAT and TLR-4/NF-κB pathways in murine models of adjuvant-induced arthritis. The separate administration of both tofacitinib and sitagliptin resulted in a reduction of anti-CCP, RANKL, TNFα and IL-6 compared to untreated mice, but the combination of both drugs produced a more significant decrease of the serological markers compared to each therapy alone (137).

Hu et al. demonstrated the anti-inflammatory effects of saxagliptin on articular chondrocytes exposed to AGEs. Saxagliptin reduced the expression of mRNA of enzymes such as MMP-1, MMP-3 and MMP-13, which are involved in type II collagen degradation. Furthermore, it significantly inhibited expression of ADAMTS-4 and 5, resulting in less aggrecan degradation. Oxidative stress was reduced by the inhibition of DPP-4 by inhibiting ROS generation and increasing levels of glutathione. Exposure to AGEs activated the p38 MAPK signaling pathway and increased the degradation of IκBα, upregulating NF-κB. In OA, saxagliptin was able to affect this pro-inflammatory process (138).

Glucagon-Like Peptide 1 Analogues

GLP-1 is an incretin mimetic hormone exerting different effects on glucose metabolism (**Table 1**). It can increase the secretion of insulin induced by glucose, delay gastric emptying and stimulate satiety. Moreover, GLP-1 plays a diuretic role and modulates the proliferation of β-cells (139). Growing evidence suggests that gut hormones act as key signals in regulating the interplay between the metabolic axis and the immune system and may also be involved in the response to immunomodulatory therapy for RA (140). In RA patients, the incretin-insulin axis and the incretin effect are impaired (64). The first study exploring the effects of GLP-1 analogues on the pathological characteristics of RA in human FLSs was conducted using lixisenatide. Lixisenatide downregulated TNFα, IL-6, IL-8 and MMPs, inhibiting the inflammatory response through blockage of cellular signaling pathways such as c-Jun N-terminal kinase (JNK), activator protein 1 (AP-1) and NF-κB. Moreover, treatment with lixisenatide caused a reduction in oxidative stress and prevented cell death in FLSs (141).

Chen et al. explored the role of GLP-1 receptor (GLP-1R) in OA demonstrating that liraglutide could protect chondrocyte apoptosis and extracellular membrane degradation by regulating endoplasmic reticulum stress. Liraglutide upregulated the anti-apoptotic marker Bcl-2 and diminished the expression of apoptotic proteins active caspase 3 and Bax (142). The activation of GLP-1R/PI3K/Akt signaling by GLP-1 analogues is involved in various metabolic processes. The inhibition of PI3K/Akt signaling impaired the protective effects of GLP-1R increasing apoptotic activity and endoplasmic reticulum stress. The activation of GLP-1R inhibited the NF-κB pathway thus decreasing the release of inflammatory mediators. The same results were obtained also in a model of knee OA (142).

Tao et al. studied the role in RA pathogenesis of GLP-1R on human FLSs using the selective GLP-1 agonist exenatide. FLSs were exposed to TNFα in the presence or absence of exenatide. Exenatide treatment significantly reduced expression of IL-1β, IL-6, MMP-3, MMP-13 and MCP-1. Moreover, by preventing IκBα degradation, the treatment inhibited activation of the p38/MAPK and NF-κB pathways (143).

Similar results were observed in RA human FLSs incubated with TNF in presence of dulaglutide. Dulaglutide treatment significantly downregulated proinflammatory mediators such as IL-1β, IL-6, MCP-1, HMGB-1, MMP-3 and MMP-13. The effects of dulaglutide were mediated by the inactivation of JNK and increased phosphorylation of IκBα, causing a reduction of NF-κB (144).

The inhibitory effect of dulaglutide on OA-related cytokines and chemokines was demonstrated also in chondrocytes treated with AGEs. In chondrocytes, the AGEs-mediated deterioration of articular extracellular matrix components, such as type II collagen and aggrecan, was reduced by treatment with dulaglutide through inhibition of MMP-3 and MMP-13 (145).

EFFECTS OF DISEASE-MODIFYING ANTIRHEUMATIC DRUGS ON GLUCOSE METABOLISM

Compared with the general population, insulin resistance is more prevalent in patients affected by RA (7, 90, 92, 146, 147). Insulin resistance is influenced by both inflammation-related and metabolic factors (95, 147–149) but, in RA patients, it can be modified by the use of anti-rheumatic drugs. The cytokines involved in the pathogenesis of RA, in particular TNFα, IL-1 and IL-6, also promote the development of insulin resistance. The topic has been extensively reviewed elsewhere (7, 9, 150) but, briefly, by acting through IRS-1, TNF-α reduces IR tyrosine kinase activity and induces serine phosphorylation leading to inhibition of IR in skeletal muscle cells and adipocytes (151, 152). Anti-TNFα agents improve insulin sensitivity and decrease insulin resistance in RA patients, also reducing the risk of developing T2D (153–157). Moreover, similar effects on the decrease of insulin resistance were shown in RA patients treated with IL-6 antagonists (158–160), anti-IL-1 agents (161, 162) or T-cell costimulation blockade (163). In summary, the introduction of disease-modifying anti-rheumatic drugs can control inflammation and exert beneficial effects on insulin resistance and insulin sensitivity in RA patients, potentially reducing the risk of developing T2D in non-diabetic individuals or aiding in the achievement of better glucose control in diabetics.

CONCLUSIONS

In summary, through its integrated signaling network, insulin regulates intracellular and intercellular pathways in immune cells, in cartilage and in synovial tissue, behaving as a crucial modulator of the inflammatory response observed in arthritis. Finally, robust *in vitro* and *in vivo* evidence outlines the

effects of glucose-lowering therapies in arthritis. Metformin, TZDs, DPP-4 inhibitors and GLP-1 analogues may exert an immunomodulatory action downregulating the expression of proinflammatory cytokines and chemokines, thus reducing synovial inflammation and potentially leading to improvement of arthritis.

AUTHOR CONTRIBUTIONS

CT, CB, RG, RM, and FU contributed to conception of the review. CT, JC, VP, PR, and NV performed the literature search and wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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B Cell Metabolism and Autophagy in Autoimmunity

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B cells are central to the pathogenesis of multiple autoimmune diseases, through antigen presentation, cytokine secretion, and the production of autoantibodies. During development and differentiation, B cells undergo drastic changes in their physiology. It is emerging that these are accompanied by equally significant shifts in metabolic phenotype, which may themselves also drive and enforce the functional properties of the cell. The dysfunction of B cells during autoimmunity is characterised by the breaching of tolerogenic checkpoints, and there is developing evidence that the metabolic state of B cells may contribute to this. Determining the metabolic phenotype of B cells in autoimmunity is an area of active study, and is important because intervention by metabolism-altering therapeutic approaches may represent an attractive treatment target.

Keywords: autophagy, metabolism, autoimmunity, B cell, SLE - systemic lupus erythematosus, B cell development and differentiation

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INTRODUCTION

B lymphocytes play a crucial role in immune responses against pathogens and tumours through the production of protective antibodies. They are also implicated in the development and control of autoimmunity, in which the immune response is directed towards self-antigens. Various B cell populations display the capacity for both protective and self-destructive activity. The best understood B lymphocytes are conventional bone marrow-derived B2 cells, which mediate adaptive humoral responses. In various autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), B2 cells are responsible for autoantibody production, the presentation of endogenous peptides to self-reactive T cells, and the secretion of proinflammatory cytokines (1–5). Additional to B2 cells are a distinct population of innate–like antibody–secreting lymphocytes, known as B1 cells. Generated in the foetal liver, B1 cells are found with the peritoneum and are capable of resting self–renewal. B1 cells produce natural IgM against bacterial polysaccharide antigens and exhibit a significant degree of self–reactivity (6, 7). The autoantibodies and cytokines produced by B1 cells can mediate autoimmunity (8, 9). However, B1 cell–derived autoantibodies also facilitate the clearance of apoptotic cells, a major source of autoantigens, and appear to promote intestinal homeostasis (10–12). Meanwhile, regulatory B cells (B_{regs}) play a well–defined role in tolerogenesis, secreting immunoregulatory cytokines such as IL–10 and TGF–β, and enacting contact–dependent suppression of self–reactive lymphocytes (13).

The causes of B cell dysfunction in autoimmunity remain incompletely defined. Metabolism, although a well–established regulator of cellular activity, has only relatively recently been appreciated as a determinant of B cell function in health and disease. Physiologically, metabolism enables proper B cell development, differentiation and antibody secretion (14, 15). Unsurprisingly,

the contrasting phenotypes of B cells at different stages of development and maturity are reflected in significant variations in metabolic activity (16, 17). Moreover, different B cell subsets, particularly B1 and B2 cells, utilise divergent metabolic pathways (18). Metabolism forms an important component of deletional and anergic checkpoints against autoimmunity, and metabolic dysregulation is associated with the escape from tolerogenic checkpoints and the enhanced functionality of self–reactive B cells (19–22). As well as traditional aspects of cellular metabolism, B cells depend on autophagy, a mechanism of degradative processing, principally of damaged cellular components. Autophagy maintains metabolic homeostasis during nutrient deprivation and supports long–term plasma cell (PC) viability (23). Pathologically, autophagy appears to support self–reactive B cells in subverting autoimmune checkpoints, to undergo activation by innate immune signals, and present autoantigens to T lymphocytes (24–26).

In this review, we describe B cell metabolism and autophagy in health, highlighting their roles in immune homeostasis. We then discuss the metabolic and autophagic abnormalities seen in autoimmune B cells, and how these may promote self–destructive responses. Finally, the potential of B cell metabolism and autophagy as therapeutic targets in autoimmunity will be explored. This review focuses primarily on conventional B2 cells, which are relatively well defined metabolically, although comparisons are made to B1 cells and B_{regs} where possible.

METABOLISM AND AUTOPHAGY IN B CELL DEVELOPMENT

The development of conventional B2 B cells must generate a vast and hugely diverse repertoire of cells capable of antibody secretion, whilst purging cells with self–reactive antigen specificities. Moreover, rapid growth and proliferation must be achieved in the metabolically challenging environment of the bone marrow, requiring the careful balancing of anabolic and catabolic signalling. The former is largely mediated by c–Myc and mitochondrial target of rapamycin complex (mTORC) signalling, which fuel protein synthesis and cell growth by upregulating glycolysis and oxidative phosphorylation (OXPHOS) (14, 27, 28). mTORC signalling plays a crucial role in successful B cell development (27, 28). In mice, deletion of the mTORC1–associated protein Raptor prevents the interleukin (IL)–7–driven development of pro–B cells (14, 29). Without mTORC1 signalling, pro–B cells are less able to transition into pre–B cells, the precursors in which the pre–B cell receptor (BCR), consisting of mature immunoglobulin heavy chains and surrogate light chains, is expressed (14, 29). Unlike mTORC1, the role of mTORC2 in early B cell development has been contested, although it appears to facilitate peripheral B cell maturation (14, 30). Specifically, mTORC2 has been implicated in regulating mTORC1 and c–Myc activity during the terminal stages of B cell development (31).

While mTORC signalling is important during B cell development, excessive anabolic activity is detrimental. Indeed, B cell development is compromised at the large pre–B cell stage following the deletion of Fnip1 (32). Fnip1 interacts with 5' adenosine monophosphate–activated protein kinase (AMPK), an energy stress sensor and catabolic regulator which opposes mTORC1. Although AMPK can promote catabolism in the absence of Fnip1, its ability to inhibit mTORC signalling is impaired (32). The metabolic stress resulting from unrestrained anabolism renders pre–B cells more vulnerable to apoptosis following pre–BCR cross–linking (32). The concept of metabolism as a regulator of cell death, controlling B cell precursor viability in response to antigen stimulation, has implications for tolerogenic checkpoints against autoimmunity (33).

The large pre–B cell stage, during which the pre–BCR is expressed on the cell surface and tested for affinity towards self–antigens in the bone marrow, represents both an autoimmune checkpoint and a period of metabolic vulnerability (34). Although its necessity during B cell development is controversial, glucose metabolism represents one example of this vulnerability (20, 32, 35). The rapid proliferation of large pre–B cells is believed to be sustained through upregulated glucose metabolism, given that large pre–B cells import more glucose than other precursor populations (16, 35). Large pre–B cells experience significant oxidative stress and are vulnerable to glycolytic inhibition, which impairs their transition into small pre–B cells (16, 35). Signalling through an autoreactive pre–BCR drives hyperactivation of the phosphoinositide 3–kinase (PI3K)–protein kinase B (Akt)–mTORC1 pathway, with the resulting metabolic stress inducing negative selection (19). Activation of a non–autoreactive pre–BCR does not affect Akt activation or viability in pre–B or leukemic pre–B (pre–B ALL) cells (19, 36). In contrast, activation of an autoreactive pre–BCR induces rapid, Akt–dependent cell death (19). In pre–B ALL cells, deletion of the PI3K inhibitor phosphatase and tensin homologue (PTEN) increases glycolytic flux, although elevated anabolism results in ATP depletion and cell death (19). These changes are reversed by the mTORC1 inhibitor rapamycin, suggesting that hyperactivation of the PI3K–Akt–mTORC1 pathway downstream of an autoreactive pre–BCR results in an energy crisis (19). PTEN plays a vital role in the development of pro–B cells, reducing their susceptibility to apoptosis (14). While PTEN deletion affects non–metabolic features of B cell precursors, such as B lymphoid transcription factor expression, these results further highlight the importance of balanced metabolic programmes during B cell development (14).

Alongside controlling other aspects of development, B lymphoid transcriptional factors themselves impose metabolic restriction upon B cell precursors, perhaps to enable hyperactivation–induced cell death (37). Mutations in the transcription factors *PAX5* and *IKZF1* are commonly seen in acute lymphoblastic leukaemia, suggesting that their expression may confer a selective disadvantage (37). The inducible reconstitution of *PAX5* and *IKZF1* in pre–B ALL cells reduces glucose uptake and ATP synthesis, promoting cell death (37). Notably, the B cell–specific expression of a non–functional *IKZF1* predisposes mice to the development of autoimmunity, supporting the idea that these transcription factors may play a tolerogenic role during B cell development (38). Metabolic

restriction is also a prominent feature of B cell anergy. Along with apoptosis and receptor editing, tolerogenesis can be exerted on self–reactive B cells and their precursors through the induction of anergy, rendering B cells hyporesponsive to antigenic stimulation. Anergy is a major mechanism of tolerising early transitional B cells following egress from the bone marrow. Anergic B cells are characterised by suppressed PI3K signalling and impaired metabolic reprogramming in response to BCR or Toll–like receptor (TLR) 4 stimulation (20, 39). Presumably, metabolic suppression increases the activation threshold of anergic self–reactive B cells.

Given its role in promoting metabolic homeostasis, the role of autophagy in B2 cell development has been explored (**Figure 1**) (40). Reconstitution of the foetal livers of *Rag1*$^{-/-}$ mice with cells lacking the key autophagy gene *Atg5* demonstrates a developmental block at the pre–B cell stage (41). When *Atg5* deletion was restricted to mature B cells, splenic and lymph node B cell populations were unaffected, implying that autophagy is necessary for the development, but not peripheral maintenance, of B2 cells (41). However, pro– to pre–B cell transition occurs in the absence of *Atg5* expression following conditional deletion using Cd79a–cre (40). In contrast, autophagy was necessary to maintain populations of mature B cells in the periphery (40). That autophagy may be dispensable in B2 cell precursors is perhaps unsurprising, given the crucial developmental role of the autophagy inhibitor mTORC1 (14).

In contrast to B2 cell development in bone marrow, innate–like B1 cells develop in the foetal liver before migrating principally to the peritoneum and pleura (42). Given that B1 cells occupy different niches and rely upon self–renewal for population maintenance, their metabolic phenotype unsurprisingly differs from that of conventional B2 cells (42). Compared to follicular B2 cells, peritoneal B1 cells are characterised by greater glucose uptake, higher rates of glycolysis and OXPHOS and heightened sensitivity to glycolytic inhibition (18). Mechanistically, elevated glucose metabolism is likely driven by high levels of c–Myc expression (18, 43). In keeping with their localisation in the lipid–rich environment of the peritoneum, B1a cells extensively acquire exogenous fatty acids, while also utilising endogenous fatty acid synthesis (18).

Autophagy has also been explored in B1 cells (**Figure 1**). The loss of autophagic flux in mature B cells leads to significant depletion of B1a, though not B1b, cells (41). The specific role of autophagy in B1 cell development has only recently been explored (18). In mice, the B1 progenitor population is unaffected by the absence of autophagy, suggesting that autophagy is not required for B1 cell development (18). This conclusion is supported by temporal changes in the murine B1a cell population in the absence of autophagy. While the B1a cell compartment is normal at two weeks of age, by 12 weeks it is dramatically smaller than in wildtype mice (18). Together, these results suggest that autophagy is needed for peripheral self–renewal but not the differentiation of B1a cells. In peritoneal B1a cells, autophagy appears to be important for controlling the expression of metabolic genes, fatty acid uptake and degradation of lipid droplets (lipophagy) (18).

B CELL METABOLISM FOLLOWING ACTIVATION

In the periphery, naïve B cells are maintained in a state of metabolic quiescence, which likely promotes their long–term

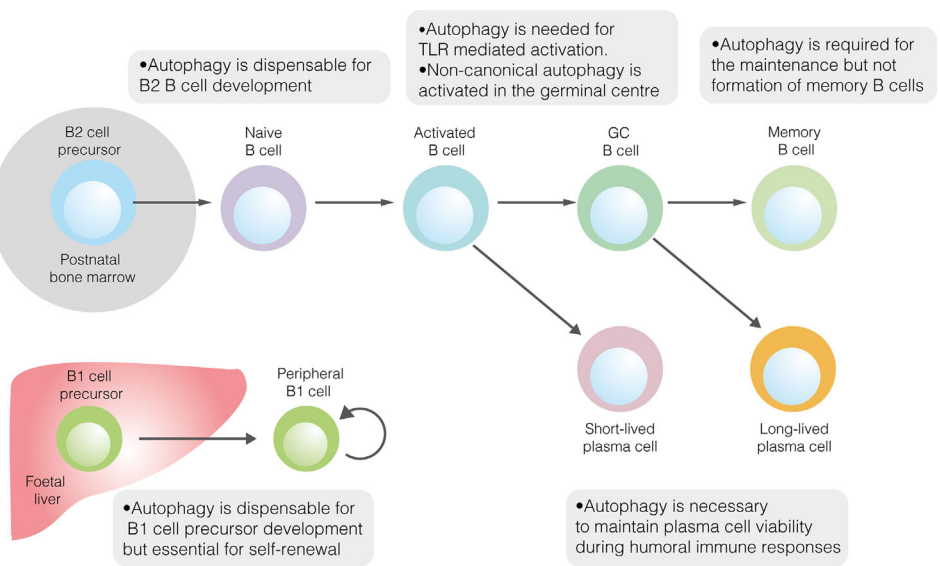

FIGURE 1 | The role of autophagy in developmental and mature B cells. Autophagy performs homeostatic roles in various B cell subsets. It is dispensable for the development of both B1 and B2 cells but is required for the peripheral self–renewal of B1 cells. Regarding B2 cells, autophagy is necessary for TLR–mediated, but not antigen–driven, activation. Autophagy also plays important roles in sustaining plasma cell immunoglobulin production and ensuring memory B cell survival.

viability (44). The maturation of transitional B cells, which egress from the bone marrow and are peripherally tolerised, to follicular cells, is associated with a reduction in OXPHOS, glycolysis and protein synthesis (44). An increased ratio of AMPK: mTORC1 activity suppresses anabolism and exposure to oxidative stress (44). However, the survival of naïve B cells requires some degree of metabolic activity. Their maintenance depends on tonic signalling *via* the BCR and B cell activating factor (BAFF) receptor, both of which activate PI3K (45, 46). Notably, BAFF signalling upregulates the expression of enzymes involved in glucose metabolism (46). IL–4, another extrinsic signal required by naïve B cells, upregulates glucose uptake and glycolysis in murine B cells in a PI3K–independent manner (47). Although these signals promote glucose metabolism, naïve B cells appear to rely heavily on fatty acid oxidation to generate ATP (20).

During B cell activation, this relatively inactive metabolic state is rapidly reversed, enabling growth and proliferation. Stimulation of murine B cells *via* their BCR increases mitochondrial mass and PI3K–dependent glucose uptake (20, 48). In mice, elevated glucose import is enabled by Glut1 upregulation, although human B cells express relatively little Glut1 and may rely on other transporters (15, 48). However, the nature of glucose metabolism in activated B cells has been contested. Activation *via* TLR4 or the BCR had been thought to upregulate both glycolysis and OXPHOS in a balanced manner, with only the former considered essential for successful antibody responses (20, 48, 49). A recent analysis, combining RNA sequencing and stable isotope tracing, has instead suggested that mitochondrial metabolism, but not glycolysis, is upregulated during B cell activation (17). While glucose did flux through glycolysis, much was diverted towards the pentose phosphate pathway, providing substrates for nucleotide synthesis and the management of oxidative stress (17, 48). While excessive oxidative stress compromises cell viability, reactive oxygen species (ROS) represent important signalling molecules in B cells. ROS levels, determined in part by mTORC1 activity, provide instructive signals during differentiation through the regulation of haem synthesis (50, 51). As well as feeding the pentose phosphate pathway, imported glucose is used for *de novo* lipogenesis (17, 52). Fatty acid oxidation, which is extensively utilised by naïve B cells, is downregulated following activation (20). Instead, glutamine appears to represent a major substrate for mitochondrial respiration following B cell activation (17). In activated B cells, mitochondrial respiratory capacity and homeostasis are maintained by AMPK (53).

Successful antigen–driven B cell activation requires T cell costimulation, creating a checkpoint against autoimmunity. T cells are subject to stringent tolerogenesis and play an important role in preventing the activation of self–reactive B cells which have escaped other tolerance mechanisms. Metabolism contributes to the function of this post–activation checkpoint (54). In murine B cells, metabolic activation following anti–IgM stimulation is not sufficiently supported by increased mitochondrial biogenesis or glucose uptake (54). This results in ROS accumulation and apoptosis driven by mitochondrial dysfunction, which is averted by the provision of T cell help (54). Overall, antigen engagement appears to create a brief window within which B cells must obtain costimulation to avoid activation–induced cell death.

Following B cell activation, antibody–secreting cells are generated through PC differentiation. The vast quantity of immunoglobulin secreted by PCs necessitates an increase in protein production capacity. During PC differentiation, the transcription factors Blimp1 and Xbp1 mediate substantial expansion of the endoplasmic reticulum (ER) (55). However, high levels of immunoglobulin production result in the accumulation of misfolded proteins and ER stress. To maintaining metabolic homeostasis, PCs utilise both antioxidant responses and the unfolded protein response (UPR), which limits mRNA translation while enhancing protein folding capacity and the ability of the ER to degrade misfolded proteins (56, 57). While Xbp1 and Blimp1 control the UPR in PCs, it has recently been shown that mTORC1 mediates a predictive UPR, which precedes antibody secretion (58, 59). Misfolded proteins are also degraded *via* the proteasome. Surprisingly, proteasome capacity decreases progressively during PC differentiation in mice, with proteasome inhibition reducing PC viability (60). Excessive ER stress has been suggested as a factor limiting the lifespan of short–lived PCs (SLPCs), although the expression ER stress response genes is equivalent in SLPCs and long–lived PCs (LLPCs) (61, 62).

PCs require a high level of metabolic activity to fuel extensive immunoglobulin production. Metabolic remodelling occurs during PC differentiation, with Blimp1 promoting oxidative metabolism (49). Basal OXPHOS is fed by long–chain fatty acids, with glucose–derived pyruvate acting as a biosynthetic substrate and providing spare respiratory capacity in LLPCs (15). The majority of glucose taken up by PCs is diverted towards antibody glycosylation *via* the hexosamine pathway, although glycolysis can supplement ATP production (15, 49). Predictably, PCs utilise amino acid metabolism: the expression of CD98, a component of many amino acid transporters, is induced by Blimp1, and is upregulated in LLPCs compared to SLPCs (58, 61). Amino acids are used during antibody synthesis, glutamine is used to generate several of these amino acids, while also acting as a substrate for oxidative metabolism (61). The activation of mTORC1, which is crucial for PC differentiation and optimising antibody output, is supported by high levels of amino acids (58, 63). In mice, AMPK is dispensable for LLPC persistence but restrains antibody synthesis, promoting metabolic homeostasis (53). As in other biological processes, a delicate balance of mTORC1 and AMPK signalling appear to provide an optimal metabolic environment for PC function.

SLPCs are generated following initial B cell activation, while LLPCs, characterised by affinity maturation and class–switch recombination, are produced within germinal centres (GC) of secondary lymphoid organs (**Figure 2**). Each GC consists of light and dark zones, defined by histological appearance. Within the light zone, B cells of different antigen specificities compete for

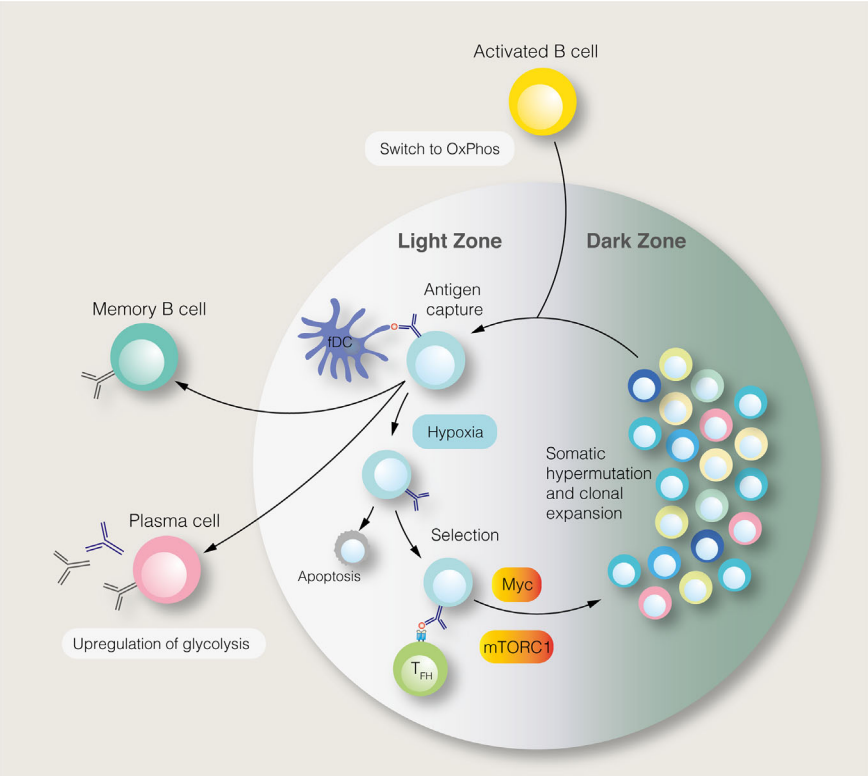

FIGURE 2 | The germinal centre reaction and its regulation by metabolic signals. In the germinal centre light zone, centrocytes capture antigens from follicular dendritic cells (fDC). Centrocytes with the highest antigen affinities are most likely to acquire antigens and present antigen–derived peptides to follicular helper T (T_{FH}) cells. Centrocytes which receive pro–survival signals from T_{FH} cells may migrate to the dark zone, where, as centroblasts (CB), they undergo somatic hypermutation and clonal expansion. This process generates B cells with new antigen specificities. The proliferation of centroblasts is enabled by mTORC1 and c–Myc signalling in the light zone, although this anabolic phenotype is restrained by germinal centre hypoxia. Instead of entering the dark zone, centrocytes, may differentiate into long–lived plasma cells and memory B cells.

pro–survival signals provided by follicular helper T (T_{FH}) cells. B cells with high antigen affinities are more likely to receive sufficient T cell help, enabling them to migrate to the dark zone, where they undergo clonal expansion and somatic hypermutation. To fuel this proliferation, light zone B cells, known as centrocytes, adopt an anabolic phenotype mediated by mTORC1 (64). Centrocytes which receive CD40–mediated signals, representing T_{FH} cell help, activate mTORC1 to promote glucose uptake, cell growth and ribosomal biogenesis (64). mTORC1 activation, specifically in the light zone, is essential for GC function (64). The activity of centrocytes also requires PI3K signalling (65). Upstream of the PI3K–Akt–mTORC1 pathway, the GTPase R–Ras2 couples T_{FH}–derived signals to mitochondrial and glycolytic metabolism (66). Recently, however, it has been shown that GC B cells rely on fatty acid oxidation for ATP production, utilising glycolysis minimally (67). Despite being highly active, GCs are characterised by nutrient deprivation and hypoxia, particularly within the light zone (68, 69). Within the GC, mTORC1 signalling is opposed by HIF–1α and glycogen synthase kinase 3, which promotes B cell viability during nutrient deprivation (68, 69). Interestingly, B cell metabolism changes as the GC reaction progresses (70). Over time, the GC goes from producing predominantly memory cells to LLPCs, with this switch thought to be partly mediated by metabolic changes (70). Indeed, a subset of B cells in the GC, characterised by reduced mTORC1 and c–Myc signalling, display a propensity for memory B cell differentiation (71).

Little is known about the metabolism of memory B cells themselves, although they are thought to be relatively quiescent metabolically. Unlike naïve B cells and PCs, memory cells are capable of BAFF–independent survival (72). AMPK appears to play a crucial role in maintaining metabolic homeostasis in memory B cells, supporting mitochondrial function and preventing excessive oxidative stress (53). Memory B cell inactivity is rapidly reversed upon antigen re–exposure, enabling PC differentiation. During secondary humoral immune responses, the balance between mTORC1 and AMPK signalling controls the nature of this differentiation (22, 53).

As with memory B cells, the metabolic phenotype of unconventional B cell subsets is largely unexplored. Recently, the production of the immunoregulatory cytokine IL–10 by B_{regs} has been shown to depend on both cholesterol metabolism and

HIF–1α (73, 74). However, more work is needed to metabolically characterise B_{regs}, as well as B1 cells.

AUTOPHAGY IN ACTIVATED B CELLS

As with other aspects of metabolism, autophagy plays a crucial role in B cell function post–activation. In the initial stages of B cell activation, the differential regulation of autophagy by BCR engagement and T cell costimulation has been proposed to create an autoimmune checkpoint (75). While BCR stimulation of primary B cells promotes autophagy and triggers apoptosis, concomitant stimulation *via* the CD40 coreceptor induces a more modest rise in autophagy levels, limiting cell death (75). Although the amelioration of B cell autophagy by CD40 engagement has been disputed, the non–optimal induction of autophagy in the absence of costimulation may sensitise self–reactive B cells to cell death pathways (75, 76). Acutely, increased autophagic flux following BCR engagement is driven by the upregulation of non–canonical autophagy, which is resistant to inhibition by bafilomycin A1 (76). In contrast, bafilomycin–sensitive canonical autophagy is temporarily inhibited following BCR activation, likely due to increased mTORC1 activity (76).

While autophagy is induced by BCR signalling, it appears to be dispensable for BCR–driven B cell activation, given that proliferation following anti–IgM stimulation is unaffected in the absence of autophagy (**Figure 1**) (40). B cell autophagy is also induced by stimulation with TLR ligands, although in this context it appears to be necessary for plasmablast differentiation and survival (**Figure 1**) (23, 76). It was initially demonstrated that plasmablast differentiation did occur in autophagy–deficient B cells following stimulation with the TLR4 ligand lipo polysaccharide (LPS), although significant cell death was incurred (23). Furthermore, cells in which *Atg5* deletion was not penetrant were selectively enriched during differentiation (23). It has been subsequently reported that stimulation with LPS or the TLR9 ligand CpG leads to impaired plasmablast differentiation and viability among B cells lacking the autophagy genes *Atg5* or *Atg7*, characterised by the accumulation of damaged mitochondria and reduced expression of B cell transcriptional regulators (25, 40, 77). The effect of autophagy on plasmablast differentiation likely varies between studies according to the extent of metabolic stress induced during different protocols.

In the context of T cell–dependent (TD) antigens, costimulation requires antigen presentation on class II major histocompatibility complexes (MHC–II) to cognate T cells. Several early studies demonstrated that the ability of B cells to present antigens on MHC–II was altered following pharmacological inhibition of autophagy or its induction through starvation (78–80). Of note, these studies were conducted with Epstein–Barr virus (EBV)–infected B cells. EBV infection modifies B cell antigen presentation, limiting the physiological relevance of this model (24). More recently, autophagy has been implicated in the polarisation of internalised antigens following BCR engagement (81). In the absence of autophagy, the colocalisation of antigen–BCR complexes with MHC–II–containing vesicles is disrupted, particularly in the case of particulate antigens (81). While partial relocalisation of the BCR–antigen complex is sufficient for B cells to present peptides from soluble antigens on MHC–II, the presentation of particulate antigen epitopes to cognate T cells is compromised (81). Overall, autophagy appears to be necessary for the presentation of some, but not all, antigens by B cells.

The GC reaction, which enables the generation of memory B cells and LLPCs, is also characterised by extensive B cell autophagy. Of note, levels of canonical autophagy are relatively low, likely due to the upregulation of mTORC1 by T_{FH} cell–derived costimulation (76). The high overall levels of autophagy in GC B cells are skewed towards non–canonical flux, regulated by WIPI2 (76). The role of autophagy in GC function has been questioned, as neither its macroscopic appearance nor the extent of B cell affinity maturation is affected by B–cell–specific *Atg5* deletion (**Figure 1**) (23, 82). However, while it has only been shown that class III PI3K–independent non–canonical autophagy occurs in the GC, some forms of non–canonical autophagy proceed in the absence of *Atg5* itself (76, 83). It is possible that non–canonical autophagy occurs in the GC, and in other B cell subsets, following the deletion of core autophagy genes.

Given its roles in metabolic homeostasis and the degradation of misfolded proteins, it is unsurprising that autophagy appears to play an important role in the maintenance of antibody responses in both SLPCs and LLPCs (**Figure 1**). However, the precise effects of its inhibition are contested. As discussed in the context of plasmablast differentiation, there is disagreement as to the necessity of autophagy for initial antibody production following LPS–induced activation. *Atg7* deletion has been shown to compromise short–term IgM production, in line with failed plasmablast differentiation (25). However, it has also been reported that B cells lacking *Atg5* display elevated short–term antibody production following LPS stimulation, although in this study plasmablasts were characterised by ATP deficiency and elevated ER stress (23). This discrepancy, as in differences seen in plasmablast differentiation, may be caused by differences in the degree and timing of metabolic stress experienced by activated B cells. The idea that autophagy may restrain short–term immunoglobulin output suggests that it instead promotes long–term PC viability (23). In support of this conclusion, IgM and IgG responses to T cell independent (TI) pneumococcal polysaccharide antigens and the TD antigen NP–CGG were diminished in B cell autophagy–deficient mice, as was long–term LLPC survival (23). In contrast, humoral immunity against the TI hapten NP–Ficoll was unaffected by the loss of B cell autophagy in mice, perhaps due to continual B cell activation by this antigen (23). At the same time, another report found that early antibody responses to hapten–conjugated TD and TI antigens, as well as helminths, were diminished in B–autophagy–deficient mice (77). Subsequently, B cell autophagy was suggested to be important to antigen–specific IgM but not IgG responses against the TD antigen ovalbumin in mice (40). Autophagy was concluded to be important for PC survival, with LLPCs displaying greater resistance to a short–term loss of autophagy than SLPCs (40).

Meanwhile, a separate study found that the murine humoral response to the TD antigen NP–KLH was largely unaffected by the loss of B cell autophagy, although anti–NP IgG1 trended towards a decrease six weeks post–immunisation (84). Clearly, there is significant heterogeneity in results, likely arising from differences in immunisation protocols. However, these results together suggest an important role for autophagy in maintaining PC viability over time.

To enable their longevity, LLPCs would logically utilise autophagy to a greater degree than SLPCs. Accordingly, the extent to which PCs utilise autophagy varies according to cellular lifespan. Among adult human bone marrow PCs, the expression of autophagy–associated genes and the prevalence of autophagic LC3B–II punctae are greater among LLPCs than SLPCs (85). In agreement with human data, autophagosomes are more prevalent in murine PCs with longer half–lives (61). Autophagy also appears to support the long–term survival of murine memory B cells, thus promoting the maintenance of immunological memory (**Figure 1**) (82, 84). Expression of autophagy–associated genes is higher in memory B cells than other B cell subsets, suggesting an important role in their function (84). In B cell autophagy–deficient mice, secondary humoral immunity against NP–KLH is affected to a much greater degree than the primary response, indicating a failure of B cell memory (84). While the number of memory cells formed two weeks post–immunisation was normal in mice lacking B cell autophagy, their number was markedly diminished by eight weeks, indicating that autophagy is not necessary for memory B cell formation, but is needed to maintain this population (82). Autophagy–deficient early, but not late, memory B cells are able to mount functional immune responses following antigen re–exposure (82). The survival of autophagy–deficient memory B cell survival is partially restored by NecroX–2, a necrosis inhibitor which reduces oxidative stress (84). Recently, it has been shown that mitochondrial autophagy, which helps to limit oxidative stress, is regulated in memory B cells by AMPK (53). Together, these results implicate autophagy as an important pro–survival mechanism in memory B cells, as well as PCs.

B CELL METABOLISM IN AUTOIMMUNITY

The past decade has seen an explosion in research exploring the association between immunometabolism and autoimmunity (86). Within this field, B cells have received relatively little attention. Nevertheless, there are clear indications that the metabolic profile of B cells is disturbed in autoimmunity, particularly in SLE. Importantly, dysregulated B cell metabolism has been implicated in promoting and exacerbating disease pathology.

The dysfunction of tolerogenic B cell checkpoints is seen in many autoimmune diseases (87, 88). As has been discussed, these checkpoints, which play a crucial role in restricting the self–reactive potential of the B cell repertoire, have a significant metabolic component (19, 54). It has therefore been investigated whether metabolic dysregulation could compromise the function of these checkpoints. One apparent mechanism underlying defective tolerogenesis is increased exposure to BAFF. Serum BAFF levels are elevated compared to health in several autoimmune diseases, including RA, IgA nephropathy and SLE (89–91). In otherwise healthy mice, autoimmune manifestations are seen following transgenic BAFF overexpression (92). Exposure to excess BAFF rescues self–reactive B cells from deletional checkpoints and supports the survival of anergic B cells (21, 93). Anergic B cells show an increased reliance on BAFF signalling and fail to compete with non–anergic B cells for the cytokine under normal conditions (94). Elevated BAFF may promote their survival, increasing the likelihood of peripheral activation. Regarding metabolism, chronic exposure to BAFF increases the glycolytic and oxidative metabolism of B cells (20). Prolonged exposure to high levels of BAFF may allow self–reactive B cells to avoid the metabolic restriction and energy crisis which contribute to anergy and deletion, respectively.

The autoimmune manifestations of TRAF3–deficient mice further evidence the importance of BAFF in autoimmunity. TRAF3 inhibits BAFF–induced activation of nuclear factor (NF)–κB2 signalling (95, 96). In mice, B cell–specific loss of TRAF3 enhances the survival of resting B cells in a BAFF–independent manner. This results in an expanded B cell compartment, increased spontaneous GC formation and autoimmunity (95). As with exposure to elevated BAFF, TRAF3 deficiency in B cells increases glucose uptake, glycolysis and OXPHOS, in an NF–κB–dependent manner (97).

Another important mechanism underlying B cell anergy is the suppression of PI3K signalling by PTEN, Src homology region 2 domain–containing phosphatase (SHP)–1 and Src homology 2 domain–containing inositol polyphosphate 5–phosphatase (SHIP)–1 (39, 98–100). In mice, PTEN deletion alters the responsiveness of B cells to tolerogenic signals, with activation and proliferation favoured over anergy (39). The loss of either PTEN or SHP–1 in B cells results in autoimmunity (39, 101). Moreover, the expression of PTEN by human B cells is reduced in patients with type 1 diabetes mellitus, autoimmune thyroiditis and SLE compared to healthy controls (98, 102). Together, these results suggest that unrestrained PI3K signalling, through the loss of negative regulators, enables self–reactive B cells to escape tolerogenesis.

Downstream of PI3K, hyperactive B cell mTORC1 signalling is seen in autoimmunity. Given its roles in B cell development, GC reactions and PC function, an association between altered mTORC signalling and autoimmunity is perhaps unsurprising (14, 64). mTORC1 hyperactivity is seen in animal models of autoimmunity, including murine models of SLE and RA (103, 104). In the latter, mTORC1 hyperactivation in B cells was associated with increased glucose metabolism, although disturbed B cell metabolism was deemed to occur downstream of T cell dysregulation (104). In human autoimmunity, elevated mTORC signalling is seen in the B cells of SLE patients, where it is correlated with disease activity, and in the salivary gland B cells of patients with Sjögren's syndrome (22, 105).

The precise metabolic consequences of dysregulated B cell signalling networks in autoimmunity have not been well defined. However, there is clear evidence to suggest that the tight

metabolic control of B cell function is lost in autoimmunity and that this has consequences for pathophysiology. More work is needed to directly investigate the nature and consequences of metabolic dysregulation seen in autoimmune B cells. Furthermore, a more complete characterisation of metabolism in different B cell subsets is needed. Comparatively little is known about the metabolism of B1 cells, B_{regs} and memory B cells in both health and disease. Given the different phenotypes and functions of different B cells, it is likely that they affected differently by metabolic disturbances. Finally, most research has focussed on B cells in SLE, with the dysfunction of B metabolism in other diseases mediated by autoantibodies and B cell cytokines warranting further investigation.

B CELL AUTOPHAGY IN AUTOIMMUNITY

As discussed above, autophagy promotes metabolic homeostasis of B cells, particularly following peripheral activation and in memory cell maintenance (23, 84). It has also been implicated in antigen presentation and deletional autoimmune checkpoints (75, 81). This has fuelled considerable interest in the role that autophagy may play in expanding the repertoire, functionality and survival of self–reactive B cells in diseases such as SLE, RA, and multiple sclerosis (MS). Although not cell–specific, early evidence of perturbed autophagy in autoimmunity came from genome–wide association studies (GWAS). Specifically, several reports linked single nucleotide polymorphisms (SNPs) in the *Atg5* gene and *Prdm1–Atg5* intergenic region to the development of SLE and, in a European population, to RA (106–109). While GWAS are unable to resolve associations in a cell–specific manner, it was subsequently demonstrated that the B cells of patients with SLE displayed elevated *Atg5* expression compared to healthy controls (110). Furthermore, an SLE–associated SNP in the *Prdm1–Atg5* intergenic region was associated with elevated expression of autophagy–associated genes in the B cells of SLE patients and healthy individuals (110). In parallel to these early GWAS, autophagy was implicated in SLE through therapeutic studies. The 21–mer peptide rigerimod (P140), which ameliorates lupus pathology, was shown to inhibit B cell autophagy (111). Specifically, rigerimod targets chaperone–mediated autophagy, which is upregulated in lupus–prone mice, suggesting that inhibiting dysregulated autophagy may underlie the efficacy of rigerimod (112). The apparent association between B cell autophagy and autoimmunity appears to be mediated by several distinct mechanisms, which may occur in a disease–specific manner.

As with metabolism, dysregulated autophagy has been implicated in the loss of tolerogenic checkpoint function in autoimmunity. While autophagy is dispensable for B2 cell development, this does not preclude a role for altered autophagy in enabling the subversion of metabolic or apoptotic checkpoints by self–reactive B cell precursors (**Figure 3**) (25, 40). Indeed, while autophagy is upregulated in lupus–prone mice compared to healthy controls, this difference is restricted to bone marrow–resident pre–B, immature and mature B cells (25). No difference is seen in among splenic B cells, including PCs. In patients with SLE, elevated autophagy compared to healthy controls is more notable in naïve B cells than PCs or memory cells (25). Both pre–B and naïve B cells are subject to tolerogenic checkpoints. Upregulated autophagy may protect self–reactive B cells against apoptotic or metabolically damaging stimuli. Similarly, the induction of autophagy has been proposed to mediate T cell escape from tolerogenic checkpoints (113).

Autophagy also appears to play a role in enabling B cells to process and present peptides derived from self–antigens to cognate T cells (**Figure 3**). Physiologically, autophagy has been shown to mediate the presentation of peptides derived from particular antigens (81). In autoimmunity, autophagy enables B cells to present citrullinated peptides on MHC–II, antibodies against citrullinated antigens feature prominently in autoimmune diseases, most notably in RA (114, 115). *In vitro*, the presentation of endogenous citrullinated peptides by B lymphoma cells was enabled by serum starvation, a state which activates autophagy, and suppressed by both the class III PI3K inhibitor 3–MA and *Atg5* knockdown (115). In contrast, neither starvation nor 3–MA affected the presentation of non–citrullinated antigens. Furthermore, stimulation of primary B cells with anti–IgM antibodies induced significant citrullinated peptide presentation and increased LC3–II levels (115). Given the frequency of anti–Ig antibodies in RA and the apparent induction of autophagy following BCR engagement, such antibodies have been proposed to trigger the presentation of endogenous citrullinated antigens on MHC–II (115). Although the exact relationship between autophagy and RA remains unclear, B cell autophagosome density is not upregulated in patients with RA compared to healthy controls, yet autophagic activity appears necessary for the presentation of certain autoantigens (116).

B cell autophagy has also been associated with the presentation of citrullinated self–antigens to cytotoxic T lymphocytes in MS. EBV, a well–established risk factor for development of MS, induces autophagy in B cells, and has recently been linked to the class I MHC (MHC–I) presentation of myelin oligodendrocyte glycoprotein (MOG)–derived peptides (24, 117–119). The role of autophagy in this process is poorly defined, although autophagosomes may protect citrullinated MOG peptides from cathepsin–mediated degradation, directing them towards MHC–I cross–presentation machinery (24).

As well as BCR ligands, autophagy has been implicated in B cell recognition of innate immune triggers, specifically nucleic acid antigens, by TLRs 7 and 9. These receptors recognise pathogen–derived ssRNA and CpG–containing dsDNA respectively, with their cellular expression restricted to endosomes to prevent cross–recognition of self–nucleic acids. The recognition of self–nucleic acids may directly activate B cells or sensitise them to BCR–mediated activation: the transgenic overexpression of *Tlr7* or *Tlr9* generates autoreactive PCs and triggers lupus–like disease in mice (26, 120, 121). Murine experimental lupus induced by transgenic *Tlr7* overexpression is ameliorated in the absence of B cell autophagy (122). Autophagy mediates the transport of endocytosed RNA–containing

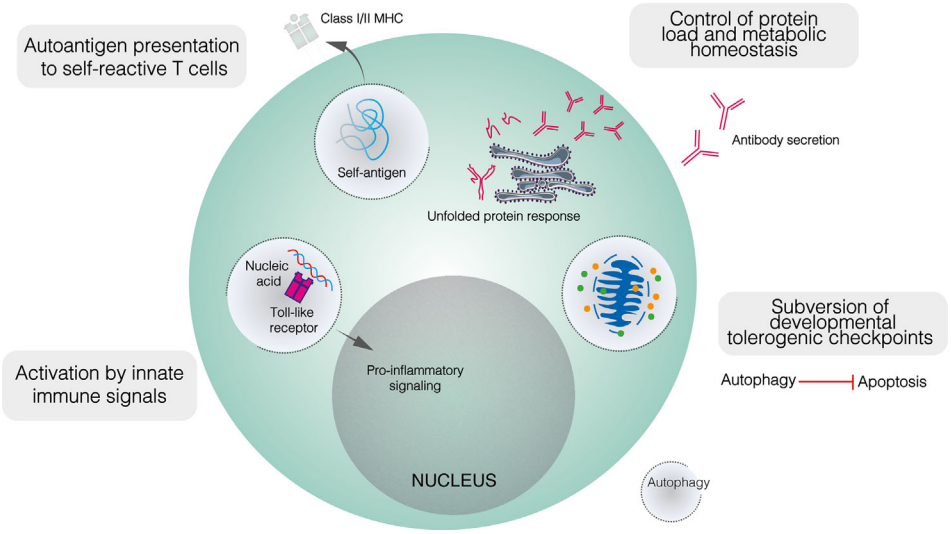

FIGURE 3 | The role of autophagy in the function of self–reactive B cells. Developmentally, upregulated autophagy is thought to enable autoimmune B cell precursors to subvert apoptotic checkpoints. In mature B cells, autophagy appears to mediate both the presentation of self–peptides by B cells, and the recognition of innate immune ligands such as nucleic acids. It is also likely that autophagy contributes to metabolic homeostasis and control of the misfolded protein load in plasma cells.

immune complexes to TLR7–containing endosomes in dendritic cells and may play a similar role in B cells (**Figure 3**) (123). Autophagy has also been implicated in TLR9 signalling (124). Following BCR engagement, TLR9–containing endosomes are recruited to autophagosomes which contain the internalised BCR–antigen complex, although this co–localisation is abolished by 3–MA (124). Anti–IgM conjugated to CpG DNA hyperactivates B cells compared to anti–IgM alone, and this additive effect was abolished when TLR9 recruitment to autophagosomes was blocked by disrupting microtubule function (124). Collectively, autophagy seems to play an important role in delivering nucleic acid antigens to endosomal TLRs, potentially impairing the discrimination between self– and non–self–nucleic acids.

Intuitively, the role of autophagy in maintaining PC viability should extend to self–reactive PCs (**Figure 3**) (23, 40, 60). While PC autophagy does not appear to be upregulated in lupus–prone mice compared to healthy controls, it contributes demonstrably to the pathology of murine experimental lupus (25, 40). In mice with *Atg5* conditionally deleted in mature B cells, anti–dsDNA IgM levels were similar to those seen in wildtype mice, whereas anti–dsDNA IgG was significantly lower (40). The splenic B cell repertoire, including the proportions of different B cell subpopulations and the appearance of germinal centres, was largely unaffected by the absence of autophagy, although bone marrow LLPCs were depleted (40). Together, these results suggest that autophagy makes little contribution to early B cell activation in autoimmunity but is important in ensuring the long–term survival of autoreactive PCs. It was proposed that SLPC survival may have been compromised in the absence of autophagy but was compensated for by increased replenishment (40). Importantly, autophagy deficiency likely also reduces the memory B cell compartment (84).

While B2 cell autophagy plays an important role in autoimmunity, its relevance in other B cell subsets remains poorly defined. Although B1 cells display great sensitivity to changes in autophagic flux, it is not clear whether changes in B cell autophagy seen in autoimmunity apply to these cells and what the effects might be (18). This is important to understand, given that self–reactive IgM produced by B1 cells is implicated both in autoimmune pathogenicity and in the clearance of apoptotic cells (8, 11). Moreover, B1 cells in gut–associated lymphoid tissue promote intestinal homeostasis through IgA and IgM production (12). The loss of B cell autophagy reduces mucosal IgA and impairs B cell responses to intestinal inflammation (77). Overall, B1 cell autophagy may be relevant to inflammatory and autoimmune diseases, particularly within the gastrointestinal tract. As in B1 cells, the effects of perturbed autophagy on B_{reg} function have yet to be defined, although their unique phenotype suggests that they may be affected differently to antibody–secreting cells. Further investigation is required to understand the role of autophagy in unconventional B cells during autoimmunity.

B CELL METABOLISM AND AUTOPHAGY AS THERAPEUTIC TARGETS

The dysregulation of B cell metabolism and autophagy in autoimmune diseases has raised the prospect of targeting these processes therapeutically. While traditional immunosuppressive

drugs such as glucocorticoids affect immunometabolism, recent efforts have focussed on disrupting metabolic pathways more specifically (125). Several drugs being explored or approved for the treatment of autoimmune diseases target B cell immunometabolism or its regulators, likely contributing to their efficacy.

As discussed above, elevated BAFF levels are seen in several autoimmune diseases (89–91). B cells exposed to high levels of BAFF have enhanced metabolic capacity and can escape from tolerogenic checkpoints (20, 21). Efforts to inhibit this dysregulated signalling culminated in the approval of the BAFF–specific monoclonal antibody belimumab as an add–on therapy in SLE, having demonstrated efficacy, including ameliorating B cell dysfunction, in phase 3 clinical trials (126). The treatment of SLE patients with belimumab induces anergy in autoreactive B cells (93). Notably, the BAFF–independent survival of memory B cells means that humoral immune responses to vaccine antigens are left intact by belimumab (127). The ability to target self–reactive B cells without compromising physiological B cell responses is clearly desirable. Excessive BAFF signalling may also be attenuated through blockade of its receptor. Recently, the BAFF receptor inhibitor ianalumab was shown to effectively deplete B cells and improve clinical parameters in patients with Sjögren's syndrome (128).

Hyperactive mTORC1 signalling, a feature of both T and B lymphocytes in autoimmunity, can be inhibited with rapamycin. In murine models of SLE, rapamycin attenuates pathology, including decreasing anti–dsDNA antibody titres (129). Although its effects have been largely attributed to changes in T cells, rapamycin inhibits BAFF–mediated mTORC1 signalling in B cells, limiting proliferation and survival (130, 131). mTORC1 inhibition also results in the induction of autophagy. Although self–reactive B cells appear to benefit from a degree of autophagy upregulation, increased autophagic flux may be detrimental to B cell survival (75). In a single–arm, open–label Phase 1/2 trial in SLE patients, rapamycin was associated with decreased disease activity and reduced levels of some autoantibodies (132).

mTORC dysregulation is also targeted by metformin. Used to treat type 2 diabetes mellitus, metformin activates AMPK and is, therefore, able to downregulate mTORC1 activity. Metformin has shown efficacy in the treatment of lupus–like autoimmunity in mice (133). As well as decreasing T_{FH} and T_{H17} populations, metformin suppressed PC differentiation and GC formation, resulting in lower autoantibody levels (133). In a separate study, metformin given alongside the glycolytic inhibitor 2–DG was shown to ameliorate autoimmune pathology in lupus–prone mice (134). A recent clinical trial into the efficacy of metformin in SLE patients found no efficacy in reducing the incidence of disease flares, although pooled analysis with a previous trial suggested a modest reduction in flare incidence was achieved, warranting further investigation (135, 136).

While some of the therapeutic effects of rapamycin and metformin may derive from targeting B cell autophagy, this process is affected more clearly by rigerimod (111). Rigerimod disrupts chaperone–mediated autophagy, likely affecting the MHC–II–restricted presentation of endogenous antigens to autoreactive T cells (112, 137). In mice, rigerimod suppresses autoimmune responses without compromising anti–viral immunity (138, 139). Although rigerimod showed efficacy in early clinical trials in SLE patients, it failed to meet its primary endpoint in a recent phase 3 clinical trial (140, 141). However, promising therapeutic potential has meant a new phase 3 trial is scheduled to commence in 2021.

Owing to their limited proteasome capacity and high levels of immunoglobulin production, PCs display exquisite sensitivity to proteasome inhibition (60). Proteasome inhibition has been investigated as a potential therapeutic strategy in diseases characterised by autoantibody production. Efforts to target the proteasome have focussed on bortezomib, which was originally developed for the treatment of multiple myeloma. In multiple myeloma, B cells with high immunoglobulin production are disproportionately vulnerable to proteasome inhibition (142). The high immunoglobulin output of self–reactive B cells in autoimmune diseases will likely confer elevated sensitivity to proteasome inhibitors. In a murine model of lupus, bortezomib alleviated autoantibody–driven pathology (143). In small numbers of human participants, bortezomib has shown promise in treating autoantibody–driven diseases, such as anti–NMDA receptor encephalitis and SLE (144, 145).

Although the metabolism and autophagy of self–reactive B cells represent promising therapeutic avenues, there are several challenges associated with such targets. Firstly, a full understanding of the metabolic regulation of B cell function is lacking in both health and disease. Moreover, much of our current knowledge comes from studying B cell metabolism in mice, not humans. A critical goal in the field is uncovering cell–specific metabolic pathways which may be targeted more selectively. Similarly, immune cells differ considerably in their metabolic plasticity and therefore resistance to metabolic pathway inhibition. However, many of the most commonly used drugs in medicine (e.g. metformin, statins, and methotrexate) all target broadly active metabolic processes. This observation suggests that tolerability of metabolic inhibition be better than supposed. As always when treating autoimmunity, excessive immunosuppression must be avoided, and the risk of infection will remain an important consideration. Finally, the extent to which specific metabolic pathways are disturbed in patients with autoimmune disease is likely to vary significantly between individuals. The use of metabolomic biomarkers may be needed to identify patients who are likely to respond to treatments targeting metabolism.

CONCLUSION

Although metabolism has emerged relatively recently as a regulator of immunological function, it is clear that it has far–reaching consequences in both the physiological state and autoimmunity. Arguably, efforts have been made to exploit B cell metabolism and autophagy therapeutically before these processes have been well characterised. Nevertheless, it appears

that they do offer real translational potential. While much focus to date has been rightly placed on understanding the role of metabolic regulators such as mTORC1 and AMPK, metabolism itself, as well as its impact on B cell function, require greater investigation. Finally, it is imperative that metabolic interactions between B cells and other leukocyte populations are explored. While this review has focussed only on B cells, exploiting the full therapeutic potential of B cell metabolism in autoimmune diseases will require it to be understood within the context of wider immune dysfunction.

AUTHOR CONTRIBUTIONS

IR wrote and edited the manuscript AC supervised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Glycolysis Inhibition Induces Functional and Metabolic Exhaustion of CD4$^+$ T Cells in Type 1 Diabetes

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In Type 1 Diabetes (T1D), CD4$^+$ T cells initiate autoimmune attack of pancreatic islet β cells. Importantly, bioenergetic programs dictate T cell function, with specific pathways required for progression through the T cell lifecycle. During activation, CD4$^+$ T cells undergo metabolic reprogramming to the less efficient aerobic glycolysis, similarly to highly proliferative cancer cells. In an effort to limit tumor growth in cancer, use of glycolytic inhibitors have been successfully employed in preclinical and clinical studies. This strategy has also been utilized to suppress T cell responses in autoimmune diseases like Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), and Rheumatoid Arthritis (RA). However, modulating T cell metabolism in the context of T1D has remained an understudied therapeutic opportunity. In this study, we utilized the small molecule PFK15, a competitive inhibitor of the rate limiting glycolysis enzyme 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3 (PFKFB3). Our results confirmed PFK15 inhibited glycolysis utilization by diabetogenic CD4$^+$ T cells and reduced T cell responses to β cell antigen *in vitro*. In an adoptive transfer model of T1D, PFK15 treatment delayed diabetes onset, with 57% of animals remaining euglycemic at the end of the study period. Protection was due to induction of a hyporesponsive T cell phenotype, characterized by increased and sustained expression of the checkpoint molecules PD-1 and LAG-3 and downstream functional and metabolic exhaustion. Glycolysis inhibition terminally exhausted diabetogenic CD4$^+$ T cells, which was irreversible through restimulation or checkpoint blockade *in vitro* and *in vivo*. In sum, our results demonstrate a novel therapeutic strategy to control aberrant T cell responses by exploiting the metabolic reprogramming of these cells during T1D. Moreover, the data presented here highlight a key role for nutrient availability in fueling T cell function and has implications in our understanding of T cell biology in chronic infection, cancer, and autoimmunity.

Keywords: glycolysis, type 1 diabetes, immunometabolism, T cell exhaustion, autoimmunity, PD-1, LAG-3

INTRODUCTION

Invasion of pancreatic islets by immune cells is a hallmark of Type 1 Diabetes (T1D), where the innate and adaptive immune systems work cooperatively to mediate damage of insulin-secreting β cells (1, 2). This attack is largely orchestrated by self-reactive $CD4^+$ T cells, which are fundamental drivers of disease pathology (2). During their life cycle, $CD4^+$ T cells rely on specific metabolic pathways to generate energy in the form of adenosine triphosphate (ATP) (3, 4). However, it has become abundantly clear that these programs are not merely for energy production, but rather are necessary for the ability of T cells to carry out specialized effector capabilities, including interferon gamma (IFNγ) secretion (5). During homeostasis, naïve $CD4^+$ T cells utilize oxidative phosphorylation (OXPHOS) to support surveillance efforts and migration in the periphery. Upon encounter with antigen (i.e., islet β cells in T1D), activated T cells undergo robust metabolic reprogramming marked by a transition to the less efficient aerobic glycolysis (3, 4). Although net energy obtained through the glycolysis pathway is far less than what is generated *via* OXPHOS (net gain of 2 versus approximately 36 ATP molecules, respectively), glycolysis is required to generate ATP quickly to support T cell activation, clonal expansion, and effector cytokine production (5).

While distinct metabolic programs dictate T cell differentiation and function, a number of studies have also implicated nutrient availability as an important determinant of T cell fitness. This is especially evident in the tumor microenvironment (TME), where reports have demonstrated that cancer cells outcompete T cells for key nutrients like glucose and amino acids, which are required for acquisition of specialized effector functions (6). This battle for metabolic substrates, along with persistent antigen exposure, has been implicated in suppressing immune responses and driving T cell exhaustion. Exhaustion is defined as a state of dysfunction characterized by increased expression of immune inhibitory receptors (IRs) like programmed cell death protein- 1 (PD-1) and Lymphocyte Activation Gene-3 (LAG-3), and a marked decrease in T cell effector functions that allow tumors to go unabated by the immune system (6, 7). Although metabolic insufficiencies demonstrate a major hurdle in reinvigorating tumor-specific T cell responses, the opposite is plausible in settings of autoimmunity, where enforcing T cell exhaustion by targeting metabolism may be a novel mechanism by which tolerance to self-antigens is restored (8).

Efforts to target the glycolysis pathway have been successfully employed to limit tumor growth and metastasis in cancer, with a number of these inhibitors undergoing FDA clinical trials (9–12). One such inhibitor, PFK15, is a small molecule inhibitor of 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3 (PFKFB3), an enzyme involved in a cells commitment to metabolize glucose *via* glycolysis (9, 12, 13). Regarding autoimmunity, researchers have successfully targeted metabolism as a means to control T cell responses in models of Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), and Rheumatoid Arthritis (RA), however the ability to target metabolic pathways to control T1D have been largely unstudied (14–17). Previously, our laboratory demonstrated that redox modulation *via* disruption of third signal reactive oxygen species (ROS) impeded T cell metabolic reprogramming to glycolysis, and altered the diabetogenic potential of autoreactive $CD4^+$ T cells (18). However, whether specifically modulating the glycolysis pathway could be used to limit the activation of autoreactive $CD4^+$ T cells and prevent attack of pancreatic β cells deserves further exploration. Based on these previous studies, we hypothesized that use of the glycolysis inhibitor PFK15 would inhibit the activation, proliferation, and effector capabilities of autoreactive $CD4^+$ T cells, thereby delaying the onset of T1D. Herein, we demonstrate that PFK15 treatment interrupts metabolic reprogramming to glycolysis upon activation with β cell antigen, and reduces T cell responses *in vitro*, while delaying the onset of T1D *in vivo*. These results were, in part, due to increased and sustained expression of checkpoint molecules PD-1 and LAG-3 and downstream functional and metabolic exhaustion of diabetogenic T cell clones. These findings support that inhibition of glycolysis drives T cell exhaustion, and that metabolic modulation may serve as a novel therapeutic target to control T cell metabolism and restore tolerance in autoimmunity.

MATERIALS AND METHODS

Animals

Non-obese diabetic (NOD), NOD.BDC2.5.TCR.Tg (BDC2.5) and NOD.*scid* mice were maintained under specific pathogen free conditions in the animal facility located at the Rangos Research Center within UPMC Children's Hospital of Pittsburgh. All animal experiments were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC; Assurance Number: D16-00118). Male and female mice aged 6-12 weeks old were used in all experiments.

Splenocyte Isolation and *In Vitro* Stimulation

BDC2.5 animals were sacrificed, and spleens harvested and homogenized into single cell suspensions as described (18). Red blood cells were lysed using RBC lysis buffer (Sigma Aldrich). For *in vitro* experiments, 2.5 x 10^6 splenocytes were plated per well in 24 well plates and stimulated with 0.05 μM of their peptide mimotope ± 5 μM PFK15 soluble drug (Selleck), 0.2-1 mM 2-DG (Sigma Aldrich), 25-50 μM YN1 (Millipore Sigma), or 2.5-5 μM PFK158 (Selleck) for 24-72 hours. Cells and culture supernatants were collected for downstream flow cytometry, western blotting, ELISA, and lactate measurement analyses.

Flow Cytometry

$5x10^5$ – $1x10^6$ cells were harvested at indicated timepoints and surface stained for flow cytometric analysis as described (18). Briefly, cells were incubated with Fc block (CD16/CD32; BD Biosciences) for 15 minutes prior to staining for flow cytometry.

Surface staining was performed at 4°C using CD4-PE, PerCP-Cy5.5, or APC (Clone RM4-5), CD69-PeCy7 (Clone H1.2F3), CD25-APC (Clone PC61), CD223-PE (LAG-3; Clone C9B7W), CD279-BV480 (PD-1; Clone J43) antibodies (BD Biosciences) in FACS buffer (1% BSA in PBS). For proliferation measurements, splenocytes were labeled with Cell Proliferation Dye Violet (BD Bioscience) per manufacturer's instructions prior to stimulation. After indicated timepoints, cells were harvested, and surface stained as described above. In some instances, cells were fixed in 2% PFA for 15 minutes at 4°C (Thermo Fisher Scientific). Cells were stored at 4°C until time of analysis.

To measure glucose uptake, splenocytes were incubated with 100 μM of the fluorescent glucose analog 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG; Cayman Chemical) for 10 minutes at 37°C prior to harvest as described (18, 19). Cells were washed with PBS and surface stained for CD4 expression and analyzed live by flow cytometry. To measure fatty acid uptake, cells were harvested, surface stained for CD4 expression, and incubated with the fluorescent fatty acid BODIPY FL C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Hexadecanoic Acid; Invitrogen) in serum-free warm PBS for 30 minutes at 37°C. Cells were washed with PBS and analyzed live by flow cytometry.

To assess mitochondrial function, Day 14 BDC2.5 T cell clones were harvested, surface stained for CD4 expression, and incubated with MitoTracker green, MitoSOX red, or TMRE (tetramethylrhodamine, ethyl ester, perchlorate; Invitrogen) in warm PBS at 37°C for 15-30 minutes. Cells were washed with PBS and analyzed live by flow cytometry as described previously (19). For all flow cytometry studies, fluorescence was measured using a FACS Aria II flow cytometer (BD Biosciences). All data were analyzed using FlowJo software (v10.5.3) and samples were gated on $CD4^+$ cells. Forward scatter of $CD4^+$ T cells was also determined by flow cytometry.

Preparation of Protein Lysates and Western Blotting

Cells were lysed by sonication in anti-pY lysis buffer (50 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM Na_3VO_4, and 1 mM PMSF). Protein concentration was determined by Bicinchoninic acid protein (BCA) assay (Thermo Fisher Scientific). 25 μg of protein per sample were boiled in 6x Lammaeli buffer (BIORAD) for 5 minutes and separated on 4-20% gradient SDS-PAGE gels (BIORAD). Samples were then transferred to PVDF membranes for 2 hours in 3% MeOH Tris-Glycine Transfer buffer (BIORAD). Western blots were blocked in 5% non-fat dry milk in Tris-buffered Saline with 1% Tween-20 (TBST). Blots were probed with the following antibodies in 5% BSA/TBST overnight at 4°C: Glut-1, CPT1α (1:1000; Abcam), HK2, PFKFB3, LDHA (1:1000; Cell Signaling), and β-actin as a loading control (1: 10,000; Sigma- Aldrich). Membranes were washed with TBST and incubated with HRP-conjugated goat anti-rabbit or rabbit anti-mouse (Jackson ImmunoResearch 1:10,000) secondary antibodies for 2 hrs in 5% non- fat milk/TBST at room temperature. Chemiluminescence was detected using SuperSignal West Pico PLUS Chemiluminescence solution (Thermo Fisher Scientific) and the iBright FL1500 imaging system (Invitrogen).

Lactate and Cytokine Measurements

Cell culture supernatants from *in vitro* experiments were harvested and used to measure IL-2, IFNγ, and TNFα by ELISA. Antibody pairs for IFNγ and IL-2 ELISAs were purchased from BD Biosciences, and TNFα ELISA kits were purchased from R&D according to the manufacturer's instructions. ELISAs were read on a SpectraMax M2 microplate reader (Molecular Devices) and data analyzed using SoftMax Pro version 7.0.2 software (Molecular Devices). Lactate, a byproduct of aerobic glycolysis, was measured using the Lactate Plus meter and test strips per manufacturer's instructions (Novus Biologics).

$CD4^+$ T Cell Isolation and *Ex Vivo* Activation and Expansion

Spleens from BDC2.5 animals were harvested and homogenized into single cell suspensions as described above. $CD4^+$ T cells were isolated by magnetic bead separation using the EasySep $CD4^+$ T cell Negative Selection isolation kit (StemCell) per the manufacturer's instructions. For *ex vivo* activation, 6 well plates were coated with plate bound αCD3 (BD Biosciences; 1 ug/mL) in PBS for at least 3 hours in a cell culture incubator (37° C, 5% CO_2). The antibody solution was decanted, and $5x10^6$ isolated cells were plated with 100 U/mL IL-2 and 1 ug/mL soluble αCD28 (BD Biosciences) for 3 days in a cell culture incubator. After 3 days in culture, cells were harvested and transferred to T-75 flasks with an additional 100 U/mL IL-2 for expansion. At the end of the 3-day expansion in IL-2, cells were isolated, counted, and washed with sterile PBS for adoptive transfer experiments.

Adoptive Transfer Model of T1D

1 x 10^7 *ex vivo* activated $CD4^+$ T cells from BDC2.5 mice (described above) were injected i.p. into NOD.*scid* recipients. For initial prevention studies, recipient animals were split into two groups. One cohort of recipients received 25 mg/kg PFK15 (Selleck) dissolved in 5% DMSO + 45% PEG300 + 1% Tween80 + 49% ddH_2O prepared fresh; the other cohort received vehicle control every other day for 2 weeks. In reversibility studies, recipient animals were placed into one of the following treatment groups: 1) Vehicle Control, 2) PFK15 + IgG (Isotype controls for αPD-1 and αLAG-3 blocking antibodies; 200 μg each per treatment), or 3) PFK15 + 200 μg αPD-1 (clone J43; BioXCell), + 200 μg αLAG-3 (clone C9B7W; BioXCell) as previously described (19, 20). Animals were treated every other day for two weeks, with checkpoint blockade or IgG treatment initiated during the second week. Body weights and blood glucose (BG) levels were monitored over the course of the experiments. Animals were deemed diabetic after two consecutive BG readings ≥ 350 mg/dL. Diabetic animals were sacrificed at indicated timepoints and peripheral blood, pancreata, and spleens were harvested for downstream analyses.

Tissue Collection and Histological Assessment

Pancreatic tissue was collected and fixed in 4% paraformaldehyde (PFA; Thermo Fisher Scientific) overnight at 4°C. Fixed tissue was processed and embedded in paraffin by the Histology Core Laboratory located at UPMC Children's Hospital of Pittsburgh's Rangos Research Center (21). Embedded tissue was sectioned at 4 μm thickness and stained with hematoxylin and eosin (H&E) for histological examination of immune infiltration in pancreatic islets. Samples were imaged using a Nikon Eclipse E800 microscope (Nikon) and associated software.

Immunofluorescent Staining

Immunofluorescent staining was performed on paraffin embedded samples prepared as described above. Antigen retrieval was performed in either sodium citrate or Tris-EDTA buffers followed by overnight incubation with primary antibodies against insulin (1:100; Santa Cruz), CD3 (1:100; Abcam), and PD-1 (1:50; Abcam) all co-stained with DAPI (1:3000; Thermo Fisher Scientific). The following day, slides were incubated with Alexa Fluor 488 conjugated donkey anti- rabbit secondary antibody (Invitrogen). Samples were imaged using a Leica DMi8 inverted microscope (Leica) and LAS X Navigator software (Leica).

Maintenance of BDC2.5 T Cell Clones

$CD4^+$ MHC-II restricted BDC2.5 T cells, a generous gift from Dr. Kathryn Haskins (University of Colorado), were maintained in supplemented DMEM as previously described (22–25). Briefly, BDC2.5 T cells were cultured in T-25 flasks with β membrane (antigen), irradiated NOD splenocytes (antigen presenting cells: APC), and EL-4 supernatant (source of IL-2) for 2 weeks in a cell culture incubator. For mechanistic studies, a subset of flasks were treated with **5** μM PFK15 every third day over the course of the restimulation period. Day 8 and 14 T cells and culture supernatants were harvested for downstream analyses. Similarly, for reinvigoration studies, untreated and PFK15 treated T cells were put into restimulation flasks **±5**μg/mL αPD-1 (clone J43; BioXCell), αLAG-3 (clone C9B7W; BioXCell), or αPD-1 + αLAG-3. Cells were treated every third day for 2 weeks.

ADP/ATP Ratio Measurements

Day 14 control and PFK15 treated T cell clones were harvested and assayed for the ADP/ATP ratio per the manufacturer's instructions (Millipore Sigma).

Statistical Analyses

All data are presented as mean values ± standard error of the mean (SEM), with n indicating the number of independent experiments or animals. Student's t-test, One- way ANOVA, or Two-way ANOVA were used where appropriate. For survival studies, Kaplan-Meier analysis was used to measure significance of diabetes incidence. A p-value of $p < 0.05$ was considered significant for all statistical analyses. Histology and immunofluorescent images were generated using Photoshop. All statistics and graphs were generated using GraphPad Prism software.

RESULTS

PFK15 Interrupts Metabolic Reprogramming to Glycolysis and Reduces T Cell Effector Functions During Activation

To determine the effect glycolysis inhibition would have on the activation and subsequent metabolic reprogramming of autoreactive $CD4^+$ T cells in T1D, we stimulated splenocytes from NOD.BDC2.5.TCR.Tg animals *in vitro* with their cognate peptide mimotope (MM) ± PFK15 (a PFKFB3 inhibitor; **Figure 1A**), as previously described (18). PFK15 treated BDC2.5 splenocytes failed to upregulate glycolysis-associated proteins glucose transporter-1 (Glut-1), hexokinase 2 (HK2), PFKFB3, and lactate dehydrogenase A (LDHA) in response to MM stimulation compared to T cells stimulated without treatment (**Figure 1B**). Additionally, PFK15 treatment decreased glucose (2-NBDG) uptake by $CD4^+$ T cells upon stimulation to levels similar to T cells in media alone (**Figures 1C, D**). A significant reduction in 2-NBDG fluorescence from treated $CD4^+$ T cells was most likely due to reduced expression of Glut-1 (**Figure 1B**), indicating decreased capacity to engage in aerobic glycolysis (18, 26). Utilization of the glycolysis pathway by $CD4^+$ T cells is accompanied by increased secretion of the by product lactate (3, 4, 18, 27). Splenocytes stimulated with MM alone displayed a significant increase in lactate secretion in cell culture supernatants as expected, indicating increased glycolytic flux (**Figure 1E**). In comparison, PFK15 treated splenocytes secreted less lactate compared to MM stimulated T cells, further confirming an inability to metabolically transition to glycolysis upon encounter with β cell antigen (**Figure 1E**).

As bioenergetics and T cell function are intricately linked, we next determined the impact of glycolysis inhibition on the activation, proliferation, and effector capabilities of BDC2.5 T cells. While activated $CD4^+$ T cells displayed increased forward scatter (FSC) compared to unstimulated controls, indicative of increased cell growth, PFK15 treatment resulted in reduced FSC compared to stimulated T cells, indicating inhibited growth (**Figure 2A**). We then assessed proliferative capacity by measuring cell proliferation dye violet (CPDV) dilution by $CD4^+$ T cells in all three treatment groups. MM stimulated T cells displayed robust proliferation in response to antigen; however, PFK15 treatment significantly reduced this response (**Figures 2B, C**).

To interrogate the activation status of PFK15 treated T cells, expression of the early activation marker CD69, and the late activation marker and high- affinity interleukin-2 (IL-2) receptor CD25, was measured 48-72 hours post stimulation on $CD4^+$ T cells by flow cytometry (**Figures 2D, E**). There were no appreciable differences in the expression of CD69 in stimulated or PFK15 treated groups indicating that PFK15 treatment does

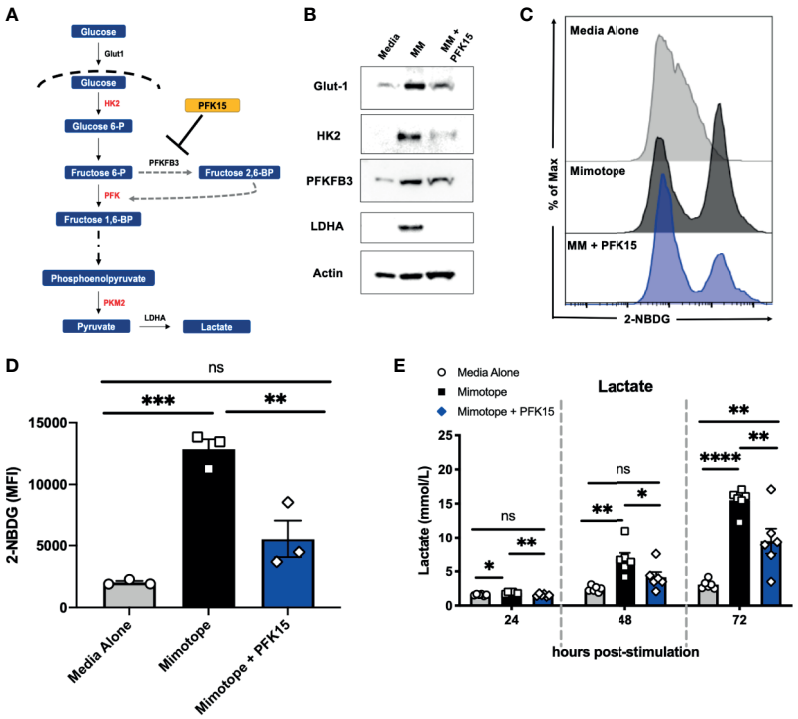

FIGURE 1 | PFK15 treatment inhibits metabolic reprogramming to glycolysis during T cell activation. NOD.BDC2.5 splenocytes were stimulated with their cognate peptide MM ± PFK15 for 24-72 hrs. **(A)** Schematic diagram displaying the mechanism of action of PFK15. **(B)** Representative western blot analysis of glycolysis proteins in untreated, MM stimulated, and MM+ PFK15 treated splenocytes after 48 hrs in culture. **(C, D)** Representative histogram and statistical analysis showing 2-NBDG fluorescence and MFI of $CD4^+$ T cells, indicative of glucose uptake 48 hrs post stimulation (n = 3). **(E)** Lactate measurements in cell culture supernatants (n = 6). All data are presented as the mean ± SEM. (not significant (ns), *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001).

not interfere with early activation (**Figure 2D**). Conversely, CD25 expression was significantly decreased with PFK15 treatment, suggesting an inability to fully transition to late activation status when glycolysis is inhibited (**Figure 2E**). Since glycolysis is required for acquisition of effector functions (5), we interrogated this ability by kinetically measuring IL-2, tumor necrosis factor alpha (TNFα), and IFNγ in cell culture supernatants (**Figures 2F–H**). ELISA analysis revealed a reduced ability to secrete all three cytokines. The phenotype we observed was due to specific targeting of PFKFB3, as treatment of BDC2.5 splenocytes with the prototypical glycolysis inhibitor 2-Deoxy-D-glucose (2-DG), a non- metabolizable glucose analog, was only capable of dampening IFNγ secretion, but not IL-2 or TNFα (**Supplementary Figure 1**). However, activation of BDC2.5 splenocytes with two other known PFKFB3 inhibitors YN1 and PFK158 recapitulated our data with PFK15 (**Supplementary Figure 1**), further implicating glycolysis, and more specifically PFKFB3, as a vital metabolic pathway required for optimal activation and cytokine secretion by autoreactive effector T cells (**Figures 2F–H**).

Targeting Glycolysis Delays the Onset of T1D in an Adoptive Transfer Model

Based on the ability of PFK15 treatment to reduce BDC2.5 T cell responses, we examined the impact of glycolysis inhibition on the onset of diabetes using an adoptive transfer model (**Figure 3A**). Here, isolated $CD4^+$ T cells from the spleens of NOD.BDC2.5.TCR.Tg animals were activated and expanded *ex vivo* with plate- bound αCD3/αCD28 and EL-4 supernatant as a source of IL-2 as previously described (28). We confirmed activation by measuring proinflammatory cytokine secretion in culture supernatants 3 days post activation, and observed significant levels of TNFα and IFNγ in BDC2.5 T cell cultures prior to adoptive transfer (**Supplementary Figure 2**). Activated T cells were transferred *via* intraperitoneal (i.p.) injection into immunodeficient NOD.*scid* recipients. A cohort of animals received 25 mg/kg of PFK15 treatment beginning on the day of the adoptive transfer. Animals were treated every other day for two weeks, and monitored for drug related toxicity (body weight) and diabetes onset (BG ≥ 350 mg/dl). 100% of control animals exhibited diabetes 7 days post-transfer, however PFK15 treatment delayed disease onset, with 57% of animals remaining diabetes free for the duration of the study, with no appreciable weight loss observed (**Figures 3B, C**). Pancreases from control animals exhibited invasive insulitis, while protected animals treated with PFK15 displayed peri- islet insulitis as observed *via* H&E staining (**Figures 3D, E**). In agreement with this, immunofluorescent (IF) staining for the T cell marker CD3 revealed reduced T cell infiltration into the islets of PFK15 treated animals corresponding to an inability for T cells to

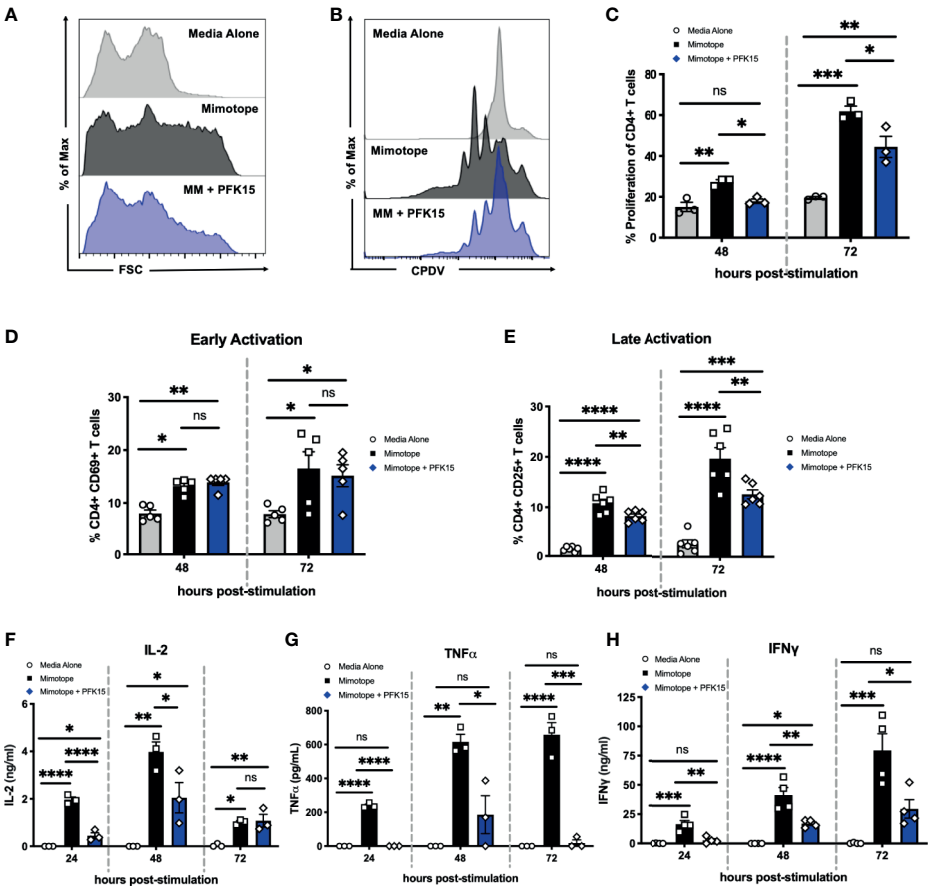

FIGURE 2 | Inhibiting glycolysis suppresses CD4$^+$ T cell responses to β cell antigen *in vitro*. Assessing the impact of glycolysis inhibition on diabetogenic CD4$^+$ T cell responses *in vitro*. **(A)** Representative histogram measuring forward scatter (FSC). **(B, C).** Representative histogram and statistical analysis of CD4$^+$ T cell proliferation assessed by cell proliferation dye violet (CPDV) dilution (n = 3). **(D)** Frequency of CD4$^+$ CD69$^+$ T cells (n = 5). **(E)** Frequency of CD4$^+$ CD25$^+$ T cells (n = 6). **(F–H)** ELISA analysis of IL-2, TNFα, and IFNγ in cell culture supernatants 24-72 hrs post stimulation (n = 3-4). All data are presented as the mean ± SEM. (not significant (ns), *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001).

completely penetrate the islets (**Figures 3F, G**). As expected, loss of insulin staining was observed in diabetic controls, with retention of insulin staining observed in PFK15 treated animals, correlating with disease status (**Figures 3H, I**). Lastly, PFK15 treatment had no impact on circulating CD4$^+$ T cell frequencies in the peripheral blood, however reduced CD4$^+$ T cell percentages were observed in the spleens of treated animals, indicating reduced expansion of PFK15 treated T cells (**Figure 3J**). Analysis of CD25 on CD4$^+$ T cells in control and PFK15 treated animals revealed a significant decrease in the percentage of CD4$^+$ CD25$^+$ T cells in the periphery, however a higher percentage of CD4$^+$ T cells expressed CD25 in the spleens of treated animals compared to controls (**Figure 3K**). Although a larger percentage of CD4$^+$ T cells in the spleens of treated animals expressed CD25, treated animals had less CD4$^+$ T cell percentages in the spleens, indicating possible sequestration of effector- like T cells in the spleen compared to control animals, as glycolysis is required for proper T cell migration to sites of inflammation (29). Together, these data indicate that metabolic modulation by PFK15 treatment alters the diabetogenic potential of activated CD4$^+$ T cells, thereby reducing the immunopathological parameters associated with disease onset.

PFK15 Treatment Increases the Expression of Checkpoint Molecules PD-1 and LAG-3 on CD4$^+$ T Cells

In the tumor microenvironment (TME), metabolic restriction leads to increased IR expression and subsequent T cell exhaustion (6). To investigate the mechanisms leading to T cell hyporesponsiveness and protection in PFK15 treated animals, we measured known markers of exhaustion, PD-1 and LAG-3, in the peripheral blood and spleens from control and PFK15 treated animals. Consistent with a hyporesponsive phenotype, PFK15 treatment led to significantly increased frequencies and expression of PD-1$^+$ (**Figures 4A–C**) and LAG-3$^+$ CD4$^+$ T cells (**Figures 4D–F**) in the peripheral blood and spleens. Since T cell exhaustion is associated with increased expression of multiple immune inhibitory receptors (IRs), we assessed co-

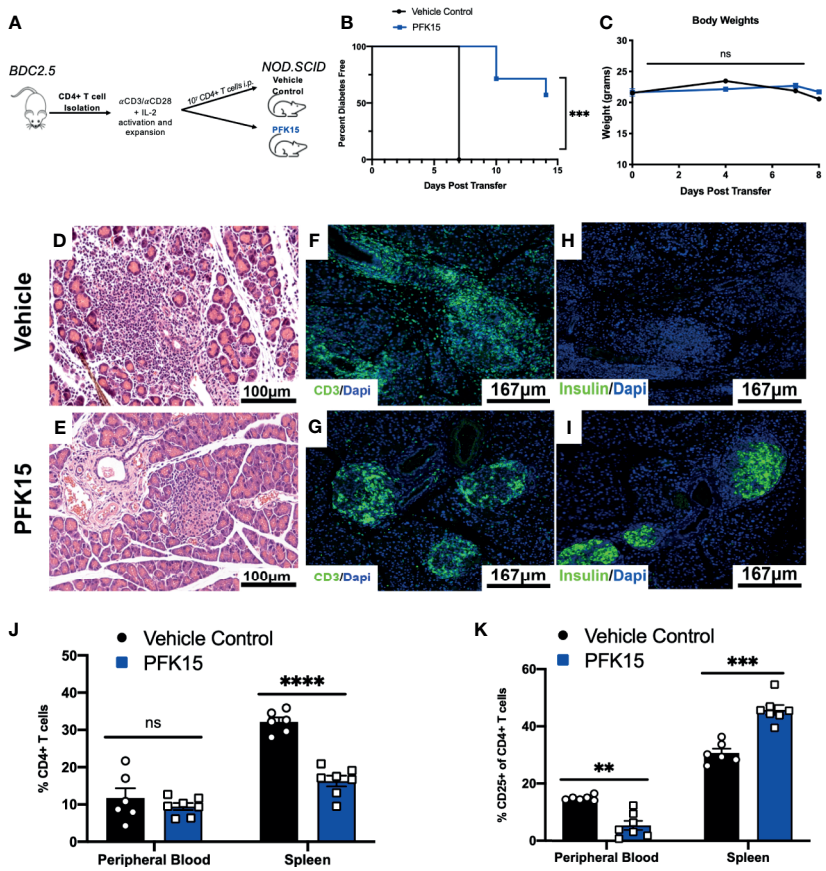

FIGURE 3 | PFK15 treatment alters the diabetogenic potential of autoreactive CD4$^+$ T cells and delays adoptive transfer of Type 1 Diabetes. The effect glycolysis inhibition has on Type 1 Diabetes onset was assessed using an adoptive transfer model. **(A)** Schematic diagram of experimental design for adoptive transfer studies. **(B)** Survival analysis of diabetes incidence in vehicle control and PFK15 treated groups. Kaplan-Meier survival analysis test was performed for statistical significance. **(C)** Body weight measurements. **(D, E)** Representative H&E staining to assess islet infiltration in pancreatic sections. **(F, G)** Representative pancreatic tissue immunostaining for the T cell marker CD3 co-stained with DAPI. **(H, I)** Representative pancreatic tissue immunostaining for insulin co-stained with DAPI. **(J)** Frequency of CD4$^+$ T cells in the peripheral blood and spleens of PFK15 treated and control animals. **(K)** Frequency of CD4$^+$ CD25$^+$ T cells in the peripheral blood and spleens. All data are presented as the mean ± SEM. (n = 6-7 animals/group; (not significant (ns), **p < 0.01, ***p < 0.005, ****p < 0.0001)).

expression of PD-1 and LAG-3 and found that T cells from PFK15 treated animals retained high expression of both PD-1 and LAG-3 on circulating CD4$^+$ T cells and T cells in the spleens (**Figures 4G, H**). Effector T cells transiently upregulate checkpoint molecules early during activation to temper their initial response and clonal burst (7, 30). To phenotypically characterize differences between PD-1$^+$ LAG-3$^+$ T cells in control and PFK15 treated animals, we measured the percentage of PD-1$^+$ LAG-3$^+$ T cells expressing the activation marker, CD25. Strikingly, we observed a higher percentage of PD-1$^+$ LAG-3$^+$ T cells in the peripheral blood of control animals expressing CD25, indicative of an effector phenotype, while PD-1$^+$ LAG-3$^+$ T cells in PFK15 treated animals had decreased CD25 expression, consistent with an exhaustion phenotype (**Figure 4I**). Similar to our analysis of CD25 on CD4$^+$ T cells in **Figure 3K**, no differences in CD25 expression were observed in the spleen of control and PFK15 treated animals, providing further evidence for sequestration of effector like T cells due to the importance of both glycolysis and PD-1 signaling in T cell trafficking (29, 31). Finally, to determine the impact glycolysis inhibition had on T cell responses in the pancreas, we stained pancreatic sections for PD-1. Indeed, PFK15 treated animals displayed increased PD-1 staining in the pancreatic islets compared to diabetic control animals (**Figures 4I, J**). These results demonstrate that PFK15 treatment results in increased frequency of CD4$^+$ T cells expressing checkpoint molecules PD-1 and LAG-3 *in vivo*, suggesting that inhibition of glycolysis induces potential T cell exhaustion thereby contributing to delayed T1D onset.

Modulating Glycolysis Leads to Functional and Metabolic Exhaustion of Diabetogenic CD4$^+$ T Cell Clones

To further substantiate evidence of T cell exhaustion induction by PFK15 treatment, we performed mechanistic studies *in vitro* using the BDC2.5 T cell clone maintained on a 2-week restimulation schedule (22–25). This 2- week period allows for

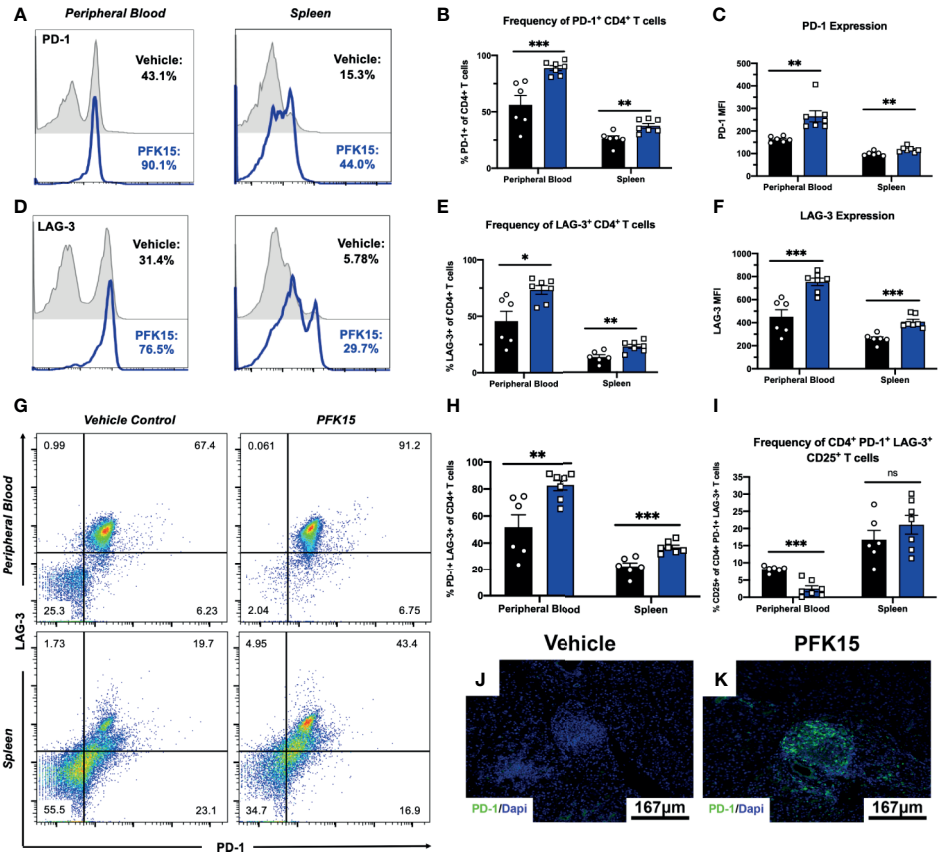

FIGURE 4 | Inhibition of glycolysis results in increased expression of PD-1 and LAG-3 on $CD4^+$ T cells. PD-1 and LAG-3 expression were assessed on $CD4^+$ T cells in PFK15 treated and control animals. **(A, B)** Representative histogram and statistical analysis measuring the frequency of PD-1^+ $CD4^+$ T cells in the peripheral blood and spleen of PFK15 treated and control animals. **(C)** Statistical analysis of PD-1 expression (MFI) on $CD4^+$ T cells in the peripheral blood and spleens. **(D, E).** Representative histogram and statistical analysis measuring the frequency of LAG-3^+ $CD4^+$ T cells in the peripheral blood and spleen of PFK15 treated and control animals. **(F)** Statistical analysis of LAG-3 expression (MFI) on $CD4^+$ T cells in the peripheral blood and spleens of PFK15 and control animals. **(G, H)** Representative flow plots and statistical analysis measuring PD-1 and LAG-3 co-expression on $CD4^+$ T cells in the peripheral blood and spleen of PFK15 treated and control animals. **(I)** Statistical analysis of the frequency of PD-1^+ LAG-3^+ $CD4^+$ T cells expressing CD25. **(J, K)** Representative pancreatic tissue immunostaining for PD-1 co-stained with DAPI. All data are presented as the mean ± SEM. (n = 6-7 animals/group; (not significant (ns), *p < 0.05, **p < 0.01, ***p < 0.005)).

the treatment of T cells with PFK15 on a similar regimen to our *in vivo* study. Consistent with our *in vivo* data, sustained PFK15 treatment of BDC2.5 T cell clones significantly increased the expression of PD-1 and LAG-3 alone on $CD4^+$ T cells (**Figures 5A–C**). However, while a hallmark of T cell exhaustion is the expression of checkpoint molecules, expression of these proteins alone is not indicative of exhaustion, as these molecules are upregulated transiently on the surface of newly activated T cells (7). We confirmed this by kinetically measuring PD-1 and LAG-3 co-expression on days 4, 8, and 14 post stimulation on $CD4^+$ T cells from untreated and PFK15 treated flasks (**Figure 5D**), and quantified the percentage of $CD4^+$ T cells co-expressing both PD-1 and LAG-3 on day 14 (**Figure 5E**). BDC2.5 T cells upregulated both PD-1 and LAG-3 early (Day 4) after activation, however by Day 8 most untreated $CD4^+$ T cells began to downregulate expression of the checkpoint molecules as predicted, with an even further downregulation evident by Day 14 (**Figures 5D, E**) (30). Interestingly, PFK15 treated T cells displayed increased and sustained expression of PD-1 and LAG-3 over the course of the 14-day restimulation, with a majority of T cells expressing both PD-1 and LAG-3 on Day 14 (**Figures 5D, E**). Concomitant with this, PFK15 treated T cell clones had decreased expression of CD25 compared to control flasks, confirming that PD-1 and LAG-3 expression on PFK15 treated T cells occurred independently of late T cell activation (**Figures 5F, G**).

During exhaustion, progressive loss of function occurs in a hierarchical manner, with high proliferative capacity and IL-2 production lost first, followed by a reduced ability to produce TNFα and IFNγ (7, 30, 32). We assessed these parameters in order to link PD-1 and LAG-3 expression with functional measures of T cell fitness. While control BDC2.5 T cells expanded 9-fold, treated T cells proliferated significantly less, with a mean 5-fold expansion (**Figure 5H**). Coinciding with this, we measured IL-2 production in culture supernatants on days 8 and 14 post stimulation by ELISA and found significantly more

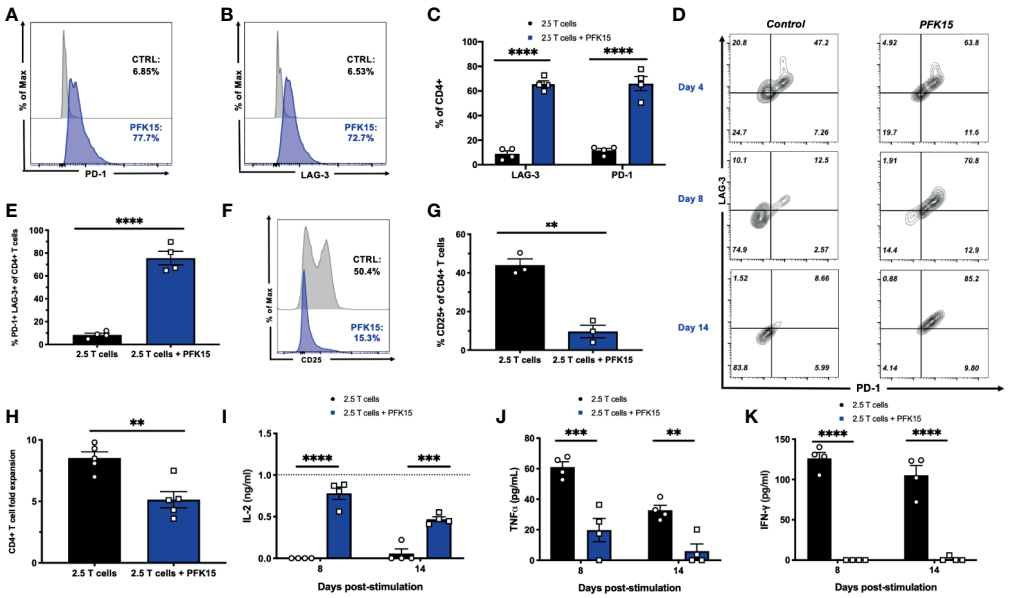

FIGURE 5 | PFK15 treated T cell clones are functionally exhausted. BDC2.5 T cell clones were treated with PFK15 over the course of a 2- week restimulation period to perform mechanistic studies investigating whether glycolysis inhibition induces functional exhaustion of $CD4^+$ T cells. **(A–C)** Representative histograms and statistical analysis assessing PD-1 and LAG-3 expression on control and PFK15 treated BDC2.5 T cell clones (n = 4). Cells were gated on $CD4^+$ T cells. **(D)** Representative flow plots measuring PD-1 and LAG-3 co-expression on PFK15 treated and control BDC2.5 T cell clones on days 4, 8, and 14 post stimulation. Cells were gated on $CD4^+$ T cells. **(E)** Statistical analysis of the percentage of BDC2.5 T cells co-expressing PD-1 and LAG-3 in control and treated flasks on Day 14 (n = 4). **(F, G)** Representative histogram and statistical analysis of the frequency of control and PFK15 treated BDC2.5 T cells expressing CD25 (n = 3). Cells were gated on $CD4^+$ T cells. **(H).** Fold expansion of treated and control BDC2.5 T cells (n = 5). **(I-K).** ELISA analysis of IL-2, TNFα, and IFNγ in culture supernatants on days 8 and 14 post stimulation (n = 4). All data are presented as the mean ± SEM. (**p < 0.01, ***p < 0.005, ****p < 0.0001).

IL-2 accumulated in cultures treated with PFK15 (**Figure 5I**), indicating a reduced ability to consume IL-2 as a growth factor compared to control T cell cultures (**Figures 5F, G**). Reduced consumption of IL-2 was likely due to the significantly decreased expression of the high affinity IL-2 receptor CD25 observed in PFK15 treated T cell cultures (**Figures 5F, G**). Further, the levels of IL-2 in PFK15 treated BDC2.5 cultures were similar to the amount of IL-2 supplemented into restimulation cultures from EL-4 supernatant (gray dotted line on the graph). We also measured the effector cytokines TNFα and IFNγ in cell culture supernatants on days 8 and 14 post stimulation, both of which were significantly reduced upon treatment with PFK15 (**Figures 5J, K**). In sum, these data indicate that targeting glycolysis leads to severe exhaustion of diabetogenic $CD4^+$ T cell clones, and suggest this as the mechanism by which PFK15 treatment delays T1D onset *in vivo* (**Figure 3**).

Functional exhaustion of T cells is associated with downstream metabolic consequences. Generally speaking, exhausted T cells are thought to be metabolically deficient, with a majority of metabolic flux supporting cell survival, and limited reserve for fueling effector functions (33). As PFK15 treated T cell clones were functionally exhausted, we wanted to determine whether this phenotype correlated with decreased metabolic fitness as has been reported in the literature (33–35). To begin our investigation, we measured relative adenosine diphosphate (ADP) and ATP levels, and calculated the ADP/ATP ratio from Day 14 control and PFK15 treated T cell clones. While there was no difference in the relative levels of ADP, relative ATP levels were significantly reduced upon PFK15 treatment, indicating decreased metabolic flux (**Figures 6A, B**). While control T cells had a low ADP/ATP ratio, indicative of cell proliferation, PFK15 treated T cell clones had a significantly higher ADP/ATP ratio, indicating an inability to generate ATP efficiently and metabolic insufficiency (**Figure 6C**). To characterize the reduced metabolic capacity of PFK15 treated T cells, we measured indicators of glycolysis, fatty acid oxidation (FAO), and mitochondrial metabolism. As expected, PFK15 treated T cells had downregulated levels of the key glycolysis proteins Glut-1, HK2, PFKFB3, and LDHA and reduced supernatant lactate levels as compared to control BDC2.5 T cell clones, confirming an inability to engage in glycolysis upon encounter with β cell antigen (**Figures 6D, E**).

PD-1 signaling has been reported to promote FAO, with early exhausted T cells having increased carnitine palmitoyltransferase 1α (CPT1α) expression, a rate limiting enzyme that regulates mitochondrial fatty acid transport (36). To investigate FAO, we first measured fatty acid uptake utilizing a BODIPY C16 fluorescent analog, and found no significant difference in uptake of fatty acids in Day 14 control or PFK15 treated T cells (**Figure 6F**). CPT1α expression was measured by western

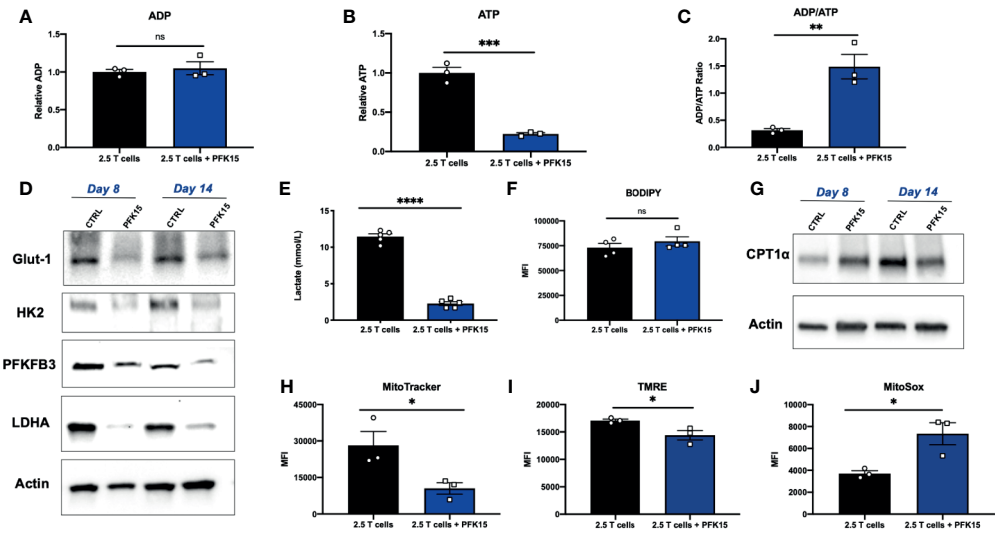

FIGURE 6 | Glycolysis inhibition during activation renders CD4$^+$ T cells metabolically insufficient. Assessing metabolic consequences of PFK15 treatment on BDC2.5 T cell clones. All analyses were performed 14 days post stimulation (Day 14), unless otherwise noted. **(A–C)** Relative ADP, ATP levels, and the ADP/ATP ratio were measured from Day 14 control and treated BDC2.5 T cells (n = 3). **(D)** Representative western blots for glycolysis proteins in control and PFK15 treated BDC2.5 T cell clones on days 8 and 14 post stimulation. **(E)** Lactate measurements in cell culture supernatants (n = 5). **(F)** Statistical significance of BODIPY fatty acid uptake of Day 14 control and PFK15 treated BDC2.5 T cells (n = 4). Cells were gated on CD4$^+$ T cells. **(G)** Representative western blot analysis of CPT1α expression in control and PFK15 treated BDC2.5 T cell clones on days 8 and 14 post stimulation. Cells were gated on CD4$^+$ T cells. **(H)** Statistical analysis of mitochondrial mass by MitoTracker green staining on Day 14 control and PFK15 treated T cell clones (n = 3). Cells were gated on CD4$^+$ T cells. **(I)** Statistical analysis of mitochondrial membrane potential by TMRE staining on Day 14 control and PFK15 treated T cell clones (n = 3). Cells were gated on CD4$^+$ T cells. **(J)** Statistical analysis of mitochondrial ROS by MitoSOX staining on Day 14 control and PFK15 treated T cell clones (n = 3). Cells were gated on CD4$^+$ T cells. All data are presented as the mean ± SEM. (not significant (ns), *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001).

blot analysis, and revealed increased expression of CPT1α in Day 8 PFK15 treated T cells, but decreased levels in treated Day 14 clones compared to untreated control T cells (**Figure 6G**). Together, these results indicate that by Day 8 control T cell clones are engaging in glycolysis in response to presentation of β cell antigen (**Figure 6D**) while PFK15 treated T cells are utilizing FAO. Although fatty acid uptake was unaltered, reduced CPT1α expression was observed in PFK15 treated clones compared to controls on Day 14, demonstrating a reduced ability to efficiently transport fatty acids into the mitochondria (**Figures 6F, G**), and likely contributing to the inefficient ATP generation we observed (**Figure 6B**).

A number of reports demonstrate that exhausted T cells have reduced mitochondrial fitness (34, 35, 37–40). To investigate the mitochondrial health of PFK15 treated T cells, we measured mitochondrial mass, reactive oxygen species (ROS), and mitochondrial membrane potential. Indeed, PFK15 treated T cell clones exhibited mitochondrial dysfunction as demonstrated by reduced mitochondrial mass (**Figure 6H**), decreased mitochondrial membrane potential (**Figure 6I**), and increased generation of mitochondrial ROS when compared to control T cells (**Figure 6J**). The mitochondrial dysfunction observed supports the reduced ATP levels measured in PFK15 treated T cells even when uptake of fatty acids was unaffected (**Figures 6F, G**), further pointing to inefficient utilization of nutrients and overall metabolic insufficiency. All in all, these data indicate that inhibition of glycolysis during the activation of autoreactive CD4$^+$ T cell clones enforces an exhausted phenotype that mediates protection from T1D onset *in vivo*.

PFK15 Treated CD4$^+$ T Cells Are Terminally Exhausted

Exhausted T cell lineages display heterogeneity amongst subsets with unique characteristics and varying abilities to become reinvigorated (41, 42). To determine the state of exhaustion observed in PFK15 treated T cells, we performed reinvigoration experiments where a subset of BDC2.5 T cell clones were treated with PFK15 every third day for two weeks to induce exhaustion. Then, T cells from control or PFK15 treated flasks (PFK15 treated T cells put into restimulation cultures termed PFK15 T_{EX}) were restimulated for another two weeks without further PFK15 treatment (**Figure 7A**). We first measured PD-1 and LAG-3 expression on PFK15 T_{EX} cells after restimulation and found that PFK15 T_{EX} sustained high expression of both PD-1 and LAG-3 compared to control cultures (**Figure 7B**), consistent with retention of an exhausted phenotype. Notably, PFK15 T_{EX} were unresponsive to IL-2 present in restimulation cultures, further confirming exhaustion and ruling out anergy (33). We also measured lactate and IFNγ in cell culture supernatants on days 8 and 14 post restimulation as indicators of re-engagement in the glycolysis pathway upon activation and effector function. We observed reduced lactate production and little secretion of IFNγ by PFK15 T_{EX} in response to restimulation, suggesting that treated T cell clones are terminally exhausted (**Figures 7C, D**).

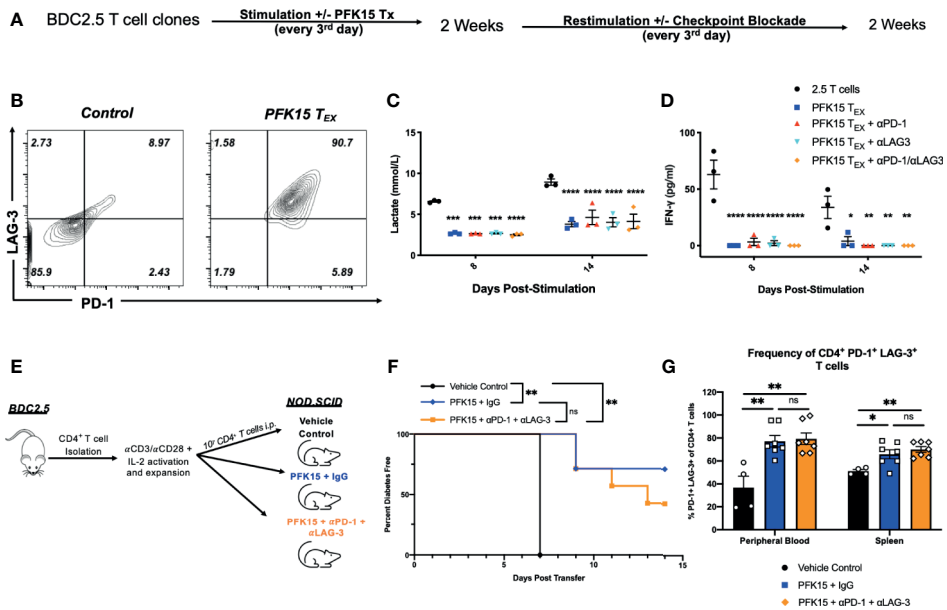

FIGURE 7 | Inhibition of glycolysis leads to terminal exhaustion of CD4$^+$ T cells that are refractory to checkpoint blockade. To determine the degree of exhaustion induced by PFK15 treatment, PFK15 treated T cell clones were restimulated ± checkpoint blockade. **(A)** Schematic diagram of experimental design for reinvigoration studies. **(B)** Representative flow plots of PD-1 and LAG-3 expression on CD4$^+$ T cells on control and PFK15 T_{EX}. **(C)** Statistical analysis of lactate secretion in Day 8 and 14 restimulation culture supernatants (n = 3). **(D)** ELISA analysis of IFNγ in culture supernatants on days 8 and 14 post stimulation (n = 3). **(E)** Schematic diagram of experimental design for *in vivo* reversibility studies. **(F)** Survival analysis of diabetes incidence in vehicle control and PFK15 treated groups. Kaplan-Meier survival analysis test was performed for statistical significance. **(G)** Statistical analysis measuring PD-1 and LAG-3 co-expression on CD4$^+$ T cells in the peripheral blood and spleen of vehicle control (n = 4), PFK15 + IgG (n = 7), and PFK15 + αPD-1 + αLAG-3 (n = 7) treatment. All data are presented as the mean ± SEM. (not significant (ns), *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001).

Reports have demonstrated that terminally exhausted T cells are refractory to checkpoint blockade (33, 34, 37). To determine whether PFK15 T_{EX} cells were responsive to checkpoint blockade, a subset of restimulation cultures were treated with αPD-1, αLAG-3, or a combination of both αPD-1/αLAG-3 blocking antibodies as described in the methods. We measured lactate and IFNγ in cell culture supernatants to determine whether checkpoint blockade treatment would rescue PFK15 T_{EX} cell's ability to utilize glycolysis and exert their effector function (**Figures 7C, D**). Neither αPD-1, αLAG-3, nor a combination of αPD-1 and αLAG-3 blocking antibody treatments were capable of rescuing PFK15 T_{EX} ability to respond to self- antigen, evidenced by significantly less lactate and IFNγ secretion measured in culture supernatants of PFK15 T_{EX} ± PD-1 and/or LAG-3 blockade compared to control T cells (**Figures 7C, D**). To confirm the induction of terminal exhaustion *in vivo*, we performed reversibility experiments using our adoptive transfer model, described in **Figure 3A**. Briefly, ex vivo activated BDC2.5 T cells were adoptively transferred into NOD.*scid* recipients, with recipient animals being separated into one of three treatment cohorts: 1) vehicle control, 2) PFK15 + IgG, and 3) PFK15 + αPD-1 + αLAG-3 (**Figure 7E**). Animals in the PFK15 treatment groups were treated every other day for two weeks, with IgG or checkpoint blockade treatment initiated in the second week. Notably, the dose of αPD-1 and αLAG-3 blocking antibodies administered have been previously shown to accelerate diabetes onset in NOD animals, therefore any delay or protection achieved with PFK15 treatment would be considered durable if irreversible with checkpoint blockade (20). As expected, 100% of control animals displayed fulminant diabetes by day 7 post- transfer, with significant delays associated with PFK15 treatment (**Figure 7F**). While 70% of animals receiving PFK15 + IgG remained diabetes free through the end of the study period, 42% receiving PFK15 + αPD-1 + αLAG-3 treatment were protected from disease (**Figure 7F**). Although more animals receiving checkpoint blockade succumbed to diabetes than PFK15 + IgG treated animals, diabetes incidence between these two groups were not statistically significant (p = 0.348), indicating an inability to reverse PFK15 induced T cell exhaustion. Finally, regardless of αPD-1 and αLAG-3 blockade, CD4$^+$ T cells in PFK15 treated animals retained high expression of PD-1 and LAG-3 in the periphery and spleens compared to control animals, consistent with an exhausted phenotype (**Figure 7G**). Together, these data support the findings that inhibition of glycolysis in diabetogenic CD4$^+$ T cells leads to terminal exhaustion, characterized by functional and metabolic dysfunction that is irreversible by restimulation or checkpoint blockade therapy *in vitro* and *in vivo*. This work demonstrates that the rate- limiting glycolysis enzyme PFKFB3 is a novel target for controlling autoreactive T

cell activation as a means to protect against the onset of T1D by enforcing exhaustion of pathogenic T cells.

DISCUSSION

In the present study, we evaluated the ability of the anti-glycolytic PFK15 to control the activation of diabetogenic $CD4^+$ T cells in T1D. To our knowledge, this is the first study testing the ability of a PFKFB3 inhibitor to prevent the onset of autoimmune diabetes. Our findings confirmed PFK15 inhibited glycolysis upon activation of $CD4^+$ T cells (**Figure 1**), dampened autoreactive $CD4^+$ T cell responses *in vitro* (**Figure 2**), and delayed disease onset *in vivo* (**Figures 3**, **4**). The protective benefits associated with PFK15 treatment are not entirely surprising as glycolysis is required for $CD4^+$ T cell activation and IFNγ secretion. In fact, targeting T cell metabolism has been successfully used to prevent and reverse disease in other autoimmune diseases, albeit without mediating durable tolerance (14–17, 43). However, our findings demonstrate an induction of terminal exhaustion by glycolysis inhibition, which to our knowledge, has not been previously reported in the literature.

Previously, use of the prototypical glycolysis inhibitor 2-DG in SLE, RA, and MS failed to generate a long- lasting benefit, as cessation of treatment was associated with disease flare-ups (14–17). Dissimilarities in the observed outcomes between our study and others is likely due to differences in the mechanisms of inhibition. 2-DG is a glucose analog that indirectly targets the action of HK2 through competition with endogenous glucose levels (44). For this reason, an effective reduction in glycolytic flux requires high dose treatments, which are associated with adverse effects and non- specific targeting (44). In comparison, PFK15 is highly selective for a defined intracellular enzyme, thus requiring a much lower concentration for effective inhibition (44). These key differences appear to have drastically different outcomes on the T cell response, and the data included in **Supplementary Figure 1** confirm this, as treatment of BDC2.5 splenocytes with 2-DG *in vitro* was only able to reduce IFNγ secretion, and failed to recapitulate PFK15's ability to dampen IL-2 and TNFα (**Supplementary Figure 1**). Strikingly, our data demonstrate an ability for PFK15 treated T cells to become early activated (**Figure 2D**). 2-DG treatment, however, leads to reduced CD69 expression upon activation, indicating maintenance of a quiescent phenotype that is reversible when treatment is stopped (17). The early activation observed in our model supports the need for T cells to lineage commit to effector subsets in order to induce a terminal phenotype. Finally, PD-1 and LAG-3 expression are induced upon TCR signaling, therefore early activation is required to upregulate IRs that ultimately render PFK15 treated T cells exhausted (**Figures 4**, **5**) (7, 30).

Although often overlooked, availability of nutrients is vital to maintaining T cell fitness. As described herein, T cells and cancer cells have a shared reliance on aerobic glycolysis, which becomes problematic in the TME when tumor cells metabolically restrict T cells, thus eliciting poor anti- tumor immunity (6, 34). Analogously, our data supports the idea that reduced glycolytic flux promotes T cell exhaustion, as PFK15 treatment induced defective effector responses upon activation (**Figures 2**, **5**). Glycolysis restriction, however, is not the only metabolic pathway dysregulated by TILs. Notably, TILs demonstrate a progressive loss of mitochondrial mass and function (34, 35, 37, 40). This, along with a low glucose environment, promote a state of metabolic insufficiency due to an inability to meet nutrient requirements; thus, leading to a permanent hyporesponsive state (6, 34, 35, 37, 40). Unexpectedly, we too observed mitochondrial dysfunction when glycolysis was inhibited (**Figure 6**). Although other factors in the TME contribute to repressed mitochondrial function, particularly hypoxia, our data strengthens the link between nutrient restriction and T cell exhaustion since glycolysis inhibition led to the development of metabolic insufficiency (45).

While the development of T cell exhaustion is detrimental in cancer and chronic infection, the opposite is true in autoimmunity, where induction of a hyporesponsive phenotype protects the host from attack (8). The onset of autoimmunity in T1D occurs due in large part to defective central and peripheral tolerance mechanisms that fail to control pathogenic T cells. This defect is due to dysregulated IR expression in T1D (46–48). In healthy individuals, binding of IR proteins to their associated ligand and subsequent downstream signaling act as a metaphorical "brake" that impedes T cell activation and protects against autoimmunity (7, 8, 30). Evidence of the importance of IRs is underscored by the accelerated diabetes observed in the absence or blockade of PD-1 or LAG-3 in NOD mice (20, 48, 49). Clinically speaking, polymorphisms in the PD-1 gene have been identified and associated with disease susceptibility (47). Concomitantly, T1D patients fail to upregulate PD-1 on T cells compared to control subjects, correlating to aberrant T cell activation and effector function (46, 50). In our study, PFK15 treatment led to increased PD-1 and LAG-3 expression on $CD4^+$ T cells that correlated with protection from diabetes onset (**Figure 3**). Importantly, expression of IRs alone is not sufficient to induce T cell exhaustion, since activated T cells transiently upregulate both PD-1 and LAG-3 upon early activation (7, 30). In fact, exhaustion can occur even in the absence of checkpoint molecules, further complicating the role these molecules play in enforcing and maintaining functional exhaustion (34). While IRs may play a lesser role in driving exhaustion in other disease settings, our data reveal a pivotal role for PD-1 and LAG-3 in maintaining tolerance against autoimmune responses. In conclusion, we have demonstrated a unique ability to correct defects in peripheral tolerance mechanisms in T1D by inducing PD-1 and LAG-3 expression on $CD4^+$ T cells. Increased IR expression and glycolysis restriction led to the functional and metabolic exhaustion of diabetogenic T cells independent of PD-1 and LAG-3 signaling, as checkpoint blockade failed to reverse this phenotype (**Figure 7**).

Therapeutic strategies for T1D have focused on two specific areas: 1) β cell replacement *via* regeneration of endogenous β cell

mass or 2) immunomodulation (51, 52). Although innovative efforts have been made to restore β cell mass, these strategies ultimately fail due to reemergence of the autoimmune response (52). Moreover, although immunomodulation has yielded positive results in preclinical studies, success in the clinic has remained limited. Unfortunately, present clinical studies have only administered immunotherapies to patients with diagnosed T1D. By diagnosis, T1D patients have endured longstanding autoimmunity, with significant β cell loss, thus highlighting a need to intervene prior to symptomatic disease (1). Interestingly, the presence of autoantibodies is known to be a strong predictor for disease onset (53, 54). In fact, the first in man prevention study was published recently, where the αCD3 antibody teplizumab was utilized in patients at risk for diabetes development (51). Teplizumab delayed T1D onset by 2 years, and similarly to our study, protection was associated with increased expression of IRs and induction of a T cell hyporesponsive state (51). However broad immunosuppression was observed in teplizumab treated patients, which is an adverse effect of most immunotherapies due to non- specific targeting. A potential benefit of modulating glycolysis is the ability to specifically target activated T cells, while leaving established memory T cells and regulatory T cell populations unaffected based on their reliance on alternative metabolic pathways (4, 55–57). Clinically speaking, we would anticipate glycolysis inhibition to delay or prevent disease onset, while use of PFK15 in conjunction with methods to restore β cell mass may be a novel way to reverse T1D.

In summary, these findings demonstrate an ability to induce terminal exhaustion of autoreactive T cells in T1D by modulating the glycolysis pathway *via* targeting of PFKFB3. On a broader note, these data support a key role for glucose utilization in T cell activation and function, since an inability to efficiently metabolize glucose enforces a hyporesponsive phenotype. In our study, this phenotype was associated with expression of PD-1 and LAG-3, which are known to be dysregulated in T1D patients. This study remains focused on the ability to restrain diabetogenic $CD4^+$ T cells due to their importance in the initiation of autoimmunity. While we would anticipate an overall benefit to other mediators of autoimmunity in T1D, like $CD8^+$ T cells, further investigations are required to fully understand the impact glycolysis inhibition would have on other immune cell subsets. However, with the ability to restore tolerance in preclinical studies, we anticipate the use of metabolic modulators, like PFK15, may have a beneficial impact in both the clinical prevention and reversal of disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CM and JP conceived the project. CM, DP, KC, and JP, designed experiments. CM, LN, EO'C, DP, KC, and IT, performed experiments and/or analyzed data. KC and SS-L provided assistance, expertise, and reagents for histology experiments. CM wrote the manuscript; CM, DP, KC, SS-L, and JP contributed to and edited the manuscript. All authors contributed to the article 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.2021.669456/full#supplementary-material

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REVIEW
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The N-Formyl Peptide Receptors and Rheumatoid Arthritis: A Dangerous Liaison or Confusing Relationship?

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Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by a progressive symmetric inflammation of the joints resulting in bone erosion and cartilage destruction with a progressive loss of function and joint deformity. An increased number of findings support the role of innate immunity in RA: many innate immune mechanisms are responsible for producing several cytokines and chemokines involved in RA pathogenesis, such as Tumor Necrosis Factor (TNF)-α, interleukin (IL)-6, and IL-1. Pattern recognition receptors (PRRs) play a crucial role in modulating the activity of the innate arm of the immune response. We focused our attention over the years on the expression and functions of a specific class of PRR, namely formyl peptide receptors (FPRs), which exert a key function in both sustaining and resolving the inflammatory response, depending on the context and/or the agonist. We performed a broad review of the data available in the literature on the role of FPRs and their ligands in RA. Furthermore, we queried a publicly available database collecting data from 90 RA patients with different clinic features to evaluate the possible association between FPRs and clinic-pathologic parameters of RA patients.

Keywords: rheumatoid arthritis, formylpeptide receptors, rheumatoid arthritis histopathotypes, pattern recognition receptors, innate immunity

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by a progressive symmetric inflammation of the joints resulting in bone erosion and cartilage destruction with a progressive loss of function and joint deformity (1). The major clinical characteristic of RA is joint swelling reflecting inflammation in the synovial membrane (2). Extra-articular symptoms such as pulmonary manifestations (e.g., lung nodules, pleural effusion, and interstitial lung disease), vasculitis, keratoconjunctivitis, hematological abnormalities (e.g., anemia, leukopenia, thrombocytopenia, or thrombocytosis), rheumatic nodules, and lymphomas are also possible, especially in later stages of the disease (1, 3, 4). It has been hypothesized that RA likely occurs in genetically predisposed subjects due to a combination of genetic, epigenetic, and environmental

factors initiated by a stochastic event such as an infection or tissue injury (2). These triggering factors may activate the previously generated autoreactive B and T cells leading to a disruptive tolerance resulting in tissue damage (1). The tissue destruction presents as inflammation of the joint capsule (synovitis) with the expansion of the synovial membrane (pannus) that may lead to periarticular bone erosions and cartilage degradation. This chronic joint inflammation is promoted and maintained by several different cell type; the cellular composition of RA synovitis includes features of both innate (e.g., monocytes, dendritic cells (DCs), mast cells, and innate lymphoid cells) and adaptive (e.g., T helper cell (Th) 1, Th17, B cells, plasmablasts, and plasma cells) immunity, together with fibroblasts, and osteoclast (2). Hence, in RA inflammatory process, both innate and adaptive immunity are pivotal pathogenetic actors. An increased number of findings supports the role of innate immunity in RA; indeed, many innate immune mechanisms are responsible for the production of a significant proportion of cytokine and chemokine synthesis involved in RA pathogenesis, such as Tumor Necrosis Factor α (TNFα), interleukin (IL)-6, and IL-1 (5, 6). In addition, macrophage numbers and the detection of TNFα in the synovial tissue of patients with RA are good predictors of the clinical course of the disease (7) and anti-cytokine therapy effectiveness. There has been a longstanding hypothesis that infection plays a role in triggering pathways that leads to RA. Molecules of bacterial or viral origin have been found in the joints of patients with RA (5, 8, 9), where they can trigger inflammatory reactions through pattern recognition receptors (PRRs).

PRRs are non-specific "sensors" of pathogen-associated (PAMPs) or damage-associated molecular patterns (DAMPs), playing a crucial role in modulating the activity of the innate arm of the immune response (10, 11). Different classes of PRRs have been characterized: the most studied being the Toll-like receptors (TLRs) and the nucleotide oligomerization domain-like receptors (NLRs) (10, 11). Our group focused its attention over the years on the expression and functions of a specific class of PRR, namely formyl peptide receptors (FPRs) (12). FPRs, like many other PRRs, are constitutively expressed on several cell types, including immune and epithelial cells (12). They sustain immune cell recruitment and activation (12) and regulate wound healing and homeostasis of epithelia (13–16).

A key function in modulating the inflammatory response has been defined for FPRs: they are classically able to sustain the inflammatory response, but, as a function of the context and/or the agonist, they can intervene in the resolution of the inflammatory response (17–19). This activity seems to be common to other PRRs (20, 21). This key role of FPRs in modulating the induction, the amplification, and the following physiologic resolution phase of the inflammatory responses prompted some research groups to study FPRs role in diseases whose pathogenesis is strictly linked to a strong imbalance between the inflammation and its resolution. TLRs and NLRs have already been defined as important for the pathogenesis of ankylosing spondylitis, psoriatic arthritis, systemic lupus erythematosus (SLE), RA, osteoarthritis (OA), and gout (22).

In the present review, we will focus on the data available in the literature on the role of FPRs and their ligands in RA, and we will discuss the results obtained querying a publicly available database collecting data from 90 RA patients with different clinical features (23).

FORMYL PEPTIDE RECEPTORS

FPRs are a group of G protein-coupled (GPCRs) chemoattractant receptors with an important role in host defense and inflammatory response (24). The *FPR* gene family can vary significantly in different mammalian species: the FPRs family includes FPR1, FPR2, and FPR3 in humans, and mFPR1, mFPR2/3, mFPR-rs1, mFPR-rs3, mFPR-rs4, mFPR-rs5, mFPR-rs6, and mFPR-rs7 in mice (25). The three genes encoding receptors mFPR1, mFPR2, and mFpr-rs1 are the best characterized. Although the complex evolution of the FPR gene family caused a high divergence between species orthologs, FPR1 is considered the mouse ortholog of human FPR1. Mouse FPR2 is a low-affinity receptor for N-formyl-methionyl-leucyl-phenylalanine (fMLF) and can be activated by several agonists of human FPR2 and FPR3. Further studies also indicate that mouse Fpr-rs1 share pharmacologic properties with human FPR2. The biological functions of other mouse *FPR* gene family members have not been clearly determined (25).

FPRs are mainly expressed in several types of innate immune cells, including neutrophils and monocytes/macrophages. In detail, macrophages express all three receptors (26, 27); neutrophils, monocytes, and natural killer cells express FPR1 and FPR2, but not FPR3 (26, 28); immature DCs express FPR1 and FPR3, while mature DCs express FPR3, but not FPR1 and FPR2 (29). The activation of FPRs in these cells induces chemotactic migration, phagocytic activity, and reactive oxygen species (ROS) production, mediating innate defense activity (25, 30). FPRs expression has also been reported in adaptive immune cells such as native CD4 T cells, human tonsillar follicular helper T cells, Th1 cells, Th2 cells, and Th17 cells (31).

Non-immune cells also express FPRs. For example, FPR1 is found in astrocytes, microglial cells, hepatocytes, and lung cells (32). FPR2 is the more ubiquitously expressed of the group, and it is found in synovial fibroblasts (33, 34), keratinocytes (35), brain cells, hepatocytes, microvascular endothelial cells (24), endocrine glands, intestinal epithelial cells (36, 37) and human bone marrow-derived mesenchymal stem cells (38–40). FPR3 is the least well-known of the three receptors, and its biological role has not been completely elucidated. This receptor is mainly expressed on monocytes and DCs, and it is located in intracellular vesicles rather than on the cell surface like the other FPRs (28, 41).

Our group described FPRs expression on basophils (42), gastric (16), and nasal (43) epithelial cells, and on fibroblasts (44).

FPRs, especially FPR1 and FPR2, have been shown to play a role in the development of several pathological conditions, such as neoplasms and inflammatory diseases. FPRs may act differently in these processes, both promoting and suppressing

the disease progression. For example, FPR1 has a dual role in cancer development, playing a promoting role in glioblastoma (45, 46) and, conversely, tumor-suppressing functions in gastrointestinal cancers (19, 37, 47).

Contradictory findings have also been observed dealing with the relationship between FPRs activation and infection response. For example, constitutively active FPRs were indispensable in the defense against the formation of biofilms by *Candida albicans* and aggressive infiltration by *Vibrio harveyi* (48, 49). Further studies are needed to elucidate this complex and apparently contradictory role to identify the different factors influencing FPRs behavior. However, one of the elements that may explain FPRs protean activity is that FPRs respond to various ligands with diverse classifications. Although most FPRs ligands are involved in the clearance of infections, mediating chemotactic migration and phagocytic activity, other ligands activate pro-resolving, anti-inflammatory pathways (24, 49). This duality in modulating inflammatory mechanisms is better expressed by FPR2, depending on ligand-specific conformational changes resulting in the switch between FPR2-mediated pro- and anti-inflammatory cell responses. In detail, it has been suggested that the binding of anti-inflammatory ligands such as Annexin A1 (AnxA1) caused FPRs to form homodimers, which led to the release of inflammation-resolving cytokines like IL-10; conversely, inflammatory ligands such as serum-amyloid alpha (SAA) did not cause receptor homodimerization (50). Generally, bacterial and mitochondrial formylated peptides are among those that classically activate a proinflammatory cell response, while AnxA1 and Lipoxin A4 (LXA4) are some of the better-known anti-inflammatory FPR2 ligands (49, 51). Many of these FPR2 ligands have also been suggested to play a promoting or protective role in RA. For example, SAA may induce several proinflammatory cytokines such as TNFα, IL-1β, IL-6, and matrix metalloproteinases-1 and -3, suggesting a role through the interaction with FPR2 in bone and cartilage destruction observed in RA (52). In turn, other FPR2 ligands such as AnxA1, LXA4, and Compound 43 (Cpd43) seem to exert a protective role in RA (**Figure 1**).

Through the years, several reports investigated the role of the FPRs or of specific members of the receptor family in the pathogenesis of RA. Gripentrog and coll. tried to establish the correlation between different FPR haplotypes and the pathogenesis of RA by analyzing 74 Caucasian RA patients and 74 controls. Although a specific FPR haplotype (i.e., 16A) was found only in the RA population, the authors had to conclude that only minor differences in haplotype distributions could be observed. It has to be taken into account, the low numbers of samples analyzed prevented from obtaining any conclusions regarding RA association to FPRs due to the lack of statistical power (53).

Other studies were conducted by evaluating the effects of receptor knock-out in mice models of arthritis or investigating the therapeutic effects of different FPR-agonists. The conclusion should consider the different experimental models used since several protocols are available to induce arthritis, but each involves different predominant mechanisms sustaining the joint inflammation and damage. We will in detail present the data published, presenting the evidence obtained by analyzing a specific component of the receptor/ligand system.

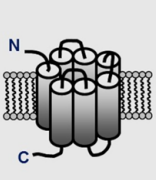
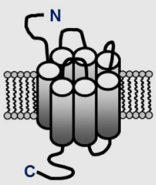
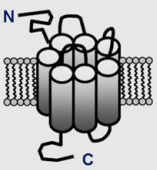
	FPR1	FPR2	FPR3
			
Pro-inflammatory role	fMLF (> 10^{-8} M) WKYMVm	Serum Amyloid A fMLF (> 10^{-5} M) uPAR84-95 WKYMVm	uPAR84-95 WKYMVm
Anti-inflammatory role	EC3 EC10	Annexin A1 Compound 43 Lipoxin A4 Scolopendrasin IX EC3 Compound 2a	EC3
Unknown role	Compound 43		

FIGURE 1 | Pro-inflammatory and anti-inflammatory N-formyl peptide receptors (FPRs) ligands in rheumatoid arthritis. FPRs respond to various ligands with diverse classifications. Although most of FPRs ligands are involved in the clearance of infections, mediating chemotactic migration and phagocytic activity, other ligands activate pro-resolving, anti-inflammatory pathways. N-formyl-methionyl-leucyl-phenylalanine (fMLF); Trp-Lys-Met-Val-D-Met hexapeptide (WKYMVm), pyridazin-3(2H)-one derivative EC3 (EC3), pyridazin-3(2H)-one derivative EC10 (EC10); urokinase plasminogen activator receptor 84-95 (uPAR84–95).

FPRS AND THEIR LIGANDS IN RA PATHOGENESIS

Few groups investigated the role of FPRs in the modulation of chronic inflammatory conditions underlying RA pathogenesis. Indeed, FPRs are essential in the activation of inflammation but are also fundamental in its resolution. Most studies focused on the potential therapeutic effects of FPR2 activation mediated by its anti-inflammatory agonists. No direct data are available in the literature on the FPR1 role in RA.

Formyl Peptide Receptor 2

Mice deficient in FPR2/3, the homologous to human FPR2, was used as a key model to address the role of FPR2 in the pathogenesis of arthritis (17). K/BxN serum transfer model in C57BL/6 mice induced arthritis by transfer of autoantibodies to glucose-6-phosphate isomerize. This model allows the generation of a synovial inflammation involving the participation of macrophages and neutrophils and the production of IL-1 and TNFα. $FPR2^{-/-}$ mice displayed an exacerbation of arthritis symptoms following K/BxN serum transfer (17), supporting the evidence that, in particular, FPR2 could mediate anti-inflammatory effects (54) that could control RA pathogenesis.

Annexin A1

Several reports regarding the protective role of FPRs in RA have been focused on the role of its ligand, AnxA1. AnxA1 is an endogenous anti-inflammatory mediator, exerting its inflammation-resolution functions by interacting with FPR2 (55).

AnxA1 has been found to be expressed in human RA synovial tissue (56–58) and has been identified as an important endogenous anti-inflammatory mediator in several animal models of RA (59).

AnxA1 and FPR2, but not FPR1, are in particular expressed by fibroblast-like synoviocytes (FLS) (54), the major cells promoting RA. It has been demonstrated that AnxA1 and FPR2 reduced FLS proliferation in an ERK and NF-kB-dependent manner and suppressed proinflammatory cytokine production from FLS (54).

In a different study, Dufton and coll. examined the effect of AnxA1 on T cell activation and differentiation and its implications for RA development, demonstrating that AnxA1 increases T cell activation in a Th1 sense. In collagen-induced arthritis (CIA) model, they administered AnxA1 to mice immediately after immunization with collagen for 12 days to evaluate the effects of AnxA1 in the early phase of RA development in which the Th1 phenotype is critical. These experiments showed that AnxA1 could increase arthritis symptoms if administered during the immunization phase of the CIA (60). Furthermore, an analysis of AnxA1 expression in T cells from RA patients and controls revealed higher protein expression levels in patients with RA than controls (60).

The authors discuss that the results mentioned above are only in apparent contrast with that obtained in AnxA1 null mice displaying an increased arthritic response. This could be due to the different etiology of joint damage and the different kinetics of the two models employed (CIA here, and antigen-induced arthritis in AnxA1 null mice) (54, 60). In the antigen-induced arthritis model, the Met-BSA induced arthritis through a Th2-response, demonstrating a protective AnxA1 role in RA (61). In the CIA model, a Th2-response reduction was described in the absence of Anx-A1, being this consistent with a Th2 pathogenic role in RA. The authors suggest to re-derivate the Anx-A1-null mice on the appropriate background to clearly define the role of Anx-A1 in the CIA model (60).

Lipoxin A4

Lipoxin A4 (LXA4) is an endogenous lipoxygenase-derived mediator produced from arachidonic acid and exerting potent anti-inflammatory and pro-resolving effects on various cell types by activating FPR2 (62). Lipoxin A4 has been shown to suppress FLS production of proinflammatory cytokines and reduce RA severity in a CIA model (63).

Compound 43

Cpd43 is a low molecular weight compound acting as an agonist for FPR2, although it has been reported to interact also with FPR1. Cpd43 exerts anti-arthritic effects in a model of K/BxN serum transfer. In particular, Cpd43 was demonstrated to be able to i) suppress TNFα expression in the joint; ii) inhibit osteoclast differentiation; iii) inhibit cytokine production in human FLS and macrophages in culture (54). Blocking FPR2, but not FPR1, abolished Cpd43 effects supporting the evidence that its protective role in the RA model is due to FPR2 (54).

A different study presents the results of Cpd43 administration to mice with CIA or antigen-induced arthritis (AIA). Cpd43 was able to reduce arthritis severity in both models: in CIA, Cpd43 decreased CD4 T cell proliferation and survival; in AIA, it increased CD4 T cell apoptosis. While inhibiting CD4 Th2 T cell proliferation and activity, Cpd43 was also able to increase the proportion of protective regulatory T cells (64). Furthermore, in both models, Cpd43 decreased TNF-sustained FLS proliferation (64).

Scolopendrasin IX

An antimicrobial peptide - scolopendrasin IX - was identified from *Scolopendra subspinipes mutilans* used in the oriental medicine as a remedy for RA. This peptide acts as an agonist to FPR2 and showed therapeutic effects in RA by inhibiting cytokine production and neutrophil recruitment into the joint.

The administration of scolopendrasin IX in K/BxN serum-injected mice significantly decreased paw thickness, the clinical score of inflammatory arthritis, and markedly ameliorated joint destruction. The results obtained by Park et al. suggest that scolopendrasin IX was effective against inflammatory arthritis by blocking joint destruction (65). Scolopendrasin IX administration was also demonstrated to inhibit neutrophils recruitment into the synovium and their activation mediation by LPS (65).

FPRs Agonists With Pyridinone and Pyrimidindione Scaffolds

Dr. Crocetti et al. identified three compounds with pyridinone and pyrimidindione scaffolds able to bind and activate, although with different affinities, the FPR family members. The pyridazin-3(2H)-one derivative EC3 (EC3) is a mixed FPR1/FPR2/FPR3

agonist; the pyridazin-3(2H)-one derivative EC10 (EC10) acts as an agonist to FPR1; and compound 2a is the most potent ligand identified with a 10-fold preference for FPR2 (66). The authors evaluated the therapeutic activity of the three compounds using a rat model of RA. All three compounds ameliorated the clinic of RA by increasing the pain threshold and reducing pain hypersensitivity (66).

Serum Amyloid A

As mentioned above, FPR2 in humans can mediate both pro- and anti-inflammatory signals depending on the specific ligand (25). Among the pro-inflammatory FPR2 agonist, the role of Serum Amyloid A (SAA) in synovial damage has been investigated (33, 52, 67). It has been demonstrated that FLS, endothelial cells, and macrophages isolated from the synovial tissue of patients with RA patients expressed increased levels of SAA and FPR2 (52). In culture, SAA sustains FLS proliferation and survival (33), stimulates metalloproteases production by FLS (52), stimulates the proliferation, migration, and tube formation of endothelial cells (33). Finally, SAA induces in rheumatoid synoviocytes the expression of Pentraxin 3 (PTX3), an acute-phase reactant involved in amplifying the inflammatory response (67). This evidence, taken together, sustains the pathogenic role of SAA in RA.

FPRs Agonists fMLF, uPAR84–95, and WKYMVm Peptide

We have recently demonstrated (44) that fibroblasts obtained from skin biopsies of patients affected by Systemic Sclerosis (SSc) express all three receptors for the N-formyl peptides. The expression of these receptors was highly increased compared to normal skin fibroblasts both at mRNA and protein levels. In addition, we conducted experiments using specific agonists [i.e., fMLF, urokinase plasminogen activator receptor 84-95 (uPAR84–95), and Trp-Lys-Met-Val-D-Met hexapeptide (WKYMVm)], demonstrating that upon stimulation, SSc fibroblasts from affected subjects were able to proliferate, migrate, and transform into a myofibroblast phenotype as assessed by ROS generation, matrix deposition, and α-smooth muscle actin (α-SMA) overexpression as compared to normal skin fibroblasts. In order to evaluate whether FPRs stimulation plays a role in some ROS-mediated processes such as tissue remodeling and fibrosis, we then conducted experiments on BJ normal fibroblasts showing that FPRs stimulation led to Rac1 and ERKs activation, promoting $gp91^{phox}$ and $p67^{phox}$ expression as well as a direct interaction between GTP-Rac1 and $p67^{phox}$ (68). However, the possible involvement of the FPRs in other more common autoimmune conditions such as RA has been only partially confirmed.

ASSOCIATION OF FPRS WITH CLINIC-PATHOLOGIC PARAMETERS OF RA PATIENTS

Rheumatoid Arthritis Histopathotypes

Heterogeneity in the quality and quantity of the synovial cellular infiltrate is well recognized, and it has been evaluated as a possible biomarker of treatment response in patients with RA (69). Recently published data reporting cellular and molecular analyses of synovial tissue from a cohort of 144 patients with treatment-naïve early RA demonstrated for the first time the presence of three pathology groups: i) lympho-myeloid dominated by lymphoid lineage infiltration (T cells, B cells, plasma cells) in addition to myeloid cells; ii) a diffuse-myeloid group characterized by macrophage or monocyte enrichment, but poor in B cells/plasma cells; and iii) a pauci-immune fibroid group showing a distinct lack of immune-inflammatory infiltrate and prevalent stromal cells (70). They also demonstrated that synovial cellular and molecular signatures define prognostic and treatment phenotypes, such as the response to disease-modifying antirheumatic drug (DMARD) therapy, clinical outcome, and radiographic joint damage (70). Moreover, integrating histological and molecular signatures into a clinical prediction model may help predict whether patients will require biological therapy. For instance, recent data by Lliso-Ribera and colleagues suggest that the lympho-myeloid pathotype, with a dense synovial infiltrate enriched in B cells and significant upregulation of T/B cell genes at disease onset, predicted poor outcome with the need for biological therapy irrespective of clinical classification (71). This evidence is in line with recently published data in early RA that reports the association between the lympho-myeloid pathotype with highly aggressive disease and worse radiographic outcomes (70). The analysis of the synovial histopathology has also been evaluated as a helpful tool to identify among clinically indistinguishable patients those with a lower probability of response to TNFα-blockade (69), especially the pauci-immune pathotype could predict an inadequate response to treatment with TNFα antagonists. In a recent study, Lewis and colleagues (23) analyzed the histology and RNA-seq of synovial biopsies from a large cohort of early treatment-naïve patients [the Pathobiology of Early Arthritis Cohort (PEAC)]. From this larger cohort, they selected 90 individuals meeting the 1997 ACR classification criteria for early RA to identify the three histological pathotypes and reveal gene modules associated with RA severity and clinical outcome. They analyzed gene expression changes at the RNA sequencing level in both blood and synovium from the same RA patient and identified transcriptional endotypes in the synovium linked to the three distinct pathotypes. They also combined RNA-seq with detailed synovial histology and correlated these molecular signatures with clinical and imaging phenotype data at disease presentation. Finally, the authors developed a data exploration website (available at https://peac.hpc.qmul.ac.uk/) to dissect gene signatures across synovial and blood compartments, integrated with deep phenotypic profiling. Herein, we used the data exploration website developed by Lewis and colleagues (23) for describing the gene expression of FPRs in both blood and synovium in patients with early RA.

FPRs Are Differently Expressed in the Distinct Rheumatoid Arthritis Pathotypes

As shown by the RNA sequencing (RNA-seq) analysis of synovial biopsies and blood of patients data available from PEAC (http://www.peac-mrc.mds.qmul.ac.uk) (23), the three

FPR receptors show a different expression in the distinct RA pathotypes: the fibroblastic pauci-immune pathotype, the macrophage-rich diffuse-myeloid pathotype, and the lympho-myeloid pathotype, suggesting different pathogenic pathways or activation disease states.

FPR1-lymphoid expression at synovial level was greater in comparison to the expression in the other pathotypes, while at blood level, FPR1 expression showed similar mean values in lymphoid, myeloid, and fibroid subgroups. In addition, the mean gene expression of FPR1 was greater in blood than that observed in the synovial samples

FPR2 expression showed an opposite pattern compared to that of FPR1. Indeed, at the synovium level, FPR2 genes were less expressed in all the pathotypes as compared to blood expression (**Table 1**). Moreover, the mean gene expression was markedly lower in synovial samples of all pathotypes than the mean expression of FPR1 (**Table 1**).

FPR3 mean gene expression was higher in comparison to FPR1 and FPR2 (**Table 1**), and it was upregulated in the lympho-myeloid pathotype.

The different expression of the FPRs genes in the three histologically identified subgroups can be visualized though the 3D volcano plot (**Figure 2**). FPR1 gene using the 3D volcano plot was depicted in blue, showing that this receptor was upregulated in the lympho-myeloid pathotype and the fold change was significant compared to the other groups ($r=0.828$ $p=5.99^{-6}$). FPR2 was depicted in grey, demonstrating a not significant difference in the expression in the pathotypes. FPR3 was upregulated in the lympho-myeloid, as confirmed by the primary color blue, which identifies the lympho-myeloid

TABLE 1 | FPRs mean gene expression in synovial and blood samples.

	FPR1		FPR2		FPR3	
	Synovial	**Blood**	**Synovial**	**Blood**	**Synovial**	**Blood**
Lymphoid	11.23	14.47	6.94	12.53	12.28	5.12
Myeloid	10.75	14.52	6.60	12.59	11.92	4.89
Fibroid	9.88	14.18	6.61	12.35	11.22	5.17

Adapted from http://www.peac-mrc.mds.qmul.ac.uk.

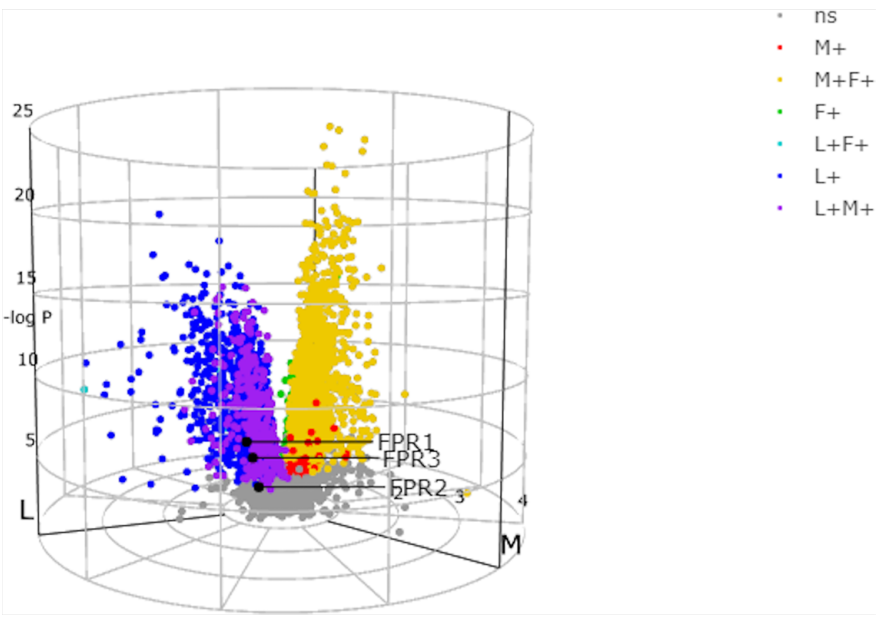


FIGURE 2 | N-formyl peptide receptors (FPRs) expression in the three histologically identified subgroups using the 3D volcano plot. In the three-way volcano plots genes, which were significantly upregulated in one group alone, were colored using primary colors, blue in the lympho-myeloid group (L+), red in the diffuse-myeloid group (M+), and green in the pauci-immune fibroid (F+). Moreover, genes upregulated in two groups (compared to the minimum reference group) were illustrated using secondary colors, i.e., genes upregulated in lympho-myeloid and diffuse-myeloid compared to pauci-immune fibroid: purple; upregulated in diffuse-myeloid and pauci-immune fibroid versus lympho-myeloid: yellow; upregulated in lympho-myeloid and pauci-immune fibroid versus diffuse-myeloid: cyan. Non-significant genes (ns) are colored gray. FPR1 and FPR3 are colored in blue, FPR2 gene is colored in gray. Adapted from http://www.peac-mrc.mds.qmul.ac.uk.

group. The fold change, used as an alternative to the Z score (indicating the vectors for pathotype per gene), showed that the upregulation of FPR3 was significant compared to the other groups (r=0.979, $p=3.71^{-5}$).

Lewis and colleagues (23) demonstrated a stark difference in the absolute quantity of differentially expressed transcripts among the pathotypes, with nearly 3,000 transcripts in synovium compared to only 8 differentially expressed transcripts in corresponding peripheral blood. All the three receptors FPRs, at blood level, showed a non-significant expression between the subgroups as demonstrated by the genes colored in grey (**Figure 3**) (23, 70, 72).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The currently available literature concerning the relationship between FPR1, FPR3, and RA is scarce. By analogy to the role played in other autoimmune diseases such as SSc (44), we can suppose that FPR1 exerts a potentially proinflammatory role in RA. However, very little work has been done to further explore this connection. Data related to FPR3 have been only partially evaluated since FPR3 is the least well-known of the three receptors, and its biological role has not been completely elucidated. FPR2 is the more ubiquitously expressed of the FPRs (26). Given its potentially protective role in RA, the interest in FPR2 and its ligands has recently grown. Indeed, a better understanding of the complex interaction between FPR2 and its ligands may help establish these molecules as potential therapeutic interventions.

In this review, we have widely discussed the potential effects and roles of FPRs ligands in different pathological models. However, this study has some limitations. Data related we extrapolated querying the online database developed by Lewis and colleagues (23), referred only to the gene expression of FPRs in both blood and synovium in patients with early RA. No comparison on the healthy subject's basal levels of FPRs can be made. In addition, data from the interactive website (https://peac.hpc.qmul.ac.uk/) report synovial, and blood gene expression, but no data are available on protein expression.

In synovial biopsies that FPR1 and FPR3 were significantly increased in all pathotypes, whereas FPR2 showed an opposite pattern of expression, being less represented. The lower FPR2 gene expression in the RA cohort patients could be related to the protective role played by this receptor in the disease pathology. Indeed, several authors described that among the three members of the N-formyl peptide receptor family, FPR2 could mediate anti-inflammatory effects (54), playing a role in the pathogenesis of RA. Moreover, AnxA1, expressed in human RA synovial tissue (56–58), has been recognized as a significant endogenous anti-inflammatory mediator in several animal models of RA.

Conversely, FPR1 is expressed at a high level in the lympho-myeloid subgroup, which is related to a high disease activity measured by DAS28-ESR/CRP and confirmed by an aggressive radiological and radiographic involvement, causing a poor clinical and therapeutic outcome.

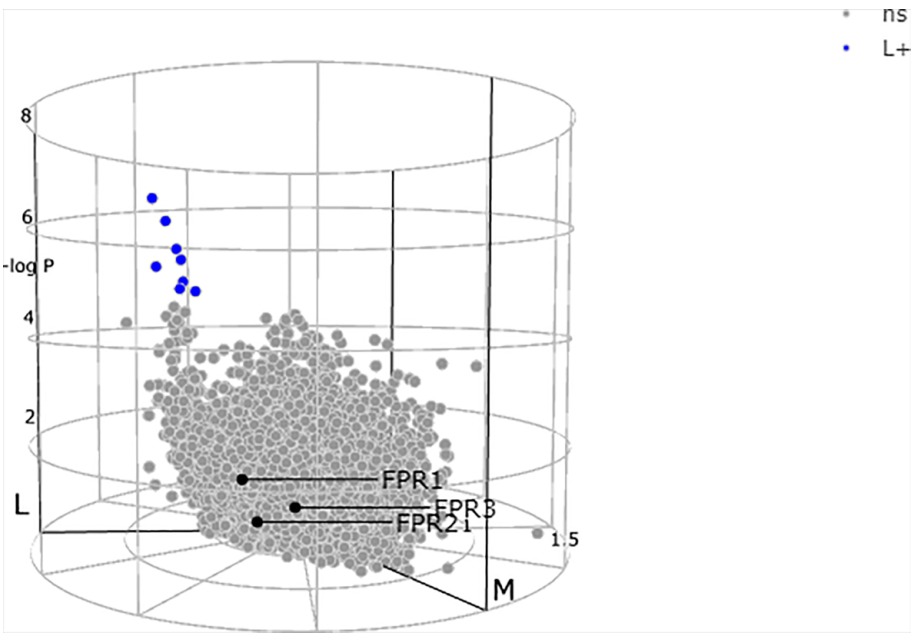


FIGURE 3 | N-formyl peptide receptors (FPRs) expression at blood level showing a non-significant (ns) expression between the subgroups as demonstrated by all the three genes colored in gray. Adapted from http://www.peac-mrc.mds.qmul.ac.uk.

In conclusion, FPRs are characterized by multifaceted roles that encourage researchers to target these receptors to treat several inflammatory and neoplastic diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. IM, NP, and FR participated in planning the study, analysis and interpretation of data, drafting the article, critical revision of the article for important intellectual content, final approval of the article. FG, VP, and AP participated in drafting the article, critical revision of the article for important intellectual content, final approval of the article.

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REVIEW
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Metabolic Control of Smoldering Neuroinflammation

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Compelling evidence exists that patients with chronic neurological conditions, which includes progressive multiple sclerosis, display pathological changes in neural metabolism and mitochondrial function. However, it is unknown if a similar degree of metabolic dysfunction occurs also in non-neural cells in the central nervous system. Specifically, it remains to be clarified (i) the full extent of metabolic changes in tissue-resident microglia and infiltrating macrophages after prolonged neuroinflammation (e.g., at the level of chronic active lesions), and (ii) whether these alterations underlie a unique pathogenic phenotype that is amenable for therapeutic targeting. Herein, we discuss how cell metabolism and mitochondrial function govern the function of chronic active microglia and macrophages brain infiltrates and identify new metabolic targets for therapeutic approaches aimed at reducing smoldering neuroinflammation.

Keywords: microglia, macrophages, metabolism, immunometabolism, mitochondria, smoldering inflammation, progressive multiple sclerosis

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INTRODUCTION

Cellular metabolism is at the foundation of all biological activities (1). While the metabolic processes that support cellular bioenergetics and survival have been extensively studied (2, 3), the role of metabolism in guiding complex cellular functions is yet to be completely understood. Extensive metabolic rewiring occurs in cells to adapt to the local microenvironment in physiological conditions (4), during development (5), and in conditions of disease (6), as cells try to preserve their functions under the shifting availability of energetic substrates.

In this review, we discuss how the regulation of nutrient uptake and consumption is regulated in myeloid cells, when instructed by physiological cues, and as they undergo polarisation in the context of neuroinflammation. Specifically, we highlight how the regulation of their metabolism changes homeostatic cell activities to guide cell activation and signalling in the persistently inflamed central nervous system (CNS).

SMOLDERING NEUROINFLAMMATION IN PROGRESSIVE MS

Multiple sclerosis (MS) is a chronic inflammatory condition of the CNS that is characterized by demyelination with axonal and neuronal degeneration (7). Most MS patients (~85%) present a relapsing-remitting course (RR), while the remaining ~15% show a primary progressive (PP) disease

course characterised by continuous neurological deterioration without definable relapses (8). As the disease evolves, the majority of RR MS patients also advance to a secondary progressive (SP) disease course, usually after 15–20 years from disease onset (9). Despite great successes in the development of therapies for RR MS and disease-modifying therapies that delay the conversion to SP MS (10), progressive MS patients still have limited treatment options (9, 11, 12). Unfortunately, effective treatment of progressive MS remains elusive due to the occurrence of specific degenerative mechanisms that characterize progressive MS, which are distinct from RR MS and are not sufficiently targeted by the approved immunomodulatory compounds (9).

In RR MS, active plaques predominate, and lesions show a diffuse perivascular and parenchymal T cell infiltration that is the substrate of clinical attacks (13). However, as the disease evolves, there is a shift from a T cell mediated adaptive immune response towards an innate immune activation (13, 14). In fact, progressive MS, like many other neurodegenerative CNS diseases [such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease], is characterized by a persistent state of CNS inflammation that is driven by myeloid cell activation (8, 15–18).

In progressive MS, myeloid cells are present in the normal appearing white matter (NAWM), in subpial cortical lesions, and, most importantly, in smoldering plaques (13, 19). Smoldering plaques are histopathologically defined as slowly expanding lesions that are characterized by a rim of activated myeloid cells and a slow expansion of the pre-existing plaque edge (19, 20). Here, increased activation of myeloid cells correlates with demyelination and axonal loss, leading to higher clinical disability in patients with progressive MS (8, 14, 18). Indeed, current magnetic resonance imaging (MRI) tools aimed at assessing chronic active and smoldering lesions have emerged as a diagnostic tool to predict secondary disease progression (21, 22), as well as clinical progression in PP MS patients (23).

These data suggest that a slowly expanding, myeloid-mediated, smoldering neuroinflammation is the core feature from which progression starts and evolves in MS. Therefore, understanding the mechanisms underpinning chronic myeloid cell activation in the CNS may hold the promise of identifying new targets to treat and/or delay disease progression (24).

MYELOID CELLS DYNAMICS IN NEUROINFLAMMATION

Far from being a homogenous cell population, the cellular makeup of CNS myeloid cells is instead spatially and temporally heterogenous, being under tight regulation by (patho)physiological cues that determine beneficial and/or detrimental immune cell activation (25). Recent single cell technologies have unveiled how the immune landscape of the brain drastically changes with fluctuations in the neuroinflammatory status (26).

In the healthy CNS, immune function is exclusively attributed to parenchymal and extra-parenchymal myeloid cells. Within the brain parenchyma, the most immune-privileged compartment of the CNS, the only resident myeloid cells are microglia (27). Microglia are specialized macrophages that are seeded into the brain from the extra-embryonic yolk sac during embryogenesis (28), and they have key roles in synaptic pruning, phagocytosis, and immune surveillance (29). During early CNS development, microglia are distinguished by several unique transcriptional markers (*Arg1*, *Rrm2*, *Ube2c*, *Cenpa*, *Fabp5*, *Spp1*, *Hmox1*, and *Ms4a7*) associated with cell cycle, phagocytosis, lipid metabolism, and surveillance, which highlights the ongoing maturation of these cells (30). Microglia exhibit dynamic heterogeneity that fluctuates throughout the life of the mouse with the highest diversity occurring in the developmental stage, followed by a decline during adulthood, and increased heterogeneity during CNS diseases (30). In fact, during CNS maturation, healthy adult parenchymal microglia lose their developmental heterogeneity and begin to express "homeostatic" markers (*P2ry12*, *Fcrls*, *C1qa*, *Selplg*, and *Tmem119*) related to lipid metabolism and immune cell interaction (30–32). Interestingly, this transition is seemingly regulated by Maf bZIP transcription factor B (MAFB), which controls myeloid cell differentiation and cellular responses to viral infection (31).

Within the CNS borders, such as the dural meninges, the cellular make-up is mostly dominated by T and B cells with a minority of cells constituting macrophages and monocytes. This diverse and complex immune surveillance network facilitates the interaction between lymphocytes and macrophages at specialized immune hubs located along the dural sinuses (33). Border-associated macrophages (BAMs) [also known as CNS-associated macrophages] localized in the leptomeninges, perivascular space, and choroid plexus, are responsible for immune surveillance, together with a small proportion of other immune cells, such as dendritic cells (DCs) and neutrophils (34). BAMs share similar transcriptional markers with microglia [*Aif1 (*encoding Iba1), *Csf1r*, and *Cx3cr1*] (30, 35) and some transcriptional signatures of developing microglia overlap with BAM clusters (*Ms4a7*, *Ccr1*, and *Mrc1*), possibly suggesting ongoing maturation status (30).

In the context of neuroinflammation, the brain immune landscape drastically changes. In mice affected by experimental autoimmune encephalomyelitis (EAE), an animal model of MS, the CNS is predominantly populated by short-lived infiltrating cells ($Ly6C^{hi}$ and $Ly6C^{lo}$ monocyte-derived cells [MdCs]) and T cells that infiltrate during the acute phase of disease through a "leaky" blood-brain barrier (BBB) (34). Here, BAM cell numbers decrease and their phenotype becomes more homogenous, with nearly all BAMs exclusively expressing MHCII and CD38 (34). At the peak of disease, microglia downregulate homeostatic markers and shift their phenotype towards a pro-inflammatory state whereby they overexpress IFN-γ-responsive genes [*H2*

(encoding MHCII) and *Sca1*], which imply increased microglia-T cell interactions. Exclusively to the peak phase of EAE, four sub-populations of disease associated microglia (DAM) emerge (daMG1-4), which are distinguished by their unique expression patterns of chemokines, cytokines, and cysteine proteases (36). Although all four populations are specific to EAE, only three are identified within demyelinating lesions (daMG2-4) and exhibit similar downregulation of homeostatic genes (*P2ry12*^{lo}, *Tmem119*^{lo}, *Md1*^{hi}). DaMG2 upregulates *Cd74*, *Ctsb*, and *Apoe* but proliferate less compared to the daMG3, whereas daMG3 expresses high levels of *Cxcl10*, *Tnf*, and *Ccl4*. Finally, daMG4 overexpress *Ccl5*, *Ctss*, and *Itm2b* (36). Further studies investigating myeloid cells in chronic EAE are required to understand whether these daMG profiles are transient. Nonetheless, similar findings are observed in the brain of patients with MS, where specific DAMs downregulate the expression of homeostatic genes (*TMEM119*, *P2RY12*, and *SLC2A5*) and upregulate *APOE* and *MAFB* in late-active demyelinating lesions (37). These clusters were highly enriched in *CTSD*, *APOC1*, *GPNMB*, *CD74*, *HLA-DRA*, and *HLA-DRB*, which further supports the notion of increased microglial heterogeneity during CNS insult not only in EAE but also in the MS brain (38). Of note, analogous DAM transcriptional changes are also confirmed in the 5xFAD animal model of AD, cuprizone-mediated demyelination, and facial nerve axotomy where downregulation of canonical microglial genes (*P2ry12/13*, *Cx3cr1*, *Tmem119*) is coupled with the upregulation of genes related to phagocytosis and lipid metabolism (*Apoe*, *Lpl*, *Cst7*, *Ctsd*, *Tyrobp*, and *Trem2*) (26).

Understanding how these unique, disease-specific, microglial phenotypes can be targeted to promote a beneficial phenotype that ultimately ameliorates smoldering CNS inflammation is a current research challenge that will certainly uncover new therapeutic avenues.

GLUCOSE AND GLUTAMINE METABOLISM

Strong evidence has revealed that changes in the reactive states of macrophages and microglia can be regulated by their cellular metabolism (24). How the unique metabolic environment of the brain regulates the effector function of myeloid cells in health and disease is only now starting to be uncovered.

The CNS has intrinsic high metabolic demands associated with neural activity, as ~20% of the body's glucose and oxygen is used by the CNS, despite only accounting for 2% of the total body weight (39). Glucose is shuttled from the blood *via* specialized glucose transporters (GLUTs) to provide fuel for cellular functions (40). Despite the high utilization of glucose by the CNS, only a small pool of nutrient reserves is stored as glycogen (41, 42). Therefore, tight regulation of glucose metabolism is critical for brain physiology, as disturbed glucose metabolism may contribute to several neurodegenerative diseases (43, 44).

Microglia require a large amount of energy to perform homeostatic functions. This is accomplished by microglia preferentially utilizing glucose as the main source of metabolic fuel, which is transported into microglia primarily by GLUT1, 3, and 5 (45, 46) to support oxidative metabolism. In oxidative metabolism, glucose is broken down into pyruvate through glycolysis, which is shuttled into the mitochondria where it is utilized by the tricarboxylic acid cycle (TCA) to drive oxidative phosphorylation (OXPHOS); ultimately producing adenosine triphosphate (ATP) (47) (**Figure 1**). Oxidative metabolism is the primary source for energy of microglia under homeostasis, as shown by transcriptomic analysis of *ex vivo* isolated mouse brain microglia, which express the full complement of genes required for both glycolytic and oxidative energy metabolism (48, 49). The ability of microglia to utilize glucose as a primary substrate for energy production has been mainly investigated *in vitro*. Primary rat microglia and the BV2 microglial cell line cultured in the presence of 2-deoxyglucose (2DG), which inhibits hexokinase 2 (HK2) and blocks glycolysis, leads to ATP depletion and cell death (50), indicating a reliance of microglia on glucose utilization for normal functioning. During experimental glucose starvation, primary microglia isolated from CD-1 IGS mice and the BV2 microglial cell line are able to maintain oxidative metabolism using other available substrates (glutamine, lactate, pyruvate, ketone bodies) (51). The reliance of microglia on oxidative metabolism through glucose has been recently confirmed *in vivo* in mice. Using endogenous fluorescence lifetime imaging (FLIM) of intracellular nicotinamide adenine dinucleotide phosphate (NADPH), as well as time-lapse two-photon imaging, microglia have been observed to maintain a glycolytic profile under resting conditions (52). Further, in conditions of aglycemia, microglia switches from glycolysis to glutaminolysis of extracellular glutamine, a major substrate for the generation of both excitatory and inhibitory neurotransmitters (53), in a mechanistic target of rapamycin (mTOR)-dependent manner to maintain OXPHOS and their immune surveillance functions (52). This suggests that microglia display bioenergetic versatility and, like peripheral macrophages, possess the ability to adapt its metabolic pathways to use substrates available in the local environment (51).

The ability of macrophages to shift or reprogram their metabolism to changes in the microenvironment is a key feature that underlies the complex, long-term changes of these cells under inflammatory conditions. In fact, immune cell polarization after inflammatory activation leads to drastic reprogramming of cellular metabolism pathways. A key finding is that macrophages exposed to an inflammatory stimulus shift their metabolism from oxidative metabolism to aerobic glycolysis (54), with a concomitant increase in the enzymatic activity of enzymes involved in glucose metabolism (55) and several transcription factors, including hypoxia-inducible factor-1 alpha (HIF1α) and mTOR (56) (**Figure 1**). This metabolic switch occurs when macrophages are first faced with an immune challenge to support and enable the rapid production of ATP (57) - regardless of the availability of oxygen - which is

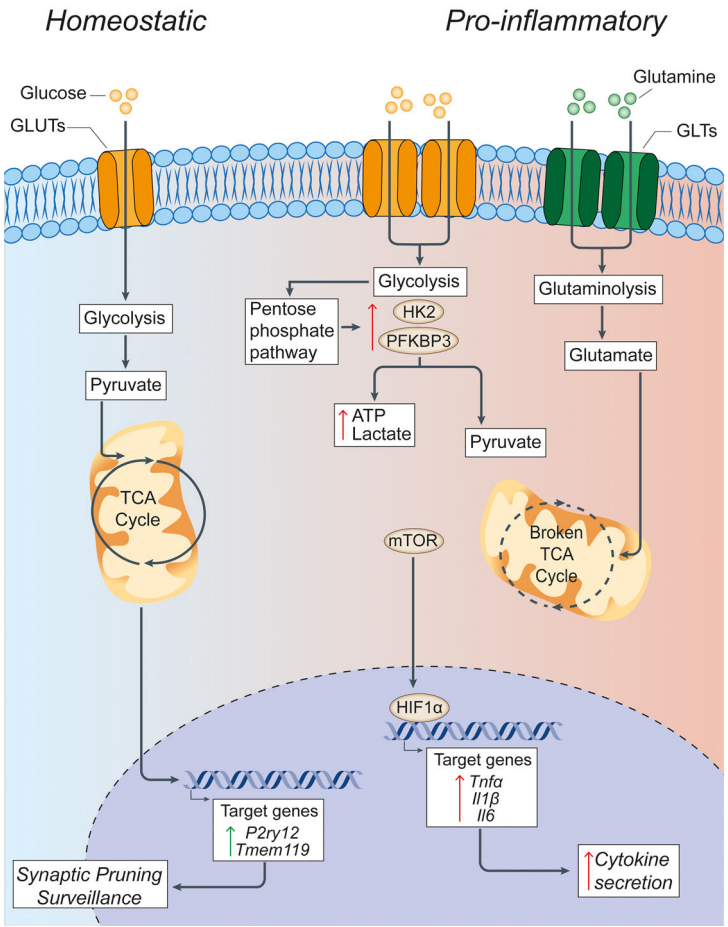

FIGURE 1 | Glucose metabolism in microglia under homeostatic and inflammatory conditions. Under homeostatic conditions, extracellular glucose is transported into microglial cells through specialized glucose transporters, where it is converted into pyruvate through cytoplasmic glycolysis. Pyruvate is then actively transported across the mitochondrial membrane to drive the TCA cycle. The energy and metabolites produced in the TCA cycle can then support the expression of the homeostatic microglial genes *P2ry12* and *Tmem119*, which facilitate microglial functions of synaptic pruning and immune surveillance. In pro-inflammatory conditions, microglia have a broken TCA cycle and increase the expression of membrane transporters to facilitate the uptake of glucose and glutamine, thus driving enhanced glycolysis and glutaminolysis. Glycolysis is supported by increased expression of the rate-limiting enzymes of glycolysis HK2 and PFKBP3. This leads to the increased generation of lactate and ATP to compensate for the broken TCA cycle, and shunting of metabolites into the pentose phosphate pathway. The increased glycolysis is sustained by the activation of nuclear transcription factors HIF1α and mTOR that support the synthesis and production of cytokines for secretion. Green arrows = homeostatic effects. Red arrows = pro-inflammatory effects. GLUT, glucose transporter; TCA, tricarboxylic acid cycle; GLT, glutamate transporter; HK2, hexokinase 2; PFKBP3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphate-3; HIF1α, hypoxia inducible factor 1 α; ATP, adenosine triphosphate; mTOR, mechanistic target of rapamycin; TNFα, tumor necrosis factor alpha; Il1β, interleukin-1β; Il6, interleukin-6.

similar to the Warburg effect described in cancer cells (58). Here, the increased consumption of glucose by activated macrophages leads to the generation of downstream products, such as glucose-6-phosphate and pyruvate, which feed into the pentose phosphate pathway (PPP) and TCA cycle, respectively. The PPP facilitates the synthesis of proteins, nucleotides, and reactive oxygen species (ROS) to support cellular function during immune challenge (59).

Despite the extensive study of metabolic reprogramming in macrophages during inflammation, very little emphasis has yet been placed on assessing metabolism in microglia under neuroinflammatory conditions. Early studies investigating the links between metabolism and microglial activation identified metabolic modifications similar to those observed in peripherally activated macrophages, such as a shift from oxidative metabolism towards a more glycolytic profile after exposure to pro-inflammatory stimuli such as the toll-like receptor (TLR) ligand lipopolysaccharide (LPS). BV2 microglia treated with LPS exhibit increased lactate production and decreased mitochondrial ATP production, which is indicative of a shift to glycolysis (60). Another study confirmed the increased glucose consumption and glycolytic enzyme activity in parallel with increased anaerobic glycolysis and PPP utilization following LPS and IFN-γ treatment (61). Mouse primary microglia

treated with IFN-γ result in a metabolic switch towards glycolysis and the retention of iron nanoparticles that is thought to be driven by 6-phosphofructo-2-kinase/fructose-2,6-biphosphate (PFKFB)3, an enzyme involved in glycolysis (62). The treatment of primary microglia with LPS for 24 hours also causes a shift from OXPHOS to glycolysis (63), which is mediated through the activation of the mTOR pathway and leads to enhanced ROS production (64). The metabolic switch is abolished following the addition of the phosphatidylinositol 3'-kinase antagonist LY294002, rapamycin or torin1, which all suppress the phosphorylation of mTOR (64). Further, 2DG treatment of primary mouse microglia in parallel with LPS stimulation inhibits glycolysis with subsequent downregulation of LPS-induced genes (*Il6*, *Il1β*, and *Nos2*) and cytokine production (IL-6 and IL-1β) (65). Recently, primary mouse microglia treated with IL-1β and IFN-γ for 24 hours not only exhibit increased glucose metabolism but also glutamine metabolism through glutaminolysis. Interestingly, within this same study, human microglia-like cells differentiated from pluripotent stem cells treated with LPS for 24 hours exhibit increased *PFKB3* gene expression and increased glycolysis. The use of two-photon FLIM imaging to interrogate the metabolic signatures of individual microglia in acutely prepared mouse hippocampal slices exposed to LPS revealed an increase in aerobic glycolysis in microglia that is blocked by the addition of 2DG (5 mM) (66).

BV2 microglia, and the B6M7 microglial cell line, treated with LPS and IFN-γ exhibit the expected metabolic shift towards enhanced glycolysis and increased gene expression of GLUT1. The inhibition of GLUT1 with STF31 in pro-inflammatory conditions specifically prevents the increase in microglial glucose uptake and attenuates the upregulation of inflammatory cytokines TNF-α, IL-1β, IL-6, and CCL2 *in vitro*, whereas an intraperitoneal injection of STF31 in a mouse model of light-induced retinal degeneration leads to reduced microglia activation and retinal degeneration *in vivo* (46). In BV2 and primary mouse microglia cultured in a hypoxic environment (1% oxygen), HK2, the first rate-limiting enzyme in glycolysis, is increased and correlates with enhanced glycolysis (67). Here, the pharmacological inhibition of HK2 with lonidamine impairs the activation profiles of both BV2 and primary microglia under hypoxia. HK2 blockade prevents ischemic brain injury by repressing microglia mediated neuroinflammation in a rat experimental model of stroke *in vivo* (67).

These studies suggest that under inflammatory conditions, microglia exhibit an increased glycolysis to OXPHOS ratio, similarly to what occurs in peripherally activated macrophages and during the Warburg effect of cancer cells. In summary, these studies strongly indicate that microglial polarization results in significant changes in the preferred metabolic pathway, from oxidative metabolism in homeostasis to a reliance on glycolysis and glutaminolysis in pro-inflammatory states.

Therefore, focusing on small molecules and/or drugs that promote oxidative metabolism over glycolysis will have profound impacts in the way we approach neuroinflammatory and neurodegenerative conditions.

LIPID SENSING AND SIGNALLING IN MYELOID CELLS

Lipids are fundamental building blocks of cell membranes and myelin in the brain (68–70). In the context of CNS damage, including demyelinating diseases, lipids play a key role in modulating inflammatory responses and contribute to metabolic dysfunction, which is an important aspect of disease pathophysiology (71, 72). In particular, the metabolism of lipids is central to both homeostasis and inflammatory responses in CNS myeloid cells, where it plays vital roles in respiration, activation, inflammatory signalling, migration, and phagocytosis (70, 73). Indeed, recent transcriptomics studies have provided indirect evidence supporting drastic changes in the lipid metabolism of activated microglia, as seen by the upregulation of lipid metabolism genes such as *Trem2, Apoe, Spp1, Cts7, Lpl*, and *Fabp5* under inflammatory conditions (30, 70, 74, 75). However, the role of these genes and pathways is still under investigation, especially in diseases where myelin deposition in the CNS parenchyma can exceed the lipid processing capacity of myeloid cells (**Figure 2**) (73, 76, 77).

The basis of the myeloid cell response to lipids is determined by the carefully regulated composition of phospholipids (PLs) in the cell membrane. PLs are formed from two fatty acids (FAs), a phosphate group, and a glycerol or sphingosine molecule. While they are best known as major components of the cell membrane, PLs are also critical for vesicle formation, apoptosis, and as metabolic intermediates for the production of both pro- and anti-inflammatory molecules (70). Sphingosine containing PLs, also known as sphingolipids, are prominent signalling molecules in the CNS. Sphingosine-1-phosphate (S1P), derived from the phosphorylation of sphingosine, can act as an intracellular intermediate for complex sphingolipid and phosphatidylethanolamine (PE) synthesis or can be released from the cell where it can act *via* autocrine or paracrine signalling through five different G-protein coupled receptors ($S1PR_{1\text{-}5}$) (78, 79). The exact role of S1P signalling in myeloid cells remains unclear. The treatment of LPS stimulated mouse primary microglia with the S1P structural analog fingolimod, results in the downregulation of pro-inflammatory cytokines and the upregulation of brain-derived neurotrophic factor and glial-derived neurotrophic factor (80). However, *in vitro* evidence suggests that both S1P and fingolimod act *via* astrocytes, rather than myeloid cells or neurons, to suppress chronic neuroinflammation (81, 82). Other data suggests, instead, that signalling through $S1PR_{1\text{-}3}$ activates the NF-κB pathway and polarizes microglia towards a pro-inflammatory, amoeboid phenotype *in vitro* and in mouse models of cerebral ischemia (83–86). Ultimately, further research is required to elucidate the myeloid-specific role of S1P signalling in chronic neuroinflammation.

Myeloid cells express specialized scavenger receptors (SCARs) that sense and uptake extracellular lipids, including FAs. The class-B SCAR CD36, also known as FA translocase, is a phagocytic receptor that is widely expressed on microglia and peripheral myeloid cells to facilitate long chain FA uptake and low-density lipoprotein binding (87). Under demyelinating neuroinflammatory conditions, such as MS, CD36 is necessary

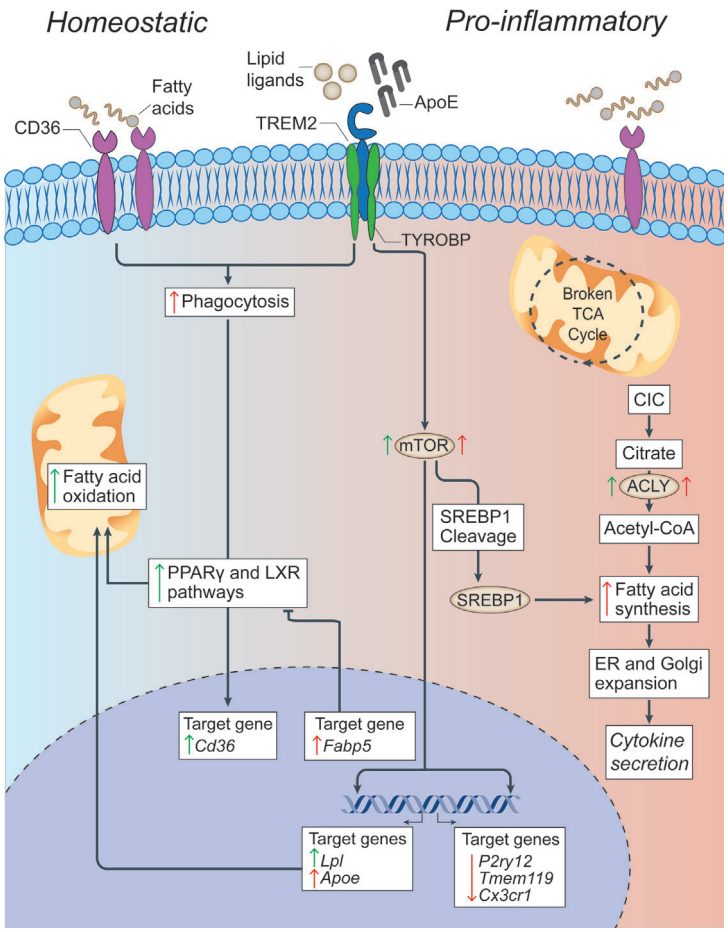

FIGURE 2 | Lipid metabolism in microglia under homeostatic and inflammatory conditions. In microglia, CD36 and TREM2 play a key role in the response to extracellular lipids. CD36 promotes lipid-responsive signalling pathways (like the PPARγ and LXR pathways), which in turn increase FAO and further upregulate *Cd36*. TREM2 activation (via ligands such as ApoE) results in the suppression of homeostatic microglial genes (*P2ry12, Tmem119, and Cx3cr1*), the activation of mTOR signalling, and the upregulation of lipid processing genes (such as *Apoe*, *Lpl*, and *Fabp5*). Despite mTOR increasing both FAS (through the cleavage and activation of SREBP-1) and glycolysis (which are canonically associated with a pro-inflammatory activation of myeloid cells), it appears that the role of TREM2 is to support correct lipid metabolism. In fact,TREM2 deficient microglia show the formation of intracellular cholesterol crystals that activate the inflammasome pathway. On the contrary, the downstream gene *Fabp5* seems to play a key role in determining the pro-inflammatory activation of myeloid cells, possibly via inhibition of PPARγ signalling and FAO. In pro-inflammatory microglia, a broken TCA cycle is coupled with an upregulated mitochondrial CIC, which increases citrate export from the mitochondria to the cytosol, where it is converted into acetyl-CoA for FAS by ACLY. The resultant increase in FAS supports the expansion of the ER and Golgi, and the increased production of pro-inflammatory cytokines. Green arrows = homeostatic effects. Red arrows = pro-inflammatory effects. PPARγ, peroxisome proliferator-activated receptor γ; LXR, liver X receptor; FAO, fatty acid oxidation; mTOR, mechanistic target of rapamycin; FAS, fatty acid synthesis; ACLY, ATP citrate lyase; CIC, citrate carrier; TCA, tricarboxylic acid cycle; SREBP-1, Sterol regulatory element binding protein 1; TYROBP, TYRO protein tyrosine kinase-binding protein; TREM2, Triggering receptor expressed on myeloid cells 2; Lpl, Lipoprotein Lipase; P2ry12, Purinergic Receptor P2Y12; Tmem119, Transmembrane Protein 119, Cx3Cr, C-X3-C Motif Chemokine Receptor 1.

for the phagocytosis of myelin debris (88, 89). Here, myelin internalization promotes anti-inflammatory lipid-responsive signalling pathways, like the peroxisome proliferator-activated receptor-γ (PPARγ) pathway, which in turn upregulates CD36 (88, 90, 91). This further supports the notion that CD36 serves an anti-inflammatory role as pro-inflammatory microglia have been demonstrated to downregulate CD36 *in vivo* (91) and the *in vitro* inhibition of CD36 in microglia and bone marrow-derived macrophages (BMDMs) promotes inflammation while reducing anti-inflammatory signalling pathways [e.g., PPARγ and liver X receptor] (88).

Another extracellular lipid sensing molecule with implications for chronic neuroinflammation is triggering receptor expressed on myeloid cells 2 (TREM2). TREM2 is a microglia-specific transmembrane receptor with several proposed ligands including ApoE (92), anionic or zwitterionic lipids, PL (93), PE, and phosphatidylserine, which become exposed on the cell surface during apoptosis (94). The binding

of TREM2 to extracellular ligands results in the suppression of homeostatic microglial genes and a shift towards an activated phagocytic state (74, 75). It also leads to the activation of mTOR signalling, a pathway that has critical implications for both glycolysis (95) and lipid metabolism (95). In fact, TREM2 has emerged as an innate immune receptor that impacts microglia metabolism through the basic activation of mTOR signalling, which supports long-term cell trophism, survival, growth, and proliferation rather than drastic metabolic reprogramming (95). A recent study used cell type-specific lipidomics to demonstrate that TREM2 is not necessary for myelin uptake by microglia, rather it is required for the upregulation of lipid processing genes involved in lysosome function, cholesterol transport, and cholesterol metabolism, such as *Apoe* and *Lpl* (96, 97). Global TREM2 deficiency hinders the efflux of cholesterol from microglia *in vitro* and *in vivo* and enhances the neurotoxic effect of cuprizone in mouse models of chronic demyelination (96).

Therefore, defective lipid metabolism in TREM2 deficient microglia could result in the accumulation of intracellular cholesterol crystals that damage lysosomes and activate the inflammasome pathway (76).

Understanding the link between lipid sensing, uptake, and intracellular metabolism is therefore key in identifying further targets for therapeutic approaches aimed at resolving chronic inflammation.

LIPID METABOLISM

FAs are transported into mitochondria and used to fuel mitochondrial OXPHOS, a process known as fatty acid β-oxidation (FAO) (**Figure 2**). Changes in FAO in response to inflammatory mediators has been well characterized in peripheral macrophages (98), and more recent studies in microglia have drawn many parallels between these myeloid cells. In peripheral macrophages *in vivo*, alternative activation *via* IL-4 increases FAO through PPARγ signalling (99). Alternatively activated microglia also increase FAO (61, 100), but the involvement of PPARγ signalling has yet to be confirmed. Most importantly, increasing FAO reduces the response to inflammatory perturbations such as LPS in SIM-A9 mouse microglia cells (101), while the inhibition of FAO in human macrophage-differentiated THP-1 monocytic cells (102) and mice microglia *in vitro* and *in vivo* (103) has the opposite effect. Therefore, FAO positively regulates anti-inflammatory responses possibly by minimizing FA metabolites that cause endoplasmic reticulum (ER) stress and act as precursors of pro-inflammatory molecules (70). In line with this, a deficiency in lipoprotein lipase (LPL), a catalyst for the release of FAs that is required for FAO in microglia, causes a shift in microglia metabolism towards glycolysis and increased pro-inflammatory activation (100).

In addition to LPL, lipid metabolism for processes such as FAO can be facilitated through fatty acid binding proteins (FABPs). FABPs are a family of 14-15 kilodalton (kDa) lipid chaperones that reversibly bind hydrophobic molecules, including FAs, and transport them to specific nuclear compartments (104). In homeostasis and activation, peripheral myeloid cells express the FABP isoforms FABP4 and FABP5 (105). Microglia express FABP5 only during development or upon activation (30), suggesting a specific role for FABP5 in activated microglia, which has yet to be discerned. In another type of immune cell, regulatory T cells (Treg), FABP5 loss of function results in decreased OXPHOS and impaired lipid metabolism, ultimately increasing Treg IL-10 production and promoting Treg immunosuppressive activity (106). Furthermore, FABP5 inhibition in $CD4^+$ T cells increases PPARγ expression and skews T cell differentiation away from effector T cells (e.g., Th1, Th17) and towards Tregs *in vitro* (107). The same study found that systemic FABP5 inhibition reduces inflammation and improves clinical scores in mouse models of EAE (107). In FABP5 knock out BMDMs, stimulation with inflammatory (LPS and IFN-γ) or anti-inflammatory (IL-4) mediators results in significantly higher expression of anti-inflammatory factors (105, 108). These findings suggest that loss of FABP5 function promotes anti-inflammatory responses in macrophages. Thus, while little is known about the role of FABP5 in microglia, it represents an interesting target that could be manipulated to alter PPARγ signalling and lipid metabolism to reduce chronic neuroinflammation.

Fatty acid synthesis (FAS) is the generation of FAs from the breakdown of the metabolite acetyl-CoA and co-factor NADPH by fatty acid synthases and acetyl-CoA carboxylase in the cytoplasm. Acetyl-CoA is generated from citrate *via* the cytoplasmic enzyme, ATP citrate lyase (ACLY), which is activated in inflammatory macrophages (109). Of note, the role of some of these players can be ambivalent, as IL-4 stimulation of macrophages activates Akt-mTORC1 pathway to phosphorylate and activate ACLY, leading to increased histone acetylation and the upregulation of a subset of M2 genes (110). Myeloid cells challenged with LPS increase FAS through a combination of metabolic and transcriptional pathways. Metabolically, *in vitro* macrophages isolated from histiocytoma and treated with LPS were shown to upregulate mitochondrial citrate carrier (CIC), which exports citrate to the cytosol where it is converted into acetyl-CoA, which is then available for FAS (111). It is also known that LPS increases glycolysis in macrophages, driving flux through the PPP which increases the availability of NADPH for FAS (112, 113). Transcriptionally, LPS activation has been demonstrated to activate mTOR signalling in primary rat microglia and mouse N9 microglia cell lines (114). mTOR activation has been widely shown to increase FAS through the cleavage and activation of sterol regulatory element-binding protein-1, the transcriptional regulator of lipogenesis (112, 113).

Together, the resultant increase in FAS supports the expansion of the ER and Golgi, allowing for increased production of pro-inflammatory cytokines such as IL-6, TNFα, and IL-12 (115). The disruption of FAS reduces both ER and Golgi expansion and pro-inflammatory cytokine secretion in DCs (115), but these findings have yet to be confirmed in myeloid cells.

The emerging field of lipidomics, the improvement of complimentary high throughput techniques, and additional

experimental work aimed at assessing the fate of lipids in intracellular organelles (e.g., mitochondria) will reveal exciting roles for lipid metabolism in regulating myeloid cell function and, ultimately, chronic neuroinflammation.

MITOCHONDRIAL DYNAMICS IN MYELOID CELLS

Mitochondrial dynamics in tissues, including the CNS and the immune system, are regulated by complex mechanisms (116, 117). Far from being isolated organelles inside cells, mitochondria participate in an active network that is regulated by the local events of fission and fusion, as well as a global control through cellular signalling and metabolic pathways (118).

Fission is mainly controlled by the GTPase dynamin-related protein 1 (DRP1), which is further regulated by several adaptor proteins, such as the mitochondrial fission factor, as well as the mitochondrial dynamics proteins of 49 and 51 kDa and the mitochondrial fission 1 protein (119). DRP1 functions by assembling into oligomeric spirals that constrict and cut the mitochondrion apart by working in concert with dynamin-2 (120). DRP1 activity is further controlled by post translational modifications whereby phosphorylation of serine (Ser) residues Ser 638 or Ser 616 blocks or enhances mitochondrial fission, respectively (121, 122). Fusion is a two-step process that is regulated by the dynamin-like GTPases mitofusin 1/2 on the outer mitochondrial membrane and optic atrophy 1 (OPA1) on the inner mitochondrial membrane. Long forms (L-OPA1) are proteolytically cleaved by peptidases to generate short forms (S-OPA1) to balance fusion (123). Given its location within the inner mitochondrial membrane, OPA1 plays a key role in maintaining cristae morphology, mitochondrial DNA (mtDNA), and supercomplex assembly (124). It is this fine balance between fission and fusion events that regulates key cellular processes, including mitophagy, mitochondrial transport, calcium homeostasis, and mitosis/apoptosis, thus modifying cell metabolic states *via* a bidirectional cross talk (125).

Upon pro-inflammatory activation, myeloid cells undergo major changes in the structure and function of their mitochondrial network that are linked with extensive metabolic rewiring (**Figure 3**). Activated amoeboid microglia in demyelinated cerebellar white matter show greater numbers of small and short mitochondria than the ramified microglia in wild-type (WT) mice suggesting a link between mitochondrial fission and microglial cell activation (126). Interestingly, these changes seem to partially differ between macrophages and microglia during neuroinflammation. Using *Ccr2rfp/*$^{+}$:: *Cx3cr1gfp/*$^{+}$mice, in which tissue-resident microglia and infiltrating monocyte-derived macrophages were labelled with green fluorescent protein and red fluorescent protein respectively, microglia are described to have longer and thinner mitochondria and spherical nuclei than monocyte-derived macrophages in spinal cord tissues at the onset of EAE (126).

Interfering with the mitochondrial dynamics of microglial cells has shown to affect their activation both *in vitro* and *in vivo*. *In vitro* studies suggest that LPS induces mitochondrial fragmentation in microglia *via* DRP1 signalling thus inducing mitochondrial ROS (mtROS) generation (126). Treating microglial cells *in vitro* with mitochondrial fission inhibitor 1 (Mdivi-1), an inhibitor of DRP1, blocks LPS-induced mitochondrial fragmentation and increases mitochondrial membrane potential, ROS production, and accumulation of intracellular TCA cycle intermediates (e.g., succinate), which is indicative of impaired OXPHOS (63). *In vivo*, microglia isolated from the brains of animals following induction of systemically driven neuroinflammation and con-current treatment with Mdivi-1 (from P1 to P3) show attenuated expression of genes related to pro-inflammatory activation (e.g., *iNOS*, *Ptgs2*) suggesting that controlling mitochondrial fission *in vivo* may intrinsically recue microglial activation (63).

Thus, modulating the mitochondrial dynamics of myeloid cells may also have extrinsic effects on neighbouring CNS cells. Indeed, fission events followed by the release of fragmented and dysfunctional microglial mitochondria propagate neuronal death through activation of naïve astrocytes to the neurotoxic A1 state (127). Following from this model, regulating fission and fusion in microglia might reduce the release of dysfunctional extracellular mitochondria, thus lessening the propagation of damage from activated microglia to astrocytes and from astrocytes to neurons. This mechanism is strictly dependent on the altered function of extracellularly released mitochondria, as intact extracellular astrocytic mitochondria instead provide neuroprotection (127–129). Recent work from our group has shown that delivering functional extracellular mitochondria (via extracellular vesicles) is effective in re-establishing normal mitochondrial function in myeloid cells *in vitro* and *in vivo* during neuroinflammation (130). Further studies will be needed to identify the applicability of these findings to the cure of progressive MS and other neurodegenerative disorders (131).

MITOCHONDRIAL METABOLISM OF MYELOID CELLS

Inhibition of mitochondrial respiration drives the pro-inflammatory activity of myeloid cells and prevents their repolarization to an anti-inflammatory phenotype (132). Reduced OXPHOS is linked with major changes to the mitochondrial metabolism that drive diverse intracellular and extracellular signalling functions (**Figure 3**) (24).

The mitochondrial metabolism of myeloid cells has been thoroughly characterized *in vitro* using pro-inflammatory BMDMs. Carbon flux analyses have identified two "breaks" in the TCA cycle: one at the level of isocitrate dehydrogenase (IDH), the enzyme that converts isocitrate to α-ketoglutarate (αKG) and another at the level of succinate dehydrogenase (SDH), which regulates the oxidation of succinate to fumarate (133). These breaks are partially compensated for *via* an

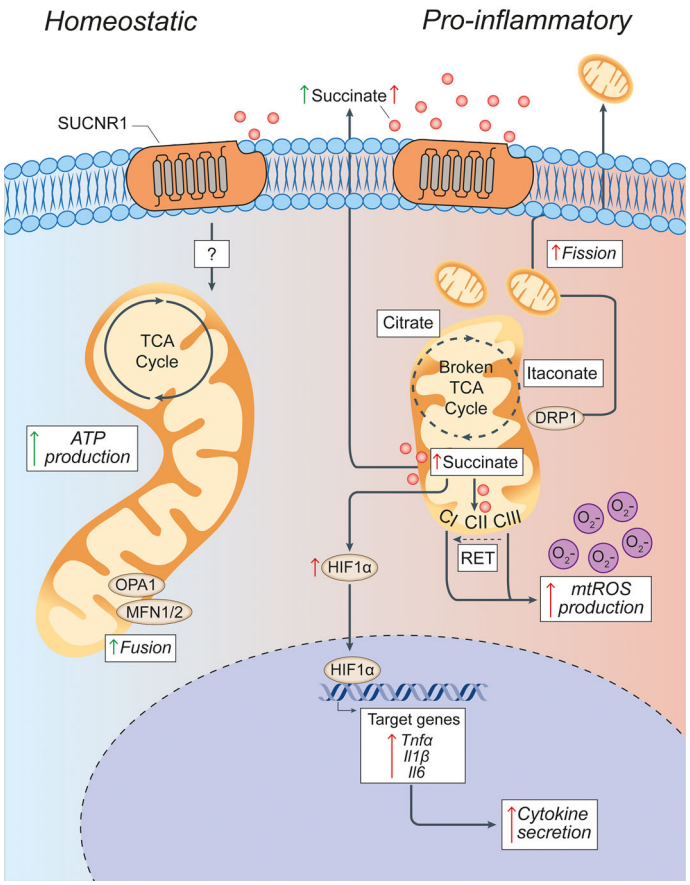

FIGURE 3 | Pro-inflammatory conditions lead to morphological and functional mitochondrial alterations of microglia. Microglia in homeostatic conditions have intact mitochondria with a functioning TCA cycle and conserved fusion of the mitochondrial network through OPA1 and MFN1/2 activities. Succinate signalling through SUCNR1 in homeostasis is presumably low, but its signalling functions in resting microglia are currently undefined. In pro-inflammatory conditions, mitochondria undergo DRP1-mediated fission and fragmentation, and show two breaks in the TCA cycle that lead to the intracellular accumulation of specific metabolites (such as succinate, citrate, and itaconate). Succinate accumulation within mitochondria can drive RET, which produces excessive mtROS through complex I. This mitochondrial dysfunction creates a pseudohypoxic state that leads to the stabilization of HIF1α and enhances cytokine production and secretion. Both fragmented mitochondria and succinate can be released into the extracellular environment where succinate can signal in an autocrine or paracrine manner via SUCNR1, thus modulating both anti-inflammatory and pro-inflammatory effects. Green arrows = anti-inflammatory effects; red arrows = pro-inflammatory effects. SUCNR1, succinate receptor 1; TCA, tricarboxylic acid cycle; OPA1, optic atrophy 1; MFN1/2, mitofusin 1/2; DRP1, dynamin-related protein 1; CI, complex I; CII, complex II; CIII, complex III; mtROS, mitochondrial reactive oxygen species; RET, reverse electron transport; ATP, adenosine triphosphate; HIF1α, hypoxia inducible factor 1 α; mtROS, mitochondrial reactive oxygen species; TNFα, tumor necrosis factor alpha; Il1β, interleukin-1β; Il6, interleukin-6.

enhanced arginosuccinate shunt that feeds into fumarate and malate or *via* increased glutaminolysis. However, they mostly lead to significant metabolic changes that include the decrease of downstream metabolites such as αKG and fumarate, with a concomitant increase of itaconate, citrate, and succinate (24).

The increase of the expression of cis-aconitic acid decarboxylase (CAD) coded by the immunoresponsive gene 1 sustains the production of itaconate from the accumulated isocitrate (134, 135). Itaconate has antimicrobial properties (by inhibiting the citrate-lyase expressed by different bacterial strains) but can also act as an inhibitor of SDH, limit the levels of inflammatory cytokines, and modulate the IkBz-ATF3 inflammatory and nuclear factor erythroid 2-related factor 2 (NRF2) signalling axis (136, 137). Citrate, instead, is used as a precursor for FAS and lipogenesis but also for prostaglandin and nitric oxide (NO) production, thus sustaining the inflammatory activity of myeloid cells (133). Finally, succinate accumulation, which has been attributed to SDH inhibition (136), glutaminolysis replenishing αKG levels (138, 139), and the gamma-aminobutyric acid shunt (140), plays major roles in regulating both extracellular and intracellular inflammatory signalling.

Extracellularly, succinate accumulates in several inflammatory conditions, including in the cerebrospinal fluid, but not in the blood, of mice with chronic EAE (141). Extracellular succinate modulates inflammation *via* binding to its cognate succinate receptor 1 (SUCNR1), thus eliciting complex responses that are tissue- and context-dependent

(142). While in DCs the succinate-SUCNR1 axis clearly potentiates the production of pro-inflammatory cytokines (143, 144), its role in the activation of other myeloid cells is still under investigation. On the one hand, a 'positive-feedback' mechanism has been described in chronic inflammation, where IL-1β triggers the production and release of succinate from macrophages, which in turn stimulates SUCNR1-expressing cells to maintain chronic inflammation *via* an autocrine and paracrine loop (145, 146). On the other hand, recent evidence suggests that SUCNR1 stimulation prompts an anti-inflammatory phenotype on adipose tissue macrophages and tumour associated macrophages (147, 148).

Intracellularly, succinate regulates signalling mostly by enhancing the pro-inflammatory activity of myeloid cells. Succinate can be transported from the mitochondria *via* the dicarboxylic acid transporters to the cytosol where in excess it impairs prolyl hydroxylase activity by product inhibition leading to HIF-1α stabilization and activation (140). This phenomenon has been defined as pseudohypoxia (149) and leads to the increased production of pro-inflammatory IL-1β (140). The progressive accumulation of succinate also drives the activity of SDH, thus promoting mtROS production (150). This process links mitochondrial activity, cell metabolism, and ROS production and could be key in treating myeloid mediated oxidative injury in chronic neuroinflammation.

MITOCHONDRIAL FUNCTION AND OXIDATIVE INJURY

Recent approaches using toxic RNA sequencing (Tox-seq), which transcriptionally profiles ROS^+ innate immune cells, has helped to identify neurotoxic CNS innate immune populations in EAE mice (151). When $CD11b^+$ cells labelled for ROS production were analysed by single cell RNA sequencing, a specific ROS^+ microglia cluster is found to display low levels of homeostatic microglia markers (e.g., *P2ry12*, *Sparc*, *Cx3cr1*, and *Tmem119*) but high levels of oxidative stress and pro-inflammatory genes [e.g., *NADPH oxidase subunit 2* (*gp91-phox*), *MhcII*, *Il1b*] (151). In addition, several genes are upregulated in ROS^+ microglia and macrophages throughout the oxidative stress network, including glutathione transferases (*Gsto2* and *Gstt2*), γ-glutathione peroxidase (*Gpx7*), and the acivicin target genes (*Ggt1* and *Ggt5*) (151). When EAE mice are treated with the compound acivicin, which inhibits the degradation of the antioxidant glutathione by targeting γ-glutamyl transferase, they show decreased oxidative stress and neurodegeneration, even when treatment is started 80 days after disease onset (151). These data suggest that targeting ROS production in innate immune cells is a promising strategy to treat active chronic neuroinflammation, such as that occurring in people with progressive MS.

Under inflammatory conditions, ROS are produced through various mechanisms. Cytosolic ROS are produced by the NADPH oxidase (NOX) family and NO synthases (NOS). Superoxide, OH^-, and H_2O_2 are instead generated in mitochondria at mitochondrial complex I (CI) and III (CIII), which are the main sites of mtROS production (152, 153). Notably, a link exists between these processes where NO regulates the abundance of TCA cycle metabolites (e.g., succinate and itaconate), as well as the catalytic subunits of CI in inflammatory macrophages (154). This oxidative response is counterbalanced by the activity of several enzymes (e.g., catalase, superoxide dismutases, sirtuin 3), coenzymes (e.g., coenzyme Q), and metabolites (e.g., glutathione) with antioxidant activities (155). In addition, transcription factors [e.g., NRF2, Kelch Like ECH Associated Protein 1] control the expression of antioxidant genes (156), while mitochondrial transporter proteins [e.g., uncoupling protein 2] shuttle H^+ from the intermembrane space to the mitochondrial matrix, leading to decreased membrane potential and mtROS production (157). When these mechanisms are saturated/inhibited, excessive intracellular ROS production can impact ATP synthesis, cytokine production, mtDNA mutation, and post-translational modification of proteins (155). Extracellularly, ROS release from CNS innate immune cells maintains inflammation, while promoting neurodegeneration and demyelination (158). Given the predominant role of mitochondria in ROS production during inflammation, key potential targets for this new approach reside in specific mitochondrial proteins and complexes (**Figure 3**).

CI is a supercomplex of 44 subunits which form three modules: N module (oxidizing NADH and electron input), Q module (electron output to ubiquinone) and P module (proton transport) (159). CI can produce ROS when electrons circulate in the forward or reverse direction, depending on multiple factors that include mitochondrial function, cell metabolism, and cellular type (150). In fact, forward electron transport (FET) can produce proton leak from CI, but a highly reduced pool of coenzyme Q and a large membrane potential can also trigger reverse electron transport (RET) from over-reduced coenzyme Q back to CI, significantly increasing superoxide production (160). During FET, blocking CI with rotenone suppresses electron transport causing electron leak and increased ROS production (161), while rotenone prevents the electron transport back from coenzyme Q and significantly reduces ROS production during RET (152, 160). In microglia and BMDMs, rotenone enhances ROS and pro-inflammatory cytokine production when cells are in a resting state (162, 163), which suggests that ROS results from impaired FET. In line with this, BMDMs displaying a knockout of the CI subunit *Ndufs4* produce more lactate and ROS than WT BMDMs (164). However, in pro-inflammatory myeloid cells excess of the SDH substrate, succinate, stimulates RET and ultimately shifts mitochondrial activity to mtROS production (150). Accordingly, in LPS-stimulated myeloid cells, especially after prolonged treatment (8–24 h), ROS and pro-inflammatory cytokine production are reduced by rotenone, and this effect may be due to decreased RET (152). Targeting this process, without altering the normal function of CI and OXPHOS, as recently shown for ischemia reperfusion injury (165), could be key in treating chronic neuroinflammatory diseases. CIII is another key site of mtROS generation, which can be modulated. Similarly to CI, blocking CIII activity with antimycin A or myxothiazol, for

example, in unstimulated BMDMs increases ROS production (166), while in pro-inflammatory BMDMs, blocking CIII reduced NFκB nuclear accumulation as well as ROS and pro-inflammatory cytokine production (167, 168).

Altogether these data suggest that interacting with CI or CIII (dys)functionality may be important to treat CNS inflammatory disorders. Indeed, the use of CI inhibitors such as rapamycin or metformin can inhibit mtROS production by inhibiting CI formation (169) and attenuate the induction of EAE by restricting the infiltration of mononuclear cells into the CNS and down-regulating the expression of proinflammatory cytokines (IFNγ, TNF, IL-6, IL-17, iNOS), cell adhesion molecules, and matrix metalloproteinase 9 (170).

Further studies will be needed to differentiate these effects from the pleiotropic effects that these molecules have on metabolic pathways (e.g., mTOR) and CNS cell types (e.g., oligodendrocytes) (171–174).

CONCLUSION

The growing interest in immunometabolism has demonstrated that myeloid cells are well-equipped to quickly adapt to varying environmental challenges, even when access to carbon sources is highly variable, such as in conditions of inflammation. Therapeutically attractive targets have emerged, with preliminary *in vitro* and *in vivo* testing of compounds proving to be promising. Within this framework, two routes to therapeutic relevance have emerged, targeted therapies using small molecules and compounds (175) and non-targeted therapies. In regard to the latter, the use of dietary intervention (e.g., through the ketogenic diet and/or exercise) may hold the most direct and clinically translatable therapeutic approach towards reprogramming myeloid metabolism from harmful to helpful (176). As previously discussed, microglia can utilize ketone bodies as an alternative energy substrate to glucose, and ketosis has been shown to modulate a range of microglial inflammatory processes and reduce Aβ and tau accumulation in AD mice (177). High-fat, low-carbohydrate ketogenic diets are thought to trigger a shift from glucose metabolism towards FA metabolism, which in turn yields increased ketone body concentrations. Interestingly, pre-treatment of mice with a ketogenic diet decreased microglia activation and pro-inflammatory cytokine IL-6, IL-1β and TNF-α levels in the MPTP mouse model of PD (178). Similarly, oral administration of ketone body metabolites such as β-hydroxybutyrate have been shown to reduce microglial inflammation (179), reduce expression of pro-inflammatory cytokines IL-1β, IL-6, CCL2/MCP-1 (180), and inhibit NLRP3 inflammasome activation (181). Metabolic reprogramming has been identified in exercise-related changes in cognition and immune functions, as exercise attenuated age-dependent inflammatory cytokine expression and cognitive decline in mice, while decreasing glycolytic enzymes and increasing phagocytosis in isolated microglia (182).

In conclusion, the impact of metabolism on both immune and non-immune cells in neuroinflammatory conditions has seen a groundswell of interest in the past decade. Ultimately, more work must be done to fully understand how the microenvironment influences the metabolism of cells and how we can better modulate these functions.

AUTHOR CONTRIBUTIONS

LP-J designed the review outline, wrote the manuscript, and outlined the figures. CW contributed to sections of the manuscript and designed the figures. RH and GK contributed to sections of the manuscript and provided input for the relative figures. SP critically reviewed manuscript. All authors contributed to the article and approved the submitted version.

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Targeting JAK-STAT Signalling Alters PsA Synovial Fibroblast Pro-Inflammatory and Metabolic Function

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Objectives: Psoriatic arthritis (PsA) is a chronic inflammatory disease associated with psoriasis. Janus Kinase inhibitors (JAKi) have emerged as an encouraging class of drugs for the treatment of PsA. Here, we compare the effect of four JAKi on primary PsA synovial fibroblasts (PsAFLS) activation, metabolic function, and invasive and migratory capacity.

Methods: Primary PsAFLS were isolated and cultured with JAKi (Peficitinib, Filgotinib, Baricitinib and Upadacitinib) in the presence of Oncostatin M (OSM). pSTAT3 expression in response to OSM was quantified by Western Blot analysis. Pro-inflammatory cytokines/chemokines were quantified by ELISA and cell migration by wound-repair scratch assays. Invasive capacity was examined using Matrigel™ invasion chambers and MMP multiplex MSD assays. PsAFLS bioenergetics was assessed using the Seahorse XF^e Extracellular Flux Analyzer, which simultaneously quantifies two energetic pathways- glycolysis (ECAR) and oxidative phosphorylation (OCR). In parallel, inflammatory, invasive, and migratory genes were quantified by RT-PCR.

Results: OSM induces pSTAT3 expression in PsAFLS. OSM-induced secretion of MCP-1 and IL-6 was inhibited by all JAKi with Peficitinib, Baricitinib and Upadacitinib showing the greatest effect. In contrast, JAKi had no significant impact on IL-8 expression in response to OSM. PsAFLS cell invasion, migratory capacity and MMP1, 3, and 9 were suppressed following JAKi treatment, with Peficitinib showing the greatest effect. These functional effects were accompanied by a change in the cellular bioenergetic profile of PsAFLS, where JAKi significantly decreased glycolysis and the ECAR/OCR, resulting in a shift to a more quiescent phenotype, with Peficitinib demonstrating the most pronounced effect.

Conclusion: This study demonstrates that JAK/STAT signalling mediates the complex interplay between inflammation and cellular metabolism in PsA pathogenesis. This inhibition shows effective suppression of inflammatory mechanisms that drive pathogenic functions of PsAFLS, further supporting the role of JAKi as a therapeutic target for the treatment of PsA.

Keywords: psoriatic arthritis, metabolism, JAK-STAT (janus kinase-signal transducer and activators of transcription), synovial fibroblast, synovial invasion

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic disease characterised by joint destruction and associated psoriasis (PsO) (1, 2). PsA synovitis is characterised by dysfunctional angiogenesis, followed by infiltration of both innate and adaptive immune cells. This leads to proliferation and activation of synovial fibroblast cells (FLS), a major source of pro-inflammatory mediators and matrix-degrading enzymes which orchestrate the persistent infiltration of immune cells and invade adjacent cartilage and bone (1–4). Furthermore, PsAFLS can further induce angiogenesis, promoting a more dysregulated endothelial cell (EC) phenotype compared to that of rheumatoid arthritis synovial fibroblast-like cells (RAFLS) (5), a phenotype consistent with the macroscopic appearance of blood vessels *in vivo* (6, 7). Indeed, PsAFLS alter the morphology, migratory and adhesive functions of ECs, in addition to their metabolic profile (5).

Many proinflammatory cytokines have been implicated in the pathogenesis of PsA, including TNF, IL-17A and IL-12/IL-23 pathways which play a key role in promoting the inflammatory response (8–11). Recently, targeted agents developed for PsA treatment include inhibitors of the Janus-Kinase (JAK) family of receptor-associated tyrosine kinases (12). Activated JAKs recruit and activate signal transducer and activator of transcription (STATs), which in turn drives gene transcription (13, 14). There are four JAK isoforms: JAK1, JAK2, JAK3 and TYK2, which depending on their stimulus induce the phosphorylation of different STAT proteins. Despite ongoing clinical trials, few studies have examined the precise effect of these agents in PsA synovial tissue cell subtypes, and thus our understanding of the comparative effect by which they reduce inflammation in the PsA joint is limited. Fiocco et al. demonstrated increased expression of the JAK1/STAT3/STAT5 transcriptional network associated with joint specific T cell populations in PsA (15). Consistent with this, studies have demonstrated in PsA that Tofacitinib, a JAKi, can regulate the frequency of pathologic $CD4^+CD11a^+CD45RO^+$ IL-17^+ T cells (16), inhibit Mo-DC differentiation through NOX5 and ROS production (17), decrease the T cell stimulatory capability of dendritic cells through suppression of type-I-IFN signalling (18), in addition to suppression of enthesitis in a $A20^{myelKO}$ animal model (19). Furthermore, we and others have shown increased expression of STAT signalling components in PsA synovial-biopsies and FLS (19, 20), with tofacitinib inhibiting spontaneous release of pro-inflammatory cytokines from *exvivo* PsA synovial explant cultures, in addition to inhibition of PsAFLS migratory and invasive capacity (21).

Current therapeutic options for PsA are mainly monoclonal antibody drugs targeting TNF, IL-23 or IL-17 (22). The therapeutic responses to these biologic disease modifying anti-rheumatic drugs (bDMARDs) can vary greatly with some patients showing inadequate responses. As the JAK/STAT pathway is central in driving both pro- and anti-inflammatory signals in immune regulation, including pathways which are involved in the pathogenesis of PsA, JAKi are now of great interest as a treatment option for PsA patients (23, 24). Currently, Tofacitinib is the only JAKi approved for PsA, with Peficitinib, Filgotinib, Baricitinib and Upadacitinib in clinical trials or undergoing pre-clinical evaluation (25). Upadacitinib (SELECT- PsA 2) and Filgotinib (PENGUIN 2), both JAK1 inhibitors, are currently in placebo controlled, double-blind phase III trials for the treatment of PsA patients with inadequate responses to at least 1 DMARD (26, 27). In addition, approximately 25% of patients with moderate-severe PsO develop PsA. Peficitinib, a pan JAKi and Baricitinib a JAK1/2 inhibitor have been shown to significantly improve both clinical and histological manifestations of this skin disease in phase II clinical trials (28, 29).

As head-to-head comparisons are difficult to perform, the aim of this study was to directly compare the effect of Peficitinib, Filgotinib, Baricitinib and Upadacitinib on PsAFLS inflammatory responses, migratory and invasive capacity, in addition to their effect on the metabolic profile of these cells.

MATERIALS AND METHODS

Patient Recruitment and Arthroscopy

PsA patients were recruited from the Rheumatology Department, St. Vincent's University Hospital. Ethics for this study was approved by the St. Vincent's University Hospital Ethics and Medical Research Committee and was performed in accordance with the Declaration of Helsinki. All patients gave fully informed written consent. PsA patients were defined according to CASPAR criteria. Baseline demographics of the PsA cohort are shown in **Table 1**. Arthroscopies were performed under local anaesthetic using a Wolf 2.7 mm needle, and synovial tissue biopsies were obtained from the site of inflammation under direct visualisation as previously described (6). Biopsies were utilised for isolation of primary PsA synovial fibroblasts (PsAFLS). Ethics approval number RS18-055.

Isolation of Primary Fibroblasts

PsA synovial biopsies were digested with 1 mg/ml collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) in RPMI-1640 (Gibco-BRL, Paisley, UK) for 4 h at 37°C in humidified air with 5% CO_2. Dissociated cells were grown to confluence in

TABLE 1 | Baseline Characteristics and clinical features of PsA Patients.

Demographic and Clinical Data	PsA (n = 14)
Female sex n (%)	10 (71.4)
Age (years)	53.8 ± 14.3
Disease duration (Years)	11.7 ± 13.1
ESR (mm/h)	25.9 ± 28.2
CRP (mg/L)	7.5 ± 12.6
No. tender joints	2 ± 1.8
No. swollen joints	1.1 ± 1.2
Pain VAS	57.5 ± 17.8
DAS28	3.1 ± 0.9
DMARDs (in last 3 months)	36%

Data presented as Mean (SD). ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; TJC28, tender joint count out of 28 joints; SJC28, swollen joint count out of 28 joints; VAS, visual analog scale; DAS28, disease activity score based on 28-joint count; DMARD, disease-modifying antirheumatic drugs.

RPMI-1640, 10% FBS (Gibco-BRL), 10 ml of 1 mmol/l HEPES (Gibco-BRL), penicillin (100 units/ml; Biosciences), streptomycin (100 units/ml; Biosciences) and fungizone (0.25 μg/ml; Biosciences) before passaging. Cells were used between passages 2–8.

Stimulation of PsAFLS

PsAFLS were seeded in 6- ($1x10^5$ cells/well), 48- ($2x10^4$ cells/well) or 96- ($2x10^4$ cells/well) well plates and allowed to attach overnight in RPMI-1640, 10% FBS, 10 ml of 1 mmol/l HEPES, penicillin, streptomycin and fungizone. Media was removed the following day and cells were serum starved by adding RPMI-1640 containing 1% FBS, 10 ml of 1 mmol/l HEPES, penicillin, streptomycin and fungizone for a further 24 h. PsAFLS were then pretreated with JAKi (Peficitinib (PEF), Filgotinib (FILGO), Baricitinib (BARI) (ACHEMBLOCK, CA,USA) and Upadacitinib (UPA) (Ambeed Inc, IL, USA); 5 μM) (JAKi were reconstituted in DMSO to 100 mM) or DMSO (5 μM; Sigma Aldrich) (vehicle control) for 1 h before being stimulated with Oncostatin M (OSM) (10 ng/ml; R&D) for 24 h. Concentration of 5 μM for all JAKi was used based on a previous study showing the dose response of all JAKi in FLS (29, 30). Additional experiments were performed to examine if JAKi alter secondary downstream effects of cytokines that do not signal through the JAK-STAT. Therefore, PsAFLS were plated in a 96- ($2x10^4$ cells/well) well as outlined above and stimulated with IL-1β (10 ng/ml; Bio-Techne LTD, UK) +/- JAKi, with a DMSO control. Additional experiments were also performed for Tofacitinib (5 μM) under OSM (10 ng/ml) or IL-1β (10 ng/ml) stimulation.

Protein Isolation and Western Blot Analysis

To determine the effect of OSM on pSTAT3 expression, PsAFLS ($1x10^5$ cells/well) were seeded in 6-well plates. Once confluent, cells were serum starved as previously described and stimulated with OSM (10 ng/ml) overnight, unstimulated (basal) PsAFLS were used as a control. Media was removed from the PsAFLS and ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma) containing 10 μg/ml phosphatase inhibitor cocktail and 10 μg/ml protease inhibitor cocktail (Sigma) was used to extract protein from the PsAFLS. Measurement of protein concentration was performed using a BCA assay (Pierce Chemical Co, Rockford, IL, USA). Protein (3 μg) was resolved by SDS-PAGE (5% stacking, 10% resolving), resolved proteins were then transferred onto nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) prior to 1 h blocking in wash buffer containing 5% non-fat milk with gentle agitation at room temperature. Membranes were incubated with rabbit polyclonal anti-pSTAT3 (Cell-Signaling Technology, UK), diluted in 5% non-fat milk containing 0.1% Tween 20 at 4°C overnight with gentle agitation. β-actin (1:5000, Sigma) was used as a loading control. Following three 15 min washes, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000) for 3 h at room temperature. The signal was detected using SuperSignal® West Pico Chemiluminescent Substrate (Amersham Biosciences). Band densities were imaged using the ChemiDoc MP Imaging System (Bio-Rad, USA).

Enzyme-Linked Immunosorbent Assay

Supernatants from treated PsAFLS and DMSO control (5 μM) ($2x10^4$ cells/well) seeded in 96-well plates were harvested and levels of IL-8, IL-6, and MCP-1 measured by specific ELISA (MCP-1: eBiosciences, USA, IL-8, IL-6; DuoSet ELISA, R&D systems, UK) according to manufacturer's protocol.

mRNA Extraction and cDNA Synthesis

To determine the effects of JAKi on specific genes in response to OSM stimulation, PsAFLS ($1x10^5$ cells/well) were seeded in 6-well plates and stimulated as previously described. Total RNA was isolated using an RNeasy Plus mini kit (Qiagen, Germany) according to the manufacturer's specifications. The integrity of the RNA samples was assessed using a bioanalyzer (Agilent, CA, USA). Samples with a 260:280 nm ratio of 1.8 or above were used in subsequent experiments. Total RNA (100 ng) was reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Cheshire, UK) and stored at -20°C until further use.

RT-PCR Analysis

Gene expression data were quantified by RT-PCR using the QuantStudio 5 Thermal Cycler (Applied Biosystem, Lewes, UK). Reaction mixtures contained 1 μl of cDNA, SYBR green PCR mastermix (Applied Biosystems) and target mRNA specific primer pairs as follows: IL-6 for 5' CCCTGAGAAAGGA GACATTGTAAC 3', IL-6 rev 5'CCTCTTTGCTGCTTT CACACATG 3', IL-8 for 5' TTGGCAGCCTTCCTGATTTC 3', IL-8 rev 5' TGGCAAAACTGCACCTTCAC 3', MCP-1 for 5' GCTCGCTCAGCCAGATGCAA 3', MCP-1 rev 5' TGGTGAA GTTATAACAGCAGGTGA 3', MMP1 for 5' GCTAACAAAT ACTGGAGGTATGATG 3', MMP1 rev 5' ATTTTGGGATAA CCTGGATCCATAG 3', ICAM for 5′ AACCAGAGCCAGGA GACACTG 3′, ICAM rev 5′ GCGCCGGAAAGCTGTAGATG 3′.

Samples lacking multiscribe reverse transcriptase formed the negative controls to ensure target-specific quantification. Data were analysed using the comparative threshold cycle (Ct) method with normalization to the expression of RPLPO (for 5′ GCGTCCTCGTGGAAGTGACATCG 3′, rev 5′ TCAGGGATT GCCACGCAGGG 3′) and HPRT1 (for 5′ ATGGACAGGAC TGAACGTCTTG 3′, rev 5′ GGCTACAATGTGATGGCCTC 3′) as endogenous controls.

Cellular Bioenergetic Function Analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), reflecting oxidative phosphorylation and glycolysis, respectively, were measured using the Seahorse-XFe96 analyser (Seahorse Biosciences, UK). PsAFLS were seeded at $12x10^3$/well in a 96-well cell culture XFe microplate (Seahorse Biosciences) and allowed to adhere overnight. Following this, cells were treated with JAKi/DMSO (5 μM) for 1 h and then stimulated with OSM (10 ng/ml) for 24 h. Additional experiments were also performed in the presence of IL-1β (10 ng/ml). Basal oxidative

phosphorylation/glycolysis were calculated by the average of five baseline OCR/ECAR measurements, respectively, obtained before injection of specific metabolic inhibitors; oligomycin (ATP-synthase-inhibitor) (2 μg/ml; Seahorse Biosciences), trifluorocarbonylcyanide phenylhydrazone (FCCP) (mitochondrial uncoupler) (5 μM; Seahorse Biosciences) and antimycin A (complex-III inhibitor) (2 μM; Seahorse Biosciences) and rotenone (2 μM; Sigma Aldrich). Oligomycin was injected to evaluate both the maximal glycolytic rate and ATP synthesis, determined by subtracting the amount of respiration left after oligomycin injection from baseline OCR. FCCP was injected to evaluate the maximal respiratory capacity (average of three measurements following injection). Maximal respiratory capacity was determined by subtracting baseline OCR from FCCP-induced OCR and the respiratory reserve (baseline OCR subtracted from maximal respiratory capacity).

Migration Assay

PsAFLS ($2x10^4$ cells/well) were seeded in 48-well plates for 24 h and serum starved as previously described. A single scratch wound was induced through the middle of each well with a sterile pipette tip and cells were subsequently treated with JAKi/DMSO (5 μM) for 1 h followed by stimulation with OSM (10 ng/ml) for 24 h.

PSA FLS migration across the wound margins was assessed and photographed using a phase-contrast microscope. Semi-quantitative analysis of cell repopulation of the wound was assessed. Briefly, cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and the number of migrating cells across the time zero margin was assessed.

Transwell Invasion Assay

BioCoat Matrigel™ Invasion Chambers (Becton Dickinson, UK) were used to assess PsAFLS invasion. Cells were seeded at $3x10^4$ cells/well in the migration chamber on 8 μM membranes pre-coated with matrigel. Cells were treated with JAKi/DMSO (5 μM) for 1 h and stimulated with OSM (10 ng/ml) for 48 h. Non-migrating cells were removed from the upper surface by gentle scrubbing. Migrating cells attached to the lower membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells from five random high-power fields for each well were counted to assess the average number of invading cells.

MMP 3-Plex MSD Assay

Supernatants from stimulated PsAFLS ($2x10^4$ cells/well) seeded in 96-well plates were harvested for MMP1, MMP3 and MMP9 analysis by MSD assay (Meso Scale Diagnostics, USA) and MMP expression was measured according to manufacturer's protocol.

Statistical Analysis

Statistical analyses were performed using Prism 8 software. Wilcoxon Signed Rank test, one-way analysis of variance (ANOVA), Friedman Test with Dunn's multiple comparison were utilised. p values of less than 0.05 ($^*p < 0.05$), 0.01 ($^{**}p < 0.01$), 0.001 ($^{***}p < 0.001$) and 0.0001 ($^{****}p < 0.0001$) were determined as statistically significant. All raw data are available on request.

RESULTS

JAK Inhibitors Alter PsAFLS Secretion of Pro-Inflammatory Mediators Induced by OSM

As OSM was utilised to active the JAK-STAT pathway, initial experiments assessed the effect of OSM on pSTAT3 expression. **Figure 1A** demonstrates that OSM stimulates pSTAT3 in n=3 separate PsAFLS. To assess the impact of JAKi, we initially determined their effect on a range of pro-inflammatory mediators. Firstly, we stimulated the PsAFLS with OSM and found that MCP-1 and IL-6 secretion (both $p < 0.05$) were significantly increased following stimulation compared to control (**Figures 1B, C**). IL-6 gene expression was also significantly increased ($p < 0.05$), with an increasing trend observed for MCP-1 gene expression (**Figures 1B, C**). MCP-1 secretion was significantly reduced by Peficitinib, Upadacitinib (both $p < 0.001$) and Baricitinib ($p < 0.05$) (**Figure 1B**). Although not significant, Filgotinib also showed a strong decrease in MCP-1 secretion. In parallel, inhibition was also observed at gene level, with Baricitinib ($p < 0.01$) and Upadacitinib ($p < 0.05$) significantly decreasing MCP-1 mRNA expression (**Figure 1B**). Similarly, JAKi also reduced OSM-induced expression of IL-6 at both the protein and gene level (**Figure 1C**). Peficitinib ($p < 0.001$) and Upadacitinib ($p < 0.01$) displayed significant inhibition of IL-6 (**Figure 1C**). This observation was also observed at gene level with Baricitinib ($p < 0.05$) and Upadacitinib ($p < 0.05$) showing significant reductions in IL-6 expression. Although not significant, Peficitinib also displayed an inhibitory capacity on IL-6 gene expression (**Figure 1C**). In contrast to both MCP-1 and IL-6 expression, OSM significantly reduced IL-8 expression ($p < 0.05$) compared to control (**Figure 1D**). JAKi showed no significant effect on IL-8 secretion, however, there was an increasing trend observed for IL-8 mRNA expression (**Figure 1D**).

JAK Inhibitors Reduce the OSM-Induced Shift to Glycolysis in PsAFLS

To examine whether the inhibitory effect of JAKi on pro-inflammatory mediators is paralleled by a shift in metabolism, we analysed the two major energy pathways: oxidative phosphorylation (OCR) and glycolysis (ECAR) in real time, using the Seahorse XFe-Analyser. **Figure 2A** displays the average bioenergetic profiles for ECAR and OCR of PsAFLS before and after injections of mitochondrial inhibitors: oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), antimycin A and rotenone in the presence of OSM. As shown in the seahorse profiles, stimulation with OSM increased the ECAR with no effect observed for OCR (**Figure 2A**). Quantification demonstrated a significant increase in baseline ECAR ($p < 0.05$) (**Figure 2B**) with no effect observed for baseline OCR (**Figure 2D**). Similarly, max glycolytic capacity ($p < 0.05$), but not max respiratory capacity was significantly increased by OSM stimulation (**Figures 2C, E**).

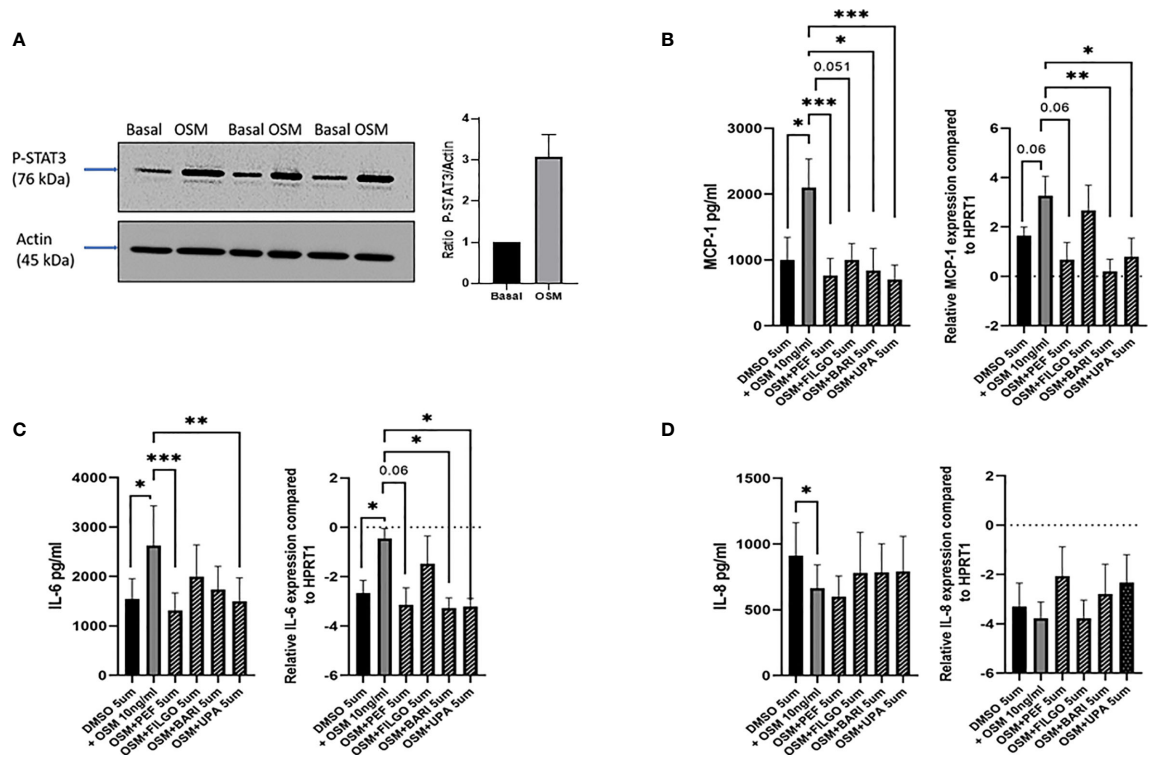

FIGURE 1 | Effect of JAKi on the OSM driven expression of pro-inflammatory mediators in PsAFLS. **(A)** Representative western blot images and bar graph showing pSTAT3 expression in PsAFLS stimulated with/without OSM for 24 h. **(B–D)** PsAFLS were treated with JAKi (5 μM) or DMSO (5 μM) for 1 h and stimulated with OSM (10 ng/ml) for 24 h. Bar graphs demonstrating secretion by ELISA (n=7) and gene expression by real-time PCR (n=4-5) for MCP-1 **(B)** IL-6 **(C)** and IL-8 **(D)**. Values expressed ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from DMSO+OSM control.

Treatment with JAKi demonstrated a decrease in the ECAR bioenergetic profile for all JAKi, with minimal change observed for the OCR bioenergetic profile (**Figure 2A**). All JAKi reduced the OSM- induced glycolytic capacity of PsAFLS with a significant decrease in basal glycolytic capacity in response to Peficitinib ($p < 0.01$) and Baricitinib ($p < 0.01$) (**Figure 2B**), and a significant decrease in Maximal Glycolytic Capacity in response to Baricitinib ($p < 0.0001$) (**Figure 2C**). No change in basal respiration (**Figure 2D**), or maximal respiratory capacity in response to JAKi was observed (**Figure 2E**). This therefore resulted in a significant decrease in the ECAR : OCR ratio, signifying a shift away from glycolytic mechanisms and towards a reliance on mitochondrial respiration. This was most significant following treatment with Peficitinib ($p < 0.01$), Baricitinib ($p < 0.05$) and Upadacitinib ($p < 0.05$) (**Figure 2F**). Filgotinib also decreased both the glycolytic capacity and ECAR : OCR ratio, but this did not reach significance (**Figure 2F**). The impact of JAKi on PsAFLS bioenergetics is clearly demonstrated in the overall metabolic profile, whereby the glycolytic profiles of PsAFLS induced by OSM, shift towards a more quiescent state following treatment with JAKi (**Figure 2G**). To examine if JAKi alter secondary downstream effects of other cytokines that do not signal through the JAK-STAT pathway, we examined their effect on IL-1β-induced PsAFLS (**Figures 3** and **4**). JAKi have no effect on IL-1β-induced MCP-1 secretion (**Figure 3A**). While some inhibitory effect was observed for IL-1β-induced IL-6 expression, this is not significant (**Figure 3B**). We also examined the bioenergetic profile of PsAFLS stimulated with IL-1β and the four JAKi (**Figures 3C–F**), in addition to Tofacitinib (**Figures 4A–C**). IL-1β increased the ECAR but not the OCR of the cells, with the JAKi's including Tofacitinib showing minimal effect on either energetic pathways (**Figures 3C–F** and **Figures 4A–C**). Tofacitinib which predominantly inhibits JAK3 and to a lesser extent JAK2 significantly inhibited both MCP-1 and IL-6 in response to OSM (**Figure 4D**). Similar to the other JAKi, Tofacitinib had no effect on IL-1β-induced MCP-1 and IL-6 secretion (**Figure 4E**).

OSM Driven PsAFLS Invasion Is Inhibited by JAKi

To further examine the effect of JAKi on PsAFLS pathogenic function, we examined the effect of JAKi on the invasive capacity of PsAFLS using Transwell Matrigel™ invasion chambers following stimulation with OSM. Representative images of PsAFLS invasion in unstimulated cells, OSM stimulated cells and OSM stimulated cells following treatment with JAKi are shown in **Figure 5A**. Quantitative analysis demonstrated the significant increase in invasive capacity following OSM stimulation ($p < 0.05$) compared to unstimulated cells (**Figure 5B**). This was significantly impacted by treatment with all JAKi, however Peficitinib ($p < 0.001$) and Filgotinib ($p < 0.05$)

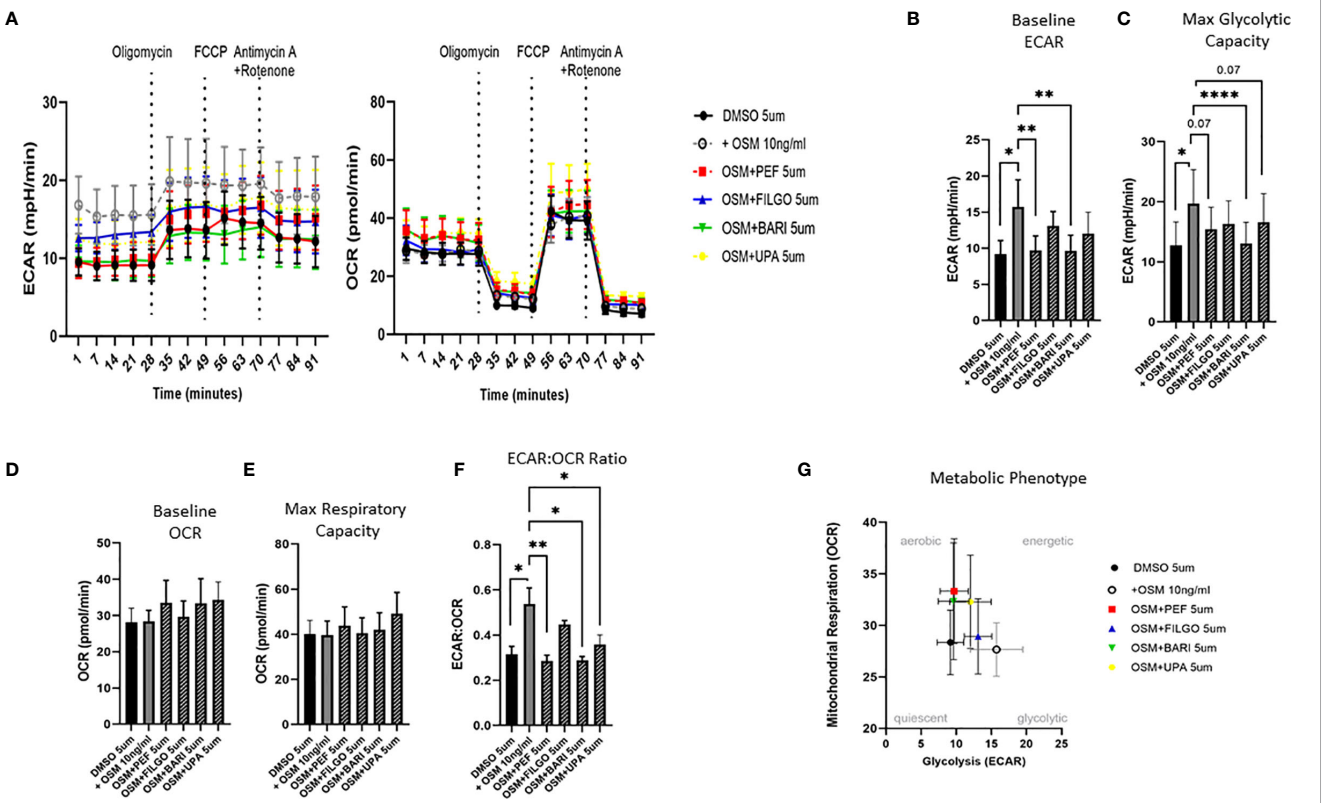

FIGURE 2 | JAKi reduce the increased glycolytic profile of PsAFLS induced by OSM. **(A)** PsAFLS were treated with JAKi (5 μM) or DMSO (5 μM) for 1 h and stimulated with OSM (10 ng/ml) for 24 h. Average seahorse profiles demonstrating extracellular acidification rate (ECAR) (glycolysis) and oxygen consumption rate (OCR) (oxidative phosphorylation). **(B)** Representative bar graphs demonstrating baseline ECAR, **(C)** maximal glycolytic capacity, **(D)** baseline OCR, **(E)** maximal respiratory capacity, and **(F)** ECAR : OCR ratio. **(G)** Metabolic phenotype profile of PsAFLS, n=6. Values expressed as mean +/- SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ significantly different from DMSO+OSM control.

had the most significant reductions (**Figure 5B**). In parallel, we examined the effect of JAKi on the secretion of the cartilage destructive matrix metalloproteinase enzymes. Peficitinib significantly decreased MMP-1 ($p < 0.05$), MMP-3 ($p < 0.05$) and MMP-9 ($p < 0.05$). While a decrease for all three MMPs was also observed in Upadacitinib, Filgotinib and Baricitinib treated cells, this did not reach significance (**Figure 5C**). In response to the OSM induced MMP-1 expression, gene analysis showed similar decreasing trends, with Peficitinib ($p < 0.01$) and Baricitinib ($p < 0.05$) displaying the greatest inhibition. Although not significant, Filgotinib and Upadacitinib both reduced MMP-1 expression (**Figure 5D**).

JAK Inhibitors Block PsAFLS Migration Promoted by OSM

Finally, the capacity of PsAFLS to migrate within the joint environment is associated with progressive and destructive joint disease, therefore, we next investigated the role of JAKi on migration of PsAFLS using a wound repair scratch assay. **Figure 6A** shows representative images demonstrating the increased migratory capacity of PsAFLS in response to OSM compared to unstimulated cells, in addition to the inhibitory effect of JAKi on PsAFLS migration, where repopulation of wound margins was inhibited by JAKi. Quantitative analysis demonstrated that OSM significantly induced migration of PsAFLS ($p < 0.01$) compared to basal control (**Figure 6B**). However, Peficitinib ($p < 0.001$), Filgotinib ($p < 0.01$) and Baricitinib ($p < 0.05$) all significantly decreased PsAFLS migration across the wound margins (**Figure 6B**). Although not significant, Upadacitinib also demonstrated strong inhibition of PsAFLS migration (**Figure 6B**). As migration of FLS is aided by adhesion molecules we also determined if JAKi influenced ICAM expression. Gene analysis showed a significant inhibition of OSM- induced ICAM expression by Upadacitinib ($p < 0.01$), all other inhibitors displayed decreases in ICAM expression, although these did not reach significance (**Figure 6C**).

A summary table outlining the main effects of each JAKi can be found in **Table 2**.

DISCUSSION

In this study, we identified the impact of four JAKi inhibitors; Peficitinib, Filgotinib, Baricitinib and Upadacitinib on the

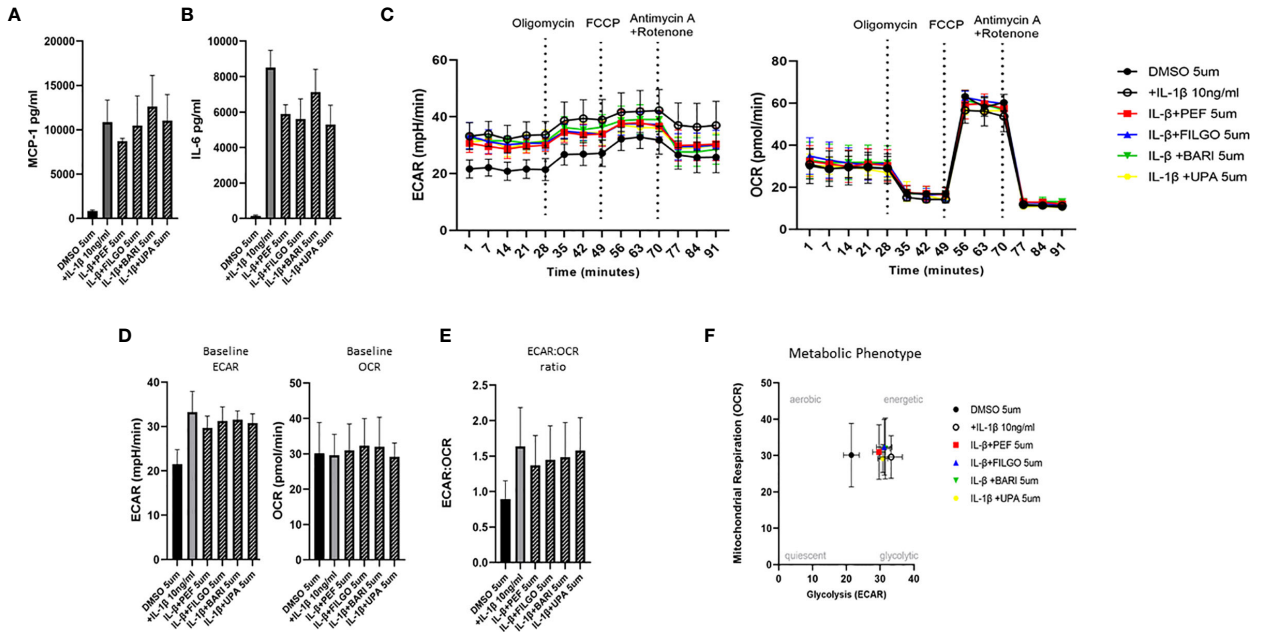

FIGURE 3 | Effect of JAKi on the IL-1β-induced expression of pro-inflammatory mediators and bioenergetic profile in PsAFLS. **(A, B)** PsAFLS were treated with JAKi (5 μM) or DMSO (5 μM) for 1 h and stimulated with IL-1β (10 ng/ml) for 24 h. Bar graphs demonstrating secretion of **(A)** MCP-1 and **(B)** IL-6 by ELISA (n = 3). **(C)** Average seahorse profiles demonstrating extracellular acidification rate (ECAR) (glycolysis) and oxygen consumption rate (OCR) (oxidative phosphorylation). **(D)** Representative bar graphs demonstrating baseline ECAR and OCR and **(E)** ECAR : OCR ratio. **(F)** Metabolic phenotype profile in PsAFLS, n = 3. Values expressed as mean +/- SEM.

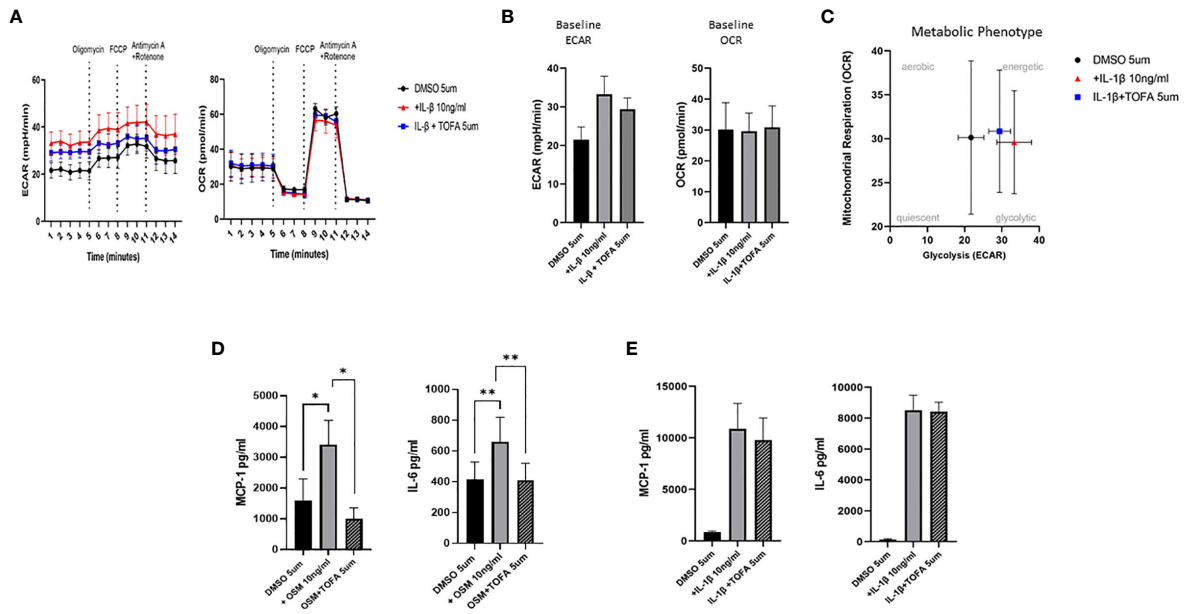

FIGURE 4 | Effect of Tofacitinib on the IL-1B or OSM induced expression of pro-inflammatory mediators and bioenergetic profile in PsAFLS. **(A)** Average seahorse profiles demonstrating extracellular acidification rate (ECAR) (glycolysis) and oxygen consumption rate (OCR) (oxidative phosphorylation). **(B)** Representative bar graphs demonstrating baseline ECAR and OCR. **(C)** Metabolic phenotype profile of PsAFLS, n=3. **(D, E)** PsAFLS were treated with Tofacitinib (5 μM) or DMSO (5 μM) for 1 h and stimulated with OSM (10ng/ml) (n=7) or IL-1β (10 ng/ml) (n=3) for 24 h. and MCP-1 and IL-6 quantified. Values expressed as mean +/- SEM $^{*}p < 0.05$, $^{**}p < 0.01$.

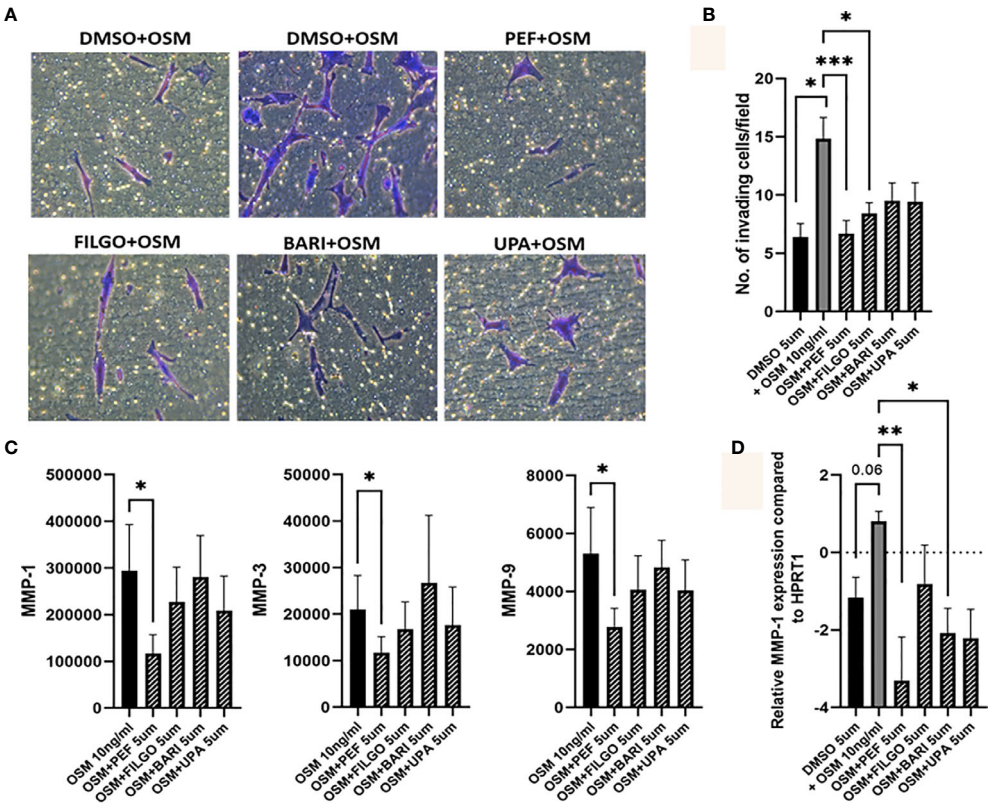

FIGURE 5 | OSM driven invasion by PsAFLS is blocked by JAKi. **(A)** Representative photomicrographs and **(B)** bar graph showing PsAFLS invasion following treatment with JAKi (5 μM) or DMSO (5 μM) for 1 hr and stimulated with OSM (10 ng/ml) for 24 h, n=6 (magnification X20). **(C)** Representative bar graph showing MMP-1, MMP-3 and MMP-9 expression by MSD ELISA (n=7) and **(D)** gene expression analysis of MMP-1 by real-time PCR (n=5). Values expressed as the mean +/- SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from DMSO+OSM control.

pathogenic phenotype observed in PsAFLS. JAKi are an encouraging class of drugs for the treatment of PsA, as evidence of increased JAK/STAT signalling has been shown at the site of inflammation (15, 21). To date, no study has examined the role of these JAKi on primary cells isolated from PsA synovial tissue. We utilised OSM as a stimulant, as it signals through the JAK-STAT pathway, specifically activating JAK1, JAK2 and to a lesser degree TYK2 (25). OSM is increased at the site of inflammation, in addition, several studies have shown that OSM drives synovial fibroblast invasive mechanisms (31–35). Using OSM to drive this inflammatory response, we demonstrate that JAKi significantly decreased the secretion of key pro-inflammatory mediators; MCP-1 and IL-6. This was accompanied by changes in the bioenergetics of the cells, whereby JAKi decreased the glycolytic profile of the PsAFLS resulting in a shift towards a more oxidative phosphorylated/quiescent phenotype. Finally, we demonstrated the ability of JAKi to inhibit the pathogenic function of PsAFLS by significantly decreasing their invasive and migratory capacity. While all JAKi inhibitors decreased pro-inflammatory and metabolic mechanisms in PsAFLS, this was most pronounced for Peficitinib. These data demonstrate that JAK/STAT signalling mediates pro-inflammatory mechanisms that drive PsA pathogenesis, an effect inhibited with the use of JAKi.

In this study, we show that JAKi significantly reduce the secretion of OSM-induced pro-inflammatory mediators MCP-1 and IL-6, while displaying an increasing trend in IL-8 secretion, this differential regulation is consistent with the pleiotropic effects of OSM (36). We also show that Peficitinib, Baricitinib and Upadacitinib displayed the most significant inhibition of MCP-1 and IL-6 secretion, while Filgotinib also decreased cytokine secretion, this did not reach significance. The role for JAK-STAT signalling in PsA is consistent with studies showing increased expression of pSTAT3 and pSTAT1 in PsAFLS and PsA synovial tissue (21). Studies in psoriasis have shown an increase in pSTAT expression localised to the epidermal hyper-proliferation layer (37, 38). Furthermore, the effect of JAKi is consistent with previous reports showing Tofacitinib, Peficitinib and Baricitinib inhibit IL-6 and MCP-1 expression in RAFLS (39, 40). As these JAKi can inhibit multiple pro-inflammatory mediators simultaneously and rescue function, they may act as a superior treatment for PsA compared to blockade of one specific cytokine.

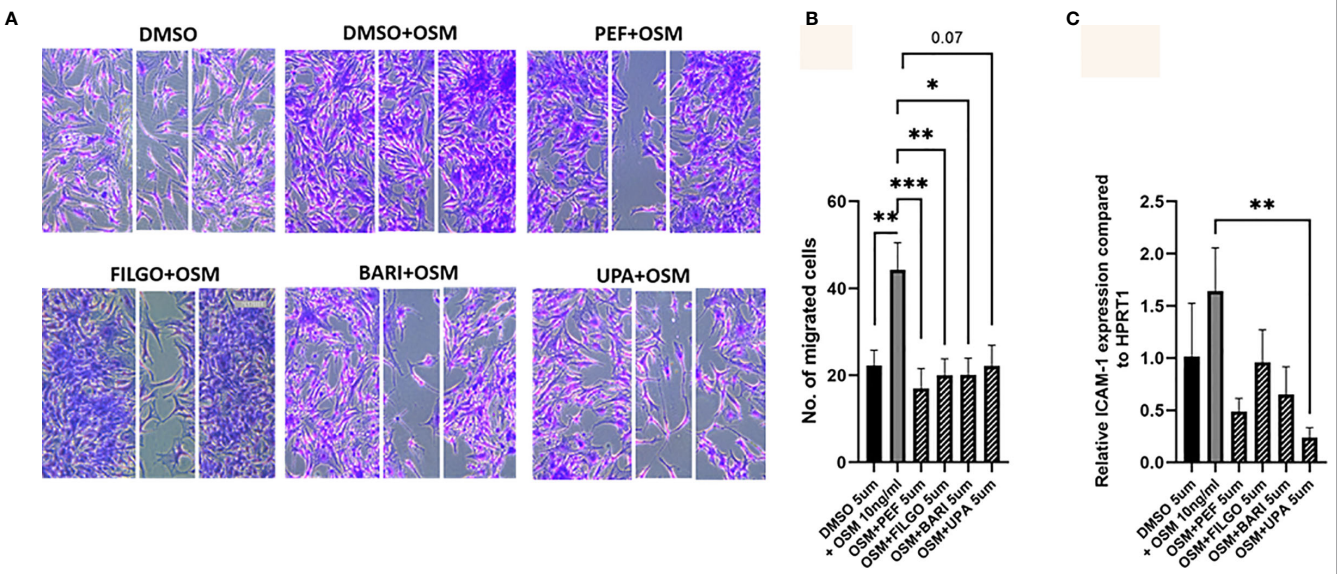

FIGURE 6 | JAKi inhibit OSM induced migration of PsAFLS. **(A)** Representative photomicrographs and **(B)** bar graph showing PsAFLS migration following treatment with JAKi (5 μM) or DMSO (5 μM) for 1 hr and stimulated with OSM (10 ng/ml) for 24 h, n=8 (magnification X10). **(C)** Gene expression analysis of ICAM by real-time PCR (n=5). Values expressed as mean +/- SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from DMSO+OSM control.

TABLE 2 | Summary of JAKi functions.

JAKi	Target	IL-6	MCP-1	ECAR/OCR Ratio	Invasion	MMPs	ICAM-1	Migration
Peficitinib	Pan JAKi	↓↓↓	↓↓↓	↓↓	↓↓↓	↓	↓	↓↓↓
Filgotinib	JAK 1	-	-	-	↓	-	↓	↓↓
Baricitinib	JAK1/2	↓	↓	↓	-	↓	↓	↓
Upadacitinib	JAK1	↓↓↓	↓↓	↓	-	-	↓↓	-

To examine if the effect of JAKi on pro-inflammatory function also alters the energy profile of PsAFLS, we investigated the two major energy pathways, glycolysis and oxidative phosphorylation using real-time Seahorse Technology. Changes in metabolism have been observed at the site of inflammation in both PsA and RA, due most likely to environmental factors within the joint resulting in a hypoxic microenvironment (41–44). Previous studies have shown a shift to a more glycolytic profile in RAFLS compared to OAFLS (45), with several studies demonstrating elevated levels of metabolic intermediates and increased activity of key glycolytic enzymes in both the RA and PsA synovium/cells (46–48). However, this is the first study to compare the bioenergetics of PsAFLS in response to treatment with JAKi. We show that JAKi reduced the glycolytic shift in favour of a more oxidative state, similar to a cell in quiescence. These findings are consistent with a report by McGarry et al. using Tofacitinib which inhibited glycolysis along with key glycolytic genes *HK2, GSK3A, PDK1* and *HIF1α* in RAFLS (39). Although all JAKi displayed inhibition, Peficitinib and Baricitinib significantly reduced the rates of glycolysis, with Peficitinib having the greatest effect on the ECAR/OCR ratio. Regulation of the metabolic pathways has been strongly linked with resolution of inflammation in the inflamed joint, with several studies demonstrating that metabolic blockade inhibits inflammation *in vitro*, *ex vivo* and *in vivo* models of arthritis (39, 42, 44, 45, 47–51).

Interestingly, interactions between JAK-STAT signalling and metabolic pathways have been demonstrated in previous studies. Blockade of the key glycolytic enzyme PFKFB3 inhibits pSTAT3 activation in RAFLS (41, 51, 52). In turn, STAT3 itself can regulate glycolysis through HK2 in cancer cells (41, 51, 52), and plays a key regulatory role in mediating interactions between HIF1α and PKM2 (41, 51–54). Interplay between STAT3 and Sirtuin-1 has also been demonstrated to regulate oxygen consumption, ETC complex activity and metabolic intermediates in the mitochondria (53, 54). Indeed, studies have suggested that this effect may be due to localised STAT3 expression in the mitochondria which modulates the activity of complex I and II (52), however, other studies suggest alternative mechanisms, either *via* additional transcriptional regulation, or indirect activation of mitochondrial signalling pathways (55). In context of the inflamed joint, STAT3 interacts with various other key signalling pathways including HIF1a, Notch and NFκB all of which regulate each other's activation through complex positive and negative feedback loops in the PsA/RA joint (56). Therefore, the use of JAKi in metabolically reprogramming these cells may aid in reducing their inflammatory aggressive phenotype in PsA.

We also determined the effect of JAKi on PsAFLS function by examining their invasive and migratory capacity. All JAKi showed a striking inhibition of invasion by PsAFLS, although Peficitinib and Filgotinib displayed the strongest effect. In parallel, all JAKi significantly inhibited PsAFLS migration. While the precise mechanism by which JAKi impacts invasion and migration is unclear, Peficitinib significantly reduced MMP-1, MMP-3, and MMP-9 secretion, while the other JAKi showed a slight decrease. Consistent with our data, several other studies using RAFLS have reported inhibition of MMP-1 and MMP-3 by both Tofacitinib and Peficitinib (29, 39, 40), in addition to the inhibitory effect of Tofacitinib on PsAFLS invasion, network migration and migration (21). Furthermore, in PsA synovial explants, Tofacitinib inhibits MMP-3 expression and the overall MMP-3/TIMP ratio (21), thus reducing the ability of FLS to invade the tissue thereby reducing joint destruction. Other pathways involved in FLS invasion and migration include Integrin-cytoskeletal pathways that bridge cell–cell and cell–ECM interactions (57), with previous studies showing that JAK-STAT signalling regulates RA-FLS lamellipodia formation and RhoGTPases, key proteins involved in cellular movement (58). As migration of these cells to the joint is aided by adhesion molecules, we also show that ICAM expression is strongly reduced following treatment with JAKi. Similar effects were seen with RANKL, where Peficitinib and Tofacitinib decreased expression in RAFLS (34). Other potential mechanisms include the YAP pathway which has been implicated in RAFLS invasiveness (59, 60). Indeed, studies in fibroblasts from other disease settings have shown complex interactions between metabolic pathways and YAP/TAZ signalling (61). Therefore, the use of JAKi in PsA may help in reducing inflammation induced by infiltrating FLS to the inflamed joint.

Finally, in this study we utilised OSM as an activator of the JAK/STAT pathway, however, it is only one of many cytokines implicated in PsA pathogenesis that acts through this pathway, including IL-12, IL-23, IL-22 and IFNγ. Thus, there are limitations to the interpretation. While outside the scope of this study, an ideal model would be to culture PsAFLS with a cocktail of all the relevant cytokines that are known to be increased in the PsA joint in the presence or absence of JAKi.

In conclusion, this study demonstrates the effect of JAKi in targeting PsAFLS function *in vitro*, inhibiting invasive, migration and metabolic mechanisms leading to resolution of inflammation. These findings support the role for JAKi in patients with inadequate responses to current PsA therapies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by St Vincent's University Hospital Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AO'B, MH, VM, SW, and KF performed the experiments, analysed the data, and prepared the manuscript. KF also processed clinical samples. UF conceived the experimental approach, analysed the data, and supervised and prepared the manuscript. DV conceived the experimental approach, collected the clinical samples, analysed the data, supervised the study, and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Inflammatory Bowel Diseases and Sarcopenia: The Role of Inflammation and Gut Microbiota in the Development of Muscle Failure

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Sarcopenia represents a major health burden in industrialized country by reducing substantially the quality of life. Indeed, it is characterized by a progressive and generalized loss of muscle mass and function, leading to an increased risk of adverse outcomes and hospitalizations. Several factors are involved in the pathogenesis of sarcopenia, such as aging, inflammation, mitochondrial dysfunction, and insulin resistance. Recently, it has been reported that more than one third of inflammatory bowel disease (IBD) patients suffered from sarcopenia. Notably, the role of gut microbiota (GM) in developing muscle failure in IBD patient is a matter of increasing interest. It has been hypothesized that gut dysbiosis, that typically characterizes IBD, might alter the immune response and host metabolism, promoting a low-grade inflammation status able to up-regulate several molecular pathways related to sarcopenia. Therefore, we aim to describe the basis of IBD-related sarcopenia and provide the rationale for new potential therapeutic targets that may regulate the gut-muscle axis in IBD patients.

Keywords: IBD, sarcopenia, gut-muscle axis, gut microbiota, probiotics, inflammation, muscle wasting, malnutrition

INTRODUCTION

The European Working Group on Sarcopenia in Older People defined sarcopenia as a progressive and generalized skeletal muscle disorder, characterized by loss of muscle mass and function, low muscle strength and poor physical performance (1). Accordingly, it represents a major health burden in industrialized country by determining the risk of physical disability, poor quality of life, increased hospital admissions and increased mortality (2, 3).

Muscle impairment represents a common pathological hallmark of common chronic gastrointestinal diseases, including inflammatory bowel diseases (IBD). Recently, it has been reported that 42% of IBD patients suffered from sarcopenia (4). In addition, in most of them sarcopenia coexists with malnutrition as results of chronic inflammation.

Inflammatory bowel disease, including Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders affecting the gastrointestinal tract, characterized by a relapsing-

remitting course. Although their etiopathogenesis is still unknown, it has been hypothesized an aberrant immune-mediated response to specific antigens of the gut microbiota (GM) in genetically predisposed individuals (5–9).

The GM represents a real ecosystem, consisted of more than 10^{14} bacteria and more than 1000 species as well as fungi, viruses, phages, parasites, and archea, that colonizes gastrointestinal tract and plays an important role in nutrient absorption, maintenance of metabolic homeostasis, protection from infections and development of systemic and mucosal immunity (10–13).

Several studies have shown significant difference in the GM composition between patients with IBD and healthy people. In particular, the phylum Firmicutes - specifically Faecalibacterium prausnitzii - is often reduced in the stool of patients with CD, while members of the Proteobacteria phylum, such as Enterobacteriaceae, including Escherichia coli, are commonly increased in patients with IBD compared to healthy individuals (5, 14–16). This contributes to a shift in the balance between commensal and potentially pathogenic microorganism that leads to dysbiosis (16).

Among several factors involved in the pathogenesis of sarcopenia, the role of GM in developing muscle wasting in IBD patients has now gained increasing interest. It has been hypothesized that GM moving from protective to pro-inflammatory effects, might alter the immune response and host metabolism, promoting a low-grade inflammation status able to up-regulate several molecular pathways related to sarcopenia, with consequent development of musculoskeletal impairment and frailty (17–19).

Therefore, this narrative review aims to describe the bases of IBD-related sarcopenia and to provide the rationale for new potential therapeutic targets that might regulate the gut-muscle axis in IBD patients.

THE GUT-MUSCLE AXIS HYPOTHESIS

Recently, growing data support the hypothesis of a "gut-muscle axis" (5, 20, 21), wherein inflammation, gut dysbiosis, and malnutrition, interplay chorally for development of muscle failure in IBD patients (**Figure 1**). In this next section we focus on these key players of the gut-muscle axis.

Inflammation

The reduction of muscle mass and strength in sarcopenia increases with age. There are several factors involved in the development of muscle atrophy and age-related sarcopenia. The persistent low-grade inflammatory status in the elderly, characterized by increased circulating levels of pro-inflammatory cytokines, such as TNF-alpha, IL-6, and myostatin, defined as "inflammaging", is crucial (22–24). To date there were conflicting data with regards to the median ages of sarcopenic IBD patients. Zhang et al. reported that IBD patients with sarcopenia were significantly younger compared with those without sarcopenian (6), while according to Pedersen et al. sarcopenic patients were more likely to be older with more medical comorbidities, such as hypertension and diabetes, than younger non sarcopenic (25).

However, the intestinal inflammatory state that characterizes patients with IBD might be considered as the starting point for the development of muscle impoverishment, by activating several pathways in common with sarcopenia (5, 17, 19).

TNF-alpha, produced by macrophages, lymphocytes, mast cells, fibroblasts and endothelial cells, is considered the key driver of intestinal damage, by stimulating macrophages to produce pro-inflammatory cytokines, inducing apoptosis of intestinal epithelial cells and Paneth cells, and stimulating the synthesis of proteases (26, 27). It is commonly believed that the disruption of intestinal epithelial tight junctions (TJ) leads to an increase of gut permeability with a consequent translocation of lipopolysaccharide (LPS) into systemic circulation. Several studies showed that the epithelial barrier function is impaired in IBD patients. Experimental models of colon biopsies of IBD patients hypothesized as a possible cause of barrier dysfunction a reduction of tight junction strands in both UC and CD (28–31). In response to the LPS stimuli, nuclear factor κB (NF-kB) translocates from cytoplasm to nucleus and stimulates dendritic cells and macrophages to produce pro-inflammatory cytokines and mediators, for instance, cyclo-oxygenase-2 (COX-2), TNF-alpha, inducible nitric oxide synthase (iNOS), and IL-6, that regulate intestinal and systemic inflammation (28–31).

Importantly, it has been shown that increased serum levels of TNF-alpha are associated with muscle impairment. Indeed, TNF-alpha regulates the activation of NF-kB signaling pathway through the expression of the "atrogenes" (atrophy-related genes) and promotes protein degradation through the transcription of ubiquitin proteasome E3 ligases: muscle RING-finger protein-1 (MurF1), and Atrogin (32, 33).

In addition, IL-6 produced by macrophages and T cells in the inflamed gut, is a pleiotropic cytokine able to upregulate the production of pro-inflammatory cytokines and inhibit T cell apoptosis through the activator of transcription 3 (STAT3) and hence contributes to the disruption of skeletal muscle proteosynthesis (34, 35).

Notably, the role of IL-6 in muscle homeostasis depends on the timing of its production. While during exercise IL-6 transient production is associated with beneficial effects, a persistent elevation of its serum levels, in particular in elderly, is associated with muscle wasting and sarcopenia (36, 37). Of note, the reduced proteosynthesis and the increase in protein degradation of the skeletal muscle tissue seem to be promoted by the activation of three different cellular signaling pathways, all starting from the binding of IL-6 and its receptor: the Janus kinasi (JAK)/STAT pathway, the Mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinases (ERK) pathway and the Phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway (38).

In particular, the JAK/STAT signaling pathway is highly involved in IBD pathogenesis, mediating the function of several inflammatory cytokines implicated in gut inflammation, such as IL-2, IL-4, IL-6, IL-7, IL-9, IL-12, IL-15, IL-21, IL-23, and IFN-gamma (39).

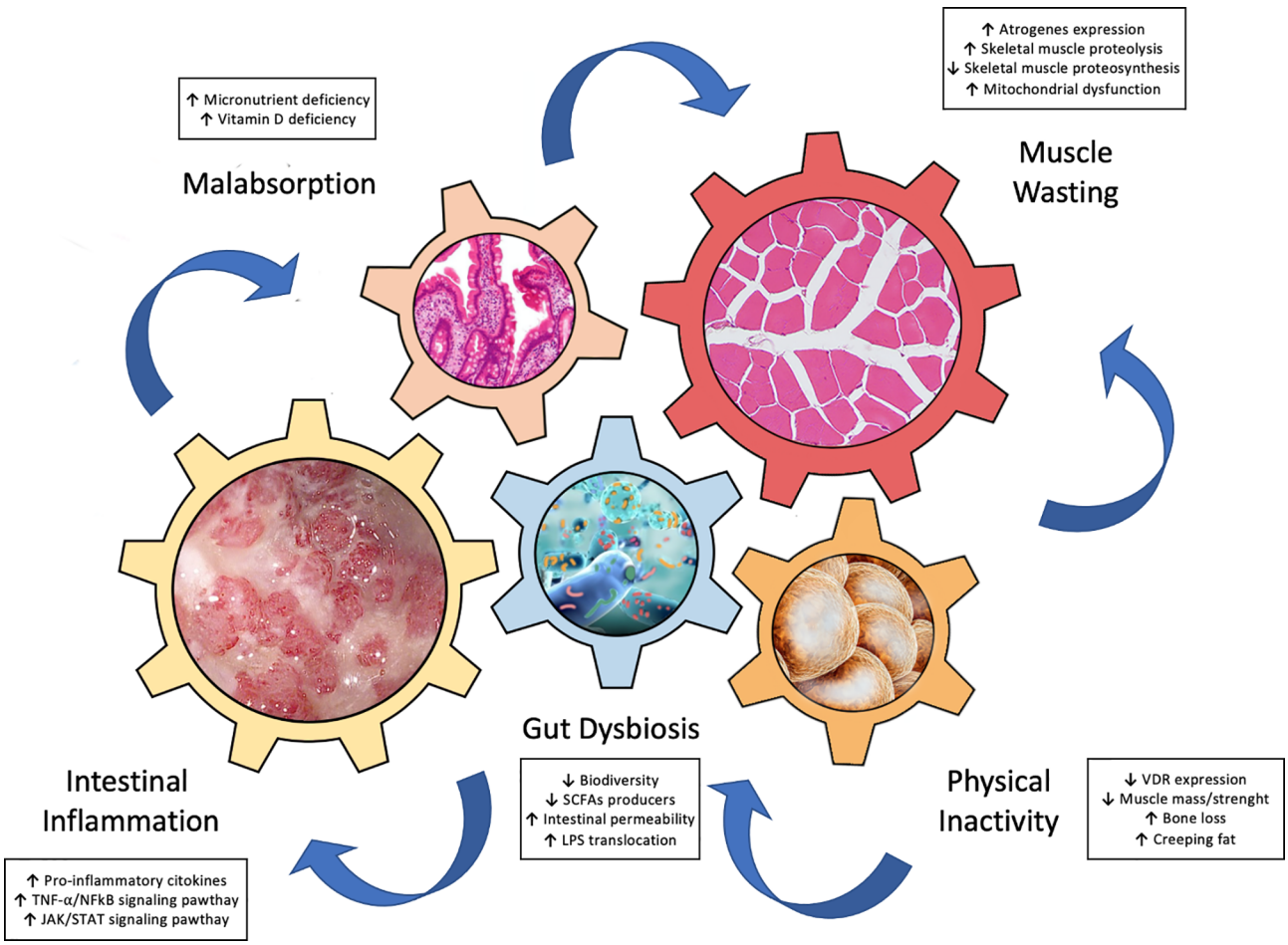

FIGURE 1 | Key drivers involved in the pathogenesis of "gut-muscle axis".

In this context, skeletal muscle fibers by expressing cytokine receptors and both the toll-like receptor (TLR)-2 and TLR-4 (which bind LPS), determine an overproduction of reactive oxygen species (ROS) and an oxidative stress (40). Given that mitochondria are the most vulnerable cellular organelle to ROS, the oxygen consumption decreases mitochondrial respiration and the ability to produce ATP. This process drives to disrupted mitochondrial dynamics and leads to "mitophagy", i.e. hyperactivation of mitochondrial degradation pathways (41).

In a number of chronic clinical disorders, such as also sepsis, heart failure and chronic obstructive pulmonary disease, mitochondrial dysfunction has been associated with increased systemic inflammation, which influences muscle protein synthesis and impairs both mitochondrial and muscle function (42). These processes are predominantly a consequence of oxidative stress secondary to ROS with a negative impact on skeletal muscle and hence are likely to contribute to the development of sarcopenia (42).

Although mitochondrial dysfunction has been founded in the intestinal epithelium of IBD patients, the role of epithelial mitochondrial stress in the pathogenesis of IBD has not yet fully understood (43). A recent study conducted by Jackson et al. identify Paneth cells as highly susceptible to mitochondrial dysfunction driven by loss of prohibitin 1 (PHB1), a major component protein of the inner mitochondrial membrane, and central to the pathogenesis of ileitis (44). This provides important translational implications for mitochondrial-targeted therapeutics in a subset of CD patients exhibiting Paneth cell defects (44).

Finally, there is a strict connection between mitochondria function and microbiota. In fact, the short chain fatty acids (SCFA) produced by gut bacteria is positively correlated with the expression of mitochondrial protein involved in the energy production, redox balance, and the modulation of the inflammatory cascade activation. However, in IBD patients, bacteria that ferment fibers and produce SCFAs are typically reduced in mucosa and feces as compared to healthy individuals. This low representation of SCFA producers in gut microbiota has been associated with increased subclinical chronic inflammation, which reinforces the skeletal muscle anabolic resistance (45).

Gut Dysbiosis

Gastrointestinal tract and skeletal muscle tissue interact each other through a complex network modulated by the GM consisting of hormones, implicated in the homeostasis of energy metabolism, protein metabolism favors breakdown, and inflammatory mediators (i.e.TNFα) which increase the permeability of the intestinal membrane and cause both local and systemic inflammatory effects (18, 20, 21).

In IBD patients, this ecosystem is altered in terms of biodiversity, microbial composition and functions, determining the so-called "gut dysbiosis", that reflects an inappropriate immune response of a complex microbial community to the intestinal inflammation (46–48). It has been reported that IBD patients had a decrease of Firmicutes and Bacteroides, and a relative increase of bacterial species belonging to Enterobacteriaceae, that disrupt the intestinal barrier integrity, and an increase in mucolytic bacteria that determine the degradation of the mucosal barrier and thereby an increased penetration of pathogens into the intestinal tissues (49). This condition, defined as "leaky gut syndrome", represents a cofactor in the onset of a pro-inflammatory status leading to sarcopenia in both IBD and elderly patients (50, 51). Several studies showed an overall decrease in alpha and beta diversity of the gut microflora in IBD patients (52, 53). It has been reported by Qin et al. that in IBD patients' mucosal microbial genes are reduced of 25% compared to healthy controls (54). Recently, a comprehensive description of qualitative alterations in the gut microbiome of 132 patients affected by IBD has shown the presence of a functional dysbiosis in the gut microbiome during flairs of the disease with an increase in facultative anaerobes over obligate anaerobes, as well as molecular dysregulation in microbial transcription, metabolites, and serum levels of antibodies in the host (55). Particularly, Joossens et al. reported an increase in Ruminococcus gnavus and a decrease in Bifidobacterium adolescentis, Dialister invisus, Faecalibacterium prausnitzii, and an unknown member of Clostridium cluster XIVa in IBD patients (56).

However, GM influences resident mucosal immune cells to produce proinflammatory cytokines and microbiota-derived metabolites. Among them, SCFAs, secondary bile acids, water-soluble B-vitamins, tryptophan, polyphenols, and urolithins, are crucial for modulation of gut-muscle axis, by promoting insulin sensitivity, biosynthesis of amino acids, mitochondrial biogenesis, and muscular anabolism (21, 57–61).

Of note, several studies investigated the modulatory role of GM on the skeletal muscle functions and amino acid bioavailability (62–64). For instance, in both frail and IBD population there is a decrease in Faecalibacterium prausnitzii, a SCFAs producer with a significant anti-inflammatory function. In addition, Bifidobacteria lactobacilli, involved in protein breakdown to amino acids within the gut, produce SCFAs for energy production, stimulate insulin growth factor -1 (IGF-1)/mTOR pathway and promote the expression of genes involved in muscle protein synthesis (45, 65, 66). Similarly, Escherichia coli and Klebsiella play a role in the skeletal muscle anabolism and cell proliferation through the stimulation of the IGF-1/mTOR pathway (67).

Recently, Picca et al. profiled GM in older adults affected by physical frailty and sarcopenia, showing that an increase of Oscillospira and Ruminococcus, and a decreased of Barnesiellaceae and Christensenellaceae microbial taxa, are associated with muscle impairment (68). Furthermore, it has been reported that exercise induces changes of GM with an important over-representation of some healthy bacterial species, such as Akkermansia, Prevotella, Faecalibacterium, and Roseburia, in fecal samples of humans with active lifestyle (69–73).

Regardless the correlation between GM and muscle function, Bjørkhaugh et al. showed that patients with chronic alcohol overconsumption had a loss of muscle strength, assessed by hand-grip strength test, associated with a higher relative abundance of Proteobacteria, Sutterella, Clostridium and Holdemania and a lower relative abundance of Faecalibacterium with reduced SCFAs fecal levels (74). In a recent non-randomized trial examining the effect of exercise on GM modulation in 32 sedentary elderly women it has been reported that the abundance of Bacteroides was positively correlated with an increased physical performance assessed by the 6-min walking distance test (75). Furthermore Fielding et al. showed that the colonization of germ-free mice with GM of high-functioning older adults is associated with an increase of muscle strength with a higher relative abundance of Prevotella and Barnesiella (76).

Therefore, the modulation of GM modulation could impact significantly on the onset of sarcopenia. Varian et al. have shown that the administration of Lactobacillus reuteri in mouse models of cancer could inhibit the development of sarcopenia and increase in muscle weight and fiber size, through an up-regulation of the transcriptional factor Forkhead Box N1 (FoxN1) (77).

In a randomized controlled study prebiotic administration compared with placebo significantly improved two frailty criteria: exhaustion and increases the grip strength in elderly people over 65 years old (78). Similarly, Munukka et al. found that supplement of probiotics (Faecalibacterium prausnitzi) increased muscle mass that is linked to enhanced mitochondrial respiration, improved insulin sensitivity, modified gut microbiota composition and improved intestinal integrity (79).

Furthermore, folate and vitamin B12 are used as nutrient substrates by intestinal microflora for the maintenance of GM homeostasis; thus, nutrient deficiency negatively impacts on the GM function and consequently on muscle protein homeostasis (80).

Malnutrition

The chronic inflammation contributes to malnutrition through associated anorexia and decreased food intake; it further impacts on metabolism with elevation of resting energy expenditure and increased muscle catabolism (81). Of note, in more than two thirds of malnourished patients, sarcopenia coexists (82).

Malnutrition is also a condition that commonly affect IBD patients. In detail it has been reported in 65–75% of patients with CD and in 18–62% of patients with UC, as results of malabsorption, side effects of steroids, and increase in basal energy expenditure due to the inflammatory status (19). Importantly, malnutrition is typically associated to a significant alteration of body composition i.e. weight loss, reduction of

skeletal muscle mass, bone loss, and expansion of visceral and "creeping" fat (83, 84).

The association of malnutrition and sarcopenia in IBD patients is controversial. Indeed, it is noteworthy that while malnutrition in IBD is characterized by weight loss during the acute phase of disease followed by a gradual recovery during disease remission, sarcopenia may be present even in IBD patients in remission and not only with low but also normal or elevated values of BMI (85–87).

However, the poor and/or inadequate oral intake is considered one of the most important determinants of IBD-related malnutrition, due to a voluntary food restrictions and symptoms such as nausea, abdominal pain, vomiting, and diarrhea occurred in case of IBD flare up (88). Importantly, macronutrient intake is usually preserved in almost all IBD patients, while micronutrient deficiency, such as iron, copper, selenium, magnesium, zinc, vitamins and antioxidants, can occur more frequently (89). In addition, an insufficient protein intake can determine sarcopenia.

It is now clear that nutrients contribute significantly for the health of the trillions of bacteria, fungi, viruses, phages, parasites, and archea that compose the GM. Metagenomics analysis revealed that diet alters microbial community structure and overwhelms inter-individual differences in microbial gene expression (90–92). For instance, high-protein diets are associated with low microbial diversity. While a high-fat diet leads to a decrease in Bacteroidetes and an increase in Firmicutes, alterations that have been associated with an increase of opportunistic bacteria, intestinal permeability, low-grade systemic inflammation and insulin resistance (93). Conversely, promotion of insulin sensitivity, mitochondrial biogenesis, energy production and modulation of inflammation are induced by SCFAs produced by gut bacteria such as Faecalibacterium, Succinivibrio, and Butyricimonas (20). Of note, human studies focused on GM and malnutrition showed that children with protein energy wasting displayed an increase in Proteobacteria and a decrease in Bacteroidetes when compared with healthy children (94, 95).

Whilst there is a large body of supporting evidence for supplemental interventions for the prevention of muscle loss, strength and function in older adults, few data analyze this effect in patients with IBD.

Leucine supplementation, as well as vitamin D, in association with physical exercise increased skeletal muscle mass and muscle strength (96). Furthermore, the supplementation of beta-hydroxy-beta-methyl butyrate is associated with preservation of muscle tissue during short period of bed rest and increased muscle mass and strength, particularly in combination with resistance training (97–99).

Furthermore, vitamin D deficiency plays a key role in the onset of IBD-related sarcopenia (18). A recent metanalysis including 938 IBD patients, showed that lower vitamin D serum levels are more frequent in patients with IBD (64%) than controls (100). Although the exact mechanism through which vitamin D affects skeletal muscle homeostasis is not totally elucidated, loss of muscle mass seems to be strictly related to the decrease of vitamin D receptor (VDR) expression (101). It has been reported that vitamin D, once interacting with VDR, can elicit two different effects: 1) a non-genomic effect, such as modulation of calcium channel activation, muscle contraction and mitochondrial function; 2) a genomic effect, up-regulating nuclear expression of gene coding contractile proteins and myogenic transcription factors (102). It has been reported that VDR expression declines in elderly and in patients affected by chronic inflammatory diseases, such as IBD, chronic obstructive pulmonary disease, renal failure, diabetes and asthma (103, 104). Therefore, vitamin D serum levels should be monitored and supplemented to prevent the onset of osteoporosis and muscle wasting in IBD patients.

STUDIES EXPLORING IBD AND SARCOPENIA

Animal Studies

The exact pathogenesis of sarcopenia in IBD patients is still uncertain. Thus, in an attempt to elucidate the interaction between gut inflammation and muscle failure, experimental animal models were reproduced. These imply to collect gastrointestinal and muscle tissue samples, but also plasma and stools to assess serum markers of inflammation and perform microbiota analysis (21). The main mouse models of experimental IBD are induced by the intrarectal administration of trinitrobenzene sulphonic acid (TNBS), leading to a transmural colonic inflammation similar to that observed in CD patients, or through the oral intake of dextran sulphate sodium (DSS), promoting a histological intestinal inflammation similar to UC in humans (105).

It has been shown that skeletal muscle mass and proteins are reduced in murine models of TNBS-induced colitis, suggesting the linkage between IBD and muscle wasting (106). In this context, gut inflammation seems to be the trigger of skeletal muscle atrophy due to an accelerated rate of protein breakdown mediated by the up-regulation of the ubiquitin proteasome proteolytic pathway and enhanced expression of atrogin-1 and Murf-1 skeletal muscle atrophy-related genes (atrogenes) implicated in muscle protein breakdown (106).

Furthermore, a transcriptional upregulation of Murf-1, and consequent myofibril degradation was observed in mice affected by DSS-induced colitis. Indeed these latter differ from controls for the loss of skeletal muscle mass and decrease in muscle function, assessed by the significant reduction of the number of gastrocnemius myofibrils and the physical performance on rota-rod test (107). Similarly, a mild chronic gut inflammation caused by DSS accelerates the muscle dysfunction evaluated by using rotarod test, gait analysis, and grip strength test in α-synuclein mutant mice (108).

The analysis of molecular pathways of IBD-related sarcopenia assessed by using immunohistochemistry showed a down-expression of IGF1-R and Phospho-mTOR, markers of muscle growth, and an over-expression of Murf-1 and Myostatin, considered markers of sarcopenia (107). Similarly, Saul et al.

showed that experimental IBD mice had a decrease in skeletal muscle weight and fiber size with a reduction of muscle protein content tested in quadriceps and gastrocnemius (109). Moreover, they observed an increased in mRNA expression of Murf-1 and Atrogin-1 suggesting an enhanced protein degradation responsible of sarcopenia (109).

However human muscle stem cells (hMuSCs) treated with IFN-γ and TNF-α for 48 hours, and then transplanted intravenously, ameliorated colitis in mice treated with DSS by producing TNF-stimulated gene 6 (TSG-6) implicated in anti-inflammatory functions (110).

Notably, animal models have been used also to evaluate the effects of gut microbiota manipulation on parameters of muscle mass and function. Indeed, probiotics seem to have a marked anti-inflammatory effect with beneficial consequences for muscle health through the promotion of anabolism. Probiotics containing Faecalibacterium prausnitzii, one of the main SCFA producers, were associated with improved liver anabolism and reduced systemic inflammation in mice models (79).

Human Studies

The assessment of nutritional status with body composition and an early detection of sarcopenia in patients with IBD is essential for providing an appropriate nutritional support, even during the remission period, and preventing sarcopenia-related surgical and negative outcome.

To date the majority of studies have defined sarcopenia as a loss of muscle mass. Typically, three imaging techniques have been employed for the assessment of muscle mass: computed tomography (CT), dual energy X-ray absorptiometry (DXA) and bioelectrical impedance analysis (BIA). While CT allows a direct estimate of muscle mass, DXA and BIA only provides indirect estimates such as lean mass. However, according to the revised European Working Group on Sarcopenia in Older People (EWGSOP), the definition of sarcopenia was also based on muscle function, quantity and physical performance (1). The muscle function is commonly evaluated through the handgrip strength, 5-times repeated chair stand test and 4-meter walking speed, while the physical performance can be measured by gait speed, the Short Physical Performance Battery, and the Timed-Up and Go test.

Ryan et al. performed a systematic review, including a total of 658 patients, to assess the prevalence of sarcopenia in IBD patients and the correlation between sarcopenia and needs of surgery and surgical outcomes in patients with IBD (4). Forty-two percent IBD patients had a diagnosis of sarcopenia detected with radiologic assessment of body mass composition and had an increased risk of requiring surgery with high rate of major complications after surgery (4).

Notably, sarcopenic IBD patients had lower preoperative serum levels of albumin and higher preoperative serum levels of C-reactive protein, deemed markers of malnutrition/inflammation and predictive factor of surgical negative outcome (86). Furthermore, a recent cross-sectional study involving 344 IBD patients in clinical remission revealed an increased risk of sarcopenia in malnourished patients (87).

However, in addition to muscle wasting, two thirds of IBD population had a lower perception of muscle strength with an increased asthenia and a decreased quality of life, similarly to geriatric controls; importantly, the combination of the quantitative and the qualitative parameters of muscular disorder configured a condition of sarcopenia in 28% of IBD patients (19).

Recently, mesenteric fat proliferation is gaining growing attention. Indeed, many studies support the active role of mesenteric fat creeping in the pathophysiology and clinical course of CD. Grillot et al. described the association between sarcopenia and visceral obesity assessed by computed tomography (CT) with adverse outcomes in severe CD patients, supporting the hypothesis that the human fat is considered as a dynamic tissue involved in the immunity regulation and consequent inflammation response (111).

Interestingly, the visceral to subcutaneous adipose tissue area ratio has been considered as a biomarker of complications of CD, such as stricture and fistula (111).

Bamba et al. observed that the muscle volume and visceral adipose tissue volume (relative to subcutaneous adipose tissue volume) are associated with the long-term outcome of intestinal resection (112). In detail, it has been shown that male sex, CD, low psoas muscle index, and high visceral adipose tissue volume are associated with bowel surgery; therefore, moderate exercise and elemental diet might be useful for maintaining muscle mass and reduce visceral fat (112).

Biologic therapy, including anti-TNF alpha agents and newer anti-interleukin, anti-integrin, and JAK inhibitors agents used for treatment of IBD, might reduce sarcopenia, blocking the catabolic effects on skeletal muscle tissue (54). Nevertheless, there are only few studies investigating the potential role of biologics in the prevention of muscle wasting in IBD patients. Subramaniam et al., showed that in active CD the treatment with infliximab can reverse IBD-related sarcopenia, leading to a significant improvement in both skeletal muscle volume and maximal isokinetic strength after 6 months of therapy (113). These highlight the key role of NF-kB signaling pathway in development of muscle impairment (113). Similarly, it was observed an improvement of BMI and muscle parameters, described as fat free mass index, after 3 months of infliximab or adalimumab, suggesting the beneficial effect of the anti-TNF alpha therapy on the nutritional status and body composition of IBD patients (114) (**Table 1**).

With regards to the effects of gut microbiota interventions on parameters of muscle mass and function, most of the available studies were carried out on animal models as we mentioned before. Few data have explored the impact of manipulating gut microbiota on skeletal muscle outcomes in humans. A randomized controlled trial (RCT) explored the effects of a mixture of inulin and fructooligosaccharides (FOS) versus placebo in ambulatory elderly residing in nursing homes for 13 weeks. Of note, the intervention group experienced significant improvements in handgrip strength and self-reported feeling of exhaustion (78). Subsequently Theou et al. conducted secondary analysis by using a similar intervention (inulin+FOS versus placebo) and showed improvements in physical function, frailty degree, nutritional status and quality of life following a 12-week intervention in frail elderly (115).

Thus, these data support the hypothesis of a modulation of muscle function by gut microbiota. However, translation of these results in IBD patients is uncertain. A clinical application of the

TABLE 1 | Principal human studies that explored the relationship between inflammatory bowel diseases and sarcopenia.

Authors, Year	Study Design	Study Population/ Number of patients	Intervention/Groups	Outcomes	Key findings
Subramaniam et al. (113), 2015	Prospective Study	99 patients with CD	MRI volume of quadriceps femoris, maximal concentric quadriceps contractions strength, physical activity, and serum levels of IL6 were assessed at week 1 (pretreatment),week 16 (post-IFX induction) and week 25 (post-first IFX maintenance dose)	Gain of muscle volume and strength after anti-TNF alpha therapy	The anti-TNF agent infliximab reverses inflammatory sarcopenia in patients with CD
Adams et al. (86), 2017	Retrospective Study	90 IBD patients	IBD patients starting a new anti-TNF alpha therapy that had CT within 3 months of initiation	Hospitalization, need for surgery, or new biological medication	45% of IBD patients were sarcopenic; of these, 19.5% were overweight/obese. CRP was higher and albumin lower in sarcopenic subjects. Sarcopenia was the only significant predictor of need for surgery in overweight and obese patients
Pizzoferrato et al. (19), 2019	Prospective Study	127 IBD patients	Four cohorts of patientswere recruited: 1. IBD patients 2. healthy controls 3. healthy elderly 4. elderly with primary sarcopenia	Rate of sarcopenia in IBD patients	36% of patients with IBD showed a significant reduction in skeletal muscle mass associated with a lower perception of muscle strength with a higher incidence of asthenia and reduction in quality of life
Ryan et al. (4), 2019	Systematic Review	658 IBD patients	Five studies	Needs of surgery and surgical outcomes	42% of IBD patients had sarcopenia. IBD patients had a higher probability of requiring surgery. The rate of major complications was significantly higher in patients with sarcopenia
Grillot et al. (111), 2020	Retrospective Study	88 CD patients	CD patients who had abdominal CT scans during hospitalization	Prevalence of sarcopenia and visceral obesity in CD patients and its association with adverse events	The prevalence of sarcopenia was 58%. Among sarcopenic patients,13.7% were overweight, and 2% were obese. Sarcopenic CD patients had significantly more abscesses, hospitalizations and surgery. Both sarcopenia and visceral obesity were associated with adverse outcomes
Bamba et al. (112), 2020	Prospective Study	187 IBD patients	IBD patients who were admitted to hospital and underwent abdominal CT	Association of skeletal muscles and adipose tissue measured at the third lumbar vertebra level on CT image with clinical outcomes in IBD patients	Male sex, CD, low psoas muscle index, and high visceral to subcutaneous adipose tissue area ratio were associated with intestinal surgery
Ünal et al. (87), 2021	Cross-sectional Study	344 patients with IBD	IBD patients in clinical remission	Nutritional status and sarcopenia in patients with IBD in clinical remission	Sarcopenia was diagnosed in 41.3% of patients. 5.5% of patients were underweight and 9.9% were malnourished. Total number of flares requiring hospitalization was the most important predictor of sarcopenia

basic science background linking gut microbiota with sarcopenia should be the challenge of future innovative research in IBD population. Hence further studies are awaited.

CONCLUSIONS

The gut-muscle axis is a very promising area of research for the management of sarcopenia in IBD. It could be hypothesized that GM targeted treatment or complementary therapy such as physical activity and nutritional supplementation could be proposed to patients suffering from IBD and sarcopenia. Since sarcopenia has a negative impact on clinical outcome in IBD patients such as the need for surgical intervention and higher risk of post-operative complications, the assessment of muscle function and nutritional status should be adopted in the daily management of IBD patients. The modulation of the immune response, GM, oxidative stress, mitochondria disfunctions and metabolic processes could give potential benefits for IBD patients to improve muscle mass, muscle function and hence clinical outcomes. An incremental clinical benefit of blocking the catabolic effects on skeletal muscle tissue could be observed in IBD patients with sarcopenia treated by biologics including anti-TNF alpha agents and newer anti-interleukin, anti-integrin, and JAK inhibitors agents used for treatment of IBD. However, the evidence for GM targeting in IBD population is exiguous.

Understanding the pathophysiology of gut-muscle axis is key to developing translational research in this area of intense interest. Hence, we hope to encourage intervention studies exploring the impact of modulation GM on muscle function in IBD patients.

AUTHOR CONTRIBUTIONS

OMN and RdS contributed to conception, design, drafting, and revised of the manuscript. VP and FS wrote sections of the manuscript. AT and GV contributed to manuscript draft. FC critically reviewed the manuscript for important intellectual content, edited, revised, and provided overall supervision. All authors contributed to the article and approved the submitted version.

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Spontaneously Resolving Joint Inflammation Is Characterised by Metabolic Agility of Fibroblast-Like Synoviocytes

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Fibroblast-like synoviocytes (FLS) play an important role in maintaining joint homeostasis and orchestrating local inflammatory processes. When activated during injury or inflammation, FLS undergo transiently increased bioenergetic and biosynthetic demand. We aimed to identify metabolic changes which occur early in inflammatory disease pathogenesis which might support sustained cellular activation in persistent inflammation. We took primary human FLS from synovial biopsies of patients with very early rheumatoid arthritis (veRA) or resolving synovitis, and compared them with uninflamed control samples from the synovium of people without arthritis. Metabotypes were compared using NMR spectroscopy-based metabolomics and correlated with serum C-reactive protein levels. We measured glycolysis and oxidative phosphorylation by Seahorse analysis and assessed mitochondrial morphology by immunofluorescence. We demonstrate differences in FLS metabolism measurable after *ex vivo* culture, suggesting that disease-associated metabolic changes are long-lasting. We term this phenomenon 'metabolic memory'. We identify changes in cell metabolism after acute TNFα stimulation across disease groups. When compared to FLS from patients with early rheumatoid arthritis, FLS from patients with resolving synovitis have significantly elevated mitochondrial respiratory capacity in the resting state, and less fragmented mitochondrial morphology after TNFα treatment. Our findings indicate the potential to restore cell metabotypes by modulating mitochondrial function at sites of inflammation, with implications for treatment of RA and related inflammatory conditions in which fibroblasts play a role.

Keywords: fibroblasts, arthritis, inflammation, metabolism, mitochondria

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INTRODUCTION

In health, quiescent fibroblast-like synovial cells (FLS) are anabolic, producing abundant collagen and hyaluronic acid and maintaining homeostasis and turnover of synovium and cartilage. However, in disease, stromal and myeloid populations expand and leukocytes are recruited to the synovial tissue contributing to an increasingly inflammatory and hypoxic environment (1, 2). In the transformation to a chronically activated and pathogenic phenotype, FLS respond and contribute to these environmental cues, forming an invasive pannus tissue and producing degradative enzymes which damage cartilage and bone. These cellular behaviours and microenvironmental features closely resemble those seen in malignancy, where they are associated with therapeutically targetable changes in nutrient and oxygen availability, metabolic demand and metabolic phenotype of cells (3, 4).

In rheumatoid arthritis (RA) patients, metabolic deviation is observed globally as an increase in basal metabolic rate (5) and symptoms of cachexia. This phenotype is thought to be orchestrated by inflammatory cytokines such as TNFα, IL-1β, IL-6, LIF, and IFNγ which are elevated in chronic inflammatory diseases (6, 7). In addition multiple cellular metabolic dysfunctions have been identified (8–10). We and others have shown that metabolic fingerprints measurable in body fluids (serum, urine and synovial fluid) correlate with hallmarks of diseases including CRP and TNFα levels, and can predict drug responses (11–15). Metabolomic studies such as these have the power to identify novel biomarkers and drug targets but have less frequently been used to investigate pathological changes in metabolism at a cellular level or the contribution of individual cell types to the overall metabolic phenotype of RA (16).

Aerobic glycolysis, a characteristic response of many cell types to pro-inflammatory stimuli, is defined by the metabolic fate of pyruvate, a product of glucose catabolism. Rather than being converted to acetyl-CoA and used to fuel mitochondrial respiration, in aerobic glycolysis pyruvate is instead reduced to lactate and exported from the cell. In RA, FLS are reported to undergo several metabolic alterations, including an increase in aerobic glycolysis (17–19); impaired mitochondrial respiration and altered mitochondrial dynamics (20, 21); as well as altered lipid metabolism (22). However, to date these studies have used mouse models of disease or tissue from very late stage RA and osteoarthritis and have not explored the mechanisms responsible for the persistence rather than resolution of inflammatory disease. It is well-established that early intervention in the course of disease progression is important for preventing joint damage and dysfunction in RA (23). Therefore knowledge of the metabolic state in early disease is critical for understanding both disease mechanisms and possible treatment options that target metabolic components.

In this study, we describe the metabolic fingerprint of human resting and primed FLS from uninflamed and inflamed joints and elucidate a metabolic phenotype of FLS which distinguishes acute, self-limiting synovitis (resolving arthritis) from very early, persistent RA (veRA).

METHODS

Patients

The study was conducted in compliance with the Declaration of Helsinki. Ethical approval was obtained from the local ethics committee and all subjects provided written, informed consent. Patients with early arthritis were seen in the BEACON cohort, details of which have been reported previously (24). Unselected, consecutive DMARD- and glucocorticoid-naïve patients with at least one clinically swollen joint within 12 weeks of the onset of any inflammatory symptoms were recruited and followed for 18 months to determine diagnostic outcome. Age and sex matched patients were classified as having very early, persistent RA (veRA) according to the 2010 ACR criteria (25). As previously described, patients with resolving arthritis were diagnosed if there was no evidence of joint related soft-tissue swelling on final examination and where no DMARD or steroid treatment was administered in the preceding 3 months (26). Consenting patients with appropriate joints underwent ultrasound-guided synovial biopsy using needle or portal and forceps techniques (27, 28). NSAID usage and ultrasound-derived inflammation were comparable between disease groups. The uninflamed control group comprised patients with no evidence of degenerative or inflammatory disease, macroscopic or microscopic joint pathology, who underwent exploratory conventional arthroscopy for knee pain. All patients underwent detailed clinical and laboratory evaluations to rule out any concomitant inflammatory, metabolic, and neoplastic disorders. FLS were maintained in culture *ex vivo* and all lines were at the same passage number when experiments were performed.

FLS Culture

FLS were grown out of tissue and maintained at 70-80% confluence in media containing 10% fetal calf serum, MEM Non-essential amino acids (0.87x), sodium orthopyruvate (0.87 mM), glutamine (1.75mM), penicillin (87U/ml) and streptomycin (87ug/ml). After 3 passages, cultures were >99.5% phenotypically homogeneous. Conditioned culture medium and cells were harvested for bioenergetic analysis, immunofluorescence staining or NMR spectroscopy at passage 6 and 5-6 cell lines from each disease group were utilized in each individual experiment.

Metabolite Preparation

For preparation of metabolites from conditioned culture medium, supernatants were collected, centrifuged at 13000g for 5 minutes and filtered to exclude species >3KDa (29). Flow through was mixed in a 1:1 ratio with an aqueous NMR buffer with final concentrations of 10% D_2O, 150mM NaCl, 1mM trimethylsilyl 2,2,3,3-tetradeuteropropionic acid (TMSP) and 20mM sodium phosphate (pH7). Intracellular metabolites were extracted using cold methanol/chloroform (30). The aqueous fraction was dried overnight and resuspended in NMR buffer as described above.

Metabolomic Analysis

One dimensional (1D) ^{1}H spectra were acquired at 300K using a standard spin-echo pulse sequence with water suppression and using excitation sculpting, on a Bruker DRX 600MHz NMR spectrometer equipped with a cryoprobe. Samples were processed and data calibrated with respect to the TMSP signal. Spectra were read into ProMetab, custom written software in Matlab (version 7, The Mathworks, Natick, MA) and truncated to a chemical shift range of 0.8-10.0 ppm (31). The water peak was removed, spectra were corrected for baseline offset and normalised to a total spectral area of unity, and a generalised log transformation was applied (31, 32). Spectra were then read into Chenomx NMR suite (Chenomx, professional version 4.0) and an inbuilt peak database was used alongside published data to identify and quantify associated metabolites (33). Pathway analysis was carried out using Metaboanalyst 3.0 software.

Partial least-squares regression analysis (PLS-R) identifies metabolites which predict a continuous variable, and was used to investigate the relationship between the metabolic fingerprint and inflammatory burden measured as C-reactive protein (CRP). This analysis yielded an R^2 value as a measure of the cross-validated goodness-of-fit of the linear regression. Permutation testing was used to assess the statistical significance of the relationship when compared to a slope of zero.

Cellular Bioenergetics

FLS were seeded in 24 well flux plates (Seahorse) at $2x10^4$/well and allowed to adhere for 24 hours in the presence or absence of 1ng/ml TNFα. Prior to assay, cells were equilibrated for 1 h in a non-CO_2 incubator at 37°C. Cellular bioenergetics were analyzed within the University of Birmingham Mitochondrial Profiling Facility using a Seahorse XFe24 extracellular flux analyzer according to the manufacturer's instructions. Both oxygen consumption rate (OCR, as a measure of oxidative phosphorylation) and extracellular acidification rate (ECAR, as a measure of aerobic glycolysis), were assessed. ECAR was measured in XF media in basal condition and in response to 10 mmol/L glucose (basal glycolysis), 2 µmol/L oligomycin (glycolytic capacity) and 50 mmol/L 2-DG. OCR was measured in XF media (non-buffered DMEM medium, containing 10 mmol/L glucose, 2 mmol/L l-glutamine, and 1 mmol/L sodium pyruvate), under basal conditions (basal respiration) and in response to 2 µmol/L oligomycin (ATP-linked respiration), 5 µmol/L of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) (maximal respiration) and 3 µmol/L antimycin and rotenone (Sigma Aldrich). Three technical replicates were carried out for each condition and a total of 17 measurements of 4 minutes duration were made. Calculations of glycolysis and respiration were established from area under the curve.

Immunofluorescence and Mitochondrial Analysis

Cells were adhered to chamber slides at $2x10^3$/well and cultured with or without 1ng/ml TNFα for 24 hours. Slides were fixed in acetone, air-dried and stored at -20°C. Slides were rehydrated in PBS, blocked in 10% normal goat serum and incubated with mouse anti-TOMM20 (4F3, Abcam, UK) prior to goat anti-mouse IgG1 (Southern Biotech, Birmingham, USA). The FITC signal was amplified with anti-FITC Alexa Fluor 488 (Life Technologies) and nuclei were stained with Hoechst 33258. Slides were mounted in Prolong Diamond (Life Technologies) before imaging. Images were captured on the Leica DM6000 using the proprietary software and processed using Fiji (34) in a method adapted from (35). In brief, the image in the TOMM-20 channel was sharpened, thresholded, converted to a mask and then skeletonized prior to running the binary connectivity plug-in as described (36, 37). For visualisation the Glasbey lookup table was used and numbers of each pixel connection type were exported. Nuclei were counted as an assessment of cell number in each field of view and these data were combined.

Statistical Analysis

Data are presented as mean ± SEM. Analysis of variance (ANOVA) was used for multiple comparisons, paired Student's t tests for comparison of resting and stimulated cells. Results were considered significant where $p<0.05$.

RESULTS

Steady State Metabolomic Analysis in FLS From Healthy and Inflamed Joints

Patients presenting with synovial inflammation underwent synovial biopsy at presentation and were subsequently classified as resolving arthritis or very early RA (veRA) (24), and were compared with uninflamed synovial samples obtained from exploratory arthroscopy as detailed in methods. Clinical characteristics of subjects are shown in **Table 1**.

To assess whether alterations in the bioenergetic responses of FLS drive the pathological transition to chronicity in RA we started our analyisis by assessing the metabolomic profile in FLS derived from uninflamed, resolving synovitis and RA patients. 1D NMR spectroscopy was carried out on 6 FLS cell lines derived from uninflamed patient synovium, 6 FLS cell lines from resolving arthritis patients and 5 FLS cell lines from veRA patients. We identified metabolic signatures from these cells and identified 31 metabolites present in all 17 conditioned media (**Figure 1A**) and a further 36 present in all 17 cell extracts (**Figure 1B**). A summary of pathways which these data implicate as important in FLS metabolism is shown in **Supplementary Figure 1**. Glycolysis is an important metabolic pathway that utilises glucose for biosynthesis and ATP generation. Lactate, which is the end product of glycolysis, and glucose were most highly represented in culture supernatants of all patient groups (**Figures 1A, C**). Glucose and lactate levels in supernatants and cell extracts did not vary between disease groups (**Figures 1C, D**).

Glycerol was significantly reduced in cell extracts of resolving and veRA synovitis when compared to uninflamed controls (**Figure 1E**). Glycerol is a major link between sugar and fatty acid metabolism (38) by reducing dihydroxyacetone phosphate (DHAP, a key triose in glucose metabolism and energy

TABLE 1 | Demographics and clinical characteristics of participants at the time of synovial biopsy.

	Uninflamed controls (n = 11)	Resolving arthritis (n = 12)	Very early RA (n = 11)
Age (years); median (IQR)	41 (38-44)	40.5 (32.25-52.5)	61 (48-70)
Female; number (%)	5 (45.5)	5 (41.7)	6 (54.5)
Symptom duration (weeks);median (IQR)	–	5 (3.25-6.75)	4 (3.5-7.5)
NSAID; number (%)	0 (0)	9 (75.0)	8 (72.7)
CRP (mg/ml); median (IQR)	–	8.5 (1.5-13)	25 (10-32)
RF positive; number (%)	–	0 (0)	5 (45.5)
Anti CCP antibody positive; number (%)	–	0 (0)	7 (63.6)
Joint biopsied			
Ankle; n (%)	0 (0)	3 (25)	3(27.3)
Knee; n (%)	11 (100)	9 (75)	5 (45.4)
MCP; n (%)	0 (0)	0 (0)	3 (27.3)
Ultrasound greyscale hypertrophy score (1-3); median (IQR)	–	2 (1-2)	3 (1.75-3)
Ultrasound Power Doppler hypertrophy score (1-3); median (IQR)	–	1(1-2)	1.5 (0.75-2)

RA, rheumatoid arthritis; IQR, interquartile range; NSAID, non-steroidal anti-inflammatory drugs; CRP, C-reactive protein; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; MCP, metacarpopharangeal. Of these patients, cells from 5-6 individuals from each disease group were used in each experiment.

generation) into glycerol-3-phosphate, which is suggestive of a potential substrate to feed lipid synthesis.

Acetate, which is also involved in lipid metabolism, showed higher trend in veRA synovitis relative to controls (p=0.06) while the amino acid glycine was at lower concentrations in FLS extracts from veRA than resolving arthritis, however this did not reach statistical significance (p=0.056, **Figure 1E**).

Although we did not identify striking differences in individual metabolites between groups, we went on to investigate whether the metabolites measured by NMR spectroscopy were linked to the inflammatory status of individuals at the time of biopsy using PLS-R analysis (**Figure 1F**). Indeed, a metabolomic profile was identified which can predict levels of the inflammatory marker C-reactive protein (CRP) in patient sera (R^2 = 0.6801, p=0.001).

Differential Metabolic Adaptation in FLS From Resolving *Versus* Persistent Inflamed Synovium

To directly investigate the balance of glycolytic and mitochondrial energy generation in resolving arthritis and veRA, we used the Seahorse XF Analyzer and metabolic inhibitors or potentiators to determine the maximal capacity of FLS to utilize these bioenergetic pathways under stress. The parameters calculated from the Seahorse glyco- and mito-stress tests are detailed in **Figures 2A** and **3A**. Seahorse analysis was carried out on 5 FLS cell lines derived from uninflamed synovium, 6 FLS cell lines from resolving arthritis patients and 6 FLS cell lines from veRA patients. We assessed both resting and TNFα stimulated FLS. Example traces measuring extracellular acidification rate (ECAR) as a measure of aerobic glycolysis and oxygen consumption rate (OCR) as a measure of mitochondrial respiration in each patient group are shown in **Figures 2B–D** and **3B–D** respectively.

Basal glycolysis was comparable in all disease groups (**Figure 2E**), consistent with the metabolomic data showing no change in glucose uptake and lactate production in the resting state (**Figures 1C, D**). Interestingly only resolving arthritis FLS showed a significant upregulation of glycolysis in response to TNFα (**Figure 2E**). Glycolytic capacity is determined using the mitochondrial ATP synthase inhibitor oligomycin (**Figure 2A**), and demonstrates the cell's maximal possible rate of glycolysis under the given conditions. Glycolytic capacity was significantly enhanced by TNFα treatment in all groups (**Figure 2F**)

Respiratory parameters were determined using oligomycin to inhibit ATP synthase; FCCP to uncouple oxygen consumption from ATP synthase; and rotenone plus antimycin A to poison mitochondrial respiration (**Figure 3A**). Basal respiration in resting FLS did not differ between patient groups, and was significantly increased by TNFα only in healthy control FLS (**Figure 3E**). Maximal respiration was significantly higher in resolving arthritis than veRA FLS, and was significantly reduced by TNFα treatment only in resolving arthritis FLS (**Figure 3F**). Spare respiratory capacity (the difference between maximal and basal respiration rates) was significantly higher in resolving arthritis than veRA FLS, and was significantly reduced by TNFα treatment in healthy control and resolving synovitis FLS, but not in veRA FLS (**Figure 3G**). Differences in respiration linked to ATP synthesis were not significant (**Figure 3H**). Thus, FLS from patients with resolving synovitis or early arthritis differed in certain basal metabolic parameters even after several passages, providing further evidence of sustained metabolic memory. The capacity of metabolic activities to respond to an inflammatory challenge was characteristic of FLS derived from patients with resolving synovitis, but lost in patients who would later be diagnosed with RA. These results suggest that mitochondrial dysfunction in FLS is an early event in RA disease, which may be linked to the development of persistent synovitis. Whether this reduced capacity is driven by specific inflammatory signals within the RA synovial environment remains to be determined.

Resolving FLS Display a Distinct Mitochondrial Morphology in Response to TNFα

We next investigated whether the metabolic differences observed above were associated with changes in mitochondrial morphology and dynamics. Immunofluorescence imaging was carried out on 5 FLS cell lines derived from uninflamed synovium, 6 FLS cell lines from resolving arthritis patients and 6 FLS cell lines from veRA

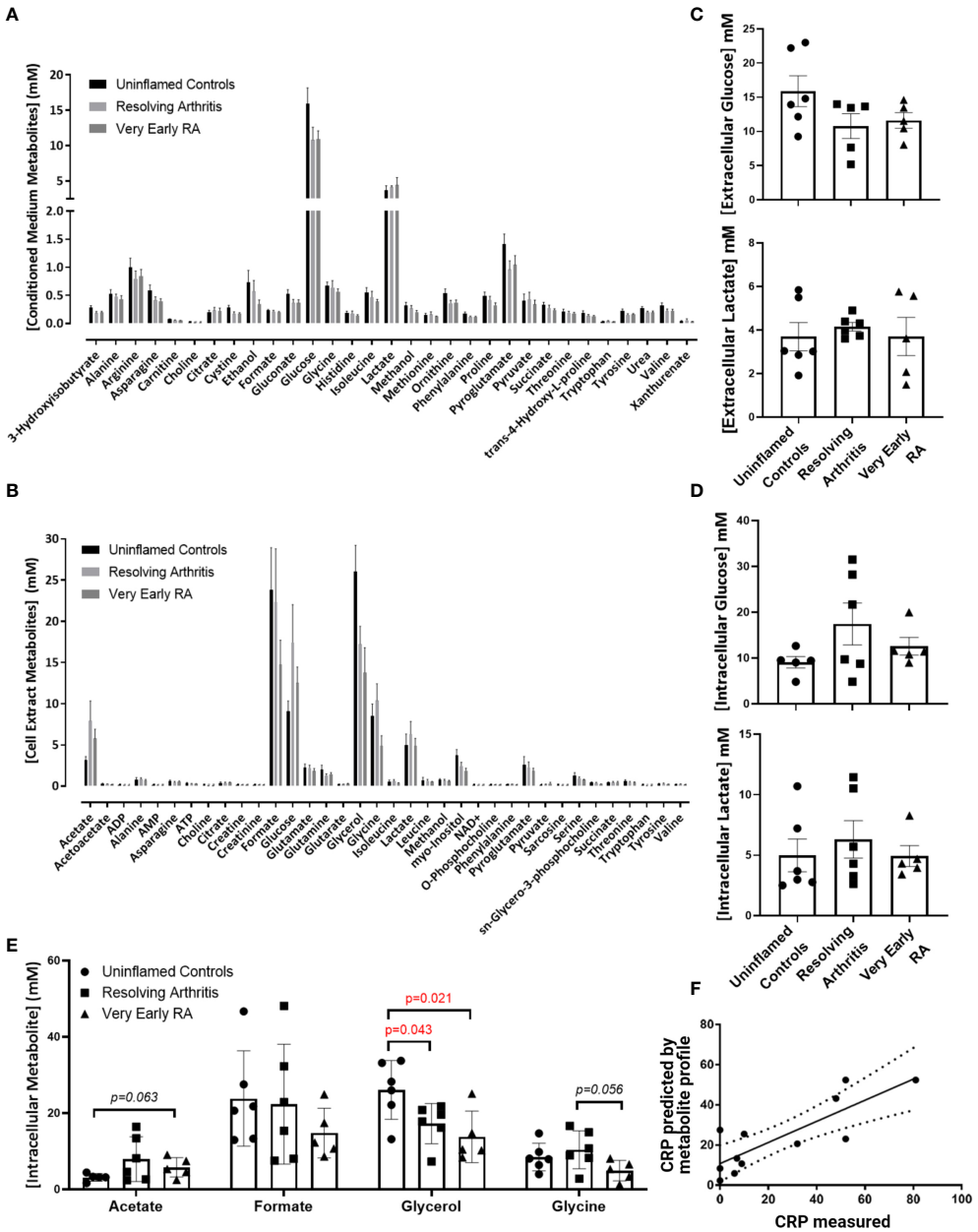

FIGURE 1 | Metabolomic fingerprinting in FLS from uninflamed and inflamed synovium. FLS were cultured from synovial biopsies of patients with no inflammation (n=6), resolving arthritis (n=6) and very early rheumatoid arthritis (RA) (n=5) and metabolites were quantified by 1D nuclear magnetic resonance spectroscopy. **(A)** All quantified metabolites measured in conditioned culture medium, **(B)** All quantified metabolites measured in cell extracts, **(C)** glucose and lactate measured in conditioned culture medium, **(D)** glucose and lactate measured in cell extracts and **(E)** other metabolites for which differences were observed between disease groups, **(F)** strong correlation between measured C-reactive protein (CRP) levels and CRP levels predicted by the FLS metabolic profile in uninflamed controls and arthritis patients. The predicted values were calculated from the concentrations of metabolites identified using partial least squares regression analysis (R^2 = 0.6801). Statistical significance was determined by one-way ANOVA test.

patients. FLS mitochondria were visualized by staining for TOMM20 (**Figure 4A**), and mitochondrial area, linearity and branching were quantified (**Figure 4B**). Total mitochondrial area per cell varied between individuals and was comparable between disease outcomes and unchanged after stimulation of cells with TNFα for 24 hours (**Figure 4C**). Linearity and branching also showed great variability between individuals (**Figures 4D–F**). However, FLS from patients with resolving arthritis showed increased mitochondrial linearity following TNFα treatment in comparison to the other groups (**Figure 4E**). Consistent with the results of the bioenergetic analysis, measurement of mitochondrial linearity also revealed that the FLS from resolving arthritis patients

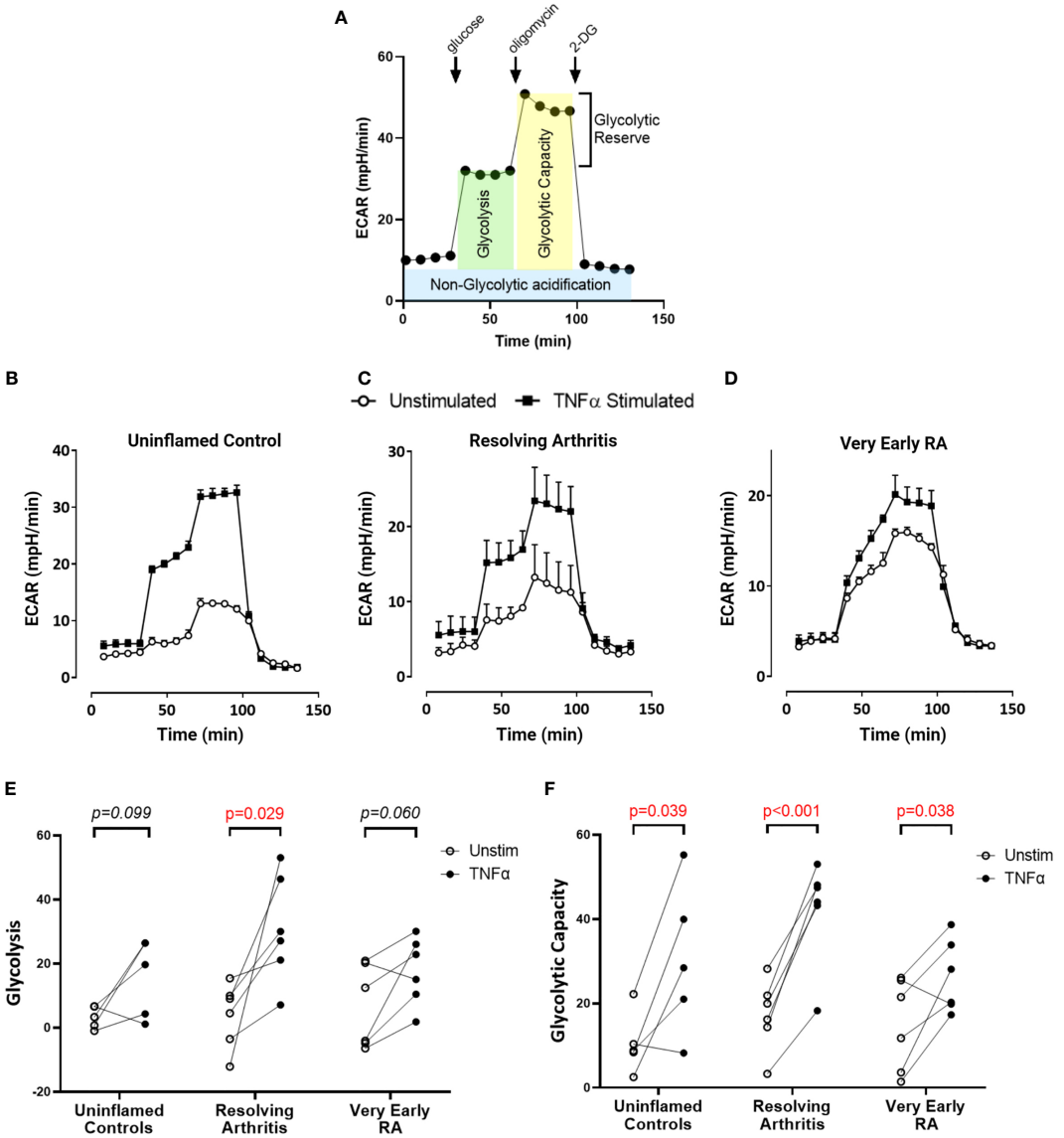

FIGURE 2 | FLS from healthy and inflamed synovium show increased glycolytic capacity in response to TNFα. FLS were cultured from synovial biopsies of patients with no inflammation (n=5), resolving arthritis (n=6) and very early rheumatoid arthritis (RA) (n=6). Extracellular acidification rate (ECAR) was measured in real time in the presence of glucose, oligomycin and 2-deoxyglucose (2DG) **(A)**. Example traces are shown for unstimulated FLS and FLS stimulated for 24 hours with tumour necrosis factor α (TNFα); **(B)** uninflamed, **(C)** resolving arthritis and **(D)** very early RA. **(E)** Glycolysis (after the addition of glucose) and **(F)** glycolytic capacity (difference of oligomycin rate and 2DG rate) were calculated as shown in **(A)**. Statistical significance between treatments was determined by paired student's t test. No statistical significance was found between patient groups.

demonstrated the greatest degree of plasticity in response to TNFα stimulation (**Figure 4E**). We therefore conclude that the capacity to spontaneously resolve synovial inflammation is associated with elevated metabolic agility of fibroblast-like synoviocytes.

DISCUSSION

Altered metabolism and dysregulated metabolic pathways are important determinants of inflammatory and destructive processes in chronic inflammatory and autoimmune diseases, including rheumatoid arthritis (RA) (1, 8–10, 40). In this study we explored the hypothesis that alterations in the bioenergetic responses of fibroblast-like synoviocytes (FLS) may be important in orchestrating the pathological transition to chronicity in RA. As the FLS were taken from patients who had a symptom duration of less than 3 months, our analysis gives unique insights into the phenotypic changes that fibroblasts undergo during the initiation of, or adaptation to inflammation. We found that FLS from patients with synovitis that went on to

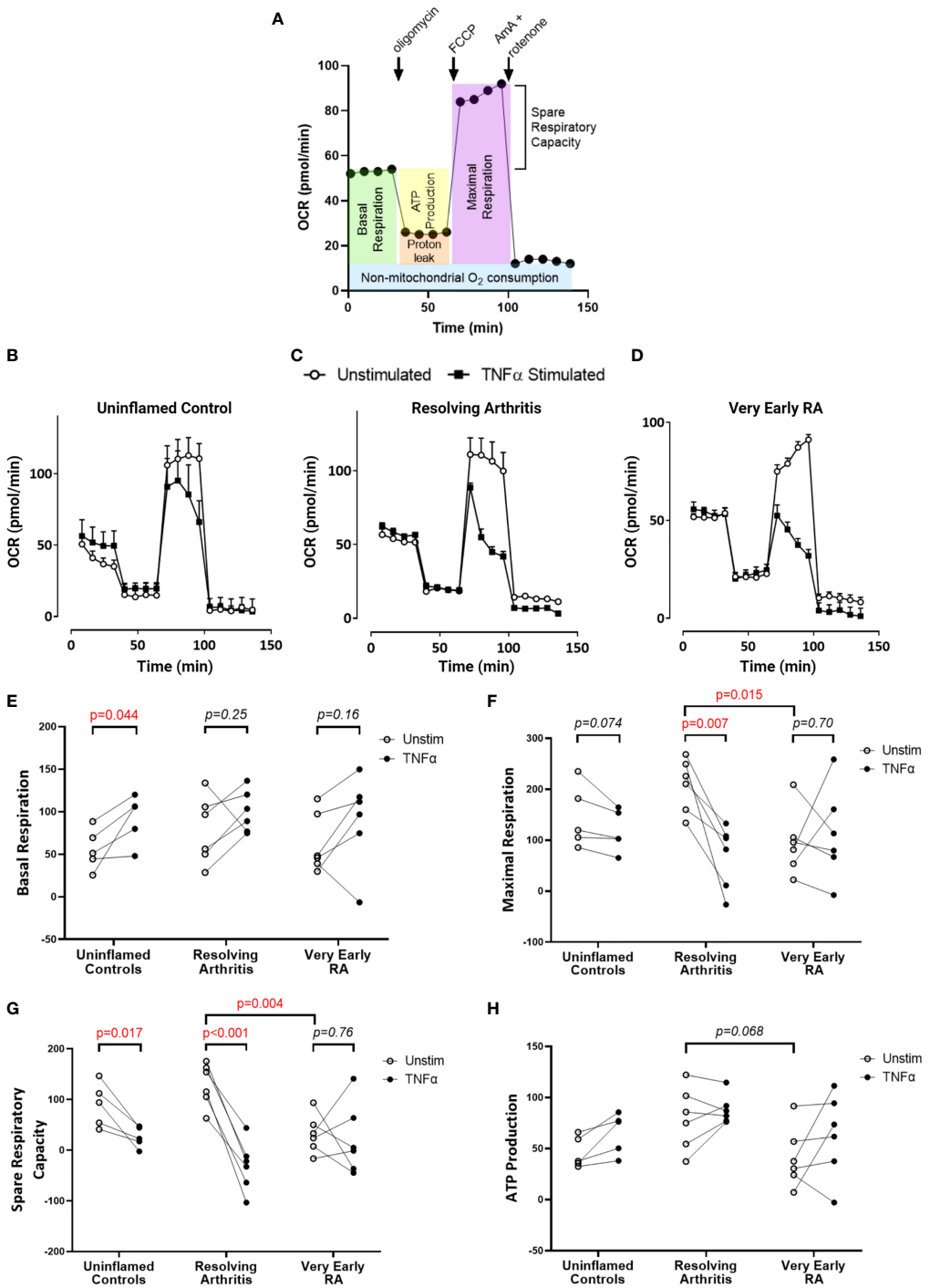

FIGURE 3 | FLS from patients with very early RA have reduced respiratory capacity compared with those from patients with resolving arthritis. FLS were cultured from synovial biopsies of patients with no inflammation (n=5), resolving arthritis (n=6) and very early rheumatoid arthritis (RA) (n=6). Oxygen consumption rate (OCR) was measured in real time in the presence of oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and antimycin A (AmA) and rotenone in combination **(A)**. Example traces are shown for unstimulated FLS and FLS stimulated for 24 hours with tumour necrosis factor α (TNFα); **(B)** uninflamed, **(C)** resolving arthritis and **(D)** very early RA. Basal respiration rate [**(E)**, before addition of oligomycin], maximal respiration rate [**(F)**, difference of FCCP rate and AmA + rotenone rate], spare respiratory capacity [**(G)**, difference between the rate of basal respiration and the maximum, FCCP-stimulated rate of respiration], and ATP-linked respiration [**(H)**, ATP production, difference of basal rate and oligomycin rate] were calculated as shown in **(A)**. Statistical significance between treatments was determined by paired student's t test. Statistical significance between groups was determined by two-way ANOVA.

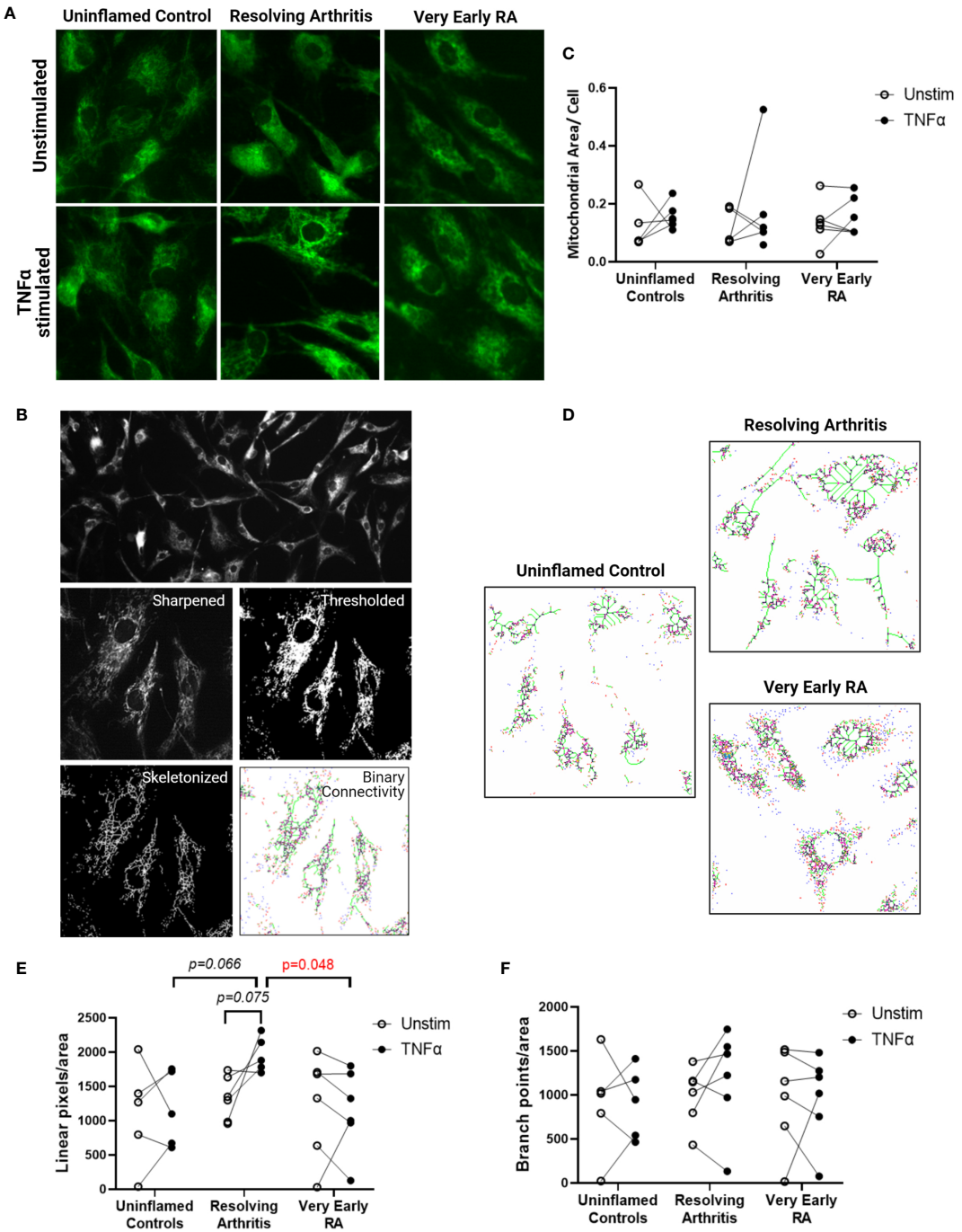

FIGURE 4 | FLS from resolving arthritis synovium display a distinct mitochondrial morphology in comparison to healthy and RA FLS in response to stimulation. FLS from patients with no inflammation (n=5), resolving arthritis (n=6) and very early rheumatoid arthritis (RA) (n=6) were labelled with TOMM-20 and visualised by fluorescence microscopy **(A)**. **(B)** Representative image showing how TOMM-20 channel was sharpened, thresholded, converted to a mask and then skeletonized prior to running the binary connectivity plug-in. **(C)** Mitochondrial area per cell was quantified in unstimulated cells and cells stimulated with TNFα for 24 hours. **(D)** Representative images of mitochondrial connectivity in TNFα-stimulated FLS from different disease groups. Filamentous/linear (green) or fragmented/punctate (purple) mitochondria are represented (39). Quantification of linear pixels **(E)** and branch point pixels **(F)** per cell normalised to mitochondrial area from skeletonized images of unstimulated cells and cells stimulated with TNFα for 24 hours. Statistical significance between groups was determined by one-way ANOVA test.

resolve (resolving arthritis) showed greater mitochondrial respiratory capacity than those from patients with very early, persistent RA (veRA), and presented with greater mitochondrial linearity upon morphological analysis. Resolving arthritis FLS also consistently demonstrated greater metabolic agility in response to inflammatory stimulation with TNFα *ex vivo*. These results suggest that the ability of FLS to metabolically adapt to their environment and to carry out mitochondrial respiration may contribute to their capacity to resolve inflammation, and that potentiating mitochondrial function could be a novel strategy to promote resolution of arthritis.

We identified a signature of 36 metabolites common to FLS regardless of disease status, highlighting pathways fundamental to the physiological function of the FLS. We observed that in particular glucose and lactate levels were not differerent between groups. These findings suggest that FLS undertake glycolytic metabolism even in the absence of inflammation. This is an important consideration given the recent interest in targeting glycolytic pathways for the treatment of autoimmune diseases including RA, however we acknowledge the potential differences between *in vitro* and *in vivo* metabolic commitments.

It is also likely that a similar metabolic profile supports the shared functions of fibroblasts at sites other than the joint, as demonstrated by findings in foreskin-derived cells (41). These cells were heavily reliant upon glucose metabolism and utilised the pentose phosphate pathway to maintain a biosynthetic phenotype even in quiescence (41). Although studies report upregulation of glucose transporters in inflammation (17, 42), we did not find differences in terms of glucose uptake and lactate production from culture medium in FLS from both resolving arthritis and veRA patients *in vitro*. Our NMR analysis in early pathogenesis is in line with a mass spectrometry based study of FLS which identified disturbance in metabolites associated with amino acid, sugar and lipid metabolism compared to osteoarthritis, but which did not identify a switch to glycolysis even in end stage RA FLS (43).

Glycerol depletion in cells from both resolving arthritis and veRA patients suggests there is also a role for lipid metabolism in the steady state and emphasises the importance of upregulation of this pathway during the inflammatory response as well as in the resolution of inflammation as previously described (44). It would be valuable to perform a lipidomic study in the future to further clarify this mechanism.

Building on these metabolomic findings using Seahorse bioenergetic analysis, we again showed no disease-associated augmentation of glycolysis. This suggests that the early stages of the disease are not characterised by commitment to glycolysis, for example as a result of epigenetic reprogramming, mitochondrial dysfunction or loss. Enhanced glycolysis may be a lasting adaptation to mitochondrial damage or to low oxygen supply (45) as is found in the inflamed joint (2). A permanent switch toward aerobic glycolysis is important to the pathogenic phenotype of tumour cells and cancer-associated fibroblasts (46), and has been described in some but not all studies of late stage RA FLS metabolism (19, 40) however our results show that this does not occur in early stages of arthritis (**Figure 2E**).

Alternatively, an upregulation of glycolysis can be a transient, physiological response to activation stimuli, as is well characterised in innate and adaptive immune cells (47). A glycolytic response in fibroblasts exposed to hypoxia (19) platelet derived growth factor (PDGF), lipopolysaccharide (LPS) (17) as well as complement (48) has been documented. An early study demonstrated upregulation of glycolysis in TNFα stimulated, late stage RA FLS (49).

Due to their metabolic agility, we showed that resolving FLS after re-exposure to TNFα were better equipped to increase glycolysis after glucose injection (**Figure 2E**); whilst TNFα-treated veRA FLS showed only a modest effect, which reached statistical significance only after the addition of oligomycin, a glycolysis stimulator (**Figure 2F**).This suggests that veRA FLS may display a delay in upregulating glycolysis in response to inflammatory stimuli as previously found in other RA cell types (50). Similarly, uninflamed controls did not show a significant increase in glycolysis but displayed enhanced glycolytic capacity; suggesting that TNFα only was not sufficient to promote a glycolytic shift in these individuals. This could be explained by the fact that they were isolated from uninflamed joints with low levels of TNFα in the surrounding environment, opposite to veRA and resolving FLS that were obtained from TNF-rich inflamed joints. These findings support the hypothesis that FLS have a "metabolic memory" and display a trained immune response (51, 52) which is maintained in *ex vivo* cultures after re-exposure to inflammatory triggers, and that the FLS of resolving arthritis patients display a greater degree of metabolic plasticity in response to repeat cues. However the fact that all FLS groups upregulated glycolytic capacity following TNFα demonstrated a shared ability to enhance glycolytic flux in response to extreme stressors such as oligomycin. The extent to which repeated stimulation re-programmes metabolic pathways in FLS is of great interest and warrants further study.

Metabolic adaptation is pivotal for FLS to maintain viability and functional competence (40) and switching from glycolysis to oxidative phosphorylation is a prerequisite to promote resolution of inflammation (53, 54). In line with this, our analysis of oxidative respiration showed low steady state mitochondrial maximal and spare respiratory capacity in FLS from veRA compared with resolving arthritis. This suggests that FLS from resolving disease are better equipped to respond metabolically to inflammatory cues than those from joints which develop RA. Other groups have reported decreased oxygen consumption and maximal respiratory capacity in fibroblasts from late stage RA (21, 22). We show that this loss of mitochondrial function represents an early change in the timeline of RA disease development, suggesting that mitochondrial capacity may be linked to the ability of the joint to resolve the inflammatory state.

We also found that the resolving phenotype correlated with more dynamic mitochondrial morphology in response to TNFα, manifesting as increased connectivity (linear pixels in green) in resolving arthritis FLS compared to veRA, which displayed a more punctate/fragmented (in purple) morphology. Mitochondrial fission has an important role in inducing mitophagy in conditions of oxidative stress (55) while

mitochondrial fusion is known to protect cells against deleterious mitochondrial DNA mutations (56) and enhance oxidative phosphorylation (57). Historical and recent studies have described altered mitochondrial macrostructure and high levels of mitochondrial DNA mutagenesis that correlates with inflammation in RA (19, 58). Wang et al. recently reported that synovial tissue and *ex vivo* FLS from late stage RA patients demonstrate shortened mitochondria and increased expression of the fission protein DNM1L (DRP1) (22). We did not find differences in Dnm1l mRNA levels between the groups (data not shown) suggesting that other mechanisms may drive the mitochondrial dysfunction in early disease that differ from those observed at late stage. In addition mitochondrial dynamics are regulated by activation/inflammatory stimuli (59) thus explaining the absence of mitochondrial modulation in unstimulated controls.

Correlations between mitochondrial function and age have also been demonstrated extensively, and several mechanisms have been proposed (60). Human studies have reported that mitochondrial decline advances with age and becomes particularly significant after 70 years old, with mild differences between 40 and 60 years old (61). Our veRA patients samples used for Seahorse and mitochondrial morphology analysis came from a marginally older age group (44-70, average 61) than the resolving arthritis group (32-64, average 43). Whilst we cannot definitively rule out an impact of this age difference on our measurements, we believe that the overall impact would be minor between these groups. We did not find a significant correlation between age and mitochondrial linearity ($p=0.10$; $R=0.4$) and branching ($p=0.92$; $R=0.03$) in our cohort; however a larger cohort is needed to better establish a correlation between age and mitochondrial dysfunction in RA.

The metabolic phenotypes we show to be associated with different disease outcomes were evident even after multiple passages *in vitro*, highlighting a metabolic transformation or 'metabolic memory' acquired by cells during the acute phase of inflammation *in vivo*. This provides a metabolic dimension to the previously described 'imprinted aggressor' role of the FLS (62) and offers future scope to investigate epigenetic changes and mutations in mitochondrial genetics which might confer the altered metabolism we and others have observed. Our findings suggest that the functionality of mitochondria in FLS may be of importance for the resolution of acute inflammation in the synovium. This is suggested by changes in mitochodrial morphology with increased connectivity which would indicate a tendency to rely on mitochondrial fusion or biogenesis, and oxidative phosphorylation in resolving FLS that would need further investigation. This is also in line with previous studies showing that fragmented mitochondria are the consequence of oxidative damage or are due to defects in fusion or fission (63–65). The specific mechanisms by which FLS of very early RA patients lose mitochondrial respiratory capacity and mitochondrial agility in response to TNF are yet to be determined, and this represents an intriguing challenge for future work with these difficult to obtain but important cell populations. The current study did not include patients treated with TNF blocking agents, as it focused on early phases of diseases pre-therapy. However, correlations between TNFα cytotoxicity and mitochondrial dysfunction have been reported (66–68). In RA TNFα signalling promotes increased ROS production (66) and treatment with TNF blocking agents has shown amelioration of synovial oxygen tension and reduction in mtDNA mutation as well as improvement of disease activity calculated as DAS28 CRP (69).

Taken together our findings show that FLS from resolving arthritis patients are distinct from healthy controls in terms of the consistency and magnitude of mitochondrial and glycolytic responses to an inflammatory challenge *in vitro*. In contrast, FLS from early RA patients display weaker and less consistent responses to challenge, particularly at the level of mitochondrial maximal respiration and spare respiratory capacity. These findings suggest that a lasting state of metabolic agility can be induced in FLS by exposure to inflammation *in vivo*, and that this agile state is associated with capacity to resolve. We postulate that potentiating mitochondrial function may represent a novel strategy for promoting resolution in RA and related conditions. We have recently defined multiple fibroblast subtypes in the RA joint with different functions (70). A better understanding of how the different synovial cell types co-ordinate their metabolism will be required to fully appreciate how metabolic changes in disease differs from that in health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical approval was obtained from West Midlands - Black Country Research Ethics Committee (12/WM/0258) and all subjects provided written, informed consent. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JF carried out metabolomic data analysis and bioenergetics experiments. JF, VP, and SC carried out data analysis and manuscript preparation. JM carried out mitochondrial imaging experiments. SR carried out cell preparations for NMR metabolomics and contributed to NMR data analysis. HA carried out cell preparations for NMR metabolomics. AP provided advice and assistance with studying cellular bioenergetics. AF and KR provided clinical samples and academic input throughout the study. SY and CB conceived and led the project. CB, SY, AF, KR, and AC provided academic

support, conceptual input and manuscript editing. All authors contributed to the article 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.2021.725641/full#supplementary-material

Supplementary Figure 1 | Pathways associated with the metabolomic profile of FLS. Pathways involved in FLS metabolism were identified using Metaboanalyst 3.0 software (71). Intensity of symbol colour and position on the y axis is representative of the pathway p value. Symbol size and position on the x axis is representative of the pathway contribution to the model. As such those pathways with high values on both axes make the greatest contribution to FLS metabolism. This model accounts for the number of metabolites represented from each pathway and not the concentration at which they are present in the samples analysed.

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2-Deoxy-D-glucose Alleviates Collagen-Induced Arthritis of Rats and Is Accompanied by Metabolic Regulation of the Spleen and Liver

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Rheumatoid arthritis (RA) is significantly associated with glycolysis. This study used 2-deoxy-D-glucose (2-DG), an inhibitor of glycolysis, to treat rats with collagen-induced arthritis (CIA) and investigate the metabolic regulatory mechanism of glycolysis in the disease. 2-DG significantly alleviated CIA. Metabolomics and transcriptomics, as well as their integrative analysis, detected significant changes in the pathways of bile secretion, cholesterol and linoleic acid metabolism in the plasma, liver and spleen during the CIA process and the opposite changes following 2-DG treatment, whereas the expression of the genes regulating these metabolic pathways were changed only in the spleen. In the rat liver, levels of (S)-5-diphosphomevalonic acid in the terpenoid backbone biosynthesis pathway were significantly decreased during CIA progression and increased following 2-DG treatment, and levels of taurochenodeoxycholic acid in the pentose and glucuronate interconversions pathway showed the opposite results. In the spleen, levels of 3-methoxy-4-hydroxyphenylglycol glucuronide in bile secretion and 12(S)-leukotriene B4 in arachidonic acid metabolism were significantly decreased during CIA progression and increased following 2-DG treatment. The changes in the gene-metabolite network of bile secretion in the spleen correlated with a decreased plasma L-acetylcarnitine level in CIA rats and an increase following 2-DG treatment. Our analysis suggests the involvement of spleen and liver metabolism in CIA under the control of glycolysis.

Keywords: rheumatoid arthritis, glycolysis, transcriptomics, metabolomics, collagen-induced arthritis, liver, spleen, 2-deoxy-D-glucose

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis. Breakdown of self-tolerance and onset of autoimmunity are the main features of the disease. Glycolysis is a metabolic process that breaks down carbohydrates and sugars to either pyruvic acid or lactic acid through a series of reactions and releases energy as two molecules of ATP. RA is significantly associated with increased glycolysis (1, 2). Many studies have detected increased glycolysis in RA synovial fluids and

inflammatory joints in a rat model of collagen-induced arthritis (CIA) (3–5). Moreover, the balance of glycolysis and oxidative phosphorylation is shifted toward glycolysis in RA fibroblast-like synoviocytes (FLSs) (6). Glycolytic enzymes such as hexokinase 2 (HK2), phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFKFB) and phosphoglycerate kinase (PGK) play essential roles in aggressive FLSs (3, 7). Glycolytic blockers are able to reduce the aggressiveness of FLSs, resulting in decreased joint damage in various arthritis models (3). The accumulation of lactate, an end product of glycolysis, is partially responsible for the establishment of an acidic environment in individuals with RA (8, 9). Lactate dehydrogenase A (LDHA) expression was dramatically increased in synovial tissue and synovial fluids in RA. Lactate promotes the switch of CD4+ T cells to an IL-17+ subset (9). The overexpression of LDHA in CD8+ T cell subsets from individuals with RA conferred cells with an increased ability to proliferate and release proinflammatory and cytolytic mediators (8). Treatment with 2-deoxy-D-glucose (2-DG), an inhibitor of glycolysis, suppressed IFN-γ production and cell proliferation in activated primary human CD4+ T cells, although combined use of 2-DG and metformin, an inhibitor of oxidative phosphorylation, showed more potent suppression on IFN-γ production and cell proliferation of the CD4+ T cells (10). 2-DG treatment attenuated the proliferation of CD4+ T cells and the Sjögren syndrome-like autoimmune response (11). Blocking glycolysis with 2-deoxyglucose was recently shown to inhibit Th17 cell differentiation while promoting regulatory T (Treg) cell generation (12). Induction of glycolysis was critical for antibody production, as glycolytic inhibition with the pyruvate dehydrogenase kinase inhibitor dichloroacetate substantially suppressed B cell proliferation and antibody secretion *in vitro* and *in vivo* (13). The above studies indicated that glycolysis is the major driver of immune activation and joint inflammation in RA. However, no study has combined metabolomics and transcriptomics to fully investigate the metabolic regulatory pathways of glycolysis in RA, and an integrative analysis combining metabolomic and transcriptomic data has not been conducted.

There is a significant epidemiologic, genetic and immunologic overlap between rheumatologic disorders and liver or spleen diseases. Accumulating evidence suggests the importance of the liver and spleen in regulating the immune response in individuals with RA. The spleen, the largest secondary lymphoid organ in the body, acts as the center of the blood defense system through innate and adaptive immunity (14). Many autoimmune diseases are associated with lymphadenopathy and splenomegaly. Abnormal splenic function has been documented in patients with RA, SLE, and Wegener's granulomatosis (15). Splenomegaly in patients with autoimmune diseases is thought to be hyperplasic and show histiocytic necrosis. A mixture of CD4+ T cells and CD8+ T cells surrounding the necrotic area was observed (16). A progressive redistribution of memory B cells in the spleen may influence autoimmune disease activity. Splenectomy is associated with the development of autoimmune phenomenon in the clinical course of patients with a prior autoimmune disease (17). The gene expression level of Toll-like receptor 3 in the spleen was reported to regulate the initiation and development of experimental arthritis (18). The liver has a major role in the control of glucose homeostasis by controlling various glucose metabolism pathways, including glycogenesis, glycogenolysis, glycolysis and gluconeogenesis (19). Many primary immune-mediated liver disorders such as primary biliary cholangitis, autoimmune hepatitis and primary sclerosing cholangitis have rheumatologic manifestations (20). Liver damage is observed in many RA patients (21, 22). Hepatic glucokinase activity and glycolysis were also increased in arthritic rats (23). These studies suggested the involvement of the liver and spleen in RA pathogenesis, although no direct data are available to support the importance of liver and spleen in RA pathogenesis. No metabolomic and transcriptomic studies or integrative analyses have been conducted to investigate the roles of the liver and spleen in RA in terms of glycolysis.

Rats with CIA share many features with RA patients. CIA rats have systemic manifestations of RA, including alterations in metabolism (24). Increased glucose uptake and glycolytic gene expression were detected in arthritic joints of a mouse arthritis model. Inhibiting glycolysis significantly decreased arthritic severity in the model (6, 25). Treatment with 2-DG significantly reduced joint inflammation and activated both adaptive and innate immune cells, as well as the production of pathogenic autoantibodies, in K/BxN mice (26). Metabolomics approaches have been successfully applied for the analysis of aqueous metabolites in CIA rats following the administration of silkworm excrement (27), Guan-Jie-Kang (28), Zushima tablets (29) and silybin (30). However, no transcriptomic or metabolomic analyses, alone or integrated, have been used to fully investigate the metabolic changes in CIA animals following 2-DG treatment. Importantly, to date, no metabolic changes in CIA animals have been measured by active intervention to alter glycolysis.

This study aimed to actively interfere with glycolysis in a CIA rat model to determine the effect of glycolysis on CIA. We treated CIA rats with 2-DG and applied metabolomics to examine the peripheral blood of the animals. We also used transcriptomic and metabolomic analyses, as well as integrative analysis, to investigate the metabolism, gene expression profiles and gene-metabolic networks in the liver and spleen of CIA rats. We aimed to determine the effect of glycolysis on RA and the importance of the liver and spleen in this process as well as the metabolic regulatory pathways that are affected by glycolysis.

MATERIALS AND METHODS

Establishment of an Animal Model With CIA

Bovine type II collagen (2 mg/ml) (Chondrex, USA) was mixed with complete Freund's adjuvant (2 mg/ml) (Sigma-Aldrich, USA) at a one to one ratio. One hundred microliters of emulsion was injected intradermally into six-week-old Sprague–Dawley (SD) rats (Shandong Laboratory Animal Center, China) at the tail root (n=12). One week later, these

rats received an intradermal booster injection with 100 μL of emulsion of bovine type II collagen and incomplete Freund's adjuvant (Sigma-Aldrich) at a one to one ratio. Other rats injected with phosphate-buffered saline (PBS) were used as healthy controls (n=12). An aqueous solution of 2-DG (100 mg/mL) was prepared by dissolving 0.1 g of 2-DG powder in sterile water and was intraperitoneally injected on 3 day before the first injection of collagen. Those rats receiving collagen injections were intraperitoneally administered PBS (n=6) or 2-DG (Solarbio, China) (n=6) (50 mg/kg) twice per week (a total of 6 times, once every 3.5 days). Additionally, those healthy rats receiving the PBS injection in place of collagen were simultaneously treated with 2-DG as a control (n=6). Clinical arthritis scores were calculated according to paw thickness measurements and histologic evidence. The rats were sacrificed on the 21 day after the first 2-DG injection. The hind paws of the rats were collected, fixed in 4% paraformaldehyde and embedded in paraffin for histochemical analysis. The study was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University (20200115). The care of the rats was carried out in accordance with the Regulations of the People's Republic of China on the Administration of Experimental Animals.

Detection of Lymphocyte Subtypes in Rats

Rats were euthanized with ketamine and xylazine, and peripheral blood was collected in anticoagulant K_2-EDTA (ethylenediamine tetraacetic acid). Peripheral blood mononuclear cells (PBMCs) were separated and collected using rat peripheral blood lymphocyte separation medium (Solarbio, China) according to the manufacturer's protocol. The concentration of PBMCs was adjusted to 10^7/ml. Flow cytometry antibodies (1.0 μg/10^6 cells/100 μl) were added to each group. The mixture was incubated at 4°C for 20 min in the dark following mixing. NovoCyte flow cytometry (American ACEA BIO, NovoCyte D2040R) was applied for phenotype analysis, and FlowJo software (Tree Star) was applied for the data analysis. Fluorescein isothiocyanate (FITC) anti-rat CD3 and adenomatous polyposis coli (APC) anti-rat CD45RA antibodies were used for B cell detection. FITC anti-rat CD3, APC anti-rat CD4 and palmar erythema (PE) anti-rat CD8 antibodies were used for CD4+ T cell and CD8+ T cell detection. APC IgG1, FITC IgG1 and PE IgG1 antibodies were used as isotype controls. These antibodies were commercially obtained from BioLegend. The gating strategy for identifying immune cell types is shown in **Supplementary File 1**. FSC/SSC gating was used to identify lymphocytes, and the CD3 and CD8/CD4/CD45RA/CD161 bivariate analysis identified the CD8+ T, CD4+ T, CD3- CD45RA+ B, CD3- CD161+ NK (bright), CD3- CD161+ NK (dim). CD3- CD45RA+ is often used to identify B cells in rats, and CD3- CD161+ is used to identify natural killer cells (31–33).

Detection of Cytokine Levels and the Th1/Th2 Ratio in Rats

Peripheral blood samples were collected and centrifuged at 10,000 rpm for 20 min. The serum levels of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-13 (IL-13), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-a) were measured using a rat Th1/Th2 cytokine assay kit (BioLegend). Each well of the detection plate was supplied with the capture beads, antibody, fluorescent reagent and the standard or tested sample, and the plate was incubated for 3 h at 4°C on a shaker at a speed of 500 rpm in the dark. Cytokines were analyzed using NovoCyte flow cytometry (American ACEA BIO, NovoCyte D2040R). Data were analyzed using LEGENDplex v8.0 (BioLegend).

Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5 and IL-13) cytokines were measured using flow cytometry analysis. The Th1/Th2 ratio was calculated based on the average fluorescence intensities of these cytokines and referred to the study by Anand (34).

Assessment of Lactate and Pyruvate

The CIA animals were anesthetized by intraperitoneal injection of 3% sodium pentobarbital. Anticoagulation blood samples were collected from the inferior vena cava. The concentrations of lactate and pyruvate in the plasma samples were measured by an ISCUS microdialysis analyzer (CMA Microdialysis, Sweden). The lactate/pyruvate ratio (LPR) was calculated based on the concentrations of lactate and pyruvate in the plasma samples.

Quantitative Real Time PCR Analysis

RNAiso Plus (TaKaRa Clontech, Kusatsu, Japan) was used to extract total RNA from the rat PBMCs according to the manufacturer's instructions. The PrimeScript™ RT reagent Kit (TaKaRa Clontech, Kusatsu, Japan) was used to synthesize complementary DNA. The SYBR Premix Ex Taq II system (TaKaRa Clontech) was used for quantitative real-time PCR analysis in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA). The relative mRNA expression level was analyzed using the $2^{-\Delta\Delta CT}$ calculation method. The β-actin mRNA expression level was used as an endogenous control. The forward primer and reverse primer were obtained from Sangon Biotech (Shanghai, China). The primer sequences for the rat genes used in this study are shown in **Supplementary File 2**.

Metabolomics Analysis

After administration of 2-DG, CIA rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital. Liver and spleen tissue samples were collected. A 20 μL aliquot of internal standard (L-2- chlorophenylalanine, 0.3 mg/ml; Lyso PC17:0, 0.01 mg/mL) and 400 μL of methanol aqueous solution (CH_3OH:H_2O v:v = 4:1) were added to 30 mg liver or spleen samples. The tissue mixture was ground with two precooled small steel balls at -20°C for 2 min. Meanwhile, a 10 μL aliquot of the internal standard and 300 μL of methanol:acetonitrile mixture (2:1) were also added to 100 μL of the rat plasma samples. The mixture was allowed to stand for 30 s. The samples were then ultrasonicated in an ice-water bath for 10 min, allowed to stand at -20°C for 20 min, and centrifuged for 10 min (13000 rpm, 4°C). A 300 μL aliquot of supernatant was collected and mixed with 400 μL of methanol-water (v:v=1:4). The mixtures were vortexed for 30 s and ultrasonicated for 2 min. Following centrifugation for 10 min (13000 rpm, 4°C), 150 μL of the

supernatant was collected with a syringe. The samples were filtered through a 0.22 μm organic phase pinhole filter, transferred to LC injection vials and stored at -80°C until LC-MS analysis. Quality control samples (QCs) were prepared by mixing the extracts of all samples in equal volumes. An LC-MS system composed of an AB ExionLC ultrahigh-performance liquid chromatography instrument coupled with a QE high-resolution mass spectrometer (SCIEX, AB ExionLC, USA) was used for the analysis. The chromatographic conditions were as follows: Chromatographic column: ACQUITY UPLC BEH C18 (100 mm× 2.1 mm, 1.7 μm), column temperature: 40°C, mobile phase A: water (containing 0.1% formic acid), mobile phase B: acetonitrile (containing 0.1% formic acid). The flow rate was 0.35 mL/min. The sample injection volume was 5 μL. The mass spectrometry conditions were set up as follows: Ion source: ESI, sample mass spectrum signal acquisition: both positive and negative ion scanning modes, spray voltage (V): 3500, capillary temperature (°C): 320, probe heater temperature (°C): 350, sheath gas flow rate (Arb): 40, Aux gas flow rate (Arb): 10, S-lens RF level: 50, mass range (m/z): 100-1000, full ms resolution: 70000, MS/MS resolution: 17500, NCE/stepped NCE: 10, 20, 40, UNIFI: 1.8.1. Data were preprocessed before pattern recognition. The original data were subjected to baseline filtering and peak identification, integration, retention time correction, alignment and normalization by Progenesis QI v2.3 metabolomics processing software (Nonlinear Dynamics, Newcastle, UK). The main parameters were as follows: precursor tolerance: 5 ppm, product tolerance: 10 ppm, product ion threshold: 5%. Compounds were identified based on accurate mass number, secondary debris and isotope distribution and were qualitatively identified by comparison with the human metabolome database (HMDA), LIPID MAPS (v2.3) and the METLIN database. Orthogonal partial least-squares discriminant analysis (OPLS-DA) and principal component analysis (PCA) were performed using the SIMCA 14.1 software package (Umetrics, Umea, Sweden). Metabolites with variable importance in projection (VIP) values > 1 and $P < 0.05$ were defined as differentially expressed metabolites (DEMs).

Transcriptomic Analysis

Total RNA was extracted from the liver and spleen using TRIzol reagent (Invitrogen), and DNA was digested with DNase. Eukaryotic mRNA was enriched with oligo (dT) magnetic beads (Invitrogen). The mRNA was broken into short segments, reversed transcribed into single-stranded cDNA using a six-base random primer (Collibri™ Stranded RNA Library Prep Kit for Illumina™ Systems, Invitrogen), and synthesized into double-stranded cDNAs using Collibri™ Stranded RNA Library Prep Kit for Illumina™ Systems (Invitrogen). The purified double-stranded cDNA was tailed with a sequencing connector. PCR amplification was carried out to amplify the sample. The library quality was verified on an Agilent 2100 Bioanalyzer. After quality inspection, transcriptome sequencing and sequence analysis were carried out with an Illumina sequencer (Illumina HiSeq X Ten). Clean reads were mapped to the rat reference genome rn6. Gene expression levels were quantified by the fragments per kb per million reads (FPKM) method. DESeq software (DESeq version 1.39.0) was used to standardize the count number of each sample gene. The base mean value was used to estimate the gene expression level, and the fold change (FC) was calculated. A negative binomial distribution test (NB) was used to test the significance of the difference in read number. The differentially expressed protein-coding genes were screened according to the FC and difference significance test results. Differentially expressed genes (DEGs) were selected from those protein-coding genes with a VIP FC > 2 or < 0.5 and $P < 0.05$. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were used to analyze the enrichment of biological processes and pathways. DESeq software was used to standardize the count number of each sample gene (35).

Integrating Transcriptomics and Metabolomics Analysis

DEGs and DEMs were subjected to pathway enrichment analysis using the KEGG database. Common pathways were determined based on the overlap between enriched pathways. Cytoscape software 3.1.1 (San Diego, CA, USA) was used to construct a gene-metabolite network.

Statistical Analysis

SPSS 17.0 software (IBM, USA) was used for statistical analysis. Student's t-test was used for comparisons between two groups. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups. Least significant difference (LSD) tests were used for pairwise comparisons. The data are shown as the mean ± SD. For all tests, $p < 0.05$ was considered statistically significant.

RESULTS

The Effects of 2-DG on CIA of Rats

We treated the CIA model rats with 2-DG. Significant joint inflammation was observed in rats injected with collagen compared with healthy rats ($p<0.0001$), indicating successful establishment of the CIA model. Compared with the CIA group injected with PBS, the rats treated with 2-DG had reduced toe swelling and redness (**Figure 1A**), while histochemical staining showed less inflammatory cell infiltration in the treated rats (**Figure 1B**). The inflammation curve also showed that toe inflammation was relieved in 2-DG-treated CIA rats at 16 days and 20 days ($p=0.0307$) after the first collagen injection ($p=0.0191$) compared with the CIA controls (**Figure 1C**). This observation demonstrated that 2-DG attenuated CIA in the model rats. Healthy rats treated with 2-DG alone did not show significant joint inflammation.

The lymphocyte subtypes in the model rats were measured using flow cytometry. The proportions of B cells were significantly increased in CIA rats compared with healthy controls ($p=0.036$), while the proportions of B cells in CIA rats treated with 2-DG were significantly decreased compared with those in CIA rats ($p=0.0002$), although the B cell proportion in the treated CIA rats was higher than that in the healthy rats

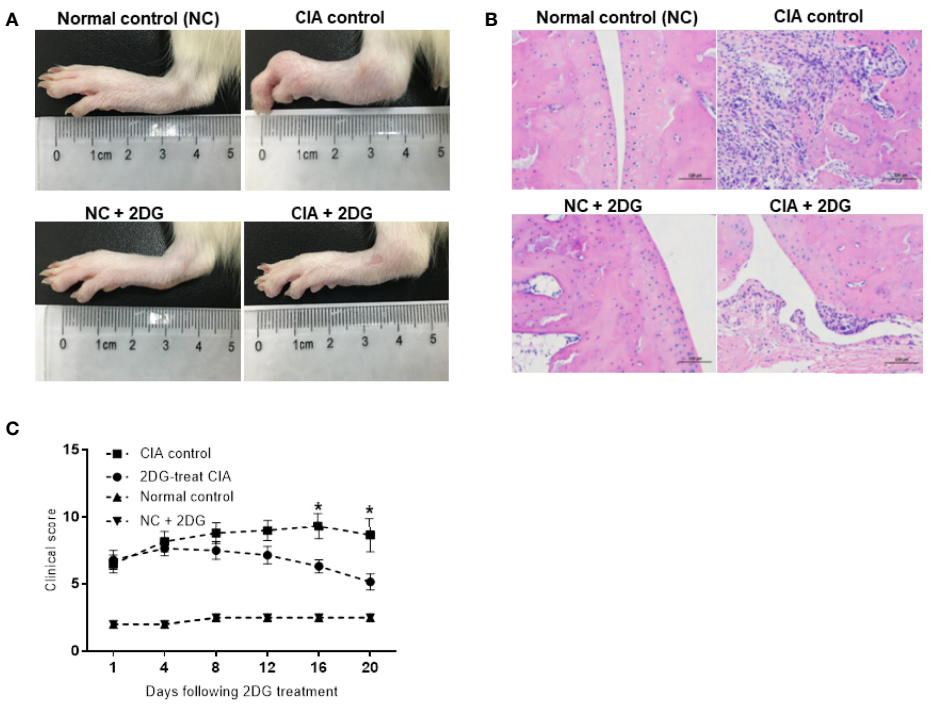


FIGURE 1 | The effect of 2-DG on CIA rats. **(A)** Photographs of paws from the CIA rats following PBS (n=6) or 2-DG (n=6) treatment. The healthy rats (NC) treated with PBS (n=6) or 2-DG (n=6) were used as controls. **(B)** Representative images of the histological examination of the rat joint synovial tissues (200 x magnification). **(C)** Clinical scores of the CIA rats treated with PBS or 2-DG. *p < 0.05.

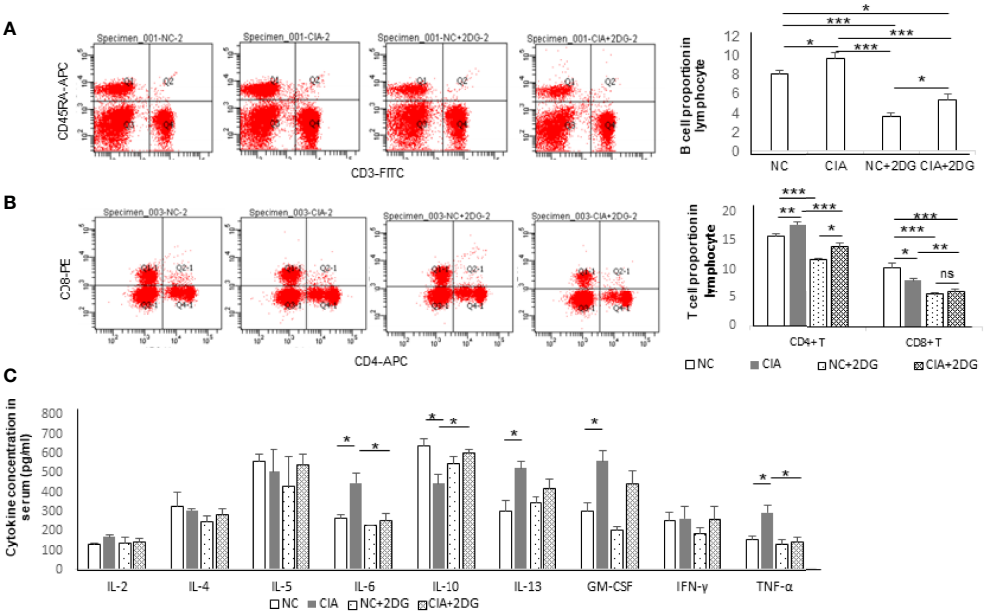
treated with 2-DG (p<0.0001) (**Figure 2A**). Additionally, the B cell proportion was significantly lower in the rats treated with 2-DG than in the heathy rats not treated with 2-DG (p<0.0001). The proportions of CD4+ T cells were significantly increased and the proportions of CD8+ T cells were significantly decreased in the CIA rats compared with healthy rats (p=0.0074 and 0.0180, respectively). The proportions of both CD4+ T cells and CD8+ T cells were significantly decreased in CIA rats treated with 2-DG compared with CIA controls (p=0.0008 and 0.0055, respectively), although the CD4+ T cell proportion in 2-DG-treated CIA rats was greater than that in 2-DG-treated healthy rats (p=0.0278) (**Figure 2B**). Both CD4+ T and CD8+ T cell proportions were significantly decreased in the healthy rats following 2-DG treatment (p<0.0001 and 0.0002, respectively).

The serum cytokine levels in the rats were measured by flow cytometry. The concentrations of IL-6, IL-13, GM-CSF and TNF-α were significantly increased (p=0.0233, 0.0248, 0.0226 and 0.0354, respectively) and the concentrations of IL-10 were significantly decreased in the serum of CIA rats compared with the healthy rats (p=0.0277). The concentrations of IL-6 and TNF-α in the peripheral blood were significantly decreased in the CIA rats treated with 2-DG compared with the CIA rats (p=0.0352 and 0.0282, respectively), and the IL-10 level was significantly elevated (p=0.017) (**Figure 2C**). The above data indicated that 2-DG significantly alleviated joint inflammation and suppressed immune reflection in the CIA model.

IFN-γ, IL-2 (Th1-related cytokines), IL-4, IL-5 and IL-13 (Th2-related cytokines) levels were measured using flow cytometry analysis. Levels of IL-2, IL-4, IL-5 and IFN-γ were not significantly changed among normal rats, CIA rats, 2-DG-treated CIA rats and rats treated with 2-DG alone. The IL-13 level was significantly increased in CIA rats compared with that in the normal rats (p=0.0248). The level decreased following 2-DG treatment, but the difference was not statistically significant (p=0.1705). The current result cannot determine the effect of 2-DG on Th1/Th2 ratio in the CIA animal model **(Figure 2D).**

The Effects of 2-DG on Glycolysis in CIA Rats

The plasma levels of lactate and pyruvate, the end products of glycolysis, as well as the lactate/pyruvate ratio (LPR), were analyzed in the CIA model rats. The levels of lactate and pyruvate were significantly increased in the plasma of the CIA rats compared with that of healthy rats (p<0.0001 and p=0.0022, respectively). Significant reductions in lactate and pyruvate levels were detected in the plasma of CIA rats treated with 2-DG compared to that of CIA rats injected with PBS (p<0.0001 and p=0.0046, respectively), although the lactate level was higher than that in healthy rats treated with 2-DG (p<0.0001 and p=0.0006, respectively) (**Figure 3A, B**). LPR was also significantly elevated in the CIA rats compared with the healthy rats (p<0.0001) and lower in the plasma of 2-DG-treated CIA rats compared to that of the CIA controls (p



D

Th1 and Th2 cytokines in the serum

		NC	CIA	NC+2DG	CIA+2DG	P (NC vs NC+2DG)	P (NC vs CIA)	P (NC+2DG vs CIA+2DG)	P (CIA vs CIA+2DG)
Th1	IL-2	129.4 ± 8.739	166.7 ± 14.25	137.9 ± 27.37	144 ± 19.07	0.7840	0.0895	0.8631	0.3935
	IFN-γ	250.4 ± 41.98	262.8 ± 64.1	182.8 ± 30.47	255.4 ± 68.53	0.2623	0.8798	0.3879	0.9411
Th2	IL-4	328.3 ± 73.9	305 ± 8.096	245.5 ± 33.84	281.4 ± 32.36	0.3663	0.7702	0.4863	0.5180
	IL-5	557.6 ± 35.97	505.9 ± 116	428.8 ± 153.4	542 ± 54.24	0.4596	0.6925	0.5247	0.7919
	IL-13	304 ± 50.32	521.9 ± 36.59	346.6 ± 30.53	415 ± 52.6	0.5089	0.0248	0.3239	0.1705

FIGURE 2 | The immune effect of 2-DG treatment on CIA rats. **(A)** B cell proportion in peripheral blood of the CIA rats treated with PBS (n=6) or 2-DG (n=6). The healthy rats (NC) treated with PBS (n=6) or 2-DG (n=6) were used as controls. **(B)** CD4+ T cell and CD8+ T cell proportions in peripheral blood of the rats. **(C)** Proinflammatory cytokine levels in peripheral blood of the rats. **(D)** Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5 and IL-13)-related cytokine levels in the rats. *p < 0.05, **p < 0.01, and ***p < 0.001.

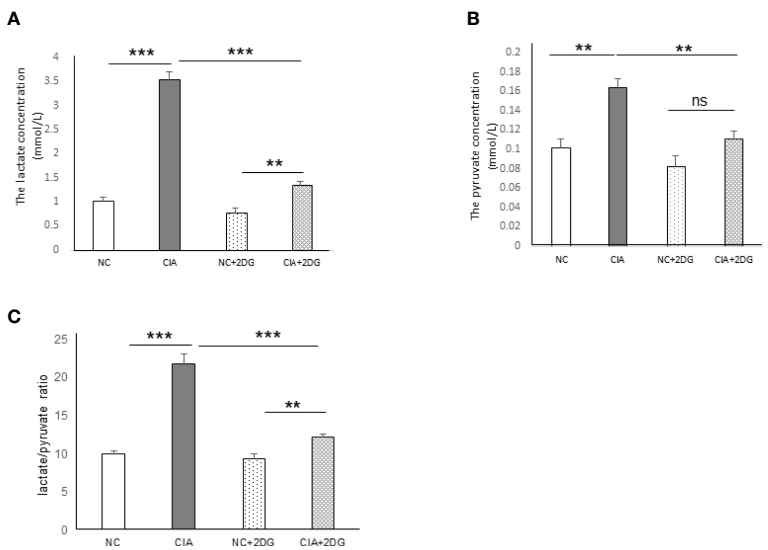


FIGURE 3 | The effect of 2-DG treatment on lactate and pyruvate production in CIA rats. The concentrations of lactate **(A)** and pyruvate **(B),** as well as the lactate/pyruvate ratio (LPR) **(C),** were measured in the peripheral blood of the rats that were treated with PBS (n=6) or 2-DG (n=6). The healthy rats (NC) treated with PBS (n=6) or 2-DG (n=6) were used as controls. **p < 0.01, ***p < 0.001 and ns, not significant .

<0.0001), although the LPR level was higher than that in the healthy rats treated with 2-DG (p<0.0001) (**Figure 3C**). This result showed increased glycolytic activity in CIA rats. The result also indicated that 2-DG significantly reduced glycolysis in the CIA rats.

The mRNA expression levels of key enzymes involved in glycolysis in rat PBMCs were analyzed using quantitative real-time PCR. The mRNA expression levels of hexokinase 2 (HK2), glucose-6-phosphate dehydrogenase (G-6-PD), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), enolase 1 (ENO1), pyruvate kinase M1/2 (PKM), lactate dehydrogenase (LDH), and glycogen synthase kinase-3 beta (GSK-3β) were significantly increased in PBMCs of CIA rats compared with those of healthy rats (p=0.0063, 0.045, 0.0002, 0.0025, 0.0031, <0.0001, 0.0002, 0.0008, and 0.0033, respectively). Phosphofructokinase (PFK) mRNA expression was not significantly different (p=0.1809) from that in the healthy controls. We detected significant reductions in levels of the HK2, GAPDH, PGK1, ENO1, PKM and GSK-3β mRNAs in the PBMCs from CIA rats treated with 2-DG compared to those from CIA rats treated with PBS (p=0.0019, 0.007, 0.0052, 0.0012, 0.0001, and 0.022, respectively). G-6-PD, PFK, TPI, and LDH mRNA expression levels were not significantly different following the 2-DG treatment (p=0.0649, 0.2109, 0.1192 and 0.2589, respectively). These data indicated that 2-DG significantly reduced the expression levels of many key glycolysis enzymes in CIA model rats (**Figure 4**).

The Metabolomic Analysis for CIA Rat Plasma

We analyzed the small molecule metabolites in CIA rat plasma using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). PCA was used to visualize the similarities and differences in the metabolomic datasets between healthy rats (P-H), CIA rats (P-C), CIA rats treated with 2-DG (P-T), and healthy rats treated with 2-DG (P-K). The PCA score plots indicated that the model discriminated differential expressed metabolites between the four groups (**Supplementary File 3**). DEMs were determined from those metabolites with VIP values > 1 and p < 0.05. Volcano plots were generated by plotting the log (base 2) of the fold change (FC) and the log (base 10) of the p values of the t-test between these four groups (**Figure 5A**). Compared with the healthy groups, levels of 7 DEMs were significantly increased in CIA rats, while levels of 12 DEMs were significantly decreased. Compared with the healthy groups, levels of 14 DEMs were significantly increased in the healthy rats treated with 2-DG, while levels of 12 DEMs were significantly decreased. Compared with the CIA rats, levels of 17 DEMs were significantly increased in CIA rats following 2-DG treatment, while levels of 3 DEM metabolites were significantly decreased. Compared with the healthy rats treated with 2-DG, levels of 14 DEMs were significantly increased in the CIA rats treated with 2-DG, while levels of 6 DEMs were significantly decreased. Detailed information on the top 20 DEMs based on VIP is shown in **Supplementary File 4**.

A metabolic pathway (top 20) analysis was conducted based on the KEGG database. The pathways were enriched with DEMs as determined by Student's t-tests with a threshold of <0.05. The analysis indicated that DEMs between the healthy rats (P-H) and CIA rats (P-C) were closely related to antifolate resistance (p=0.0014) and regulation of lipolysis in adipocytes (p= 0.00095), among which all DEM levels were significantly increased in CIA rats; bile secretion (p<0.0001) and primary bile acid biosynthesis (p=0.00037), among which all DEM levels were significantly decreased; and central carbon metabolism in cancer (p=0.00011), cholesterol metabolism (p<0.0001), choline metabolism in cancer (p<0.0001), Kaposi's sarcoma-associated herpesvirus infection (p=0.0017), linoleic acid metabolism (p= 0.00049) and mTOR signaling (p=0.0011), among which levels of some DEMs were increased and some were decreased. Additionally, DEMs between the healthy rats (P-H) and healthy rats treated with 2-DG (P-K) are closely related to linoleic acid metabolism (p=0.000027) and PPAR signaling (p=0.0017), among which all DEM levels were significantly increased in the 2-DG-treated rats; cortisol synthesis and secretion (p=0.00045) and Cushing syndrome (p=0.00058), among which all DEM levels were significantly decreased; and bile secretion (p=0.00026), biosynthesis of amino acids (p=0.0064), central carbon metabolism in cancer (p=0.00011), cholesterol metabolism

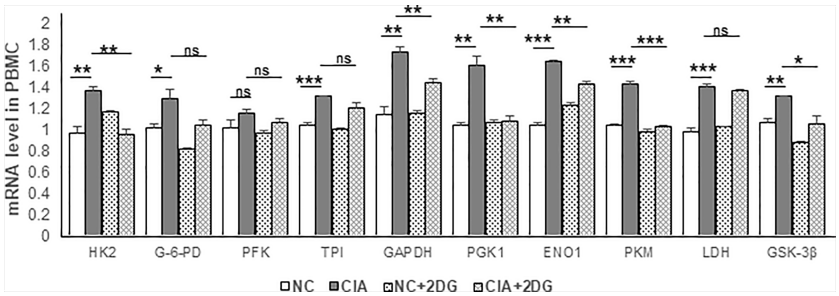


FIGURE 4 | The effect of 2-DG treatment on the mRNA expression of glycolytic enzymes in PBMCs of CIA rats. The CIA rats were treated with PBS (n=6) or 2-DG (n=6). The healthy rats (NC) treated with PBS (n=6) or 2-DG (n=6) were used as controls. The mRNA levels were determined using quantitative real-time PCR. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.

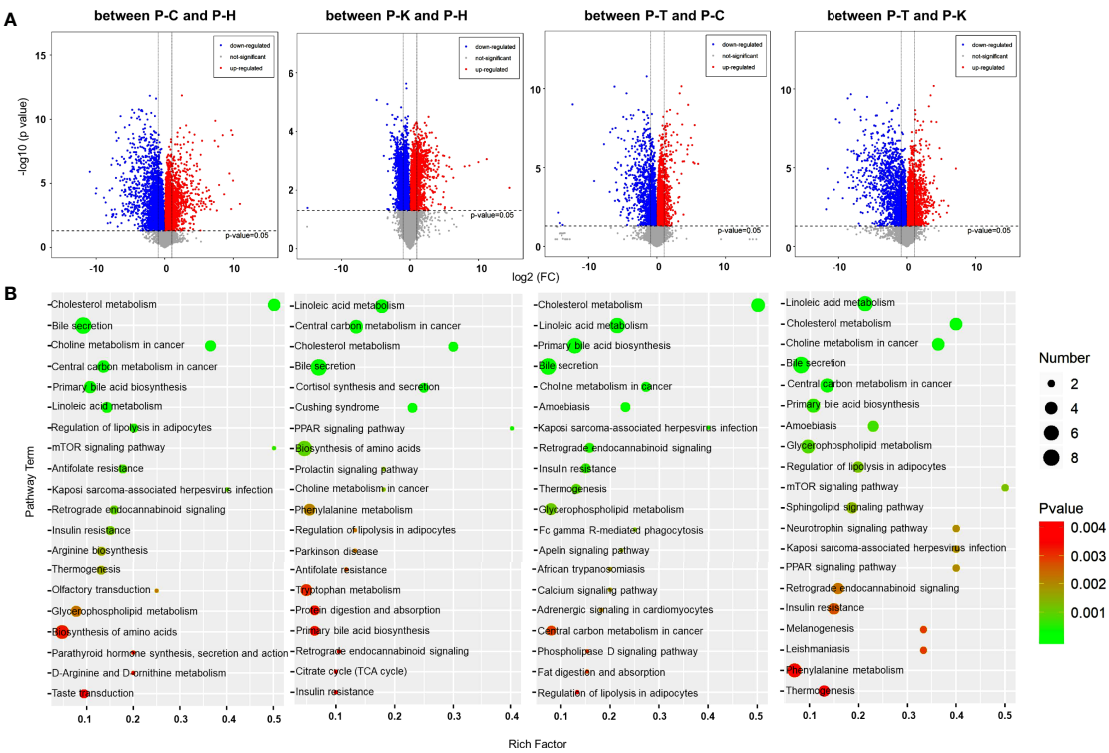


FIGURE 5 | Metabolomic analysis of the rat plasma. **(A)** Volcano plots were prepared by plotting the log (base 2) of the FC and p values of t-test results (base 10) for differentially expressed plasma metabolites in rats between pairs of the four groups including healthy control (P-H) (n=6), CIA rats (P-C) (n=6), CIA rats treated with 2-DG (P-T) (n=6), and healthy rats treated with 2-DG (P-K). **(B)** The pathway analysis was performed based on the KEGG pathway database. The bubble chart shows the pathways that were enriched with DEMs in the plasma between pairs of the four groups.

(p=0.00025), choline metabolism in cancer (p=0.0088) and prolactin signaling (p=0.00088), among which levels of some DEMs were increased and some were decreased. The analysis indicated that DEMs between CIA rats (P-C) and CIA rats treated with 2-DG (P-T) were closely related to amoebiasis (p=0.00048), bile secretion (p=0.00017), cholesterol metabolism (p<0.00001), linoleic acid metabolism (p<0.00001), choline metabolism in cancer (p=0.00028), insulin resistance (p= 0.0018), Kaposi's sarcoma-associated herpesvirus infection (p=0.0015), primary bile acid biosynthesis (p=0.000019), retrograde endocannabinoid signaling (p=0.0015) and thermogenesis (p=0.0027), among which levels of some DEMs were increased and the levels of some DEMs were decreased. DEMs between the CIA rats treated with 2-DG (P-T) and healthy rats treated with 2-DG (P-K) were closely related to choline metabolism in cancer (p=0.000011), glycerophospholipid metabolism (p=0.00079) and regulation of lipolysis in adipocytes (p=0.0011), among which levels of DEMs were significantly increased in healthy rats treated with 2-DG, and to amoebiasis (p=0.00073), bile secretion (p=0.000062), central carbon metabolism in cancer (p=0.00015), cholesterol metabolism (p<0.00001), linoleic acid metabolism (p<0.00001), mTOR signaling (p=0.0012) and primary bile acid biosynthesis (p=0.0005), among which levels of some DEMs were increased and some were decreased. The analysis described above is shown graphically in **Figure 5B**, and detailed information on the metabolic pathway analysis (top 10) is shown in **Supplementary File 5**.

Metabolomic Analysis for CIA Rat Liver and Spleen

We investigated the effects of 2-DG on metabolism in the liver and spleen of CIA rats using metabolomic analysis. PCA was used to visualize the similarities and differences in the metabolomic datasets between the livers of healthy rats (L-H), CIA rats (L-C), CIA rats treated with 2-DG (L-T), and healthy rats treated with 2-DG (L-K). The PCA score plots indicated that the model identified differences in the metabolite levels in rat livers between the L-H and L-C groups, between the L-K and L-H groups, between the L-T and L-C groups, and between the L-T and L-K groups (**Supplementary File 6**). To discover DEMs in the liver, volcano plots were prepared by plotting the log (base 2) of the FC and p values of the t-test results (base 10). DEMs were selected from those metabolites with VIP>1 and $P < 0.05$. Compared with the healthy group, the levels of 35 DEMs were significantly increased in CIA rat livers, while the levels of 26 DEMs were significantly decreased. Compared with the healthy group, levels of 40 DEMs were significantly increased in livers of the healthy rats treated with 2-DG, and the levels of 22 DEMs were significantly decreased. Compared with the CIA rats, levels of 23 DEMs were significantly increased in the livers of CIA rats

treated with 2-DG, while the levels of 28 DEMs were significantly decreased. Compared with the healthy rats treated with 2-DG, the levels of 27 DEMs were significantly increased in the livers of CIA rats treated with 2-DG, while the levels of 33 DEMs were significantly decreased (**Figure 6A**). Detailed information on DEMs in rat livers is provided in **Supplementary File 7**.

The metabolic pathways (top 20) enriched among the DEMs in rat livers were analyzed based on the KEGG pathway database. The significant pathways enriched with DEMs were determined using Student's t-test with a threshold of <0.05. The analysis indicated that DEMs between the livers of healthy rats (L-H) and CIA rats (L-C) were closely related to arachidonic acid metabolism (p=0.0014) and serotonergic synapse (p=0.0051), among which the levels of all DEMs were significantly decreased in CIA rats; and ABC transporters (p=0.0050), bile secretion (p=0.00020), cholesterol metabolism (p=0.000014), choline metabolism in cancer (p=0.000022), glycerophospholipid metabolism (p=0.00020), Parkinson disease (p= 0.0018), primary bile acid biosynthesis (p=0.0076) and protein digestion and absorption (p=0.001), among which the levels of some DEMs were increased and some were decreased. DEMs between the livers of healthy rats (L-H) and the livers of healthy rats treated with 2-DG (L-K) were closely related to serotonergic synapse (p=0.0053), among which the levels of all DEMs were significantly increased in the livers of healthy rats treated with 2-DG; carbohydrate digestion and absorption (p=0.01), among which the levels of all DEMs were significantly decreased; and ABC transporters (p=0.00025), arachidonic acid metabolism (p=0.0086), bile secretion (p= 0.0061), cholesterol metabolism (p= 0.00053), galactose metabolism (p= 0.0074), primary bile acid biosynthesis (p=0.008), retrograde endocannabinoid signaling (p=0.00025) and vascular smooth muscle contraction (p=0.0094), among which the levels of some DEMs were increased and some were decreased. The analysis indicated that DEMs between the livers of CIA rats (L-C) and the livers of CIA rats treated with 2-DG (L-T) were closely related to retrograde endocannabinoid signaling (p=0.027), among which the levels of all DEMs were significantly increased in the 2-DG-treated CIA rats; cholesterol metabolism (p<0.00001), among which the levels of all DEMs were significantly decreased; and alcoholism (p=0.0079), arachidonic acid metabolism (p=0.019), bile secretion (p<0.00001), choline metabolism in cancer (p=0.0095), glycerophospholipid metabolism (p=0.0054), cGMP-PKG signaling (p=0.015), Parkinson disease (p= 0.018) and primary bile acid biosynthesis (p=0.000036), among which the levels of some DEMs were increased and some were decreased. DEMs between the livers of CIA rats treated with 2-DG (L-T) and the livers of healthy rats treated with 2-DG (L-K) were strictly related to carbohydrate digestion and absorption (p=0.0091), among which the levels of all DEMs were significantly increased in 2-DG-treated healthy rats; arachidonic acid metabolism (p=0.0012), among which the

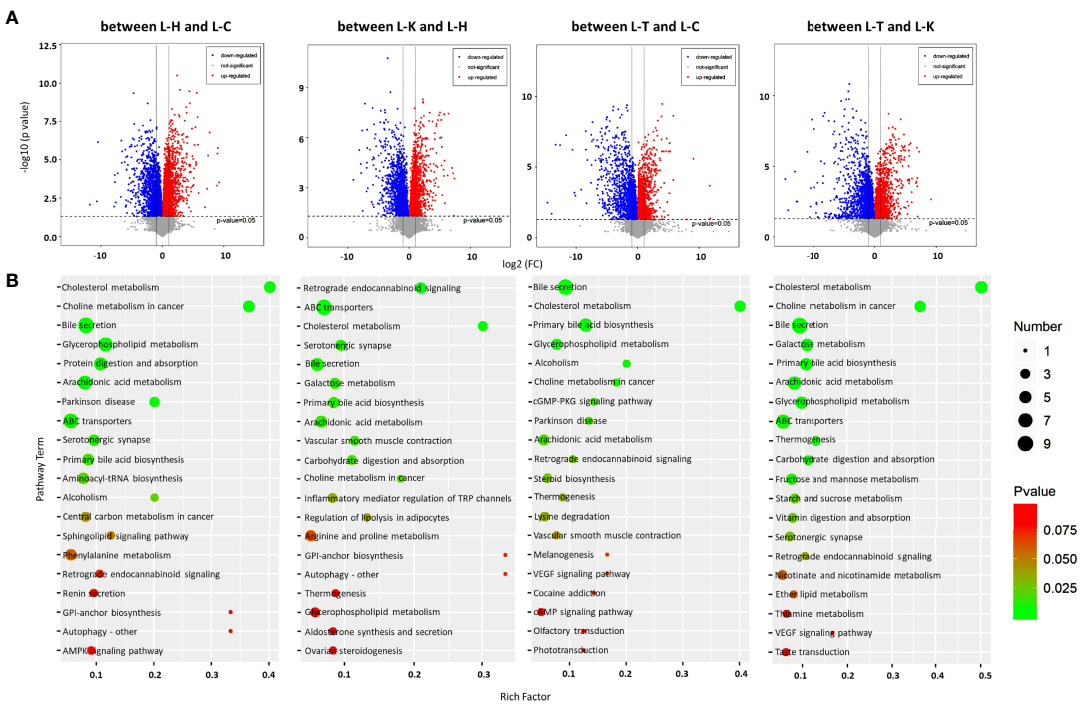


FIGURE 6 | Metabolomic analysis of the rat livers. **(A)** Volcano plots were prepared by plotting the log (base 2) of the FC and p values of t-test results (base 10) for differentially expressed metabolites in rat livers between pairs of the four groups including the healthy control (L-H) (n=6), CIA rats (L-C) (n=6), CIA rats treated with 2-DG (L-T) (n=6), and healthy rats treated with 2-DG (L-K) (n=6). **(B)** The pathway analysis was performed based on the KEGG pathway database. The bubble chart shows the pathways that were enriched with DEMs in the liver between the four rat groups.

levels of all DEMs were significantly decreased; and ABC transporters (p=0.0041), bile secretion (p=0.000022), cholesterol metabolism (p=0.00000023), choline metabolism in cancer (p=0.000019), galactose metabolism (p= 0.0008), glycerophospholipid metabolism (p=0.0014), primary bile acid biosynthesis (p=0.00089) and thermogenesis (p=0.0058), among which the levels of some DEMs were increased and some were decreased (**Figure 6B**). Detailed information on the metabolic pathways (top 20) based on the KEGG analysis is provided in **Supplementary File 8**.

PCA was also used to analyze metabolomic datasets from the spleens of healthy rats (S-H), CIA rats (S-C), CIA rats treated with 2-DG (S-T), and healthy rats treated with 2-DG (S-K). The PCA score plots indicated that the model identified differences in metabolite levels in the rat spleen between the S-H and S-C groups, between the S-K and S-H groups, between the S-T and S-C groups, and between the S-T and S-K groups (**Supplementary File 9**). Volcano plots were prepared by plotting the log (base 2) of the FC and the p values of t-tests (base 10) to discover the DEMs in the spleen. Compared with those in the healthy rats, the levels of 28 DEMs were significantly increased in spleens of CIA rats, while the levels of 34 DEMs were significantly decreased. The levels of 31 DEMs were significantly increased and levels of 30 DEMs were significantly decreased in the healthy rats treated with 2-DG compared with those in the healthy rats. Compared with the CIA rats, levels of 56 DEMs were significantly increased in spleens of CIA rats treated with 2-DG, while levels of 40 DEMs were significantly decreased. Levels of 52 DEMs were significantly elevated and levels of 37 DEMs were significantly decreased in spleens of CIA rats treated with 2-DG compared with the healthy rats treated with 2-DG (**Figure 7A**). Detailed information on the DEMs in the rat spleen is shown in **Supplementary File 10**.

The pathway analysis (TOP 20) based on the KEGG database indicated that DEMs between spleens of healthy rats (S-H) and CIA rats (S-C) were closely related to ether lipid metabolism (p=0.00079), among which levels of all DEMs were significantly increased in the CIA rats; glycosylphosphatidylinositol (GPI)-anchor biosynthesis (p=0.00088), among which levels of all DEMs were significantly decreased; and arachidonic acid metabolism (p<0.00001), asthma (p= 0.000049), choline metabolism in cancer (p<0.00001), Fc epsilon RI signaling (p=0.00075), glycerophospholipid metabolism (p<0.00001), linoleic acid metabolism (p=0.000098), retrograde endocannabinoid signaling (p<0.00001) and serotonergic synapse (p<0.00001), among which levels of some DEMs were increased and some were decreased. DEMs in spleens between healthy rats (S-H) and healthy rats treated with 2-DG (S-K) were closely related to the oxytocin signaling pathway (p=0.000053), among which levels of all DEMs were significantly decreased in the 2-DG-treated healthy rats; and aldosterone synthesis and secretion (p<0.00001), arachidonic acid metabolism

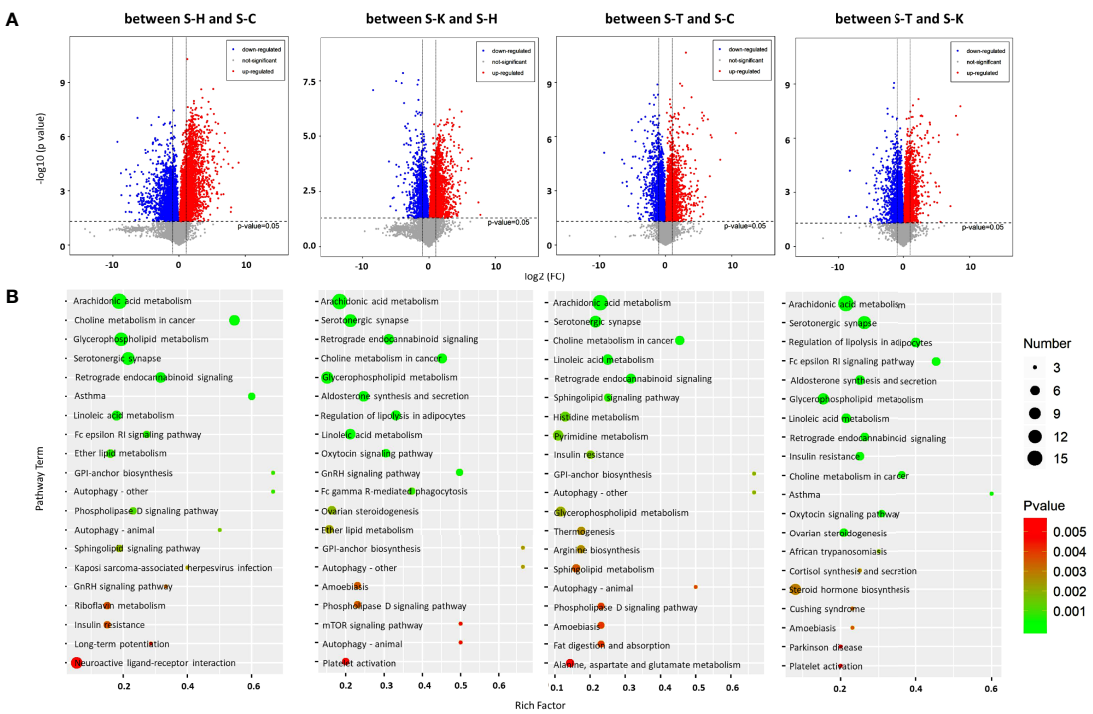


FIGURE 7 | Metabolomic analysis of the rat spleens. **(A)** Volcano plots were prepared by plotting the log (base 2) of the FC and p values of t-test results (base 10) for rat spleen metabolites between pairs of the four groups, including the healthy controls (S-H) (n=6), CIA rats (S-C) (n=6), CIA rats treated with 2-DG (S-T) (n=6), and healthy rats treated with 2-DG (S-K) (n=6). **(B)** The pathway analysis was performed based on the KEGG pathway database. The bubble charts show pathways that were enriched with DEMs in the spleen between the four rat groups.

(p<0.00001), choline metabolism in cancer (p<0.00001), glycerophospholipid metabolism (p<0.00001), GNRH signaling (p<0.00001), linoleic acid metabolism (p<0.00001), retrograde endocannabinoid signaling (p<0.00001), regulation of lipolysis in adipocytes (p<0.00001) and serotonergic synapse (p<0.00001), among which levels of some DEMs were increased and some were decreased. DEMs in spleens between CIA rats (S-C) and CIA rats treated with 2-DG (S-T) were closely related to sphingolipid signaling (p=0.00064), among which levels of all DEMs were significantly decreased in 2-DG-treated CIA rats; and arachidonic acid metabolism (p<0.00001), choline metabolism in cancer (p<0.00001), glycosylphosphatidylinositol (GPI)-anchor biosynthesis (p=0.002), histidine metabolism (p=0.0012), insulin resistance (p=0.0015), linoleic acid metabolism (p<0.00001), pyrimidine metabolism (p=0.0014), retrograde endocannabinoid signaling (p<0.00001) and serotonergic synapse (p<0.00001), among which levels of some DEMs were increased and some were decreased. Moreover, DEMs in spleens between CIA rats treated with 2-DG (S-T) and healthy rats treated with 2-DG (S-K) were closely related to arachidonic acid metabolism (p<0.00001), Fc epsilon RI signaling (p<0.00001), linoleic acid metabolism (p=0.00005) and retrograde endocannabinoid signaling (p=0.000077), among which levels of all DEMs were significantly increased in the healthy rats treated with 2-DG, and aldosterone synthesis and secretion (p=0.000019), choline metabolism in cancer (p=0.00011), glycerophospholipid metabolism (p=0.000034), insulin resistance (p=0.0001), regulation of lipolysis in adipocytes (p<0.00001) and serotonergic synapse (p<0.00001), among which levels of some DEMs were increased and some were decreased (**Figure 7B**). Detailed information on the metabolic pathways (top 20) in rat spleen based on the KEGG analysis is presented in **Supplementary File 11**.

Transcriptomic Analysis for CIA Rat Liver and Spleen

We investigated the effects of 2-DG on gene expression in the rat liver and spleen using transcriptomics. PCA was used to visualize the similarities and differences in the transcriptomic datasets of the livers between healthy rats (L-H), CIA rats (L-C), CIA rats treated with 2-DG (L-T), and healthy rats treated with 2-DG (L-K) (**Supplementary File 12**). Compared with the healthy rats, the mRNA expression levels of 544 DEGs were significantly increased and the levels of 298 DEGs were significantly decreased in the livers of CIA rats, while the expression levels of 479 DEGs were significantly increased and levels of 518 DEGs were significantly decreased in the livers of healthy rats treated with 2-DG. Compared with the CIA rats, the mRNA expression levels of 365 DEGs were significantly increased and the levels of 448 DEGs were significantly decreased in the livers of CIA rats treated with 2-DG. The mRNA expression levels of 684 DEGs were significantly increased and the levels of 440 DEGs were significantly decreased in the livers of CIA rats treated with 2-DG compared with the healthy rats treated with 2-DG. DEGs in the liver between different groups are shown in MA (M-versus-A) plots in **Figure 8A**. Detailed information is shown in **Supplementary File 13**.

DEGs were enriched in alternative gene regulation pathways, as determined using Student's t-test with a threshold of <0.05. The gene regulatory pathways (top 20) in those rats were

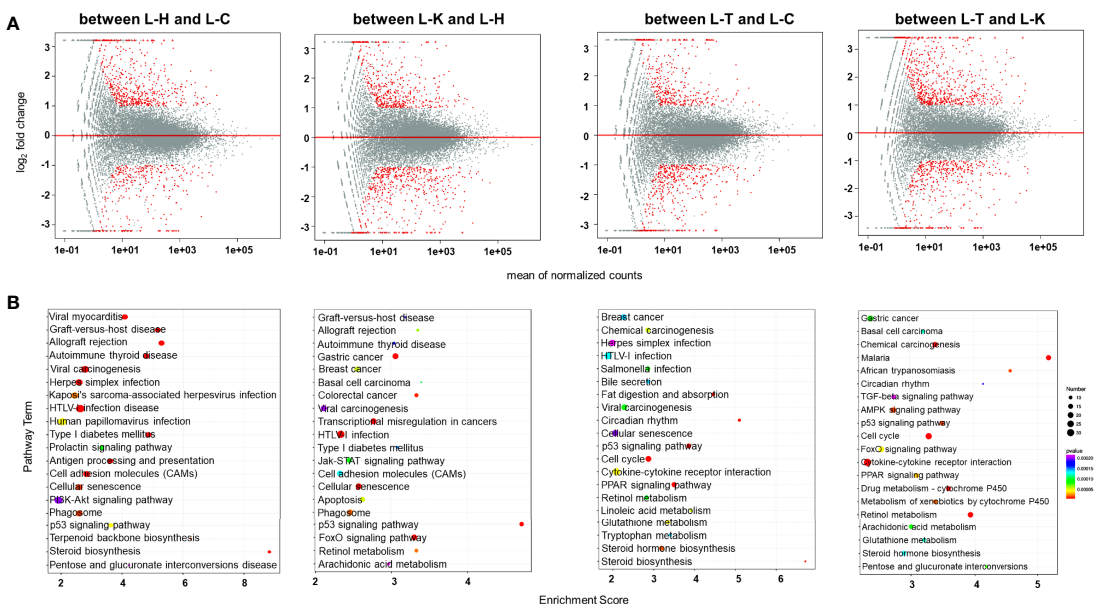


FIGURE 8 | Transcriptomic analysis of the rat livers. **(A)** MA (M-versus-A) plots showing differential expressing genes in rat livers between the four rat groups including healthy control rats (L-H) (n=6), CIA rats (L-C) (n=6), CIA rats treated with 2-DG (L-T) (n=6), and healthy rats treated with 2-DG (L-K) (n=6). **(B)** Bubble charts showing the top 20 differentially enriched pathways in the rat liver among the four rat groups.

analyzed based on the KEGG pathway database. The enrichment analysis indicated that DEGs in livers between healthy rats (L-H) and CIA rats (L-C) were closely related to steroid biosynthesis (p<0.00001) and terpenoid backbone biosynthesis (p=0.000028), among which the levels of all DEGs were significantly increased in CIA rats; and allograft rejection (p<0.00001), antigen processing and presentation (p<0.00001), autoimmune thyroid disease (p< 0.00001), cell adhesion molecules (CAMs) (p<0.00001), cellular senescence (p=0.000027), graft-versus-host disease (p<0.00001), herpes simplex infection (p= 0.000012), HTLV-I infection (p<0.00001), human papillomavirus infection (p=0.000080), Kaposi's sarcoma-associated herpesvirus infection (p=0.000044), p53 signaling (p=0.000077), phagosome (p=0.000027), pentose and glucuronate interconversions (p=0.00041), PI3K-Akt signaling (p= 0.00038), prolactin signaling (p= 0.00017), type I diabetes mellitus (p<0.00001), viral carcinogenesis (p<0.00001) and viral myocarditis (p<0.00001), among which the levels of some DEGs were increased and some were decreased. The enrichment analysis indicated that DEGs in livers between healthy rats (L-H) and healthy rats treated with 2-DG (L-K) were closely related to allograft rejection (p=0.000084), apoptosis (p=0.000071), arachidonic acid metabolism (p= 0.00029), autoimmune thyroid disease (p=0.00022), basal cell carcinoma (p=0.00013), breast cancer (p=0.00007), cell adhesion molecules (CAMs) (p=0.00017), cellular senescence (p=0.0000083), colorectal cancer (p=0.000014), FoxO signaling (p<0.00001), gastric cancer (p<0.00001), graft-versus-host disease (p=0.00024), HTLV-I infection (p<0.00001), p53 signaling (p<0.00001), Jak-STAT signaling (p=0.00012), phagosome (p=0.000025), retinol metabolism (p=0.000028), transcriptional misregulation in cancers (p<0.00001), type I diabetes mellitus (p=0.00019), and viral carcinogenesis (p=0.00026), among which the levels of some DEGs were increased and some were decreased. DEGs in livers between CIA rats (L-C) and CIA rats treated with 2-DG (L-T) were closely related to bile secretion (p=0.0017), chemical carcinogenesis (p=0.00067), breast cancer (p=0.0017), retinol metabolism (p=0.0011) and steroid biosynthesis (p=0.000061), among which the levels of all DEGs were significantly decreased in CIA rats treated with 2-DG; and cellular senescence (p=0.0024), cytokine-cytokine receptor interaction (p=0.00054), linoleic acid metabolism (p=0.00069), cell cycle (p=0.000094), circadian rhythm (p=0.00012), Cushing syndrome (p=0.0011), fat digestion and absorption (p=0.00013), glutathione metabolism (p=0.00056, not sure), herpes simplex infection (p=0.0026), HTLV-I infection (p=0.0016), p53 signaling (p=0.000044), PPAR signaling (p=0.000058), *Salmonella* infection (p=0.0011), steroid hormone biosynthesis (p=0.00026) and tryptophan metabolism (p=0.0017), among which the levels of some DEGs were increased and some were decreased. DEGs in livers between 2-DG-treated CIA rats (L-T) and 2-DG-treated healthy rats (L-K) were strictly related to African trypanosomiasis (p=0.000015), among which the levels of all DEMs were significantly increased in CIA rats treated with 2-DG; metabolism of xenobiotics by cytochrome P450 (p=0.000019), among which the levels of all DEGs were significantly decreased; and AMPK signaling (p=0.000015), arachidonic acid metabolism (p=0.000083), basal cell carcinoma (p=0.00012), cell cycle (p<0.00001), chemical carcinogenesis (p<0.00001), circadian rhythm (p=0.00018), cytokine-cytokine receptor interaction (p<0.00001), drug metabolism-cytochrome P450 (p<0.00001),FoxO signaling (p=0.000044), gastric cancer (p=0.000084), glutathione metabolism (p=0.00012), malaria (p<0.00001), p53 signaling (p=0.000014), PPAR signaling (p=0.000032), pentose and glucuronate interconversions (p=0.000078), retinol metabolism (p<0.00001), steroid hormone biosynthesis (p=0.00013), and TGF-beta signaling (p=0.00022), among which the levels of some DEGs were increased and some were decreased. The top 20 pathways significantly related to DEGs in the liver are summarized in **Figure 8B**. Detailed information is presented in **Supplementary File 14**.

Transcriptomic methods were also used to examine the gene expression in the spleens of the rats. PCA confirmed significant differences in transcriptomic datasets between healthy rats (S-H), CIA rats (S-C), CIA rats treated with 2-DG (S-T) and healthy rats treated with 2-DG (S-K) (**Supplementary File 15**). Compared with the healthy group, transcript levels of 1076 DEGs were significantly increased and the levels of 260 DEGs were significantly decreased in the spleens of CIA rats, while transcript levels of 194 DEGs were significantly increased and the levels of 1034 DEGs were significantly decreased in the spleens of healthy rats treated with 2-DG. Compared with the CIA rats, transcript levels of 1110 DEGs were significantly increased and the levels of 1703 DEGs were significantly decreased in the spleens of CIA rats treated with 2-DG. Transcript levels of 855 DEGs were significantly increased and levels of 483 DEGs were significantly decreased in the spleens of CIA rats treated with 2-DG compared with the healthy rats treated with 2-DG. DEGs in spleen tissues between the four groups are shown in MA (M-versus-A) plots in **Figure 9A**. Detailed information is presented in **Supplementary File 16**.

The pathway enrichment analysis (TOP 20) using the KEGG database indicated that DEGs in the spleen between healthy rats (S-H) and CIA rats (S-C) were closely related to ascorbate and aldarate metabolism (p=0.0000051), chemical carcinogenesis (p<0.00001), cholesterol metabolism (p<0.00001), complement and coagulation cascades (p<0.00001), drug metabolism-cytochrome P450 (p<0.00001), drug metabolism-other enzymes (p<0.00001), glycine serine and threonine metabolism (p=0.000025), linoleic acid metabolism (p<0.00001), porphyrin and chlorophyll metabolism (p<0.00001), PPAR signaling (p<0.00001) and primary bile acid biosynthesis (p<0.00001), among which the levels of all DEGs were significantly increased in CIA rats; and arachidonic acid metabolism (p<0.00001), bile secretion (p<0.00001), malaria (p=0.000011), metabolism of xenobiotics by cytochrome P450 (p<0.00001), nitrogen metabolism (p=0.000063), retinol metabolism (p<0.00001), *Staphylococcus aureus* infection (p=0.000084), steroid hormone biosynthesis (p<0.00001) and systemic lupus erythematosus (p<0.00001), among which the levels of some DEGs were increased and some were decreased. The enrichment

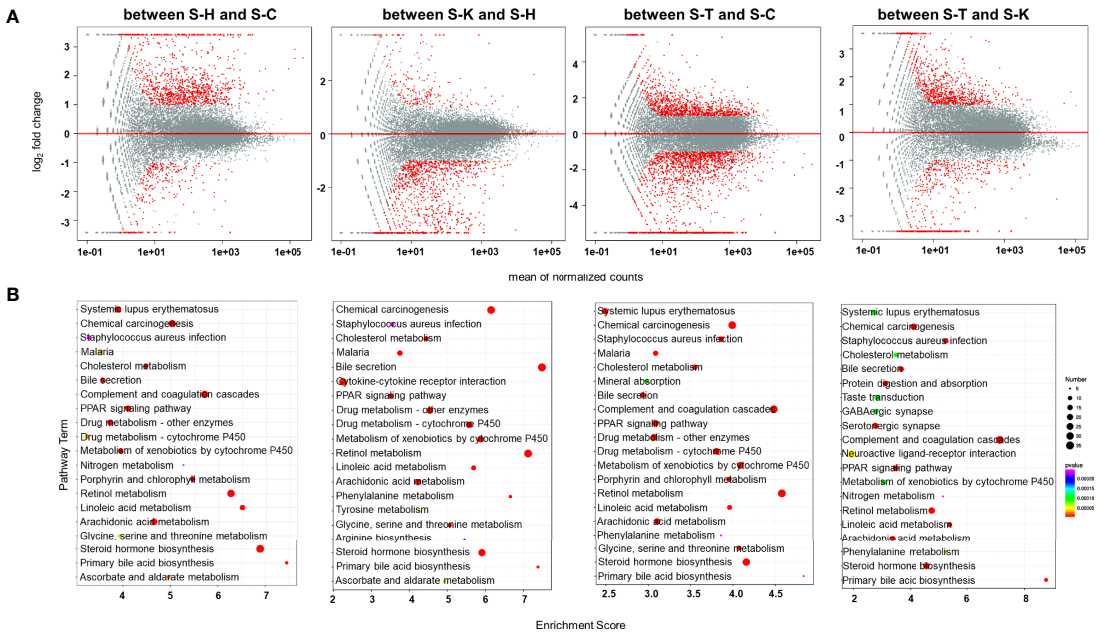


FIGURE 9 | Transcriptomic analysis of the spleens of rats. **(A)** MA (M-versus-A) plots of differential expressing genes in spleens between the four rat groups including healthy controls (S-H) (n=6), CIA rats (S-C) (n=6), CIA rats treated with 2-DG (S-T) (n=6), and healthy rats treated with 2-DG (S-K) (n=6). **(B)** Bubble charts showing the top 20 differentially enriched pathways in the rat spleen among the four rat groups.

analysis indicated that DEGs in the spleen between healthy rats (S-H) and healthy rats treated with 2-DG (S-K) were closely related to arginine biosynthesis (p=0.000016), ascorbate and aldarate metabolism (p<0.00001), bile secretion (p<0.00001), cholesterol metabolism (p<0.00001), complement and coagulation cascades (p<0.00001), drug metabolism-cytochrome P450 (p<0.00001), drug metabolism-other enzymes (p<0.00001), glycine (p<0.00001), serine and threonine metabolism (p<0.00001), metabolism of xenobiotics by cytochrome P450 (p<0.00001), phenylalanine metabolism (p<0.00001), PPAR signaling (p<0.00001), primary bile acid biosynthesis (p<0.00001), retinol metabolism (p<0.00001), *Staphylococcus aureus* infection (p=0.000018), steroid hormone biosynthesis (p<0.00001) and tyrosine metabolism (p<0.00001), among which the levels of all DEGs were significantly decreased in healthy rats treated with 2-DG; and arachidonic acid metabolism (p<0.00001), chemical carcinogenesis (p<0.00001), cytokine-cytokine receptor interaction (p<0.00001) and linoleic acid metabolism (p<0.00001), among which the levels of some DEGs were increased and some were decreased. DEGs in the spleen between CIA rats (S-C) and CIA rats treated with 2-DG (S-T) were closely related to malaria (p<0.00001), porphyrin and chlorophyll metabolism (p<0.00001) and primary bile acid biosynthesis (p<0.00001), among which the levels of all DEGs were significantly decreased in CIA rats treated with 2-DG; and arachidonic acid metabolism (p<0.00001), bile secretion (p<0.00001), chemical carcinogenesis (p<0.00001), cholesterol metabolism (p<0.00001), complement and coagulation cascades (p<0.00001), drug metabolism-cytochrome P450 (p<0.00001), drug metabolism-other enzymes (p<0.00001), glycine (p<0.00001), serine and threonine metabolism (p<0.00001), linoleic acid metabolism (p<0.00001), metabolism of xenobiotics by cytochrome P450 (p<0.00001), mineral absorption (p<0.00001), PPAR signaling (p<0.00001), phenylalanine metabolism (p<0.00001), retinol metabolism (p<0.00001), *Staphylococcus aureus* infection (p<0.00001), steroid hormone biosynthesis (p<0.00001) and systemic lupus erythematosus (p<0.00001), among which the levels of some DEGs were increased and some were decreased. The enrichment analysis indicated that DEGs in the spleen between CIA rats treated with 2-DG (S-T) and healthy rats treated with 2-DG (S-K) were strictly related to steroid hormone biosynthesis (p<0.00001), among which the levels of all DEGs were significantly decreased in CIA rats treated with 2-DG, and arachidonic acid metabolism (p<0.00001), bile secretion (p<0.00001), chemical carcinogenesis (p<0.00001), cholesterol metabolism (p=0.000092), complement and coagulation cascades (p<0.00001), GABAergic synapse (p=0.00011), linoleic acid metabolism (p<0.00001), neuroactive ligand-receptor interaction (p=0.000047), metabolism of xenobiotics by cytochrome P450 (p=0.00011), nitrogen metabolism (p=0.00024), phenylalanine metabolism (p=0.000077), PPAR signaling (p<0.00001), primary bile acid biosynthesis (p<0.00001), protein digestion and absorption (p<0.00001), retinol metabolism (p<0.00001), *Staphylococcus aureus* infection (p<0.00001), serotonergic synapse (p=0.000011), systemic lupus erythematosus (p=0.000095) and taste transduction (p=0.000091), among which the levels of some

DEGs were increased and some were decreased. The top 20 pathways significantly related to DEGs in the spleen are summarized in **Figure 9B**. Detailed descriptions of results from the integrating analysis are provided in **Supplementary File 17**.

Integrating Analysis of Metabolomic Data and Transcriptomic Data

The transcriptomic data and metabolomic data described above were integrated to identify active gene-metabolite networks in the rat liver or spleen following 2-DG treatment. The active gene-metabolite networks were constructed by overlapping those enriched pathways that were derived from a pairwise comparison of metabolomic data or transcriptomic data. The overlaps between healthy rats (NC) and CIA, between NC and NC rats treated with 2-DG (NC+2-DG), between CIA rats and CIA rats treated with 2-DG (CIA+2-DG), and between healthy rats treated with 2-DG (NC+2-DG) and CIA rats treated with 2-DG (CIA+2-DG) were analyzed as follows. Integrating transcriptomic data and metabolomic data from healthy rat livers (L-H) and CIA rat livers (L-C) revealed significant changes in gene-metabolite networks including the prolactin signaling pathway, pentose and glucuronate interconversions, retinol metabolism, steroid biosynthesis, and terpenoid backbone biosynthesis. Integrating transcriptomic data and metabolomic data from healthy rat livers (L-H) and the livers of healthy rats treated with 2-DG (L-K) indicated significant changes in gene-metabolite networks including arachidonic acid metabolism and retinol metabolism. Integrating transcriptomic data and metabolomic data from CIA rat livers (L-C) and livers of 2-DG-treated CIA rats (L-T) revealed significant changes in gene-metabolite networks including bile secretion, chemical carcinogenesis, linoleic acid metabolism, retinol metabolism, steroid biosynthesis, steroid hormone biosynthesis and tryptophan metabolism. Integrating transcriptomic data and metabolomic data from 2-DG- treated CIA rat livers (L-T) and 2-DG-treated healthy rat livers (L-K) revealed significant changes in gene-metabolite networks including AMPK signaling pathway, arachidonic acid metabolism, glutathione metabolism, chemical carcinogenesis, drug metabolism-cytochrome P450, metabolism of xenobiotics by cytochrome P450, pentose and glucuronate interconversions, retinol metabolism and steroid hormone biosynthesis. The alternative gene expression levels and metabolism in the aforementioned gene-metabolite networks are summarized in **Supplementary File 18**. The pathway analysis of these gene-metabolite networks is shown **Supplementary File 19**. The data used to construct the gene-metabolite networks are provided in **Supplementary File 20**.

An integrating analysis was also performed with transcriptomic data and metabolomic data obtained from the rat spleen. Integrating transcriptomic data and metabolomic data from healthy rat spleens (S-H) and CIA rat spleen (S-C) indicated significant changes in gene-metabolite networks including arachidonic acid metabolism, bile secretion, chemical carcinogenesis, drug metabolism-other enzymes, drug metabolism-cytochrome P450, glycine, serine and threonine metabolism, linoleic acid metabolism, metabolism of xenobiotics by cytochrome P450, nitrogen metabolism and porphyrin and chlorophyll metabolism. Integrating transcriptomic data and metabolomic data from healthy rat spleens (S-H) and 2-DG-treated healthy rat spleens (S-K) revealed significant changes in gene-metabolite networks including arachidonic acid metabolism, arginine biosynthesis, bile secretion, chemical carcinogenesis, drug metabolism-cytochrome P450, glycine, serine and threonine metabolism, linoleic acid metabolism, phenylalanine metabolism and retinol metabolism. Integrating transcriptomic data and metabolomic data from CIA rat spleens (S-C) and 2-DG-treated CIA rat spleens (S-T) indicated significant changes in gene-metabolite networks including arachidonic acid metabolism, bile secretion, chemical carcinogenesis, cholesterol metabolism, drug metabolism-cytochrome P450, glycine, serine and threonine metabolism, linoleic acid metabolism, metabolism of xenobiotics by cytochrome P450, PPAR signaling, porphyrin and chlorophyll metabolism, primary bile acid biosynthesis, retinol metabolism and steroid hormone biosynthesis. Integrating transcriptomic data and metabolomics data from 2-DG-treated CIA rat spleens (S-T) and 2-DG-treated healthy rat spleens (S-K) revealed significant changes in gene-metabolite networks including arachidonic acid metabolism, bile secretion, chemical carcinogenesis, cholesterol metabolism, metabolism of xenobiotics by cytochrome P450, neuroactive ligand-receptor interaction, phenylalanine metabolism, PPAR signaling, primary bile acid biosynthesis, protein digestion and absorption, retinol metabolism, serotonergic synapse, steroid hormone biosynthesis, systemic lupus erythematosus and taste transduction. The alternative gene expression levels and metabolism in the aforementioned gene-metabolite networks are summarized in **Supplementary File 21**. The pathway analysis of these gene-metabolite networks is showed in **Supplementary File 22**. The data used to construct gene-metabolite networks are provided in **Supplementary File 23**.

DISCUSSION

2-DG, a glucose analog, blocks glycolysis by inhibiting hexokinase 2 (HK2) activity (23). In the present study, we used 2-DG to treat CIA rats. Treatment with 2-DG significantly alleviated joint inflammation in the rats. The treatment also decreased the levels of IL-6 and TNF-α and increased IL-10 levels in the peripheral blood of CIA rats. Moreover, the proportion of B cells was significantly decreased in the CIA rats following 2-DG treatment. This observation demonstrated that 2-DG had a therapeutic effect on CIA and suppressed immune reactions in CIA animals. The results described above also indicated an important role for glycolysis in the CIA process. 2-DG has been reported to ameliorate experimental autoimmune encephalomyelitis and to modulate Th17/Treg cell differentiation (36). This chemical directly alleviated joint inflammation by inhibiting glycolysis in RA fibroblast-like synoviocytes (RA FLSs) (6).

Our real-time PCR analysis detected significantly increased expression of HK2, G-6-PD, TPI, GAPDH, PGK1, ENO1, PKM, LDH and GSK-3β, key enzymes involved in glycolysis, in PBMCs from CIA rats. The mRNA levels of HK2, GAPDH, PGK1, ENO1, PKM and GSK-3β were considerably decreased in the CIA rats following 2-DG treatment, indicating that 2-DG suppressed the expression of genes encoding glycolysis enzymes. Our previous analysis also detected an increase in TPI, ENO1, HK2, and PGK1 expression in synovial tissues from RA and CIA rats (37, 38). Several studies have reported that high glycolysis flux in tumor cells depends on the overexpression of glycolysis-related genes, including HK2, G-6-PD, PFK, TPI, GAPDH, PGK1, ENO1, PKM, LDH and GSK-3β (39). This high glycolysis flux could result in an overproduction of pyruvate and lactate. Lactate and the lactate/pyruvate ratio (LPR) are markers for anaerobic glycolysis, and a high LPR represents a metabolic crisis (40). Lactate and pyruvate can stimulate abnormal cell proliferation, angiogenesis and pannus formation in RA synovial tissues (41). The present study also revealed increased lactate and pyruvate levels and an increased LPR in CIA rats, and the ratio was dramatically decreased following 2-DG treatment. We suggest that glycolysis is elevated in CIA by increasing expression of the key enzyme-encoding genes.

We measured small-molecule metabolites in plasma samples from CIA rats using LC-MS/MS. The level of L-acetylcarnitine was significantly decreased in the plasma of CIA rats (FC<0.5) and increased following 2-DG treatment (FC>2). Compared with healthy rats, pathways of bile secretion, cholesterol metabolism, choline metabolism in cancer, Kaposi's sarcoma-associated herpesvirus infection, linoleic acid metabolism and primary bile acid biosynthesis showed significant changes in the plasma of CIA rats. These pathways were further changed in the plasma of 2-DG-treated CIA rats compared with the untreated CIA rats. Thus, L-acetylcarnitine and these 6 metabolic pathways were involved in CIA progression through the effect of glycolysis, because their alterations were correlated with CIA and 2-DG treatment.

We also measured small molecule metabolites in rat livers using LC-MS/MS. Compared with the healthy rats, arachidonic acid metabolism, bile secretion, cholesterol metabolism, choline metabolism in cancer, glycerophospholipid metabolism, Parkinson disease and primary bile acid biosynthesis were significantly changed in livers of CIA rats. These pathways were further changed in the livers of CIA rats following 2-DG treatment. Because the activation of bile secretion, cholesterol metabolism, choline metabolism in cancer and primary bile acid biosynthesis pathways were also detected in the rat plasma, we suggested that these four metabolic pathways in the CIA rat liver represented a connection between the rat liver and peripheral blood. The integrative analysis of transcriptomic data and metabolomic data derived from the rat livers indicated that levels of (S)-5-diphosphomevalonic acid in terpenoid backbone biosynthesis were significantly decreased in the CIA rat liver (FCs <0.5) and significantly increased in CIA rat liver following 2-DG treatment (FC>2) compared with the healthy rat liver. In contrast, levels of taurochenodeoxycholic acid and (23S)-23, 25-dihydroxy-24-oxovitamin D3 23-(beta-glucuronide)/(23S)-23,25-dihydroxy-24-oxocholecalciferol 23-(beta-glucuronide) in pentose and glucuronate interconversions pathway were significantly increased in CIA rat livers (FC>2) and significantly decreased in CIA rat livers following the 2-DG treatment (FC<0.5). Moreover, the expression of genes related to the activation of terpenoid backbone biosynthesis and pentose and glucuronate interconversions pathway were specifically detected in the rat livers, and thus we suggest that the production of (S)-5-diphosphomevalonic acid in terpenoid backbone biosynthesis and taurochenodeoxycholic acid and (23S)-23, 25-dihydroxy-24-oxovitamin D3 23-(beta-glucuronide)/(23S)-23,25-dihydroxy-24-oxocholecalciferol 23-(beta-glucuronide) in pentose and glucuronate interconversions in the rat liver were occurred in the rat liver and involved in CIA under the effect of glycolysis.

We also measured small molecule metabolites in the spleens of CIA rats using LC-MS/MS. Compared with the healthy rats, pathways of arachidonic acid metabolism, choline metabolism in cancer, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, linoleic acid metabolism, retrograde endocannabinoid signaling and serotonergic synapses were significantly altered in CIA rat spleens. These pathways were further changed in the spleens of CIA rats following 2-DG treatment. Because active choline metabolism in cancer and linoleic acid metabolism were also detected in the rat plasma and arachidonic acid metabolism and choline metabolism in cancer were also detected in liver during the CIA process and after 2-DG treatment, our analysis suggested that the activation of these four metabolic pathways in the rat spleen, plasma and liver correlated with each other and corresponded to CIA progression and the effects of 2-DG treatment. Due to the specific detection of changes in the expression of the genes related to bile secretion, cholesterol metabolism and linoleic acid in the rat spleen, we suggested that bile secretion, cholesterol metabolism and linoleic acid metabolism were activated by glycolysis in the CIA rat spleen and that their products were then released into peripheral blood to subsequently affect the CIA process and were altered by 2-DG treatment. Although the activation of bile secretion and cholesterol metabolism was also detected in the rat liver, the genes regulating these two metabolic pathways did not show changes in expression in the liver. We also suggested that bile secretion and cholesterol metabolism were initially activated in the rat spleen but not the liver. The integrative analysis of transcriptomic data and metabolomic data from the rat spleen also indicated that levels of 3-methoxy-4-hydroxyphenylglycol glucuronide in bile secretion and 12(S)-leukotriene B4 in arachidonic acid metabolism were significantly decreased in the CIA rat spleen (FC<0.5), and their levels were significantly increased following 2-DG treatment (FC<2). Additionally, changes in the expression of genes regulating bile secretion and arachidonic acid metabolism were specifically detected in the rat spleen, and we suggested that the production of 3-methoxy-4-hydroxyphenylglycol glucuronide in bile secretion and 12(S)-leukotriene B4 in arachidonic acid metabolism occurred in the rat spleen and was involved in CIA progression under the effect of glycolysis.

D, L-acetylcarnitine and L-acetylcarnitine are related to L-carnitine metabolism. As described above, L-acetylcarnitine production was decreased in CIA rat plasma and increased following 2-DG treatment. L-carnitine production also declined in CIA rat spleens and was elevated following 2-DG treatment. The integrative analysis showed that L-carnitine was produced in bile secretions, and regulatory genes showed altered expression in the rat spleen rather than in the liver in response to CIA and 2-DG treatment. This measurement suggested that bile secretion is affected by glycolysis and regulates L-carnitine production in CIA rat spleens. L-Carnitine was then released to the peripheral blood to be continually metabolized to contribute to CIA and 2-DG treatment.

Studies by other researchers previously reported the important roles of some metabolites and metabolic pathways we detected in the current study. Some studies reported that taurochenodeoxycholic acid was related to inflammation, bone destruction and FLS apoptosis in CIA rats (42, 43). Carnitine is essential for energy production in muscle and is required for the transport of long-chain fatty acids and acyl coenzyme A derivatives across the inner mitochondrial membrane. Carnitine levels are decreased in RA patients (44). Organic cation/carnitine transporter 1 (OCTN1, SLC22A4) is expressed in synovial tissues of patients with RA and inflamed joints of CIA mice. The expression of OCTN1 is regulated by RUNX1, inflammatory cytokines and NF-kappa B, all of which are related to RA pathogenesis (45). Leukotriene B4 is a potent inflammatory mediator derived from arachidonic acid. Leukotriene B4 receptor is expressed in type 1 helper T cells, type 2 helper T cells, type 17 helper T cells, effector CD8(+) T cells, dendritic cells, granulocytes, eosinophils, macrophages and osteoclasts. Leukotriene B4 receptor-deficient mice show substantially reduced phenotypes in models of various inflammatory diseases, such as RA (46). Mevalonate or mevalonate phosphates are necessary for lymphocyte proliferation (47). Fibrin/fibrinogen degradation products exhibit resistance to plasmin proteolysis. Abnormal bile acid metabolism has been detected in RA (48). Metabolites of bile acid metabolism, such as chenodeoxycholic acid and deoxycholic acid, resulted in complete plasmin degradation by promoting protein unfolding or through their properties as steroid detergents (49). Treatment with anti-rheumatic drugs changes cholic acid levels (29). Linoleic acid, a member of the family of polyunsaturated fatty acids, inhibits bone resorption and increases bone formation, thereby decreasing prostaglandin-dependent bone resorption. Linoleic acid also enhances calcium absorption and may improve bone formation in animals (50). Although studies by other groups have sporadically reported the importance of some small-molecule metabolites and metabolic pathways in RA and CIA rats, those studies have not systematically investigated the mechanism of metabolic regulation and the metabolite origin in CIA and RA models from the perspective of glycolysis. So far, no direct data are available to support the involvement of liver and spleen in CIA and RA processes.

The current study does not support the hypothesis that each metabolic pathway is directly regulated by glycolysis. Activation of some metabolic pathways might result from the changes in circulating cytokine levels. Undoubtedly, the liver and spleen are not the only target organs of metabolic regulation in CIA and 2-DG-treated-CIA rats. Some studies reported variable results for metabolite levels in patients with RA. For example, Krähenbühl et al. showed that plasma carnitine level was not decreased in patients with RA, whereas urinary excretion of carnitine was lower in the patients than controls (51). Yang et al. detected an increased carnitine level in the synovial fluid of patients with RA (52).

In conclusion, the glycolytic inhibitor 2-DG exerted a therapeutic effect on CIA, reduced glycolysis and inhibited the excessive immune response in CIA rats. Moreover, metabolomics and transcriptomics, as well as their integrated analysis, revealed significant disturbances in (S)-5-diphosphomevalonic acid production in terpenoid backbone biosynthesis and taurochenodeoxycholic acid production in pentose and glucuronate interconversions in the rat liver, as well as in levels of L-carnitine and 3-methoxy-4-hydroxyphenylglycol glucuronide production in bile secretion and 12(S)-leukotriene B4 production in arachidonic acid metabolism in rat spleen in response to CIA occurrence and subsequent 2-DG treatment, which suggested the importance of these metabolites and their gene-metabolite networks in the development of CIA by regulating glycolysis. Additionally, significant alterations in bile secretion, cholesterol metabolism and linoleic acid were detected in rat plasma, spleen and liver during CIA development and 2-DG treatment, whereas the altered expression of the genes related to these 3 metabolic pathways was only detected in the rat spleen. This measurement suggested that bile secretion, cholesterol metabolism and linoleic acid metabolism were activated in the CIA rat spleen through the effect of glycolysis, and their products were then released into the peripheral blood and even the liver to contribute to CIA and the effects of 2-DG therapy. Our studies systematically investigated the effect and metabolic regulatory mechanism of glycolysis in CIA rats. This study also suggests the importance of spleen and liver metabolism in CIA and RA pathogenesis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of The Affiliated Hospital of Qingdao University.

AUTHOR CONTRIBUTIONS

All authors conceptualized the study and edited the manuscript. HW, NZ, and KF performed the experiments and data analysis.

XC and KF wrote the manuscript. All authors contributed to the article 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.2021.713799/full#supplementary-material

Supplementary File 1 | Gating strategy for identifying immune cell types. **(A)** FSC/SSC gating was used to identify lymphocytes. **(B)** The CD3 and CD8/CD4/CD45RA/CD161 bivariate analysis identified the CD8+ T, CD4+ T, CD3- CD45RA+ B fractions, respectively. FSC means forward scatter, and SSC means side scatter.

Supplementary File 2 | The primer sequences of rat genes.

Supplementary File 3 | PCA score plots for the metabolomic analysis of the rat plasma. PCA was used to visualize the similarities and differences in the plasma metabolites between healthy rats (P-H) (n=6), CIA rats (P-C) (n=6), CIA rats treated with 2-DG (P-T) (n=6), and healthy rats treated with 2-DG (P-K) (n=6).

Supplementary File 4 | DEMs in plasma between the four rat groups.

Supplementary File 5 | The pathway analysis (top 20) based on DEMs in rat plasma.

Supplementary File 6 | PCA score plots for the metabolomic analysis of the rat livers. PCA was used to visualize the similarities and differences in the liver metabolites between healthy control rats (L-H) (n=6), CIA rats (L-C) (n=6), CIA rats treated with 2-DG (L-T) (n=6), and healthy rats treated with 2-DG (L-K) (n=6).

Supplementary File 7 | DEMs in livers between the four rat groups.

Supplementary File 8 | Pathway analysis (top 20) based on DEMs in rat livers.

Supplementary File 9 | PCA score plots for the metabolomic analysis of the rat spleen. PCA was used to visualize the similarities and differences in the spleen metabolites between healthy rats (S-H) (n=6), CIA rats (S-C) (n=6), CIA rats treated with 2-DG (S-T) (n=6), and healthy rats treated with 2-DG (S-K).

Supplementary File 10 | DEMs in spleens between the four rat groups.

Supplementary File 11 | Analysis (top 20) based on DEMs in rat spleens.

Supplementary File 12 | PCA score plots for the transcriptomic analysis of the rat livers. PCA was used to visualize the similarities and differences in the liver transcript levels between healthy control rats (L-H) (n=6), CIA rats (L-C) (n=6), CIA rats treated with 2-DG (L-T) (n=6), and healthy rats treated with 2-DG (L-K) (n=6).

Supplementary File 13 | DEGs in the liver between the four rat groups.

Supplementary File 14 | The pathway analysis (top 20) based on DEGs in rat livers.

Supplementary File 15 | PCA score plots for the transcriptomic analysis of the rat spleen. PCA was used to visualize the similarities and differences in the spleen transcript levels between healthy control rats (S-H) (n=6), CIA rats (S-C) (n=6), CIA rats treated with 2-DG (S-T) (n=6), and healthy rats treated with 2-DG (S-K) (n=6).

Supplementary File 16 | DEGs in spleens between the four rat groups.

Supplementary File 17 | The pathway analysis (top 20) based on DEGs in rat spleens.

Supplementary File 18 | Summary of alternative gene-metabolite networks in the rat liver.

Supplementary File 19 | The pathway enrichment analysis integrating transcriptomic and metabolic data of the rat liver. The enrichment analyses were completed by integrating data from healthy rat (NC) and CIA rat livers **(A)**, healthy rat (NC) and 2-DG-treated NC rat livers (NC+2-DG) **(B)**, CIA rat (CIA) and 2-DG-treated CIA rat livers (CIA+2-DG) **(C)**, or 2-DG-treated healthy rat livers (NC+2-DG) and 2-DG-treated CIA rat livers (CIA+2-DG) **(D)**. Triangles represent genes, circles represent metabolites, and squares represent pathway names. Red indicates genes or metabolites with increased levels, and green indicates genes or metabolites with decreased levels.

Supplementary File 20 | Alternative gene expression and metabolites in the rat liver.

Supplementary File 21 | Summary of alternative gene-metabolite networks in rat the spleen.

Supplementary File 22 | The pathway enrichment analysis integrating transcriptomic and metabolic data of the rat spleen. The enrichment analyses were completed by integrating data from healthy rat (NC) and CIA rat spleens **(A)**, healthy rat (NC) and 2-DG-treated NC rat spleens (NC+2-DG) **(B)**, CIA rat (CIA) and 2-DG-treated CIA rat spleens (CIA+2-DG) **(C)**, or 2-DG-treated healthy rat spleens (NC+2-DG) and 2-DG-treated CIA rat spleens (CIA+2-DG) **(D)**. Triangles represent genes, circles represent metabolites, and squares represent pathway names. Red indicates genes or metabolites with increased levels, and green indicates genes or metabolites with decreased levels.

Supplementary File 23 | Alternative gene expression and metabolites in the rat spleen.

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IL-17 and CCR9$^+$α4β7$^-$ Th17 Cells Promote Salivary Gland Inflammation, Dysfunction, and Cell Death in Sjögren's Syndrome

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Previous studies have evaluated the roles of T and B cells in the pathogenesis of Sjögren's syndrome (SS); however, their relationships with age-dependent and metabolic abnormalities remain unclear. We examined the impacts of changes associated with aging or metabolic abnormalities on populations of T and B cells and SS disease severity. We detected increased populations of IL-17-producing T and B cells, which regulate inflammation, in the salivary glands of NOD/ShiLtJ mice. Inflammation-induced human submandibular gland cell death, determined based on p-MLKL and RIPK3 expression levels, was significantly increased by IL-17 treatment. Among IL-17-expressing cells in the salivary gland, peripheral blood, and spleen, the α4β7 (gut-homing integrin)-negative population was significantly increased in aged NOD/ShiLtJ mice. The α4β7-positive population markedly increased in the intestines of aged NOD/ShiLtJ mice following retinoic acid (RA) treatment. A significant increase in α4β7-negative IL-17-expressing cells in salivary glands may be involved in the onset and progression of SS. These results suggest the potential therapeutic utility of RA in SS treatment.

Keywords: Sjögren's syndrome, aging, interleukin 17, gut-homing, retinoic acid

INTRODUCTION

Sjögren's syndrome (SS), first described in 1933 (1), is among the most common long-term autoimmune disorders, and is characterized by the infiltration of lymphocytes into exocrine glands, mainly the salivary and lacrimal glands, leading to the destruction of glandular tissue, followed by glandular secretion dysfunction, and both ocular dryness (keratoconjunctivitis sicca) and oral dryness (xerostomia) (2, 3).

SS has been reported to affect approximately 3–4% of adults aged 18–75 years in the general population (4). The primary symptoms of SS are dryness of the mouth, eyes, and skin and joint and muscle pain (5). Although life expectancy is unaffected by SS, it causes great inconvenience in daily

life (6). SS can extend from disease confined to the exocrine glands to various extraglandular manifestations and the development of non-Hodgkin B cell lymphoma (7, 8). The pathogenesis of SS is mediated by complex mechanisms involving the infiltration of lymphocytes (mainly T and B cells) into target organs during a dysregulated adaptive immune response. T and B cell-containing ectopic lymphoid structures in the salivary and lacrimal glands include hyperactivated B cells associated with the presence of autoantibodies such as anti-SSA/Ro and anti-SSB/La autoantibodies (9). The activation of B cells by follicular helper T (T_{FH}) cells is crucial for their clonal selection and affinity maturation of B cells (10).

Immune cells play crucial roles in chronic diseases related to obesity or metabolic abnormality. Altered metabolism also affects the immune system and systemic autoimmune inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, and gout (11, 12). The inflammatory process plays a critical role in the development of comorbidities such as hypertension, dyslipidemia, diabetes mellitus (DM), and metabolic syndrome (13). DM is the second most common metabolic disorder in SS patients, with an incidence rate 10% higher than that in the healthy population (14). DM and hypertriglyceridemia exacerbate SS *via* vascular damage followed by inflammatory processes (15). However, the pathological mechanisms of T and B cell actions in SS and metabolic disorders remain unknown.

T helper 17 (Th17) cells are crucial players in mucosal and inflammatory diseases (16). Interferon (IFN)-γ-producing Th17 cells, which are also known as Th1-like Th17 cells, have been shown to promote chronic inflammation in various autoimmune diseases and may also contribute to the pathogenesis of SS (17). Levels of IL-17, which is a characteristic cytokine of Th17 cells, are elevated in the peripheral blood of SS patients (18). Notably, vitamin A levels are significantly lower in patients with severe SS than in those with mild SS (19). Retinoic acid (RA) is a metabolite of vitamin A that mediates human growth and development (20). It plays a major role in the pathophysiology of inflammation and has an immunosuppressive effect in autoimmune diseases (21, 22). RA is an important regulatory factor for the induction of immune tolerance in the intestine (23) and has been reported to regulate reciprocal differentiation of regulatory T (Treg) cells and Th17 cells (24). It has been shown that RA has immunosuppressive effect on Th1/Th17 cells in multiple sclerosis (MS) (25). In addition, RA can repress the expression of inflammatory chemokines and cytokines, inhibiting inflammatory responses triggered by obesity (26). It shows a potential modulatory role of RA in metabolic diseases. Vitamin A is metabolized into RA in the intestine, and RA regulates important signaling pathways in the intestinal environment (27, 28). RA-induced gut-homing molecules such as CCR9 and α4β7 show tissue tropism of T cells for migration into the small intestine (29–31). Administration of α4β7 blocking-antibodies increased the peripheral availability of Th17 cells, resulting in increased experimental autoimmune encephalomyelitis (EAE) secerity (32). However, the role of RA in the immune response in SS has not been elucidated. Thus, RA is an important regulatory factor inducing immune tolerance in the intestines of SS patients. In the present study, we hypothesized that RA promotes anti-inflammatory factors and suppresses proinflammatory factors in SS patients with spontaneous type 1 diabetes (T1D).

In a recent study, we found that SS symptoms were more severe when accompanied by metabolic abnormalities, such as DM; invasion of Th17 and T_{FH}17 cells into spleen and salivary gland tissues was dramatically increased in SS mice with DM (33). In the present study, we investigated whether Th17 cells express CCR9 and α4β7, which are a chemokine receptor and an integrin of intestinal origin, respectively. We also explored whether homing from the periphery to the intestine is helpful for improving SS symptoms in aged NOD/ShiLtJ mice following RA treatment. Finally, we compared IL-17 expression levels between young and old mice.

MATERIALS AND METHODS

Animals

We purchased 7-week-old female NOD/ShiLtJ mice from Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed under specific-pathogen-free conditions at the Catholic Research Institute of Medical Science, Catholic University of Korea, and were fed a gamma ray-sterilized diet (TD 2018S; Harlan Laboratories, Tampa, FL, USA) and autoclaved water. All animal procedures were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experiments provided by the Institutional Animal Care and Use Committee of the School of Medicine, The Catholic University of Korea (approval no.: CUMS-2020–0271–01).

Patients

Patients were diagnosed with pSS, according to the American–European Consensus Group criteria for pSS or the 2012 American College of Rheumatology criteria. Informed consent was obtained from all patients according to the principles of the Declaration of Helsinki. Serum samples were obtained from age/sex-matched healthy volunteers, who served as controls. This study was approved by the Institutional Review Board of Seoul St. Mary's Hospital (KC13ONMI0646).

Measurement of Blood Glucose and Salivary Secretion in NOD/ShiLtJ Mice

Mice were anesthetized by inhalation of isoflurane (2%), and blood glucose levels were determined using an Accu-Check Compact glucometer (Roche, Indianapolis, IN, USA). Whole saliva was collected for 7 min from the oral cavity, starting at 90 s after intraperitoneal injection of pilocarpine (100 μg/mouse; Sigma-Aldrich, St. Louis, MO, USA). Saliva flow rates were expressed as μL saliva secreted per g body weight per min (μL/g/min).

Histopathological Assessment of Inflammation

Tissues were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H & E) and Masson's trichrome. Salivary gland and small intestine inflammation were scored as previously described (34, 35). Scoring criteria for salivary gland: score 0, no infiltrates; score 1–1.5, 1–2 foci per section; 2–2.5, 3–5 foci per section; score 3, 6–10 foci per section; score 4, more than 10 foci per section. Immunohistochemistry was analyzed using Vectastain ABC kits (Vector Laboratories, Burlingame, CA, USA). Tissue sections were first incubated with primary antibodies to IL-6 and IL-17 (Abcam, Cambridge, UK) overnight at 4°C, each primary antibody was detected using a biotinylated secondary antibody, followed by incubation with streptavidin–peroxidase complex for 1h. DAB chromogen (Dako, Carpinteria, CA, USA) was added as the substrate. Double immunohistochemistry was performed using Polink DS-MR-Ms A Double IHC staining kits (GBI Labs, Mukilteo, WA, USA). The stained cells were visualized by microscopy (Olympus, Center Valley, PA, USA).

Intracellular Staining and Flow Cytometry

Cells were isolated from spleens, salivary glands, and peripheral blood of NOD/ShiLtJ mice, and stimulated with 25 ng/mL phorbol myristate acetate and 250 ng/mL ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences, San Jose, CA, USA) for 4 h. The cells were stained with surface PerCP or PB450-conjugated anti-CD4, APC-conjugated anti-CD25, PerCP-conjugated anti-C-X-C chemokine receptor type 5 (CXCR5), PE-Cy7-conjugated anti-Inducible T-cell COStimulator (ICOS), and PE-Cy7-conjugated anti-CD19 (eBioscience, San Diego, CA, USA) antibodies. Gut-homing molecules were stained with surface phycoerythrin (PE)-conjugated anti-α4β7 and allophycocyanin (APC)-conjugated anti-CCR9 antibodies (eBioscience). Surface-labeled cells were permeabilized using Cytofix/Cytoperm solution (BD Pharmingen, Franklin Lakes, NJ, USA), and then intracellular staining for IL-17 was performed using PE or Fluorescein isothiocyanate (FITC)-conjugated anti-IL-17 and PE-conjugated anti-Foxp3 (eBioscience). All samples were analyzed using the FACS Calibur (BD Pharmingen), fluorescence-activated cell sorting (FACS) instrument, and data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

Microarray Analysis

Microarray analysis was performed using the Affymetrix Mouse Gene 2.0 ST Array by (Macrogen Co., Seoul, Korea) according to the manufacturer's instructions. Differentially expressed genes from salivary gland cells were compared between 21-week-old NOD/ShiLtJ mice and NOD/ShiLtJ mice with T1D (Accession Number: GSE179654).

Western Blotting

A human submandibular gland (HSG) cell line was cultured with recombinant human TNF-α (2 ng/mL) and in the presence or absence of recombinant human IL-17 (20 or 40 ng/mL) for 48 h, and cell lysates were prepared. The protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA), and samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Amersham Pharmacia, Uppsala, Sweden). Primary antibodies to p-MLKL (Abcam), RIPK3, and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) were diluted with 0.1% skim milk in Tris-buffered saline and incubated for 20 min at room temperature. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 15 min at room temperature.

Isolation and Culture of SGSCs

Sphere-forming murine SGSCs were isolated from mouse submandibular glands using a previously described method (36). The thin fascia covering the submandibular glands were carefully removed to expose the submandibular tissues. The glands were washed three times in phosphate-buffered saline (PBS, Gibco, Grand Island, NY, USA) with 3% penicillin/streptomycin and then chopped into small tissue fragments, which were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/mL collagenase I (Gibco) under an atmosphere of 5% CO_2 for 30 min. The fragments were then centrifuged, and the pellets were resuspended in DMEM. The cells were filtered through a 40 μm cell strainer (BD Pharmingen) to produce a single-cell suspension. After centrifugation, the cells were cultured in DMEM/F12 culture medium (1:1 mixture, v/v; Gibco) supplemented with 20 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 20 ng/mL fibroblast growth factor-2 (PeproTech), 1% N-2 Supplement (Gibco), 1% insulin–transferrin–selenium (Gibco), 1 μm dexamethasone (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich). SGSCs were cultured in Matrigel (Corning Inc., Corning, NY, USA) or suspension culture for 1 week. SGSCs were treated with 10 or 20 ng/mL IL-17.

α-Amylase Assay

SGSC α-amylase activity was determined using an α-amylase assay kit (Abcam) according to the manufacturer's instructions.

Real-Time Polymerase Chain Reaction (qPCR)

mRNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. PCR amplification was performed using the Applied Biosystems StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). All reactions were performed using SensiFAST SYBR Hi-ROX (Bioline USA Inc., Taunton, MA, USA) according to the manufacturer's instructions. The following primers were used to amplify the human genes (37, 38): Aquaporin 5 (AQP5), 5′-GCC CTC TTA ATA GGC AAC CAG-3′ (sense) and 3′-GCA TTG ACG GCC

AGG TTA C-5′ (antisense); amylase1 (Amy1), 5′-AAC CCA AAT AAC AGG GAC TTT CC-3′ (sense) and 3′-GGT AGT TCT CGA TAC CTC CAC TT-5′ (antisense); keratin18 (Krt18), 5′-ACT CCG CAA GGT GGT AGA TGA-3′ (sense) and 3′-TCC ACT TCC ACA GTC AAT CCA-5′ (antisense); and Nanog, 5′-CAC AGT TTG CCT AGT TCT GAG G-3′ (sense) and 3′-GCA AGA ATA GTT CTC GGG ATG AA-5′ (antisense); β-actin, 5′-GAA ATC GTG CGT GAC ATC AAA G-3′ (sense) and 3′-TGT AGT TTC ATG GAT GCC ACA G-5′ (antisense). All expression values were normalized to β-actin expression in the same RNA sample and calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Isolation and Culture

Splenic CD4$^+$ T cells were isolated from 6-week-old NOD/ShiLtJ mice. For Th17 cell differentiation, the cells were stimulated with anti-CD3 (0.5 μg/mL), anti-CD28 (1 μg/mL), anti-IFN-γ (10 μg/mL) and anti-IL-4 (10 μg/mL) antibodies, IL-6 (20 ng/mL), and transforming growth factor-β (TGF-β) (2 ng/mL) for 3 days. Recombinant mouse IL-6 and antibodies to IFN-γ and IL-4 were purchased from R&D Systems (Minneapolis, MN, USA), and TGF-β was purchased from PeproTech. Cells were pretreated with RA (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 0.2–1 μM for 2 h and then stimulated under the required conditions.

ELISA

Serum samples were stored at –20°C until use. Total IgA levels in sera were measured using IgA ELISA quantification kits (Bethyl Lab Co., Montgomery, TX, USA). The IL-17 levels in culture supernatants were determined using sandwich ELISA (DuoSet; R&D Systems, Lille, France). Horseradish peroxidase-conjugated streptavidin (HRP-Streptavidin) was used for color development. Serum levels of RA were determined using an RA ELISA kit (MyBioSource, San Diego, CA, USA). Horseradish peroxidase-conjugated RA (HRP-RA) was used for color development. Absorbance at 450 nm (A_{450}) was measured on an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Confocal Microscopic Analysis

Small intestine tissues were stained with anti-CD4 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-IL-17 (Abcam), and anti-α4β7 (Biolegend, San Diego, CA, USA) primary antibodies at 4°C overnight, followed by secondary antibodies conjugated with FITC (Santa Cruz Biotechnology, APC (Thermo Fisher Scientific, Rockford, IL, USA) and PE (Thermo Fisher Scientific) incubated at room temperature for 2 h. Nuclei were stained with 4,'6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Confocal images were analyzed using an LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) at 200× magnification.

RA Treatment

We injected 12-week-old NOD/ShiLtJ mice intraperitoneally with 1 mg/kg RA dissolved in corn oil three times per week for 6 weeks. Control mice received intraperitoneal injection of corn oil according to the same schedule.

Statistical Analyses

Data are presented as means ± standard errors of the mean (SEM). All statistical analyses were performed using the GraphPad Prism ver. 5 software for Windows (GraphPad Software, San Diego, CA, USA). Normally distributed continuous data were analyzed using the parametric Student's *t*-test. Differences in means among groups were subjected to one-way analysis of variance (ANOVA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

Blood Glucose and IL-17 Expression Levels Are Higher in NOD/ShiLtJ Mice With Aging and T1D Development

To investigate the effects of aging and metabolic alterations on SS progression, we compared metabolic alteration and SS progression in differnet aged NOD/ShiLtJ mice. Blood glucose levels were higher in NOD/ShiLtJ mice with T1D (**Figure 1A** and **Supplementary Figure 1A**). NOD/ShiLtJ mice with T1D also showed body weight loss (**Supplementary Figure 1A**). Salivary secretion decreased in an age-dependent manner in mice with T1D (**Figure 1A** and **Supplementary Figure 1B**). Lymphocytic infiltration into the salivary glands gradually increased between 8 and 16 weeks, and fibrosis occurred at 21 weeks (**Figure 1B, C**). Focal inflammation and fibrosis of the salivary gland were exacerbated in NOD/ShiLtJ mice with T1D (**Supplementary Figures 1C, D**). The infiltration of proinflammatory cytokines, such as IL-6 and IL-17 into the salivary glands increased with age (**Figure 1D**) or metabolic abnormalities (**Supplementary Figure 1E**). Flow cytometric analysis showed that Th17 (CD4$^+$IL-17$^+$), T_{FH}17 (CD4$^+$CXCR5$^+$ICOS$^+$IL-17$^+$), and B17 (CD19$^+$IL-17$^+$) cells increased with age or T1D in salivary gland cells, peripheral blood mononuclear cells, and splenocytes (**Figure 1E** and **Supplementary Figure 1F**). These data suggest that increased proliferation of IL-17-producing cells causes inflammation in salivary gland tissue and exacerbates SS symptoms.

IL-17 Causes Inflammation-Induced Cell Death and Inhibited Self-Renewal in Salivary Gland Stem Cells (SGSCs)

Profiles of gene expression in salivary gland cells were compared between NOD/ShiLtJ mice with or without T1D (**Figure 2A**). Among 41,345 analyzed genes, 1,051 were upregulated and 695 were downregulated in NOD/ShiLtJ mice with T1D compared with those without T1D. The expression of IL-17 receptor signaling pathway-related genes such as CCL11, CCL7, Fos, Jun, and Lcn2, was elevated in NOD/ShiLtJ mice with T1D (**Figure 2B**). These data suggest that activation of the IL-17

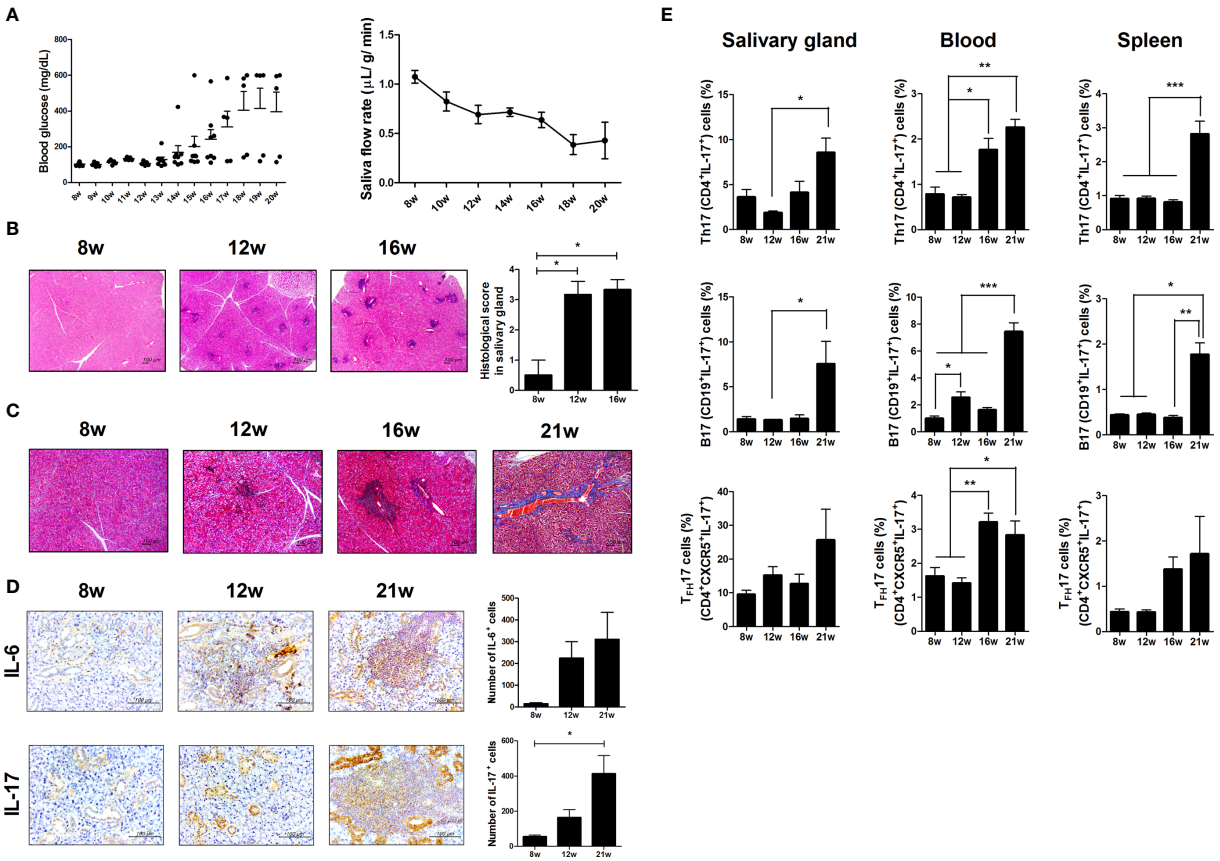

FIGURE 1 | Investigation of Sjögren's syndrome (SS) and T1D symptoms in NOD/ShiLtJ mice. **(A)** Blood glucose levels (left) and salivary flow rates (right) were measured in NOD/ShiLtJ mice at the indicated ages (N=20 at 8 wk, 15 at 9 to12 wk, 10 at 13 to16 wk, and 5 at 17 to 20 wk). **(B)** Representative hematoxylin and eosin (H&E)-stained images of salivary glands from NOD/ShiLtJ mice at the indicated ages. Scale bar = 100 μm. Bar graph shows average histological scores (N=5). **(C)** Representative Masson's trichrome-stained images of salivary glands from NOD/ShiLtJ mice at the indicated ages (N=5). Scale bar = 100 μm. **(D)** Representative IL-6- (top) and IL-17- (bottom) stained images of salivary glands of NOD/ShiLtJ mice at the indicated ages. Bar graphs show average numbers of IL-6- (top) and IL-17- (bottom) positive cells (N=5). **(E)** Bar graphs show average frequencies of $CD4^+IL\text{-}17^+$ (Th17), $CD19^+IL\text{-}17^+$ (B17), and $CD4^+CXCR5^+IL\text{-}17^+$ ($T_{FH}17$) cells among cells isolated from salivary glands (left), peripheral blood (center), and spleens (right) of NOD/ShiLtJ mice at the indicated ages (N=5). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 h and GolgiStop for the final 2 h, and then stained with the indicated antibodies for flow cytometry analysis. Values are means ± SEM from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

signaling pathway accelerates SS progression. To determine the effects of IL-17 on salivary glands, we investigated the expression levels of the inflammatory cell death-related markers p-MLKL and RIPK3 in IL-17-treated human submandibular gland (HSG) cells. The expression levels of p-MLKL and RIPK3 increased similarly to those under necroptotic conditions (TNF-α + z-VAD) after IL-17 treatment (**Figure 2C**). We also investigated the effects of IL-17 on the self-renewal and differentiation potential of SGSCs. Salisphere morphology numbers were used as an indicator of SGSC self-renewal potential. Following IL-17 treatment, the salisphere population and α-amylase activity of SGSCs were reduced (**Figure 2D**) and the transcript levels of *AQP5*, *Amy1*, *Krt18*, and *Nanog* were decreased in SGSCs (**Figure 2E**). These data suggest that IL-17 caused inflammation-induced cell death in salivary gland cells and downregulated the stem cell properties of SGSGs.

Th17 Cells Expressing Gut-Homing Molecules Decrease With Age in NOD/ShiLtJ Mice

To investigate whether Th17 cells return to the gut, the expression levels of gut-homing molecules such as CCR9 and α4β7 were examined in Th17 cells of the salivary glands, peripheral blood, and splenocytes of NOD/ShiLtJ mice. The numbers of $\alpha4\beta7^-$ Th17 cells increased in the salivary glands, peripheral blood, and splenocytes of aged NOD/ShiLtJ mice (**Figure 3A**) and intestinal damage severity increased in an age-dependent manner (**Figure 3B**). IgA levels were significantly higher in 21-week-old mice and mice with T1D (**Figure 3C**). These data suggest that intestinal damage is caused by an imbalance between intestinal tolerance and increased serum levels of IgA. RA plays a crucial role in the pathophysiology of inflammation. To investigate the role of RA in SS, we measured RA levels in NOD/ShiLtJ mice and found that RA levels gradually decreased in an age-dependent manner (**Figure 3D**).

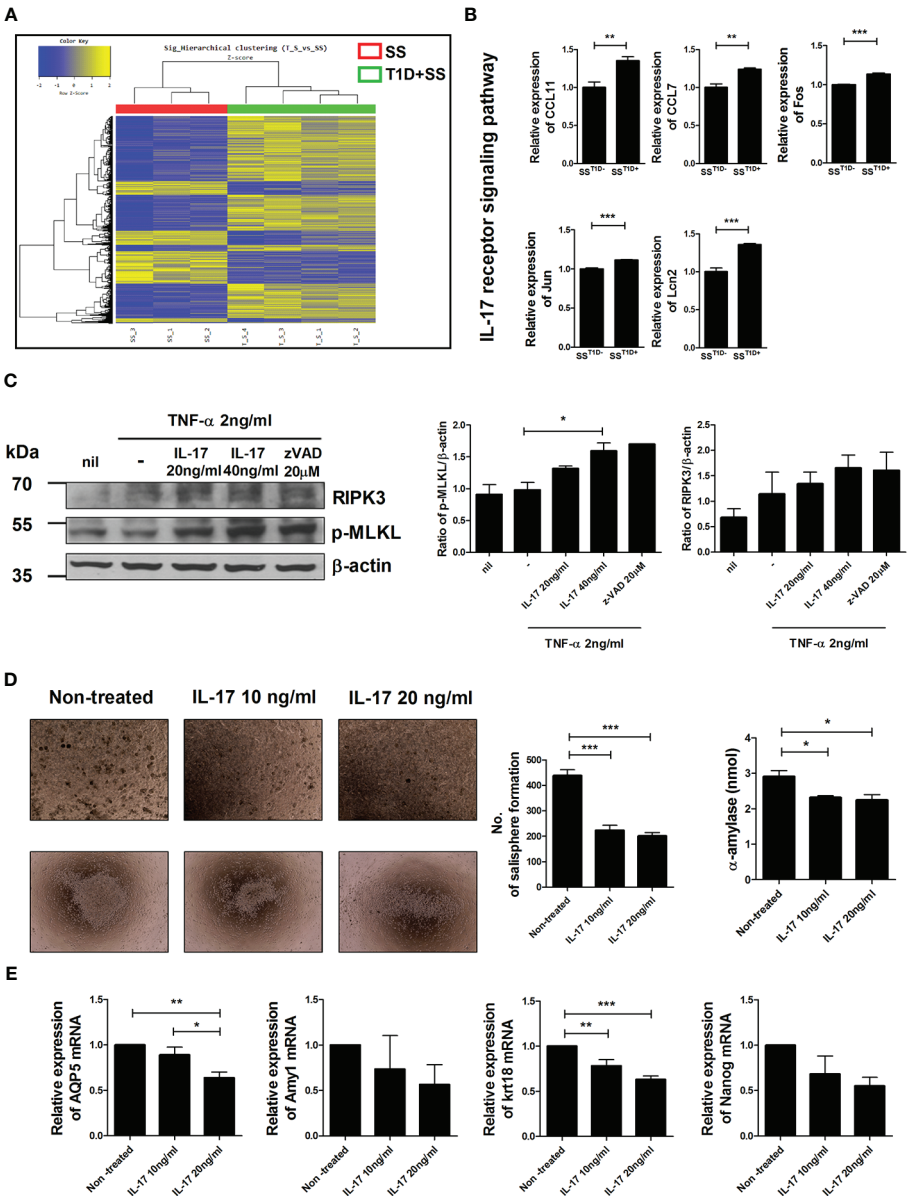

FIGURE 2 | Effects of IL-17 in salivary gland cells. **(A)** Hierarchical clustering shows gene expression in isolated salivary gland cells from NOD/ShiLtJ mice with or without T1D. Yellow and blue regions in cells indicate high and low relative expression levels, respectively. **(B)** Bar graphs show expression levels of indicated genes involved in the IL-17 receptor signaling pathway according to microarray analysis. **(C)** Human salivary gland cells were cultured with TNF-α in the absence or presence of recombinant human IL-17 or z-VAD for 48 h, and the expression levels of p-MLKL (left), RIPK3 (right), and β-actin were examined by Western blotting. Bar graphs show average expression levels of p-MLKL (left) and RIPK3 (right). **(D)** Micrographs show mouse salisphere sizes in cultured salivary gland stem cells (SGSCs) with IL-17. Original magnifications were 200× (top) and 40× (bottom). Bar graphs show average numbers of salispheres (left) and average secretion of α-amylase (right) under the indicated conditions. **(E)** Bar graphs show average transcription levels of *AQP5*, *Amy1*, *Krt18*, and *Nanog* under the indicated conditions. Values are means ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RA Regulates the Expression of IL-17 and Gut-Homing Molecules *In Vitro*

To determine the role of RA in the induction of gut-homing molecules expression in Th17 cells, we sorted CD4^{+} T cells from spleen and cultured cells under Th17 differentiation with RA for 3 days. The number of Th17 cells decreased significantly, whereas the number of Treg cells increased in the RA treatment group (**Figure 4A**). IL-17 secretion was examined in the culture supernatants using an enzyme-linked immunosorbent assay (ELISA) and found to be lower in the RA treatment groups (**Figure 4B**). Interestingly, RA increased the expression levels of gut-homing molecules CCR9 and α4β7 in Th17 cells, and

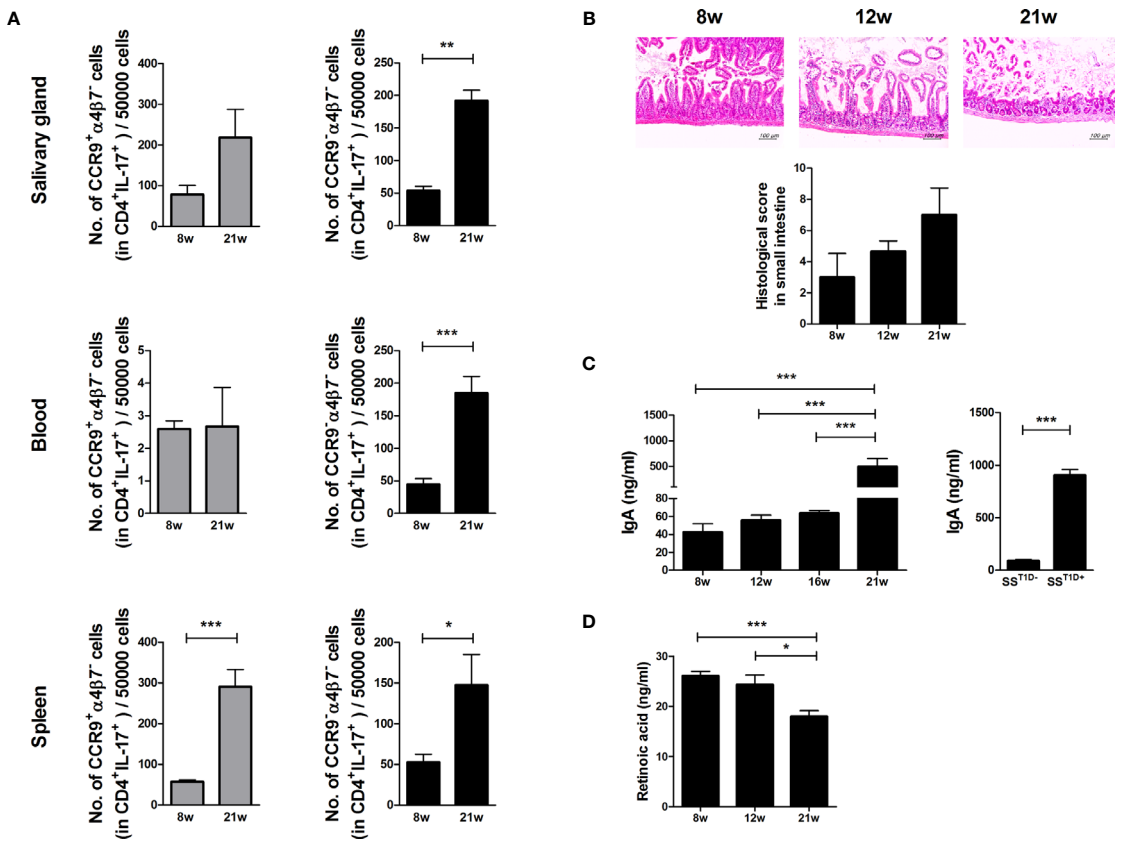

FIGURE 3 | Regulation of small intestinal inflammation and gut-homing molecules expression in Th17 cells due to retinoic acid (RA) deficiency in aged mice. **(A)** Bar graphs show average numbers of CCP9^{+}α4β7^{-} (left) and CCP9^{-}α4β7^{-} (right) cells among cells isolated from salivary glands (top), peripheral blood (middle), and spleens (bottom) of 8- and 21-week-old NOD/ShiLtJ mice. Cells were stimulated with PMA and ionomycin for 4 h and GolgiStop for the final 2 h, and then stained with the indicated antibodies for flow cytometry analysis (N=5). **(B)** Representative H&E-stained images of small intestines from NOD/ShiLtJ mice at the indicated ages. Bar graph shows average histological scores (N=5). Scale bar= 100 μm. **(C)** Bar graphs show average IgA levels in sera from NOD/ShiLtJ mice at the indicated ages (left) and SS mice with or without T1D (right) (N=5). **(D)** Bar graph shows average retinoic acid levels in sera from NOD/ShiLtJ mice at the indicated ages (N=5). Values are means ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

populations of CCR9 and α4β7 double-positive Th17 cells increased markedly in the RA treatment groups (**Figure 4C**). These data demonstrate that RA regulated the numbers of Treg cells and Th17 cells expressing gut-homing molecules.

RA Administration Regulates Blood Glucose, Salivary Secretion, and Inflammation in the Salivary Glands and Small Intestines of NOD/ShiLtJ Mice

To investigate whether RA ameliorates SS symptoms in NOD/ShiLtJ mice, RA was injected intraperitoneally three times per week at a dose of 1 mg/kg into NOD/ShiLtJ mice. RA levels were significantly higher in the sera of RA-treated mice (**Figure 5A**). Blood glucose levels were lower and the salivary flow rate was higher in the RA-treated group than in the vehicle-treated group (**Figure 5B**). RA also attenuated the infiltration of lymphocytes into the salivary glands compared with the vehicle-treated group (**Figure 5C**). The numbers of Th17 (CD4^{+}IL-17^{+}), $T_{FH}17$ (CD4^{+}CXCR5^{+}IL-17^{+}), and B17 (CD19^{+}IL-17^{+}) cells were lower in the salivary glands of RA-treated mice, whereas those of Treg (CD4^{+}CD25^{+}Foxp3^{+}) cells were higher in the salivary glands of RA-treated mice (**Figure 5D**). The numbers of Th17 (CD4^{+}IL-17^{+}) and B17 (CD19^{+}IL-17^{+}) cells were lower in the peripheral blood mononuclear cells of RA-treated mice (**Figure 5E**). IgA levels were significantly lower in the sera of RA-treated mice (**Figure 5F**) and the numbers of α4β7^{-} Th17 cells were lower in the salivary glands and spleens of RA-treated mice (**Supplementary Figure 2A**). Furthermore, small intestine damage was ameliorated by RA treatment (**Figure 5G**) and α4β7^{+} Th17 cell proliferation increased in the RA-treated group (**Figure 5H**). These data suggest that RA ameliorated SS symptoms and high blood glucose by reducing the infiltration of IL-17-producing cells into the salivary gland and increasing the numbers of gut-homing Th17 cells in the small intestine. Levels of serum IgA, a factor involved in the intestinal barrier were reduced in RA-treated mice, alleviating barrier damage.

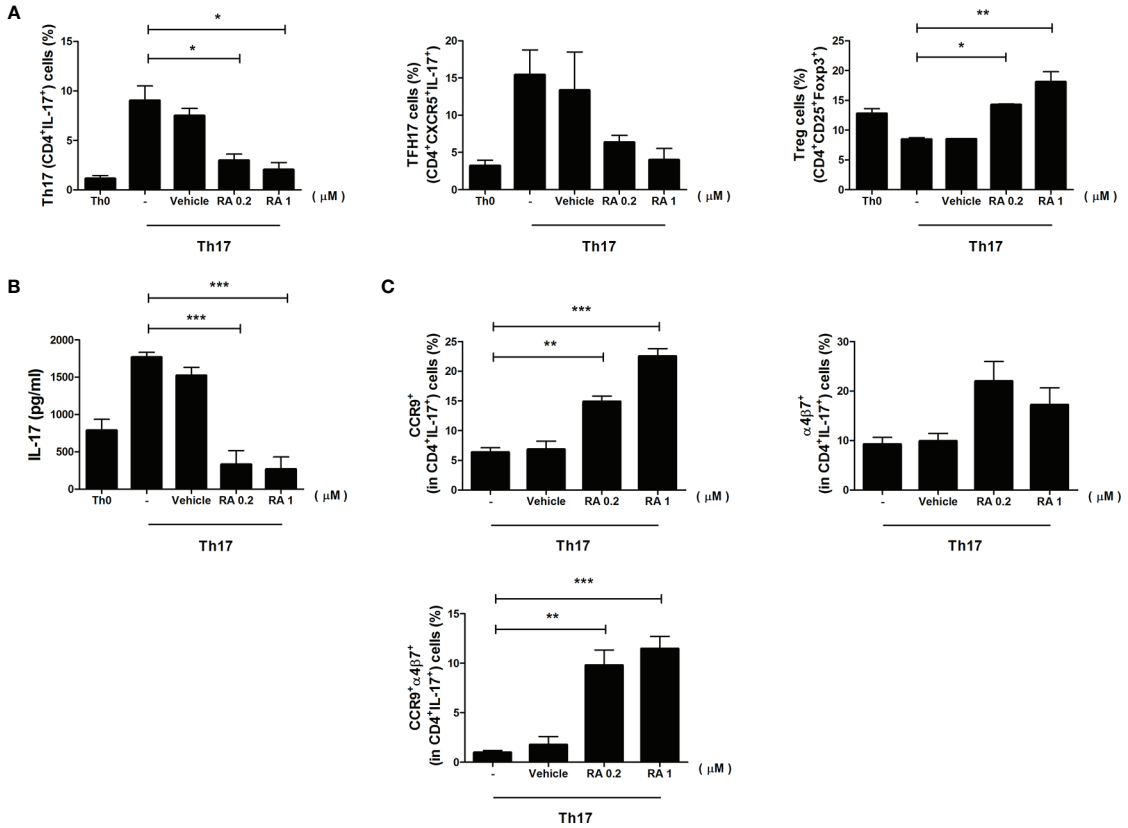

FIGURE 4 | The role of RA in the regulation of CD4+ T cells and gut-homing molecules. Splenic CD4$^+$ T cells were isolated from NOD/ShitJ mice and then cultured under Th17 differentiation conditions with or without RA for 3 days. **(A)** Bar graphs show average frequencies of CD4$^+$IL-17$^+$ (Th17), CD4$^+$CXCR5$^+$ IL-17$^+$ (T_{FH}17), and CD4$^+$CD25$^+$Foxp3$^+$ (Treg) cells under the indicated conditions. **(B)** Bar graph shows average levels of secretory IL-17, as determined in the culture supernatant by enzyme-linked immunosorbent assay (ELISA) under the indicated conditions. **(C)** Bar graphs show average frequencies of CCR9$^+$ (top and left), α4β7$^+$ (top and right), and CCR9$^+$α4β7$^+$ (bottom) in CD4$^+$IL-17$^+$ cells under the indicated conditions. Data are means ± SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

IL-17 Levels Are Higher and RA Levels Are Lower in SS Patients

Based on these findings, we examined CCR9 and IL-17 expression in human salivary gland biopsy specimens acquired from non-SS patients and patients with a histological score of either 0 or 1. Higher scores were associated with greater numbers of CCR9 and IL-17 double-positive cells in salivary gland tissues (**Figure 6A**). IL-17 production was significantly higher in sera from SS patients than in those of healthy controls (**Figure 6B**) and IgA levels were generally higher in SS patient sera (**Figure 6C**). As expected, RA concentration was lower in sera from SS patients than in healthy controls (**Figure 6D**). These findings demonstrate the therapeutic potential of RA in SS patients.

DISCUSSION

Lymphoid follicles consist of Peyer's patches and isolated lymphoid follicles beneath the intestinal epithelium. Within the follicles, a variety of immune cells including B and T cells, dendritic cells, and neutrophils regulate immune responses by presenting antigens, secreting cytokines, and producing antigen-binding antibodies (39). Another component of the immunological barrier is secretory IgA, which is present primarily on intestinal mucosal surfaces and is an important factor for interactions with commensal bacteria to protect against pathogens (40). Recent studies have shown that intestinal barrier dysfunction may be an important causative factor in autoimmune diseases (41, 42). Some studies have demonstrated a role of IL-17 in the pathogenesis of SS (43, 44). IL-17 has been reported to promote SS pathogenesis in an age-dependent manner, but this process has not been examined in detail.

Aging is characterized by a loss of cellular function, which is associated with the loss of adaptive response to stress and increasing probability of death (45). Aging leads to metabolic abnormalities, which can include DM, and biological changes in the immune system (46). Most SS patients are around 50 years of age at the time of diagnosis (47). In this study, we investigated the

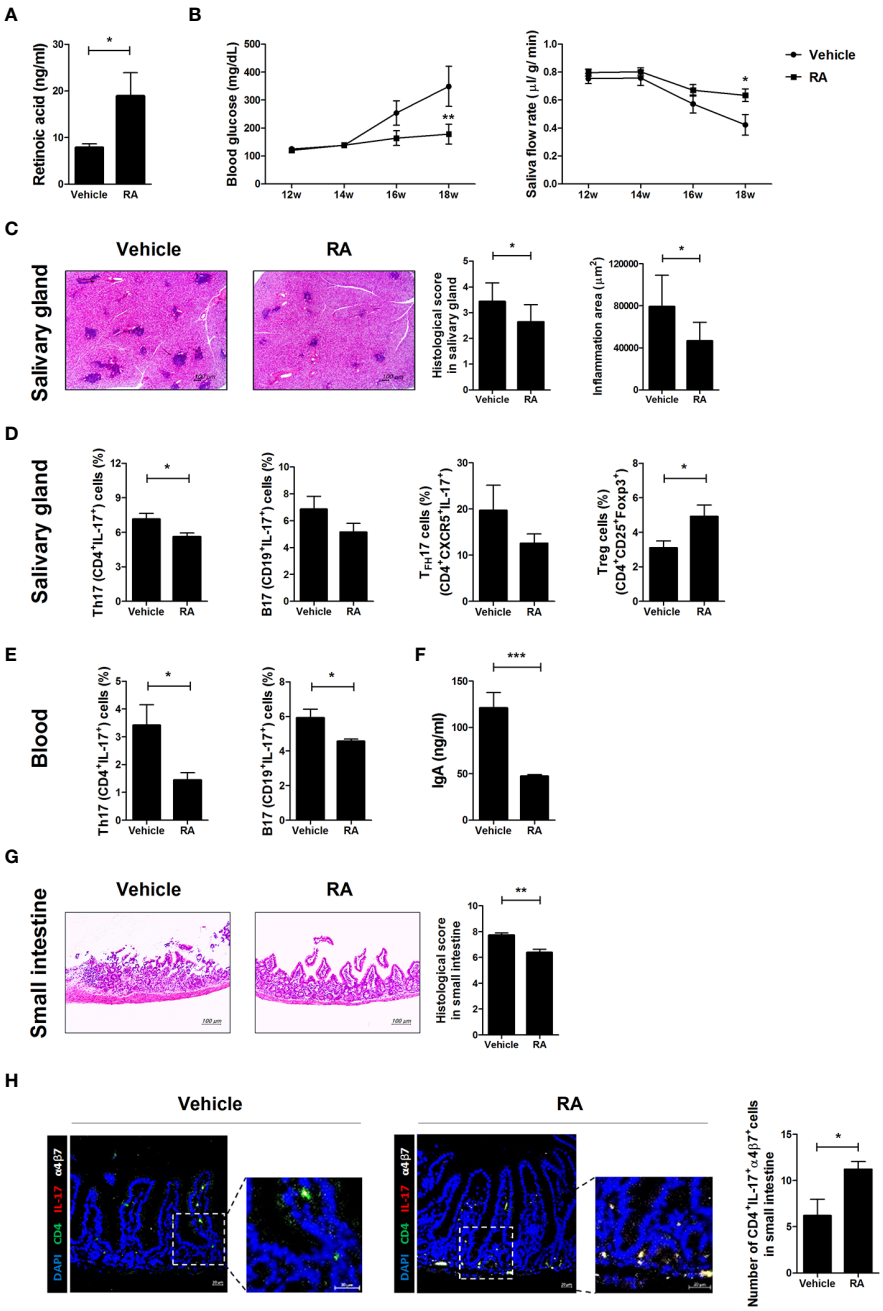

FIGURE 5 | Treatment with RA ameliorated SS symptoms in NOD/ShiLtJ mice. We injected 12-week-old NOD/ShiLtJ mice intraperitoneally with 1 mg/kg RA three times per week for 6 weeks. **(A)** Bar graph shows average RA levels in sera from vehicle- (N=5) and RA-treated mice (N=5). **(B)** Blood glucose levels (left) and salivary flow rates (right) were measured in vehicle- (N=10) and RA treated-NOD/ShiLtJ mice (N=10) at the indicated ages. **(C)** Representative H&E-stained images of salivary glands from vehicle- (left) (N=10) and RA treated- (right) NOD/ShiLtJ mice (N=10). Bar graph shows average inflammation area of salivary glands. Scale bar= 100 μm. **(D)** Bar graphs show average frequencies of Th17 ($CD4^{+}IL\text{-}17^{+}$), B17 ($CD19^{+}IL\text{-}17^{+}$), $T_{FH}17$ ($CD4^{+}CXCR5^{+}IL\text{-}17^{+}$), and Treg ($CD4^{+}CD25^{+}Foxp3^{+}$) cells among cells isolated from salivary glands of vehicle- (N=10) and RA-treated NOD/ShiLtJ mice (N=10). Cells were stimulated with PMA and ionomycin for 4 h and GolgiStop for the final 2 h, and then stained with the indicated antibodies for flow cytometry analysis. **(E)** Bar graphs show average frequencies of Th17 ($CD4^{+}IL\text{-}17^{+}$) and B17 ($CD19^{+}IL\text{-}17^{+}$) cells in peripheral blood mononuclear cells of vehicle- (N=10) and RA-treated NOD/ShiLtJ mice (N=10). Cells were stimulated with PMA and ionomycin for 4 h and GolgiStop for the final 2 h, and then stained with the indicated antibodies for flow cytometry analysis. **(F)** Bar graph shows average IgA levels in sera from vehicle- and RA-treated mice. **(G)** Representative H & E-stained images of small intestines from vehicle- (left) (N=10) and RA treated- (right) NOD/ShiLtJ mice (N=10). Bar graph shows average histological scores for small intestine tissues. Scale bar= 100 μm. **(H)** Representative immunofluorescence images for CD4 (green), IL-17 (red), α4β7 (white) and DAPI counterstaining (blue) in small intestine tissues of vehicle- (N=5) and RA-treated mice (N=5). Original magnification was 200×. Values are means ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

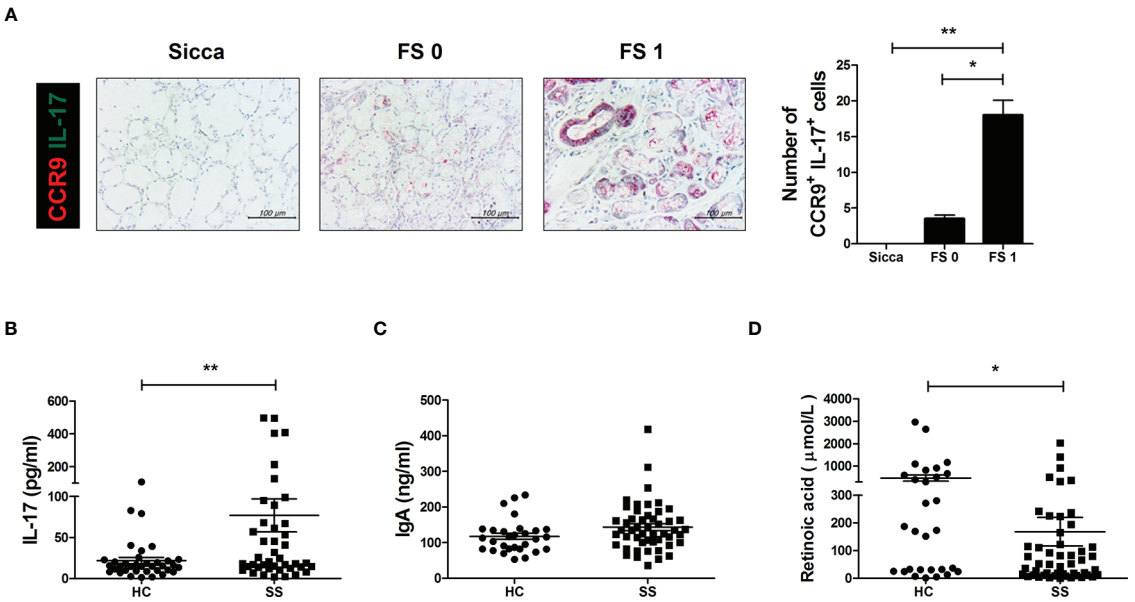

FIGURE 6 | IL-17 cells expressing gut-homing receptors in salivary glands and RA, IgA, and IL-17 levels in SS patient sera. **(A)** Representative CDR9- and IL-17-stained salivary glands from the indicated patient groups. Bar graph shows average numbers of CCR9^{+}IL-17^{+} cells in salivary gland tissues. **(B–D)** Bar graphs show average levels of IL-17 **(B)**, IgA **(C)**, and RA **(D)** in sera from healthy control (HC) and SS patients. Values are means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

effects of metabolic abnormalities related to age on immune cells in NOD/ShiLtJ mice with SS and T1D. We found that blood glucose levels increased and salivary flow rates decreased with aging. Infiltration of lymphocytes into the salivary glands and salivary gland fibrosis were more prevalent among aged mice. Furthermore, greater numbers of IL6- and IL-17-producing cells infiltrated the salivary glands, and greater numbers of Th17, $T_{FH}17$, and B17 cells were observed in the salivary glands, peripheral blood, and spleen with aging. Among aged NOD/ShiLtJ mice, we observed lower body weight and salivary flow rates and higher blood glucose levels, and infiltration of inflammatory cells into the salivary glands in mice with spontaneous T1D than in those without T1D. NOD/ShiLtJ mice with spontaneous T1D showed higher infiltration of IL-6- and IL-17-producing cells into the salivary glands than in mice without T1D. Furthermore, more Th17 and B17 cells were observed in the salivary glands, peripheral blood, and spleen. These data suggest that metabolic abnormalities associated with aging may contribute to the development of SS by increasing infiltration of IL-17-producing immune cells into the salivary glands.

The expression of IL-17 receptor signaling pathway-related genes was significantly higher in the salivary gland cells of NOD/ShiLtJ mice with spontaneous T1D. There have been several reports that TNF-α and IFN-γ induce apoptosis in HSG cells (25, 48). However, there were no reports about role of IL-17 in cell death of HSG. In this study, we investigated the role of IL-17 in cell death of HSG. The necroptotic markers (49), p-MLKL and RIPK3, were highly expressed in IL-17-treated salivary gland cells. Salisphere formation, saliva secretion, and transcript levels of a salivary functional gene (*Amy1*), acinar cell marker (*AQP5*), ductal cell marker (*Krt18*), and stem cell marker (*Nanog*) (37) were lower in IL-17-treated SGSCs. These data suggest that increased IL-17 expression induced by metabolic abnormalities may cause cell death and inhibit tissue regeneration in the salivary glands of SS patients.

RA is a critical factor for maintaining intestinal homeostasis by directly modulating effector cytokines (22). Integrin α4 and integrin β7 form gut-homing integrin α4β7 in T cells. Recent studies have shown that the regulation of homing molecules during T cell activation affects T cell function in a variety of positive and negative signals (50, 51). Suppression or upregulation of RA causes differential expression of gut-homing molecules (CCR9 and α4β7) in Th17 cells (52). Our results, based on *in vitro* and *in vivo* experiments and data from SS patients, suggest that decreased RA levels exacerbate SS by retaining IL-17-producing immune cells in the peripheral blood, salivary glands, and spleen instead of their migration to the gut.

In conclusion, the inflammation of salivary gland tissue in NOD/ShiLtJ mice, a model of spontaneous SS development, was increased by the infiltration of Th17, Tfh17, and IL-17-expressing B cells into the salivary glands, resulting in salivary gland cell apoptosis and tissue dysfunction. Since our results demonstrated that RA levels were lower in NOD/ShiLtJ mice, we used RA to treat this SS mouse model to improve SS symptoms. The infiltration of IL-17^{+} cells and the number of α4β7^{-} Th17 cells were decreased in RA-treated mice. We also showed that RA ameliorated imbalance in the Th17/Treg cell population in SS and that IL-17 levels were lower, and Foxp3 levels higher, in RA-treated SS mice. Intestinal damage occurred in NOD/ShiLtJ mice, but was alleviated by RA treatment. RA not only recruits Th17

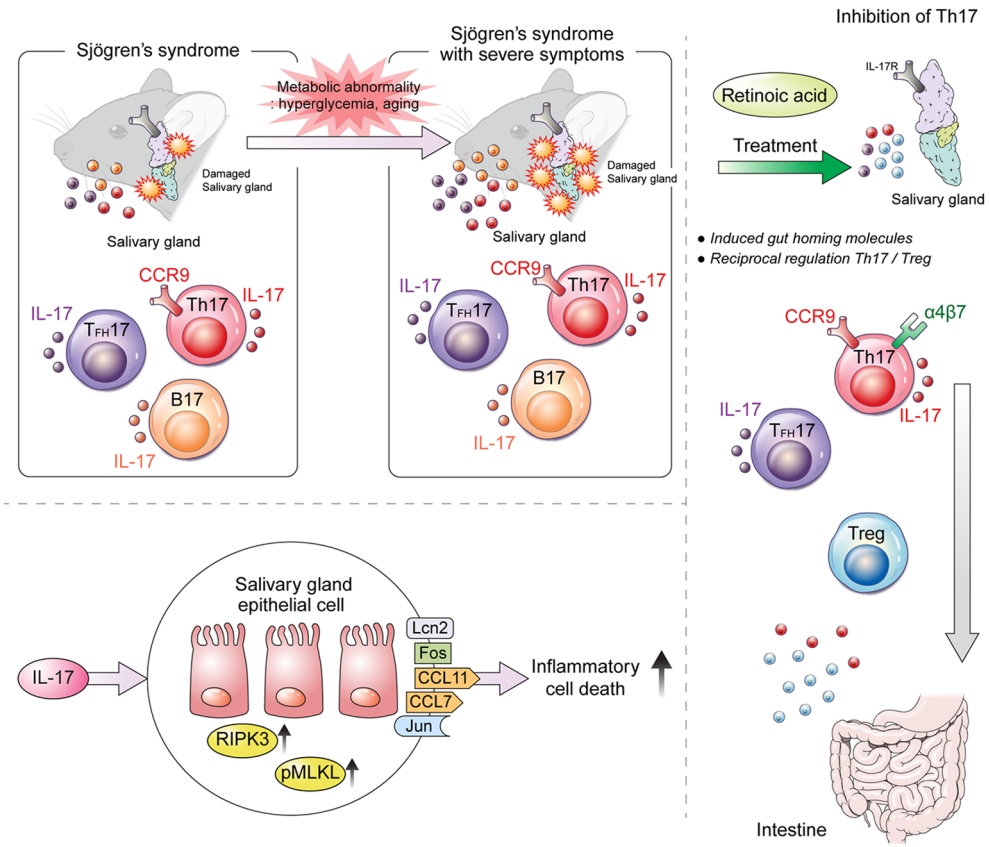

FIGURE 7 | Graphical summary of the findings of the present study. Our study demonstrates that IL-17 and CCR9^{+}α4β7^{-} Th17 cells promote salivary gland inflammation and dysfunction in SS. RA ameliorates aging- and metabolic-abnormality-induced salivary gland inflammation by inducing gut-homing CCR9^{+}α4β7^{+} Th17 and Treg cells in SS.

into the gut but also seems to relieve barrier damage by increasing the Treg cell population.

The results of the present study suggest that RA treatment may be helpful for relieving and recovering from SS symptoms. Metabolic abnormalities associated with aging exacerbate SS by increasing both the number of IL-17-producing immune cells and their infiltration into salivary glands due to reduced RA levels (**Figure 7**). Our findings suggest that RA may be a key regulator of the development of IL-17-mediated SS.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Seoul St. Mary's Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Research Ethics Committee of the Catholic University of Korea.

AUTHOR CONTRIBUTIONS

S-HH and M-LC: conception and design of study. S-HH, SY, JL, JC, and KL: acquisition data. S-HH, JM, and J-SP: analysis and interpretation of data. S-HH, JM, JW S-KK, S-HP, and M-LC: drafting the article. All authors contributed to the article 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.2021.721453/full#supplementary-material

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Relationship Between Inflammation and Metabolism in Patients With Newly Presenting Rheumatoid Arthritis

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Background: Systemic inflammation in rheumatoid arthritis (RA) is associated with metabolic changes. We used nuclear magnetic resonance (NMR) spectroscopy–based metabolomics to assess the relationship between an objective measure of systemic inflammation [C-reactive protein (CRP)] and both the serum and urinary metabolome in patients with newly presenting RA.

Methods: Serum (n=126) and urine (n=83) samples were collected at initial presentation from disease modifying anti-rheumatic drug naïve RA patients for metabolomic profile assessment using 1-dimensional ^{1}H-NMR spectroscopy. Metabolomics data were analysed using partial least square regression (PLS-R) and orthogonal projections to latent structure discriminant analysis (OPLS-DA) with cross validation.

Results: Using PLS-R analysis, a relationship between the level of inflammation, as assessed by CRP, and the serum (p=0.001) and urinary (p<0.001) metabolome was detectable. Likewise, following categorisation of CRP into tertiles, patients in the lowest CRP tertile and the highest CRP tertile were statistically discriminated using OPLS-DA analysis of both serum (p=0.033) and urinary (p<0.001) metabolome. The most highly weighted metabolites for these models included glucose, amino acids, lactate, and citrate. These findings suggest increased glycolysis, perturbation in the citrate cycle, oxidative stress, protein catabolism and increased urea cycle activity are key characteristics of newly presenting RA patients with elevated CRP.

Conclusions: This study consolidates our understanding of a previously identified relationship between serum metabolite profile and inflammation and provides novel evidence that there is a relationship between urinary metabolite profile and inflammation as measured by CRP. Identification of these metabolic perturbations provides insights into the pathogenesis of RA and may help in the identification of therapeutic targets.

Keywords: inflammation, metabolism, rheumatoid arthritis, glycolysis, citrate cycle, urea cycle, oxidative stress, cachexia

INTRODUCTION

RA is a systemic inflammatory disease characterised by synovial inflammation and bone damage. Early RA appears to be a unique entity, with evidence that it is phenotypically distinct from established RA (1). Rapid initiation and escalation of treatment in early RA is associated with improved outcomes (2–4) and as such, the early stages of RA represent a unique "window of opportunity" (5). Systemic inflammation associated with early RA is responsible for significant extra-articular morbidity with an increased prevalence of stroke, heart failure (6), chronic obstructive pulmonary disease, asthma and interstitial lung disease (7) amongst early RA patients compared to matched controls. Furthermore, there is evidence of systemic changes in metabolism several years prior to onset of overt disease, which may be driven by early immune processes (8). Increased understanding of the relationship between inflammation and metabolism in early RA is thus important, in particular as therapies which target metabolic pathways emerge (9–11).

In RA, there is evidence of systemic immune activation and immune cell infiltration into synovium (12, 13). Synovial fibroblasts take on an aggressive inflammatory, matrix regulatory, and invasive phenotype. These fibroblasts, together with increased chondrocyte catabolism and synovial osteoclastogenesis, promote articular destruction (14, 15). In addition, inadequate lymphangiogenesis, which limits cell egress, together with local fibroblast activation, promotes the establishment of synovial inflammation. Immune cells involved in this inflammation are metabolically active (16). The resulting metabolic perturbations can lead to downstream effects (17).

Several metabolomics analyses of the serum and urine of patients with rheumatic diseases have been performed to date. A study of early inflammatory arthritis patients with a symptom duration of ≤3 months showed a relationship between CRP and the serum metabolome as assessed using NMR metabolomics with lactate and lipids as discriminators of inflammation (18). PLS-R models showed a relationship between the serum metabolome and CRP in two separate groups of early arthritis patients (r^2 = 0.671, p<0.001 and r^2 = 0.4157, p<0.001). Metabolomics has also been used to assess the relationship between low-grade inflammation and both the serum and the urinary metabolome in healthy individuals (19). Inflammation as measured by hsCRP was associated with multiple changes in metabolomes associated with oxidative stress and the urea cycle (19). Although urinary metabolomics has been shown to distinguish elevated disease activity in those with rheumatic diseases (20) and to predict responses to anti-TNF therapy in RA patients (21), urinary metabolomics has not been used to study the effect of inflammation as measured by CRP on metabolism in early RA before. However, findings from the serum metabolome are typically applicable to the urinary metabolome for instance the prediction of response to anti-TNF therapy in RA patients (21–23) and distinguishing healthy individuals with elevated inflammatory markers (19). Nevertheless, the relationship between the urinary metabolome and inflammation in patients with RA remains an understudied area.

We hypothesized that a metabolomics approach using serum, filtered to remove confounding high molecular weight species, and urine could identify a relationship between metabolic dysfunction and inflammation in patients with newly presenting RA.

MATERIAL AND METHODS

Patients

Patients were recruited from the Birmingham Early Arthritis Cohort (BEACON). BEACON includes patients presenting with DMARD naive inflammatory arthritis; details have been reported previously (16). This study focuses on patients with RA [classified using established criteria (24, 25)] recruited between January 2013 and September 2015. Patients with an unclassified arthritis (UA), recruited to BEACON during the same time period, were used as a non-RA inflammatory arthritis control group. All samples were defrosted and analysed by NMR spectroscopy at the same time, minimising magnetic field drift. Collectively, these considerations allow mitigation from some confounding factors while allowing assessment between systemic inflammation and metabolome in RA and a control inflammatory arthritis population.

The study was approved by the Black Country Research Ethics Committee and all patients gave written informed consent. The study was conducted over two sites: City Hospital, Sandwell and West Birmingham NHS Trust, Birmingham and Queen Elizabeth Hospital Birmingham, University Hospitals Birmingham NHS Foundation Trust. The following data were collected at baseline: age, gender, symptom duration, current medications, tender (26) and swollen (27) joint counts. Blood and urine were collected at presentation and processed as described below.

Serum Samples

Samples of sera (RA; n=126 and UA; n=41) and urine (RA; n=83 and UA; n=25) were collected from patients at baseline, prior to initiating DMARD therapy. Blood was collected at presentation in vacutainer tubes containing clotting accelerator (Greiner Bio-one) and subsequently centrifuged at 600g for 10 minutes. Serum was removed and stored at minus 80°C until analysis. Serum samples were thawed at 4°C and centrifuged at 15,000g at 4°C for 5 minutes. To remove proteins, 200µl from the middle of the sample was placed into a Nanosep® Omega 3000 Da (Pall Lifesciences, UK) molecular weight cut-off (MWCO) and centrifuged at 10,000g at 4°C for 15 minutes. Immediately prior to use, to remove the preservative glycerol, the filters were washed 6 times in distilled water at 37°C by centrifugation at 3000g for 15 minutes (28). The resulting filtrate was diluted in a 1 + 3 ratio with NMR buffer containing 1.6mM Difluorotrimethylsilylmethylphosphonic acid (DFTMP, Manchester Organics, Manchester, UK), 400mM phosphate, 40% D_2O, 0.4% azide and 2mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d6, all from Merck, Southampton, UK). An aliquot (60ul) was removed to glass champagne vials (Cole-Parmer, Saint Neots, UK) and stored at -80°C until analysis.

Urine Samples

Mid-stream urine samples were collected from patients at presentation to the clinic, centrifuged at 600g for 10 minutes and stored at minus 80°C. Samples were prepared using a standard protocol that has been used in other studies of urine (29). After thawing, urine samples (1ml) at 4°C were centrifuged at 15,000g for 5 minutes. A cleared sample (0.5ml) was mixed at 1:3 ratio with the 4x NMR buffer as for the serum above. The pH was adjusted (twice over a period of 30minutes) to pH 7.0. The samples were centrifuged at 15000g for 5 minutes and a sample (60ul) was removed to glass champagne vial and frozen at -80°C prior to NMR spectroscopy.

NMR Spectroscopy

Samples were defrosted and transferred to 1.7mm NMR tubes (Bruker Biospin, Coventry, UK) using an Anachem Autosampler. After capping the tubes and wiping with dust-free paper, one-dimensional ^{1}H spectra were acquired at 300K using a standard 1D-^{1}H-Nuclear Overhauser Effect spectroscopy (NOESY) pulse sequence with water saturation using pre-sat in a Bruker AVANCE II 600 MHz NMR spectrometer (Bruker Corp., USA) equipped with a 1.7 mm cryoprobe. Spectral width was set to 12 ppm and the scans were repeated 128 times. Samples were loaded into racks and held at 6°C in the SampleJet sample handing device until processed. Two-dimensional 1H J-resolved (JRES) spectra were also acquired to aid metabolite identification (30).

Spectra were read and processed with Metabolab software (Version 2018.x; Birmingham, UK) (31). Each spectrum was phased according to the DSS-d6 peak, then aligned and corrected for baseline offset. The spectra were truncated to a range of 0.6 - 8.6 ppm (parts per million) and the water peak removed. Spectra were divided into chemical shift "bins" of 0.005 ppm and the spectral area of each bin integrated then scaled with probabilistic quotient normalization (PQN) to account for differences in sample dilutions (24) and normalised with a generalised log transform ($\lambda = 1e^{-08}$) to equalize the weightings of smaller and larger peaks. Data were then compiled into a matrix where each row represented an individual sample before statistical analysis. Binning of spectra was performed as opposed to individual metabolite identification and quantification. This approach allows multivariate analysis on the entirety of the metabolomic data as opposed to on only the limited number of metabolites that can be definitively identified from the NMR spectra (25, 32).

Statistical Analyses

Principal Components Analysis

The data bins from groups of spectra were mean centred and then assessed by PCA using Soft Independent Modeling of Class Analogy (SIMCA) version 14 (Umetrics) (33). PCA is an unsupervised multivariate mathematical analysis that extracts components in order of decreasing variance from multivariate datasets, enabling an understanding into the causes and effects behind these relationships.

Supervised Multivariate Analysis: Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) & Partial Least Square Regression (PLS-R) Analysis

Whilst PCA describes the relationship between possibly correlated variables in a single large multivariate matrix (matrix X) of data using PCs, partial least square is a multivariate analysis which attempts to describe the relationship between two different matrices of data using a latent variable (LV) approach to modelling the covariance in these two spaces. OPLS-DA was used to perform supervised clustering of samples using SIMCA version 14 (Umetrics) (33, 34). The OPLS-DA models were cross-validated using Venetian blinds (34), a method which reassigns randomly selected blocks of data to the OPLS-DA model to determine the accuracy of the model in correctly assigning class membership. The application of such methods to clinical studies is well established and guards against over fitting the model (35).

A PLS-R finds a linear regression model by projecting a predicted variable, which is created following application of an algorithm using latent variables to describe the covariance between the X and Y matrix, and the continuous variable in the Y matrix. Data bins were also subjected to PLS-R using the PLS Toolbox (version 5.8) (Eigenvector Research) in MatLab (release 2018b; MathWorks). This method identifies which metabolites can predict a continuous variable. This analysis yields an r^2, a measure of the cross-validated goodness-of-fit of the linear regression, while permutation testing performed by multiple analyses using random data subsets, was used to assess the significance of this prediction. Models can be further optimised using a forward selection approach, which identifies a proportion of the metabolome that correlates with the continuous variable.

Identification of Metabolites & Pathway Analysis

Bins of interest, which may represent biomarkers, were identified for each statistically significant analysis. Weightings for each bin in PLS-R analysis models were assigned using regression coefficients Potential biomarkers were identified using +/- 2 standard deviations of the mean regression coefficient of the entire dataset (36). NMR spectra were annotated using Chenomx NMR suite (Chenomx, professional version 8.5) (37) programme. The Human Metabolome Database version 4.0 (38) and published lists of metabolites detectable by NMR spectroscopy of serum (25) and urine (32) were also used for labelling spectra.

Functional interpretation of the biomarkers implicated by the models was undertaken using MetaboAnalyst version 4 (39). A combination of both enrichment analysis and pathway analysis was used. Both analyses rely upon the identification of a metabolite as a biomarker, however they do not account for the direction of change of the metabolite. The enrichment analysis is an "over-representation" analysis. This tests whether a group of compounds involved in a pathway is enriched compared by random hits using a reference metabolome (40), thus are represented more than would be expected by chance. A hypergeometric test is used to generate a p value, which represents the probability of observing at least a specific number of metabolites from a certain metabolite set in a compound list. Pathway analysis incorporates both over representation analysis as discussed above and pathway

topological analysis to determine which pathways are more likely to be involved by considering the pathway structure.

RESULTS

The baseline characteristics of patients included in the serum and urinary metabolomics analyses are shown in **Table 1**. PCA was used to generate an unbiased overview to investigate differences in metabolite profiles. OPLS-DA and PLS-R were used to perform supervised multivariate analyses. For both the PCA and OPLS-DA, a comparison was made between those individuals with low and high CRP values comparing patients in the lowest and highest CRP tertile groups. PLS-R analyses included all patients.

Relationship Between Serum Metabolite Profile and CRP

PCA showed no separation between patients in the lowest CRP tertile and the highest CRP tertile groups (**Figure 1A**). However, a supervised analysis using OPLS-DA showed a strong separation with 1 + 1+0 LV (**Figure 1B**; p=0.033). To investigate this further, the relationship between the serum metabolite profile and CRP was assessed using the regression analysis PLS-R. Using all 590 bins, a PLS-R analysis of metabolite data (**Figure 1C**) showed a statistically significant relationship between the serum metabolite profile and CRP (r^2 = 0.29, 7 LV, p<0.001). Forward selection was carried out to produce a model containing the top 36 NMR bins (**Figure 1D**). This enhanced the relationship between metabolite profile and CRP (r^2 = 0.551, 6 LV, p=0.001) compared to the original PLS-R. Spectral fitting to identify metabolites was performed using Chenomx (see **Figure 2**) and a published list of metabolites (25, 32). Potential metabolites identified by this model are shown in **Table 2**. Univariate analysis did not reveal a relationship between the concentrations of the metabolites identified in the bins with the three greatest regression coefficients (see **Table 2**) and CRP, except for citrate (R_s=-0.302, p<0.001).

Functional metabolomics analysis based on the biomarkers identified by PLSR analysis showed alanine, aspartate and glutamate metabolism, arginine and proline metabolism, pyruvate metabolism and glycine, serine and threonine metabolism are altered in the serum of RA patients with elevated CRP (**Figure 3**). Over-representation analysis (**Figure 4**) in pathway-associated metabolite sets indicated that amongst the multiple pathways which were implicated, methylhistidine metabolism, the urea cycle and the glucose alanine cycle were the most overrepresented in the serum of patients with elevated CRP. These results suggested that perturbed energy and amino acid metabolism in the serum are key characteristics of RA patients with elevated CRP.

Relationship Between Urinary Metabolite Profile and CRP

PCA was used to generate an unbiased overview to identify differences between patients in the lowest CRP tertile and the highest CRP tertile (**Figure 5A**). There was no discernible separation between these groups. However, a supervised analysis using OPLS-DA (**Figure 5B**) showed a strong separation with

TABLE 1 | Baseline characteristics of serum & urine metabolomics analysis of RA patients.

	RA patients included in sera metabolomics analysis (n = 126)	RA patients included in urinary metabolomics analysis (n = 83)	UA patients included in sera metabolomics analysis (n = 41)	UA patients included in urinary metabolomics analysis (n = 25)
Age, median (IQR) years	55 (47-62)	48 (55-60)	51 (42-60)	51 (38.5-60)
Missing (%)	0	0	0	0
Sex, no. (%) females	88 (69.8)	55 (66.3)	26 (63.4)	16 (64)
Missing (%)	0	0	0	0
Symptom duration, median (IQR) weeks	20.5 (11-47)	24 (12-45)	21 (12-42)	28 (14.5-50.5)
Missing (%)	0	0	0	0
CRP, median (IQR) mg/L	8 (3-16.3)	8 (3-16)	6 (3-21)	5 (3-11.5)
Missing (%)	0	0	0	0
DAS28CRP (IQR)	5 (4.3-5.8)	4.9 (4.2-5.7)	3.4 (2.7-4.5)	3.3 (2.7-4.4)
Missing (%)	2 (1.6)	2 (2.4)	0	0
RF positive, no. (%)	76 (60.3)	51 (61.4)	7 (17.1)	7 (28)
Missing (%)	0	0	0	0
ACPA positive, no. (%)	66 (52.4)	45 (54.2)	1 (2.4)	0 (0)
Missing (%)	0	0	0	0
NSAIDs, no. (%)	49 (38.9)	41 (49.4)	22 (53.7)	11 (44)
Missing (%)	0	0	0	0
Steroids, no. (%)	7 (5.6)	18 (21.7)	13 (31.7)	1 (4)
At baseline	3 (2.4)	3 (3.6)	2 (4.8)	0 (0)
Within last 3 months	4 (3.2)	15 (18.1)	11 (26.9)	1 (4)
Missing (%)	0	0	0	0

RA, Rheumatoid arthritis; IQR, Interquartile range; CRP, C reactive protein; DAS28CRP, Disease activity score 28 using C reactive protein; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody.

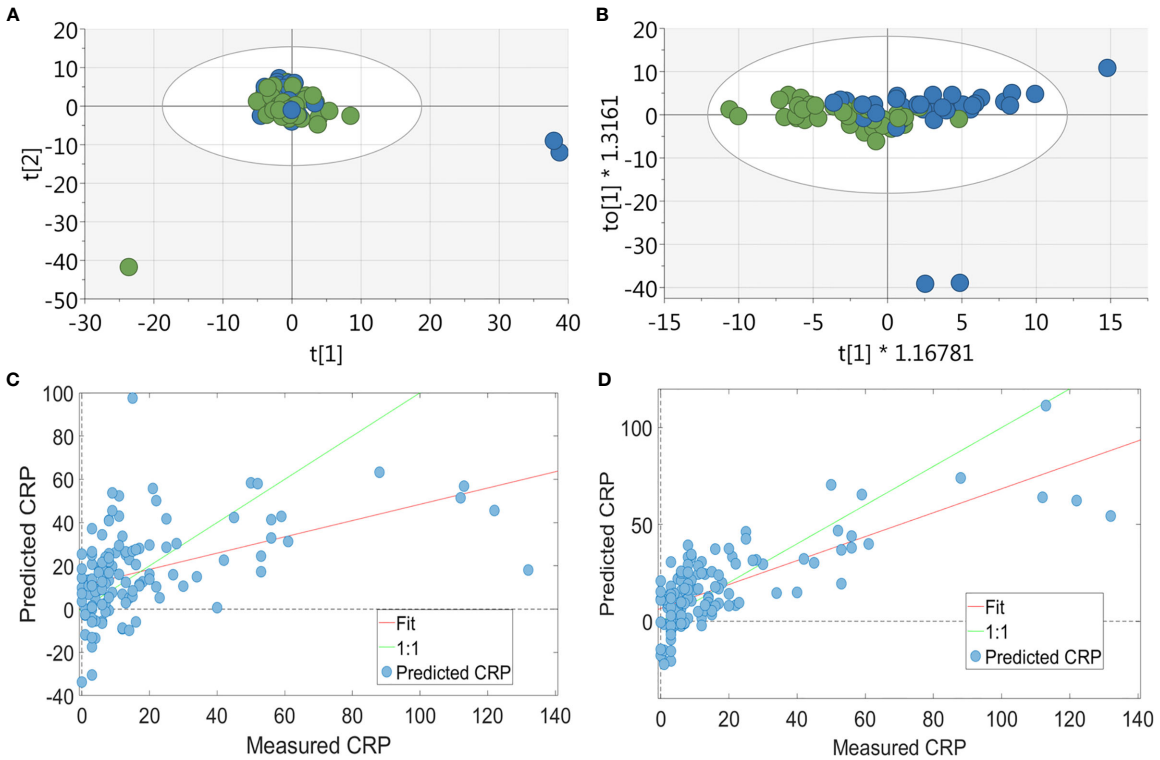

FIGURE 1 | Multivariate analysis of RA patients' serum metabolite profile. For the PCA & OPLSDA, patients were split into tertiles according to CRP values, with data shown for the highest and lowest tertile: **(A)** PCA plot of metabolic data derived from RA patients' (n = 84) sera (green = CRP <5 and blue = CRP>13; 19 PC, r^2 = 0.673) showing no separation between the two groups. **(B)** OPLS-DA plot of metabolic data derived from RA patients' (n = 84) sera (green = CRP <5 and blue = CRP>13; 1 + 1+0 LV, p value= 0.033) showing a strong separation between the two groups. PLS-R analysis showed a relationship between serum metabolite profile and CRP. Using the full 590 serum metabolite binned data (n = 126) **(C)** there was a correlation between metabolite data and CRP on PLS-R analysis (r^2 = 0.29, 7 LV, p < 0.001). Using forward selection, 36 bins were identified which correlated with inflammation and a subsequent PLS-R analysis using these bins **(D)** showed a stronger correlation between serum metabolite profile and CRP (r^2 = 0.551, 6 LV, p = 0.001).

1 + 0+0 LV (p value<0.001). Using all 900 bins, PLS-R analysis (**Figure 5C**) showed a correlation between urinary metabolite profile and serum CRP (r^2 = 0.095, 1 LV, p=0.008). Using a forward selection approach, a PLS-R using 144 urinary NMR bins (**Figure 5D**) produced the most optimal correlation with CRP (r^2 = 0.429, 3 LV, p<0.001). Metabolites identified by this model are shown in **Table 3**. Univariate analysis assessing the relationships between CRP and the concentrations of the metabolites identified in the bins with the three greatest regression coefficients (see **Table 3**) showed a relationship between CRP and 3-aminoisobutyrate (R_s=0.504, p=0.001), alanine (R_s=0.302, p=0.004), cystathionine (R_s=0.579, p<0.001), phenylalanine (R_s=0.593, p<0.001), cysteine (R_s=0.442, p=0.003), and 3-methylhistidine (R_s=0.383, p<0.001) respectively.

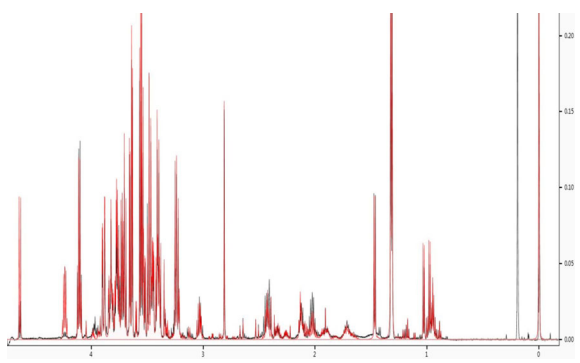

FIGURE 2 | Spectral fitting to identify metabolites. NMR spectra were annotated using Chenomx NMR suite (Chenomx, professional version 8.5).

Figure 6 shows that alanine, aspartate and glutamate metabolism and beta-alanine metabolism were the most impacted metabolic pathways. **Figure 7** shows the enrichment analysis using metabolites identified by PLS-R analysis of RA patients' urinary metabolite data and CRP. Beta-alanine metabolism, glycine and serine metabolism, homocysteine degradation and methylhistidine metabolism were the only overrepresented metabolic pathways that reached statistical significance.

Further analyses assessed the correlations between metabolic data derived from RA serum/urine and ESR (**Supplementary Figure 1**), autoantibody status (**Supplementary Figures 2A, B**) and symptom duration (**Supplementary Figures 2C, D**). PLS-R

TABLE 2 | Metabolites responsible for the relationship seen in PLS-R analysis between CRP and serum metabolite profile.

Order	Metabolite	Chemical shift of peak (ppm)	Regression coefficient
1	Citrate	2.534, 2.5048, 2.6511, 2.5106, 2.6745, 2.5282, 2.6394, 2.6453, 2.6886, 2.6687	↓↓↓↓↓↓↓↓↓
1	Glutamine	2.534, 2.5048, 2.5106, 2.5282, 2.3643, 2.3701, 2.0846, 3.6813	↓↓↓↑↑↑↓
2	Lactate	1.2585, 1.2349, 1.1116, 1.3633, 1.4921	↑↓↑↑↓
2	Threonine	1.2585, 1.2349, 1.3633, 3.6111	↑↓↑↓
2	Isoleucine	1.2585, 1.2349, 1.4921, 0.89505, 3.6813	↑↓↓↑↓
3	Glucose	5.1799, 2.7916, 3.1948, 5.174, 2.8911, 2.7858, 3.6111, 3.336, 3.3418, 3.6813, 2.8741, 3.1253	↑↑↓↑↑↑↓↑↑↓↓
4	Pyruvate	2.5048, 2.5106, 2.6687	↓↓↓
5	Aspartate	2.7916, 2.6511, 2.6745, 2.6394, 2.6453, 2.7858, 2.6886	↑↓↓↓↓↑↓
5	Methylguanidine	2.7916, 2.8911, 2.7858, 2.8741	↑↑↑↑
6	Formate	8.4462	↑
7	Carnitine	3.1948, 3.336, 3.3418, 3.1253	↓↑↑↓
7	Glycerol	3.1948, 3.6111, 3.336, 3.3418, 3.6813, 3.1253	↓↓↑↑↓↓
7	Betaine	3.1948, 3.336, 3.3418	↓↑↑
7	3-methylhistidine	3.1948, 7.0472, 3.336, 3.6813	↓↓↑↓
7	Arginine	3.1948, 3.336, 1.7482	↓↑↓
7	Tyrosine	3.1948, 7.1291, 7.1463, 3.1253	↓↑↑↓
7	Cystine	3.1948, 3.336, 3.3418	↓↑↑
7	Choline	3.1948	↓
8	Methionine	2.6511, 2.6394, 2.6453, 2.0846, 2.6687	↓↓↓↑↓
9	3-hydroxybutyrate	1.2349	↓
9	Isopropanol	1.2349	↓
10	Asparagine	2.8911, 2.8741	↑↑
11	Phenylalanine	7.3164, 7.3223, 3.1253, 7.4042, 7.4159, 7.3106	↑↑↓↑↑↑
12	Histidine	7.0472, 3.1253	↓↓
13	Proline	2.3643, 2.3701, 3.336, 3.3418, 2.0846	↑↑↑↑↑
13	Succinate	2.3643, 2.3701	↑↑
13	Glutamate	2.3643, 2.3701, 2.0846	↑↑↑
14	Valine	1.1116, 3.6111, 0.89505	↑↓↑
14	Propylene glycol	1.1116	↑
15	Alanine	1.3633, 1.4921	↑↓
15	Lysine	1.3633, 1.4921, 3.6813, 1.7482, 3.1253	↑↓↓↓↓
16	Glycine	3.6111	↓
17	Methanol	3.336, 3.3418	↑↑
18	2-hydroxybutyrate	0.89505, 1.7482	↑↓
18	Leucine	0.89505, 3.6813, 1.7482	↑↓↓
19	Ornithine	1.7482	↓
20	Malonate	3.1253	↓
20	Cysteine	3.1253	↓
21	Tryptophan	7.3106	↑

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP).

analysis showed a correlation between serum metabolite data and ESR (n=120, r^2 = 0.15, 5 LV, p=0.013). Likewise, a PLS-R analysis showed a correlation between urinary metabolite data and ESR (n=79, r^2 = 0.19, 5 LV, p=0.014). OPLS-DA showed no separation between seropositive (for either ACPA or RF or both) and seronegative RA patients based on either serum (p=1) or urinary (p=1) metabolic data. Additionally, OPLS-DA showed no separation between early (symptom duration of ≤12 weeks) and established (symptom duration of >12 weeks) RA patients based on either serum (p=0.556) or urinary (p=1) metabolic data.

In order to assess whether the relationship between the metabolome and CRP was specific to RA or was seen in non-RA inflammatory arthritis, serum and urine were analysed from patients with UA. Similar to the correlations between CRP and metabolic data derived from RA patients' serum and urine samples, a relationship was also seen between CRP and metabolic data derived from the sera (n=41, r^2 = 0.7209, 9 LV, p<0.001) and urine (n=25, r^2 = 0.6117, 8 LV, p=0.025) of UA patients (**Supplementary Figure 3**).

DISCUSSION

In this study, we applied ^{1}H-NMR metabolomics to assess the relationship between systemic inflammation, as assessed by the serum CRP, and the serum and urinary metabolome in a group of DMARD naïve newly presenting RA patients. Young et al. (18) have previously shown a significant relationship between metabolites identified in unfiltered serum and CRP in two groups of early inflammatory arthritis patients. The metabolites which contributed to that relationship included low-density lipoprotein lipids, lactate, glucose, methylguanidine and amino acids and their derivatives (taurine, acetylglycine, choline, threonine and methylhistidine) (18). Furthermore, a relationship between CRP,

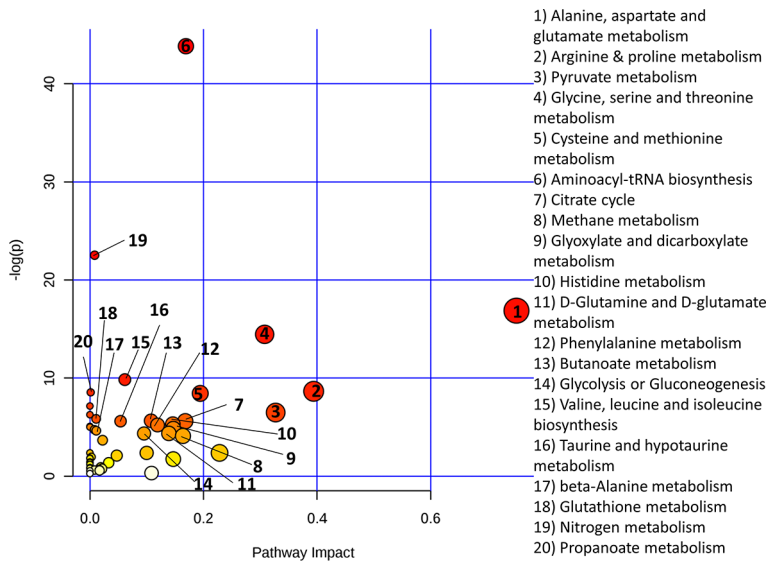

FIGURE 3 | Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and patients' serum metabolites.

measured using a high sensitivity assay, and metabolites in plasma and urine of healthy individuals has been previously seen, with permutations of metabolites related to oxidative stress and the urea cycle observed (19).

In the present study, filtered serum was used which is devoid of large proteins and lipoproteins. This was done to avoid the significant overlap of the broad NMR signals of proteins and lipoproteins with the metabolites in the spectra (41), which can lead to difficulty in identifying individual metabolites. Despite losing information provided by proteins and lipoproteins, filtration of serum results in spectra with fewer overlapping metabolites which can make metabolite identification less problematic. Loss of lipoproteins notwithstanding, PLS-R analysis of filtered serum identified a significant relationship between serum metabolites and CRP ($r^2 = 0.551$, 6 LV, p=0.001). The most highly weighted metabolites in the model included glucose, amino acids, lactate, and citrate. This validates the previously identified relationship between metabolites and CRP (18). Furthermore, it shows a definitive relationship between CRP and metabolites which persist in filtered serum.

Our study also showed a relationship between urinary metabolites and CRP. Blood concentrations of metabolites are strictly regulated, while urine metabolite concentrations can vary widely and can provide complementary information about systemic metabolism. In addition to filtration, the kidney has important role in the generation, breakdown, and active reabsorption and secretion of metabolites, which together determine urinary metabolite concentrations (42, 43). Urinary metabolomics has previously been used to predict responses to anti-TNF treatment in patients with RA (21) and to facilitate diagnosis (20, 44) in patients with inflammatory rheumatic conditions. Pietzner et al. demonstrated a serum and urinary metabolic signature of chronic low grade inflammation in apparently healthy individuals (19). Our findings extend this observation showing a relationship between clinically apparent inflammatory states and the urinary metabolome. The functional interpretation of biomarkers generated by PLS-R analysis largely confirmed the findings seen in the serum analysis, namely increased urea cycle activity, oxidative stress and protein catabolism.

In addition to showing a relationship between CRP and metabolic data derived from RA patients' serum and urine samples, our study showed a relationship between ESR and metabolic data derived from RA patients' serum and urine samples. Furthermore, we were able to show the relationship between CRP and metabolome is not specific to RA but is also present in non-RA inflammatory arthritis. This suggests the relationship between inflammation and the metabolome is present independent of the underlying of inflammatory arthritis. In patients with RA, there were no significant differences in the metabolome between patients with very early or longer standing disease or between patients with RA related autoantibodies compared with patients who were seronegative. These important RA related disease features thus do not appear to influence the metabolome. Finally, some metabolites identified as biomarkers in multivariate models of RA patients' metabolic data and CRP did not show a statistically significant univariate correlation between the metabolite concentration and CRP. This provides further evidence of the well-established importance of multivariate analysis in the field of metabolomics (45), as important relationships between metabolites and variables of interest could be overlooked through univariate analysis alone.

Figure 8 summarises how the metabolic changes we observed to be correlated with CRP at clinical presentation relate to increased urea cycle activity, oxidative stress, increased glycolysis, and skeletal muscle degradation related to cachexia.

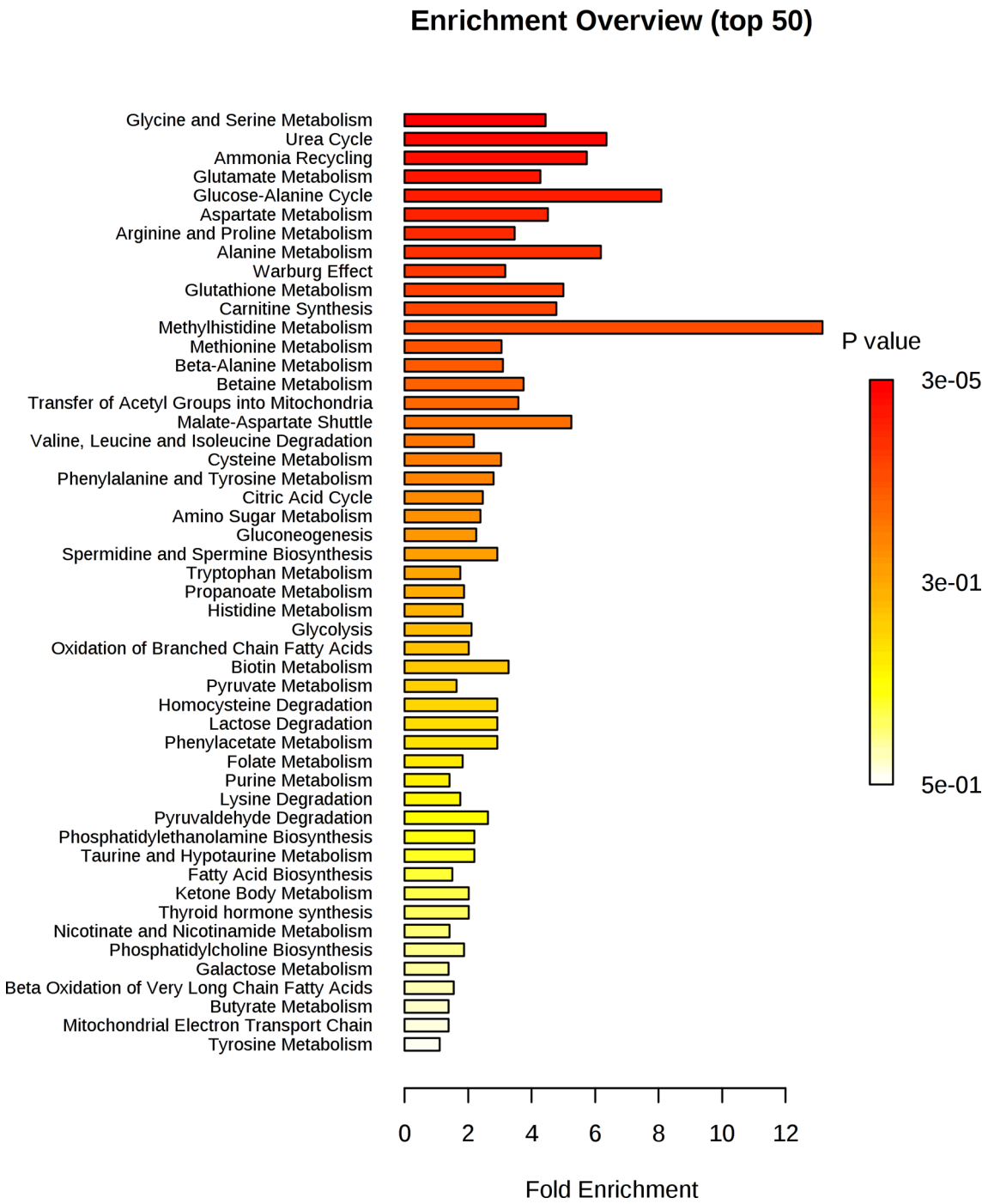

FIGURE 4 | Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of CRP and patients' serum metabolites.

A positive correlation was observed between CRP and several amino acids including glutamate and phenylalanine. This could represent amino acid mobilization from protein stores such as skeletal muscle. In support of this, a positive correlation exists between CRP and 3-methylhistidine. 3-methylhistidine, the methylated analogue of histidine, is an amino acid which is present in actin and myosin (46–48). Catabolism of this complex results in 3-methylhistidine excretion and thus urinary and plasma 3-methylhistidine has been suggested as marker of skeletal muscle turnover (49–53). Leucine and valine, which are amongst the most abundant amino acids in skeletal muscle (54), also showed a positive correlation with CRP. Unlike other amino

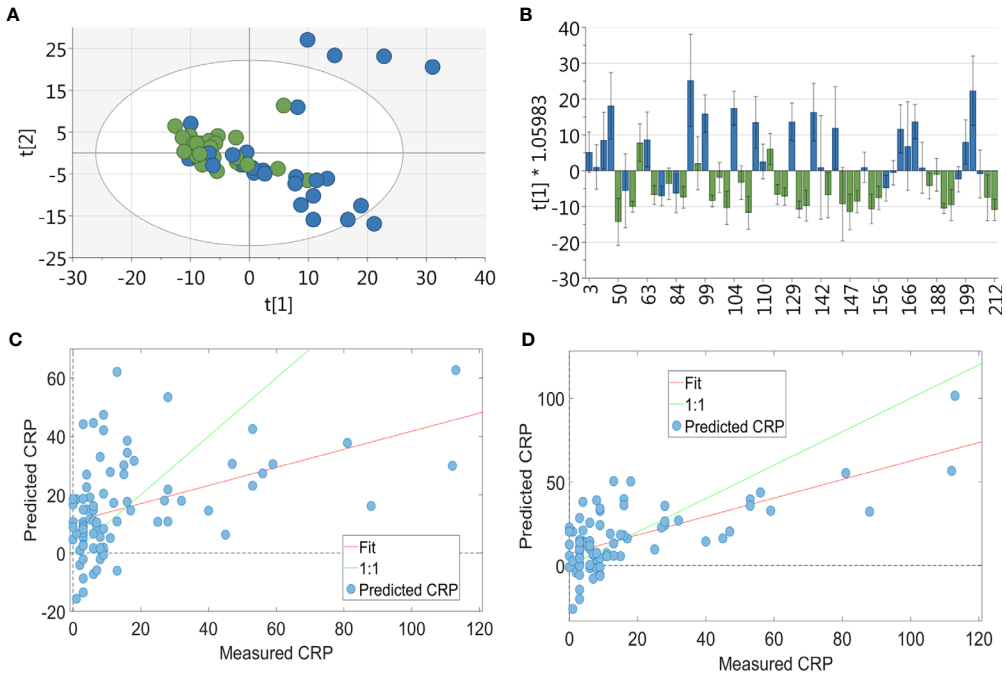

FIGURE 5 | Multivariate analysis of RA patients' urinary metabolite profile. For the PCA & OPLSDA, patients were split into tertiles according to CRP values, with data shown for the highest and lowest tertile (n = 54): **(A)** PCA plot of metabolic data derived from RA patients' urine (green = CRP <5 and blue = CRP>11; 19 PC, r^2 = 0.673) showing no separation between the two groups. **(B)** OPLS-DA plot of urinary metabolic data (n = 83, green = CRP <5 and blue = CRP>11; 1 + 0+0 LV, p value < 0.001) showing a strong separation between the two groups. PLS-R analysis showing the relationship between urinary metabolites and CRP. Using the full 900 NMR urinary metabolite bins for RA patients (n = 83) **(C)** there was a correlation between metabolite profile and CRP (r^2 = 0.095, 1 LV, p = 0.008). Using forward selection, 144 bins were identified which most strongly correlated with CRP and a subsequent PLS-R using these bins **(D)** showed a correlation between urinary metabolite profile and CRP (r^2 = 0.429, 3 LV, p < 0.001).

acids, a negative correlation is seen between CRP and cysteine, cystathionine, and methionine. It is possible that these amino acids are being utilised to produce glutathione. This suggests the presence of oxidative stress, as glutathione is used to reduce reactive oxygen species (55, 56).

As a result of protein catabolism there will be an increase in tissue nitrogen load. Despite the requirement for nitrogen for acute phase proteins, there appears to be a tendency to increase nitrogen excretion through upregulation of the urea cycle in proinflammatory states (57–59). Our findings suggest urea increases and aspartate decreases as CRP increases, which supports the finding of increased urea cycle activity during systemic inflammation.

A negative correlation was seen between CRP and citrate. Immune metabolic reprogramming could be responsible for this. Activation of innate immune cells, such as M1 macrophages and dendritic cells, leads to an upregulation of glycolysis and the pentose phosphate pathway, in addition to downregulation of the citrate cycle, oxidative phosphorylation and fatty acid oxidation (60). This increased glycolytic flux may represent a need to generate more ATP and other intermediates from the citrate cycle. As the citrate cycle switches from a primarily catabolic to an anabolic pathway, one consequence is the accumulation of both citrate and succinate in mitochondria (61). Citrate is transported to the cytosol and broken down to acetyl-CoA for both fatty-acid synthesis and protein acetylation, both of which have been linked to macrophage and DC activation (61). A positive correlation was seen between CRP and succinate; this has been reported previously (62). The increase in succinate, by glutamine-dependent anaplerosis, leads to HIF 1α activation and ultimately enhanced IL-1β production during inflammation (63). In addition, succinate mediated post-translational protein modification (succinylation), also perpetuates the inflammatory response.

A positive correlation was seen between CRP and glucose and lactate. Furthermore, a negative correlation was seen between CRP and pyruvate. Multiple mechanisms are likely to be responsible for the elevated glucose in proinflammatory states (26, 27, 64–69), which may serve to meet the demand for highly active immune cells. Highly active immune cells have high rates of glucose uptake and rely on glycolysis as their main form of energy production. Pyruvate is reduced to lactate even in an aerobic environment, *via* aerobic glycolysis (also known as the Warburg effect), to produce ATP rapidly (70). Lactate has significant downstream effects which propagate the inflammatory response (71–73).

Our analysis has focused on the independent analysis of the patient urine and serum metabolites and their correlation with the inflammatory process. Analysis of any relationship between the serum and urine metabolites was not possible since in this retrospective cohort we had an insufficient number of paired samples to allow a valid assessment. Nevertheless, this

TABLE 3 | Metabolites responsible for the relationship seen in PLS-R analysis between CRP and urinary metabolite profile.

Order	Metabolite	Chemical shift of peak (ppm)	Regression coefficient
1	3-Aminoisobutanoic acid	1.1431, 1.1376, 1.1303, 1.127, 3.0865, 1.0904, 1.1019, 1.1607, 3.0923, 1.1468, 1.1665, 3.0982, 1.155, 3.104	↓↓↓↓↑↑↓↓↓↓↓↓
1	Propylene glycol	1.1431, 1.1376, 1.1303, 1.127, 1.1468	↓↓↓↓↓
2	Lysine	1.4943, 1.4885, 1.506, 1.5002, 1.4416	↑↑↑↑↑
2	Azelaic acid	1.4943, 1.4885, 1.506, 1.5002, 2.1733	↑↑↑↑↓
2	Sebacic acid	1.4943, 1.4885, 1.506, 1.5002, 2.1733	↑↑↑↑↓
2	3-Methyladipic acid	1.4943, 1.4885, 1.506, 1.5002, 2.1733	↑↑↑↑↓
2	Suberic acid	1.4943, 1.4885, 1.506, 1.5002, 2.1733	↑↑↑↑↓
2	Alanine	1.4943, 1.4885, 1.506, 1.5002, 1.4416	↑↑↑↑↑
2	3-Methyl-2-oxovaleric acid	1.4943, 1.1303, 1.127, 1.4885, 1.0904, 1.1019, 1.5002, 1.4416, 1.0558	↑↓↓↑↑↑↑↓
3	L-Cystathionine	3.0865, 3.0923, 3.0982, 2.7353, 2.7294, 2.1733, 2.706, 2.7587, 3.104, 3.8474, 3.8416	↓↓↓↑↑↓↑↓↓↓
3	Creatinine	3.0865, 2.7879, 3.0923, 2.8172, 3.0982, 2.7353, 2.7294, 3.1567, 2.8406, 2.706, 2.7587, 2.7938, 3.104	↓↑↓↑↓↑↑↓↑↑↑↓
3	Phenylalanine	3.0865, 3.0923, 7.2191, 3.0982, 7.3819, 7.2484, 7.5001, 7.2425, 7.225, 7.3771, 3.104, 7.4123	↓↓↑↓↑↑↑↑↑↓↓
3	Cysteine	3.0865, 3.0923, 3.0982, 3.104	↓↓↓↓
3	3-methylhistidine	3.0865, 7.7518, 3.0923, 3.1567	↓↑↓↓
4	2-Ketobutyric acid	1.0904, 1.1019, 2.7879, 2.7587, 1.0558	↑↑↑↑↓
4	Methylsuccinate	1.0904, 1.1019, 1.0558	↑↑↓
5	Hippuric acid	7.7518, 7.9918, 7.7768, 7.9332, 7.8513, 7.5001, 7.9976, 7.781	↑↓↑↑↑↑↓↑
5	Tryptophan	7.7518, 7.2484, 7.5001, 7.225	↑↑↑↑
5	Phenylglyoxylic acid	7.7518, 7.9918, 7.7768, 7.9497, 7.9332, 7.9976, 7.781, 7.947	↑↓↑↑↑↓↑↑
5	Urea	7.7518, 7.9918, 7.7768, 7.2191, 7.3819, 7.9497, 7.9332, 7.2484, 7.5001, 7.9976, 7.781, 7.2425, 7.225, 7.3771, 7.947	↑↓↑↑↑↑↑↑↓↑↑↑↑↑
5	7-Methylxanthine	7.7518, 7.9918, 7.7768, 7.9332, 7.8513, 7.9976, 7.781, 3.8474, 3.8416	↑↓↑↑↑↓↑↓↓
6	Dihydrothymine	1.1607, 2.7879, 1.1665, 3.1567, 1.155, 2.7587, 2.7938	↓↑↓↓↓↑↑
7	Quinolinic acid	7.9918, 7.9976	↓↓
7	Carnosine	7.9918, 2.7294, 3.1567, 7.9976, 2.706	↓↑↓↓↑
7	Picolinic acid	7.9918, 7.8981, 7.9497, 7.9332, 7.9976, 7.947	↓↑↑↑↓↑
7	Histidine	7.9918, 7.8981, 7.9332, 3.1567, 7.9976	↓↑↑↓↓
8	Succinylacetone	2.7879, 2.8172, 2.8406, 2.7938, 3.8474, 3.8416	↑↑↑↑↓↓
8	Aspartate	2.7879, 2.8172, 2.8406, 2.7938	↑↑↑↑
8	Methylguanidine	2.7879, 2.8172, 2.7938	↑↑↑
8	Citrate	2.7879, 2.8172, 2.7353, 2.7294, 2.8406, 2.706, 2.7587, 2.7938	↑↑↑↑↑↑↑
8	5-Aminolevulinic acid	2.7879, 2.7587, 2.7938	↑↑↑
8	Levulinic acid	2.7879, 2.7587	↑↑
9	Malonate	3.0923, 3.0982, 3.104	↓↓↓
10	Symmetric dimethylarginine	2.8172	↑
11	4-Hydroxybenzoic acid	7.7768, 7.781	↑↑
12	Indoleacetate	7.2191, 7.2484, 7.5001, 7.2425, 7.225	↑↑↑↑↑
13	trans-Ferulic acid	7.2191, 7.225	↑↑
13	Tyrosine	7.2191, 7.225	↑↑
13	Ortho-Hydroxyphenylacetate	7.2191, 7.225	↑↑
13	Indoxyl sulfate	7.2191, 7.3819, 7.5001, 7.225, 7.3771	↑↑↑↑↑
13	Tryptophan	7.2191	↑
13	Phenylacetate	7.2191, 7.3819, 7.2484, 7.2425, 7.225, 7.3771	↑↑↑↑↑↑
14	Mandelic acid	7.3819, 7.3771, 7.4123	↑↑↓
15	Cinnamic acid	7.3819, 7.5001, 7.3771, 7.4123	↑↑↑↓
16	Cystine	3.3792, 3.1567, 3.385	↓↓↓
16	4-Hydroxyproline	3.3792, 2.1733, 3.385	↓↓↓
16	Pantothenic acid	3.3792	↓
17	Anserine	2.7353, 2.7294, 2.706	↑↑↑
17	Sarcosine	2.7353, 2.7294, 2.7587	↑↑↑
17	Citramalic acid	2.7353, 2.7587	↑↑
18	Kynurenic acid	7.8981, 7.9332, 7.5001	↑↑↑
18	3-Methylhistidine	7.8981, 7.9332	↑↑
19	Benzoic acid	7.9332, 7.8513, 7.5001	↑↑↑
20	3-Hydroxyphenylacetate	7.2484, 7.2425, 7.225	↑↑↑
21	L-Kynurenine	7.8513, 7.4123	↑↓
22	Ethanolamine	3.1567, 3.8474, 3.8416	↓↓↓
22	Beta-Alanine	3.1567	↓
23	Asparagine	2.8406	↑

(Continued)

TABLE 3 | Continued

Order	Metabolite	Chemical shift of peak (ppm)	Regression coefficient
24	Vanillic acid	7.5001	↑
24	Uracil	7.5001	↑
24	4-Pyridoxic acid	7.5001	↑
24	Cytosine	7.5001	↑
25	Monomethyl glutaric acid	2.1733	↓
25	Pimelic acid	2.1733	↓
25	Methionine	2.1733, 3.8474, 3.8416	↓↓↓
25	Isovalerylglycine	2.1733	↓
25	Glutamate	2.1733	↓
25	Methylglutaric acid	2.1733	↓
25	L-2-Hydroxyglutaric acid	2.1733	↓
25	Glutamine	2.1733	↓
26	Isoleucine	1.4416	↑
27	Dihydrouracil	2.706	↑
28	Valine	1.0558	↓
29	L-Arabitol	3.8474, 3.8416	↓↓
29	Serine	3.8474, 3.8416	↓↓
29	N-Acetylneuraminic acid	3.8474, 3.8416	↓↓
29	D-Maltose	3.8474, 3.8416	↓↓
29	Pseudouridine	3.8474, 3.8416	↓↓
29	Thymidine	3.8474, 3.8416	↓↓
29	Hydroxypropionic acid	3.8474, 3.8416	↓↓
29	Alpha-Lactose	3.8474, 3.8416	↓↓
29	Adenosine	3.8474, 3.8416	↓↓
29	Sorbitol	3.8474, 3.8416	↓↓
29	D-Galactose	3.8474, 3.8416	↓↓
29	Homovanillic acid	3.8474, 3.8416	↓↓
29	D-Xylitol	3.8474, 3.8416	↓↓
29	Gluconic acid	3.8474, 3.8416	↓↓
29	L-Arabinose	3.8474, 3.8416	↓↓
29	Sucrose	3.8474, 3.8416	↓↓
29	Dehydroascorbic acid	3.8474, 3.8416	↓↓
29	1-Methyladenosine	3.8474, 3.8416	↓↓
29	Glyceric acid	3.8474, 3.8416	↓↓

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP).

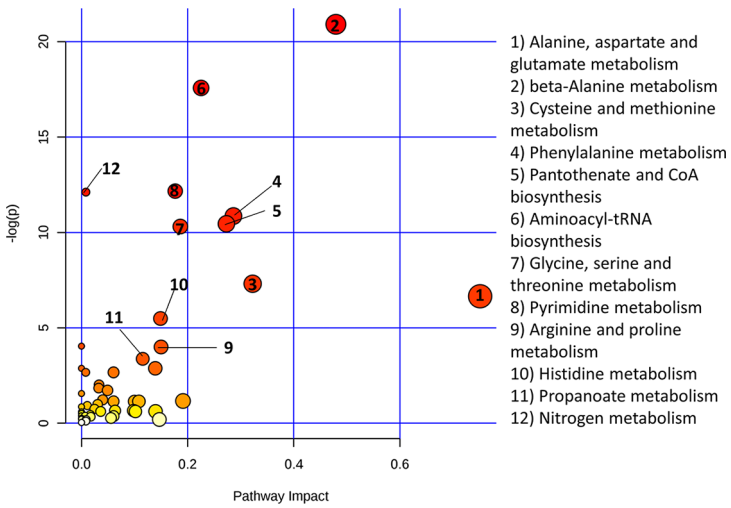

FIGURE 6 | Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and patients' urinary metabolites.

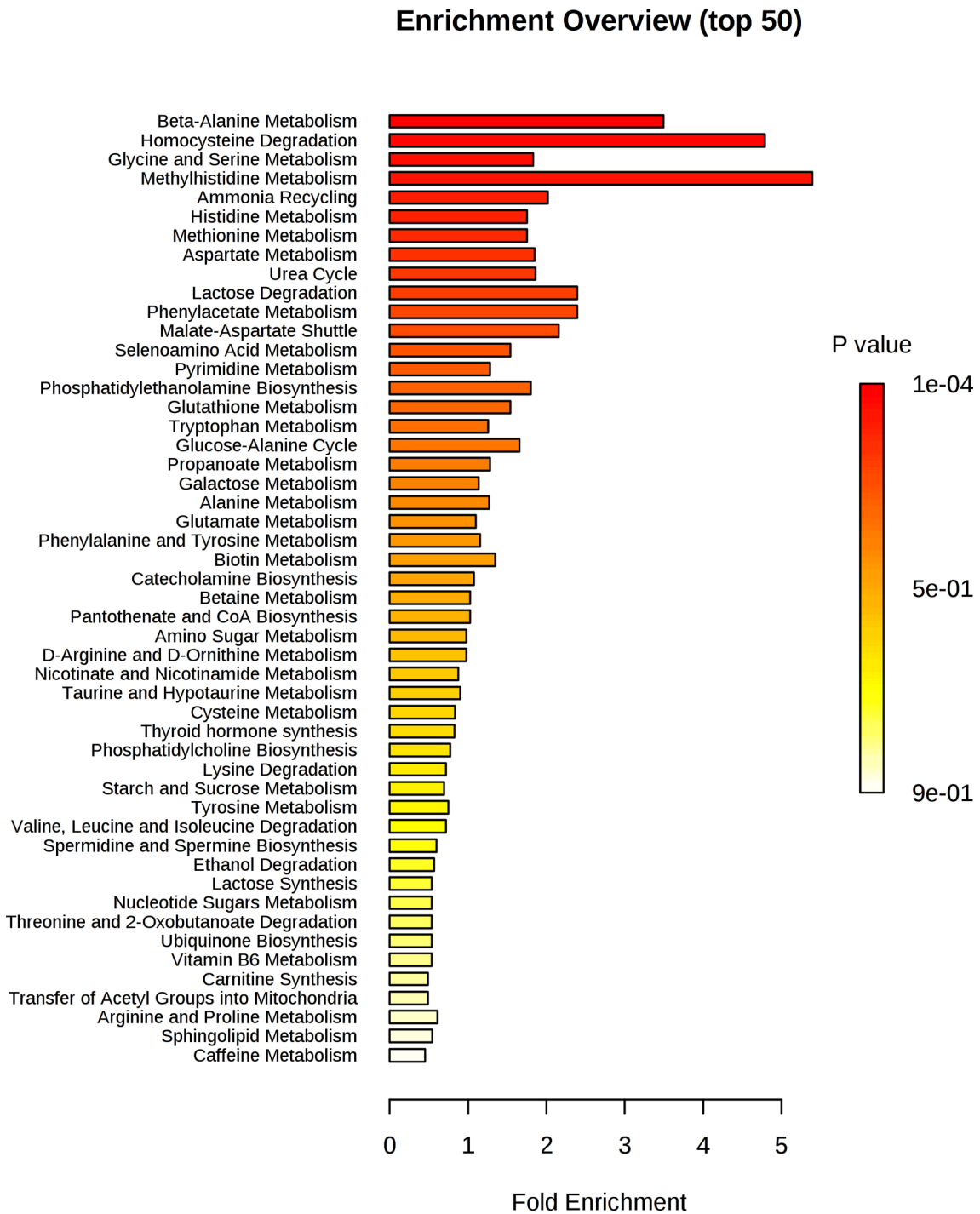

FIGURE 7 | Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and RA patients' urinary metabolites.

comparison might be useful in future studies since a correlation of a small number of metabolites has been seen in a large biobank study of urine and serum from children (74). However, in that study serum was seen to provide stronger correlates with important biological features such as age, sex, BMI and ethnicity while urine metabolites were more strongly influenced by diet. In adults, serum metabolites have been shown to be less variable than urine metabolites and so they may provide a more reliable comparator in disease states (75). However, our previous work (21), has shown that urine metabolites were able to predict responses to anti-TNF

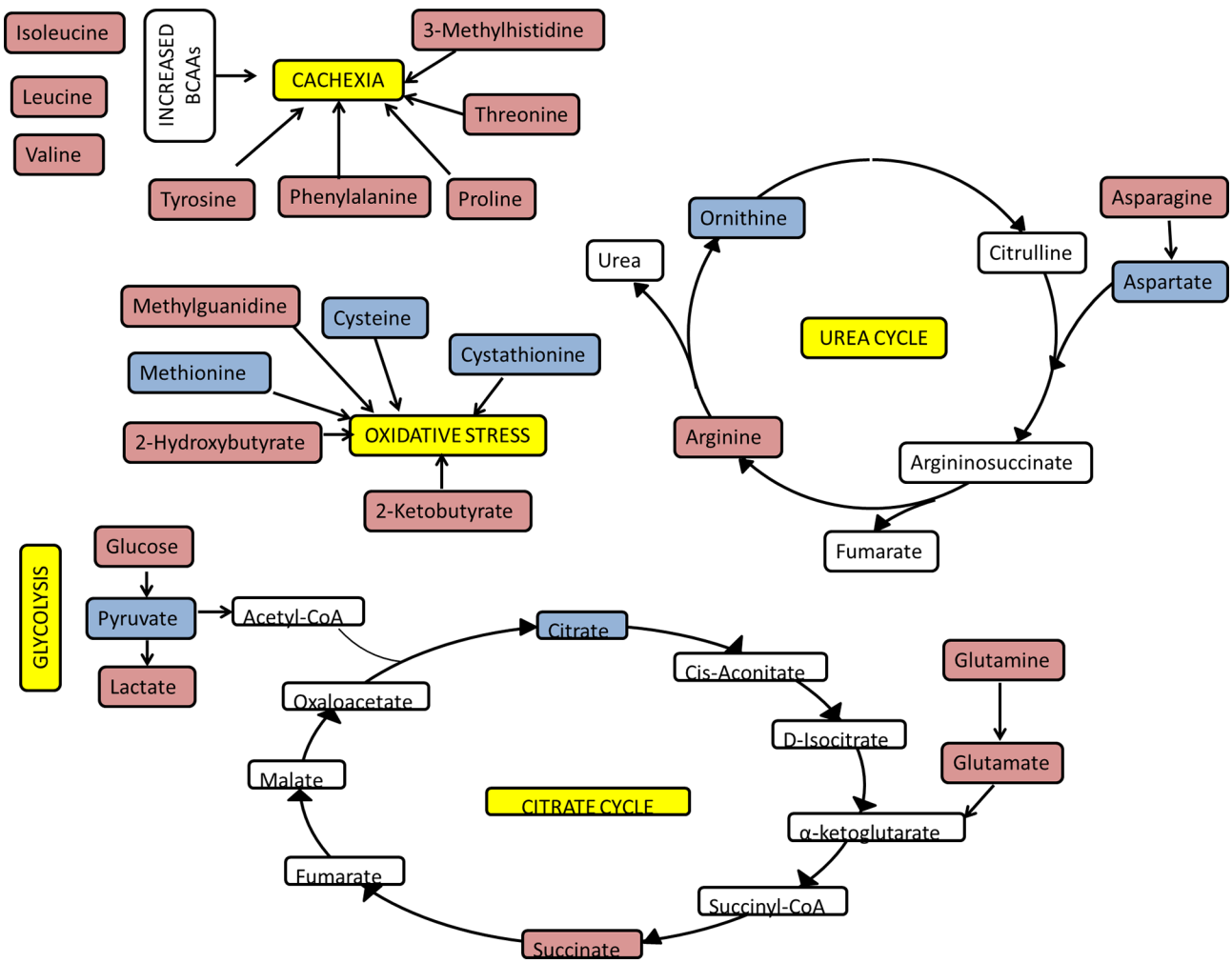

FIGURE 8 | Overview of key pathways and metabolites correlating with CRP. The functional analysis of PLS-R analysis of the serum and urinary metabolome of newly presenting RA patients as assessed by ^{1}H NMR spectroscopy. Red metabolites had a positive correlation with CRP and blue metabolites had a negative correlation with CRP.

therapy in a small cohort of RA patients. Since TNF is a major driver of the inflammatory process in RA this would support our observation in the current paper that urinary metabolites do indeed reflect the inflammatory state in RA patients and as such may provide easily accessible source of useful biomarkers in RA and other inflammatory states.

LIMITATIONS

Despite the advantages of using NMR spectroscopy to assess the metabolome, including minimal, non-destructive sample preparation, relatively low cost, good direct quantitation and high reproducibility (76), there are several important limitations. Firstly, there is low sensitivity for identifying metabolites, leading to failure to identify metabolites present at a lower concentration. Secondly, overlapping ^{1}H signals can make metabolite identification difficult. For example, individual spectral peaks may be a result of a combination of metabolites rather than a single metabolite. Thus, it can be difficult to determine which of a range of possible metabolites represented within that peak is driving the association between the magnitude of that peak and the clinical variable under consideration (in this case CRP). Our use of filtered serum limited the assessment of lipid metabolism, as large proteins and lipoproteins are removed by filtration. Other confounders which are known to influence metabolism were not controlled for including comorbidities, medications, diet and time of sample collection.

CONCLUSION

The PLS-R models assessing relationships between metabolite profiles and CRP have provided insight into metabolic

derangements during inflammatory states such as oxidative stress, cachexia and impaired glycolytic metabolism. These findings validate a known relationship between serum metabolite profile and inflammation as measured by CRP and shows there is relationship between the urinary metabolite profile and inflammation. Serum and urine are easily accessible and easily studied by NMR spectroscopy. The focus of this paper was to examine the relationship between CRP and the serum and urinary metabolome of RA patients. Future work should examine the relationships between the metabolome and other important clinical variables including levels of pain and fatigue. Furthermore, future work should assess the impact of anti-inflammatory therapies assessing whether therapeutic response is associated with alterations in specific pathways.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Black Country Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GJ: data collection and manuscript composer. KS: quantified metabolites and edited manuscript. IS: data collection. AF: composed and edited manuscript. SY: composed and edited manuscript. KR: composed and edited manuscript. TA has been involved in the interpretation of the data and has reviewed, edited and approved the manuscript. All authors contributed to the article 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.2021.676105/full#supplementary-material

Supplementary Figure 1 | PLS-R analysis showing the relationship between metabolic data derived from RA patients and ESR. **(A)** Using the full 590 serum metabolite binned data (n = 120) there was a correlation between metabolite data and ESR on PLS-R analysis (r^2 = 0.15, 5 LV, p = 0.013). **(B)** Using the full 900 NMR urinary metabolite bins for RA patients (n = 79) there was a correlation between metabolite profile and ESR (r^2 = 0.19, 5 LV, p = 0.014).

Supplementary Figure 2 | OPLS-DA of RA patients' metabolome by autoantibody status and symptom duration. **(A)** OPLS-DA plot of serum metabolic data (n = 126, blue is seronegative*, green is seropositive*; 1 + 0+0 LV, p = 1) showing no separation between the two groups. **(B)** OPLS-DA plot of urinary metabolic data (n = 83, blue is seronegative*, green is seropositive*; 1 + 0+0 LV, p = 1) showing no separation between the two groups. **(C)** OPLS-DA plot of serum metabolic data (n = 126, blue is symptom duration of ≤12 weeks, green is symptom duration of >12weeks; 1 + 0+0 LV, p = 0.556) showing no separation between the two groups. **(D)** OPLS-DA plot of urinary metabolic data (n = 83, blue is symptom duration of ≤12 weeks, green is symptom duration of >12weeks; 1 + 1+0 LV, p = 1) showing no separation between the two groups. *seropositive for either ACPA or RF or both.

Supplementary Figure 3 | PLS-R analysis showing the relationship between metabolic data derived from UA patients and CRP. **(A)** UA patients have a statistically significant relationship between serum metabolite profile and CRP (n = 41, 98 NMR bins post forward selection, r^2 = 0.7209, 9 LV, p < 0.001). **(B)** UA patients have a statistically significant relationship between urinary metabolite profile and CRP (n = 25, 90 NMR bins post forward selection, r^2 = 0.6117, 8 LV, p = 0.025).

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REVIEW
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Glycolysis Rate-Limiting Enzymes: Novel Potential Regulators of Rheumatoid Arthritis Pathogenesis

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Rheumatoid arthritis (RA) is a classic autoimmune disease characterized by uncontrolled synovial proliferation, pannus formation, cartilage injury, and bone destruction. The specific pathogenesis of RA, a chronic inflammatory disease, remains unclear. However, both key glycolysis rate-limiting enzymes, hexokinase-II (HK-II), phosphofructokinase-1 (PFK-1), and pyruvate kinase M2 (PKM2), as well as indirect rate-limiting enzymes, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), are thought to participate in the pathogenesis of RA. In here, we review the latest literature on the pathogenesis of RA, introduce the pathophysiological characteristics of HK-II, PFK-1/PFKFB3, and PKM2 and their expression characteristics in this autoimmune disease, and systematically assess the association between the glycolytic rate-limiting enzymes and RA from a molecular level. Moreover, we highlight HK-II, PFK-1/PFKFB3, and PKM2 as potential targets for the clinical treatment of RA. There is great potential to develop new anti-rheumatic therapies through safe inhibition or overexpression of glycolysis rate-limiting enzymes.

Keywords: glycolysis, rate-limiting enzymes, rheumatoid arthritis, RA, pathogenesis

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory diseases. It involves chiefly the joints, but can present extra-articular manifestations, such as rheumatoid nodules, pulmonary involvement, vasculitis, and systemic comorbidities (1). RA's prevalence has remained relatively stable in many populations (ranging from 0.5% to 1%) and it is at least twice more common in women than in men. The disease can occur at any moment, but the peak incidence arrives at approximately 50 years of age (2, 3). RA is mainly characterized by an inflammatory infiltration with abnormal vascular proliferation in the synovial membrane and pannus formation; recurrent attacks to the joints lead to cartilage destruction and bone erosion, eventually leading to deformation of the affected joint and even complete loss of its motor function (4). The immune cells in RA share an altered proliferative capacity that is evident in the joint-resident cells that form the synovial pannus (5). Synovial proliferation, neoangiogenesis, and leukocyte extravasation transform

the normal noncellular synovium into an invasive tumor-like pannus (6). Pannus formation is one of the driving pathological processes leading to RA joint erosion (7). Energetic and biosynthetic precursors are on high demand during biomass construction, implying that metabolic control is fundamental during the pathogenesis of RA (5).

Glucose metabolism involves a 10-step cytoplasmic reaction called glycolysis, or more formally, the glycolytic pathway. This 10-step reaction converts one molecule of glucose into two molecules of 3-carbon pyruvate. Glycolysis also creates two molecules of ATP and reduces two NAD+ molecules to NADH, these two types molecules are metabolic fuels that drive other biological reactions (8). Under aerobic conditions, the pyruvate in mitochondria can be used during aerobic respiration *via* the tricarboxylic acid cycle (TAC) to produce more ATP for the cell (9). Under low partial oxygen pressure, pyruvate is fermented by lactate dehydrogenase (LDH) in the cytoplasm into lactate while converting NADH to NAD+ (10). However, in the presence of sufficient oxygen and functional mitochondria, some cells display an enhanced and accelerated metabolic conversion of glucose into lactate, a phenomenon named the Warburg effect (11, 12), which can also be referred to as aerobic glycolysis (13). Chronic inflammatory disorders, such as rheumatosis, require a good deal of energy supplied and distributed to the activated immune system (14). RA is a classical rheumatic disease with high metabolic demands (15), but its multiple pathological alterations maintain the affected tissues in a hypoxic state (16, 17). Therefore, complex alterations must be present in tissue and cellular metabolic pathways. Studies have shown that glycolysis, an important process during glucose metabolism, plays a significant role in the pathogenesis of RA (18, 19). Herein, we systematically describe the association between glycolysis and RA from the perspective of glucose metabolism and the key rate-limiting enzymes in the glycolytic pathway (**Figure 1**).

GLYCOLYSIS AND RA

During RA, hypoxia alters the cellular bioenergetics by inducing mitochondrial dysfunction and promoting a switch to the glycolytic pathway that leads to abnormal angiogenesis, cellular invasion, and pannus formation (20). Angiogenesis is a result of the hypoxic state and is a prominent feature of rheumatoid synovitis (21). Although some neovascularization delivers oxygen to the increased inflammatory cell mass, the neovascular network is dysfunctional and unable to restore tissue oxygen homeostasis, that's why the joints in RA remain in a rock-ribbed hypoxic environment (22). The synovial fluid oxygen tension of the knee in patients with RA is significantly lower than that in patients with osteoarthritis (OA) with synovial hyperplasia (23). RA synovial fluid presents significant elevations in lactate and decreases in glucose concentrations as a consequence of the high synovial cell metabolic demands (24–26). By applying magnetic resonance spectroscopy (MRS), it was found that anaerobic metabolism becomes more pronounced

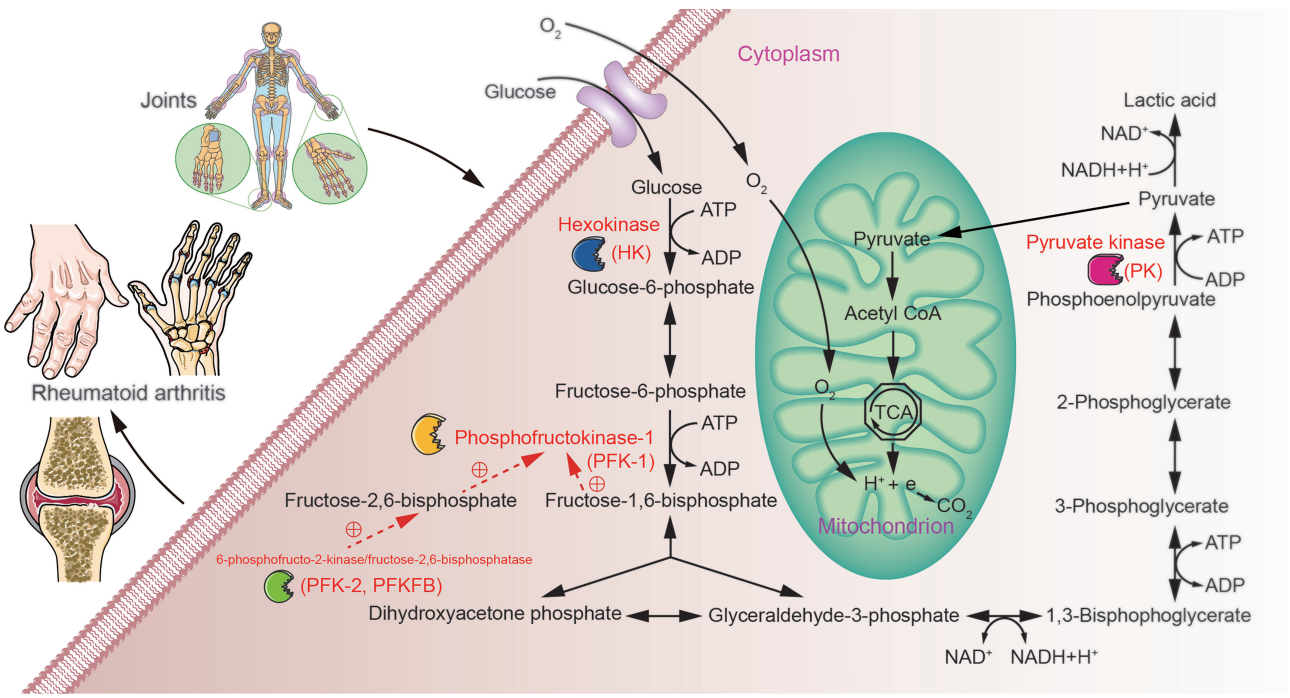


FIGURE 1 | Possible role of glucose metabolism during RA. Over- or under-expression of glycolysis rate-limiting enzymes may contribute to metabolic differences in the tissues of patients with RA through aberrant regulation of glycolytic pathways.

with the progressive increase in the degree of inflammation and vascularity of the RA synovium (27). Magnetic resonance spectroscopic imaging of hyperpolarized [1-^{13}C]-pyruvate metabolism has also demonstrated an increase in the lactate-to-pyruvate ratio within hind paws of the complete Freund's adjuvant (CFA) RA arthritis model. This soar indicates enhanced glycolysis and an increased lactate concentration associated with acidosis, as observed in the synovial fluid of patients with RA (28). In addition, roles for glycolysis, fatty acid, and amino acid metabolism, and other related pathways (TCA and urea cycle) in RA have been revealed by gas chromatography-mass spectrometry (GC-MS) experiments showing decreased levels of amino acids and glucose and increased levels of fatty acids and cholesterol in sera of patients with RA (29). Metabolic changes of glucose, phospholipids, and bioactive lipids (such as sphingosine-1-phosphate and lysophosphatidic acid) are needed during activation of fibroblast-like synoviocytes (FLSs) and may contribute to initiation of immune responses or abnormal immune responses that trigger RA and contribute strongly to joint destruction (30).

In RA, FLSs, which form the articular lining, exert a significant part in pathological processes and are epigenetically imprinted with an aggressive phenotype (31). FLS are the most common cell type at the pannus-cartilage junction, triggering joint destruction through the production of cytokines, chemokines and matrix degradation molecules as well as migration and invasion (30, 32). These pathological features are inevitably linked to tumor-like phenotypes of FLSs. The rate of proton efflux, proton (H+) production rate (PPR), and the mitochondrial respiration rate, oxygen consumption rate (OCR), were measured in RA and OA FLSs cell lines using a Seahorse XF analyzer, and the ratio of PPR to OCR represents an estimate of the relative balance between glycolysis and oxidative phosphorylation. RA FLSs have a higher PPR : OCR ratio than OA FLSs, suggesting that the balance between glycolysis and oxidative phosphorylation shifts to the glycolytic pathway in RA FLSs compared to the balance in human OA FLSs (33). Activation of FLSs by CD4 T cells increases their energy demands. The cells shift rapidly from oxidative phosphorylation to anaerobic glycolysis to support abnormal behaviors (proliferation, invasion, and conglutination) and to accelerate cytokine release (34). Substantial flux control is performed in four steps: glucose import, hexokinase (HK), phosphofructokinase (PFK), and lactate export. These four flux control steps are specifically upregulated by the Ras oncogene: optogenetic Ras activation rapidly induces transcription of isozymes that catalyze these four steps, thereby enhancing glycolysis (35). T cells from patients with RA express significantly high levels of K-RAS, and present a hyperactivated Ras/MEK/ERK pathway (36, 37). Multiple Ras proteins are expressed in both synovial tissue (ST) and cultured FLSs, and Ras protein expression and activation changes lead to the pathological phenotype of FLSs in RA (38). Additionally, IgG immune complexes sensitize human monocytes for overactive inflammation through transcriptomic and epigenetic reprogramming in RA (39). This suggests that RA specific autoantibodies can train monocytes in inflammatory lesions as early as the asymptomatic stage, when enhanced glycolysis in RA diseased region is already occurring (40). An alteration of energy metabolism is clear from the initial stages of RA; glucose, the main energy supplier of the body, is preferentially utilized. The associations between altered glucose metabolism, glycolysis, and RA should be understood first.

GLYCOLYSIS RATE-LIMITING ENZYMES AND RA

Studies have implicated numerous energy metabolic enzymes such as glucose phosphate isomerase (41), α-enolase (ENO1) (42, 43), aldolase (25), and triosephosphate isomerase in RA glycolysis mechanisms (44). However, most of the 10 reactions of the glycolytic pathway are reversible, and the direction and rate of these reactions are controlled by the concentrations of their substrates and products. Changes in the activity of the enzymes that catalyze these reversible reactions do not determine the direction of the reactions (45). Instead, the control of the flow of glycolysis depends primarily on the activity of three key rate-limiting enzymes, hexokinases (HKs), phosphofructokinase-1 (PFK-1), and pyruvate kinases (PKs) (46, 47). These three key enzymes are regulated by irreversible reactions within the cell and the rate of these reactions is slow (45). By regulating glucose metabolism, glycolytic rate-limiting enzymes become crucial regulators of the RA pathogenesis.

HKs are the first rate-limiting enzymes in the glycolytic pathway. Glucose transported into the cell *via* glucose transporter (GLUT) is phosphorylated by HKs to glucose-6-phosphate (G-6-P) (48), a process that is efficient and irreversible, and it has evident flux control (35, 49). There exist four isoforms of HKs in mammalian tissues, HK-I, HK-II, HK-III and HK-IV, which differ in their major distribution in various tissues of the body (50). The ubiquitous HK-I isoform seems to be constitutively expressed in most tissues. HK-II is a major regulated isoform in many cell types and is widely expressed in insulin-sensitive tissues such as muscle and adipose, which are the bulk of peripheral glucose utilization. HK-II is also widely expressed in many highly glycolytic cancers (51). For instance, overexpression of HK-II promotes cell migration and invasion *via* the FAK/ERK1/2/MMP-9 pathway and enhances stemness properties *via* the FAK/ERK1/2/NANOG/SOX9 cascade. HK-II abrogation inhibits tumor growth and spread *in vivo* (52).

PFK-1 is the second key rate-limiting enzyme of glycolysis. PFK-1 is a tetrameric protein with three genes encoding human isoforms: PFK-M (muscle), PFK-L (liver), and PFK-P (platelet) (53). PFK-1 phosphorylates fructose 6-phosphate (F-6-P) into fructose 1,6-bisphosphate (F-1,6-BP), in another irreversible reaction that is far from equilibrium and constitutes a critical control point for the regulation of glycolytic flux (54). The rate of PFK-1 is influenced by a variety of allosteric effectors in the cytoplasm. The most potent allosteric effector of PFK-1 is fructose 2,6-bisphosphate (F-2,6-BP) (55). The interplay between F-2,6-BP levels, the enzymes that produce and degrade it, and PFK-1 activity have important implications for different aspects of cellular metabolism as well as for systemic

metabolic conditions (56). F-2,6-BP levels are closely related to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2, PFKFB), a bifunctional enzyme containing a phosphatase domain that can consume F-2,6-BP (35), and is responsible for the synthesis and degradation of F-2,6-BP. PFKFB controls the glycolytic flux by limiting the intracellular concentration of F-2,6-BP (55, 57). The human body has four isozymes of PFKFB, namely PFKFB1, PFKFB2, PFKFB3 and PFKFB4, which display tissue-specific expression patterns and different kinase-to-phosphatase activities. All four isozymes are induced by hypoxia *in vivo*, however, the hypoxia responsiveness varies in different organs (58).

PKs catalyze the tenth and final reaction of glycolysis: the irreversible conversion of ADP and phosphoenolpyruvate to ATP and pyruvate, both of which are essential for cellular metabolism (59). The PK family contains four isoforms encoded by two distinct PK genes (60). PKR is the only isoform expressed in erythrocytes; PKL is the dominant isoform in liver; and PKM1 is the dominant isoform in differentiated skeletal muscle, heart, and brain; unlike all other isoforms, PKM2 is present in numerous differentiated adult tissues (61). PKM1 and PKM2 are encoded by alternative splicing of the PKM gene, which is significantly regulated by three heterogeneous nuclear ribonucleoproteins (hnRNPA1, A2, and I) dependent on c-Myc (60). PKM2, a crucial rate-limiting enzyme of glycolysis, is normally overexpressed in proliferating and tumor cells, it regulates glycolysis and the Warburg effect (62). PKM2 is associated with some cancers and contributes to the direction of the glycolytic pathway into fermentation and the lactate formation (63). PKM2 contributes to TLR-mediated inflammation and autoimmunity and may be a promising target for controlling inflammation and autoimmunity (64). In addition, PKM2 is required for Th1 and Th17 differentiation *in vitro* and *in vivo*. PKM2 also represents a therapeutic target for T cell-dependent autoimmune diseases (65).

CD4+ T lymphocytes play a role in the pathogenesis of RA, forming a regulatory and functionally multitudinous population within the immune system (66). CD4+ T secretory factors induce a metabolic shift in FLSs from an oxidative metabolism to a more glycolytic phenotype. In primary RA FLSs activated by CD4 T cell-conditioned medium (CM), the mRNA expression levels of GLUT1, GLUT3, HK-II, PFKFB3, LDHA, and GSK3A are increased (34). Oncostatin M (OSM) is highly expressed in RA joints (67). OSM is a member of the IL-6 subfamily produced by inflammatory cells and some tumor cells and shares a common receptor signaling subunit (gp-130) with IL-6-type cytokines (68–70). OSM regulates metabolic reprogramming in RA FLSs in conjunction with TNFα, and it boosts mRNA expression of GLUT1, HK-II, PFKFB3, PKM2, and LDH through STAT3 phosphorylation (67). Many glycolytic pathway enzymes (including HK-III, PFK, PKM2, and ENO) have been shown to be upregulated in plate-bound human IgG-trained monocytes compared to their levels in blank controls (40). Upon encountering ICOSL+ B cells, activated effector memory TH cells from patients with RA spontaneously differentiate into inflammatory TH subsets. ICOSL-induced glucose uptake is involved in inflammatory TH polarization by B cells, and glycolysis is significantly upregulated in T cells polarized by B cells. Key enzymes of the glycolytic pathway (HK, PFKL, PFKFB3, and PKM) get significantly overexpressed in TH cells that have interacted with B cells (71). Identifying the exact role, such as inflammation, invasion, and proliferation, of the key rate-limiting enzymes of glycolysis pathway in RA, is crucial for understanding the relationship between energy metabolism and the occurrence and development of RA, and it is essential to completely elucidate the pathogenesis of RA.

HK-II

Immunohistochemistry (IHC) methods have shown higher HK-I, HK-II, and HK-IV expression levels in the STs of patients with RA than in those of patients with OA (72). A quantitative real-time polymerase chain reaction (RT-qPCR) comparison of the expression levels of glycolytic related genes in RA FLSs and OA FLSs revealed that the mRNA levels of HK-II are significantly higher in the RA FLSs than in the OA FLSs (73). Moreover, HK-II is more differentially expressed in RA STs than the other HK isoforms (72), being very dominant in both the lining and sublining (18). The expression of GLUT1 mRNA is higher in RA FLSs than in OA FLSs, and the level is closely correlated with that of HK-II (33). Higher expression of metabolic enzymes and proteins associated with glycolysis, HK-II, GLUT1, LDHA, monocarboxylate lactate transporter 4 and HIF-1α, was also observed in RA synovial membrane CD8+ T cells (74). Primary bovine synovial cell cultures treated with different lipopolysaccharide (LPS) concentrations for 12 hours showed enhanced HK-II mRNA expression (starting at 100 ng/ml of LPS) increasing in a dose-dependent manner; the expression boost was maximal at 1 μg/ml of LPS (75). Double immunohistochemistry can be used to determine whether FLSs express HK-II in the RA synovium, these cells can be identified by their positivity for vimentin, vascular cell adhesion protein 1 (VCAM-1), or podoplanin (PDPN) (76–78). HK-II positive staining co-localized with all the diverse FLS markers in the lining (18). Stimulation with activated-Th cells-conditioned media (ThCM) results in a significant increase in HK-II mRNA expression in the RA synovial fibroblasts (SFs) compared with the levels under unstimulated conditions (79). Adjuvant arthritis (AA) is a commonly used animal model for human RA that can be used to score synovial proliferation, seep inflammatory cells, and cartilage destruction at different stages of the progressive disease (PD). The severity of pathological changes increases gradually with PD, while the expression of HK-II in ST was detected, and HK-II expression soared significantly with PD (80). Moreover, OSM significantly induces angiogenic networks formation, adhesion, and invasion mechanisms that are accompanied by changes in the bioenergetic profile of cells, in which OSM significantly increases the extracellular acidification rate (ECAR)/OCR ratio of primary RA FLSs (favoring glycolysis) and it induces the expressions of

GLUT1 and of the key glycolytic rate-limiting enzymes HK-II and PFKFB3 (67).

Intra-articular injection of adenovirus carrying murine HK-II (ad-mHK-II) in the knee of healthy mice significantly increases the synovial lining thickness, and promotes FLS activation and proliferation. Overexpression of HK-II in the synovium of healthy mice transforms thin linings into hypertrophic synovial membranes, which also enhances FLS migration to the cartilage. HK-II causes healthy natural synovial linings to become hypertrophic with upregulated α-sma and matrix metalloproteinase-3 (MMP-3) expressions (18). K/BxN arthritis mice are a spontaneous model driven by T cell receptor transgenic CD4+ T cells from the KRN strain that are activated by G-6-P isomerase peptides presented by the H-2g7 allele from the NOD strain (81). HK-II is highly expressed in the synovial lining after K/BxN serum transfer arthritis (18). HK-II plays a pivotal role converting glucose into subsequent products of the glycolytic pathway and enhancing the cellular metabolic activity. Fortified HK-II activity is associated with major pathways of inflammation, angiogenesis, migration, invasion, and cell survival in FLSs (82). HK-II also increases the extracellular lactate production (83). Both HK-I and HK-II similarly increase the extracellular lactate levels, but only HK-II overexpression triggers an aggressive FLS phenotype, this suggests the presence of a glycolytic-independent mechanism (18).

Silencing of HK-I/II, siHK-I/II, or the use of an HK inhibitor like lonidamine (LND) decreases cell viability and reduces the production of proinflammatory cytokine and chemokines, IL-6, IL-8, CXCL9, CXCL10, and CXCL11 (72). Under normoxic conditions, Hif-1α knockdown reduces glycolytic metabolism and induces apoptosis in SFs (84). LND also induces apoptosis in RASF (72), but HK-I- or HK-II-silencing does not alter cell viability or shape (18). Inhibition of HK-II inhibits synovial cell activation *via* the AMPK/NF-κB pathway to improve arthritic symptoms in AA (80). 2-DG is a synthetic glucalogue in which the 2-hydroxyl group has been replaced by a hydrogen (85). 2-DG inhibits phosphorylation of other available sugars, such as glucose, and may act as a noncompetitive antagonist of HKs to restrain the cellular glycolytic activity and regulate glycolysis, leading to a decrease in intracellular ATP production (86, 87). 2-DG (200 mg/kg) treatment has been shown to increase the expression of p-AMPK proteins and to decrease the expression of p-p65 and p-IκBα proteins, suggesting that 2-DG may activate the AMPK pathway (80). RA FLSs pretreated with 2-DG and then cultured in the presence of platelet-derived growth factor-BB (PDGF-BB) for 4 days, exhibited a significantly diminished cell proliferation rate as measured by an MTT assay; the cellular levels of cell migration, IL-6, and MMP-3 were also significantly reduced (88). 2-DG treatment reverses LPS-induced increases in GLUT1 and HK-II mRNA expression. The compound reduces the dependence of synovial cells on the glycolytic pathway for ATP production by enhancing the contribution of mitochondrial respiration (MR) to ATP production and inhibiting LPS-stimulated lactate production (75). Human monocyte-derived dendritic cells (moDC) stimulated in the presence of 2-DG exhibit an impaired glycolysis due to HK activity inhibition. 2-DG prevents the cytokine production induced by individual Toll-like receptor (TLR) stimulation. Additionally, 2-DG also strongly restrains cytokine production after co-stimulation with complexed IgG and Pam3CSK4 (89). HK-II^{Col1} mice (HK-II deleted in HK-II^{Col1} joint FLSs) present a significant reduction in arthritis severity, and in bone and cartilage damages as compared to manifestations in control mice (18).

3-Bromopyruvate (BrPA), a specific HK-II inhibitor, significantly reduces the arthritis and the histological scores in the SKG mouse model (a genetic model with many RA features) while significantly increasing the number of regulatory T cells (Treg). *In vitro*, BrPA promotes differentiation of Treg cells, suppresses interleukin-17-producing T cells (Th17), and inhibited the activation of dendritic cells (90). Treatment with LND in a type II collagen-induced arthritis (CIA) in DBA-/1 mouse model reduces the production of antibodies against IgG1, IgG2a, and IgG2b, thereby reducing articular inflammation and destruction. LND delays illness in an animal model of RA, and it may get developed into a novel class of anti-rheumatic drug complementary to biological therapies targeting immunity (72). Treatment of AA rats with 2-DG significantly reduces joint swelling, diminishes bone destruction, inhibits synovial cell proliferation and migration, and decreases synovial cell secretion. 2-DG (200 mg/kg) significantly decreases TNF-α, IL-1, and nuclear factor κB ligand (RANKL) levels secreted by synovial cells, while it significantly increases osteoprotegerin (OPG) expression (80). RANKL belongs to the TNF superfamily and constitutes the dominant regulator of osteoclast formation and bone resorption, it binds RANK to activate osteoclasts (91). OPG, an osteoclast inhibitory factor, decreases osteoclast differentiation and activation by inhibiting RANKL-RANK interactions (92). 2-DG reverses CD4 CM-induced FLS activation, decreases the rate of glycolysis, and downregulates H-II, GLUT1, GLUT3, LDHA, PFKFB3, VEGF, and MMP-3 (34). 2-DG, LND, and BrPA are latent agents for the treatment of RA that inhibit HK-II through a variety of modes of action.

PFK-1/PFKFB3

Analyses of FLSs and ST by RT-qPCR, Western Blot, and IHC have shown increased transcription and expression rates of PFKFB3 in patients with RA compared with the rates in patients with OA. PFKFB1 mRNA expression has not been detected in FLSs, and the expression levels of PFKFB2 and PFKFB4 are similar in the FLSs of patients with RA and OA (93). By contrast, Saeki N's research demonstrated the mRNA expression levels of Pfkfb1 and Pfkfb3 are up-regulated in murine arthritis tissue-derived synovial macrophages (ADSM) treated with arthritis tissue-derived SF (ADSF)-CM compared to the levels in ADSM treated with ADSM-CM and normal tissue-derived SF (NDSF)-CM (94). PFK15 is a small molecule PFKFB3 inhibitor with potent anti-PFKFB3 activity (95). PFKFB3 inhibition by PFK15 or PFKFB3 siRNA have been used to evaluate the role of PFKFB3 in the pathogenesis of RA. PFKFB3 inhibition reduces the expression of the pro-inflammatory cytokines IL-8 and IL-6, and of the

chemokines CCL2 and CXCL10 in RA FLSs; also, PFKFB3 inhibition prevents cell proliferation, migration, and invasion (93). PFK15 inhibits the TNF-α-induced activation of NF-κB, p38, JNK, and ERK MAPK signaling in RA FLSs (93). TNF-α can promote PFKFB3 mRNA expression in primary RA FLSs through STAT3 phosphorylation in combination with OSM (67). In addition, lactate levels in RA FLSs increase after stimulation with TNF-α. However, this increase is inhibited by PFK15 or PFKFB3 siRNA treatment (93). During RA, lactate accumulation regulates the inflammatory immune response (26). To assess the association between lactate and pro-inflammatory cytokines and migration, FLSs were treated with PFK15 for 3 hours and subsequently incubated with 10 mM lactate; results showed that lactate reverses the inhibitory effect of PFKFB3 on the TNF-α-stimulated pro-inflammatory cytokines and chemokines in RA FLSs (93).

Studies have revealed that PFKFB3 silencing inhibits the nuclear translocation of NF-κB-p65 (96), while MAPK encourages PFKFB3 gene transcription and allosteric activation (97, 98). Addition of lactate to PFK15-pretreated RA FLSs reverses the observed reduction in nuclear translocation of p65 and the phosphorylation of IKK and IκBα. The p38, JNK, and ERK activity reductions induced by PFK15 are also reversed by the addition of lactate. This suggests that lactate is involved in the PFKFB3-mediated activation of NF-κB and MAPK in RA FLSs (93). Moreover, selective inhibition of STAT3 using STATTIC, a small molecule JAK/STAT inhibitor (99), in LPS-stimulated RA CD14+ monocytes results in almost complete suppression of the expression of inflammatory markers (such as TNF-α, IL-6, IL-1β, IL-27) and chemokines (such as CXCL10 and CXCL11), and it further diminishes the expression of the PFKFB3, HK-II, and GLUT1 proteins (100). In an animal model of RA, PFK15 treatment reduced the inflammatory cell infiltration and synovial proliferation and reduced the infiltration of pannus into calcified cartilage and bone of CIA mice compared with those of dimethyl sulphoxide (DMSO)-treated mice. The levels of IL-6 in serum and synovium decrease in PFK15-treated CIA as compared with the levels in the DMSO group mice (93).

The immunogenetics of RA suggest that abnormal T cell activation pathways exert a crucial part in the disease onset and/or persistence (101). Determinants of T cell differentiation and survival include antigen recognition, and metabolic mechanisms that provide energy and biosynthetic molecules for the cell building (102). A crucial RA feature of T cells is the transcriptional inhibition of the glycolytic enzyme PFKFB3, resulting in a slow glycolytic flux, reduced ATP and pyruvate production, and reduced extracellular environment acidification (103). Hohensinner PJ et al. summarized this as: in essence, they are "hungry" and energy deprived (104). Glycolytic activation in normal naive CD4+ T cells occurs in response to upregulation of GLUT, which increases glucose uptake and the activity of several rate-limiting enzymes, including PFK-1 (105). The evidence supports the hypothesis that the T cells from RA patients adopt a disparate metabolic program than healthy T cells, which is consistent with autoimmune effector functions dependent on specific energy sensing, energy production, and energy utilization pathways (106). Primary CD4 T cells from RA patients do not metabolize equal amounts of glucose, produce less intracellular ATP, and are inclined to apoptosis as are age-matched control cells (107). This differences are attributed to insufficient induction of PFKFB3. PFKFB3 deficiency occurs in the early stages of the T cell life cycle in patients with RA and has profound metabolic and functional consequences (5). However, this is dissimilar from some other types of autoimmune diseases. For example, CD4+ T cells exert a significant role in pathogenesis of Type 1 Diabetes (T1D) (108). In T1D, CD4+ T cells undergo metabolic reprogramming to the less efficient aerobic glycolysis, similar to that of highly proliferative malignant cells. Inhibition of PFKFB3 *via* PFK15 induces functional and metabolic exhaustion of CD4+ T cells in T1D (109).

To investigate whether RA T cells have an intrinsic glycolysis defect, Zhen Yang et al. measured the expression of 29 glycolytic related genes in activated CD4 T cells from patients with RA and matched controls 72 hours after T cell activation. They found RA T cells were defective in upregulating PFKFB3 compared to controls, with 50% lower transcript levels of PFKFB3 in T cells (107). Mimicking RA T cells, PFKFB3 knockdown decreased PFKFB3-specific transcripts by 50%, encouraging aggressive T cell infiltration with subsequent intense innate and adaptive inflammation, including TNFSF11 expression, indicating recruitment and retention of RANKL+ T cells and tissue production of IL- 1β, IL-6 and TNF (110). The expression of SH3PXD2A, the gene encoding the scaffold protein TKS5, is highly sensitive to metabolic interference. The PFKFB3 inhibitor 3PO (3-[3-pyridinyl]-1-[4-pyridinyl]-2-propen-1-one) on healthy CD4+ T cells mimics the slow glycolytic breakdown of RA T cells, which in turn increases the transcript level of SH3PXD2A (110). The dominating metabolite in inflamed joints is lactate, a decomposition product of glucose produced by metabolically active stromal, endothelial, and invading immune cells (26, 106). Lactate uptake into CD4+ T cells (mediated by the lactate transporter protein SLC5A12) induces remodeling of their effector phenotype, boosting IL-17 production and enhancing fatty acid synthesis *via* nuclear PKM2/STAT3 (111). However, RA T cells do not generate as much ATP and lactate as natural control T cells, they proliferate vigorously instead (112). Three outcome parameters were assessed after knockdown of PFKFB3 in normal T cells: lactate production, intracellular ATP levels, and apoptosis susceptibility. Intracellular ATP production and lactate output were reduced by 25-35%, similar to spontaneous PFKFB3-deficient RA T cells. When PFKFB3 is knocked down, the frequency of Annexin V+ and 7AAD+ cells increases significantly from 5% to 20% (107). Thus, CD4+ T cells in RA produce less lactate and ATP levels than T cells in healthy individuals, suggesting that glycolytic ATP production is the leading source of energy for CD4+ T cells (5).

Mitochondrial and lysosomal abnormalities ultimately lead to the generation of short-lived tissue-invasive effector T cells. This differentiation defect is established on a metabolic platform that shunts glucose from energy production to cell building and motility programs (113). Naive CD4 T cells from patients with RA express an altered pattern of glucose metabolizing enzymes,

resulting in slower glycolytic breakdown and increased pentose phosphate pathway (PPP) shunting, favoring anabolic over catabolic reactions (102, 114). A biological consequence of the PPP is the production of NADPH, which is essential for the reduction of oxidized glutathione to glutathione (GSH) and maintains the cellular redox homeostasis (115). What's more, NADPH protects cells from oxidative toxicity by decreasing ROS levels (116). The disproportionate increase in NADPH (50% higher NADPH levels in RA-derived cells) and reduced oxidized glutathione result in cells that are overconsumed with reactive oxygen species (ROS) and under reductive stress (107). ROS are by-products of oxygen metabolism known for their destructive potential, but contemporary evidence suggests that they play a role as secondary messengers regulating cellular functions through redox-activatable signaling systems (117). Activated and abundant intracellular ROS can regulate cell cycle progression, proliferation efficiency, and naive-to-memory conversion (114). Anti-proliferative measures that induce cell cycle arrest may represent therapeutic approaches against RA (118). ROS-deficient RA T cells are unable to maintain their naive phenotype and bypass the G2/M cell cycle checkpoint to overproliferate (due to surplus reducing equivalents that do not adequately activate the redox-sensitive kinase ATM) (114). CD4 + T cells are able to differentiate into Th1 cells that promote cellular immunity, Th2 cells that support humoral immunity, Th17 cells that promote mucosal immunity, or Treg cells that inhibit the function of effector T cells (26). Th1 and Th17 cells are involved in many autoimmune diseases (119). Insufficient activation of ATM leads to differentiation of T cells towards the Th1 and Th17 lineages, resulting in an excessive inflammatory phenotype (114). The expressions of phosphofructokinase p (PFKp) mRNA and protein are enhanced in RASF in response to ThCM stimulation (79). Inhibition of pro-inflammatory T cell differentiation by correcting reductive stress may suppress synovial inflammation (114).

PFKFB3 deficiency also diminishes the capacity of RA T cells to rely on autophagy as an alternative means of energy and biosynthetic precursor molecules. Knockdown and overexpression of PFKFB3 in TCR-stimulated T cell parental cells. PFKFB3-specific RNA interference inhibits autophagy. In contrast, forced overexpression of PFKFB3 rapidly accelerates autophagic activity (107). PFKFB3 silencing in healthy T cells and overexpression in RA T cells confirm the mechanistic link between glycolytic regulation and autophagy. These studies question the simplistic notion that posits autophagy as the default process for energy production in starving cells. Instead, in T cells, energy production appears to be a coordinated process in which multiple pathways function in parallel (120). To test whether PFKFB3 overexpression can protect RA T cells from autophagy-associated apoptosis, exogenous PFKFB3 reconstitution was combined with treatment with the autophagy inhibitor 3-MA. PFKFB3 overexpression partially rescued RA T cells from 3-MA-induced apoptosis, confirming the upstream position of PFKFB3 in the autophagy regulation stream (107). Moreover, the reduction of cellular ROS inhibits the activation-induced boost of H_2O_2 and superoxide. ROS have been associated with enhanced autophagy as a survival strategy. Thus, decreased ROS levels may be an additional mechanism impairing autophagy in RA T cells (57). Essentially, the T cells of patients with RA (even those cells in a naive state) undergo a metabolic reorganization in the presence of insufficient upregulation of the glycolytic enzyme PFKFB3 that leaves them energy deficient, ROS and autophagy deficient, apoptosis sensitive, and senescence prone (107).

The expression levels of PFKFB3 can vary in different tissues. PFKFB3's expression in the normal human body is in a homeostatic state that gets disrupted by either overexpression in FLSs or underexpression in RA T cells (**Figure 2**). By contrast, inhibition of PFKFB3 expression in RA FLSs may effectively improve RA symptoms by inhibiting glycolysis (93). Forced overexpression of PFKFB3 in RA T cells restores the glycolytic flux and protects cells from excessive apoptosis (107). Therefore, controlling the glycolytic pathway by targeting PFKFB3 to treat RA may be possible, but the choice of therapeutic modality and the dosages need to be further investigating.

PKM2

A MALDI-TOF-MS analysis of 1633 and 1603 protein spots in the synovial FLSs of patients with RA and controls (respectively) showed that the expression of PKM1/M2 protein was more than 3-fold higher in RA FLSs than in control FLSs. In addition, a Western blot assay demonstrated that the PKM2 expression results were consistent with the proteomic analysis (121). PK expression was high in RA synovial cells, indicating that RA STs present increased glycolytic activity (122). Phosphorylated PKM2 (p-PKM2) expression is increased in FLSs and the ST of patients with RA (123). PKM2 expression is higher in the lining and sublining layer, and vascular system of the RA ST than in the OA ST (124). siRNA transfection of PKM2 results in decreased expression of PKM2 and p-PKM2, which in turn results in decreased migration, invasion, expression of inflammatory factors (such as IL-1β, IL-6, and IL-8), glucose uptake and lactate secretion, and expression of LDHA, PDK1, and GLUT1 (123). Studies have described an altered glucose metabolism in RA. Pkm2 has been found overexpressed in ED1-positive macrophages (Mφ) in the spleen and ST of pristane-induced arthritis (PIA) rats by immunofluorescence, Western blots, and RT-qPCR; the overexpressed Pkm2 promotes Mφ activation *via* Stat1 signaling (125). Patient-derived Mø created higher levels of ATP with an interesting RA Mø hierarchy (126). Glycolysis is also upregulated in RA patient-derived Mφ, as are rate-limiting enzymes such as PKM2, PFKFB3, and HK-II, and GLUT1 and GLUT3 (127). GSK-3β inhibition promotes mitochondrial activity, and it enhances ATP synthesis and ROS release in RA patient-derived Mφ. This metabolic constellation leads to a cytoplasmic-to-nucleus translocation of PKM2 with functional consequences that include PKM2-dependent activation of STAT3, which promotes the production and secretion of pro-inflammatory cytokines such as IL-6 and IL-1 (128). Toll-like receptor 2 (TLR2)-activation regulates bioenergetic profile

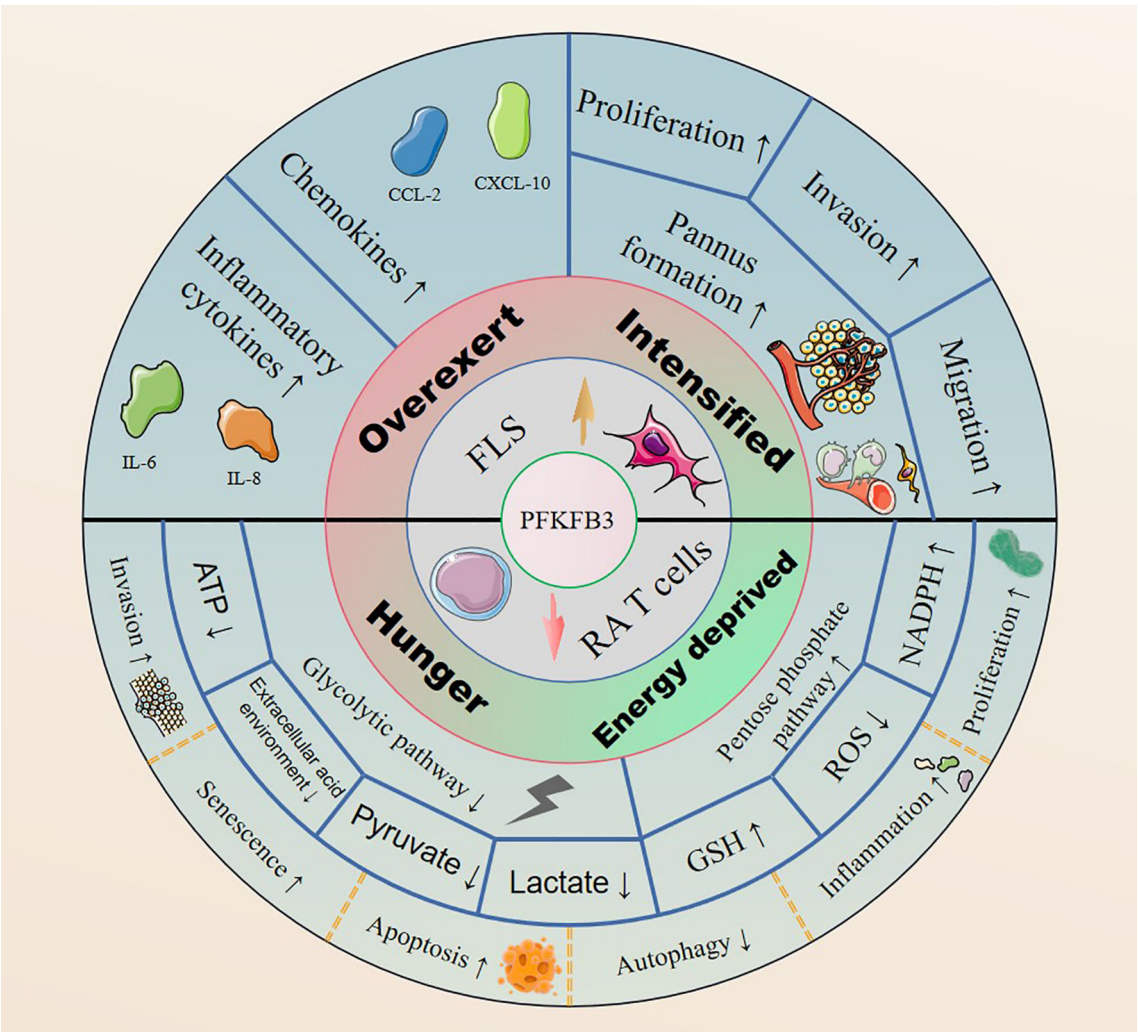


FIGURE 2 | PFKFB3 is highly expressed in RA FLSs, it upregulates the expression of inflammatory cytokines and chemokines, and facilitates cell proliferation, invasion and migration by promoting pannus formation. In contrast, PFKFB3 is expressed at low levels in RA T cells, generating an RA pathological phenotype with an inhibited glycolytic pathway and enhanced PPP shunting.

changes in primary RASFs consisting of PKM2 nuclear translocation, mitochondrial respiration and ATP synthesis reductions, and glycolysis increases (124). Inflammatory tissue lactate levels in CD4+ T cells induce IL17 expression through nuclear PKM2 and fatty acid synthesis-mediated STAT3 phosphorylation (111). PKM2 is significantly upregulated in activated CD4+ T cells and is required for pro-inflammatory Th17 and Th1 cell differentiation (129). The increase in aerobic glycolysis mediated by activated T helper cells pushes SFs towards an inflammatory phenotype. In response to ThCM stimulation, the mRNA and protein expressions of PKM2 and lactate dehydrogenase A (LDH-A) in RASFs get induced (79).

PDPN is highly expressed in cadherin-11-positive cells throughout the RA synovial lining layer. This expression is most pronounced in sections with hyperplasia and high matrix metalloproteinase-9 (MMP-9) expression, where it coincides with upregulation of α-smooth muscle actin (α-sma) (130). CD45-PDPN+ FLS cells from the K/BxN mouse serum transfer model of arthritis and enriched in culture show significantly higher expressions of PKM2 mRNA, GLUT1, LDHA and ENO1 than CD45-PDPN- cells (33). A comparative transcriptomic analysis has shown PMK2 upregulation in human IgG-trained monocytes compared to its expression in controls (40). SUMOylation is an important modification with a regulatory role in cellular responses to various types of stress including osmotic, hypoxic and oxidative stress (131). SUMOylation occurs through a series of stress-induced biochemical responses (132). Oxidative stress is a contributing factor in the pathogenesis of RA and influences the development of the RA process through multiple pathways (133, 134). RA as an autoimmune disease and SUMOylation is a novel pathway involving its phenotypic differences (135). Wang C et al. found increased expression of SUMO-activating enzyme subunit 1 (SAE1)

and ubiquitin like modifier activating enzyme 2 (UBA2) in FLSs and ST of patients with RA, where SAE1/UBA2 regulated the glycolytic pathway and biological functions of the RA FLSs through SUMOylation-mediated PKM2 phosphorylation (123). For instance, in lung cancer cells, SUMO1 promotes PKM2-dependent glycolysis. SUMO1 modification of PKM2 has been proposed as a therapeutic target against lung cancer (136). In RA FLSs, treatment by siRNA knockdown of SAE1 or UBA2 with GA, an inhibitor of SAE1/UBA2-mediated SUMOylation, resulted in reduced glycolysis, inflammatory and aggressive phenotype (123).

In an experiment where Dark Agouti (DA) rats were treated intraperitoneally with either shikonin or an RNA-interfering plasmid of PKM2 and a negative control plasmid, respectively (125). Shikonin is a specific PKM2 inhibitor that inhibits cellular aerobic glycolysis and cell proliferation by reducing PKM2 activity (137, 138). Pkm2 intervention reduced the severity of PIA, including macroscopic arthritis scores, perimeter changes of midpaw, synovitis, and bone and cartilage destruction, and reduced ST in rat ED1 and p-Stat1-positive cell populations (125). Inhibition of PKM2 reduces phosphorylation levels of STAT1 and STAT3 and inhibits the transcription of downstream genes regulating pro-inflammatory cytokines, thereby alleviating experimental arthritis (129). In classically activated rat and mouse Mφ, silencing Pkm2 by RNA interference results in less production of TNF-α and Il-1β *via* Stat1 signaling (125). Blocking ICOS (a member of the CD28 superfamily) signaling during TH cell and B cell co-culture successfully inhibits the upregulation of the key rate-limiting glycolytic enzymes PKM2, HK-II, PFKFB3, and PFKL in TH cells (71).

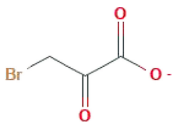
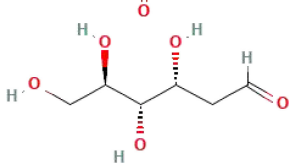
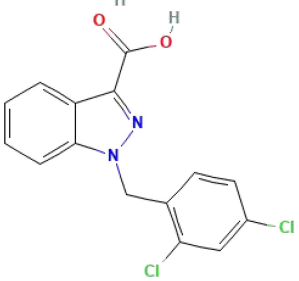
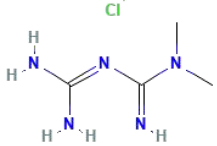
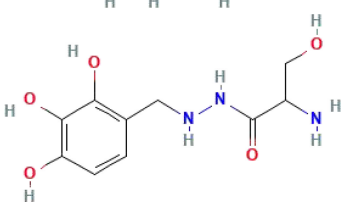
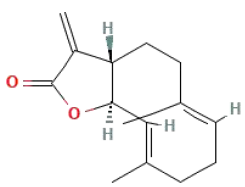
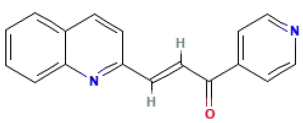
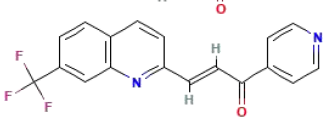
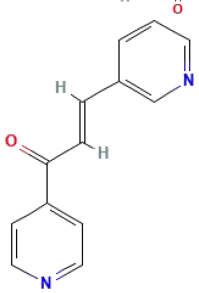
FUTURE DIRECTION

Metabolic disorders and changes in the intracellular levels of specific metabolites are associated with an inflammatory phenotype of immune cells that has been associated with autoimmune diseases such as systemic lupus erythematosus, RA, multiple sclerosis, and diabetes mellitus (139). Targeting key metabolism players (such as mTOR by rapamycin, HKs by 2-DG, and AMP-activated protein kinase by metformin) can improve autoimmune inflammation (139–142). Tofacitinib is the first disease-modifying anti-rheumatic drug (DMARD) approved for the treatment of RA (143). Its efficacy and safety profile as an oral Janus kinase inhibitor for the treatment of RA is promising (144). Tofacitinib inhibits the mRNA expression of HK-II, GLUT1, PFKFB3, 3′-phosphoinositide-dependent protein kinase 1 (PDK-1), and GSK-3α in RA whole-tissue synovial organotypic explants *ex vivo*, as well as the expression of the pro-inflammatory cytokines IL-6, IL-8, and IL-1β, the key adhesion molecule soluble intercellular adhesion molecule 1 (sICAM), and the growth factors TIE-2 and vascular endothelial growth factor (VEGF) (145). The successful application of DMARDs has provided insights for the potentially effective development of therapeutic agents for RA based on modulators of key glycolysis rate-limiting enzymes (**Table 1**).

HK-II blockade represents a novel therapeutic strategy for RA that ameliorates inflammation and cartilage damage in a K/BxN arthritis model (164). 2-DG, a non-competitive inhibitor of HKs, reverses LPS-induced enhancement of glycolytic activity and inhibits the expression of vital inflammatory cytokines (IL1-β, IL6) and matrix metalloproteinases (MMP-1, MMP-3) in the RA pathogenesis (75). Targeting the cAMP response element binding protein (CREB) is a potential idea to treat RA (165). Aberrant cAMP/CREB signaling has a crucial role in inducing imbalance in Mø polarization (166), and in promoting osteoclast differentiation in RA (167). 2-DG inhibits phosphorylation of the LPS-enhanced transcription factor CREB (75). A preventive glycolytic pathway inhibition with 2-DG significantly limits antibody-mediated pathology in the K/BxN model of RA, most likely *via* its effects on Tfh cells. Metformin2 has been shown to inhibit HK-II activity and induce HK-II dissociation from mitochondria, but it has no inhibitory effects on HK-IV (47, 148). However, the combination of metformin 2 and 2-DG has little beneficial effects in the course of RA treatment. The addition of metformin 2 inhibits the compensatory switch to the oxidation of other substrates, limiting the efficacy of the glycolytic pathway inhibition (81). 3-BrPA is a halogenated analogue of pyruvate known for forty years as an alkylating agent that reacts with the thiol groups of many proteins. It is based on impairing the energy metabolism of tumor cells by inhibiting enzymes in the glycolysis, HK-II, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, and oxidative phosphorylation, succinate dehydrogenase (168). 3-BrPA effectively ameliorates RA-related symptoms of CIA due to the overlap between ICOS signaling, phosphoinositide 3-kinase (PI3K) signaling and glucose metabolism (169). Addition of 3-BrPA to block glycolysis in Th cell-stimulated SF significantly reduces lactate production and the ratio of glycolysis to oxidative glucose metabolism. In addition, 3-BrPA suppresses the pro-inflammatory phenotype by strongly reducing the secretion of IL-6 and MMP-3 in ThCM-stimulated SF (79). In conclusion, overexpression of HK2 in FLS is closely related to inflammatory phenotypes of RA, and therapeutic interventions targeting HK2 by inhibitors such as 2-DG and 3-BrPA are important for the future DMARDs exploitation. The discovery of novel inhibitors with their specific mechanisms of action is of biomedical importance to explore optimal therapies for the prevention and treatment of RA.

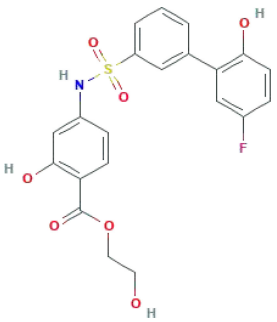
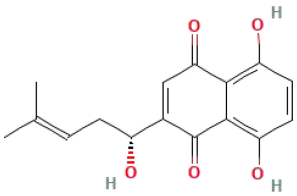
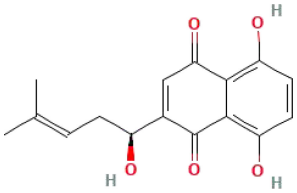
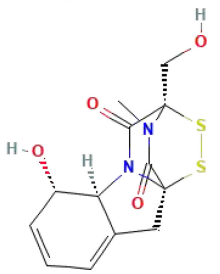
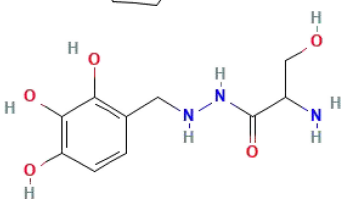
The *in vivo* effects of PFK15 (a selective PFKFB3 inhibitor) on RA synovial inflammation and joint destruction have been evaluated in CIA mice: Intraperitoneal injection of PFK15 reduces the increase in clinical scores compared to DMSO treatment (93). In RASFs, blockade of glycolysis by 3PO, another PFKFB3 inhibitor (47), reverses TLR2-induced pro-inflammatory mechanisms, including invasion, migration, and secretion of IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), normal T-cell expressed and secreted (RANTES), and growth-regulated oncogene alpha (GRO-α) (124). Attempts to modify the pathological process of RA by targeting PFKFB3 are attractive, but the diverse expression of PFKFB3 in different tissues of RA poses a challenge to such studies. Treatment of RA with

TABLE 1 | Some common molecular inhibitors of glycolysis rate-limiting enzymes.

Molecules	Molecular Formula	Molecular Weight	2D Structure	Notes	References
HK-II					
3-BrPA	$C_3H_3BrO_3$	166.96		The structural similarity to lactate	(146)
2-DG	$C_6H_{12}O_5$	164.16		Noncompetitive antagonist	(75, 85)
Lonidamine	$C_{15}H_{10}Cl_2N_2O_2$	321.2			(147)
Metformin	$C_4H_{11}N_5$	129.16		Metformin could offset the increased compensatory effect in oxidative respiration in HK-II silenced cells	(148, 149)
Benserazide	$C_{10}H_{15}N_3O_5$	257.24			(150)
Costunolide	$C_{15}H_{20}O_2$	232.32		A well-characterized sesquiterpene lactone compound	(151)
PFKFB3					
PFK-15	$C_{17}H_{12}N_2O$	260.29			(47, 152)
PFK-158	$C_{18}H_{11}F_3N_2O$	328.3		The first-in-man PFKFB3 inhibitor to be evaluated in a phase I clinical trial	(47, 153, 154)
3PO	$C_{13}H_{10}N_2O$	210.23		The mechanism of action is controversial	(155–157)

(Continued)

TABLE 1 | Continued

Molecules	Molecular Formula	Molecular Weight	2D Structure	Notes	References
KAN0438757	$C_{21}H_{18}FNO_7S$	447.4			(158, 159)
PKM2					
Shikonin	$C_{16}H_{16}O_5$	288.29		Currently the most commonly used PKM2 inhibitors	(47, 160)
Alkannin	$C_{16}H_{16}O_5$	288.29		The enantiomeric isomer of shikonin	(161)
Gliotoxin	$C_{13}H_{14}N_2O_4S_2$	326.4		IC_{50} = 22.64 μM	(162)
Benserazide	$C_{10}H_{15}N_3O_5$	257.24			(163)

PFKFB3 inhibitors such as PFK15 or 3PO may reduce pathological phenotypes of RA, but whether this process further aggravates the intrinsic PFKFB3 defect in RA CD T cells, further studies are needed to gain insight into the detailed mechanism of action.

Daurinol is a novel topoisomerase II inhibitor isolated from the traditional medicinal plant Haplophyllum dauricum (170). Real-time PCR analysis has shown that daurinol treatment of murine CD4+ T cells cultured under Th17-polarizing conditions downregulates genes encoding for various molecules involved in aerobic glycolysis, such as HK-II, PKM, GLUT1, monocarboxylic acid transporter member 4 (MCT4), GPI, triosephosphate isomerase (TPI), Eno1, compared to vehicle-treated cells. Moreover, treatment with daurinol reduces the development of inflammatory arthritis in a dose-dependent manner and inhibits osteoclastogenesis *in vitro* and *in vivo* (171).

Studies have found no significant changes in kidney, serum creatinine, liver, ALT and AST, or serum glucose in mice treated with PFK15. Additionally, there were no significant histopathological alterations in the liver or kidneys removed from PFK15-treated mice compared to those in DMSO-treated groups. These data demonstrate the safety of PFK15 treatment in CIA mice (93). The viability of SFs was not affected by the 3-BrPa concentrations used for experiments (79). However, other

studies have reported bloody ascites, abdominal distention, and organ cirrhosis in some rats treated with 3-BrPA (172). In rabbits, selective intra-arterial injection of 25 mM 3-BrPA can cause considerable toxicity in the liver and in the gastrointestinal system, and this dose-dependent toxicity can lead to death at high doses (173). Therefore, toxicological studies during the development of these drugs are needed to be able to direct necessary drug modifications while maintaining their efficacy and minimizing adverse effects.

CONCLUSION

Studies have emphasized the association between inflammatory tissue damage (induced by cytokines, chemokines and ROS) and RA pathogenesis. However, the overwhelming majority of life activities are energy-dependent, and metabolism changes are usually prominent disease effectors. "Pull one hair and the whole body is affected"; therefore, exploring the pathogenesis of RA from a metabolic energy point of view is a valid approach. Our understanding of the role of glucose metabolism in RA is incomplete, but studies on glycolytic rate-limiting enzymes have greatly expanded our understanding of the energy metabolism interaction network. The glycolysis rate-limiting enzymes HK-II, PFK-1/PFKFB3, and PKM2 can act as regulators of inflammatory factors, chemokines, and growth factors, which in turn play important roles in the development of RA. Studies on HK-II, PFK-1/PFKFB3 and PKM2 in the pathogenesis of RA have facilitated the development of new DMARDs targeting rate-limiting enzymes of the glycolytic pathway to implement new anti-rheumatic therapies. In conclusion, verifying whether glycolysis is important in the pathogenesis of RA and exploring the mechanisms of key glycolytic pathway rate-limiting enzymes in RA is a valid and promising avenue.

AUTHOR CONTRIBUTIONS

JZ and JT have contributed equally to this work and share first authorship. JZ, JT, and PS contributed to conception and design. JZ and JT wrote the manuscript and figures. ML and ZZ collected the data and designed the figures. YL, HT, EL, BG, and TL performed literature search, and provided valuable comments. All authors contributed to the article and approved the submitted version.

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The Role of Immunometabolism in the Pathogenesis of Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder in which pathogenic abnormalities within both the innate and adaptive immune response have been described. In order to activated, proliferate and maintain this immunological response a drastic upregulation in energy metabolism is required. Recently, a greater understanding of these changes in cellular bioenergetics have provided new insight into the links between immune response and the pathogenesis of a number of diseases, ranging from cancer to diabetes and multiple sclerosis. In this review, we highlight the latest understanding of the role of immunometabolism in SLE with particular focus on the role of abnormal mitochondrial function, lipid metabolism, and mTOR signaling in the immunological phenomenon observed in the SLE. We also consider what implications this has for future therapeutic options in the management of the disease in future.

Keywords: systemic lupus erythematosus (SLE), immunometabolism, mitochondria, lipid metabolism, T cell, B cell, monocyte, autoimmunity

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1 INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder characterized by the formation of autoantibodies directed against nuclear components. Clinically it may present with a wide array of manifestations and a variety of immunological phenomenon. In spite of recent advances in the management of the disease, therapeutic options remain limited and are often untargeted (1).

The underlying pathogenesis of the disease is poorly understood although abnormal innate and adaptive immune responses have been implicated (2) and is summarized in **Figure 1**. Observed pathogenic innate responses include dysfunction of macrophages that appear to be defective in removing apoptotic material. It has been suggested that a result of this impaired clearance induces antigenicity to exposed cellular debris including nuclear components (3, 4). Macrophages (and their precursors, monocytes) have also been noted to display abnormal polarization in both animal models and in patients with SLE (5, 6). Abnormal neutrophil function has also been observed in the pathogenesis of SLE (7), with recent evidence also implicating the production of neutrophil extracellular traps (NETs) disease development (8, 9). In addition, plasmacytoid dendritic cells

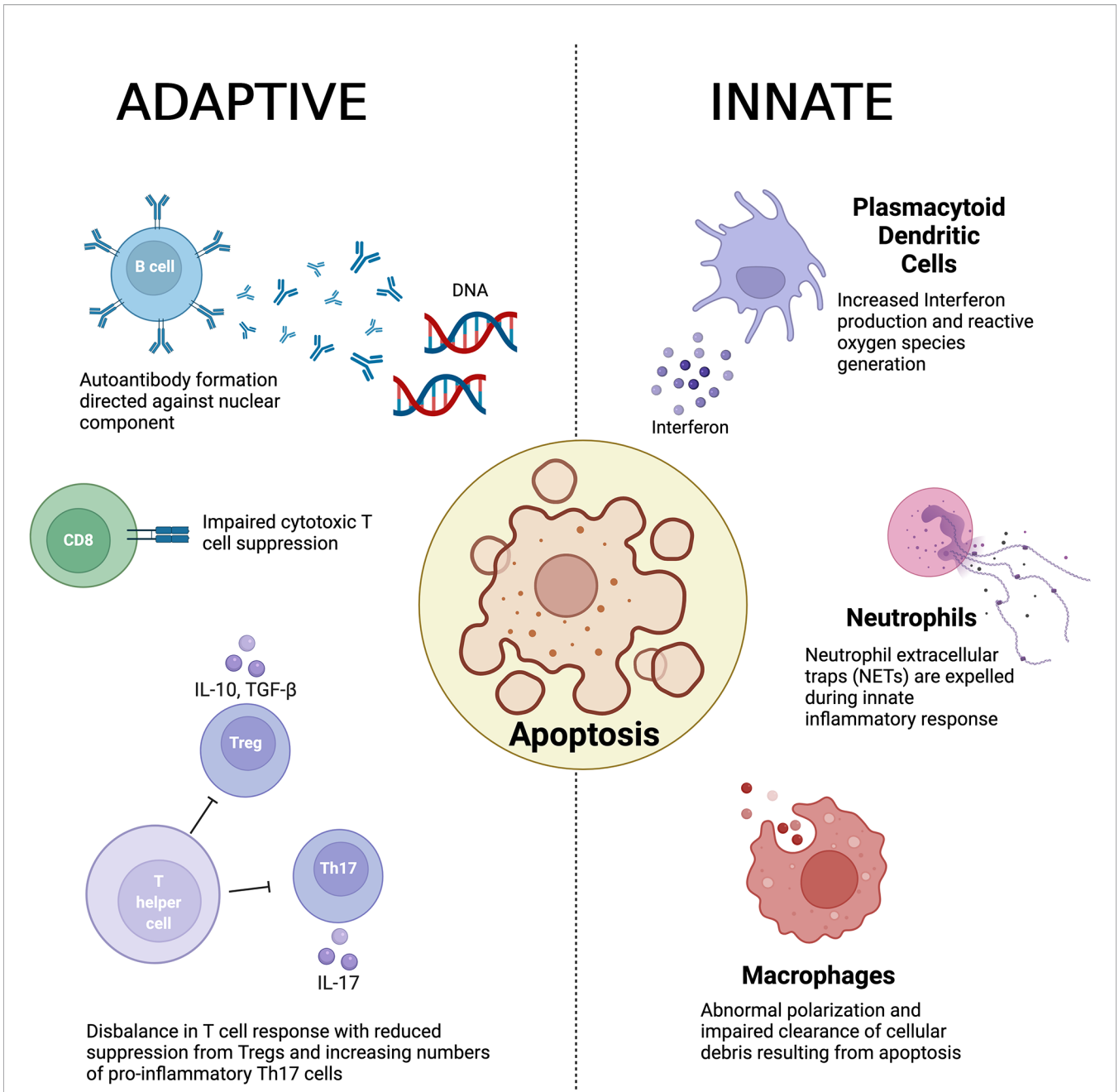

FIGURE 1 | A summary of the key immunological abnormalities described in the pathogenesis of systemic lupus erythematosus.

(pDCs) have been identified as another key innate immune driver that has been shown to play a key role in the production in interferon (INF) and generate reactive oxygen species (ROS) (10, 11).

The defective clearance of apoptotic matter by dysfunction in the innate immune response is believed to result in a loss of self-tolerance and in turn culminates in auto-antibody formation by B cells, which have been noted to show abnormal activation as well as aberrant expression. In turn this results in generation of anti-nuclear antibodies (ANA) and anti-double stranded DNA (anti-dsDNA) antibodies (4), a hallmark of the disease (12). Furthermore, B cells play a vital role in the development of immune complexes that contain self-antigen, which are deposited within various tissue. The resultant engagement of the Fc receptor and activation of the complement cascade in turn promotes inflammation (12, 13).

T cells also play a central role in the adaptive immune response and a number of abnormalities have been observed in the pathogenesis of SLE in both propagation and maintenance of the immune response. Regulatory T cells (Tregs) play a vital role

in maintaining immune homeostasis in health through suppressing a hyperactive immune response. In SLE, an imbalance between pro-inflammatory T helper 17 (Th17) cells and Tregs has been demonstrated as a key contributor to the loss of self-tolerance (14, 15). Double negative T cells derived from patients with SLE have been shown to be an important producer of Interleukin(IL)-17 (16), whilst $CD8^+$ cytotoxic T cells also demonstrate impaired suppressive function in SLE (17, 18).

The precise mechanism through which this occurs is not known, however, it is felt to involve a combination of genetic factors (19) and environmental triggers (including ultraviolet radiation and possible virus exposure) (20). In addition, given that the disease has a marked female predominance (9:1) there is a growing appreciation of the influence of sex hormones on the autoimmune responses observed (21).

More recently, abnormalities in immunometabolism have been detailed in the defective immune response seen in a variety of disease states including malignancy (22), diabetes (23, 24) and multiple sclerosis (25). This has shed new light on the way in which interactions between immunological and metabolic processes may induce the disease state. Immunometabolism also presents a variety of novel therapeutic targets for treatment in the future. In this review, we highlight the latest knowledge in the field of immunometabolism in SLE and describe how this may in turn translate into future clinical care.

2 ENERGY METABOLISM

Immune cell activation and proliferation requires significant upregulation in terms of energy metabolism in order to induce and maintain the immunological response. Energy metabolism is dependent on two key pathways to generate adenosine triphosphate (ATP); glycolysis and oxidative phosphorylation (OXPHOS). In health, glycolytic pathways convert glucose to pyruvate and hydrogen ions that are essential for ATP synthesis. In comparison to glycolysis, OXPHOS occurs at the site of the electron transport chain (ETC) on the inner mitochondrial membrane.

3 MITOCHONDRIAL DYSFUNCTION IN SLE

Mitochondria are double membrane-bound organelles that generate cellular in the form of ATP, as well as regulating apoptosis. They cannot be replicated by the cell but are formed by binary fission. Each mitochondrion contains a set of circular genome that encode for RNA and proteins which are essential for mitochondrial oxidative phosphorylation. Here we explore the role of mitochondrial dysfunction in the immunopathogenesis of SLE.

3.1 B Cells (Auto-Antibodies)

The release of mitochondrial DNA (mtDNA) is a noted marker of acute and chronic disease (26, 27). MtDNA activates the innate immune system and can be a target for SLE associated autoantibodies. To identify mitochondrial autoantibodies, a study of 86 SLE patients and 30 healthy controls determined the occurrence of AmtRNA-IgG and Amt-IgM by quantitative ELISA. Both mtRNA immunoglobulins were significantly increased in the SLE patients ($p=0.0002$ and $p=0.0493$, respectively) (28). Antimitochondrial-M2 antibodies (AMA-M2) are associated with Primary biliary cirrhosis (PBC) and have been detected at increased levels in subacute cutaneous lupus erythematosus (SCLE) patients (29). In a study of 204 SLE patients, plasma samples were analyzed by ELISA for levels of anti-wMITO. Increased levels correlated to measures of disease activity SLEDAI-2K ($p<0.0001$) and SLAM ($p=0.006$), anti-dsDNA ($p<0.0001$) and other clinical measures (30). The presence of mitochondrial autoantibodies supports the role of mitochondrial damage in the pathology of SLE. Abnormal mitochondrial function in B cells derived from patients with SLE has more recently been identified. A study of 41 SLE patients and 29 healthy controls found that B cells derived from patients with lupus showed enhanced mitochondrial membrane hyperpolarization, suggesting that these cells are primed for activation. Furthermore, the degree of hyperpolarization correlated with SLEDAI-2K. The authors also noted that glutaminolysis, which generates essential metabolites for OXPHOS, played a key role in the differentiation into plasmablasts (31).

3.2 T Cells

T cell dysfunction in SLE could be attributed to mitochondrial hyperpolarization, reactive oxygen intermediates and reduced levels of ATP (32). Previous studies have demonstrated that T cells are dependent upon glycolytic energy production for the induction of the inflammatory effector response. However, mitochondrial metabolism has also been implicated in the more chronic activation of T cells observed in SLE (33). There is also evidence that in SLE, T cells have increased mitochondrial mass and size both due to defective mitophagy and increased biogenesis (34). Mitochondria contain a reservoir of Ca^{2+} ions. Increased mitochondrial mass and membrane potential ($\uparrow\Delta\psi_m$) in SLE T-cells can increase intracytosolic Ca^{2+} fluxing when stimulated, in rapamycin treated SLE this was regulated (35). SLE but not healthy control T cells undergo necrosis after CD3/CD28 stimulation due to chronic mitochondrial hyperpolarization (MHP) (36). SLE T cell necrosis can also be caused by increased production of ROS and ATP depletion. Necrotic debris can induce a pro-inflammatory interferon response in plasmacytoid dendritic cells (pDCs) (37). Nitric oxide is released by monocytes which is a driver of MHP. In turn, T cells express intrinsic nitric oxide synthase (iNOS). A meta-analysis showed that there is higher expression of iNOS at both the mRNA and protein level (38). In T cells there is an increased response to IL-15 which in turn could contribute to increased mitochondrial biogenesis, though further studies need to be conducted to establish the role of cytokines in mitochondrial dysfunction (39). The status of T cell metabolic programming can be determined by mitochondrial remodeling as a signaling

mechanism. This remodeling can change mitochondrial fusion to fission and equally oxidative phosphorylation to aerobic glycolysis. These mechanisms are distinct between effector and memory T cells (40). In SLE T cell oxidative stress is pronounced, increased expression of the mitochondrial protein genes VDAC1 and SOD2 are associated with an increase in mitochondrial mass and oxidative stress (36, 41). Other genes associated with mitochondrial dysfunction in SLE are ESRRG, a mitochondrial metabolism regulator, and UCP2, involved in ROS generation and ATP production (42, 43). It has been shown that due to oxidative stress, surface glycoprotein CD3ζ chain is damaged and replaced by FcεRIγ chain in SLE T cells. The TCR/CD3/FcεRIγ complex is up-regulated in effector T-cells and has been shown to be increased in SLE T cells (44).

More recently there is growing evidence to suggest that targeting T cell metabolism may be a potential therapeutic target for the management of SLE in the future. N-acetylcysteine (NAC) is used clinically as an anti-oxidant therapy and could have a role in targeting oxidative stress in SLE. In a randomized, double-blind, placebo trial of NAC in 36 SLE patients there was significant clinical improvement on 2.4 g and 4.8 g dose in terms of Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) at 1 months (p=0.0007), 2 months (p=0.0009), 3 months (p=0.003) and 4 months (p=0.0046). This study showed that NAC successfully blocks the mammalian target of rapamycin (mTOR) in T cells (45). Combination treatment with Metformin (a mitochondrial metabolism inhibitor, more commonly used in the treatment of diabetes) and 2-deoxy-d-glucose (2DG) in lupus prone mice has also shown promise, resulting in reduced INF-γ production. In addition, mice treated with this therapy showed a reversal of the disease process and reduction in both anti-dsDNA and ANA titers (33). Furthermore, targeting T cell glycolysis has also been demonstrated to specifically reduce the production of follicular helper T (T_{FH}) cells, which have been implicated in the pathogenesis of SLE (46). Glycolysis has also been investigated as a therapeutic target in another subtype of $CD4^+$ T cells, Th17 cells that predominantly use glycolysis for energy metabolism. In a study of T cell derived patients with SLE, it was found that by blocking pyruvate dehydrogenase phosphatase catalytic subunit 2 (PDP2), a vital enzyme in the glycolytic pathway, it was possible to limit Th17 differentiation (47). Inhibition of glutaminolysis (a key source of energy for effector T cells) has also been shown to impact on glycolytic pathways and result in a similar reduction in Th17 differentiation in samples derived from both patients and lupus prone mice, thus suggesting this could also be a potential metabolic therapeutic target in the future Furthermore, the authors also found that inhibition of glutaminolysis reduced Th17 Hypoxia Inducible Factor (HIF)-1α levels, which plays a central role in Th17 development (48). These studies suggest that through augmentation of T cell metabolic pathways it may be possible impair abnormal T cell cytokine production and differentiation.

3.3 Neutrophils

Neutrophils taken from SLE patients and healthy control INF primed neutrophils extrude high levels of oxidized mitochondrial nucleoids that act as potent interferogenic complexes, this affect could be due to failed mitophagy. TFAM enables neutrophil-derived mtDNA to be internalized and in turn can become a potent pDC activator. INF/αRNP can divert extruded oxidized mtDNA into lysosomes. In turn this drives the formation of ox mtDNA/TFAM complexes which then accumulate in the cytosol and the mitochondria itself. In SLE there are high levels of these oxidized nucleoids in the blood and the neutrophils themselves. In addition, autoantibodies against oxidized mtDNA are present in some SLE patients, proposing ox mtDNA as an autoantigen (49). In SLE and juvenile dermatomyositis (JDM) there are increased levels of neutrophil extracellular traps (NETs) and these have been found to contain mtDNA (50). In SLE, mitochondrial ROS are necessary for NETosis of low density granulocytes (8). Inhibiting mtROS may reduce the INF response in these diseases. In SLE, neutrophils are key to activating the inflammatory mechanism of mtDNA.

3.4 Monocytes

A complex study of SLE monocytes showed that excessive INFα in SLE damaged mitochondrial respiration. In the monocytes, SLE compared to healthy control, the results showed increased mitochondrial membrane potential ($p<0.0005$), PINK1 mRNA ($p<0.005$), mtDNA content ($p<0.005$) and JC1 aggregates ($p<0.05$). These results were re-produced when healthy donor monocytes were cultured with INFα for 18hrs (51). This delineates the cyclical relationship of INFα with mitochondrial dysfunction.

Across the innate and adaptive immune cells there is strong evidence that mitochondrial dysfunction plays an important role in SLE immunopathogenesis. Therefore, is an important therapeutic target to consider.

4 ABNORMAL mTOR SIGNALING IN SLE

Another important group of substrates involved immune cell metabolism are proteins, peptides and amino acids. There is now a growing appreciation of their role in autoimmunity, in particular in relation to their effects on T cell differentiation and function. This relies upon the activation of the serine-theonine protein kinase, mTOR, which exists in two separate complexes known as mTORC1 and mTORC2 (52). Furthermore, mTOR is essential in the maintenance of immune cell homeostasis through its roles in inducing metabolic signals that in turn drive cell growth, activation, proliferation and survival (52–55).

In health, mTORC1 plays a key role in the suppressive function of Tregs, a mechanism that has been demonstrated to be abnormal in many autoimmune conditions (55–57). In SLE, abnormalities within mTOR pathways have been shown to induce immune cell differentiation and proliferation, secretion of pro-inflammatory cytokines and increased ROS production (58). Previous studies have demonstrated the role of mTORC1 activation in $CD4^+$ T cells derived from patients with SLE (53) and has suggested that this may be due to mitochondrial dysfunction (45). More specifically, mTOR abnormalities have been reported to alter the balance between Th17 T cells and

Tregs to the extent that it promotes a state autoimmunity (59). This increase in mTORC1 activity has been demonstrated following increased glycolysis and also associates with reduced levels of autophagy (54), which is impaired in the pathogenesis of SLE (60).

There is growing evidence that targeting mTOR may also be an effective treatment in the management of SLE clinically. Sirolimus (Rapamycin), an immunosuppressive agent used in preventing graft rejection in solid organ transplantation and known mTORC1 inhibitor, has already been studies as a potential treatment for SLE. Inhibition of mTOR with Rapamycin has already been shown to reduced INF production by monocytes derived from patients with SLE *in vitro* (61). In a previous open-label study in 43 patients with active SLE found that following 12 months of treatment with Sirolimus, disease activity was significantly reduced, and concurrent steroid dose was also significantly lower following one year of treatment. Immunologically, Sirolimus was also noted to induce increased numbers of Tregs, which suggests a recovery immune homeostasis with the treatment. Furthermore, T cell produced IL-4 and IL-17 levels were also significantly lower following treatment (62). Although there is a lack of large randomized, placebo-controlled trials of the drug in lupus, a recent meta-analysis of nine studies containing a total of 145 patients concluded that Sirolimus showed promise as a treatment option. It was suggested that the drug was well tolerated (although hematological and mucocutaneous adverse events were the most frequently reported) (63). Inhibition of mTOR with Sirolimus additionally was associated with higher rates of dyslipidemia, which is important given the growing evidence for abnormalities in lipid metabolism in SLE.

5 LIPID METABOLISM IN SLE

The metabolism of lipids is a fundamental process used by immune cells for different energy demands, cell signaling and function. Lipids serve as precursors for bioactive metabolites and components of cellular membranes, which have both direct and indirect regulatory implications for signal transduction, gene regulation and cellular activation. Immune cell subsets have different metabolic demands for lipids, such as mitochondrial beta-oxidation of lipids for anti-inflammatory functions in regulatory T cells, against a higher dependency on glycolytic pathways for growth and proliferation in effector T cells (64). Dysregulated lipid metabolism has been heavily implicated in SLE at both the systemic and cellular level and both have been described in the context of cardiovascular comorbidities.

5.1 Lipid Metabolism and Cardiovascular Disease in SLE

Patients with SLE have an increased risk of developing cardiovascular disease (CVD) beyond traditional risk factors and CVD is a leading cause of mortality for patients (65). This CVD risk is largely due to dyslipidemia (altered lipid metabolism), a common feature of SLE (66). Dyslipidemia can accelerate atherosclerosis, the lipid build-up and chronic inflammation of the large arteries (67). This involves an imbalance between atherogenic low and very low density lipoproteins (LDL and VLDL), and atheroprotective high density lipoproteins (HDL) known to transport lipids too and away from atherosclerotic plaques respectively. Dyslipidemia in SLE includes both elevated LDL and reduced HDL (66, 68–70) which, along with chronic inflammation, accelerates atherosclerotic processes.

5.2 Lipid Metabolism in Immune Cell Function in SLE

Lipoprotein metabolism can also influence immune cell function and inflammation in SLE (71). It is well established that innate immune cells, including macrophages, take up oxidized (ox)LDL particles *via* scavenger receptors in atherosclerotic plaques, leading to lipid saturation, pro-inflammatory cytokine production, and recruitment of other inflammatory cells (72). This process could be exacerbated in SLE due to the increased circulating levels of LDL, thus, increasing atherosclerosis progression. In addition, macrophage function is likely to be altered *via* direct lipid activation of the nuclear liver-X-receptors (LXRs), which regulate cellular cholesterol levels and immune functions through transcriptional changes, such as those involved in IL-23 and IL-17 production and phagocytic pathways (73). The direct effect of a hyperlipidemic environment on the T cell inflammatory profile in SLE has also been investigated (74, 75) and oxLDL has been shown to increase T cell activation indirectly through monocyte uptake (76). T cells are key for the adaptive immune system and upon activation, T cells proliferate, migrate to inflamed sites, such as atherosclerotic plaques, and acquire functions that mediate the immune response (77). The T cell plasma membrane (PM) is made up abundantly of lipids, such as cholesterol and phospholipids, and proteins, both of which are essential to facilitate cellular signaling for inflammatory outcomes such as cytokine production and proliferation (78). Patients with SLE and other autoimmune diseases have altered T cell membrane cholesterol and glycosphingolipid levels (79, 80). This alters the composition of signaling platforms called lipid rafts, where T cell receptors provide stimulatory signals to control cellular function and inflammation (81, 82). This is partly due differences in the expression of genes responsible for lipid metabolism in SLE (71), however, this could also be due altered cellular altered uptake of cholesterol from LDL/VLDL and efflux of cholesterol to HDL; this process has been speculated in pathogenic mechanisms of multiple sclerosis (83). Altered lipid rafts have also been described in the context of dysfunctional B cell signaling in SLE (84). Altered lymphocyte function through dyslipidemia in SLE is also likely to be mediated through LXRs (82, 85, 86). Together, research strongly suggest that lipid metabolism could be targeted therapeutically to control cellular functions and inflammation, highlighting the need for a better use of lipid modification strategies in SLE.

5.3 Lipid Metabolism as a Therapeutic Target

Some conventional therapeutics currently being used to treat SLE have shown beneficial effects on lipids, including hydroxychloroquine on LDL lowering (87). Despite this, deaths associated with cardiovascular comorbidities are still high (65, 88) suggesting that additional, more specific lipid modifying therapies are in demand for patients with SLE. Despite statin trials in SLE showing mixed results regarding cardiovascular outcome measures (89–91), therapeutically lowering circulating lipid levels has been shown to improve autoimmune disease symptoms (92) and using these therapies to directly modify lipid rafts *in vitro* has also been shown to normalize signaling in T cells from SLE patients (80, 93).

Taken together, differences in lipid metabolism in patients with SLE contributes to disease pathogenesis, inflammation and CVD risk through atherosclerosis. Therapeutic intervention with lipid modifying drugs already approved for use worldwide, such as statins, could be promising strategies to control atherosclerosis and inflammation in SLE. The success of future clinical trials and the therapeutic application of these treatments is likely to be dependent on correct patient stratification. Reducing CVD risk in SLE patents from a young age will be a huge breakthrough for long term patient outcomes and quality of life.

6 CONCLUSIONS

The field of immunometabolism has enhanced our understanding of the key changes in cellular homeostasis and how this can result in autoimmune conditions including SLE. Observed mitochondrial dysfunction has implications for immune cell energy metabolism and also ROS generation. Abnormalities within mTOR signaling may induce promote immune cell differentiation and proliferation, whilst also stimulating pro-inflammatory cytokine production. Lipid metabolism has been shown to potentially play a role in immune cell signaling. **Figure 2** summarizes the key changes in immunometabolism observed in SLE to date.

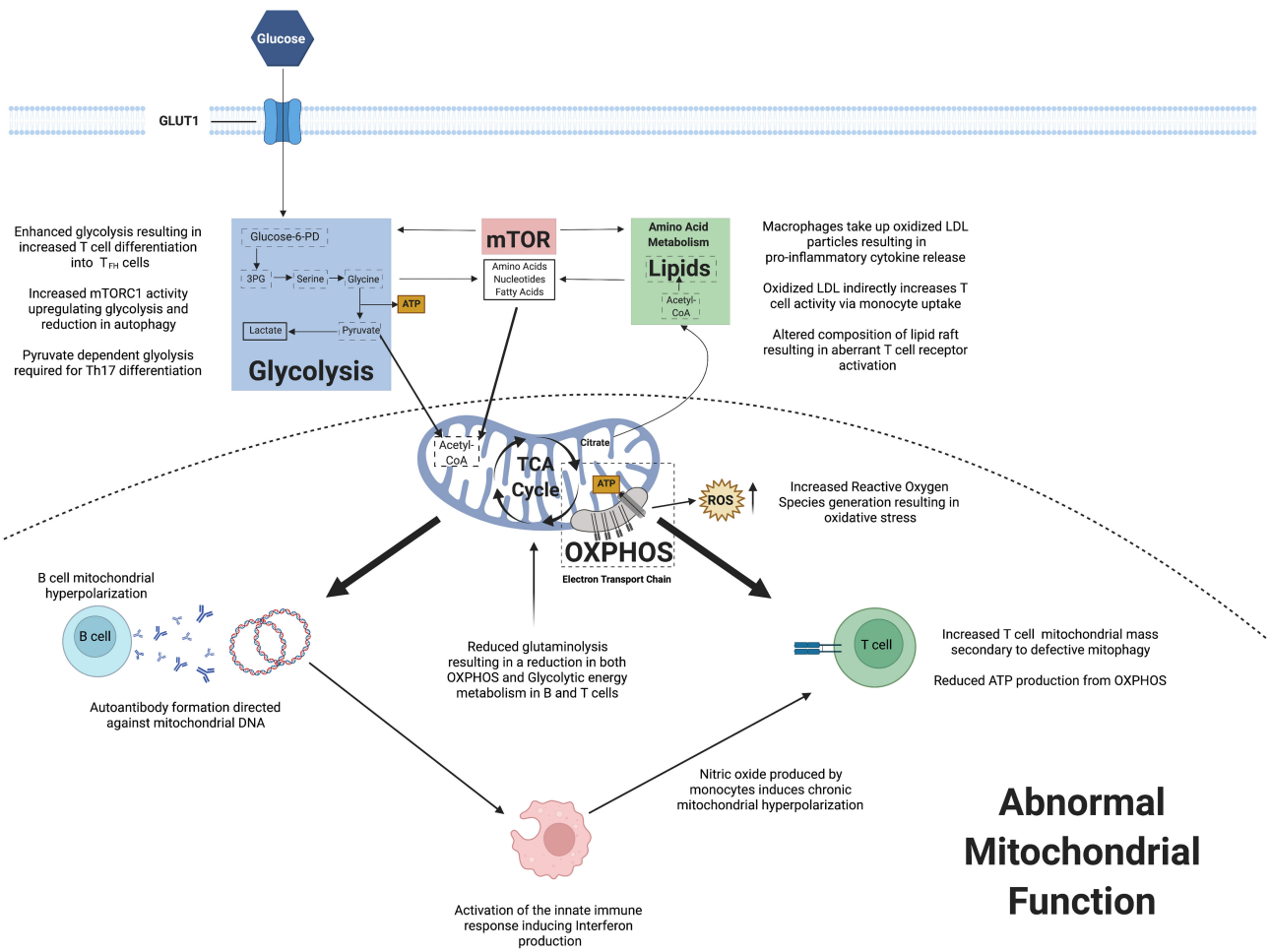

FIGURE 2 | A summary of observed changes in cellular metabolism reported in systemic lupus erythematosus.

In conclusion, our understanding of immunometabolism in SLE is rapidly increasing and main soon translate to newer agents being developed specifically to restore immune cell homeostasis in the disease.

AUTHOR CONTRIBUTIONS

MW was responsible for writing Section 3 (Mitochondrial dysfunction in SLE). GR was responsible for writing Section 4 (Lipid metabolism in SLE). CW was responsible for the remaining sections and editing the final manuscript. All authors contributed to the article and approved the submitted version.

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Ferroptosis in Rheumatoid Arthritis: A Potential Therapeutic Strategy

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Ferroptosis is one of the newly discovered forms of cell-regulated death characterized by iron-dependent lipid peroxidation. Extensive research has focused on the roles of ferroptosis in tumors, blood diseases, and neurological diseases. Some recent findings have indicated that ferroptosis may also be related to the occurrence and development of inflammatory arthritis. Ferroptosis may be a potential therapeutic target, and few studies *in vitro* and animal models have shown implications in the pathogenesis of inflammatory arthritis. This mini review discussed the common features between ferroptosis and the pathogenesis of rheumatoid arthritis (RA), and evaluated therapeutic applications of ferroptosis regulators in preclinical and clinical research. Some critical issues worth paying attention to were also raised to guide future research efforts.

Keywords: ferroptosis, rheumatoid arthritis, lipid peroxidation, reactive oxygen species, therapeutic strategy

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INTRODUCTION

Rheumatoid arthritis (RA) is the most specific systemic immune system disease among autoimmune diseases (1), invading many joints, such as knee and elbow joints. Its main clinical manifestations are joint swelling and stiffness in the morning. RA has an incidence of 0.5% to 1%, with an apparent reduction from north to south (in the northern hemisphere) and from urban to rural areas (2). Some Native American populations have a very high prevalence. The incidence of RA is high in 30-50 years of age, and the incidence in women is about three times that of men. RA may be related to various cell types and cytokines (3), and the origin of its pathology is autoantibodies (4, 5). RA is characterized by infiltration of macrophages and lymphocytes, the proliferation of synovial fibroblasts, joint inflammation, progressive cartilage destruction, and bone erosion, as well as degenerative manifestations (6).

Disease-modifying antirheumatic drugs (DMARDs) are conventional drugs in the treatment of RA. Depending on the symptom severity, RA can be treated with a single drug or a combination of 2, 3, or 4 drugs (7, 8). When the disease is refractory, some biologic DMARDs are recommended. The use rate of biological agents in the treatment of RA in North America has been as high as 50.7% (9). However, the clinical efficacy rate is inconsistent, ranging from 50% to 70% (10). In clinical practice, we should make changes in the therapeutic strategy when the arthritis is resistant to initial therapy. Hence, there is an urgent need to develop drugs with new targets or new mechanisms of action to meet the clinical needs of patients.

In RA, mature B cells and dendritic cells present antigens to T cells, leading to T cell activation. Different immune cells secrete unique cytokines and jointly stimulate the expression of cytokine

TRANCE/receptor activator of nuclear factor-kappa B ligand (RANKL), which is necessary for osteoclast differentiation. B-T cell interaction leads to the activation of plasma cells responsible for producing and secreting autoantibodies. Autoantibodies, cytokines, and RANKL stimulate osteoclasts to cause bone resorption and induce cartilage damage driven by chondrocytes. In addition, compared with activated B cells, transitional B cells can inhibit the formation of osteoclasts in an immunomodulatory manner by providing IL-10. It is reported that ferroptosis plays a vital role in the occurrence and development of many diseases such as Parkinson's disease, ischemia-reperfusion injury, and tumors (11, 12). Recent studies have shown that ferroptosis plays a critical regulatory role in autoimmune and inflammatory diseases (13, 14). New strategies for targeting ferroptosis are to regulate the immune response homeostasis, and in some cases, the reactions can influence each other. Studies have found that ZIP14, a ferroptosis-related metal transporter, may play a regulatory role in the immune system (15). Early studies have confirmed that glutathione peroxidase (GPX) activity in polymorphonuclear leucocytes of RA patients with high persistent disease activity is reduced (16). Luo et al. (17) found that RSL3, a ferroptosis activator, can induce ferroptosis in synovial cells and aggravate synovitis. Transferrin receptor 1 (TFR1) and nuclear receptor coactivator 4 (NCOA4) were upregulated, but system Xc- (an amino acid transporter mediating the exchange of extracellular cystine and intracellular glutamate) and GSH-glutathione peroxidase 4 (GPX4), as well as nuclear factor erythroid 2-related factor 2 (Nrf2, a transcriptional factor that induces antioxidative and cytoprotective responses), were downregulated by RSL3 treatment. Herein, ferroptosis may be a potential therapeutic target for inflammatory arthritis in the future.

This mini review discussed the common features between ferroptosis and the pathogenesis of RA, and evaluated therapeutic applications of ferroptosis regulators in preclinical and clinical research. Some critical issues worth paying attention to were also raised to guide future research efforts.

FERROPTOSIS IN CELL DEATH

Cell death is a sophisticated process, and its mechanisms have traditionally been divided into two types, programmed cell death (PCD) mechanisms that require energy, and necrotic cell death mechanisms that do not (18). In addition, necrotic cell death typically causes a strong immune response, whereas PCD does not (19, 20). In 2012, Dixon et al. (21) discovered a unique iron-dependent form of nonapoptotic cell death when studying the mechanism of the small molecule compound (named erastin) against RAS mutant tumors, which was called ferroptosis. It is significantly different from other death patterns in morphology, biochemistry, and genetics (22). It is characterized by the accumulation of lethal reactive oxygen species (ROS) arised from the reaction between iron and lipid peroxides, which are themselves generated by the oxidation of polyunsaturated fatty acids (PUFAs)-containing phospholipids (PUFA-PLs) (23). PUFAs are essential for ferroptosis due to their sensitivity to lipid peroxidation (24). Free PUFAs are involved in ferroptosis after they are esterified into PUFA-PL and PUFA-PLOOH. Ferroptosis does not have morphological characteristics of apoptosis, such as cell shrinkage, chromatin agglutination, formation of apoptotic bodies, disintegration of cytoskeleton, and other phenomena. However, it can be observed that the volume of mitochondria decreases and the membrane's density increases (25), which are not observed in apoptosis. At the same time, along with mitochondrial morphology alterations accompanying ferroptosis, a common morphological feature is cell ballooning/blistering followed by plasma membrane rupture (26). In terms of biochemical characteristics, ferroptosis is mainly triggered by glutathione (GSH) depletion and glutathione peroxidase 4 (GPX4) inactivation. It is mainly related to lipid peroxidation metabolism and intracellular iron balance, and several genes are involved in the regulation of ferroptosis. Ferroptosis inducers mainly include erastin, FINO2, and RSL3. Ferroptosis inhibitors mainly include liproxstatin-1, iron chelator, and ferrostatin-1. Liproxstatin-1 free radicals, which were formed by removing lipid peroxides from liproxstatin-1, can be reduced by other antioxidants (such as ubiquinone). Zika et al. found that liproxstatin-1 may reduce the accumulation of intracellular toxic lipid ROS, thereby inhibit the occurrence of cell ferroptosis (27). At present, the understanding of ferroptosis is not comprehensive enough, and its mechanism is still in the exploratory stage.

THE POTENTIAL ROLE OF FERROPTOSIS IN RA

Ferroptosis might play a role in the onset of RA and may be used as a treatment option in the future. Recently, it has been found that RA and ferroptosis have similar characteristics, mainly in the following aspects.

IRON HANDLING

Abnormal iron metabolism is an important cause of ferroptosis. Regulatory pathways of intracellular iron homeostasis mainly include ferroportin and TFR1 to regulate iron export and absorption (23). Iron ions induce the body to produce a large amount of lipid ROS through fenton reaction, promote lipid peroxidation, and lead to ferroptosis. Under oxidative stress conditions, superoxide will be produced in a short time, reducing Fe^{3+} stored in ferritin to Fe^{2+}, resulting in the release of iron ions. Fe^{3+} enters the cell under the transport of TFR1, and then is converted into Fe^{2+}. The excess iron ions are stored in ferritin to control the storage of iron ions. In addition, both iron response element binding protein 2 (IREB2) and Nrf2 are involved in regulating Fe^{2+} in cells. NCOA4 recognizes and relies on the autophagy pathway to degrade intracellular ferritin, releasing free iron ions (28) (**Figure 1**).

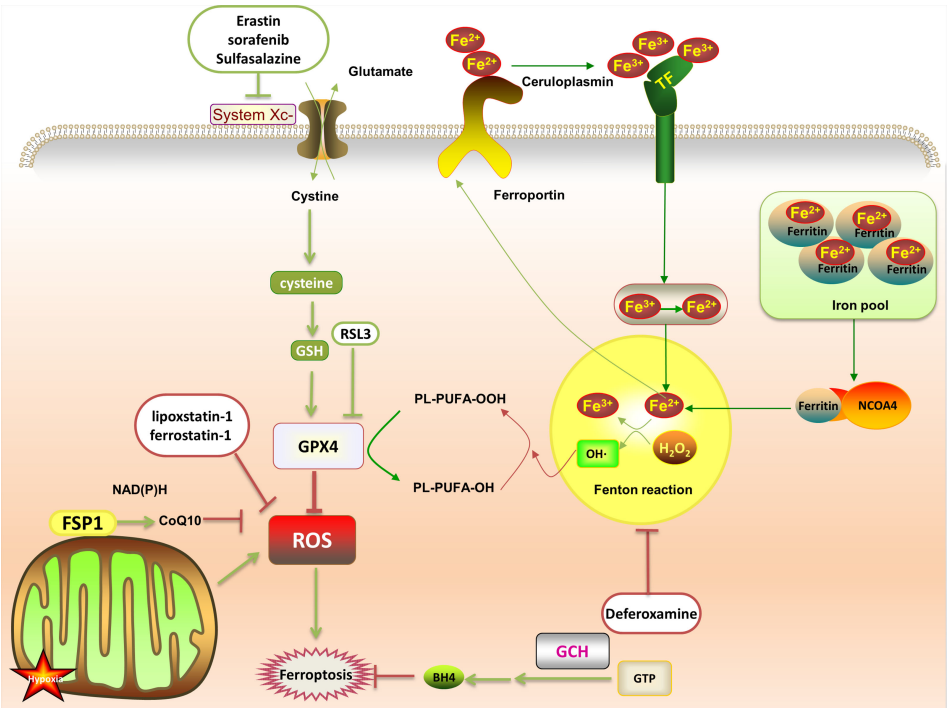

FIGURE 1 | After the transferrin binds to the transferrin receptor on the plasma membrane, the plasma membrane forms a vesicle that takes Fe^{3+} carrying transferrin into the cell. Then, the low pH in the vesicle promotes the separation of Fe^{3+} from the transferrin and the shedding of Fe^{3+}. It is reduced to Fe^{2+} and free in the cytoplasm, or is combined with ferritin to form an iron pool. The ferritin in the iron pool can be encapsulated by autophagy lysosomes under the mediation of NCOA4, and then degraded and release a large amount of Fe^{2+}. Fe^{2+} and H_2O_2 generate PLOOH through the fenton reaction, which promotes ferroptosis by promoting further lipid peroxidation and self-peroxidation. In the GSH/GPX4 pathway, with the help of GSH, GPX4 down-regulates ROS and inhibits ferroptosis. This can be suppressed by RSL3. System Xc- (cystine/glutamate antiporter) promotes synthesis of glutathione, which can be offset by erastin, sulfasalazine and sorafenib. In the FSP1 protection pathway, FSP1 can catalyze the reduction of CoQ10 to panthenol and consume NAD(P)H to inhibit ROS. In the GCH1 protection pathway, GCH1 acts as a rate-limiting enzyme to manage the biosynthesis of BH4 and reduce ferroptosis.

The proliferation and activation of osteoclasts lead to RA bone damage and bone metabolism disorders. *In vitro* and *in vivo* studies have shown that iron overload can induce osteoclast differentiation and inhibit osteoblast proliferation by increasing ROS generation (29). Low concentrations of iron ions can promote the growth of osteoblast precursor cells (MC3T3-E1), while high concentrations of iron ions inhibit their growth and increase ROS levels (30). Iron overload can inhibit the activity of osteoblasts to a certain extent, thereby affect their differentiation process. Simultaneously, it can also activate the differentiation of osteoclasts and cause bone destruction (31). Studies have shown that excessive iron ions can activate p38-MAPK and block PI3K/AKT and JAK/STAT3 signaling pathways to induce MC3T3-E1 cell death (30). In addition, iron ions initiate the growth of synovial pannus by regulating the expression of critical genes such as *c-myc* and *mdm2*. These genes are responsible for the proliferation of synovial cells and promote the occurrence and development of vascular synovitis (32).

Studies have demonstrated that iron deposits are found in osteoarthritis and RA (33, 34). Both osteoarthritis and rheumatoid synovia contained iron, but in the latter greater quantities were present. However, none of the controls with normal synovia had iron deposition. Another study found that the iron metabolism is different in RA than in general health (35). It is worth noting that iron deficiency is common (64%) in RA patients with high disease activity. RA patients had lower hepcidin, lower transferrin, and lower ferritin. Icariin has antibacterial, anti-inflammatory and antioxidant effects (36). It has been suggested that icariin counteracts the effects of RSL3 on iron content, lipid peroxidation, and relative protein (SLC7A11, SLC3A2L, GPX4, TRF, NCOA4, and Nrf2) in synoviocytes given the observation that icariin might play a role in protecting synovial cells from ferroptosis (17). Herein, it can be exploited as a new therapeutic strategy for RA.

MEMBRANE LIPID ANTIOXIDANT SYSTEM

GSH is a tripeptide containing sulfhydryl groups combined with glutamic acid, cysteine, and glycine, which has an antioxidant effect. Under normal circumstances, the cystine entering the cell is reduced to cysteine to participate in the synthesis of GSH, which helps reduce the accumulation of lipid peroxides. However, when system Xc- is inhibited, GSH synthesis disorder will promote the

decline of cellular antioxidant capacity and the accumulation of lipid ROS. Abnormally elevated lipid ROS levels can be controlled by GPX4 (37). GPX4 can effectively repair the oxidative damage of unsaturated fatty acids in mammals, thereby inhibiting ferroptosis. Stockwell et al. determined that the GPX4/GSH axis and the system Xc- regulate ferroptosis, considered a classic pathway (38). Earlier studies have shown that erastin, a system Xc- inhibitor, inhibits GSH synthesis and increases lipid ROS, leading to ferroptosis (21). Erastin has been shown to contribute to cartilage tissue damage by promoting matrix metalloproteinase 13 (MMP-13) expression and inhibiting type II collagen expression in chondrocytes (39), which may aggravate RA.

In addition to GPX4/GSH axis, ferroptosis suppressor protein 1 (FSP1)- Coenzyme Q10 (CoQ10) pathway has also been found to be related to ferroptosis. GPX4 and FSP1 are two parallel membrane lipid antioxidant pathways. FSP1 is one of the CoQ10 oxidoreductases, most of which are attached to the outer mitochondrial membrane (40). The FSP1-CoQ10 pathway can improve lipid peroxidation through free radical capture, and the process of ferroptosis is also blocked. Some studies speculate that FSP1 may improve RA through the TNF-α/ROS positive feedback loop (41, 42). However, the specific contribution of ferroptosis as a mode of cell death was not addressed in these studies.

It is worth noting that the GTP cyclohydrolase-1 (GCH1)-tetrahydrobiopterin (BH4) pathway is parallel but independent of the GPX4 and FSP1 pathways (43). GCH1 is the primary rate-limiting enzyme for the synthesis of BH4. Overexpression of GCH1 can enhance the production of BH4, and then reduce ferroptosis. So far, few such evidence has been provided for RA. Although the GCH1-BH4 protective pathway is closely related to ferroptosis, the interaction between GCH1-BH4 and RA is still not fully understood, and further research is needed.

OXIDATIVE STRESS AND LIPID PEROXIDATION

Studies have shown that oxidative stress plays a vital role in the progression of RA. ROS, as a product of oxidative stress, exists in the articular cavity of RA patients in large quantities. ROS can be used as a potential marker for the progression of RA patients (44). Excess ROS can be converted to hydrogen peroxide through the fenton reaction. In this process, Fe^{3+} can be reduced to Fe^{2+}, generating hydroxyl (-OH) or alkoxy (RO-) free radicals, and causing cell ballooning/blistering followed by plasma membrane rupture (45). ROS in cells can activate the NLRP3 receptor protein. Activated NLRP3 is polymerized by ATP to form highly ordered NLRP3 protein oligomers. Under the action of ASC, NLRP3 and pro-caspase-1 are connected to form a complex pro-caspase-1 that can be activated to form an enzymatic activity. The heterodimer caspase-1 cuts the inactive pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, aggravating RA. When the local inflammation of RA joints accelerates, it can be used as an endogenous signal regulator to expand the synovial inflammation response (46). ROS is a key element of the ROS/TNF-α feedback loop. The production of TNF-α depends on the activation of NF-κB signaling pathway stimulated by ROS, which in turn activates the p38/JNK signaling pathway to accelerate the progression of RA (41) (**Figure 2**). ROS can induce activation of metalloproteinases, inhibit the synthesis of cartilage proteoglycans, and promote chondrocyte apoptosis, which eventually leads to cartilage destruction and bone erosion. This is consistent with pathogenic manifestations of RA. A study detected a strong positive correlation between the ROS level and the severity score in RA patients (47). The levels of lipid peroxidation in the serum and synovial fluid are increased in RA patients, and the antioxidant system has also changed (48). Hence, ROS production in excess is more likely to inhibit osteoblast differentiation and lead to bone destruction.

In addition, studies have found that ROS production can also be induced by activation of the phagocyte NADPH oxidase 2 (NOX2) complex in a process generally referred to as an oxidative burst. NOX2-derived ROS have been shown to suppress antigen-dependent T-cell reactivity and remarkably to reduce the severity of experimental arthritis in both rats and mice (49). In addition, NOX2 also plays a role in antigen presentation and regulation of adaptive immunity. In $CD4^+$ T cells, the lack of NOX2 induces the production of Th17 cells and reduces regulatory T cells in a ROS-dependent manner by affecting Foxp3 and RORγt (50). The immunosuppressive properties of $CD4^+CD25^+Foxp3^+$Treg cells play a vital role in maintaining the body's immune tolerance and immune response homeostasis. Early studies have demonstrated that regulatory T cells are functionally compromised in RA (51). The $CD4^+CD25^+$Treg cells in the joint synovial fluid of RA patients are significantly increased (52). In addition, the reduction of NOX2 will increase Th1, Th2, and Th17 cells, leading to inflammatory arthritis. Antigen-presenting cells (APCs) are known to produce NOX2-derived ROS. A study found that the NOX2-dependent processing of the redox-sensitive autoantigens by APCs modified T cell activity and induced development of RA in mouse models (53). Despite many unknown facts, drugs targeting ferroptosis may represent a potential strategy for treating RA.

INFLAMMATION

Ferroptosis can also trigger the body's innate immunity, release inflammatory mediators, and activate the body's inflammatory response (14). Changes in the synovial membrane's typical physiological and metabolic properties can produce many inflammatory mediators, such as IL-1β, TNF-α, and IL-6, which increase uptake of transferrin and non-transferrin-bound iron by monocytes and increase the uptake of transferrin-bound iron by synovial fibroblast (54). Increased iron intake accelerates the vicious cycle of hemorrhage-synovitis-hemorrhage, and the proliferated synovial tissue spreads to the surface of the articular cartilage. Cartilage matrix is degraded by connective cathepsin released by

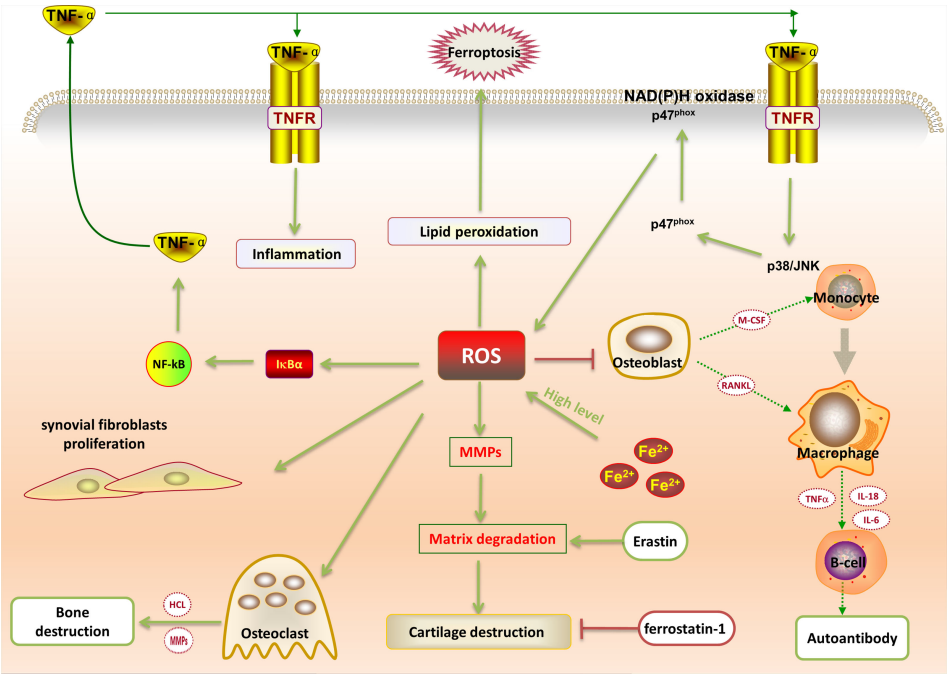

FIGURE 2 | ROS is a critical element of the ROS/TNF-α feedback loop. The production of TNF-α depends on the activation of ROS-stimulated NF-κB signaling, which activates the p38/JNK signaling pathway to accelerate the progression of RA inflammation. High levels of iron ions can catalyze the production of ROS. Excessive ROS will aggravate the proliferation of synovial fibroblasts; induce osteoclast differentiation and inhibit osteoblast proliferation; activate metalloproteinases, as well as lead to cartilage destruction and bone erosion. Excessive ROS will also promote lipid peroxidation, leading to cell ferroptosis. Moreover, the ferroptosis inducer (erastin) can promote the expression of matrix metalloproteinase 13 and promote cartilage destruction, while the ferroptosis inhibitor ferrostatin-1 can reduce cartilage degradation.

hypertrophic synovial tissue, chondrocytes and intravascular tissue, which will eventually lead to the destruction of articular cartilage and bone (55, 56).

FERROPTOSIS: A RISING STAR WITH GREAT THERAPEUTIC POTENTIAL IN RA

P53 is an excellent tumor suppressor gene that can inhibit system Xc- uptake of cystine by down-regulating the expression of SLC7A11 and result in a decrease in antioxidant capacity. Previous studies have confirmed that p53 protein is expressed in RA fibroblast-like synovial cells, and its overexpression is a characteristic of RA (57). It plays a role in controlling the balance between Th17 cells and Treg (58). Aberrant p53/p21 activation-mediated aging-related secretory phenotype can accelerate destruction of cartilage tissue (59). Studies have shown that endogenous p53, which is inducible in rheumatoid synovial cells, is functionally active based on the findings that its expression blocks the G1/S transition by inhibiting the CDK-mediated phosphorylation of Rb *via* p21 induction (60). Clinical studies have found that the expression of p53 in lymphocytes is lower in RA patients than that of healthy people (61, 62). Acyl–CoA synthetase long–chain family member 4 (ACSL4, an enzyme involved in the activation of PUFAs) is located in peroxisomes and mitochondria, which can determine the sensitivity of cells to ferroptosis activation. Doll et al. found GPX4-ACSL double-knockout cells showed marked resistance to ferroptosis (63). Mechanistically, ACSL4 enriched cellular membranes with long polyunsaturated ω6 fatty acids. ACSL4 has a marked preference for activating PUFAs (64), therefore, deletion of ACSL4 prevents PUFAs from being incorporated into membrane PLs where they would become oxidized following GPX4 inactivation (26). Clinical studies have found that the ACSL4 is down-regulated in RA patients (65). BECN1 is a crucial regulator of autophagy, which can promote ferroptosis by regulating the activity of the system Xc-. BECN1-dependent ferroptosis requires the formation of BECN1-SLC7A11 complex. Studies have found that autophagy of osteoblasts affects bone metabolism, and BECN1 may become a new target for the treatment of bone metabolism diseases (66). CoQ10, a fat-soluble antioxidant, is a crucial regulator of ferroptosis. Studies have shown that CoQ10 has anti-inflammatory effects on autoimmune diseases. Jhun et al. used CoQ10-encoded liposome/gold hybrid nanoparticles targeting STAT3/Th17 to slow RA's progression (67). Although the FSP1-CoQ10 protective pathway is closely related to RA, the interaction between CoQ10 and other ferroptosis regulators is still not fully understood, and further research is needed.

In addition, many studies have focused on the relationship between oxidative stress metabolism and ferroptosis regulators. For example, Nrf2 and heme oxygenase-1 (HO-1) level can regulate

ferroptosis (68). A study found that reduced levels of the Nrf2 factor can lead to RA (69). Targeted activation of Nrf2 can inhibit ROS production, which in turn inhibits the proliferation and migration of RA fibroblast-like synovial cells (70). Luo H et al. found that RSL3 can reduce Nrf2 and GPX4 in synovial cells (17). In addition, lack of Nrf2 can lead to changes in the expression of SLC7A11, which further leads to oxidative stress damage and aggravates joint destruction (71). Studies have found that ferroptosis can be induced through the Nrf2-SLC7A11-HO-1 pathway, which may play a regulatory role in joint destruction (71, 72).

The study found that FDA-approved RA drugs such as sulfasalazine and auranofin can prevent cell growth and induce ferroptosis. Sulfasalazine and auranofin activity were largely mitigated by the ferroptosis inhibitor ferrostatin-1, antioxidants, or by the iron scavenger deferoxamine (DFO). DFO can inhibit ferroptosis by preventing iron ions from supplying electrons to oxygen to form ROS. However, the specific mechanism is still unclear. Dixon et al. made synthetic ferrostatin-1 (ferroptosis inhibitor) and proved that it could specifically inhibit ferroptosis, but it did not impede other oxidative substances and apoptosis-induced death (21). Yao et al. found that intra-articular injection of ferrostatin-1 increased the expression of collagen II, promoted the activation of the Nrf2 antioxidant system, and reduced cartilage degradation, which is beneficial to alleviate joint inflammation (39). Some natural polyphenol compounds can also significantly inhibit ferroptosis, such as baicalein, curcumin, and gastrodin (73, 74). Baicalein was demonstrated to suppress T cell proliferation in collagen–induced arthritis model mice and significantly improve T cell-mediated autoimmune diseases (75). Studies have confirmed that curcumin alleviates inflammation, synovial hyperplasia, and the other main features involved in the pathogenesis of collagen-induced arthritis (76). Targeting ferroptosis regulators may be a new direction for developing therapeutic drugs for RA.

DISCUSSION

In summary, ferroptosis is a recently discovered significant regulatory cell death pattern, and three protective pathways have been successively confirmed. An in-depth study of the underlying mechanism of ferroptosis is of great significance for mapping its role in various related autoimmune diseases. It is worth noting that different cell types (synovial cells, chondrocytes, osteoclasts, and macrophages) may have different susceptibility to ferroptosis. The expression profile of specific genes related to the ferroptosis pathway may not be observed in all cells. Moreover, attention should be paid to the phenomenon of ferroptosis, and the judgment criteria may vary depending on the trigger mechanism. Herein, it is necessary to study the mechanism of ferroptosis regulators. At present, the inner link between ferroptosis and RA has not been studied in depth. With an in-depth understanding of the relationship between ferroptosis and other biological processes, people will find that ferroptosis, apoptosis, autophagy, and other cell death patterns have some common characteristics in their regulations. Although simultaneous regulation of multiple cell death pathways is vital for the treatment of RA, the relationship between these different types of cell death is not yet fully elucidated. This requires further exploration to validate whether they are integrated into a complex regulatory network.

In short, with the unprecedented prosperity of research on ferroptosis, ferroptosis regulation represents a potential future avenue of investigation in the effort to identify novel therapeutic targets for RA.

AUTHOR CONTRIBUTIONS

TZ wrote the manuscript. DQ revised the manuscript. ZFL raised the idea for the article. YX, ZX, QY, JS, and ZML performed the literature search and data analysis. All authors contributed to the article and approved the submitted version.

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