# FOCUSING ON T-CELLS FOR NOVEL TREATMENTS OF SYSTEMIC LUPUS ERYTHEMATOSUS

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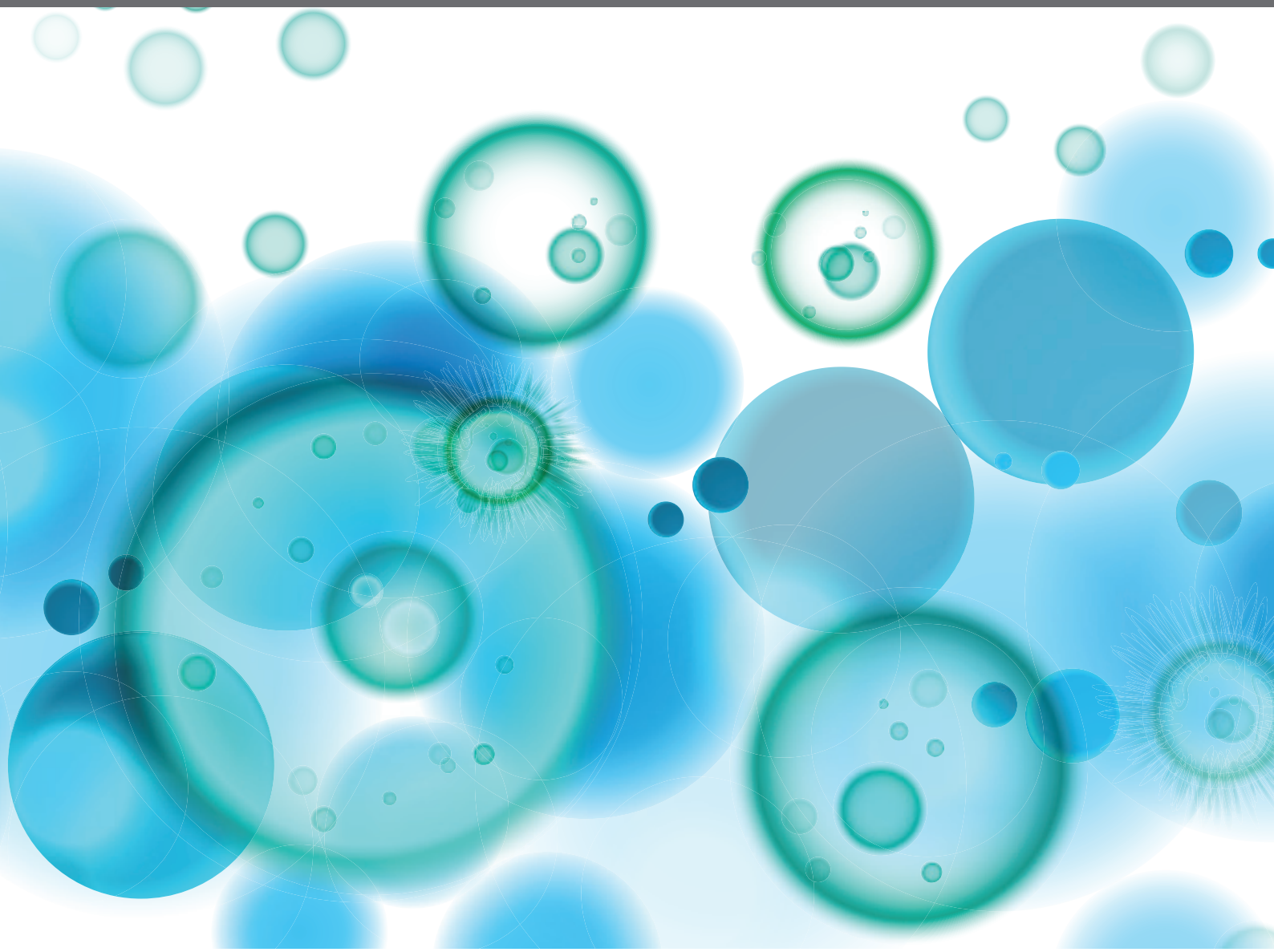

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# FOCUSING ON T-CELLS FOR NOVEL TREATMENTS OF SYSTEMIC LUPUS ERYTHEMATOSUS

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# Table of Contents

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EDITORIAL
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# Editorial: Focusing on T-Cells for Novel Treatments of Systemic Lupus Erythematosus

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**Keywords: lupus T-cells, immunometabolism, innate lymphocytes, post-transcriptional regulation, Th17/Treg balance**

## Editorial on the Research Topic

## Focusing on T-Cells for Novel Treatments of Systemic Lupus Erythematosus

The pathogenesis of Systemic Lupus Erythematosus (SLE) involves all components of the immune system, including immune cells (T cells, B cells, antigen presenting cells), autoantibody production and immune-complex deposition. As autoantibody-mediated inflammation and damage are downstream events in SLE pathophysiology, investigating molecular mechanisms driving immune cell alterations will deliver disease mechanisms and treatment targets.

Dysregulated immune responses to self- and foreign antigens can cause and/or amplify multi-organ complications. This is addressed by the manuscript from Spihlman et al. who review clinical and immunological parallels between COVID-19 and SLE. Immune responses against self-antigens in SLE have much in common with those against SARS-CoV-2, and a variety of therapeutic approaches, including the use of corticosteroids and immunosuppressive agents, are effective in both. Thus, understanding immune dysregulation in SLE may aid in the treatment of COVID-19 and vice versa.

This Research Topic 'Focusing on T-cells for novel treatments of SLE' focuses on summarizing current knowledge on lymphocyte dysregulation in SLE, centering on T-cells. Over recent years, lupus-prone mice have improved our understanding of SLE in humans, including the involvement of effector $CD3^+CD4^+$ and $CD3^+CD4^-CD8^-$, so-called "double-negative" (DN), T cells. Although numerically expanded DN T cell populations are a characteristic of lupus in mice and humans, their exact roles and origin remain controversial (1). Liu et al. found that CD138 (Syndecan-1) positive DN T cells, dominantly derived from $CD4^+$ T cells, have a central memory phenotype and are involved in the activation of autoreactive B cells in the MRL/lpr mouse, a fulminant mice lupus model. By contrast, Flores-Mendoza et al. showed that DN T cells were induced only when Fas/FasL-deprived $CD8^+$ T cells were re-stimulated with self-antigens. Furthermore, Fas/FasL on $CD8^+$ T cells is involved in the retention of CD8 expression after antigen re-stimulation. Therefore, loss of CD8 (or CD4) expression may limit excessive immune responses. However, as they express high levels of effector cytokines, DN T cells may also exacerbate and/or maintain autoimmunity (1, 2). Further studies are warranted to elucidate the exact role and function of DN T cells in autoimmune/inflammatory disease.

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The importance of $CD3^+CD4^+$ Th17 cells in lupus pathogenesis is widely accepted. Usually, to maintain immune tolerance, the development of Th17 is in balance with that of regulatory T cells (Treg), especially in the intestinal tract (3). In autoimmune/inflammatory disease, such as SLE, the Th17/Treg imbalance may be altered and contribute to the immune pathology. As reviewed by Koga et al., molecules such as Protein phosphatase 2A (PP2A), Calcium/calmodulin kinase IV (CaMK4), and cAMP-responsive element modulator (CREM)/CREM inducible cAMP early repressor (ICER), which are upregulated in T cells of SLE patients, are important for Th17 differentiation and involved in the development of SLE in mice. On the other hand, IL-17A-deficient lupus-prone mice and animals treated with anti-IL-17A antibodies still develop lupus (4). Thus, the exact role of IL-17A and Th17 cells, and the balance between Th17 and Treg populations in SLE pathophysiology remains unclear.

Indeed, the balance between Th17 and Treg cells, and mechanisms controlling their balance, including immunometabolism, is currently in the focus of research and reviewed by Shan et al. in this topic. Glycolysis and lipid synthesis are required for Th17 differentiation and inhibition of these metabolic signals alleviates disease activity in lupus-prone mice and SLE patients. Furthermore, Kono et al. described the importance of amino acid metabolism, including glutaminolysis, for lymphocyte activation and differentiation. Amino acid availability is crucial for mechanistic target of rapamycin (mTOR) activation, which is required for Th17 differentiation (5). The Nuclear factor erythroid 2-related factor 2(NRF2)/Kelch ECH associating protein 1(Keap1) pathway, a critical regulator of the antioxidant system and REDOX metabolism, is also involved in lupus pathogenesis. NRF2 deficient female mice are prone to develop lupus nephritis (6). Lupus-prone B6/lpr lacking NRF2 aggravates glomerulonephritis with increased numbers of Th17 cells (7). Indeed, dimethylfumalate, an activator of the NRF2 pathway, is now widely used for the treatment of multiple sclerosis, another Th17-driven autoimmune/inflammatory disease. In their review in this special topic, Ohl et al. discuss the possibility of targeting NRF2/Keap1 for the treatment of SLE.

In this special topic, Iwata et al. investigated fatty acid synthesis in T cells from SLE patients, and identified alterations in Th1 subsets of SLE patients and their involvement in disease pathology. Authors dissected the role of lipid metabolism in the induction of inflammatory subsets of Th1 cells, including inhibition of fatty acid synthesis that effectively altered the phenotype of peripheral T cells in SLE, while rapamycin was not as effective. While T-$bet^{hi}CXCR3^{lo}$ effector cells and T-$bet^+Foxp3^{lo}$ non-suppressor cells (which produce large amounts of IFN-γ) are abundant in SLE, T-$bet^+Foxp3^{hi}$ activated Treg cells (which do not produce IFN-γ) are lacking. These changes may be involved in the therapeutic resistance as treatment of stimulated memory $CD4^+$ T cells with rapamycin and 2-deoxy-D-glucose (2DG) suppressed T-$bet^+Foxp3^-$ cells *in vitro* and induced T-$bet^+Foxp3^+$(lo/hi) cells. Interestingly, rapamycin alone enhanced lipid metabolism and induced IFN-γ-producing T-$bet^+Foxp3^{lo}$ cells, while 2DG induced non-IFN-γ-producing T-$bet^+Foxp3^{hi}$ cells. In memory $CD4^+$ cells from SLE patients, inhibition of fatty acid synthesis suppressed IFN-γ production and enhanced Foxp3 expression in T-$bet^+Foxp3^+$ cells. Thus, in SLE, metabolic abnormalities, such as enhanced fatty acid synthesis, contribute to the overproduction of IFN-γ by Th1 cells and an imbalance of Th1 subsets.

Innate lymphocytes and natural killer cells (NK) are lymphocytic cells that cannot solely be attributed to the innate or adaptive immune system. An involvement of innate lymphocytes in lupus pathology has emerged recently (8). Among three sub-types of innate lymphoid cells (ILCs), Hu et al. reported that type 3 ILCs (ILC3s) produce IL-22 in the kidney of lupus-prone MRL/lpr mice and induce chemokine expression in tubular epithelial cells. As this is the first report investigating ILC3 in the kidney, further research is warranted. Humbel et al. investigate the role of NK cells in SLE. Authors report that NK cell numbers in the peripheral blood is reduced in SLE patients, and analyzed an array of surface markers, including SLAMF1, SLAMF7 and CD38. The expression of CD38 is increased lupus NK cells. Both the anti-SLAMF7 antibody elotuzumab and the anti-CD38 antibody daratumumab can enhance NK cell function in SLE. Notably, anti-CD38 was recently reported to be effective for the treatment of SLE (9), therefore the involvement of NK cells in anti-CD38 treatment should be considered.

Lastly, evidence is accumulating to suggest that post-transcriptional regulators, such as micro-RNAs, other non-coding RNAs, and RNA-binding proteins, play a critical role in lupus pathogenesis (10, 11). Here, Hiramatsu-Asano et al. focused on miR-223-3p, which is upregulated in lupus-prone mice, and found that it regulates the expression of sphingosine-1-phosphate receptor (S1PR1), a pivotal receptor for peripheral T cell circulation. Authors show that loss of miR223 exacerbates the lupus phenotype by increasing the population of $S1PR1^+CD4^+$ T cells and promoting their infiltration into inflamed kidney tissue. In addition to regulating S1PR1 function, miR223 may be effective for SLE treatment by targeting $S1PR1^+CD4^+$ T cells. Since both SLE-specific upregulation and downregulation of miRNAs are potential therapeutic targets, more reports and targets are expected in the future.

Excellent reports were provided covering a wide spectrum of research areas in SLE, including immunometabolism, innate lymphocyte biology and function, post-transcriptional regulation, and basic T-cell biology. The central role of T-cells in SLE is underscored by reports on voclosporin, a T-cell targeted calcineurin inhibitor, to be effective in the treatment of lupus nephritis (12). The current collection of manuscripts and future reports will help us to better understand the pathophysiology of SLE. Novel molecular and cellular candidates proposed in this special topic are expected to be further investigated for their suitability as biomarkers and/or treatment targets in SLE.

## AUTHOR CONTRIBUTIONS

KI and MM wrote the first draft of the editorial. This was revised by CH and VM with valuable comments and suggestions. All authors contributed to the article and approved the submitted version.

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# T Cell Metabolism: A New Perspective on Th17/Treg Cell Imbalance in Systemic Lupus Erythematosus

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The Th17/T-regulatory (Treg) cell imbalance is involved in the occurrence and development of organ inflammation in systemic lupus erythematosus (SLE). Metabolic pathways can regulate T cell differentiation and function, thus contributing to SLE inflammation. Increasingly, data have shown metabolism influences and reprograms the Th17/Treg cell balance, and the metabolic pattern of T cells is different in SLE. Notably, metabolic characteristics of SLE T cells, such as enhanced glycolysis, lipid synthesis, glutaminolysis, and highly activated mTOR, all favored Th17 differentiation and function, which underlie the Th17/Treg cell imbalance in SLE patients. Targeting metabolic pathways to reverse Th17/Treg imbalance offer a promising method for SLE therapy.

**Keywords: cell metabolism, T helper 17 cells, regulatory T cells, systemic lupus erythematosus, mTOR signaling**

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## INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by tissue inflammation and profound damage to multiple organs. The pathological mechanisms of SLE remain unclear; however, it has been reported that the imbalance between T helper 17 (Th17) and regulatory T cells (Tregs) underlies the pathogenesis of SLE (1–4). Th17 cells have pro-inflammatory effects, and the proportion of Th17 cells is higher in SLE patients, and the content is positively correlated with the activity of SLE disease (5). Tregs, however, have immunosuppressive function and play an important role in the induction and maintenance of self-tolerance. The reduced content and dysfunction of Tregs are closely related to the occurrence and development of SLE (6–8). Tregs injected into lupus mice can control the inflammatory response and alleviate pathological damage (9). Thus, improving the Th17/Treg cells imbalance shows some promise for the treatment of SLE.

Th17 and Treg cells are mutually antagonistic in function and differentiation. Many mechanisms regulating Th17/Treg cells balance have been reported. Besides the molecular signaling network, accumulating evidence has shown that cellular metabolism is also critically involved in Th17 and Treg differentiation (10). These two cell subsets are dictated by distinct metabolic pathways, and manipulating metabolic pathways can regulate Th17/Treg cells balance. In T-cells from SLE patients and lupus-prone mice, metabolic abnormalities linked to Th17/Treg cell imbalances have been reported. Here, we reviewed how cellular metabolism influences Th17 and Treg cell differentiation, summarized metabolic abnormalities of SLE T cells,

and we here propose that metabolic abnormalities of SLE T cells is the mechanism by which the Th17/Treg imbalance emerges in SLE patients. Manipulating cellular metabolism to correct aberrant immune responses may be a suitable means of treating SLE.

## METABOLIC CONTROL OF Th17/Treg BALANCE

T-cell metabolism is highly dynamic. The metabolic pattern changes during the process of activation, proliferation, and differentiation. Naive T cells have low energy requirements. They import a small amount of glucose and generate ATP mainly through the TCA cycle and OXPHOS. Upon activation, T cells start to proliferate and differentiate. They need to reprogram their metabolic pattern to meet their bioenergetic and biosynthetic requirements. A variety of metabolic substrates (glucose, amino acids, and fatty acids) and metabolic pathways (glycolysis, oxidative phosphorylation, the pentose phosphate pathway, fatty acid synthesis and oxidation, and glutamine metabolism) are mobilized to adapt to their biological functions. Glycolysis can rapidly produce ATP to provide the energy needed to rapidly proliferate, and a large number of intermediate products of various metabolic pathways allow biosynthesis (11–14).

Studies have shown that T helper cell subsets have different metabolic patterns, of which those of Th17 cells and Tregs are the most distinct. One study evaluated 400 energy metabolites, metabolism-related genes, and proteins in Th17 and Tregs and results indicated that Th17 cells contain high levels of pyruvate, lactic acid, early glycolysis, and pentose phosphate pathway intermediates, and they express key proteins in glycolysis pathway, like Glut-1 and HK-2, at high levels. Tregs had higher C2 and C4-OH carnitine levels and more expression of fatty acid transporter CPT1A and electron transport chain component cytochrome c, which suggested that Th17 cells were mainly powered by glycolysis, and the pentose phosphate pathway was also active; Tregs, on the other hand, were found to rely more on fatty acid oxidation and oxidative phosphorylation to supply energy (15). Glycolysis deprivation was found to impair Th17 differentiation dramatically, while defective glycolysis supported the development of Treg cells. Replacement of glucose with galactose, treatment with 2-DG (an inhibitor of hexokinase, the first rate-limiting enzyme of glycolysis), and lack of HIF1α, Cdc42, ICER, and mTOR (crucial regulators of T cell glycolytic metabolism) all resulted in diminished Th17 development but enhanced Treg cell differentiation (16–20). Conversely, inhibition of fatty acid oxidation results in diminished differentiation to Th17 cells, but increased development of Tregs (21).

Glutaminolysis is also preferentially increased in Th17 cells. TCA-cycle intermediates produced after α-ketoglutarate (α-KG) in Th17 cells were more plentiful than in Tregs. α-kG is also a metabolite of glutamine (15), suggesting increased glutaminolysis in Th17 cells. ICER, a transcriptional factor that enhances glutaminase1 and promotes glutaminolysis, is also expressed in large quantities in Th17 cells (22). In addition, glutamine metabolite 2-hydroxyglutarate could hypermethylate Foxp3 gene locus and inhibit Foxp3 transcription, thus promoting the differentiation of Th17 cells, which regulates Th17/Treg balance by an epigenetic mechanism (23).

Th17 cells have considerable fatty acid synthesis activity. The expression of ACC1, a key enzyme in fatty acid synthesis, was found to be significantly higher in Th17 cells than in Tregs. Drug inhibition or T-cell specific knockout of ACC1 could inhibit Th17 differentiation and promote the induction of Tregs both *in vivo* and *in vitro* (24, 25). Cholesterol intake and synthesis were also significantly higher in Th17 cells (26), leading to the accumulation of the cholesterol precursor, desmosterol, which acts as a potent endogenous RORγ agonist and dictates Th17 differentiation (27). Statins, a class of drugs that inhibit cholesterol biosynthesis, are reported to target Th17/Treg imbalance and alleviate Th17-mediated inflammatory response (28). The distinct metabolic patterns of Th17 and Treg cells provide a basis for intervention of Th17/Treg imbalance.

## METABOLIC ABNORMALITIES IN SLE T CELLS

Cell metabolism regulates the differentiation and function of T cells, thereby participating in the occurrence and development of SLE inflammation. Metabolic abnormalities in T cells from SLE patients and lupus-prone mice were reported (29, 30). These are characterized by the following:

(**1) Mitochondrial dysfunction:** T cells from SLE patients showed elevated mitochondrial transmembrane potential, increased ROS production and reduced ATP synthesis (31).

(**2) Hyperactivated glucose metabolism:** $CD4^+$ T cells from SLE patients and lupus-prone mice have higher OCR and ECAR levels (32, 33), suggesting they have elevated levels of both glycolysis and oxidative phosphorylation. The metabolites of pentose phosphate pathway, such as R5P and F6P, were also higher in peripheral blood lymphocytes of SLE patients (34). These results suggested that three main pathways of glucose metabolism—aerobic glycolysis, pentose phosphate pathway, and oxidative phosphorylation—are involved in T cell activation in SLE patients.

(**3) Lipid synthesis enhancement:** there was more synthesis of lipid rafts in $CD4^+$T cells of SLE patients than in normal controls, and inhibiting the synthesis of lipid rafts could alleviate the pathological manifestations of lupus in mice (35–37). Glycosphingolipids and cholesterol are important components of lipid rafts, and the levels of synthesis were significantly higher in CD4+T cells in SLE patients than in normal controls (38).

**(4) Increased glutaminolysis:** There is more expression of ICER, the transcriptional factor that promotes glutaminolysis and Th17 generation in $CD4^+$T cells from SLE patients than in healthy controls (39). Glutaminase 1 inhibition improved autoimmune pathology in MRL/lpr mice, and suppressed Th17 differentiation of T cells from patients with SLE but not in those from healthy donors (40). Those data suggested increased glutaminolysis in SLE T cells.

**(5) Highly activated mTOR:** mechanistic target of rapamycin (mTOR) is a hub in the cellular metabolic signal network, regulating cellular growth and energy utilization. $CD4^+$ T cells from SLE patients and lupus-prone mice showed increased mTOR activation (32, 33, 41, 42). The hyperpolarization of mitochondria and the overactivity of pentose phosphate pathway led to enhanced mTOR activity (43). In turn, highly activated mTOR can enhance glycolysis and fatty acid synthesis, thus promoting Th17 differentiation (44, 45). It can be seen that high mTOR expression is an important signaling mechanism leading to abnormal T cell metabolism and Th17/Treg imbalance in SLE patients. Many clinical trials in 2018 showed that sirolimus, an mTOR inhibitor, could alleviate the disease activity of SLE patients (46–48), expand their $Foxp3^+$ Treg cells, and inhibit the secretion of cytokines such as IL-17 (46).

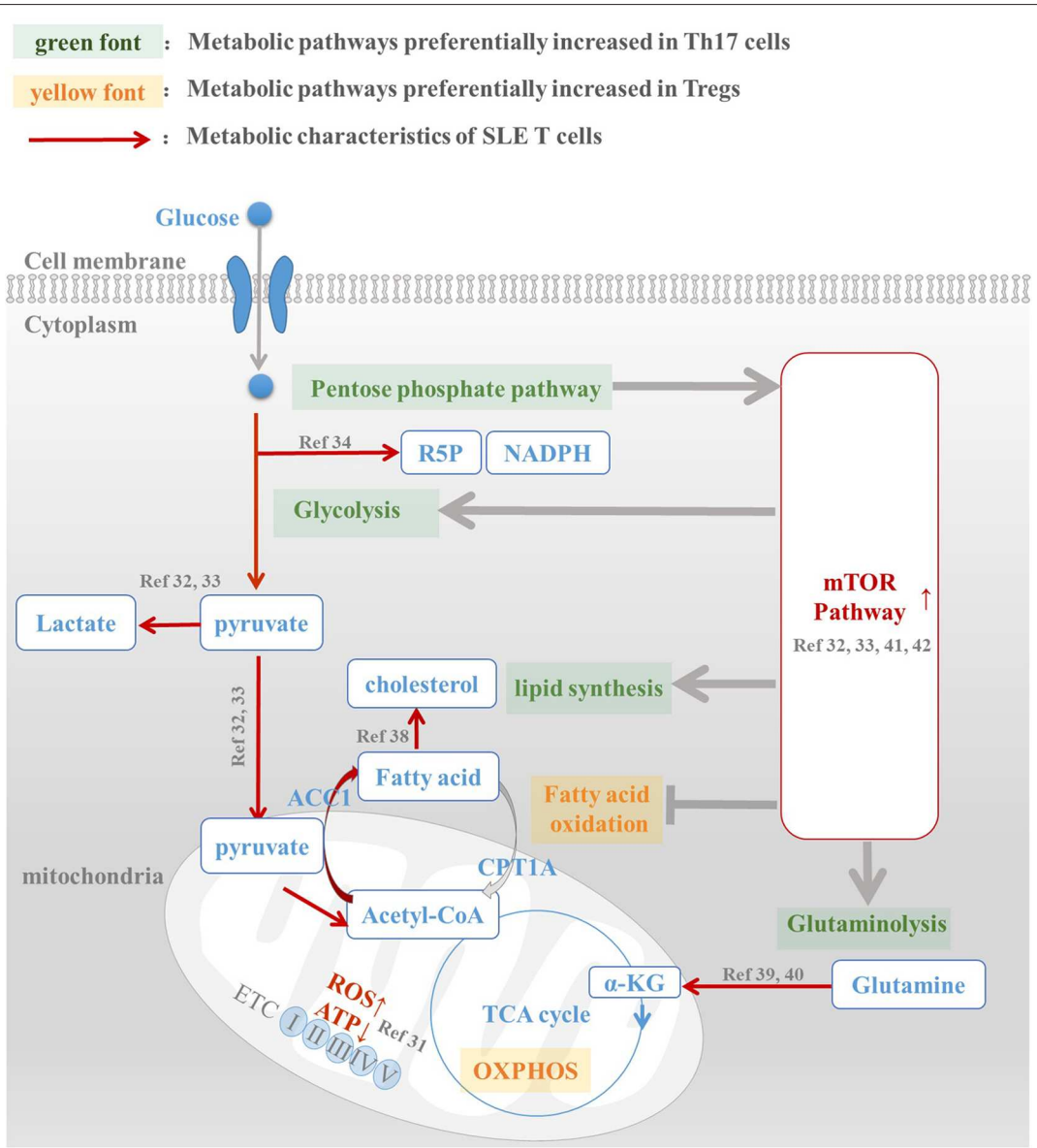


**FIGURE 1 |** Metabolic abnormalities is the underlying mechanism of Th17/Treg imbalance in SLE patients. Th17 and Treg cells have a distinct metabolic pattern: pentose phosphate pathway, glycolysis, fatty acid synthesis, and glutaminolysis are preferentially increased in Th17 cells, while fatty acid oxidation and oxidative phosphorylation are active in Treg cells. However, metabolic abnormalities of T cells in SLE patients, including enhanced glycolysis, active lipid synthesis, increased glutaminolysis, and high mTOR activation, are all conducive to Th17 differentiation and function. For this reason, we speculated that abnormal T cell metabolism was the mechanism underlying Th17/Treg imbalance in SLE patients. The intervention of metabolic pathways to reprogram T cell metabolic patterns in SLE patients, reduce their overactivated glycolysis and lipid synthesis levels, and promote the oxidation of fatty acids, is expected to reverse the Treg/Th17 imbalance in patients and restore their normal immune function.

## DICUSSION

The abovementioned metabolic characteristics of SLE T cells, such as enhanced glycolysis, lipid synthesis, glutaminolysis, and highly activated mTOR, all favored Th17 differentiation and function, which suggest the metabolic abnormalities of SLE T cells is the underlying mechanism of Th17/Treg imbalance in SLE patients (**Figure 1**). Inhibition of glycolysis (32, 33), lipid synthesis (28, 36–38), and mTOR signaling (46–48) can control inflammation and alleviate disease activity in lupus mouse and SLE patients. Manipulating cellular metabolism to correct aberrant immune responses offers promising method for SLE therapy. Further studies are still needed to explore the metabolic abnormalities occurring in T cells of SLE patients and their role in disease progression, as well as how they response to therapies, especially those have potential role in intervening Th17/Treg cell imbalance.

In addition to drugs targeting metabolic pathways, dietary habits, and nutritional factors can also modulate Th17/Treg balance by affecting T cell metabolism. Low cholesterol diet could improve Th17/Treg balance by the activation of LXRs (49), nuclear receptors that modulate cholesterol metabolism (50). And high glucose intake was found to exacerbate autoimmunity by inducing Th17 cells via upregulation of mitochondrial ROS in T cells (51). The long-chain fatty acids enhanced differentiation of Th17 cells, while the short-chain fatty acids derived from a fiber-rich diet expanded Treg cells and reduce IL-17 production (52–54). Thus, a balanced diet could be helpful in the prevention and management of SLE (55).

## AUTHOR CONTRIBUTIONS

JS, HJ, and YX wrote the manuscript. JS generated themes and ideas, guided, and edited the manuscript.

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# Disease Stage-Specific Pathogenicity of CD138 (Syndecan 1)-Expressing T Cells in Systemic Lupus Erythematosus

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CD138 (syndecan 1), a member of the heparan-sulfate proteoglycan family, regulates diverse biological responses by interacting with chemokines, cytokines, growth factors, and adhesion molecules. Expression of CD138 has been detected on T cells from both healthy and sick mice mimicking systemic lupus erythematosus (SLE) disease. However, the characteristics and the role of CD138+ T cells in SLE pathogenesis remain largely unknown. We analyzed the lupus-prone MRL/Lpr mice and the control MRL/MpJ strain as well as the common laboratory strains Balb/c, and C57BL/6 for CD138-expression and found that only the MRL/Lpr strain harbored TCRβ+CD138+ cells in various organs. The frequency of TCRβ+CD138+ cells progressively expanded in MRL/Lpr mice with age and correlated with disease severity. Majority of the TCRβ+CD138+ cells were CD4 and CD8 double-negative and 20% were CD4. At least a portion of TCRβ+CD138+ cells originated from CD4+ cells because substantial number of CD4+TCRβ+CD138- cells expressed CD138 after *in vitro* cultivation. Compared to TCRβ+CD138- cells, TCRβ+CD138+ cells exhibited central memory (Tcm) phenotype with reduced ability to proliferate and produce the cytokines IFNγ and IL-17. When co-cultured with B cells, the ability of TCRβ+CD138+ cells to promote plasma cell formation and autoreactive antibody production was dependent on the presence of autoantigen, CD4 co-receptor expression and cell-to-cell contact. Surprisingly, adoptively transferred TCRβ+CD138+ T cells slowed down disease progression in young recipient MRL/Lpr mice but had the opposite effect when DNA was co-administered with TCRβ+CD138+ T cells or when TCRβ+CD138+ cells were transferred to older MRL/Lpr mice with established disease. Thus, CD138-expressing T cells with Tcm phenotype enhance disease progression in SLE by rapidly activating autoreactive B cells when self-antigens are exposed to the immune system.

**Keywords: lupus, syndecan-1, T cells, MRL/Lpr mouse, immunopathogenesis**

## INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies and inflammatory cell infiltration in multiple tissues (1). T cells are critical for SLE pathogenesis as they, not only provide help to autoreactive B cells, but also infiltrate and damage the target organs, such as the skin, joints, brain, lung, heart, and kidneys (2). T cells from SLE patients and lupus-prone mice have expanded T helper 1 (Th1), Th17, follicular, and extrafollicular helper T (Tfh and eTfh) cell subsets, which contribute to the inflammation and autoreactive antibody production through increased IFNγ, TNFα, IL-6, IL-17, and IL-21 secretion (3–7). Diminished populations of T regulatory cells (Treg), T follicular regulatory (Tfr) cells, and cytotoxic CD8+ T cells were also thought to be contributing to the pathogenesis of SLE (8–11). In addition, increased circulating TCRαβ+CD4-CD8- double negative T (dnT) cells and renal accumulation of the minor γδT population have been associated with autoantibody production and lupus nephritis (12). Besides the phenotypic and functional alterations in effector T cells, terminally differentiated memory T cells may also be contributing to the tissue damage as these cells accumulate in SLE patients with high disease activity (13–15).

Syndecans, type I transmembrane heparan sulfate proteoglycans (HSPG), regulate diverse biological processes, such as tissue wound repair, angiogenesis, epithelial-mesenchymal transformation, and inflammation, by modifying the local concentration, stability, and accessibility of extracellular matrix components, cytokines, chemokines, and growth factors (16). Syndecan family consists of four distinct members that are mostly expressed on epithelial, endothelial, neural, or fibroblastic cells, but they are also detected on haemopoietic cells (17, 18). Syndecan 2 and syndecan 4 are up-regulated upon CD4+ T cell activation and act as inhibitors by promoting T cell receptor (TCR) clearance or by activating tyrosine phosphatase CD148 (19, 20). Syndecan 1 (CD138) is commonly used as a marker to identify plasmablasts and plasma cells (21). Recently, CD138 has been suggested to be a marker to distinguish IL-17 producing natural killer T17 (NKT17) cells from other invariant NKT cells based on its selective expression on NKT17 cells but not on NKT1 and NKT2 cells (22, 23). Moreover, CD138+ T cells were observed accumulated in gut epithelia of aged C3H wild type as well as in the spleen and lymph nodes of FasL loss-of-function C3H gld mice (24). Similarly, CD138-expressing T cells were detected in spleen and lymph nodes of lupus prone μMT/lpr mouse but these cells were only present in the lymph nodes, and not in the spleen, of another lupus-prone strain, B6/lpr mouse (25). Thus, accumulating evidence indicate the existence of CD138+ T cells in both healthy and diseased mice. However, the characteristics and pathologic roles of CD138+ T cells in lupus disease remain to be elucidated.

Here, we detected the presence of TCRβ+CD138+ cells in various organs of the lupus-prone MRL/Lpr mice. The numbers of TCRβ+CD138+ cells increased as the disease progressed. We also identified CD4+ T cells among the TCRβ+CD138- population as an important source of TCRβ+CD138+ cells. These accumulating TCRβ+CD138+ cells manifested mostly central memory phenotype (Tcm) and promoted lupus disease progression only when autoantigens were present, despite exhibiting slower activation kinetics, reduced proliferation, and diminished cytokine production after stimulation with anti-CD3/CD28 antibodies.

## MATERIALS AND METHODS

### Mice

MRL/MpJ-*Fas*$^{lpr}$/J (referred to as MRL/Lpr throughout the manuscript), MRL/MpJ (referred to as MRL throughout the manuscript) mice, and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Balb/c mice were purchased from Charles River Laboratories (Wilmington, MA). Only age-matched female mice were used for experiments. All mice were bred and maintained under specific pathogen-free conditions in the animal facility of US Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER) Veterinary Services. The breeding and use of animals were approved by the US FDA, CBER Institutional Animal Care and Use Committee (permit numbers 2002-37 and 2017-47).

### Detection of Anti-dsDNA and SM Antibodies in Sera

Serum anti-dsDNA and anti-Smith antigen (SM) antibodies were measured by ELISA as described previously (26). Briefly, calf thymic DNA (Sigma-Aldrich, St. Louis, MO) or SM (Immunovision, Springdale, AR) were coated on 96-well microtiter plates (Dynatech Immulon 4 HBX; Dynatech Labs., Chantilly, VA) at 1 μg/ml with 0.1 M of carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Plates were blocked for 30 min at room temperature in 5% BSA in PBS, then washed with 0.05% Tween-20 in PBS. Diluted serum samples were added to wells in triplicates and incubated at 37°C for 2 h. Plates were washed with 0.05% Tween-20 in PBS and further incubated with HRP-conjugated goat antibodies directed against mouse IgG (Southern Biotech, Birmingham, AL) for 1 h at room temperature. Finally, plates were washed with 0.05% Tween-20 in PBS and measured at 405 nm absorbance after developing with ABTS solution (Invitrogen, Carlsbad, CA). Antibody titers were recorded as the last titration corresponding to the OD that is twice the mean OD of blank wells.

### Flow Cytometry

Single cell suspensions of spleen, bone marrow, lymph nodes, and thymus were obtained by mechanic dissociation of tissue through a 40 μm cell strainer. The dissociated cells were filtered through a 100 μm cell strainer. Red blood cells were then lysed using ACK lysing buffer (Lonza, Wallersville, MD). In addition, mouse blood leukocytes were collected by lysing red blood cells with ACK lysing buffer and centrifugation at 300 × *g* for 5 min. Cells were stained with fluorescent-conjugated anti-mouse antibodies after blocking CD16/CD32 with Fc Block (BD Biosciences, San Jose, CA). For intracellular staining, Brefeldin A (BD Biosciences)-treated cells were stained with the surface markers and LIVE/DEAD™ Fixable Near-IR Dead

cell kit (IR-Red) (Thermo Fisher,Waltham, MA) before fixation, permeabilization, and intracellular staining as per manufactures instructions (BD Biosciences). The following antibodies were used in flow cytometry analysis: Pacific blue anti-CD19, BV421 anti-CD19, BV421 anti-TCRβ, APC anti-CD138, APC anti-TCRβ, BV605 anti-CD3, FITC anti-CD3, Percp Cy5.5 anti-CD44, FITC anti-62L, PE-Cy7 anti-PD-1, APC anti-CXCR5, Percp Cy5.5 anti-B220, PE-Cy7 anti-CD8, PE anti-CD21, PE anti-CD22, BV421anti-CD23, Alexa647 anti-CD40, FITC anti-CD80, FITC anti-CD86, Percp Cy5.5 anti-CD25, FITC anti-CD69, APC anti-CD95, Percp Cy5.5 anti-IL17, Percp Cy5.5 anti-CCR7, FITC anti-Foxp3 (all purchased from BioLegend, San Diego, CA). PE-anti-CD138 was purchased from BD Biosciences. In addition, FITC anti-BCMA, PE anti-TACI, FITC anti-IFNγ, Annexin V, (R&D system, Minneapolis, MN), ATTO 488 anti-BAFFR (Enzo life Science Inc., Farmingdale, NY), CellTrace™ CFSE Cell Proliferation Kit and Qdot605 anti-CD4 antibody (Thermo Fisher). Stained cells were acquired using LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJo (Tree Star, Ashland, OR) version 10.1 for PC.

## Quantitative Real-Time PCR

Total RNA was extracted from flow cytometry-sorted cells using the RNeasy Mini kit (Qiagen, Germantown, MD). Two hundred nanograms of total RNA were reverse-transcribed into cDNA using random hexamers with the Taqman Reverse transcription kit (Invitrogen). The expression of targeted genes and GAPDH were determined using Taqman Gene Expression assays and CFX96 Touch Real-Time System (BioRad, Hercules, CA). Relative expression values were determined by the 2-ΔCt method where samples were normalized to GAPDH gene expression.

## T Cell Isolation, Cultivation, and Adoptive Transfer Experiments

Splenic T cells from MRL/Lpr mice were purified with Dynabeads™ FlowComp™ Mouse Pan T (CD90.2) Kit and dissocated from beads as per manufacture's instructions (Thermo Fisher). Purified T cells were staind with PE-conjugated anti-CD138 antibody, and TCRβ+CD138+ and TCRβ+CD138- cells were further separated with anti-PE magnetic MicroBeads (Miltenyi Biotec, Auburn, CA). After three washes with PBS, the purity of isolated TCRβ+CD138+ cells was >95% in all experiments as determined by flow cytometry. For *in vivo* transfer, purified TCRβ+CD138+ and TCRβ+CD138- cells were suspended in PBS and $1 \times 10^7$ cells in 100 μl were i.v. injected into recipient mice. For *in vitro* culture, CD4+TCRβ+CD138- cells were further isolated from purified TCRβ+CD138- cells using the CD4 (L3T4) MicroBeads (Miltenyi Biotec), and unbound cells were identified as CD8+TCRβ+CD138- cells (over 94% purity). To block mTOR, isolated CD4+TCRβ+CD138- cells were cultured in the presence of 100 nM rapamycin (Tocris Biosciences, Minneapolis, MN). After 3 days of incubation cell viability as well as CD138 and CD4 expression levels were assessed in flow cytometry.

## Co-culture of B Cells With T Cells

Splenic B cells were isolated from 5 or 12 weeks old MRL/Lpr mice using B Cell Isolation Kit (Miltenyi Biotec). The purity of isolated B cells was over 97%. B cells were stained with CSFE before co-culturing with purified TCRβ+CD138+ or TCRβ+CD138- cells in the presence of anti-CD3/CD28 antibodies (BD Biosciences), phorbol 12-myristate 13-acetate (PMA)/ionomycin, or autoantigens [1 μg/ml of DNA or SM (Immunovision)]. DNA was isolated from MRL/Lpr splenocytes by hyperthemo treatment at 42°C for 4 h. After 3 to 4 days of incubation, cells were analyzed for CFSE dilution by flow cytometry. In other assays, after 10 days of culture, culture supernatants were analyzed for antibody production as well as IL-2 and IFNγ secretion by ELISA (R&D Systems). In CD4 blocking expriments, Ultra-LEAF™ purified CD4 antibody (clone GK1.5, Biolegend), or control rat IgG (Sigma-Aldrich) were added to T and B cell co-cultures. In some co-culture experiments, T and B cells were incubated either as mixed or separated with 0.4 μm pore sized polyester Corning Transwell® membrane insert (Sigma-Aldrich).

## Pristane-Induced Lupus Model

Nine weeks old female Balb/c or C57BL/6 mice received a single i.p. injection of 0.5 ml pristane (Sigma-Aldrich) or 0.5 ml of sterile PBS. Fourteen weeks later, sera were collected and spleens were harvested. Sera were analyzed for auto-antibodies by ELISA, and splenocytes were subjected to flow cytometry for the presence of TCRβ+CD138+ cells.

## Evaluation of Disease Progression and Histopathological Assessment of the Kidneys

Proteinuria was measured using Fisherbrand™ Urine Reagent Strips (Fisher scientific, Hampton, NH) and scored on a scale of 0–5 (0, none; 1, trace; 2, 30 mg/dl; 3, 100 mg/dl; 4, 300 mg/dl; and 5, ≥2,000 mg/dl).

## Histopathological Analysis

Mouse kidneys were fixed in 10% buffered formalin overnight, processed, and embedded in paraffin. Sections were processed as previously described (26). Hematoxylin and eosin (H&E) and Masson trichrome stainings were performed. Stained slides were scanned by Nanozoomer XR (Hamamatsu corporation, Japan) and data was store as ndpi files for analysis. Overall severity, glomerular sclerosis, inflammatory cell accumulation, and interstitial fibrosis were evaluated and scored semiquantatively between 0 and 3 (0 = within normal limits, 1 = mild pathology, 2 = moderate pathology, and 3 = severe pathology). Average scores were analyzed by GraphPad Prism software (GraphPad, San Diego, CA).

## Statistical Analysis

Data from groups were compared using GraphPad Prism software and non-parametric testing was performed by the Mann-Whitney rank sum two-tailed test for two groups and by two-way ANOVA on ranks for three or more groups.

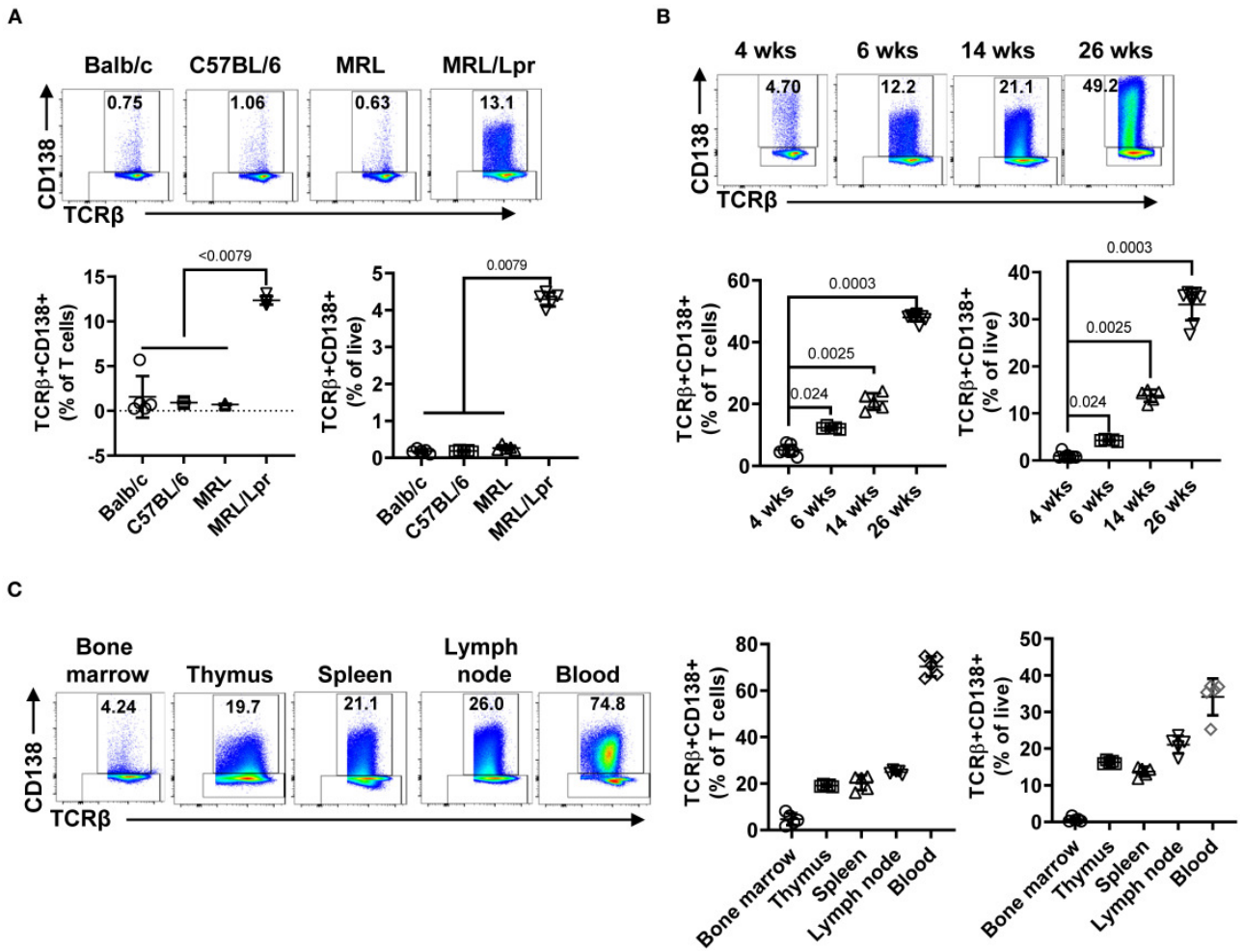


**FIGURE 1 |** TCRβ+CD138+ cells populate the spleen, lymph nodes, thymus, and blood of MRL/Lpr mice, and their numbers increase with age. In each flow cytometry experiment TCRβ+CD138+ cells were quantified after gating out dead cells and CD19+ B cells. **(A)** Splenic TCRβ+CD138+ cells from 6 weeks old Balb/c, C57BL/6, MRL, and MRL/Lpr mice were quantified. Representative pseudocolor plots from each mouse strain are shown. Mean percentages ± *SD* of five mice from two independent experiments are plotted. Two-way ANOVA test was used to calculate statistical significance. The means of the four groups were statistically significantly different ($p < 0.0001$) in 2-way ANOVA test. Mann Whitney test was used to calculate the exact *p*-value for the comparisons between MRL/Lpr and other groups. **(B)** Splenic TCRβ+CD138+ cells from 4, 6, 14, to 26 weeks old MRL/Lpr mice were quantified. Representative pseudocolor plot from each time point is shown. Mean percentages ± *SD* of five to eight mice from three separate experiments are plotted. Two tailed Mann-Whitney rank sum test was used to calculate statistical significance. **(C)** TCRβ+CD138+ cells in bone marrow, thymus, spleen, lymph nodes, and blood of 14 weeks old MRL/Lpr mice were quantified. Representative pseudocolor plots from each organ are shown. Mean percentages ± *SD* of five mice from two independent experiments are plotted.

Corresponding *p*-values for the test data points were listed on the figure.

## RESULTS

### Frequency of CD138-Expressing TCRβ+ Cells Increases Parallel to Disease Progression in MRL/Lpr Mice

The presence of CD138-expressing αβ T cells has been reported in Fas and FasL mutant mouse strains (C3H gld, μMT/lpr, and B6/lpr mice) manifesting lupus-like disease, but the physiological significance of CD138-expressing T cells in lupus pathogenesis remains unexplored (24, 25). Here, we investigated the immunopathological role of CD138-expressing T cells in MRL/Lpr mouse, a widely used lupus-prone strain (27). First, we found a large fraction of CD138-expressing cells among CD19-TCRβ+ gated splenic population in MRL/Lpr mice (6 weeks old), even before the onset of autoimmune manifestations (**Figure 1A**, **Supplemental Figure 1A**). Measurement of CD138 mRNA in qRT-PCR assay on sorted TCRβ+CD138+ cells confirmed the expression of CD138 (**Supplemental Figure 1B**). However, the frequency of TCRβ+CD138+ cells in the spleens of age-matched Balb/c, C57BL/6 as well as the parental MRL mice remained negligible (**Figure 1A**). Thus, the expression of CD138 on T cells appears to be uniquely associated with Fas

signaling deficiency (24, 25). Second, we investigated a possible correlation between TCRβ+CD138+ cells and the progression of disease in MRL/Lpr mice and found that the frequency of TCRβ+CD138+ cells gradually increased with the age of mice (**Figure 1B**). Moreover, the increase in TCRβ+CD138+ cell population was also detected in pristine-injected Balb/c and C57BL/6 mice (**Supplemental Figures 1C,D**), which also develop lupus-like autoimmune symptoms (27). It is important to note that although TCRβ+CD138+ cells were previously reported to be confined to the lymph nodes in B6/lpr mice (25), we found high percentage of these cells also in the thymus, spleen, lymph nodes, and blood of MRL/Lpr mice (**Figure 1C**). As in MRL/Lpr mice, higher frequencies of TCRβ+CD138+ cells were also found in the thymus, lymph node and blood of pristane-treated Balb/c mice (**Supplemental Figure 1E**). The increase in the frequency of blood TCRβ+CD138+ cells in 14-week old MRL/Lpr and pristane-treated mice suggest extensive circulation of TCRβ+CD138+ cells in lupus mice when they develop severe lupus disease.

## TCRβ+CD138+ Cells Derive From CD138-CD4+ T Cells

Next, we further characterized the MRL/Lpr mice TCRβ+CD138+ cells by assessing the expression of surface markers and the genes associated with B and T cell lineages. As shown previously in μMT/lpr and B6/lpr mice (24, 25), most of the TCRβ+CD138+ cells and only about 5% of TCRβ+CD138- cells in MRL/Lpr mice expressed B220 (**Supplemental Figure 2A**). There was no apparent difference in the expression of other B cell-related surface proteins BAFFR, BCMA, IgM, CD21, CD23, CD40, CD80, or CD86 as well as mRNA for *CD19*, *IGHM, Myc*, *tnfrsf13c* (BAFFR), and *tnfrsf13b* (TACI) between TCRβ+CD138+ and TCRβ+CD138- cells (**Supplemental Figures 2B–D**). In addition, the expression levels of transcription factors (*Pax5*, *PU1*, *Irf4*, and *Xbp1*) associated with B and plasma cell-differentiation were comparable between TCRβ+CD138+ and TCRβ+CD138- cells, although *Bcl-6* was higher and *Prdm1* was lower in CD138-expressing cells (**Supplemental Figure 2E**). CD138 expression did not affect the expression of the T cell marker CD3 on TCRβ+ cells as both TCRβ+CD138+ and TCRβ+CD138- were CD3+ (**Figures 2A,B**). In μMT/lpr and B6/lpr mice, all TCRβ+CD138+ cells were reported to be negative for CD4 and CD8 (24, 25). Although the majority of TCRβ+CD138+ cells were negative for CD4 and CD8 in MRL/Lpr mice also, ∼20% of TCRβ+CD138+ were CD4+, while only ∼2% were CD8+ (**Figures 2A,B**). Further confirming the association of TCRβ+CD138+ cells with T-cell but not with B-cell lineage, we measured comparable levels of *GATA3* and *Tbet* expression in TCRβ+CD138+ and TCRβ+CD138- cells, the transcription factors associated with Th2 and Th1 subsets (28), respectively (**Figure 2C**). Interestingly, CD138- cells expressed the Treg cell transcription factor *Foxp3* but not CD138-expressing cells (**Figure 2C**). To assess whether CD138 expression changed over time in incubated TCRβ+ T cells, we assessed the changes in the percentage of CD138+ cells in cultured TCRβ+CD138+ and TCRβ+CD138- cells *in vitro*. As shown in **Figure 2D**, CD138 levels remained high on TCRβ+CD138+ cells throughout the 7-days culture period. By contrast, there was a gradual increase in the frequency of CD138+ cells among the cultured TCRβ+CD138- cells from day 1 to 3. We next sought to determine whether CD138+ cells emerged from CD4+TCRβ+CD138- or CD8+TCRβ+CD138- subsets. Incubation of highly purified CD4+TCRβ+CD138- and CD8+TCRβ+CD138- for 5 days indicated that a substantial number of CD138+ cells derived from CD4+ cells. As shown in **Figure 2E** and paralleling the kinetics of CD138 expression among the TCRβ+CD138- population in **Figure 2D**, a significant increase in CD138 expression was observed among the CD4+ cells on day 1 which plateaued on day 3, while the increase in CD138+ population among the CD8+ cells remained limited. These results suggested that a portion of CD4+TCRβ+CD138- cells expresses CD138 after culture.

Multiple transduction pathways, including (mammalian target of rapamycin) (mTOR), phosphatidylinositol-3-kinase (PI3K), and mitogen-activated protein kinase (MAPK), control T cell survival, metabolism, and differentiation (29). Besides, elevated mTOR activity was found to control the expansion of dnT cells (30–32). Moreover, mTOR promotes the differentiation of CD138-expressing plasma cells (33). To test whether these pathways were also critical for CD138 expression on lupus T cells, we treated CD4+TCRβ+CD138- cells with rapamycin, a specific mTOR inhibitor, and assessed the increase in CD138 expression. As reported previously (34), rapamycin significantly reduced cell survival compared to media or DMSO treated cells after 3 days of incubation (**Supplemental Figure 2F**). As observed in **Figure 2E**, around 30% of the CD4+TCRβ+CD138- cells incubated in media expressed CD138 after 3 days of incubation (**Supplemental Figure 2G**). A similar increase in CD138 expression was observed in cells incubated in DMSO. Although, rapamycin treatment did not reduce the CD4 expression on the cell surface, as suspected, the inhibition of mTOR pathway significantly decreased both the CD138 expression level and the frequency of CD138+ cells (**Supplemental Figure 2G**). Thus, mTOR signaling pathway may be involved in the expression of CD138 on CD4+TCRβ+CD138- cells.

## TCRβ+CD138+ Cells Respond Less to TCR Engagement and PMA/ionomycin Activation Compared to CD138-Negative Counterparts

After TCR activation, T cells undergo a series of events, including up-regulation of cell surface markers, proliferation, apoptosis, and cytokine secretion (35). We assessed the differences in proliferation of TCRβ+CD138+ and TCRβ+CD138- cells following stimulation with anti-CD3/CD28 antibodies. Compared to TCRβ+CD138- cells, TCRβ+CD138+ cells proliferated significantly less at 48- and 72-h time points (**Figure 3A**). Stimulated cells were also assessed for apoptosis by flow cytometry. Compared to TCRβ+CD138- cells, TCRβ+CD138+ cells had higher number of live cells accompanied by lower number of early apoptotic and necrotic

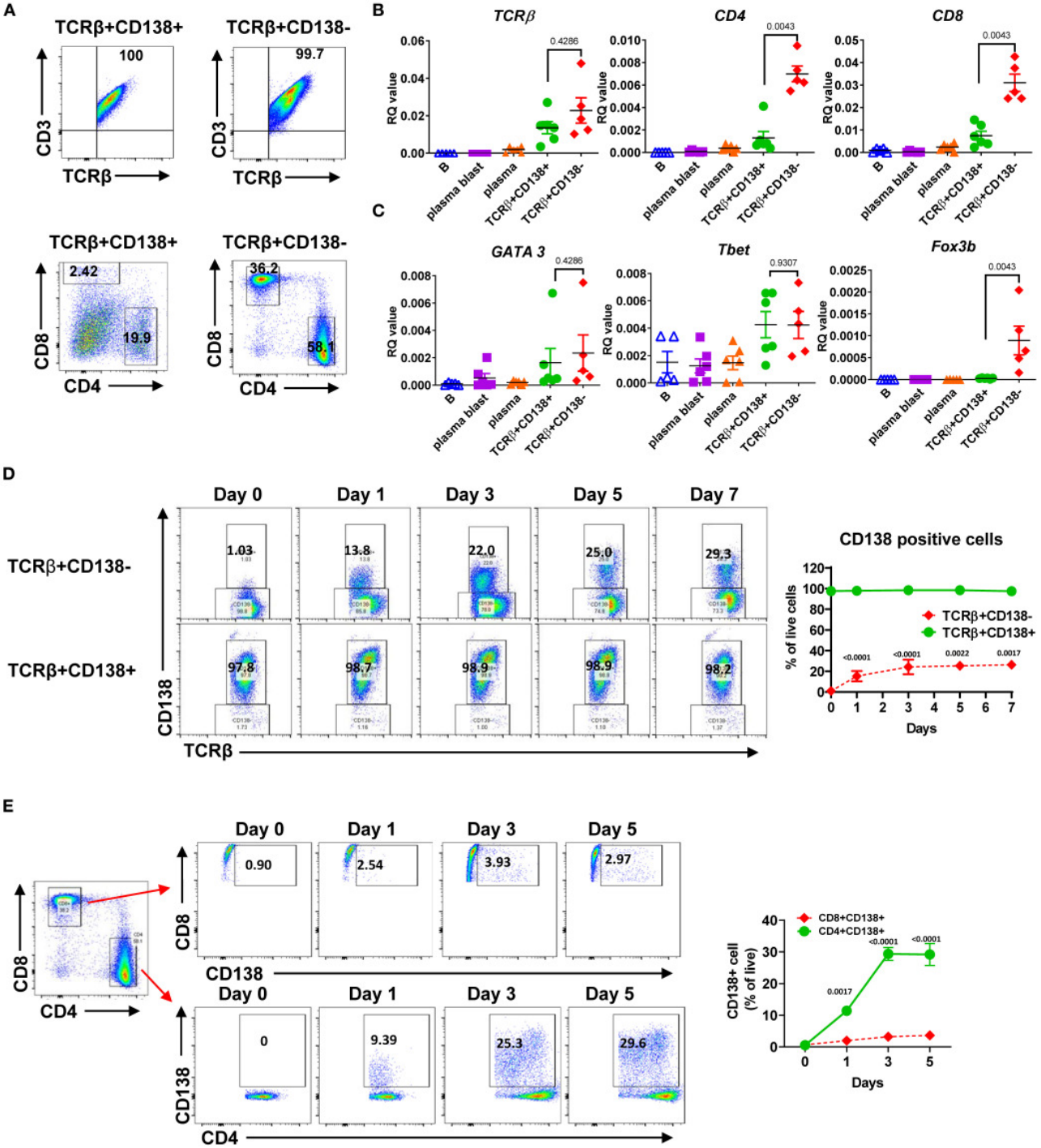


**FIGURE 2 |** Most of the TCRβ+CD138+ cells are CD4 and CD8 negative while some derive from CD4+ T cells. **(A)** Splenocytes were harvested from 14 weeks old MRL/Lpr mice. After pre-gating live and single cells, TCRβ+CD138+ and TCRβ+138- cells were further analyzed for CD3, CD4, and CD8 expression by flow cytometry. Representative pseudocolor plots from out of five mice are shown. **(B,C)** Splenocytes were collected from 14 weeks old MRL/Lpr mice. CD19+ B cells, CD19+CD138- plasmablasts, CD19-CD138+ plasma cells, CD19-TCRβ+CD138- cells, and CD19-TCRβ+CD138+ cells were sorted by flow cytometry. The mRNA expression levels of cell surface molecules *TCRβ*, *CD4*, *CD8* **(B)** and transcription factors *GATA3*, *Tbet*, and *Foxp3* **(C)** were quantified in Q-PCR. Mean ± *SD* of five to six mice from three independent experiments are plotted. **(D)** Splenocytes were collected from 14 weeks old MRL/Lpr mice. After removing CD19+ B cells, TCRβ+CD138-, and TCRβ+CD138+ cells were sorted with magnetic beads and then cultured separately for 5 days. Frequencies of CD138-expressing TCRβ+ cells were quantified by flow cytometry. Representative pseudocolor plots are shown. Mean percentages ± *SD* of six mice from three independent experiments are plotted. **(E)** Splenocytes were collected from 14 weeks old MRL/Lpr mice. After removing CD19+ B cells, CD4+TCRβ+CD138-, and CD8+TCRβ+CD138- cells were sorted with magnetic beads and then cultured separately for 5 days. Frequencies of CD138-expressing CD4+ and CD8+ T cells were measured by flow cytometry. Mean percentages ± *SD* of six mice from three separate experiments are plotted. Two tailed Mann-Whitney rank sum test was used to calculate statistical significance.

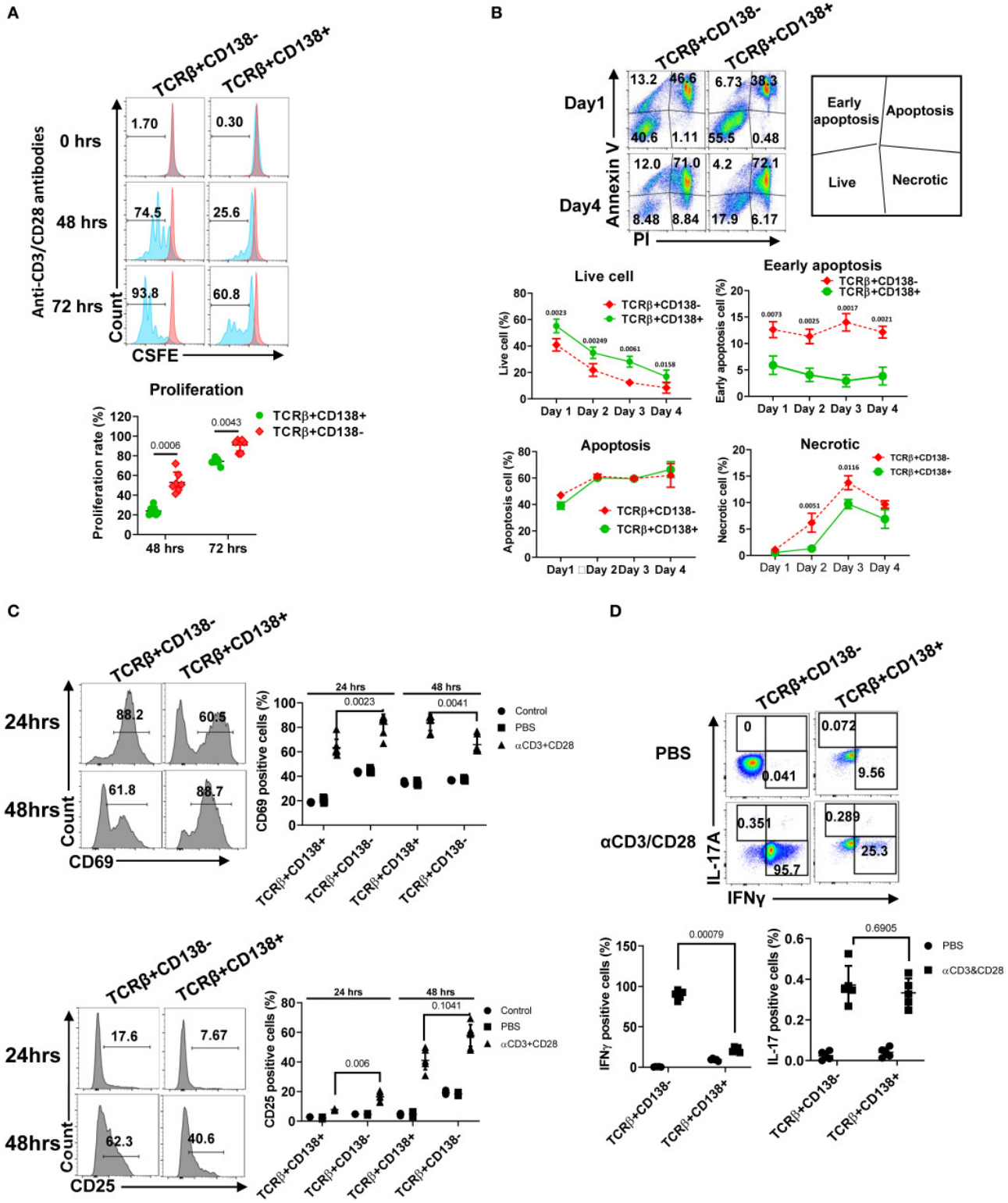


**FIGURE 3 |** TCRβ+CD138+ cells proliferate less and resist early apoptosis after activation. **(A)** Splenic TCRβ+CD138+ and TCRβ+CD138- were sorted with magnetic beads and pre-stained with CSFE prior to stimulation with anti-CD3/CD28 antibodies for 3 days. The proliferation was assessed by flow cytometry.

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**FIGURE 3 |** Representative histogram images are shown. Mean percentages ± *SD* of seven mice from three separate experiments are plotted. **(B)** Sorted splenic TCRβ+CD138+ and TCRβ+CD138- were stimulated for 4 days with anti-CD3/CD28 antibodies. Live, early apoptotic, apoptotic, and necrotic cells were measured by flow cytometry. Representative pseudocolor plots are shown. Mean percentages ± *SD* of six mice from three independent experiments are plotted. **(C)** Sorted splenic TCRβ+CD138+ and TCRβ+CD138- cells were activated with anti-CD3/CD28 antibodies for 24 or 48 h. Cells were stained with CD69 and CD25 antibodies to assess activation kinetics in flow cytometry. Representative histogram images indicating the frequency of positive cells are shown. Mean percentages ± *SD* of seven mice from three independent experiments are plotted. **(D)** Sorted splenic TCRβ+CD138+ and TCRβ+CD138- cells were activated with anti-CD3/CD28 antibodies for 16 h. Representative pseudocolor plots show intracellular IFNγ, and IL-17 staining. For each cytokine, mean ± *SD* of five mice are plotted. Two tailed Mann-Whitney rank sum test was used to calculate statistical significance.

cells (**Figure 3B**). The early and late activation state of TCR-stimulated T cells were assessed by CD69 and CD25 expression, respectively (36). Overall, anti-CD3/CD28 antibody-induced activation of TCRβ+CD138+ cells were delayed compared to TCRβ+CD138- cells. At 24-h time point, CD69 expression was higher on TCRβ+CD138- cells than on TCRβ+CD138+ cells, but by 48 h its expression decreased compared to TCRβ+CD138+ cells (**Figure 3C**). Conversely, the increase in CD69 expression on TCRβ+CD138+ cells peaked with a delay at 48 h. A similar delay in the activation markers were observed in PMA/ionomycin-stimulated TCRβ+CD138+ cells (**Supplemental Figure 3A**). In SLE, activated T cells participate in the inflammatory process through the production of cytokines such as IFNγ, TNFα, and IL-17 (2–4, 7, 37, 38). We found that after TCR engagement and PMA/ionomycin stimulation more than 90% of TCRβ+CD138- cells were positive for IFNγ, while <30% of TCRβ+CD138+ cells produced IFNγ (**Figure 3D**, **Supplemental Figures 3B,F**). Similarly, TCRβ+CD138+ cells expressed less TNFα and IL-21 compared to TCRβ+CD138- cells (**Supplemental Figures 3C,D**). Unlike IFNγ and TNFα, IL-17 production was not different between the two subsets after TCR stimulation (**Figure 3D**, **Supplemental Figure 3E**). However, TCRβ+CD138+ cells produced less IL-17 than TCRβ+CD138- cells after PMA/ionomycin stimulation (**Supplemental Figure 3F**). These results indicate that the phenotype of TCRβ+CD138+ cells activated with either PMA/ionomycin or through TCR are markedly different than their CD138 deficient counterparts.

## TCR-Stimulated TCRβ+CD138+ Cells Are Less Efficient in Activating B Cells Than TCRβ+CD138- Cells

An important function of T cells is to enhance antibody-mediated immunity by driving B cell proliferation and development into long lived memory B cells or antibody-secreting plasma cells (39). To investigate the capacity of TCRβ+CD138+ in helping B cells, we co-incubated splenic B cells purified from 6 weeks old (disease free) MRL/Lpr mice with splenic TCRβ+CD138+ or TCRβ+CD138- cells from 10 to 12 weeks old MRL/Lpr mice in the presence of anti-CD3/CD28 antibodies and determined the proliferation of B cells (**Supplemental Figure 4A**). We found significantly less proliferation of B cells co-cultured with TCRβ+CD138+ cells than those co-cultured with TCRβ+CD138- cells (**Figure 4A**). Although TCRβ+CD138+ cells harbor higher *Bcl-6* (**Supplemental Figure 2E**), a transcription factor for CXCR5+PD-1+ T follicular helper (Tfh) cells, than TCRβ+CD138- cells, in B cell co-culture experiments the increase in Tfh cell numbers among TCRβ+CD138+ cells was modest and remained less than the increase in TCRβ+CD138- cells (**Supplemental Figures 4A,B**). Moreover, TCRβ+CD138+ cells were less efficient in inducing the generation of plasma cells (**Figure 4B**) and the production of IgM and IgG than TCRβ+CD138- cells (**Figure 4C**).

To further characterize TCRβ+CD138+ cells, we tested the immunomodulatory effect of TCRβ+CD138+ cells in SLE by adoptively transferring TCRβ+CD138+ or TCRβ+CD138- cells into MRL/Lpr mice and evaluating the disease progression. We conducted the adoptive transfer experiments in two different recipient mice age groups, 7 to 8 weeks old mice with minimal lupus symptoms and 11 to 12 weeks old mice with established lupus symptoms. We chose these two age groups to assess whether the CD138-expressing cells impact the lupus progression differently in MRL/Lpr mice from different stages of disease. To our surprise, TCRβ+CD138+ cells had completely opposite effect in the recipient mice at different ages. The 7 to 8 weeks old recipient mice without lupus symptoms manifested slower progression of disease when they were transferred with TCRβ+CD138+ cells from 10 to 12 weeks old MRL/Lpr (sick) mice, compared to those that were injected with PBS. The increase in anti-dsDNA antibody and proteinuria levels were slower compared to those that were injected with PBS (**Figure 4D**, **Supplemental Figure 4C**). Kidney histopathological findings showed the most severe changes in recipients of TCRβ+CD138- group, including end stage glomeruloscrerosis with severe inflammation and interstitial fibrosis. The glomerular histopathological changes of TCRβ+CD138+ cell-recipient mice were reduced as compared to PBS-injected mice 2 weeks after the transfer of cells, but the difference in histopathological scores did not reach statistical significance (**Figure 4E**). However, histopathological changes were significantly less in TCRβ+CD138+ cell-injected mice than those injected with TCRβ+CD138- cells. In sharp contrast to its effect in young recipient mice, TCRβ+CD138+ cells significantly increased the development of anti-dsDNA antibodies and proteinuria in older MRL/Lpr mice with existing disease symptoms (**Figures 4F**, **Supplemental Figure 4D**). The TCRβ+CD138- cells accelerated the disease progression in recipient animals from both age groups but their impact on older recipient mice was not as pronounced as those that received TCRβ+CD138+ cells (**Figures 4D–F**, **Supplemental Figures 4C,D**). Together with the *in vitro* B cell co-incubation data (**Figures 4A–C**), the adoptive transfer experiments in young MRL/Lpr mice suggest an immunosuppressive function for TCRβ+CD138+ cells on B

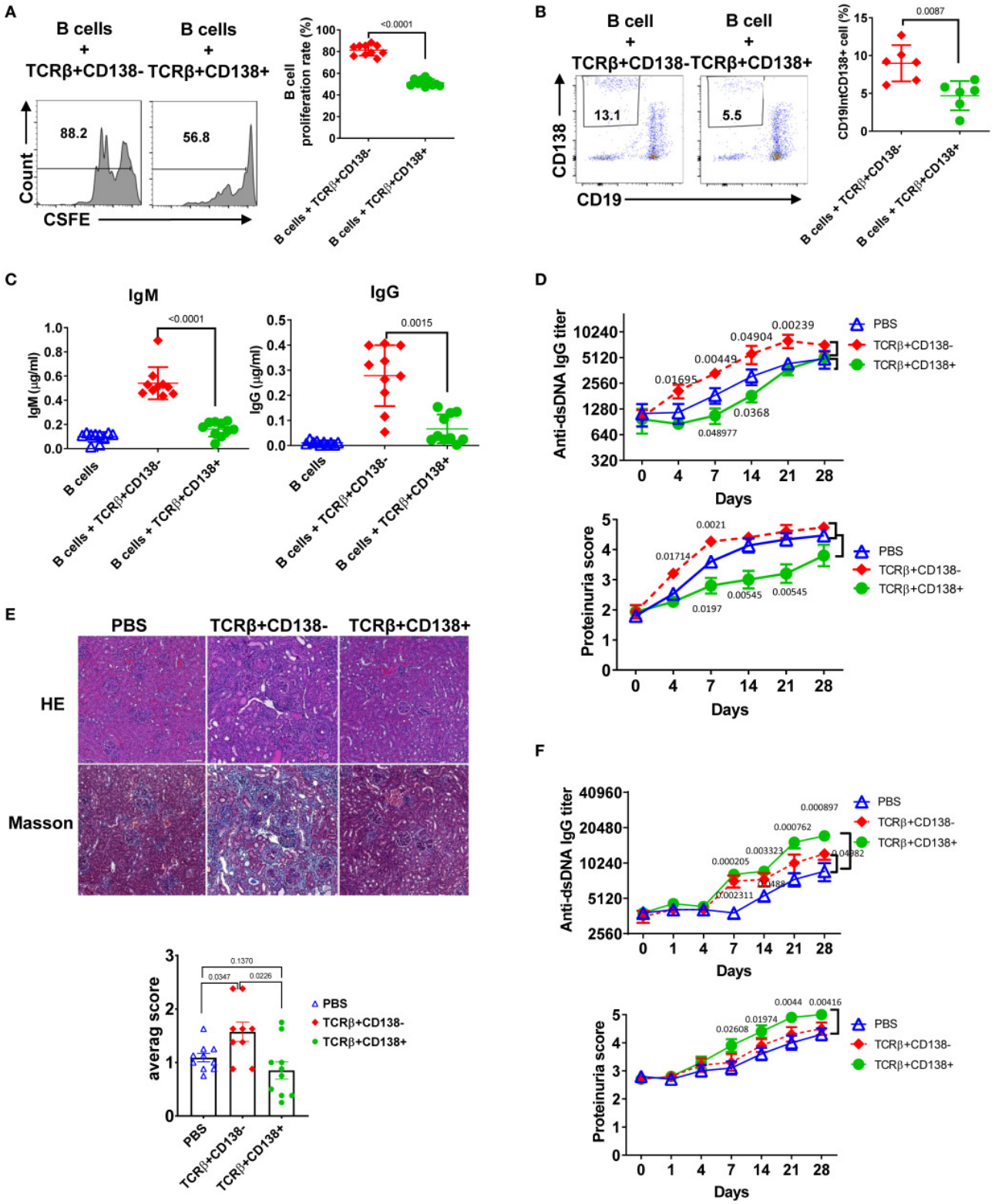


**FIGURE 4 |** TCRβ+CD138+ cells are unable to promote lupus diseases development in young MRL/Lpr mice. **(A–C)** Sorted splenic TCRβ+CD138+ and TCRβ+CD138- cells from 10 to 12 weeks old MRL/Lpr mice were co-cultured with purified splenic B cells from 6 weeks old mice in the presence of anti-CD3/CD28

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**FIGURE 4 |** antibodies for 5 days. After gating-out TCRβ+ T cells, the proliferation of B cells **(A)** and the frequency of plasma cells ($CD19^{int}$CD138+) **(B)** were measured by flow cytometry. Mean ± *SD* of 10 mice **(A)** or six mice **(B)** from three independent experiments are plotted. **(C)** Culture supernatants from the above co-culture experiment were analyzed for total IgM and IgG concentrations by ELISA. Mean ± *SD* of 10 mice from three independent experiments are plotted. **(D,E)** Splenic TCRβ+CD138+ and TCRβ+CD138- cells were sorted from 10 to 12 weeks old MRL/Lpr mice and then adoptively transferred into 7 to 8 weeks old MRL/Lpr mice without disease symptoms. **(D)** Autoreactive IgG antibody (dsDNA) and proteinuria levels were measured on indicated days. Mean ± *SEM* of 15 mice from three independent experiments are plotted. **(E)** Kidneys were collected 2 weeks after the transfer of cells and histopathological evaluations were performed on H&E and Masson stained specimens. Average pathology scores of 10 mice from two separate experiments are plotted. **(F)** Splenic TCRβ+CD138+ and TCRβ+CD138- cells were sorted from 10 to 12 weeks old MRL/Lpr mice and then adoptively transferred into 11 to 12 weeks old MRL/Lpr mice with existing disease symptoms. Autoreactive IgG antibody (dsDNA) and proteinuria levels were measured on indicated days. Mean ± *SEM* of 10 mice from two independent experiments are plotted. Two tailed Mann-Whitney rank sum test was used to calculate statistical significance.

cell differentiation. However, TCRβ+CD138+ cells contribute to the acceleration of disease progression if the recipient host has established lupus disease.

## TCRβ+CD138+ Cells Augment Autoreactive B Cell Responses When Auto-Antigens Are Present

Next, we sought to explore the underlying mechanism for the dramatic difference in the disease-stage-specific impact of TCRβ+CD138+ cells to lupus progression in the adoptive transfer experiments. An important distinction between the young and older recipient MRL/Lpr mice in the adoptive transfer experiments is the exposure of the immune system of older mice (11–12 weeks old), but not the younger (7–8 weeks old) mice, to self-antigens as a result of apoptosis (40). As observed with the younger mice in the adoptive transfer experiments, *in vitro* TCR stimulation of TCRβ+CD138+ cells also resulted in less activation of autoreactive B cells than those culture conditions containing TCR-stimulated TCRβ+CD138- cells. A common feature of these *in vitro* and *in vivo* experiments is the absence of auto-antigens in the system. Earlier studies have highlighted the importance of B cells in presenting auto-antigens to T cells in activating autoreactive T cells (41). We therefore repeated the *in vitro* co-culture experiments in the presence of apoptotic DNA instead of anti-CD3/CD28 antibodies. Interestingly, when B cells from 12 weeks old MRL/Lpr mice with established disease were co-incubated with TCRβ+CD138+ cells from age matched MRL/Lpr mice in the presence of DNA from apoptotic cells, culture supernatants contained significantly higher autoreactive (**Figure 5A**) and total IgG and IgM antibodies than those co-cultured with TCRβ+CD138- cells (**Supplemental Figure 5A**). Inclusion of DNA in the co-culture experiments resulted in a higher percentage of Tfh cell formation from TCRβ+CD138+ cells than when cells were stimulated with anti-CD3/CD28 antibodies (**Supplemental Figure 4B** vs. **Supplemental Figure 5B**). Despite this increase, the frequencies of Tfh cells among TCRβ+CD138- and TCRβ+CD138- cells were comparable (**Supplemental Figure 5B**). Nevertheless, consistent with the difference in antibody responses, higher percentage of plasma cells (CD3-CD19-CD138+) emerged from the cultures containing TCRβ+CD138+ cells than from the cultures with TCRβ+CD138- cells (**Figure 5B**). Increased plasma cell generation and autoreactive antibody production with TCRβ+CD138+ cells were not restricted to self-DNA-containing cultures, because replacement of self-DNA with the auto-antigen SM also resulted in higher percentage of plasma cell development and increased production of anti-SM antibodies with TCRβ+CD138+ cells than with TCRβ+CD138- cells (**Figure 5C**, **Supplemental Figure 5C**). Thus, regardless of the nature of the autoantigen, TCRβ+CD138+ cells are more potent in aiding autoreactive B cells to produce self-reactive antibodies than TCRβ+CD138- cells.

We next sought to determine whether TCRβ+CD138+ cell-enhanced B cell differentiation is mediated by direct cell contact, especially because the majority of the MRL/Lpr mice TCRβ+CD138+ cells were CD4 and CD8 negative (**Figure 2A**). Separation of B and TCRβ+CD138+ cells in the co-culture experiments with a Transwell® system resulted in a significant reduction in plasma cell generation compared to cells cultured without a Transwell® system (**Figure 5B**). Moreover, although CD4+ cells constituted only ∼20% of the TCRβ+CD138+ cells, the B cell help provided by TCRβ+CD138+ cells required CD4 because inclusion of anti-CD4 blocking antibodies in the co-culture system severely reduced plasma cell development and production of anti-dsDNA IgG and IgM antibodies (**Figure 5D**, **Supplemental Figure 5D**). These experiments established the CD4+TCRβ+CD138+ cells as more potent autoreactive B cell-activating T cell subset when self-antigens are present in the culture environment.

## TCRβ+CD138+ Cells Promote Disease in MRL/Lpr Mice Only When Self-Antigens Are Exposed

We showed that in order for TCRβ+CD138+ cells to augment autoreactive B cell responses, they need to be stimulated by B cell-presented self-antigens (**Figures 5A–D**). The fact that the recipient MRL/Lpr mice used in the adoptive transfer experiments in **Figure 4D** were too young to have sufficient amounts of circulating self-antigens, such as DNA, could be the reason why the transferred TCRβ+CD138+ cells were not activated and did not exacerbate SLE symptoms in the recipient mice. Conversely, the acceleration of disease progression in older MRL/Lpr mice after the transfer of TCRβ+CD138+ cells could be due to the presence of circulating self-antigens. To test this possibility, we co-administered TCRβ+CD138+ cells with DNA into young (5 to 6 weeks old) MRL/Lpr mice. As observed previously (**Figure 4D**), the increase in anti-dsDNA IgG and IgM antibodies as well as proteinuria were significantly slower in mice injected only with TCRβ+CD138+ cells compared to PBS-injected mice or control mice injected with DNA only

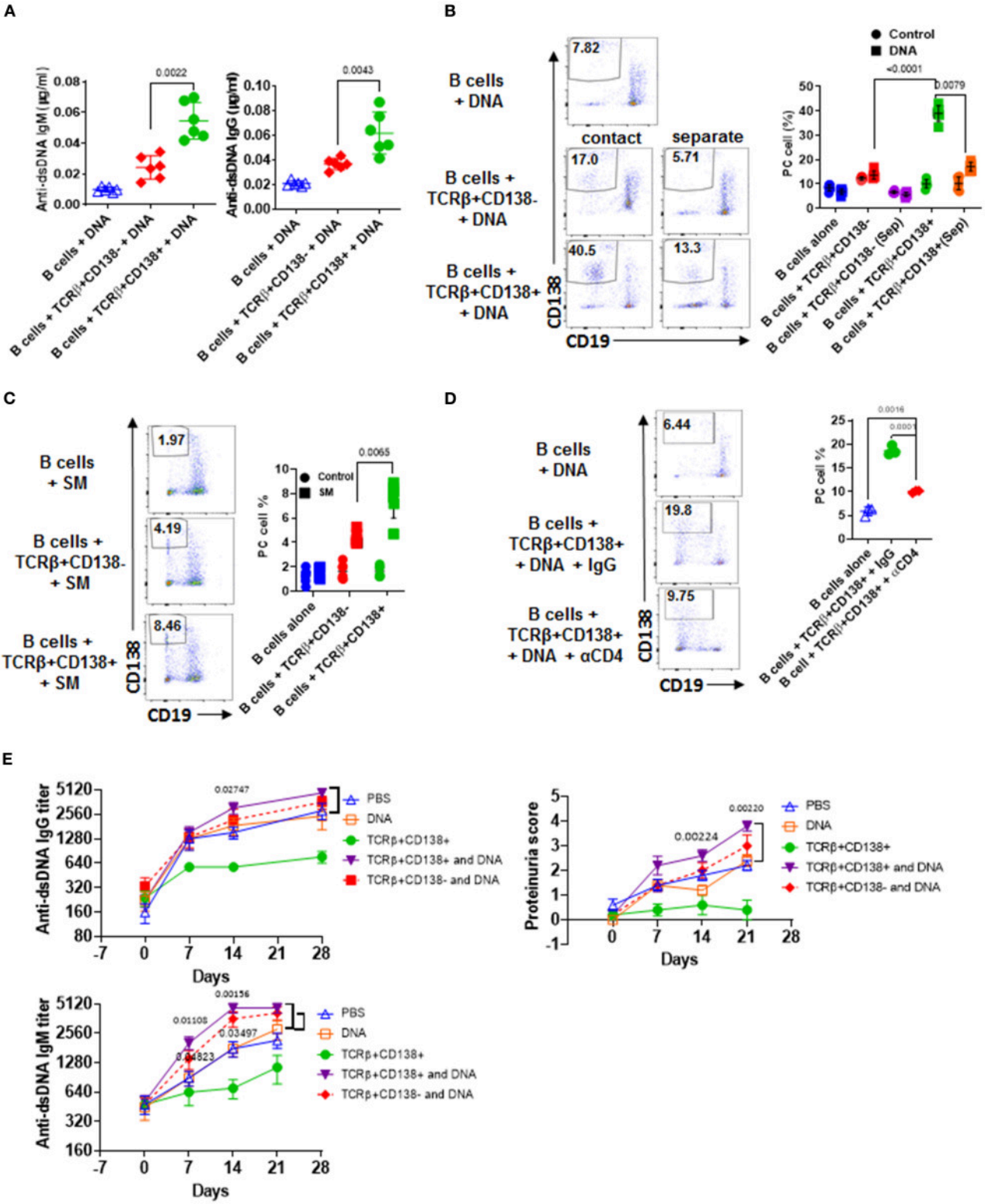


**FIGURE 5 |** TCRβ+CD138+ cells activate autoreactive B cells when auto-antigens are included in the culture. **(A–D)** Sorted splenic TCRβ+CD138+ and TCRβ+CD138- cells from 12 weeks old MRL/Lpr mice were co-cultured with purified splenic B cells from the same mice for 5 days. **(A)** DNA was included in the

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**FIGURE 5 |** co-cultured cells and culture supernatant anti-dsDNA IgM and IgG antibodies were measured by ELISA. Mean ± *SD* of six mice from three independent experiments are plotted. **(B)** T and B cells were incubated as mixed (contact) or separated with Transwell® (separate) in the presence of DNA. After gating-out TCRβ+ T cells, the differentiation of B cells into plasma cells ($CD19^{int}CD138+$) was quantified by flow cytometry. Mean percentages ± *SD* of six mice from three independent experiments are plotted. **(C)** SM was included in the co-cultured cells and differentiation of B cells into plasma cells ($CD19^{int}CD138+$) were quantified by flow cytometry after gating-out TCRβ+ T cells. Mean percentages ± *SD* of six mice from three experiments are plotted. **(D)** Cells were incubated in the presence of antibody against CD4 or control IgG and the differentiation of B cells into plasma cells was quantified by flow cytometry. Mean percentages ± *SD* of three independent experiments are plotted. **(E)** Splenic TCRβ+CD138+ and TCRβ+CD138- cells from 10 to 12 weeks old MRL/Lpr mice were sorted and then adoptively transferred into 5 to 6 weeks old MRL/Lpr mice with or without DNA. Mice that received PBS or DNA only served as control. Serum anti-dsDNA IgG and IgM antibody as well as proteinuria levels at indicated days were measured. Mean ± *SEM* of five mice are plotted. Two tailed Mann-Whitney rank sum test was used to calculate statistical significance.

(**Figure 5E**). In contrast, and as hypothesized, anti-dsDNA antibody, and proteinuria levels were significantly higher in mice co-administered with DNA and TCRβ+CD138+ cells than in control mice injected with DNA only (**Figure 5E**). Taken together, TCRβ+CD138+ cells can modulate lupus development in MRL/Lpr mice in a disease-stage-dependent manner; they slow down the symptoms prior to the emergence of self-antigens and accelerate the disease progression when self-antigens are exposed.

## TCRβ+CD138+ Cells Are Central Memory Biased T Cells

Abnormal accumulation and differentiation of memory T cells have been reported in lupus patients (13). Memory T cells confer immediate protection and mount recall responses upon reencounter with antigens. Since adoptively transferred TCRβ+CD138+ cells promoted disease progression in an autoantigen dependent manner (**Figure 5**), we asked whether TCRβ+CD138+ cells have a memory T cell phenotype. The circulating memory T cell compartments are divided into Tcm and effector memory T cells (Tem) subsets based on the expression of cell surface molecules, such as CD44, CD62L, and CCR7 (42). We first characterized the expression of CD44 and CD62L on splenic TCRβ+CD138+ and TCRβ+CD138- cells in 12 weeks old MRL/Lpr mice. Using these two markers, we identified CD44-CD62L+ naïve T (Tn) cells, CD44+CD62L- Tem, and CD44+CD62L+ Tcm subsets. Among the TCRβ+CD138- cells, ∼10% were Tn, 50% Tem, and 40% Tcm (**Figure 6A**, **Supplemental Figure 6A**). Interestingly, Tcm cells comprised the vast majority (95%) of the population among the TCRβ+CD138+ cells (**Figure 6A**, **Supplemental Figure 6A**). Typically, memory T cells are either CD4+ or CD8+ (43), but in MRL/Lpr mice, the majority of the CD138-expressing CD4+, CD8+ as well as CD4-CD8- cells exhibited Tcm memory phenotype, based on the elevated expression of CD44 and CD62L (**Supplemental Figure 6B**).

The chemokine receptor CCR7, which enables cells to home to secondary lymphoid organs where they encounter antigen, are highly expressed on Tcm cells (44). Further confirming their Tcm phenotype, TCRβ+CD138+ cells expressed higher CCR7 levels than TCRβ+CD138- cells (**Figure 6B**). The Tcm and Tem memory subsets can also be distinguished based on the expression of transcription factors *Bcl-6* and *Bim* for Tcm and BLIMP1 (*Prdm1*) for Tem, respectively (45, 46). We found higher *Bcl-6* and *Bim1* but lower *Prdm1* mRNA expression in TCRβ+CD138+ cells, compared to TCRβ+CD138- cells (**Figure 6C**). We also assessed Tcm phenotype based on IFNγ and IL-2 production because in humans, Tcm cells produce more IL-2, while Tem cells are distinguished by high IFNγ and TNFα production (47). Consistent with data in humans upon stimulation with anti-CD3/CD28 antibodies, MRL/Lpr mice TCRβ+CD138+ cells secreted significantly higher IL-2 but lower IFNγ than TCRβ+CD138- cells (**Figure 6D**). The Tcm specific IL-2 and IFNγ production profile in TCRβ+CD138+ cells were also observed in co-culture system with autoantigens and B cells (**Figure 6E**). Moreover, we found that in MRL/Lpr mice, the Tcm phenotype of TCRβ+CD138+ cells were not restricted to the spleen or the age because cells isolated from various organs of mice at different ages, all predominantly exhibited the Tcm phenotype (**Figure 6F**, **Supplemental Figures 6B,C**). Collectively, these data established the memory phenotype of TCRβ+CD138+ cells as Tcm in MRL/Lpr mice. Finally, to assess whether the Tcm phenotype of TCRβ+CD138+ is unique to MRL/Lpr mouse, we compared the memory phenotype of TCRβ+CD138+ cells from Balb/c and C57BL/6 mice to those of MRL/Lpr mice. Again, regardless of the mouse strain, TCRβ+CD138+ were CD44+CD62L+ Tcm cells (**Figure 6G**). Thus, the Tcm phenotype of TCRβ+CD138+ cells is conserved among different mouse strains.

## DISCUSSION

In this study, we uncovered a disease-stage dependent accumulation of TCRβ+CD138+ cells in various organs of lupus-prone MRL/Lpr mice, which are overwhelmingly positive for B220, but negative for CD4 and CD8 expression. Although these cells are less efficient in responding to non-specific T cell stimuli, such as PMA/ionomycin and anti-CD3/CD28 antibody engagement, they are more potent in aiding antibody production from autoreactive B cells *in vitro* as well as *in vivo* when autoantigens are present. Further characterization established the phenotype of these cells as Tcm based on high expression levels of CD62L, CD44, CCR7, and Bcl-6.

CD138 is widely expressed on epithelial cells as well as on other adherent cells, but its expression on normal lymphoid cells has been thought to be restricted to plasma cells and pre-B cells. However, recent studies reported that CD138 is also present on NKT17 and GC B cells, where it may be involved in host defense or autoimmunity through IL-17 secretion or binding to death receptor 6 on Tfh cells (22, 48). In aged C3H mice, accumulation of CD138-expressing T cells was

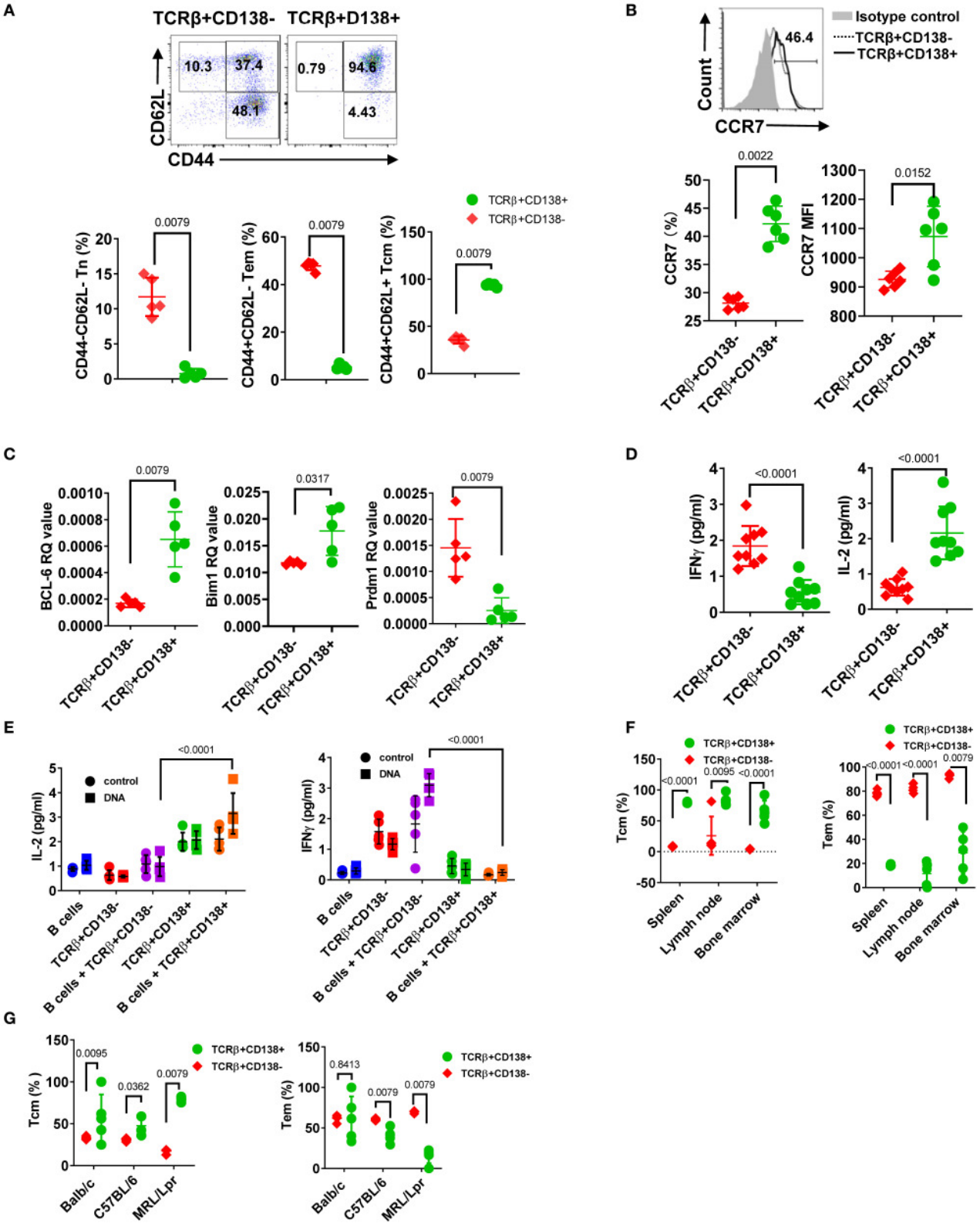


**FIGURE 6 |** CD138+ T cells exhibit central memory T cell phenotype. **(A,B)** Splenocytes were collected from 12 weeks old MRL/Lpr mice. **(A)** The expression of CD44 and CD62L on TCRβ+CD138- and TCRβ+CD138+ cells were measured by flow cytometry. Representative pseudocolor plots are shown. Mean percentages

*(Continued)*

**FIGURE 6 |** ± *SD* of five mice from two separate experiments are plotted. **(B)** Representative flow cytometry histogram of CCR7 expression on TCRβ+CD138- and TCRβ+CD138+ cells are shown. Mean ± *SD* percentages and MFIs for CCR7 expression from six mice in two separate experiments are plotted. **(C)** Splenic TCRβ+CD138+ and TCRβ+CD138- cells were sorted from 12 weeks old MRL/Lpr mice and *BCL-6*, *Bim1*, and *Prdm1* mRNA were quantified by Q-PCR. Mean ± *SD* values of five mice from two separate experiments are plotted. **(D)** Splenic TCRβ+CD138+ and TCRβ+CD138- cells were sorted from 12 weeks old MRL/Lpr mice and cultured with anti-CD3/CD28 antibodies overnight. Culture supernatant IFNγ and IL-2 levels were measured by ELISA. Mean ± *SD* of nine mice from three independent experiments are plotted. **(E)** Splenic TCRβ+CD138+, TCRβ+CD138- cells were sorted from 12 weeks old MRL/Lpr mice and co-cultured with sorted B cells from the same mice in the presence of DNA for 3 days. Culture supernatant IFNγ and IL-2 levels were measured by ELISA. Mean ± *SD* of five mice from two separate experiments are plotted. **(F)** Cells were collected from spleen, lymph nodes, and bone marrows of 12 weeks old MRL/Lpr mice. Frequencies of Tcm (CD44+CD62L+) and Tem (CD44+CD62L-) cells among TCRβ+CD138- and TCRβ+CD138+ cells were measured in flow cytometry. Mean percentages ± *SD* of five mice from two independent experiments are plotted. **(G)** Splenocytes were collected from 12 weeks old Balb/c, C57BL/6, and MRL/Lpr mice. Frequencies of Tcm (CD44+CD62L+) and Tem (CD44+CD62L-) cells among TCRβ+CD138- and TCRβ+CD138+ cells were measured in flow cytometry. Mean percentages ± *SD* of five mice from two independent experiments are plotted. Two tailed Mann-Whitney rank sum test was used to calculate statistical significance.

shown to be restricted to the gut epithelium, although they can expand to peripheral organs, such as lymph nodes and spleen when Fas ligand (gld) is ablated. Similar to C3H gld mice, a large population of CD138-expressing T cells accumulate in peripheral organs of Fas receptor mutant μMT/Lpr, B6/Lpr (25) and MRL/Lpr mice (**Figure 1A**). As previously described, the majority of CD138+ T cells also express CD3 and B220 and are negative for CD4 and CD8 (dnT). Previous reports suggested that dnT cells derive from exhausted autoreactive CD8+ cells or continuously stimulated CD8+ cells (49, 50). Differing from these reports, our observations reveal that a substantial portion of the TCRβ+CD138+ cells in MRL/Lpr mice are converted from CD4+ cells rather than CD8+ cells, as CD138+ cells emerged from *in vitro* cultured CD4+ cells, but not from CD8+ cells (**Figure 2D**). Interestingly, we found that the exposure of TCRβ+CD138- cells to rapamycin severely reduced the increase in CD138 expression on these cells (**Supplemental Figure 2H**). Rapamycin was also reported to inhibit the generation of CD138-expressing plasma cells (33). These observations point to mTOR-mediated regulation of CD138 expression on lymphocytes. mTOR is also implicated in the pathogenesis of SLE, especially because systemic rapamycin administration alleviates lupus symptoms (51, 52). Also, inhibition of mTOR is shown to reduce the production of dnT cells and favors the expansion of therapeutic CD4+CD25+Foxp3+ Treg cells in lupus-prone mice and SLE patients (30–32). Further studies will be needed to test whether the beneficial effect of rapamycin in SLE is through its inhibition of CD138 expression on T cells.

Studies in normal mice or autoimmune-prone lpr mice have shown that dnT cells are able to dampen CD4+ and CD8+ T cell-mediated autoimmune responses both *in vitro* and *in vivo* (53, 54). Consistent with these studies, adoptively transferred TCRβ+CD138+ T cells, of which the majority were CD4-CD8- cells, slowed down the disease progression in young MRL/Lpr mice when auto-antigens are not exposed. The TCRβ+CD138+ cell-mediated suppression of disease progression is unlikely to be due to the apoptosis of host CD4+ and CD8+ T cells induced by the transferred cells as this mechanism requires functional Fas-FasL interaction (54). Suppression of dnT cell-mediated alloimmune responses are also attributed to elevated levels of perforin and granzyme B produced by these cells (55). This mechanism is also unlikely to be at play in the slowing down of disease progression by TCRβ+CD138+ cells in young MRL/Lpr mice because we found comparable levels of perforin and granzyme B production by TCRβ+CD138+ and TCRβ+CD138- cells (**Supplemental Figure 7**). Although the exact molecular mechanism and cascade of events need to be deciphered, we augur that the suboptimal proliferation capacity of TCRβ+CD138+ cells as well as their diminished production of pathogenic cytokines, IL-17, TNFα, and IFNγ (56) may be responsible for the delay in disease progression afforded by these cells in young MRL/Lpr mice.

In active lupus patients or NZBxSWR mice, CD4+ as well as dnT cells augment the production of anti-dsDNA antibodies when they are co-cultured with oligoclonal autoreactive B cells (41, 57, 58). Consistent with these early observations, both TCRβ+CD138+ and TCRβ+CD138- cells enhanced plasma cell development and amplified autoreactive antibody production from MRL/Lpr mice B cells when auto-antigens were present. However, TCRβ+CD138+ were more potent than TCRβ+CD138- cells in activating autoreactive B cells both *in vitro* and *in vivo*. Interestingly, CD4-expressing TCRβ+CD138+ cells were responsible for the activation of autoreactive B cells, despite comprising <20% percent of the total TCRβ+CD138+ population. This rapid recall response upon antigen re-encounter is a typical characteristic of memory T cells (42, 47). Indeed, compared to TCRβ+CD138- cells, over 90% of TCRβ+CD138+ cells are CD44+CD62L+ Tcm cells. Although the other Tcm marker CCR7 is also higher on TCRβ+CD138+ cells than on TCRβ+CD138- cells, the frequency of CCR7- expressing TCRβ+CD138+ cells is <50%. The discrepancy between the percentage of CD44+CD62L+ cells and CCR7+ cells may be due to possible loss of CCR7 expression on TCRβ+CD138+ cells after repeated exposure to autoantigens, a phenomenon reported for CCR7+CD27+ memory T cells in lupus patients which lose CCR7 expression following repeated stimulation (13). Interestingly, clinical studies indicate that the expansion of dnT cells as well as the CD62L- CD197- effector-memory T cells and the CD62L+CD197+ central-memory cells are controlled by mTOR since rapamycin treatment decreased these populations in SLE patients (31, 32). The predominance of TCRβ+CD138+ cells presenting with memory phenotype and the suppression of CD138-expression with rapamycin (**Supplemental Figure 2H**) in MRL/Lpr mice suggest that mTOR mediated expansion of CD138-expressing memory T cells may be associated with lupus pathogenesis in MRL/Lpr mice as well as in SLE patients. Of note, the Tcm characteristic of TCRβ+CD138+ cells is independent of disease activity as TCRβ+CD138+ cells from healthy MRL/Lpr

mice as well as different healthy mouse strains also exhibit similar phenotype. Thus, TCRβ+CD138+ cells manifest Tcm phenotype, regardless of mouse strain and disease status.

Taken together, we have identified and characterized the phenotype of TCRβ+CD138+ cells in MRL/Lpr mice and unveiled a pathogenic role for these cells in lupus disease. Both *in vivo* adoptive transfer experiments and *in vitro* co-culture experiments underpin the role of TCRβ+CD138+ cells in activating autoreactive B cells when autoantigens are exposed. Although how CD138 expression renders TCRβ+ T cells more pathogenic is still enigmatic, the discovery of a novel Tcm subset with enhanced pathogenic properties may be useful in assessing and monitoring disease severity.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

## ETHICS STATEMENT

The animal study was reviewed and approved by US FDA, CBER Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

LL and MA conceived the study and contributed to the manuscript writing. LL and KT did the laboratory work. LL, KT, and MA did the statistical analysis and contributed to the data analysis and interpretation. All authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

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REVIEW
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# COVID-19 and Systemic Lupus Erythematosus: Focus on Immune Response and Therapeutics

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The SARS-CoV-2 novel coronavirus has caused the COVID-19 pandemic with over 35 million cases and over a million deaths worldwide as of early October 2020. The populations most affected are the elderly and especially those with underlying comorbidities. In terms of race and ethnicity, black and hispanic populations are affected at disproportionately higher rates. Individuals with underlying conditions that cause an immune-compromised state are considered vulnerable to this infection. The immune response is an important determinant in viral infections including coronaviruses, not only in the antiviral defense but also in the disease progression, severity, and clinical outcomes of COVID-19. Systemic lupus erythematosus is a chronic autoimmune disease which also disproportionately afflicts black and hispanic populations. In lupus patients, an aberrant immune response is characterized by the presence of circulating autoantibodies, lymphopenia, aberrant T cells, and proinflammatory cytokines along with defective regulatory mechanisms, leading to immune-mediated damage to tissues. Lupus patients are often treated with immune-suppressants and therefore are immune-compromised and more susceptible to infections and may be vulnerable to coronavirus infection. While the anti-viral immune response is important to protect from coronavirus infection, an uncontrolled proinflammatory cytokine response can lead to cytokine storm which causes damage to the lungs and other organs, causing significant morbidity and mortality. Better understanding of the underlying immune response and therapeutic strategies in lupus and COVID-19 is important to guide management of this deadly infectious disease in the context of lupus and vice-versa.

**Keywords: COVID-19, systemic lupus erythematosus, immune response, therapeutics, coronavirus infection**

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## INTRODUCTION

COVID-19 caused by the novel coronavirus severe acute respiratory syndrome (SARS) coronavirus-2 (CoV-2) is a highly contagious infection with high morbidity and mortality (1). The SARS-CoV-2 is a single stranded enveloped RNA virus with the spike glycoprotein which can bind to the angiotensin converting enzyme 2 (ACE2) receptor to enter host cells (2). ACE2 is abundantly found on the type II pneumocytes of the lung alveolar epithelium and therefore the lungs are a major site of infection. However, ACE2 is highly expressed in the gastrointestinal tract, vascular endothelium

and in other tissues. Acute respiratory illness spreads through inhalation of the virus leading to respiratory signs and symptoms which commonly include cough, shortness of breath, and fever but can also include gastrointestinal symptoms and neurological symptoms such as loss of taste or smell. While some patients are asymptomatic, and some may have mild illness, others with severe cases need hospitalization, intensive care and assisted mechanical ventilation and many suffer from respiratory complications which can be fatal. Accordingly, the disease course may range from a few days or weeks with full recovery, to several months with residual tissue damage thereafter. The immune response to viral infections including the novel coronavirus is two-fold—an appropriate early antiviral defense response and a subsequent inflammatory repair response. However, inadequate or delayed antiviral responses and/or an uncontrolled inflammatory response leading to cytokine storm can lead to the inability to clear virus and cause host organ tissue damage leading to worse disease outcomes. A number of abnormalities of the immune/inflammatory response have been observed in coronavirus infections (3–6). While high white blood cell (WBC) and neutrophil counts have been observed, low lymphocyte counts (lymphopenia) is a prominent finding reported in a majority of cases, and cytokine storm with elevated proinflammatory cytokine levels can precipitate complications and worse outcomes. While there is an antibody response with neutralizing antibodies against epitopes of the spike protein of the coronavirus, some reports suggest the short-lived nature of these antibodies (7).

The populations with an overwhelming preponderance of cases and deaths are the elderly >60 years of age, and of black and hispanic race/ethnicities. In most countries, higher cases and death rates are observed in men than women. In addition, those with underlying comorbidities are at a higher risk and suffer worse disease outcomes. While the most common underlying comorbidities are reported to be obesity, hypertension, diabetes, underlying cardiovascular and lung disease, people with immune-mediated diseases such as autoimmune disease, and those on immune-suppresants or immune-modulating drugs may also be more vulnerable to this infection.

Systemic lupus erythematosus (SLE) is a chronic painful autoimmune disease which afflicts predominantly women and is among the leading causes of death in young women (8). Complex interactions of genetics, hormones and environmental factors lead to loss of self-tolerance with aberrant immune responses of the innate and adaptive immune systems. Abnormal levels of innate inflammatory cytokines, autoantibodies produced by B cells and aberrant proinflammatory T cell responses lead to inflammation and tissue damage. While skin disease and joint inflammation are common, inflammation may affect virtually any organ leading to a myriad of clinical manifestations and organ damage. There is no cure for lupus and treatment with corticosteroids are often necessary to control disease activity. In addition, patients are frequently treated with immunosuppressive agents and cytotoxic drugs to control abnormal immune responses and tend to be immunocompromised and more susceptible to infections. Therefore, people with SLE are considered a vulnerable population for coronavirus infections and COVID-19. Here we discuss COVID-19 in the context of lupus, focusing on features of the immune response (**Figures 1**, **2**) including lymphopenia, cytokine storm, antibody response, T cell response, and therapeutic management strategies.

## AUTOIMMUNE DISEASE, SLE, AND COVID-19

Several autoimmune rheumatic diseases such as systemic lupus erythematosus (SLE) result from immune-mediated inflammation

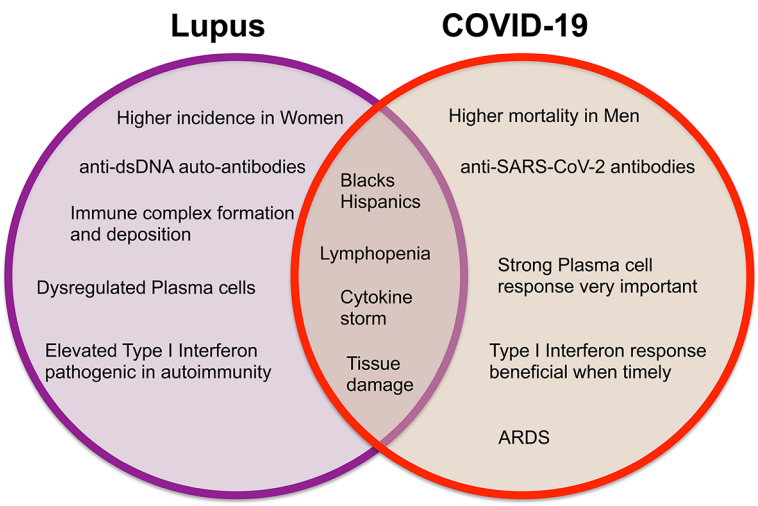


**FIGURE 1** | Venn diagram displaying the differences and similarities between incidence, clinical findings, and immune responses in systemic lupus erythematosus and COVID-19.

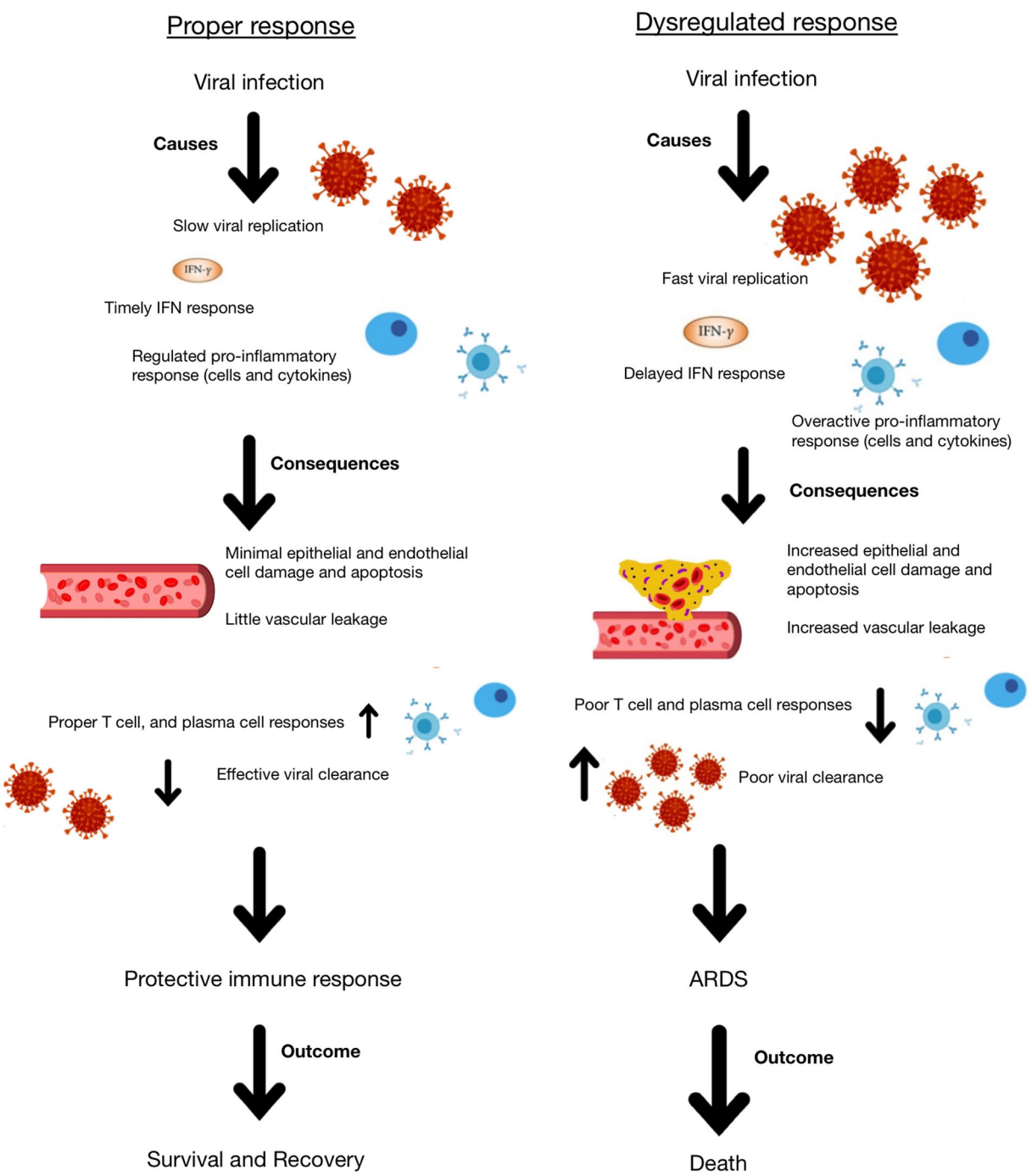


**FIGURE 2** | Schematic representation of appropriate and dysregulated host immune responses to coronavirus infection and the consequences of each.

and tissue damage caused by immune system dysregulation. As a result, patients with autoimmune rheumatic disease have been identified as a vulnerable population at risk for severe COVID-19 illness. Although a strong antiviral immune response is needed for viral clearance, hyperactive immunity has been linked to cytokine storm and tissue damage in COVID-19 patients (9). Both SLE and COVID-19 have been shown to manifest multi-organ complications of interstitial pneumonia, cytopenia, arthralgia, myocarditis, and hemophagocytic lymphohistiocytosis (10). Due to similarities in disease characteristics, treatment of patients with SLE may provide insights into development of therapeutic options for COVID-19.

The mainstay of treatment for SLE involves corticosteroids and other immunosuppressive therapies that may cause further

vulnerability to COVID-19 infection (9). Paradoxically, immunosuppressants have been investigated as a means of dampening inflammation and reducing likelihood of acute respiratory distress syndrome (ARDS) in patients already infected with coronavirus (11). The anti-rheumatic drugs, hydroxychloroquine and baricitinib, have also been identified as possible targets for COVID-19 treatment due to their proposed antiviral effects (9). However, despite initial buzz about the use of hydroxychloroquine in COVID-19 treatment, current research suggests no benefit when compared to placebo or standard care (12, 13). Prospective studies of baricitinib therapy in COVID-19 patients have yielded more success, but are limited by poor experimental design, small sample size, and lack of results reproducibility (14). Possible adverse effects associated with baricitinib, such as secondary infection and elevated creatine kinase, have also been identified (15). Additional clinical trials are needed to elucidate the risks and benefits of these medications.

Diabetes, respiratory disease, and cardiovascular disease have been linked to more complex care and higher mortality rates in COVID-19 patients (9). However, the correlation between rheumatic disease and COVID-19 is less well-studied. Barriers to research include the relatively few individuals with rheumatic disease which limits study sample sizes. As a result, more evidence is needed to definitively determine the risk to individuals with rheumatic disease.

Current research primarily focuses on hospitalized patients which introduces bias towards those with severe COVID-19 illness. Literature suggests little if no correlation between rheumatic disease and COVID-19 disease severity in hospitalized patients (9, 16, 17). One case-control study of patients analyzed differences in disease course between patients with musculoskeletal or rheumatic diseases (cases) and those without musculoskeletal or rheumatic disease (controls) (16). In patients with severe symptoms requiring hospital stay, no significant differences in duration of symptoms, length of hospital stay, chest X-ray findings, or mortality rate were observed between cases and controls. As a result, there was insufficient evidence to support a link between musculoskeletal or rheumatic disease and COVID-19 disease course in hospitalized patients. However, due to the nature of the study, it was not possible to determine whether patients with musculoskeletal or rheumatic disease were more likely to develop pneumonia and require hospitalization. Another retrospective study reported that approximately half of reported cases of patients with both rheumatic disease and COVID-19 did not require hospitalization (17). This proportion, which included comprehensive patient data from 40 countries, appeared to be higher than that observed in the general population. However, researchers acknowledged potential bias due to data collection methods, which limited the ability to determine whether this discrepancy was statistically significant. A third study found that patients with rheumatic disease controlled with immunosuppressive medication tested positive for COVID-19 at higher rates than their family members without rheumatic disease (63 versus 34%) (9). Overall, the rate of COVID-19 in subjects with autoimmune rheumatic disease was 0.43%. Because the study selected subjects based on the criteria of having rheumatic disease or living with a family member with rheumatic disease, no conclusions can be drawn about the rate of COVID-19 infection in the general population. Though the impact of hydroxychloroquine on susceptibility of coronavirus infection remains uncertain, SLE was identified as a possible confounder in this study, as patients with SLE were more likely to take hydroxychloroquine than patients with other rheumatic diseases.

Further evidence indicates that patients with SLE test positive for COVID-19 at lower rates than individuals with other autoimmune diseases such as Sjögren's syndrome (SS) and systemic sclerosis (SSc) (18). This is contradictory to what might be expected, as SLE is frequently treated with corticosteroids and other forms of immunosuppression which could increase vulnerability in early stages of COVID-19 infection. However, administration of low-dose steroids has also been linked to lower mortality in critically ill COVID-19 patients, which suggests that steroids may have variable effects dependent upon dosage and disease severity, further complicating understanding of how COVID-19 affects patients with SLE (19). Additionally, differences in prevalence between SLE and other autoimmune or immune-mediated disease subsets raise the possibility that anti-rheumatic therapies, such as hydroxychloroquine, might be effective in the prevention or treatment of COVID-19. Unfortunately, studies of HCQ as a preventive agent or a treatment have been disappointing (12, 13).

While more research is needed to reach a consensus on the relative risk of severe COVID-19 in individuals with SLE, evidence of abnormal clotting in some COVID-19 cases may implicate additional considerations for clinical care of COVID-19 patients who have SLE. Manifestations such as pulmonary embolism and the "covid toe" rash, stemming from thrombosis formation in the microvasculature of the feet, suggest that SARS-CoV-2 may induce a hypercoagulable state (20). In addition, prolonged activated partial-thromboplastin time (aPTT) has been observed in COVID-19 patients at higher rates than other patients, suggesting that COVID-19 may cause or exacerbate blood clotting abnormalities (21). Lupus anticoagulant (LA), a type of autoantibody which can cause hypercoagulability, was identified as the primary cause of prolonged aPTT in 91% of patients with aberrant results. These findings were replicated in a report that found 45% of severe COVID-19 patients and 87.7% of ICU patients with an abnormal aPTT also had positive LA results (22). While LA is not diagnostic of SLE, antiphospholipid antibodies, including LA, are found in approximately 50% of SLE patients and SLE is considered a risk factor for both venous and arterial thrombosis (23). Although studies have not yet confirmed an elevated risk of thrombosis among the subset of COVID-19 patients with SLE, abnormal clotting observed in both COVID-19 and SLE suggests that patients who possess both risk factors may require additional monitoring for thrombosis.

## DEMOGRAPHICS RELATED FACTORS IN SLE AND COVID-19

Studies of demographic factors including ethnicity and sex have suggested that specific populations may be predisposed to more severe SLE and COVID-19 disease presentation (24). Men experience greater hospitalization and mortality rates due to COVID-19 while women experience greater incidence rates of SLE. This finding is consistent with the observation that men are biased towards infections, while women are biased towards autoimmune disorders. It has been postulated that hormonal factors which heighten ability to clear infection may also increase likelihood of autoimmune disease in women (24). Additional contributions from genetic components and microbiota may cause women to produce more pro-inflammatory cytokines which, in turn, may lead to a more aggressive immune response (24). While the process by which race and ethnicity influence risk is less clear, evidence suggests that SLE and COVID-19 disproportionately affect racial and ethnic minorities and result in worse outcomes in people of color.

Women typically mount a greater innate and adaptive immune response to infections and vaccine immune challenges (25). While a robust immune response is beneficial to fighting infection, hyperactive immunity may predispose women to autoimmune diseases such as SLE. SLE is far more common among women with a female to male ratio of 9:1. The sex-based bias in autoimmune disorders is relevant to the discussion of COVID-19 because biological factors which protect men from autoimmunity may cause worse clinical outcomes in men. This is evidenced by the more than doubled COVID-19 mortality rate observed in men compared to women, despite sometimes similar prevalence across gender (26). Studies indicate a possible genetic basis to this disparity, as many immune-related genes are X chromosome-linked (24). Typically, the silencing of one copy of the X chromosome occurs in women to ensure similar gene dosage between sexes. However, some genes may fail to undergo silencing and thus escape inactivation, producing biallelic expression of gene product (24). Biallelic expression of immune-related genes has been shown to increase T- and B-cell activation, which may both predispose women to SLE and provide protective advantages in the COVID-19 immune response.

A second explanation for the sex bias observed in both COVID-19 and SLE suggests that sex hormones may promote inflammation in women. Estrogen produces immunoactivating effects, while androgens such as testosterone yield immunosuppressive effects (25). Estrogen has been linked to increased activation of CD4 T cells and higher expression of pro-inflammatory cytokines IL-1β and IFN-γ, which may allow women to more effectively combat viral infections including COVID-19. A proposed mechanism of action suggests that estrogen may promote diversity of the microbiome, causing upregulation of certain cytokines (24). Conversely, testosterone dampens expression of pro-inflammatory cytokines while upregulating expression of anti-inflammatory cytokine IL-10, leading to a less aggressive immune response (24). Therefore, increased expression of pro-inflammatory cytokines in women which promote immune dysregulation in autoimmune disorders may simultaneously promote antiviral activity against coronaviruses.

Knowledge of disparities in disease outcome can aid clinicians and policymakers in understanding how to best treat and support patients during the COVID-19 pandemic. Emerging evidence indicates a troubling pattern of inequities in COVID-19 case rate and outcome among various populations. For instance, racial/ethnic minority communities are at increased risk of COVID-19 infection and generally suffer worse outcomes after being infected. This trend is reflected in patients with SLE. In both the United States and United Kingdom, data indicates that ethnic minorities are more susceptible to infection and mortality from COVID-19. US counties with majority black residents showed three times the rate of COVID-19 cases and six times the rate of deaths as counties with majority white residents (27). A study conducted in the UK similarly found that mortality rates in COVID-19 patients were twice as high in Bangladeshi communities and 10–50% higher in other ethnic minority communities when compared to the white British population (28).

SLE is also more prevalent in black and hispanic individuals compared to white individuals (29). In the UK, prevalence of SLE was 5–9 times greater in the Afro-Caribbean group and 2–2.4 times greater in the South Asian group than the white group. A study comparing American Indian and Alaska Native populations with black Americans found similar rates of SLE in both populations, which were higher than that observed in the white population (29). In addition, ethnic minorities may experience renal complications at higher rates, as the renal disease incidence rate for black and hispanic patients is 68.9 and 60.6% respectively compared to only 29.1% in white patients. Studies indicate similarly elevated rates of lupus nephritis in South Asian and East Asian populations compared to white populations (29).

The explanation for the increased disease risk experienced by ethnic minorities is likely multifactorial, involving both biological aspects and systemic issues which reflect pre-existing health and socioeconomic disparities in communities of color (30). Several biological models for greater disease burden in ethnic minorities have been proposed. SLE has been shown to be highly heritable, suggesting possible genetic predispositions which place ethnic minorities at higher risk (31). Higher prevalence of cardiovascular risk factors in ethnic minorities, potentially due to variable expression of ACE2, also poses additional risk of acute kidney and cardiac failure from COVID-19 (27). While more evidence is required to determine a genetic cause for this bias, preliminary data indicates that some black Americans may carry a shorter CAG repeat polymorphism in the androgen receptor gene (32). Short CAG repeat polymorphisms have been linked to increased coronavirus uptake by the ACE2 receptor and worsened COVID-19 symptoms (32).

Social and economic influences, such as those which limit healthcare access in minority communities, must also be considered. For example, lower income and education attainment are associated with higher rates of preventable

disease, reduced access to health services, and shorter life expectancy. As a result, systemic inequalities which disadvantage ethnic minorities in both financial and healthcare spheres may also be reflected in trends in COVID-19 data. Patterns in employment have found that people of color are more likely to be employed in frontline jobs limiting ability to adhere to social distancing measures and increasing likelihood of disease exposure (30). While further analysis of each of these factors is needed, the impact of a pandemic which disproportionately affects already vulnerable populations must be acknowledged and addressed.

## LYMPHOPENIA

Lymphopenia, or a decreased lymphocyte count is one of the most common features in patients with SLE with a predominant effect on T cells (33). Although it is not entirely known what causes lymphopenia in SLE patients, there are many different pathophysiological theories. The first is the presence of lymphocytotoxic antibodies. Many SLE patients show higher levels of IgM and/or IgG autoantibodies, which exhibit cytotoxicity *via* the classical complement pathway and antibody-dependent cell cytotoxicity, respectively. Some of the recognized antigens are CD4, CD45, MHCI/II, glycophospholipids, and ribosomal P protein. The majority of these are present on T cells, which may explain why T cells are more affected than B cells (33). The second theory is excess apoptosis, which may be due to a decrease in glutathione in lymphocytes. Glutathione is a powerful antioxidant that is naturally occurring in many somatic cells. In the absence of this molecule, cells exhibit an increase in reactive oxygen species and resulting apoptosis. Additionally, increased apoptosis may be due to hyper-expression of Fas on naive and memory T cells. Fas is a receptor that induces the extrinsic pathway of apoptosis. Another theory is increased susceptibility to complement-mediated cytolysis. Complement proteins are naturally occurring in the body and work to destroy pathogens or infected cells. Because of their hyperreactivity, most cells on the body have multiple complement regulatory receptor proteins on their surfaces in order to prevent complement activation on healthy cells. T cells in many SLE patients exhibit decreased numbers of these receptors, specifically CD55, CD59, and CD46. A final theory is decreased lymphocyte production (lymphopoiesis) and subsequent sequestration following production. Some SLE patients show lower numbers of CD34+ hematopoietic progenitors, leading to lower numbers of lymphocytes. Additionally, IFN-γ seems to have a negative role on stem cell production because it limits self-renewal of hematopoietic stem cells by inducing hyper-expression of transcription factor PU.1 which blocks B lymphopoiesis and blocks Pro-T cell stages in the thymus. Following production of lymphocytes, there seems to be a sequestration of these cells in secondary lymphoid organs and inflammatory sites rather than active circulation in the periphery.

Whatever the cause, lymphopenia is an important factor often associated with SLE patient susceptibility to bacterial and viral infections (33–35). Interestingly, many COVID-19 patients without SLE exhibit lymphopenia (36). 35–75% (study-dependent) of patients who have COVID-19 develop lymphopenia, defined as a lymphocyte count of <1.5 x 10^9/L (37). Additionally, this clinical finding is a more common feature in patients who died from COVID-19 (36). Furthermore, there seems to be a geographical difference in lymphopenia rates. More patients in Italy show lymphopenia when compared to places like Singapore and the Zhejiang Province. There are many reasons why this may happen, but one theory is that the viral genome has mutated and is sparking different immune responses as a result of those mutations (36). Overall, a decrease in the number of B and T cells may lead to a worse patient outcome if their ability to fight off the virus is diminished. A rapid blood test of lymphocyte count in infected patients may be useful in determining cases that are more severe, allowing physicians to respond accordingly (37). It is important to keep in mind the increased risk that COVID-19 poses to SLE patients.

## CYTOKINE STORM

Cytokine storm occurs when there is an excessive and uncontrolled release of pro-inflammatory cytokines and can be associated with infectious or non-infectious diseases, and the term became popular during the avian H5N1 influenza infection (38). Normally, cytokines are a critical component of the immune response, kickstarting the innate immune system and coordinating the adaptive immune response in order to ensure the rapid destruction of a pathogen, followed by tissue repair. A cytokine storm usually begins with the innate immune response to a particular pathogen (39). Toll-like receptors (TLRs) and pattern recognition receptors (PRRs) on innate immune cells recognize pathogen-associated molecular patterns (PAMPs) on pathogens. Following this interaction, innate immune cells release cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ in order to stimulate the proper proinflammatory response to a pathogen. This response is normal and critical when it tapers off as expected. In a cytokine storm, however, there is an "autoamplifying phenomenon", which is essentially a heightened response to the pathogen. It ends up causing much more harm than good, leading to host tissue damage. The most common cytokines implicated in a cytokine storm are IL-1β, IL-6, IL-12, IL-17, TNF-α, and COX-2, and endothelial cell damage is the most common feature of cytokine storm (39).

In patients who make autoantibodies or antibodies against self, such as SLE patients, cytokine storm can be induced much more readily due to increased cell damage and a subsequent increase in damage-associated molecular patterns (DAMPs) that can activate the innate immune response (39). Additionally, it is possible to see macrophage activation syndrome (MAS) in patients with SLE (40). MAS is an acute episode of inflammation marked by activation and expansion of CD8 T lymphocytes and hemophagocytic macrophages. The pathogenesis of this disease is marked by a cytokine storm of cytokines including IL-1, IL-6, IL-18, TNF-α, and IFN-γ. IL-1, produced by macrophages, causes leukocyte and endothelial

activation. IL-6 drives the acute-phase response and may amplify the inflammatory response that contributes to a cytokine storm. TNF-α may induce tissue damage. IL-18 increases IFN-γ production by natural killer (NK) cells and T cells and increases TNF-α production by macrophages. IFN-γ is a prominent activator of inflammatory cytokine release (40). Many of these cytokines overlap with the classical cytokines implicated in cytokine storm and thus have the same effects on the patient. SLE patients show DNA hypomethylation of cytokine genes, particularly Type 1 interferon genes, which causes elevated levels and leaves these individuals more susceptible to a cytokine storm and tissue damage in response to a viral infection such as COVID-19 (41).

It is believed that the most severe manifestation of COVID-19 infection—acute respiratory distress syndrome (ARDS) is caused by excessive host inflammatory response rather than by the viral infection itself (42). Correlative evidence from SARS and MERS patients suggest that hyper-inflammatory responses play a strong role in coronavirus-related ARDS pathogenesis. In response to SARS-CoV-1 infection, many patients show elevated TNF, IL-6, and IFN cytokines and elevated chemokines CCL3, CCL5, CCL2, and CXCL10. These cytokine levels were particularly high in patients with severe disease when compared to patients with uncomplicated SARS-CoV-1 infection. MERS patients show similar pathogenesis to SARS patients (42).

There are many possible reasons for the initiation of the hyperinflammatory response to coronavirus infection, but it is likely due to a combination of rapid virus replication, delayed IFN response, macrophage and neutrophil accumulation, and infection of alveolar epithelial cells (42). All of these factors may lead to increased cytokine release and potential resulting cytokine storm. As a result of cytokine storm, whether in response to a coronavirus or any other infection, patients may exhibit epithelial and endothelial apoptosis, which damages the blood:air barrier in the lungs, leading to edema and hypoxia. Furthermore, there may be a diminished T cell response as a result of the delayed but hyperactive IFN response, which leads to a dysregulated immune response to the virus. As a result of this dysregulation and increased cytokine production, the tissue homeostasis in the lungs is often disrupted, leading to increased fibrin deposition that further disrupts the blood:air barrier and causes ARDS, hypoxia and permanent damage (42). IL-6, IL-8, and IL-1β are particularly important in mediating ARDS.

When compared to other causes of ARDS, COVID-19 patients with ARDS exhibited plasma IL-6 cytokine levels far below those exhibited in other non-COVID-19 ARDS patients (43). IL-6 is the main mediator of cytokine storm, and based on a meta-analysis of studies of COVID-19 patients, it is clear that the most severe cases exhibited elevated levels of IL-6, but those levels were 10–200 times lower than those seen in non-COVID-related ARDS. This argues that the lung damage and poor outcomes seen in COVID-19 patients may not be a result of a cytokine storm as seen in SARS-CoV-1 cases, but rather likely a result of a severe viral pneumonia (43). A post-mortem study of patients with COVID-19 ARDS identified severe vascular injury that was 9 times more prevalent than that seen in influenza ARDS. Given this information, cytokine storm may be a misleading description of the cause of COVID-19 ARDS and thus the treatments that patients receive may not be the best treatment option (43). The COVACTA trial, a multi-institution clinical trial that analyzed the efficacy IL-6 receptor inhibitor Tocilizumab in the treatment of COVID-19 patients, failed to meet its primary endpoint of improved clinical status. Additionally, despite Tocilizumab-treated patients spending approximately seven less days in the hospital than control patients, there was no improvement in mortality between the two groups. These trials call the use of Tocilizumab for COVID-19 patients into question and further suggest that the cytokine storm seen in COVID-19 patients is different than the classical description of cytokine storm (44).

## ANTIBODY RESPONSE

One marker for the diagnosis of SLE is the presence of anti-dsDNA autoantibodies (45). These are formed in many SLE patients because they respond to self dsDNA as if it is non-self and thus launch an immune response against their own tissues, leading to the formation and deposition of immune complexes throughout the body. This deposition has a damaging effect on multiple tissues throughout the body, most notably the joints and vascular and renal systems.

The etiology of the production of autoantibodies is not entirely known, but it is clear that these antibodies may be formed against true self chromatin or formed due to infection-related DNA or DNA-binding proteins through molecular mimicry (45). Most commonly, the viruses that stimulate dsDNA-specific B cells and helper CD4 T cells belong to the polyomavirus group. This group of viruses remain latent after the primary infection in an immunocompetent host, which often can lead to the stimulation of these B and T cells that will attack self rather than non-self (46). Additionally, these polyomavirus infections are linked to antibodies against T antigens and transcription factors like cAMP response-element binding protein (CREB) (45). Despite various studies, the clinical significance of infection-induced antibodies to the pathogenesis of SLE is uncertain.

The interplay of environment, epigenetic modifications, production of autoantibodies, and dysregulated immune system leaves SLE patients highly susceptible to infections, with 50% of SLE patients hospitalized with an infection during the course of their disease (34). Generally, SLE patients are more susceptible to bacterial infections such as Streptococcus pneumoniae, Escherichia coli, and Staphylococcus aureus because of the lymphopenia that disproportionately affects T cell counts over B cell counts. T cells are much more important for coordinating the correct response against a bacterial pathogen. SLE patients are also more susceptible to contracting a few viruses, the most notable being Epstein-Barr virus and cytomegalovirus (34). Some of these causes of susceptibility include breakdown of epithelial barriers due to rashes and ulcers, which allow easier access for pathogens, and

impairment of immune function due to the development of autoantibodies and immune complexes, which leads to low neutrophil count and dysfunction of neutrophils, basophils, and eosinophils. In addition to a hyperactive inappropriate immune response, SLE patients also exhibit increased autoreactivity of helper and cytotoxic T cells along with dysfunctional naive, memory, and plasma B cells. The dysfunctional plasma B cells, in addition to incorrect stimulation by helper T cells, can result in hypogammaglobulinemia and increased infection risk (34). Patients are sometimes given glucocorticoids to control disease activity, but these drugs are powerful immunosuppressive drugs that further increase a patient's risk of infection and a poor immune response.

It remains uncertain how SLE patients will respond to COVID-19 and whether they are at an increased risk to contract the infection. Although it may seem as though SLE patients are more susceptible to contracting COVID-19 because of their maladaptive immune system, it is interesting to note that there have been few cases of COVID-19 patients who also have SLE (35). The Global Rheumatology Alliance registry, as of April 1, 2020, noted 19 patients over multiple continents who have SLE and diagnosed COVID-19. A follow-up study found that individuals with rheumatic disease who took >/= 10 mg/day of Prednisone were more likely to be hospitalized, whereas individuals with rheumatic disease who took TNFα were at a decreased risk of hospitalization. Individuals with rheumatic disease using NSAIDs or anti-malarials, such as hydroxychloroquine (HCQ) saw no difference in hospitalization risk. Because many SLE patients are on medications such as glucocorticoids (Prednisone) and HCQ, it is possible that this population is at an increased risk for hospitalization, should they contract COVID-19 (17).

Although studies have noted a production of anti-SARS-CoV-2 antibodies in patients with mild, moderate, and severe COVID-19, it is uncertain the role these antibodies play in mediating the immune response to the disease (47). Because SLE patients show abnormally high levels of autoantibodies, impaired generation of antibodies against pathogens, and are frequently using DMARDs such as glucocorticoids, they may be at high risk for both contraction of COVID-19 and hospitalization should they become infected.

## T CELL RESPONSE

SLE is characterized by excessive production of autoantibodies from B cells which instigates systemic inflammation. Because of this, SLE was originally thought of as a disease mediated by abnormal B cells and plasma cells (48). In reality, aberrant T cells are equally if not more important key initiators of the observed systemic inflammation, as they stimulate proliferation, maturation, and differentiation of B cells, thereby enhancing autoantibody production and class-switching in SLE. Hyperactivation of T cells in this autoimmune disease is relevant in the context of COVID-19, as stimulation of the adaptive immune system after infection may predispose SLE patients to more severe outcomes.

In SLE, there are several phenotypic and physiological changes that are observed in T cell receptors and signaling. CD3ζ, also known as CD247, is a T cell surface glycoprotein responsible for coupling antigen recognition with downstream signal transduction. This surface protein has poor expression in patients with SLE and other inflammatory disorders, causing preferential activation of the Syk pathway for intracellular signaling. This alternative pathway confers much stronger activation of signaling molecules and calcium flux which all intensify the T cell response (48). This process leads to downstream transcription factors which upregulate production of CD40 ligand (CD40L) in patients with SLE. CD40L is a costimulatory molecule on T cells and interacts with CD40 on B cells to promote differentiation, proliferation, antibody production, and class switching in B cells (49).

A common pathway of T cell activation in SLE patients has been outlined in the previous paragraph, as many of these intermediates have been targeted as sites of dysregulation in COVID-19 disease, where T cell hyperactivation is implicated in the infamous cytokine storm. For example, CD40L enables collaboration of T cells with B cells. In patients with SLE, this ligand is upregulated by activation of the Syk pathway (48, 49). This can be problematic as CD40L has additional functions within several cell types of the vasculature, including endothelial cells, myocytes, and platelets. They have strong roles in positive feedback mechanisms of inflammation, as they are released from platelets to activate thrombosis (50, 51). Having SLE is actually an independent risk factor for development of arterial and venous thrombosis that is tied to this upregulation.

As related to COVID-19 patients, CD40L elevation is actually a marker of progression to critical illness. ICU patients with COVID-19 compared to controls have significantly higher levels of platelet and T-lymphocyte CD40L (51). This is concerning as SLE patients may already be in a hypercoagulable state, and development of pulmonary emboli (PE) and deep vein thromboses (DVT) are common complications of COVID-19 disease (10, 21). A recent study (21) found that over 90% of COVID-19 patients that had a prolonged activated partial thromboplastin time (aPTT) were at increased risk of thrombosis. These patients actually had lupus anticoagulant proteins that although function as anticoagulants *in vitro*, are strong procoagulants *in vivo*. The results of this study are clinically significant, as anticoagulation therapy should not be avoided in COVID-19 patients despite their prolonged aPTT, as lupus anticoagulants are responsible for many of these paradoxical test results (21). While not all patients with lupus anticoagulant are diagnosed with SLE, patients with SLE are much more likely to produce this antibody than the general population and should be monitored for thrombosis if they develop COVID-19.

Th17 cells are a newly discovered, differentiated subset of CD4 T lymphocytes defined by their production of the proinflammatory cytokines IL-17 and IL-22. They are crucial mediators of local inflammation, and typically attract other proinflammatory cell types such as neutrophils and Th1 cells

at later stages of inflammation (52). SLE patients with active symptoms are found to have a higher proportion of Th17 cells and serum IL-17 levels compared to the healthy controls, and Th17 lymphocyte numbers are positively correlated with SLE disease activity and severity (53). In addition, autoantibody production is also mediated by IL-17 activation of peripheral blood mononuclear cells (PBMCs) from patients with lupus nephritis (54). To compound effects, IL-17 is capable of upregulating IL-6 production, creating an easily stimulated positive feedback loop between these inflammation-prone cytokines (55). For these processes, Th17 cells are the lymphocytes most implicated in autoimmunity and inflammatory disorders.

The mammalian or mechanistic target of rapamycin complex (mTORC) is a nutrient sensor within multiprotein complexes that control the cell cycle. Dysfunction in mTOR signaling is involved in many pathological states such as cancer and especially autoimmunity, as mTORC has important roles in the activation and polarization of naive T cells (48, 56, 57). In several studies, mTORC was shown to promote Th0 divergence into Th1 and Th17 lymphocytes, and *in vivo* inhibition of mTORC reduces the proportion of these cell types in the lamina propria and lymph nodes. In SLE T cells, the activity of mTORC1 is enhanced compared to T cells from healthy patients. In a human case study of a knockout for a negative regulator of mTORC1, the patient developed an unusually severe case of SLE that led to death (48).

Th17 lymphocytes have been linked to increased disease severity in a variety of coronavirus diseases including SARS, MERS, and COVID-19 (24). As mentioned, this relation is strongly connected to the hallmark IL-17 levels that are upregulated in infected hosts. However, people with SLE and other autoimmune disorders are at special risk of secondary complications due to unique T cell dysregulations. Hyperactivity of mTORC is only one of the many possible reasons behind the disproportionate complications in COVID-19 patients with lupus and other pre-existing autoimmune diseases (48).

## THERAPEUTIC STRATEGIES

Vasculitis, leukopenia, lymphopenia, thrombocytopenia, and several other complications that are typically associated with autoimmune diseases seem to be mirrored by the novel coronavirus COVID-19. It is for these similarities and their disease pathophysiology that there is an interesting overlap between SLE and COVID-19 therapy. This also introduces the question of how to treat COVID-19 patients with pre-existing SLE. Steroids and other immune-suppressive drugs are the mainstays of therapy for SLE patients rendering them immune-compromised and potentially vulnerable to COVID-19.

### Antimalarials

Chloroquine (CQ) and its less toxic derivative hydroxychloroquine (HCQ) have been used for years as treatment for autoimmune and inflammatory disorders, and are typically considered first- line treatment for SLE. Their mechanisms of action are through dual manipulation of antigen-presenting cell signaling—alkalinization of lysosomes and blocking of toll-like receptor (TLR) signaling (10). Their use reduces disease activity of SLE, reduces inflammatory damage, and improves patient survival (58). CQ/HCQ have also been shown to have an *in vitro* antiviral effect on SARS-CoV-2 *via* ACE-2-SARS-CoV-2 binding interactions (59), leading many to consider their use as a treatment for COVID-19. However, there is little valid scientific evidence to support their use, as studies of HCQ as a preventative agent or a treatment for COVID-19 have been disappointing (58, 60, 61). In fact, the World Health Organization (WHO) discontinued its use during its Solidarity trial (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/global-research-on-novel-coronavirus-2019-ncov/solidarity-clinical-trial-for-covid-19-treatments) when it proved to make no significant improvement in the complications of COVID-19 patients (60). Other clinical trials done in China and France suggested HCQ use with azithromycin could result in a rapid decrease in viral shedding, but these studies were also faulty as they were uncontrolled and underpowered. The few pieces of conclusive evidence on CQ/HCQ in combination with azithromycin recognize their potentially dangerous impact on the electrochemical properties of the heart. To elucidate, these drugs are capable of lengthening the QT interval *via* blocking of K+ channels, precipitating cases of ventricular arrhythmia (62). Another recent randomized controlled trial has shown no clinical benefit of HCQ for COVID-19 (63). The lack of evidence-based recommendation of CQ/HCQ coupled with media speculation on its use as a COVID-19 therapy led to a worldwide shortage of these essential medications for patients suffering from SLE (58). Therefore, it is not recommended that antimalarial drugs be administered to prevent or treat COVID-19. Patients with SLE who are experiencing low availability in their prescriptions should consult a physician and consider tapering their dosage or increasing drug interval time until international drug shortages are ameliorated (58, 60).

### Glucocorticoids

Treatment for SLE may include a combination of antimalarials with a glucocorticoid such as prednisone (64). In the context of COVID-19, steroid use as a prophylaxis or treatment has been controversial through several conflicting studies. One of the most well-known clinical trials from the RECOVERY Collaborative Group found that the daily dexamethasone in hospitalized COVID-19 patients led to reduced incidence of death for those receiving invasive mechanical ventilation (19). On the other hand, reports from the COVID-19 Global Rheumatology Alliance have shown that people who use glucocorticoids such as prednisone at concentrations >10 mg/day are more likely to be hospitalized for COVID-19 (10, 17). A recent metanalysis found that use of glucocorticoids as a COVID-19 treatment may increase the risk of death in patients with coronavirus infections of a mild course (65). Because of this, most providers have suggested tapering dosage of glucocorticoids to below 10 mg/day in patients with rheumatic diseases or using a minimal possible dose to reduce

risk of complications. For patients already taking corticosteroids, completely stopping use of glucocorticoid medications is strongly advised against (17).

### IL-2 Agonists

IL-2 agonists have great potential in T cell manipulation, as they have a strong ability to redirect the host immune system towards tolerance by the upregulation of Tregs (64). In several clinical trials in SLE patients (66, 67), low-dose IL-2 therapy was shown to have strong potential of expanding Treg populations (68). There is currently a clinical trial in Paris studying the use of low-dose IL2 in COVID-19 patients (https://clinicaltrials.gov/ct2/show/NCT04357444). However, to the best of the authors' knowledge, there is still no widely available IL-2 agonist on the market for SLE or COVID-19 therapy.

### IL-6 Antagonist

IL-6 is a cytokine that is strongly correlated with inflammation and severity in SLE and COVID-19. As a therapy for rheumatic illnesses, this connection was utilized *via* development of recombinant human monoclonal antibody tocilizumab targeting the IL-6 receptor that could be used for rheumatoid arthritis, certain types of juvenile arthritis, and giant cell arteritis. Additionally in 2017, the Federal Drug Administration (FDA) approved the use of tocilizumab, a humanized anti-IL6 receptor antibody, for the treatment of cytokine release syndrome (69). Recent studies on the use of tocilizumab as a therapy for COVID-19 disease have had mixed results, although this drug has been used frequently in acute care settings as a therapy for the COVID-19 cytokine storm (64, 68).

### mTOR Antagonists

Rapamycin is the most well-known mTOR pathway inhibitor, resulting in immediate and delayed inhibition of mTORC1 and 2, respectively. In a mouse model, rapamycin was able to ameliorate lupus nephritis as well as increase levels of circulating IL-2. Rapamycin has also shown these effects in clinical trials in human subjects, although it is not commonly used to treat lupus (64, 70). There are currently clinical trials using rapamycin to protect elderly patients from COVID-19 complications, although there is still little information on the effects of rapamycin use against coronaviruses (71).

### NSAIDs

Many patients with SLE use nonsteroidal anti-inflammatory drugs (NSAIDs) to alleviate joint pain and other symptoms (17). The COVID-19 Global Rheumatology Alliance has found no increased risk of poor COVID-19 outcomes related to the use of NSAIDs (10). However, a report mentioned that NSAIDs can modulate ACE2 and aggravate COVID-19 symptoms (72). From this article, the U.S. Food & Drug Administration (FDA) advised patients to be cautious of the use of NSAIDs and advocated for use of an alternative such as ibuprofen/paracetamol (https://www.fda.gov/drugs/drug-safety-and-availability/fda-advises-patients-use-non-steroidal-anti-inflammatory-drugs-nsaids-covid-19).

### TNF Antagonists

Antibodies against TNF have been used for years as treatment for autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease. This blockage of TNF in patients with autoimmune disease leads to a rapid decrease in IL-1, IL-6, and subsequent leukocyte trafficking (73). In this regard, literature promoted by the COVID-19 Global Rheumatology Alliance suggests that patients with rheumatic illness taking anti-TNF therapy had a significantly reduced odds of hospitalization (17). TNF antagonism has been strongly advocated for in COVID-19 research development, although it has not yet been tested as a therapy in clinical trials (73).

## CONCLUSIONS

Patients with autoimmune diseases such as lupus are not only vulnerable to infections because of the aberrant immune responses inherent to the disease, but also due to the fact that they often are treated with steroids, other immune-suppressants and immune-modulator drugs. These together lead to an immune-compromised state and an increased risk for infections. Many aspects of lupus and COVID-19 are shared including some demographics of patient populations affected and aberrant immune responses while some such as gender-bias are strikingly distinct in the two diseases, with lupus predominantly afflicting women and COVID-19 with worse outcomes in men. Aberrant cellular, humoral and cytokine immune responses including lymphopenia, proinflammatory cytokines, aberrant B and T cell responses may likely influence the severity and disease outcomes of COVID-19 in patients with immune-mediated and autoimmune diseases. Better understanding of the intricacies of the immune response will be important in guiding management strategies for these patients.

## AUTHOR CONTRIBUTIONS

VM conceptualized the review. AS, NG, SW, and VM performed literature reviews, synthesized relevant information, and wrote the manuscript. AS prepared the figures. All authors contributed to the article and approved the submitted version.

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# Enhanced Fatty Acid Synthesis Leads to Subset Imbalance and IFN-γ Overproduction in T Helper 1 Cells

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Recent reports have shown the importance of IFN-γ and T-bet$^+$ B cells in the pathology of SLE, suggesting the involvement of IFN-γ-producing T-bet$^+$ CD4$^+$ cells, i.e., Th1 cells. This study determined the changes in Th1 subsets with metabolic shift and their potential as therapeutic targets in SLE. Compared with healthy donors, patients with SLE had higher numbers of T-bet$^{hi}$CXCR3$^{lo}$ effector cells and T-bet$^+$Foxp3$^{lo}$ non-suppressive cells, which excessively produce IFN-γ, and lower number of non-IFN-γ-producing T-bet$^+$Foxp3$^{hi}$ activated-$T_{reg}$ cells. These changes were considered to be involved in treatment resistance. The differentiation mechanism of Th1 subsets was investigated *in vitro* using memory CD4$^+$ cells obtained from healthy donors and patients with SLE. In memory CD4$^+$ cells of healthy donors, both rapamycin and 2-deoxy-D-glucose (2DG) suppressed T-bet$^+$Foxp3$^-$ cells, and induced T-bet$^+$Foxp3$^{+(lo/hi)}$ cells. Rapamycin induced IFN-γ-producing T-bet$^+$Foxp3$^{lo}$ cells accompanied with enhanced lipid metabolism, whereas 2DG induced IFN-γ-non-producing T-bet$^+$Foxp3$^{hi}$ cells. In memory CD4$^+$ cells of SLE patients, inhibition of fatty acid synthesis, but not β-oxidation, suppressed IFN-γ production, and up-regulated of Foxp3 expression in T-bet$^+$Foxp3$^+$ cells. Metabolic regulators such as fatty acid synthesis inhibitors may improve the pathological status by correcting Th1 subset imbalance and overproduction of IFN-γ in SLE.

**Keywords: systemic lupus erythematosus, T-bet, IFN-γ, fatty acid synthesis, immunometabolism**

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a common autoimmune disease. That nonspecific treatment of steroids or immunosuppressants is still used as the main therapeutic modality stresses the need to explore the pathological mechanisms and design new therapeutic strategies for SLE.

IFN signature plays an important role in SLE (1). In recent years, modular transcriptional repertoire analysis has demonstrated a different type I/II IFN signature and the importance of not only type I IFN

but also type II IFN (IFN-γ) (2). While helper T (Th) cells are known to play an important role in the pathogenesis of SLE, their differentiation and functional abnormalities remain unclear (3).

In the 1990–2010, SLE pathology was discussed in the context of a balance between Th1 and Th2, together with the involvement of Th1 (4, 5). However, Th1 is only defined as an IFN-γ-producing cell, and other important markers for Th1 such as CXCR3 and T-bet, have not been simultaneously examined in these papers. Recently, the diversity of Th cells has been reported, and CXCR3, T-bet and IFN-γ have been reported to be expressed not only in Th1 but also in other diverse Th subsets. In particular, it was reported that T peripheral helper (TPH) cells and Th10 cells, which attract much attention and are important for B cell help, produced IFN-γ and expressed CXCR3 and T-bet (6, 7). Recent studies have emphasized the roles of T-bet$^+$ B cells in SLE pathology, such as the production of autoantibodies and renal involvement (8–11). TPH cells and Th10 cells were known to be correlated with T-bet$^+$ B cells in SLE (6, 12).

T-bet is known as a master transcription factor that regulates Th1 differentiation, and it was reported previously that T-bet plays a positive role in the regulation of IFN-γ production by effector Th1 cells (13, 14). Interestingly, various new functions of T-bet as a transcription factor have been recognized in recent years, including the presence of T-bet$^+$Foxp3$^+$ $T_{reg}$, which specifically inhibits Th1 in mice (15) and inhibition of aberrant autocrine type I IFN and its downstream signaling in Th1 (16). However, the role of T-bet-expressing CD4$^+$ cells, i.e., Th1 subsets, in SLE pathology remains unknown.

During the process of tissue infection, lymphocytes, including Th cells, rapidly change into the effector phase upon antigen stimulation. These activities require massive energy and rapid synthesis of biological components using amino acids, lipids, and nucleic acids (17). Recent studies in rodents have demonstrated enhanced glycolysis under aerobic conditions associated with such anabolic processes in the activation of various immunocytes (18–21). The differentiation regulation mechanism through immunometabolism involved in Th cell differentiation is being clarified in many studies mainly in mice; however, it is largely unknown in human, particularly in the pathology of autoimmune diseases, such as SLE.

The aim of this study was to shed light on the pathogenic process of SLE. Specifically, we determined the changes in Th1 subsets in SLE patients and the roles of these cells in the differentiation regulation mechanism *via* immunometabolism.

# MATERIALS AND METHODS

## Cell Isolation and Differentiation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adults using lymphocyte separation medium (Lympholyte-H; Cedarlane, Burlington, NC), and CD4$^+$ T cells were purified by negative selection using CD4 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and magnetic separation (Miltenyi Biotec) for negative selection CD45RA$^-$ memory T cells were further purified by negative selection using MACS Naive CD4$^+$ T cell isolation kit; (Miltenyi Biotec). We confirmed that the purity of the obtained CD45RA$^-$ memory CD4$^+$ T cells was higher than 90%, as determined by flow cytometric analysis. CD45RA$^-$ memory CD4$^+$ T cells were activated by plate-bound anti-CD3 (2 μg/ml; eBioscience) and anti-CD28 (0.5 μg/ml; eBioscience) with anti-IFNGR Abs (10 μg/ml; R&D Systems, Inc.), tofacitinib (JAK inhibitor) (300 nM, kindly provided by Pfizer), rapamycin (mTORC1 inhibitor) (10 nM, Selleck Chemicals, Houston, TX), or 2-DG (3 mM, Wako Pure Chemical Industries, Osaka, Japan), C75 (racemic) (10 μM: Santa Cruz Biotechonology), Etomoxir (10 μM, 50 μM: Sigma Aldrich) cultured for 3 days in RPMI-1640 (Wako Pure Chemical Industries) supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, CA), 100 U/ml penicillin and 100 U/ml streptomycin (Thermo Fisher Scientific, Carlsbad, CA).

## Patients

PBMCs were obtained from patients with SLE and healthy subjects. The clinical characteristics of the patients are summarized in **Table 1**.

## Flow Cytometry

After washing the cells (PBMCs or CD4$^+$CD45RA$^-$ T cells), they were suspended in 100 ml of FACS solution (0.5% human albumin and 0.1% $NaN_3$ in PBS) and stained with the following antibodies: V500-conjugated anti-CD4 Abs (#560769), PerCP-Cy$^{TM}$5.5-conjugated anti-CD25 Abs (#560503), FITC-conjugated anti-CD28 Abs (#555728), PerCP-Cy$^{TM}$5.5-conjugated anti-CXCR3 Abs (#580832), FITC-conjugated anti-CD38 Abs (#555459), and V500-conjugated anti-HLA-DR Abs (#561224) (all from BD PharMingen, San Diego, CA) for 30 min at 4°C. For intracellular staining of T-bet (V450)-conjugated labeled, #561312, BD PharMingen), Foxp3 (Alexa Fluor 488)-conjugated labeled, #560047, BD PharMingen), mTOR (pS2448) (#O21-404) (PE)-conjugated labeled, #563489, BD PharMingen), IFN-γ (APC)-conjugated labeled, #554702, BD PharMingen), PBMCs were fixed and permeabilized with Perm Buffer III (BD Phosflow$^{TM}$) or Transcription Factor Buffer Set (BD Biosciences) before intracellular staining, then analyzed on FACSVerse (BD Biosciences). Isotype-matched mouse IgG controls (BD, Phosflow) were used to evaluate the background. Finally, the cells were washed three times with FACS solution and analyzed with a FACSVerse (BD, San Jose, CA) and FlowJo software (Tomy Digital Biology, Tokyo). For IFN-γ staining, PBMCs were incubated with PMA (50 nG/ml, 1544-5, Wako), ionomycin (1 μg/ml, 10634, SIGMA-Aldrich, MO) and breferdin (2.5 μg/ml, B7651, SIGMA-Aldrich) for 1 h at 37°C.

## Lactate Assay

CD45RA$^-$ memory CD4$^+$ T cells were cultured alone or under stimulation/treatment for 3 days in 96-well plates. The culture medium was later collected and diluted properly for measurement of lactate concentration using Lactate Assay Kit II (BioVision, Milpitas, CA), and the protocol supplied by the manufacturer.

## Cytokine Production

IFN-γ, IL-2, IL-4, IL-6, and IL-17 levels in the culture media were determined by the BD Cytometric Bead Array human Flex set, according to the instructions provided by the manufacturer (BD PharMingen).

**TABLE 1** | Baseline characteristics of the study subjects.

| | SLE (n = 60) | HD (n = 31) | p-value |
|---|---|---|---|
| Age, years | 41.4 ± 13.6 | 44.0 ± 19.3 | 0.8735 |
| Gender | M:F 5:55 | M:F 5:26 | 0.1366 |
| Disease duration, months | 142.3 ± 123.6 | | |
| Corticosteroid use | 40/60 | | |
| SLEDAI score | 11.5 ± 9.0 | | |
| BILAG score | 12.9 ± 11.0 | | |
| BILAG A1 and/or B2 | 36/60 | | |
| clinical relevant organ involvement (BILAG A or B) | | | |
| constitutional | 24/60 (40.0%) | | |
| mucocutaneous | 26/60 (43.3%) | | |
| central nervous system | 14/60 (23.3%) | | |
| acute confusional state | 4 | | |
| cerebrovascular disease | 4 | | |
| seizure disorders | 2 | | |
| headache | 2 | | |
| mood disorder | 2 | | |
| cognitive dysfunction | 1 | | |
| aseptic meningitis | 1 | | |
| neuropathy, cranial | 1 | | |
| musculoskeletal | 10/60 (16.7%) | | |
| cardiovascular/respiratory | 2/60 (3.3%) | | |
| abdominal | 4/60 (6.7%) | | |
| renal | 20/60 (33.3%) | | |
| ophthalmic | 0/60 (0%) | | |
| haematological | 20/60 (33.3%) | | |
| anti-dsDNA Abs (IU/ml) | 76.6 ± 124.9 (55.0%) | | |
| anti-Sm Abs (U/ml) | 40.4 ± 137.2 (10.9%) | | |
| IgG (mg/dl) | 1,798 ± 606 | | |
| CH50 (U/ml) | 38.2 ± 17.2 | | |
| CRP (mg/dl) | 0.8 ± 1.9 | | |
| ESR (mm/h) | 47.2 ± 29.8 | | |
| WBC (/μl) | 4,727 ± 2,040 | | |
| Lymph (/μl) | 968 ± 668 | | |
| history of treatment | | | |
| Immunosuppressants | 1.5 ± 1.6 | | |
| IVCY | 20 | | |
| AZ | 17 | | |
| TAC | 19 | | |
| CsA | 11 | | |
| MTX | 8 | | |
| MZ | 10 | | |
| RTX | 1 | | |
| HCQ | 7 | | |
| ABT | 3 | | |
| MMF | 1 | | |
| High-dose corticosteroid (times) | 0.8 ± 0.8 | | |
| Steroid pulse (times) | 0.5 ± 1.3 | | |

*Data are mean ± SD or number of patients.*
*See **Table 1** for abbreviations. IVCY, intravenous cyclophosphamide; AZ, azathioprine; TAC, tacrolimus; CsA, cyclosporin; MTX, methotrexate; MZR, mizoribine; RTX, rituximab; HCG, Hydroxychloroquine; ABT, abatacept; MMF, mycophenolate mofetil. High-dose corticosteroids were defined as the equivalent of prednisolone ≥0.8 mg/kg/day. Low-dose corticosteroids were defined as the equivalent of prednisolone ≤10 mg/day.*

## Intracellular ROS Levels

After 3-day culture, $CD45RA^-$ memory $CD4^+$ T cells were gently washed twice with 37°C PBS, then incubated with 100 μl of 1X DCFH-DA medium solution at 37°C for 60 min. They were repeatedly washed thereafter with PBS at 37°C, then cultured for 5 min in wells each containing 100 μl of culture medium with 100 μl of 2X Cell Lysis Buffer. Finally, 150 μl of the culture mixture was transferred to a 96-well plate black culture fluorometric plates. Fluorescence was read with a fluorometric plate reader at 480/530 nm.

## Quantitative Real-Time PCR

Total RNA was prepared by using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). First-strand cDNA was synthesized, and quantitative real-time PCR was performed in the Step One Plus instrument (Applied Biosystems, Foster City, CA) in triplicate wells of 96-well plates. TaqMan target mixes for *Enhancer of Zeste homolog 2 (*EZH2*)* (Hs00544830_m1), *basic leucine zipper transcription factor 2 (BACH2)* (Hs00222364_m1), *interferon regulatory factor 4 (IRF4)* (Hs01056533_m1), *PR domain containing 1 (PRDM1)* (Hs00153357_m1), carnitine palmitoyltransferase *IA* (*CPT1A*)(Hs00912671_m1), *carnitine palmitoyltransferase II* (*CPT2*)(Hs00988962_m1), fatty acid synthase (*FASN*) (Hs01005622_m1), sterol regulatory element-binding transcription factor 1 (*SREBF1*) (Hs01088679_g1), were purchased from Applied Biosystems. The mRNA expression level was normalized to the level of the endogenous control (*GAPDH* ribosomal RNA, #Hs99999905-m1, Applied Biosystems), and the relative quantity, compared with the PBMC sample as a reference, was calculated by using the quantification-comparative cycle threshold (DDCT) formula. The relative quantity was calculated by using the DDCT formula–referenced sample of PBMCs.

## Extracellular Flux Analysis

The XF96 Extracellular Flux analyzer (Seahorse Bioscience, North Billerica, MA) was used to quantify the oxygen consumption rate (OCR). $CD45RA^-$ memory $CD4^+$ T cells stimulated for 3 days were resuspended in XF media and then plated on XF96 cell culture microplates ($2 \times 10^5$ cells per well) coated with Cell-Tak (BD Biosciences). The OCR was measured in XF media (Agilent Technologies, Santa Clara, CA) supplemented with 1 mM sodium pyruvate, 10 mM glucose, and 2 mM L-glutamine under both basal conditions and in following the addition of 2 μM oligomycin, 2 μM carbonyl cyanide-*p*-trifluoromethoxy-phenylhydrazone (FCCP), and rotenone/antimycin A (Rot/AA).

## Electron Microscopy

Cells were collected and immersed in 2% glutaraldehyde solution at 4°C for 4 h. The samples were washed three times with 1 mol/L phosphate buffer, followed by 1% osmium tetroxide for 2 h, dehydrated in graded concentrations of alcohol (50, 70, 80, 90, and 100%), and embedded in epoxy resin. The embedded samples were then cut into ultra-thin serial sections (80 nm, Ultramicrotome, Leica UC-7, Leica, Germany) and stained with lead citrate and uranyl acetate. The samples were subsequently visualized using an electron microscope (JEOL, JEM-1200EX, Japan) at 80 kV at the Department of Electron Microscopy Center of University of Occupational & Environmental Health.

## Gas Chromatography Mass Spectrometry

$2\times10^5$ $CD45RA^-CD4^+$ cells metabolome under different conditions were extracted with 50% acetonitrile-water. Dried powder was dissolved in 20 mg/ml pyridine in methoxylamine-HCl, followed

by N-Methyl-N-(trimethylsilyl)trifluoro-acetamide (MSTFA) reagent. The metabolome was identified using GC/MS (JMS-Q1500, 7890GC, JEOL, Japan) equipped with a direct capillary column DB–5MS sized 30 m×0.25 mm×0.25 μm film thickness. The column temperature was set at 80°C for 2 min and gradually increased at 15°C/min until it reached 320°C then held for 12 min. The temperature of the injector was adjusted to 230°C and the MS transfer line was adjusted to 250°C. Helium gas was used as the carrier gas, which was injected at a constant flow rate of 1.5 ml/min. In the next step, 1 μl of the sample was injected with a solvent delay at 2 min. An autosampler (7650A) was coupled with the GC and set in split mode for automatic injection of the samples and solvents. The effluent of GC column was transferred directly into the source of the MS through the transfer line. Electron ionization mass spectrometry fragments were initiated at 70 eV within the range of 30–500 m/z at a full scan mode. The temperature of the ion source was adjusted to 200°C. NIST mass spectral libraries were used to identify the obtained mass spectra of the active molecules in the extract.

### Statistical Analysis

All data were expressed as mean ± SD, unless otherwise indicated. Differences between groups were examined for statistical significance by the paired- or unpaired-*t* test. Pearson correlation coefficient was used to test the relation between two variables of interest. A *p*-value of <0.05 denoted the presence of statistical significance. Statistical analyses were conducted using the Prism software (Prism Software, Irvine, CA).

## RESULTS

### Accumulation of IFN-γ Producing-CXCR3$^{lo}$T-bet$^{hi}$ Effector Memory CD4$^+$ Cells in SLE

First, we used T-bet, CXCR3, and IFN-γ, which are originally known as typical markers of Th1 cells, to examine possible abnormalities of Th1 cells in the peripheral blood of SLE patients. **Table 1** summarizes the clinical background of the Japanese 60 participating patients. They included 5 males and 55 females, with a mean disease duration of 142 months, SLEDAI of 11.5 ± 9.0, BILAG 12.9 ± 11.0, clinical relevant organ involvement (lupus nephritis 33.3%, central nervous system 23.3%). We also enrolled 31 age- and gender-matched healthy donors (the control group) (**Table 1**).

CD4$^+$ T cells were separated into three populations of CXCR3$^-$ T-bet$^-$, CXCR3$^{hi}$T-bet$^{lo}$, and CXCR3$^{lo}$T-bet$^{hi}$ cells. The percentage of CXCR3$^{lo}$T-bet$^{hi}$ cells among CD4$^+$ T cells was significantly higher in SLE than the control (HD: 1.9 ± 2.8%, SLE: 5.6 ± 8.5%) (**Figure 1A**). CXCR3$^-$T-bet$^-$ cells mainly consisted of CCR7$^+$CD45RA$^+$ naïve cells, CXCR3$^{hi}$T-bet$^{lo}$ cells consisted of CCR7$^+$CD45RA$^-$ central memory cells and CCR7$^-$CD45RA$^-$ effector memory cells, and CXCR3$^{lo}$T-bet$^{hi}$ cells consisted mainly of CCR7$^-$CD45RA$^-$ effector memory cells (**Figure 1B**). CXCR3$^{lo}$T-bet$^{hi}$ cells which did not express CD28, were CXCR5$^-$CCR6$^-$ cells (**Figures 1C, D**). The percentage of HLA-DR$^+$CD38$^+$ cells among CD4$^+$ T cells was significantly higher in SLE than the control. In addition, the percentage of HLA-DR$^+$CD38$^+$ cells among CD4$^+$ T cells was significantly higher in CXCR3$^{lo}$T-bet$^{hi}$ cells than CXCR3$^-$ T-bet$^-$ and CXCR3$^{hi}$T-bet$^{lo}$ cells in SLE (**Figures 1E, F**). CXCR3$^{lo}$T-bet$^{hi}$ cells were Foxp3$^-$ cells (**Figure 1G**). CXCR3$^{lo}$T-bet$^{hi}$ cells showed high potential for IFN-γ production selectively in patients with SLE but not in the control (**Figure 1H**).

Next, we examined the relation of CXCR3$^{lo}$T-bet$^{hi}$ cells to the clinical background of patients with SLE. The results showed that disease duration and treatment resistance were the parameter that significantly related to the percentage of CXCR3$^{lo}$T-bet$^{hi}$ cells (**Figure 2**).

### High IFN-γ Producing T-bet$^+$Foxp3$^{lo}$ Non-Suppressive Cells and Low IFN-γ Non-Producing T-bet$^+$Foxp3$^{hi}$-Activated-T$_{reg}$ Cells Percentages in SLE

Next, we analyzed the changes in T-bet$^+$Foxp3$^+$ cells in SLE patients. The clinical characteristics of the patients are summarized in **Supplementary Table 1**. For this purpose, peripheral blood FoxP3$^+$CD4$^+$ cells were divided to three subsets of CD45RA$^+$Foxp3$^{lo}$ naïve-T$_{reg}$, CD45RA$^-$Foxp3$^{lo}$ non-suppressive cells, and CD45RA$^-$Foxp3$^{hi}$ activated-T$_{reg}$ (22). There were no differences in the percentages of CD45RA$^+$FoxP3$^{lo}$ naïve-T$_{reg}$, CD45RA$^-$FoxP3$^{hi}$ activated-T$_{reg}$ between SLE and the control. However, the percentage of CD45RA$^-$FoxP3$^{lo}$ non-suppressive cells was significantly higher in SLE than the control (**Figure 3A**). Considering in detail of these populations, the percentage of IFN-γ producing-T-bet$^+$ cells among CD45RA$^-$Foxp3$^{lo}$ non-suppressive cells was higher while the percentage of IFN-γ non-producing-T-bet$^+$ cells among CD45RA$^-$FoxP3$^{hi}$ activated-T$_{reg}$ cells was lower in patients with SLE, compared to the control (**Figures 3B, C**).

Activation of mTOR, which is known to induce various anabolic processes, such as aerobic glycolysis, is important in the maintenance of a balance between T$_{eff}$ and T$_{reg}$ differentiation (23). Phosphorylation of mTOR in peripheral CD4$^+$ T cells of patients with SLE was significantly higher than the control. Phosphorylation of mTOR was significantly correlated with T-bet expression in CXCR3$^{lo}$T-bet$^{hi}$CD4$^+$ cells, but not in total CD4$^+$ cells (**Figure 3D**). These results suggest that activation of mTOR can affect the imbalance between Th1 subsets.

### Rapamycin Induced IFN-γ-Producing-T-bet+Foxp3lo Cells, Whereas 2DG Induced IFN-γ-Non-Producing-T-bet+Foxp3hi Cells by Different Effects on Lipid Metabolism

In the next step, we examined *in vitro* the effects of rapamycin, a mTORC1 inhibitor, and 2DG, a glycolysis inhibitor, on cell metabolism, differentiation, and function in stimulated memory CD4$^+$ cells obtained from HDs, as only a few CD4$^+$CD45RA$^-$ memory T cells were obtained from the patients with SLE. Stimulation with anti-CD3 Abs and anti-CD28 Abs induced mTOR phosphorylation and T-bet expression selectively in CD45RA$^-$CD4$^+$ memory cells (**Figure 4A**). The addition of rapamycin or 2DG reduced the percentage of CD4$^+$CD25$^+$Foxp3$^-$ T$_{eff}$ cells and increased CD4$^+$CD25$^+$Foxp3$^{hi}$ T$_{reg}$ cells among CD45RA$^-$CD4$^+$ cells, with a resultant significant increase in the

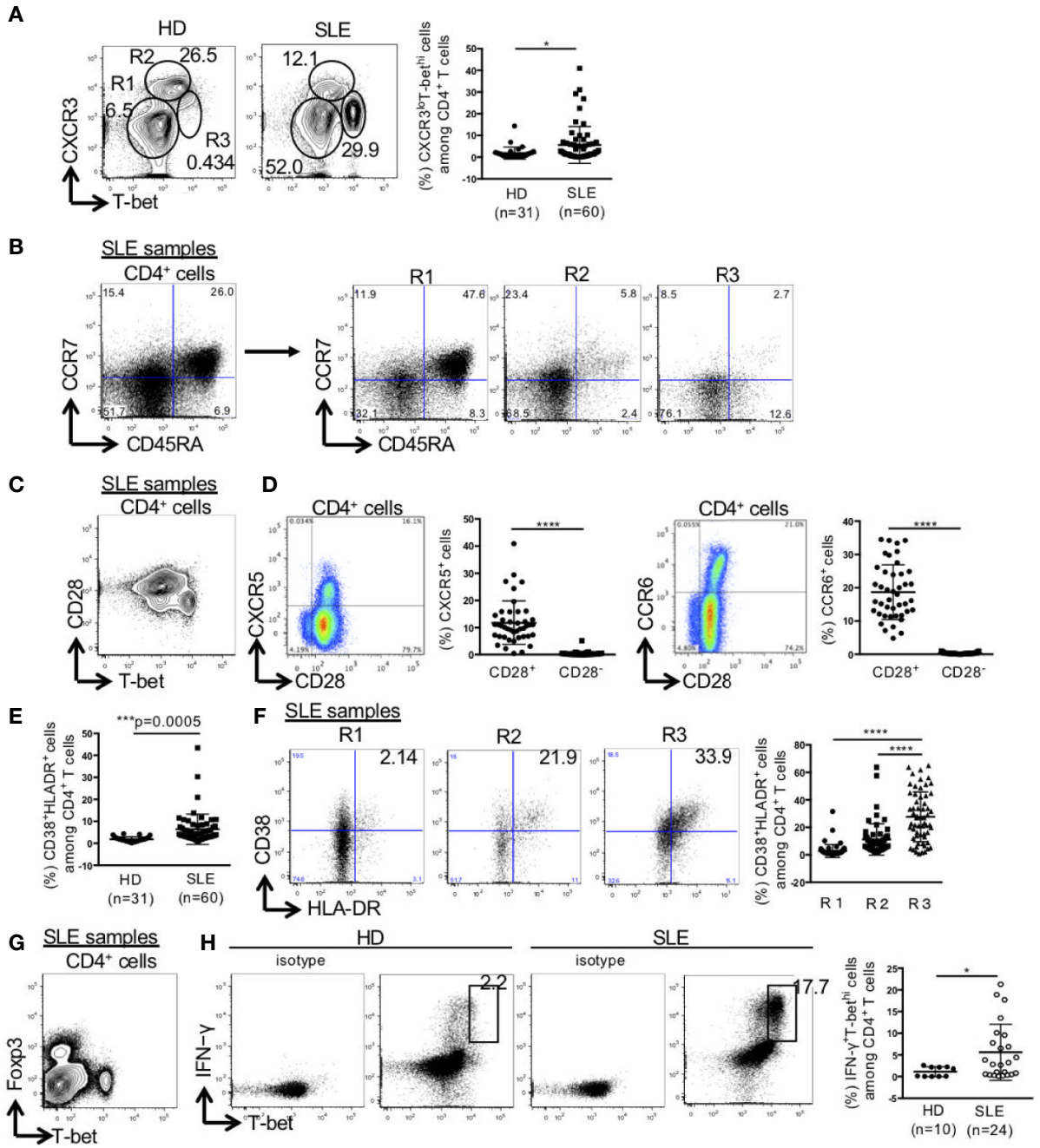


**FIGURE 1** | High percentage of CXCR3$^{lo}$T-bet$^{hi}$ effector memory CD4$^+$ cells in patients with SLE. PBMCs were obtained from 31 healthy donors (HDs) and 60 SLE patients, and CD3$^+$CD4$^+$ T cells were gated. **(A)** Expression levels of T-bet and CXCR3 in CD4$^+$ T cells were analyzed by intracellular staining using flow cytometry and shown in representative dot plots (left panel) and scatter plots of percentage of CXCR3$^{lo}$T-bet$^{hi}$ cells (right panel). **(B)** CCR7 and CD45RA were double-stained in CD4$^+$ T cells and gated in R1 (CXCR3$^-$T-bet$^-$), R2 (CXCR3$^{hi}$T-bet$^{lo}$), and R3 (CXCR3$^{lo}$T-bet$^{hi}$) in CD4$^+$ T cells in patients with SLE. **(C)** T-bet and CD28 in CD4$^+$ T cells were double-stained in CD4$^+$ T cells. **(D)** CD28 and CXCR5/CCR6 were double-stained in CD4$^+$ T cells. Percentages of CXCR5$^+$ cells in CD28$^{+/-}$CD4$^+$ cells and CCR6$^+$ cells in CD28$^{+/-}$CD4$^+$ cells of SLE patients (n = 43) were shown in scatter plots. **(E)** Percentages of CD38$^+$HLA-DR$^+$ among CD4$^+$ T cells were analyzed for HDs and SLE patients by flow cytometry and shown in the scatter plots. **(F)** Representative dot plots (left) and scatter plots (right) of expression of CD38 and HLA-DR in R1, R2 and R3 in CD4$^+$ T cells from patients with SLE. **(G)** Expression of T-bet and Foxp3 in CD4$^+$ T cells from patients with SLE was shown in the representative dot plots. **(H)** Representative dot plots of expression of T-bet and IFN-γ in CD4$^+$ cells in HDs and SLE patients were shown (left). Percentages of T-bet$^{hi}$IFN-γ$^+$ cells among CD4$^+$ T cells from HDs and SLE patients were shown in scatter plots (right). For T-bet and IFN-γ staining, PBMCs were incubated with PMA (50 ng/ml, ionomycin (1 μg/ml) and breferdin (2.5 μg/ml) for 1 h at 37°C. *p < 0.05, ***p < 0.001, ****p < 0.0001.

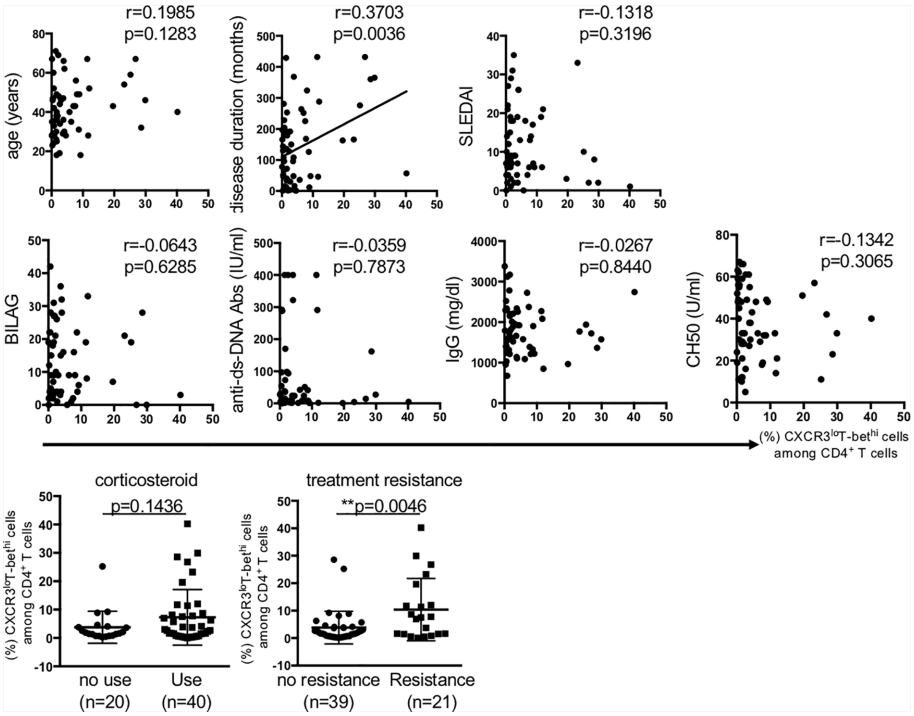


**FIGURE 2** | The relation of percentage of $CXCR3^{lo}T\text{-}bet^{hi}CD4^{+}$ cells to clinical background in patients with SLE. Pearson correlation coefficient was used to test the relation between the percentage of $CD4^{+}CXCR3^{lo}T\text{-}bet^{hi}$ cells and each factor. Treatment resistance was defined as lack of clinical response to ≥3 kinds of immunosuppressants and/or ≥2 re-increase in high-dose corticosteroid. SLEDAI; SLE disease activity score, BILAG; British Isles Lupus Assessment Group, ANA; Anti-nuclear antibody, Anti-Sm Abs; anti-Smith antibody, Anti-double stranded DNA antibody. The relation of the percentage of $CXCR3^{lo}T\text{-}bet^{hi}$ $CD4^{+}$ cells to the factors of corticosteroid use and treatment resistance were analyzed using unpaired t-test. A *p*-value of <0.05 denoted the presence of statistical significance. Statistical analyses were conducted using the Prism software (Prism Software, Irvine, CA). **p < 0.01.

$T_{reg}/T_{eff}$ ratio (**Supplemental Figure 1A**). *BACH2, IRF4, PRDM1, and EZH2* are transcriptional factors important for the differentiation and function of effector $T_{reg}$ cells (24–27). Both rapamycin and 2DG induced upregulation of *BACH2, IRF4, PRDM1*, and *EZH2* in $CD45RA^{-}CD4^{+}$ cells (**Supplemental Figure 1B**). Evaluation of the changes in Th1, Th2, and Th17 cytokines demonstrated that rapamycin and 2DG had different effects on these cytokines, especially their effects on Th1 cytokines. For example, anti-CD3 Abs and anti-CD28 Abs-stimulated IFN-γ was more strongly inhibited by 2DG than by rapamycin. In contrast, interleukin (IL) 2 was not induced by anti-CD3 Abs and anti-CD28 Abs stimulation but strongly induced only by 2DG (**Supplemental Figure 1C**).

Next, we assessed the effects of rapamycin and 2DG on $T\text{-}bet^{+}Foxp3^{+}$ cells differentiation. Rapamycin induced IFN-γ-producing $T\text{-}bet^{+}Foxp3^{lo}$ cells. On the other hand, 2DG induced IFN-γ-non-producing $T\text{-}bet^{+}Foxp3^{hi}$ cells (**Figures 4B, C**). Both rapamycin-induced $T\text{-}bet^{+}FoxP3^{lo}$ cells and 2DG-induced $T\text{-}bet^{+}FoxP3^{hi}$ cells produced IL-10, and the production tended to be slightly larger in the latter (**Figure 4D**). Since rapamycin and 2DG have different effects on $T\text{-}bet^{+}Foxp3^{+}$ cell differentiation, we next examined the mechanism from the perspective of cell metabolism using $CD4^{+}CD45RA^{-}$ memory T cells obtained from HDs. Although rapamycin and 2DG had similar effects on the dynamics of cellular metabolism, such as aerobic glycolysis, mitochondrial function and glutaminolysis (**Supplemental Figures 2, 3**), their effect on lipid metabolism was different. The expression levels of fatty acid oxidation-related enzymes such as *CPT1a* and *CPT2* were increased by both rapamycin and 2DG, while the expression levels of fatty acid synthesis-related enzymes such as *FASN* and *SREBF1* were more highly increased by rapamycin compared with 2DG. This effect of rapamycin was coupled with larger increases in palmitic and stearic acid levels compared with 2DG, suggestive of enhancement of lipid metabolism, selectively in fatty acid synthesis (**Figures 4E–G**). These results indicate that rapamycin, but not 2DG, enhanced lipid metabolism, resulting in distinct effect in $T\text{-}bet^{+}Foxp3^{+}$ cell differentiation and its IFN-γ production.

## Inhibition of Fatty Acid Synthesis in Memory CD4+ Cells Obtained From SLE Patients Resulted in Suppression of IFN-γ Production and Up-Regulated Foxp3 Expression in T-bet+Foxp3+ Cells

We also investigated the role of dyslipidemia in the observed changes in $T\text{-}bet^{+}Foxp3^{+}$ cell differentiation and IFN-γ production by memory $CD4^{+}$ T cells of HDs and SLE patients. The results showed that stimulation of the memory $CD4^{+}$ T cells with anti-CD3 Abs and anti-CD28 Abs induced IFN-γ-producing

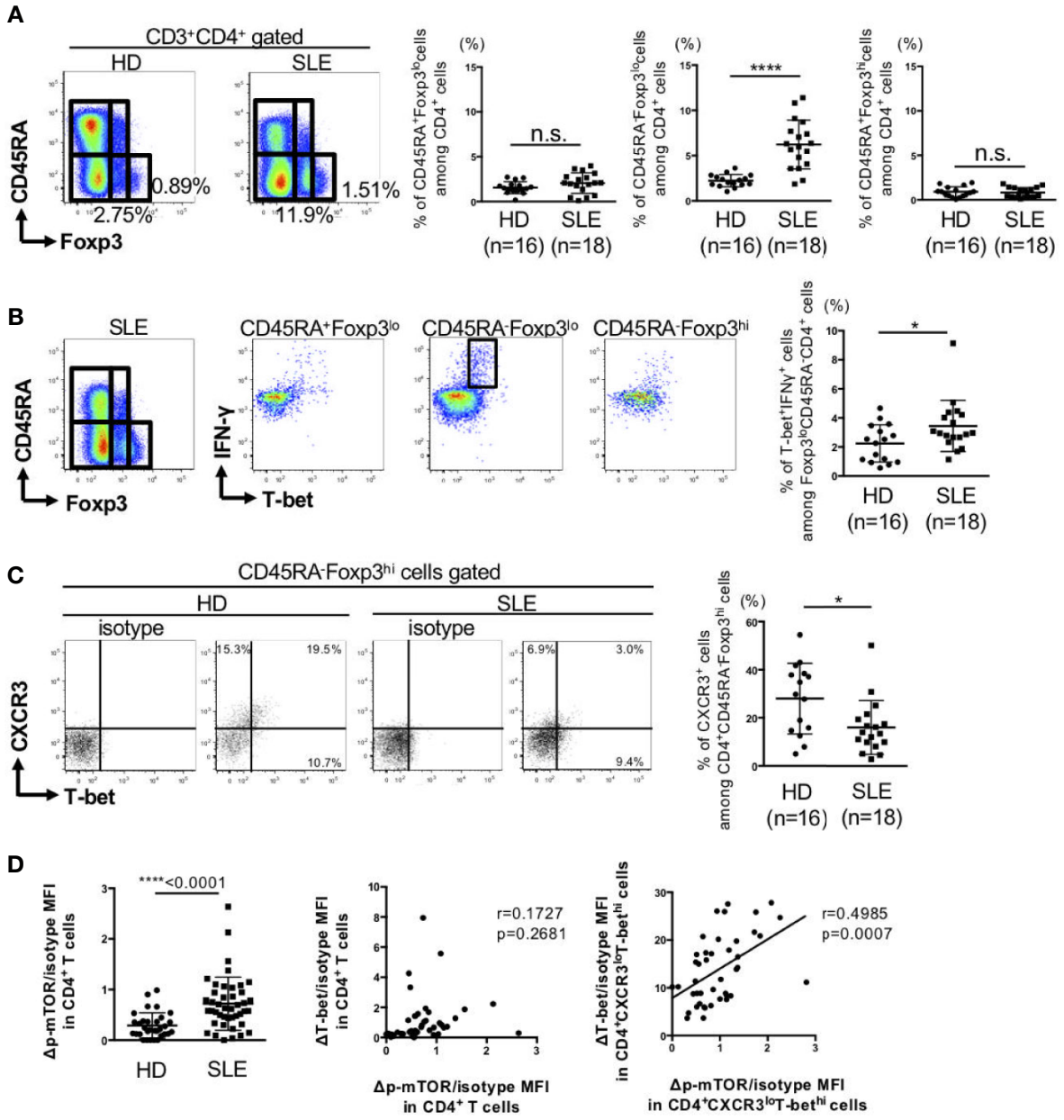


**FIGURE 3** | IFN-γ producing-T-bet$^{+}$Foxp3$^{lo}$ non-suppressive cells were increased, whereas IFN-γ nonproducing-T-bet$^{+}$Foxp3$^{hi}$ activated-T$_{reg}$ cells were decreased in patients with SLE. PBMCs were obtained from 16 HDs and 18 SLE patients, and CD3$^{+}$CD4$^{+}$ T cells were gated. **(A)** Expression of Foxp3 and CD45RA in CD4$^{+}$ T cells were analyzed by intracellular staining using flow cytometry and shown in the representative dot plots (left panels) and scatter plots of percentage of CD45RA$^{+}$Foxp3$^{lo}$ cells (naïve-Treg), CD45RA$^{-}$Foxp3$^{lo}$ cells (non-suppressive cells), and CD45RA$^{-}$Foxp3$^{hi}$ cells (activated-Treg) (right panels). **(B)** Double-staining of T-bet and IFN-γ expression in CD4$^{+}$ T cells and gated in CD45RA$^{+}$Foxp3$^{lo}$ cells (naïve-Treg), CD45RA$^{-}$Foxp3$^{lo}$ cells (non-suppressive cells) and CD45RA$^{-}$Foxp3$^{hi}$ cells (activated-Treg) in HDs and SLE patients. Data are representative dot plots (left panels) and scatter plots of percentage of T-bet$^{+}$IFNγ$^{+}$ cells among Foxp3$^{lo}$CD45RA$^{-}$CD4$^{+}$ cells (right panel). **(C)** Double-staining of T-bet and CXCR3 expression in CD4$^{+}$ T cells and gated in CD45RA$^{+}$Foxp3$^{hi}$ cells (activated-Treg) in HDs and SLE patients. Data are representative dot plots (left panels) and scatter plots of percentage of CXCR3$^{+}$ cells among CD4$^{+}$CD45RA$^{-}$Foxp3$^{hi}$ cells (right panel). **(D)** The ΔMFI/isotype MFI of mTOR phosphorylation in CD4$^{+}$ T cells from HDs and SLE patients was analyzed by flow cytometry and shown in the scatter plots. Right panel: Correlation between ΔT-bet/isotype MFI and Δp-mTOR/isotype MFI in CD4$^{+}$ T cells and CD4$^{+}$CXCR3$^{lo}$T-bet$^{hi}$ cells from SLE patients. Data are mean ± SD. Data are mean ± SD. *p < 0.05, ****p < 0.0001. n.s., not significant.

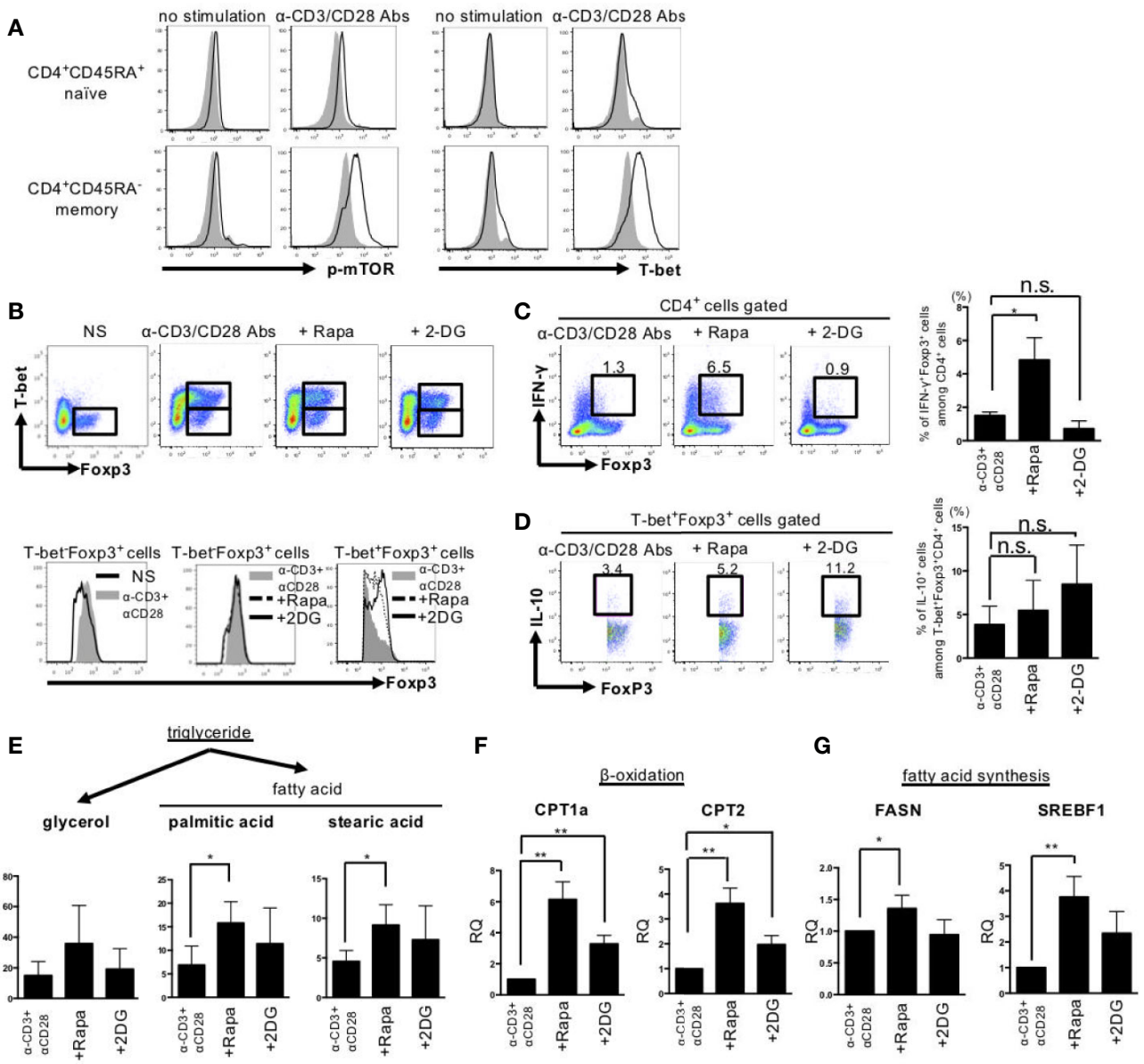


**FIGURE 4** | Rapamycin induced IFN-γ producing-T-bet$^{+}$Foxp3$^{lo}$ cells, whereas 2DG induced IFN-γ nonproducing-T-bet$^{+}$Foxp3$^{hi}$ cells by different effects on fatty acid metabolism. CD45RA$^{+}$ naïve and CD45RA$^{-}$ memory CD4$^{+}$ T cells from peripheral blood of healthy donors were stimulated with anti-CD3 Abs and anti-CD28 Abs or rapamycin (10 nM) or 2DG (3 mM) for 72 h. **(A)** Representative results of mTOR phosphorylation and T-bet expression measured by intracellular staining and flow cytometry. **(B)** Upper panel; Expression of Foxp3 and T-bet in CD45RA$^{-}$ memory CD4$^{+}$ T cells after anti-CD3 Abs and anti-CD28 Abs stimulation with or without agents for 72 h. Lower panel; Foxp3 expression in gated regions (Left: T-bet$^{-}$Foxp3$^{+}$ cells in no stimulation or anti-CD3 Abs and anti-CD28 Abs stimulation, Middle: T-bet$^{-}$Foxp3$^{+}$ cells in anti-CD3 Abs and anti-CD28 Abs stimulation or with rapamycin or 2DG, Right: T-bet$^{+}$Foxp3$^{+}$ cells in anti-CD3 Abs and anti-CD28 Abs stimulation or with rapamycin or 2DG). **(C)** Data are representative results of Foxp3 and IFN-γ expression (further treated with PMA, ionomycin and Breferdin for 1 h) estimated by intracellular staining (left panel) and bar graph of the percentage of IFN-γ$^{+}$Foxp3$^{+}$ cells (right panel). **(D)** Representative dot plots of expression of IL-10 and Foxp3 in CD45RA$^{-}$ T-bet$^{+}$Foxp3$^{+}$ cells treated with anti-CD3 Abs and anti-CD28 Abs and rapamycin or 2DG and bar graph of the percentage of IL-10$^{+}$ cells among T-bet$^{+}$Foxp3$^{+}$CD4$^{+}$ cells (right panel). Pooled data (mean ± SD of MFI) of three independent experiments in **(A–D)**. **(E)** The concentrations of glycerol, palmitic acid and stearic acid were measured by GC/MS. GC/MS data are mean ± SD of five independent experiments using cells from different healthy donors. **(F, G)** *FASN, CPT1A, CPT2* and *SREBF1* gene expression levels were determined by RT-PCR. These data are mean ± SD of four independent experiments using cells from different donors were shown. Data are mean ± SD. *p < 0.05. **p < 0.01. n.s., not significant.

T-bet$^+$Foxp3$^+$ cells in SLE samples (**Figure 5A**). Furthermore, the addition of rapamycin increased IFN-γ-producing T-bet$^+$Foxp3$^+$ cells in HDs cells. However, unlike the results in HDs cells, IFN-γ-producing T-bet$^+$Foxp3$^+$ cells remained unchanged by the addition of rapamycin in SLE patients (**Figures 5B, C**). Moreover, IFN-γ-producing T-bet$^+$Foxp3$^+$ cells did not change following the addition to rapamycin of etomoxir, a fatty acid β oxidation inhibitor, but was significantly inhibited by C75, a fatty acid synthesis inhibitor in both HDs and SLE patients (**Figure 5B**, **Supplementary Figure 4A**). The addition of C75 to rapamycin increased Foxp3 expression in T-bet$^+$Foxp3$^+$ cells to a level similar to that seen with 2DG (**Figure 5C**, **Supplementary Figure 4B**).

Finally, we evaluated the effects of various metabolic regulators on the production of IFN-γ and IL-2, which are two Th1 cytokines produced by memory CD4+ T cells in HDs and SLE. IFN-γ production induced by anti-CD3 Abs and anti-CD28 Abs stimulation was inhibited by rapamycin and 2DG and further inhibited by the combination of C75 plus rapamycin in both HDs and SLE patients (**Figure 5D**, **Supplementary Figure 4C**). Interestingly, 2DG, but not anti-CD3 Abs and anti-CD28 Abs stimulation, induced IL-2 production in both HDs and SLE patients (**Figure 5D**).

## DISCUSSION

We investigated the changes in Th1 subsets in SLE and their involvement in SLE pathology. Our results showed abundance of T-bet$^{hi}$CXCR3$^{lo}$ effector cells and T-bet$^+$Foxp3$^{lo}$ non-suppressive cells (which produce large amounts of IFN-γ) in SLE, compared with deficiency of T-bet$^+$Foxp3$^{hi}$ activated-T$_{reg}$ cells (which do not produce IFN-γ). These changes were considered to be involved in treatment resistance. In the *in vitro* arm of the study, we showed that treatment of stimulated memory CD4$^+$ cells with rapamycin and 2DG resulted in suppression of T-bet$^+$Foxp3$^-$ cells and induction of T-bet$^+$Foxp3$^{+(lo/hi)}$ cells. Interestingly, rapamycin alone enhanced lipid metabolism and induced IFN-γ-producing T-bet$^+$Foxp3$^{lo}$ cells, whereas 2DG induced IFN-γ-non-producing T-bet$^+$Foxp3$^{hi}$ cells. In memory CD4$^+$ cells of SLE patients, inhibition of fatty acid synthesis suppressed IFN-γ production and enhanced Foxp3 expression in T-bet$^+$Foxp3$^+$ cells. Thus, our results demonstrated that SLE is associated with IFN-γ overproduction by Th1 cells and Th1 subset imbalance due to metabolic abnormalities including enhanced fatty acid synthesis.

In the 1990–2010, SLE pathology was discussed in the context of a balance between Th1 and Th2, together with the involvement of Th1 (4, 5). However, Th1 is only defined as an IFN-γ-producing cell, and other important markers for Th1 such as CXCR3 and T-bet, have not been simultaneously examined in these papers. Recently, the diversity of Th cells has been reported, and CXCR3, T-bet, and IFN-γ have been reported to be expressed not only in Th1 but also in other diverse Th subsets. In particular, it was reported that TPH and Th10, which attract much attention and are important for B cell help, produced IFN-γ and expressed CXCR3 and T-bet (6, 7). In our study, the simultaneous confirmation of CXCR3, T-bet, and IFN-γ expression in CD4$^+$ cells revealed that CXCR3$^{lo}$T-bet$^{hi}$ cells, rather than CXCR3$^{hi}$T-bet$^{lo}$ cells, overproduced IFN-γ and was closely involved in the pathogenesis of SLE, including treatment resistance (**Figures 1**, **2**). In addition, our results suggest that not only the aerobic glycolysis but also fatty acid synthesis may be involved in the subset imbalance and overproduction of IFN-γ in Th1 cells (**Figure 5D**).

In 2002, Gergely and colleagues (28) were the first to describe the metabolic changes in CD4$^+$ T cells in SLE, including increased mitochondrial membrane potential (hyperpolarization) and increased ROS production. Activation of mTORC1 in CD4$^+$ T cells has also been reported in a lupus mouse model as well as in SLE patients (29, 30), suggesting that differentiation to Th1 and Th17 is enhanced by mTORC1 in wild-type and autoimmune mice. Previous studies also discussed the importance of lipid metabolism in Th17 differentiation (31). Furthermore, various metabolic abnormalities have been described in CD4$^+$ T cells of SLE patients, but many aspects of SLE pathology remain unknown (32, 33). Our study highlighted the pathogenic roles of IFN-γ overproduction and imbalance of T-bet$^+$Foxp3$^-$ and T-bet$^+$Foxp3$^+$ cells, and that abnormal fatty acid synthesis is an important metabolic abnormality mediating the above changes.

The functional properties of mTORC1 include enhancement of mRNA translation, ribosome biogenesis, and glycolysis in the downstream, as well as inhibition of catabolic metabolism, such as autophagy, fatty acid oxidation, and oxidative phosphorylation in the mitochondria (34, 35). Rapamycin is known as an mTORC1 inhibitor whereas 2-deoxy-D-glucose (2DG) is a glycolysis inhibitor. Studies in experimental animals have demonstrated that mTORC1 activity and inhibition of aerobic glycolysis by rapamycin and 2DG are associated with suppression of T$_{eff}$ cell and induction of T$_{reg}$ cells (36–38). However, the effect of rapamycin and 2DG to human CD4$^+$ T cells, especially memory CD4$^+$ T cells remained unclear. In addition, there is no consensus on the role of mTORC1 in the induction of T$_{reg}$ differentiation (39, 40). In related studies, it was demonstrated that T$_{reg}$ cells induced by selective mTORC1 deficiency by deletion of TSC and phosphatase and tensin homolog (PTEN) and persistent activation, lacked immunosuppressive capacity (40, 41). Furthermore, mTORC1 deficient mice exhibited inhibition of T$_{reg}$ induction, setting a series of inflammatory processes (42). Several studies reported that T-bet$^+$ T$_{reg}$ cells inhibit Th1 differentiation and Th1-related autoimmunopathologies (15, 43–46), while others indicated that these cells do not possess such suppressive activity (47). Thus, fractionation of (T-bet$^+$) Foxp3$^+$ CD4$^+$ cells includes populations of plastic and non-functional or rather pro-inflammatory cells. It is suggested that rapamycin may promote the differentiation of these cells.

It has been reported that CD45RA$^-$Foxp3$^{lo}$ non-suppressive T cells are increased in SLE patients and that these cells produce IFN-γ and IL-2 and exhibit little suppressive activity (48). On the other hand, CD45RA$^-$Foxp3$^{hi}$ cells (activated-Treg) have not been found to be consistent (48, 49). In the present study, CD45RA$^-$Foxp3$^{lo}$ non-suppressive T cells were increased in patients with SLE as previously reported (**Figure 3A**). By focusing on Th1 markers such as T-bet, IFN-γ, we found that T-bet$^+$IFN-γ$^+$CD45RA$^-$Foxp3$^{lo}$ non-suppressive T cells were increased, while T-bet$^+$IFN-γ$^-$

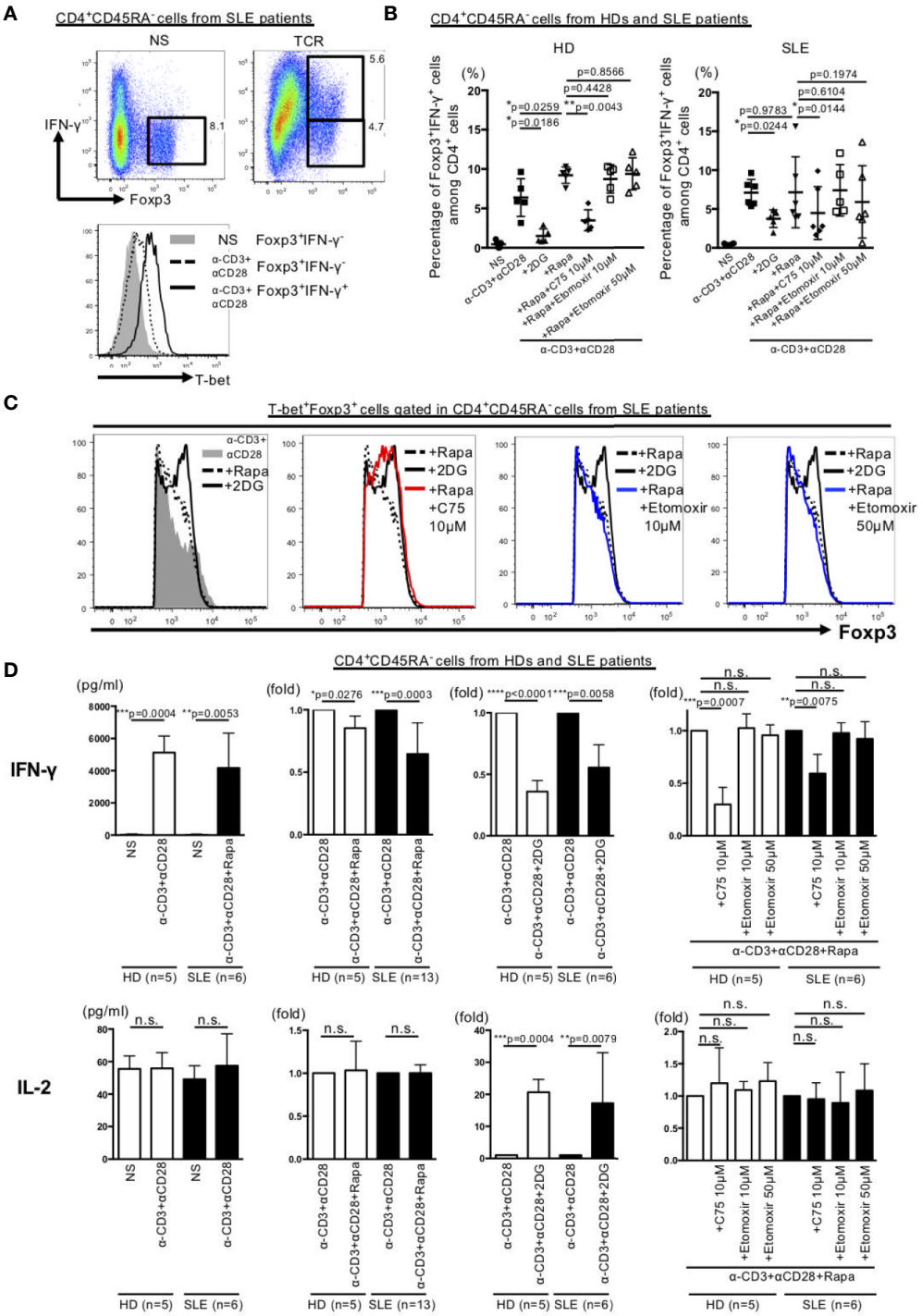


**FIGURE 5** | Inhibition of fatty acid synthesis suppressed IFN-γ production and increased Foxp3 expression in T-bet$^+$Foxp3$^+$ cells in memory CD4$^+$ T cells in SLE. CD45RA$^-$ memory CD4$^+$ T cells from peripheral blood of HDs and patients with SLE were stimulated for 72 h with anti-CD3 Abs and anti-CD28 Abs or with 2DG (3 mM), rapamycin (10 nM), C75 (10 μM) or etmoxir (10 or 50 μM). **(A)** Expression of Foxp3 and IFN-γ in CD45RA$^-$ memory CD4$^+$ T cells from patients with SLE. Representative experiment depicting Foxp3 and IFN-γ expression in SLE samples (upper panel) and histogram of T-bet expression (lower panel) in three independent experiments. **(B)** Bar graph of the percentage of Foxp3$^+$IFN-γ$^+$ cells among CD45RA$^-$CD4$^+$ cells from HDs and patients with SLE. Data are mean ± SD of three experiments using CD45RA$^-$ memory CD4$^+$cells from each six different donors of HDs and SLE patients (paired T test). **(C)** Representative data of Foxp3 expression gated in T-bet$^+$Foxp3$^+$ cells in CD4$^+$CD45RA$^-$ cells from SLE samples in three independent experiments. **(D)** Concentration and fold change of cytokines by cytometric bead array in HDs and SLE samples. Data are mean ± SD of using CD45RA$^-$ memory CD4$^+$cells from each different donors of HDs and SLE patients (paired T test). *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. n.s., not significant.

$Foxp3^{hi}CD45RA^{-}$ activated-Treg cells were decreased (**Figures 3B, C**), and the imbalance of these subsets was caused by enhanced fatty acid synthesis (**Figures 5B, C**).

Although the mTOR signaling inhibitors, including rapamycin, have been reported to be important metabolic control drugs in SLE patients, it has limited effectiveness (50). Our study demonstrated that, unlike rapamycin, 2DG induced IFN-γ non-producing $T\text{-}bet^{+}Foxp3^{hi}$ activated-$T_{reg}$ cells differentiation and IL-2 production. In this regard, previous studies reported that a decrease in serum IL-2 level in SLE patients is associated with inhibition of $T_{reg}$ function with consequent enhancement of inflammatory pathology (51, 52). In fact, previous studies showed that administration of a small amount of soluble IL-2 was associated with an increase in $T_{reg}$ cells with a resultant control of SLE disease activity (53). Evidence suggests that the primary cells that produce IL-2 are activated Th cells (54, 55). Our study demonstrated that 2DG induced the production of IL-2 from memory $CD4^{+}$ cells in patients with SLE (**Figure 5D**), suggesting that 2DG does not only correct the abnormality of Th1 subsets through the control of abnormal cell metabolism but also improve the pathological status of SLE through the induction of IL-2 production.

Limitations of this study were that a limited number of memory $CD4^{+}$ T cells can be isolated from the peripheral blood in patients with SLE, and therefore, detailed analysis of the mechanisms occurring in the memory $CD4^{+}$ T cells derived from the patients was unfeasible. Furthermore, the annual incidence of SLE without treatments for the disease is too limited to allow us to perform these studies.

Taken together, SLE patients exhibited IFN-γ overproduction by Th1 cells and subset imbalance of these cells, suggesting the involvement of Th1 cells in SLE pathology such as treatment resistance. Metabolic regulators, particularly fatty acid synthesis inhibitors, may be therapeutically beneficial in SLE by correcting these abnormalities.

## DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Ethics Review Committee of the University of Occupational and Environmental Health, Japan, and each subject provided a signed consent form (H29-045). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

SI wrote the manuscript and designed the experiments. SI, MZ, HH, GT, MH, YM, NO, YS-K, YaT, HM, MU, AN, SN, and KS performed the experiments. SI, MZ, HH, YM, and NO analyzed the data. YoT supervised the project. 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.2020.593103/full#supplementary-material

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# Deletion of *Mir223* Exacerbates Lupus Nephritis by Targeting *S1pr1* in *Fas*$^{lpr/lpr}$ Mice

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**Objective:** The micro RNAs (miRNAs) and their target mRNAs are differentially expressed in various immune-mediated cells. Here, we investigated the role of *Mir223* and sphingosine-1-phosphate receptor 1 (*S1pr1*) in the pathogenesis of systemic lupus erythematosus.

**Methods:** We analyzed miRNA and mRNA profiling data of CD4$^+$ splenic T cells derived from MRL/MpJ-*Fas*$^{lpr}$/J mice. We performed 3′ untranslated region (UTR) luciferase reporter gene assay using human umbilical vein endothelial cells (HUVECs). We generated the B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice and the lupus phenotypes were analyzed.

**Results:** In CD4$^+$ splenic T cells, we identified upregulation of miR-223-3p and downregulation of the possible target, *S1pr1* by RNA sequencing of MRL/MpJ-*Fas*$^{lpr}$/J mice. The transfection with miR-223-3p mimic significantly suppressed a luciferase activity in HUVEC treated with a Lentivirus vector containing 3′ UTR of *S1pr1*. The mRNA levels of *S1pr1* were significantly decreased after miR-223-3p overexpression. In B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice, the proportion of CD3$^+$ T cells, CD3$^+$CD4$^-$CD8$^-$ cells, B cells, plasma cells, and S1PR1$^+$CD4$^+$ T cells in the spleen was significantly increased compared with that in B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice by flow cytometry. B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice demonstrated the elevation of glomerular and renal vascular scores associated with enhanced intraglomerular infiltration of S1PR1$^+$CD4$^+$ T cells.

**Conclusion:** Unexpectedly, the deletion of *Mir223* exacerbated the lupus phenotypes associated with increased population of S1PR1$^+$CD4$^+$ T in spleen and the enhanced infiltration of S1PR1$^+$CD4$^+$ T cells in inflamed kidney tissues, suggesting compensatory role of *Mir223* in the pathogenesis of lupus nephritis.

**Keywords: miR-223-3p, *S1pr1*, S1PR1$^+$CD4$^+$ T cells, lupus nephritis, MRL/MpJ-*Fas*$^{lpr}$/J mice**

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease associated with the overproduction of autoantibodies and infiltration of autoreactive B and T lymphocytes in lymphoid and non-lymphoid organs. Despite therapeutic advancements, the improvement of mortality or the development of end-stage renal disease has not been demonstrated and the development for new effective and targeted drugs is urgently required (1). Genetic, environmental, hormonal, epigenetic, and immunoregulatory factors are associated with the pathogenesis of SLE (2). DNA methylation, histone modification, and altered micro RNA (miRNA) are widely recognized as the key epigenetic mechanisms. miRNAs target specific mRNAs for mRNA stability and can fine-tune the expression of multiple mRNAs. In the patients with SLE and MRL/MpJ-*Fas*$^{lpr}$/J (MRL/lpr) lupus-prone mice, several identified miRNAs affect the central pathway of SLE (3); for instance, upregulated miR-21 regulates lymphocyte signaling (4) and miR-155 deficiency which suppresses lupus activity by targeting sphingosine 1-phosphate (S1P) receptor 1 (*S1pr1*) (5). We previously performed global miRNA and mRNA profiling in CD4$^+$ T cells purified from the spleen of MRL/lpr mice and compared with the C57BL6/J (B6). We identified miR-200a-3p and reported that it is involved in the hypoproduction of IL-2 in T cells by targeting CtBP2 complex in MRL/lpr mice (6). Therefore, the study of the role of miRNAs may provide important clues for potential future therapies in SLE patients.

In the present study, we investigated the roles of miR-223-3p and *S1pr1* mRNA in the MRL/lpr lupus-prone mice. *Mir223* is highly expressed and tightly regulated in hematopoietic cells, especially myeloid cells, for controlling excessive innate immune responses (7). *Mir223* in CD4$^+$ T cells inhibit the human immunodeficiency virus (HIV) activity by targeting 3′ end of HIV genome (8). In autoimmune diseases, upregulation of *Mir223* in peripheral CD4$^+$ T cells, especially Th17, has a potential role in progression of multiple sclerosis (9). In SLE patients, *Mir223* has been only explored as immunological biomarkers for disease pathophysiology. Although upregulation of *Mir223* in peripheral plasma was reported (10, 11), the expression of *Mir223* in peripheral plasma was significantly decreased in SLE patients with active nephritis (12).

*S1pr1* has been known to be expressed in several cell types of the immune system. Especially, S1PR1 on T cells plays a pivotal role in T cell circulation among secondary lymphoid organs dependent on S1P concentration (13–15). *S1pr1* conventional knockout mice exhibited intrauterine death by incomplete vascular maturation (16). T cell-specific *S1pr1* knockout mice showed a block in the egress of T cell from thymus into circulation and a reduction of lymphocytes, especially CD3$^+$ cells, in secondary lymphoid organs (13). The S1P and S1PR1 signaling in T cells is also essential for T cell survival (17). The *S1pr1* expression of peripheral blood mononuclear cells was decreased in SLE patients and B6.MRL-*Fas*$^{lpr}$ mice (5). Agonists of S1PR1 suppressed the development of autoimmunity and renal injury by inducing sequestration of peripheral lymphocytes including autoreactive T cells into secondary lymphoid organs, reducing their infiltrates in target organs, and inducing its apoptosis in MRL/lpr mice (18–23). As mentioned above, miR-155 knockout lupus-prone B6.MRL-*Fas*$^{lpr}$ mice showed milder SLE clinical features than B6.MRL-*Fas*$^{lpr}$ mice by targeting *S1pr1* (5). Although the role of *S1pr1* in SLE is not completely understood, the miRNAs regulating the expression of *S1pr1* are expected critically involved in the pathogenesis of SLE. Here, we demonstrated that miR-223-3p plays a critical role in the regulation of T cell circulation and apoptosis by targeting S1PR1 in lupus prone mouse. The current investigation provides new data on the epigenetic control of T cell circulation in SLE.

## MATERIALS AND METHODS

### Mice

B6.Cg-*Ptprc*$^a$*Mir223*$^{tm1Fcam}$/J (referred to as B6-*Mir223*$^{-/-}$*Ptprc*$^{a/a}$) on background of C57BL/6J (*Ptprc*$^a$) (24), genetically lupus-prone female MRL/MpJ-*Fas*$^{lpr}$/J (referred as MRL/lpr or MRL-*Fas*$^{lpr/lpr}$*Ptprc*$^{b/b}$), B6.MRL-*Fas*$^{lpr}$/J (referred to as B6/lpr or B6-*Fas*$^{lpr/lpr}$*Ptprc*$^{b/b}$) on background of C57BL/6J (*Ptprc*$^b$) (Jackson Laboratory), and C57BL/6J (*Ptprc*$^b$) (referred as B6) (Charles River Laboratories) were purchased. The *Mir223* gene is located on the X chromosome. The animals were maintained in a 12-h light/dark cycle, with free access to water and standard rodent chow. The following animal experiments were approved by the Animal Care and Use Committee of the Department of Animal Resources, Advanced Science Research Center, Okayama University under the approval numbers of OKU-2013092, OKU-2015569, OKU-2015658, OKU-2015661, OKU-2015662, OKU-2015663, OKU-2015664, OKU-2016191, OKU-2016192, OKU-2016365, OKU-2016383, OKU-2016384, OKU-2016385, OKU-2017499, OKU-2017500, OKU-2017501, OKU-2017502, OKU-2018474, OKU-2018475, OKU-2018582, OKU-2018583, OKU-2018583, and OKU-2018585. All animal experiments were performed in accordance with relevant guidelines and regulations.

### mRNA and miRNA Expression Profiling by RNA Sequencing

Total RNA, including miRNA, was purified from CD4$^+$ T cells of MRL/lpr mice and B6 mice using a miRNeasy mini kit (Qiagen). mRNA and miRNA sequencing and expression profile data were prepared as previously described (6). All raw and processed data are freely accessible in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE87219.

### 3′ Untranslated Region Luciferase Reporter Gene Assay

Human Umbilical Vein Endothelial Cells (HUVECs; CC-2519, Lonza) were cultured in EGM-2 medium (CC-3162, Lonza) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% $CO_2$ at 37°C. The culture medium was replaced every 1–2 days, cells at 85–90% confluence were passaged at a ratio of 1:3 confluence. The cells were used between passages two and five in the following

experiments. MISSION 3′UTR Lenti GoClone containing 3′UTR of *S1PR1* (HUTR09173; S1PR1-3′UTR) and MISSION 3′UTR Lenti GoClone-Controls (HUTR001C-004C; Controls) were purchased from Sigma-Aldrich. The Lentivirus particles and 8 μg/ml Hexadimethrine bromide® (H9268, Sigma-Aldrich) were added to HUVECs for 10 h and then replaced with fresh medium. After 24 h, the infected cells were subjected to selection with 0.25 μg/ml puromycin. The selected cells were further co-transfected with miR-223-3p mimic and its negative control (nontargeting miRNA) into HUVECs using RNAiMAX (Invitrogen). The cells were harvested after 24 h of transfection, and Renilla luciferase activity was measured using the Luciferase Reporter Assay System Kit (MLS0001, Sigma-Aldrich) and GloMax® 20/20 Luminometer (Promega).

## Evaluation of Active and Chronic Lesions of Lupus Nephritis

For histology, mouse kidneys were fixed in 10% formalin for 24 h at 4°C, and 4 μm paraffin sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS) stain. Glomerular hypercellularity was evaluated by counting the number of nuclei per glomerular cross-section in 10 randomly selected glomerular cross-sections per mouse. The glomerular lesions were graded on a scale of 0–3 as previously described (25). The glomerular lesion index was calculated from the sum of the scores for 40 randomly selected glomerular cross-sections per mouse. Renal vascular lesions were graded on a scale of 0–3 as previously described (25). The vascular lesion index was calculated from the sum of the scores for all vessels per section. Tubulointerstitial lesions were graded on a scale of 0–4 as previously described (26). The tubulointerstitial lesion index was calculated from the sum of the scores for 30 consecutive high-power fields (HPF) at a magnification of ×400 in the cortex per section.

## Immunofluorescence

Mouse kidneys were immediately frozen at −80°C in OCT compound (Sakura, Japan), and 4 μm cryostat sections were stained with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG or rabbit anti-mouse C3 (Cappel). Staining of all sections was visualized with a fluorescence microscope (BX51; Olympus). Immunofluorescence intensity (measured as the number of pixels/$\mu m^2$) was quantified using cellSens software (version 1.16; Olympus). At least five glomeruli per section were analyzed.

For immunofluorescence with CD4, CD8, and S1PR1, mouse kidneys were embedded in OCT compound (Sakura, Japan), and 4 μm cryostat sections were fixed in 4% paraformaldehyde (PFA). After blocking in 5% BSA, the sections were incubated with the appropriate primary antibodies; rat monoclonal anti-CD4 (1:50; RM4-5, 100505), rat monoclonal anti-CD8a (1:50; 53-6.7, 100701) (BioLegend), and rabbit polyclonal S1PR1(1:100; ab137467, Abcam). After overnight incubation, the sections were further treated with Alexa Fluor® 647 conjugated goat anti-rat secondary antibody (ab150167, Abcam) or Alexa Fluor® 594 conjugated goat anti-rabbit secondary antibody (ab150084, Abcam). Nuclear staining was performed using 4′,6-diamidino-2-phenylindole (DAPI; 422801, BioLegend). Staining of all sections was visualized with a fluorescence microscope (BZ-X700; Keyence). Quantitation of staining was graded based on the number of positive cells per glomerulus or per HPF for tubular infiltrates, with a minimum of 10 glomeruli of HPF per mouse examined.

## RNA Isolation and Real-Time RT-PCR

Total cellular RNAs from human and mouse samples were extracted with an RNeasy mini kit (Qiagen). cDNAs were reverse transcribed from mRNAs with a high-capacity cDNA RT kit (Thermo Fisher Scientific), while cDNAs from miRNAs with a TaqMan miRNA reverse transcription kit (Thermo Fisher Scientific). Real-time PCRs for miR-223-3p (002295), sno-202 (001232), sno-234 (001234) and RNU48 (001006) were performed using TaqMan primer/probes with TaqMan miRNA assays (Thermo Fisher Scientific). The expression of miRNAs was normalized to sno-202 and sno-234 for mice and RNU48 for human samples by the ΔΔCt method. Real-time PCRs for *S1pr1* (Mm02619656_s1), *Gapdh* (Mm99999915_g1), *Cxcl9* (Mm00434946_m1), *Cxcl10* (Mm00445235_m1), *Cxcl11* (Mm00444662_m1), *Ccl2* (Mm00441242_m1), *Ccl4* (Mm00443111_m1), *Ccl5* (Mm01302427_m1), *S1PR1* (Hs01922614_s1) and *GAPDH* (Hs02786624_g1) were performed using ABI TaqMan gene expression assays (Applied Biosystems) according to the manufacturer's protocol and normalized to *GAPDH* by the ΔΔCt method.

## Systemic Lupus Erythematosus Patients and $CD4^+$ T Lymphocyte Purification

The 15 SLE patients (10 females and five males) who fulfilled at least four of the 11 revised criteria of the American College of Rheumatology for the classification of SLE and 6 healthy controls were enrolled and peripheral $CD4^+$ T lymphocytes were purified as previously described (27). The studies were approved by the Ethical Committee, Okayama University Hospital (#1779) and the written informed consent was obtained.

## Statistical Analyses

All results are shown as the mean ± standard error (SE) of data from at least three separate experiments, each performed with more than triplicate samples. Normal distribution of the data was assessed by Shapiro–Wilk test, and statistically significant differences between groups were determined using the Student's 2-tailed *t*-test or Wilcoxon signed-rank test, as appropriate. The data were also analyzed with one-way analysis of variance and Tukey's honestly significant difference test when multiple comparisons against the control were required. Pearson's $\chi 2$ test was used to compare the distribution of glomerular lesions with grading scores from 0 to 3 (25), renal vascular lesions with grading scores from 0 to 3 (25) and tublointerstitial injury with grading scores from 0 to 4 (26). P values less than 0.05 were considered significant. All statistical analyses were performed using the JMP 11.2.0 software package (SAS Institute).

# RESULTS

## Upregulated miR-223-3p and Repressed S1pr1 mRNA Levels in CD4$^+$ T Cells of *MRL/lpr* Mice

To identify new candidate miRNAs and their target mRNAs involved in the pathogenesis of SLE, we integrated miRNA and mRNA sequencing data in splenic CD4$^+$ T cells isolated from MRL/lpr and B6 mice (GSE87219). A total of 19 miRNAs were upregulated in MRL/lpr compared with B6 mice with a cut-off value of >10-fold (**Figure 1A** and **Supplementary Table 1**). We further screened the sets of upregulated miRNA and >2-fold downregulated mRNAs, which were retrieved from miRDB (http://mirdb.org.) as predicted targets. Among them, we identified that upregulation of miR-223-3p was associated with downregulation of 23 mRNAs by RNA sequencing data (**Figure 1B and Supplementary Table 2**). We further investigated the downregulation of predicted (*S1pr1*) target mRNAs in MRL/lpr, B6/lpr and B6 mice. By quantitative real-time PCR, miR-223-3p was upregulated and *S1pr1* was most significantly downregulated in MRL/lpr and B6/lpr mice compared with B6 mice (**Figures 1C, D**). Although the mRNA levels of *S1pr1* were significantly decreased in splenic CD4$^+$ T cells from MRL/lpr compared with those from B6, the protein levels of S1PR1 were rather increased in splenic CD4$^+$ T cells from MRL/lpr compared with those from B6 (**Supplementary Figure 1A**). S1PR1 protein expression on surface of CD4$^+$ T cells is known to be downregulated in the blood and upregulated in lymphoid organ (28). It was reported that S1P induces S1PR1 internalization *via* endosomal pathway (29), and it subsequently undergoes ubiquitylation and proteasomal degradation by the ubiquitin ligase WW domain containing E3 ubiquitin protein ligase 2 (Wwp2) (30, 31). The S1PR1 protein was supposed to be regulated by posttranslational modification in MRL/lpr mice and we further examined ubiquitination of S1PR1 protein in splenic CD4$^+$ T cells from MRL/lpr and B6. The starting materials (SMs) demonstrated higher expression of S1PR1 protein in CD4$^+$ T cells from MRL/lpr compared with B6, while it was barely detected in the eluted fractions (ELs) of highly purified ubiquitinated proteins by UbiQapture-Q Kit (**Supplementary Figure 1B**). Taken together, upregulation of S1PR1 protein in splenic CD4$^+$ T cells from MRL/lpr was mediated by reduced ubiquitination of the S1PR1, although B6 was not an appropriate control for MRL/lpr.

As shown in **Supplementary Table 3**, the 3′UTR region of *S1pr1* was predicted to serve as binding site for miR-223-3p in humans and mice. To determine whether *S1pr1* was a direct target of miR-223-3p, Lentivirus of S1pr1-3′UTR and Controls were infected to HUVECs for luciferase miRNA target assays. Co-transfection of miR-223-3p mimic demonstrated a significant reduction in luciferase activity, while nontargeting miRNA did not alter the luciferase activity (**Figure 2A**). To further confirm that *S1pr1* is a target of miR-223-3p, we detected the endogenous *S1pr1* mRNA and protein level after transiently transfecting mimic miR-223-3p into EL4 cells. As shown in **Figure 2B**, the expression level of *S1pr1* mRNA and protein decreased after miR-223-3p overexpression. Taken together, the miR-223-3p is a negative regulator for *S1pr1*.

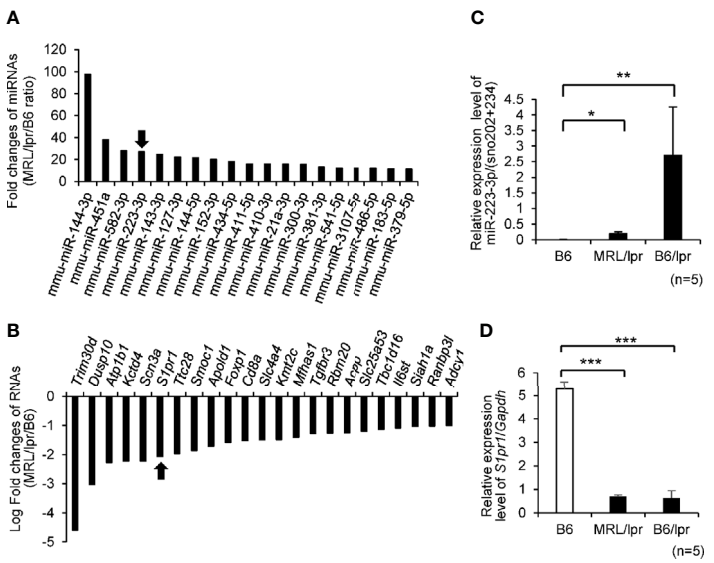


**FIGURE 1** | Top 20 differential expression levels of miRNAs and mRNAs in CD4+T cells isolated from MRL/lpr lupus-prone (MRL/lpr) *vs* C57BL/6 (B6) mice by microarray analysis. **(A)** Fold change (ratio between MRL/lpr/B6) in miRNA expression MRL/lpr *vs* B6. We focused on mmu-miR-223-3p which was indicated by arrow. **(B)** Candidate target genes for upregulated mmu-miR-223-3p were identified using the commonly used prediction algorithm, miRDB. These targets and our mRNA expression profiles were integrated. Fold change (ratio between MRL/lpr/B6) in mRNA expression MRL/lpr *vs* B6. We focused on *S1pr1* which was indicated by arrow. **(C)** The expression level of miR-223-3p was evaluated by TaqMan quantitative PCR in MRL/lpr mice and B6/lpr lupus-prone (B6/lpr) mice compared with B6 mice (n = 5 per group, 16-week-old female). **(D)** The expression of *S1pr1* in CD4$^+$T cells of mice spleen was evaluated by TaqMan quantitative PCR (n =5 per group, 16-week-old female). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, by Student's t-test.

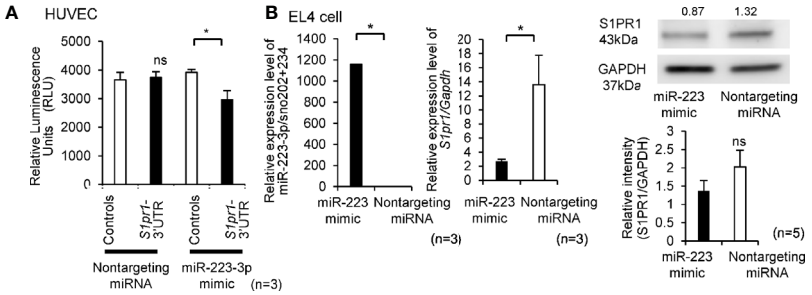


**FIGURE 2** | The miR-223-3p is a negative regulator for *S1pr1*. **(A)** HUVECs were co-transfected with nontargeting miRNA and miR-223-3p mimic with the no UTR or 3′UTR. Luciferase activity was assayed 24 h after transient co-transfection. Renilla luciferase activity was measured. (n = 3 per group). Data are presented as mean ± SEM. *$p < 0.05$, ns, not significant, by Wilcoxon signed-rank test. **(B)** The mRNA and protein level of *S1pr1* in EL4 cells was analyzed by TaqMan quantitative PCR and Western blot analyses after transfection with miR-223-3p mimic or nontargeting miRNA. The transfection efficacy of miR-223-3p mimic was confirmed by TaqMan quantitative PCR. miR-223-3p was transfected by lipofection, and the expression was compared with the nontargeting miRNA after 24 h transfection. The mRNA and protein levels of *S1pr1* after the overexpression of miR-223-3p was confirmed by TaqMan quantitative PCR and Western blot analyses. Numbers above the Western blots indicate band intensity (normalized to total GAPDH) measured by using ImageJ software. Quantification of Western blot results (right side). (n = 3 per group). Data are presented as mean ± SEM. *$p < 0.05$, ns, not significant, by Wilcoxon signed-rank test.

## Reduction of S1PR1 mRNA in CD4$^+$ T Cells From the Patients With Systemic Lupus Erythematosus

We investigated the expression levels of *S1PR1* mRNA and miR-223-3p in circulating CD4$^+$ T cells isolated from SLE patients and healthy subjects. The demographics of the enrolled SLE patients are shown in **Supplementary Table 4**. The patients with SLE also showed similar trends like MRL/lpr mice; the expression level of *S1PR1* mRNAs in CD4$^+$ T cells is significantly downregulated in SLE patients than healthy control, while miR-223-3p tended to upregulate in SLE patients without statistical differences (**Figure 3A**). Given the critical role of *S1PR1* mRNA and miR-223-3p in SLE, we investigated the simple correlations with clinical parameters (**Supplementary Table 4**). miR-223-3p expression levels in SLE patients with skin disorders, such as malar rash, were significantly lower than SLE patients without skin symptoms. However, in the SLE patients with lung disorders, such as pleural effusion, miR-223-3p expression level was significantly higher than SLE patients without lung symptoms (**Figure 3B**). *S1PR1* mRNA expression level in SLE patients with skin disorder was significantly higher than SLE patients without skin involvement, while it was lower in the SLE patients with central nervous system (CNS) involvement compared with SLE patients without this manifestation (**Figure 3C**). These results indicated that miR-223-3p upregulation linked to *S1PR1* mRNA downregulation in SLE, in patients with skin involvements.

## Systemic Lupus Erythematosus Phenotypes in Mir223$^{-/-}$Fas$^{lpr/lpr}$ Mice

To explore the involvement of miR-223-3p in the pathogenesis of SLE, we generated B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice. We observed B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice until 44 weeks of age and found that there were no differences in body weight, survival rate, skin score, organ weight, total cell numbers in spleen and lymph nodes, and circulating blood cell counts between two groups (**Supplementary Figures 2A–F**). The significant gain of weight was reported in B6-*Mir223*$^{-/-}$*Ptprc*$^{a/a}$ compared to C57BL/6J (*Ptprc*$^{a}$) (32) and the number of circulating neutrophils in *Mir223* knockout mice significantly increase and spontaneously develop lung inflammation marked by neutrophil infiltration (24). However, the presence of *Fas*$^{lpr/lpr}$ in mice canceled such *MiR223* deficiency-induced phenotypes. Similarly, in MRL/lpr background, no differences were observed in body weight, survival rate, skin score, organ weight, and total cell numbers in spleen and lymph nodes between MRL-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and MRL-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figures 3A–E**). Serum levels of total IgG and anti- dsDNA antibody were not altered in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ (**Supplementary Figure 2G**). We further examined the serum levels of immunoglobulin subclasses (IgA, IgM, IgG1, IgG2b, IgG2c, and IgG3). Although IgG2b levels at 44 weeks of age significantly elevated in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$, rest of them demonstrated no significant increase (**Supplementary Figure 2G**). Similarly, there were no differences in gamma globulin and autoantibody production between MRL-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and MRL-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figure 3F**).

## Changes in Lymphocyte Population in Spleen and Lymph Nodes From Mir223$^{-/-}$Fas$^{lpr/lpr}$ Mice

MRL/lpr mice are characterized by increased numbers of activated CD4$^+$ T cells, CD3$^+$CD4$^-$CD8$^-$ cells (double-negative T cells; DNT), B cells, and plasma cells as compared with B6 (33, 34). We next investigated the cell population in spleen and lymph nodes by flow cytometry. The proportion of CD4$^+$ and CD8$^+$ T cells, memory and effector T cells, and activated CD4$^+$ (CD69$^+$CD4$^+$) T cells in both spleen and lymph nodes did not differ between B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Figure 4A**). Compared with B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice, B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ showed a significantly higher proportion of CD3$^+$ T cells, DNT cells, B cells, and plasma cells in the spleen

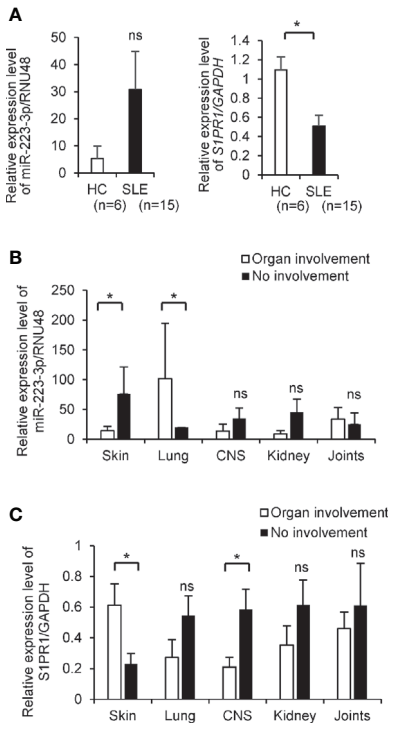


**FIGURE 3** | The decreased level of *S1PR1* and increased level of miR-223-3p transcripts in CD4$^+$ T cells from SLE patients. **(A)** mRNA expression of *S1PR1* and miR-223-3p in CD4$^+$ T cells isolated from healthy (n = 6) and the patients with SLE (n = 15). Data are presented as mean ± SEM. *$p < 0.05$, ns, not significant, by Wilcoxon signed-rank test. **(B, C)** The miR-223-3p and *S1PR1* transcript level in CD4$^+$ T cells isolated from the SLE patients with organ involvement and no involvement. SLE patients with skin involvement (n = 11) and without involvement (n = 4), with lung involvement (n = 2) and without involvement (n = 13), with central nervous system involvement (n = 4) and without involvement (n = 11), with kidney involvement (n = 6) and without involvement (n = 9), with joints involvement (n = 10) and no involvement (n = 5). Data are presented as mean ± SEM. *p < 0.05, ns, not significant, by Student's t-test.

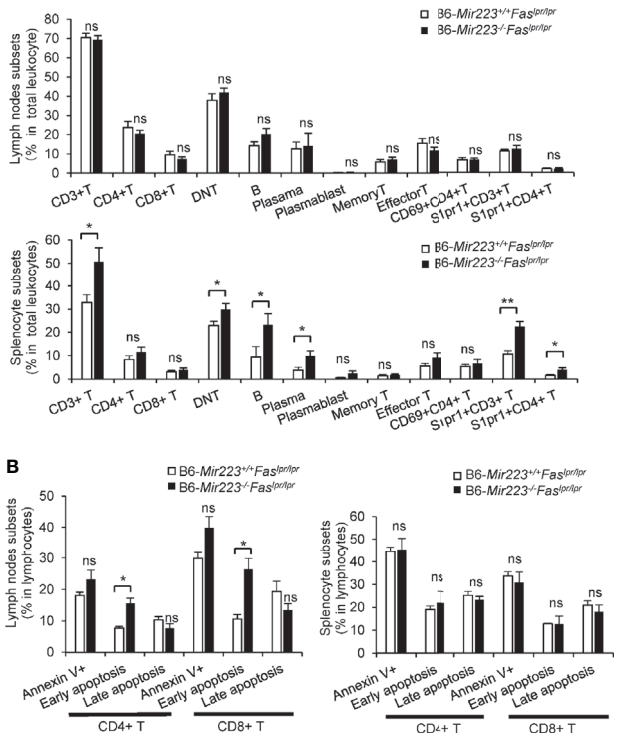


**FIGURE 4** | The proportion of CD3$^+$ T cells, CD3$^+$CD4$^-$CD8$^-$ T cells, CD19$^+$ B cells, CD19$^-$CD138$^+$ cells (Plasma cells), CD3$^+$S1PR1$^+$ T cells and CD3$^+$CD4$^+$S1PR1$^+$ T cells in spleen and early apoptotic cells in CD4$^+$ and CD8$^+$ T cells in lymph nodes were significantly increased in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice. **(A)** Various cellular subsets in lymph nodes and spleen. CD3$^+$CD4$^+$ (CD4 T cells), CD3$^+$CD8$^+$ (CD8 T cells), CD3$^+$ CD4$^-$CD8$^-$ (Double negative (DN) T cells), CD19$^+$ (B cells), CD19$^-$CD138$^+$ (Plasma cells), CD19$^+$CD138$^+$ (Plasmablasts), CD4$^+$CD44$^+$CD62L$^+$ (Memory T cells), CD4$^+$CD44$^+$CD62L$^-$ (Effector T cells), CD69$^+$CD4$^+$ cells, S1PR1$^+$CD3$^+$ cells, S1PR1$^+$CD4$^+$ cells. Distribution of subsets in total cells isolated from whole cervical lymph nodes and spleen were indicated in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ (n=7) and B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ (n = 8) mice. Absolute number of total splenocyte and lymph nodes cell had no difference between two genotypes (**Supplementary Figure 2E**). **(B)** Apoptotic cells in lymph nodes and spleen. Annexin V$^+$7-AAD$^-$ (Early apoptosis cells), Annexin V$^+$7-AAD$^+$ (Late apoptotic cells). Distribution of subsets in CD3$^+$CD4$^+$ T cells or CD3$^+$CD8$^+$ T cells isolated from whole cervical lymph nodes and spleen were indicated in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ (n = 3) and B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ (n = 3) mice. *$p < 0.05$, **$p < 0.01$, ns, not significant, by Student's t-test in **(A)**, by Wilcoxon signed-rank test in **(B)**.

(**Figure 4A**). The S1PR1$^+$CD3$^+$ and S1PR1$^+$CD4$^+$ population in the spleen increased in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mouse (**Figure 4A**), suggesting *Mir223* deficiency contributed to the increased cell surface expression of S1PR1. The low S1P concentration in secondary lymphoid organs and relatively higher concentrations in lymphatic fluid promotes S1PR1-dependent movement of T cells from secondary lymphoid organs back into the lymphatic circulation and then into blood (35). Next, we measured the concentration of S1P in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice; however, there were no significant differences between two genotypes (**Supplementary Figure 2G**), suggesting that surfaced S1PR1 on splenic T cells through *Mir223* deficiency may promote to egress from lymphoid organ to blood and infiltrate to inflamed tissue. In contrast, there were no differences in T and B cell populations in the spleen and lymph nodes between *Mir223* knockout lupus-prone MRL-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and MRL-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figure 3E** and **Supplementary Figure 5E**).

## Gene Expression and Apoptosis in CD4$^+$ and CD8$^+$ T Cells From Spleen and Lymph Nodes of Mir223$^{-/-}$Fas$^{lpr/lpr}$ Mice

The impairment of apoptosis due to *Fas* mutation is one of the mechanisms underlying the pathogenesis of *Fas*$^{lpr/lpr}$ mice. Therefore, the induction of apoptosis has been reported to ameliorate the clinical features in the mice (36). MRL/lpr mice treated with a selective agonist of S1PR1 (KRP-203) showed enhancement of lymphocytes apoptosis in lymph nodes and reduction of T cell infiltrates in kidney (23). We further examined whether apoptosis is enhanced in the lymph nodes of

B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice. Although the population of Annexin V$^+$ and late apoptotic cells were unaltered, the elevated rate of early apoptotic (Annexin V$^+$ and 7-AAD$^-$) CD4$^+$ and CD8$^+$ T cells in the lymph nodes of B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ (**Figure 4B**) and MRL-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice were observed (**Supplementary Figure 5B**). However, anti-apoptotic Bcl-2, Bcl-xL, and proapoptotic Caspase-3 protein expression levels in lymph nodes and spleen were not altered by the deficiency of *Mir223* (**Supplementary Figure 4 and Supplementary Figure 5C**). Since the ratio of late apoptotic CD4 T cells and the cleavage of caspase-3 was not altered, the promotion of whole apoptosis process was not confirmed as the mechanisms to explain the phenotypes in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and MRL-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice.

Although miR-223-3p in CD4$^+$ and CD8$^+$ T cells and B cells was barely detected in the lymph nodes and spleens of B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figure 6B**), the *S1pr1* mRNA and protein expression in CD4$^+$ T cells from spleen and lymph nodes were not altered in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ compared with that in B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figures 6A, C**). The increased population of S1PR1$^+$CD4$^+$ cells in spleen in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ suggested that S1PR1$^+$CD4$^+$ T cells induced by Mir223 deficiency may migrate from lymphatic tissues to non-lymphatic inflamed tissues.

## Exacerbation of Lupus Nephritis in Mir223$^{-/-}$Fas$^{lpr/lpr}$ Mice

Immune complex glomerulonephritis is the hallmark of the non-lymphatic tissue inflammation in MRL/lpr mice and human SLE patients (30, 37). In light microscopic examination of the tissue sections, an exacerbation of histologic damage in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice was evidenced by an expansion in glomerular size, increased cellularity, and kidney weight (**Figure 5A**, **Supplementary Figures 7C and 8B**), although mesangial matrix area demonstrated no significant difference between two genotypes (**Supplementary Figure 7C**). The distribution of glomerular and renal vascular lesions with grading scores in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice significantly exacerbated (**Figure 5B**). Immunofluorescence study showed S1PR1$^+$CD4$^+$ T cells in glomerular lesion and CD4$^+$ T cells in interstitial fibrosis lesion were significantly increased in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ compared to B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Figures 6A, B**, **Supplementary Figure 8C**). We also assessed the effect of *Mir223* deficiency on glomerular immune complex formation/deposition. We found an enhanced deposition of C3 in the glomerular immune complex in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice compared to B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ littermates (**Supplementary Figures 7A, B**) and the deposition of IgG demonstrated no significant changes (**Supplementary Figure 7B**). The tendency for the increase of S1PR1$^+$CD4$^+$ T cells in glomerular lesions and CD4$^+$ T cells in interstitial fibrosis lesions was seen in MRL-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figures 9** and **10**). The daily urinary protein excretions tended to be higher in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ than B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figure 8A**).

Chemokines contribute to renal damage by recruiting inflammatory cells to the kidney. The CXCR3 ligands, CXCL9

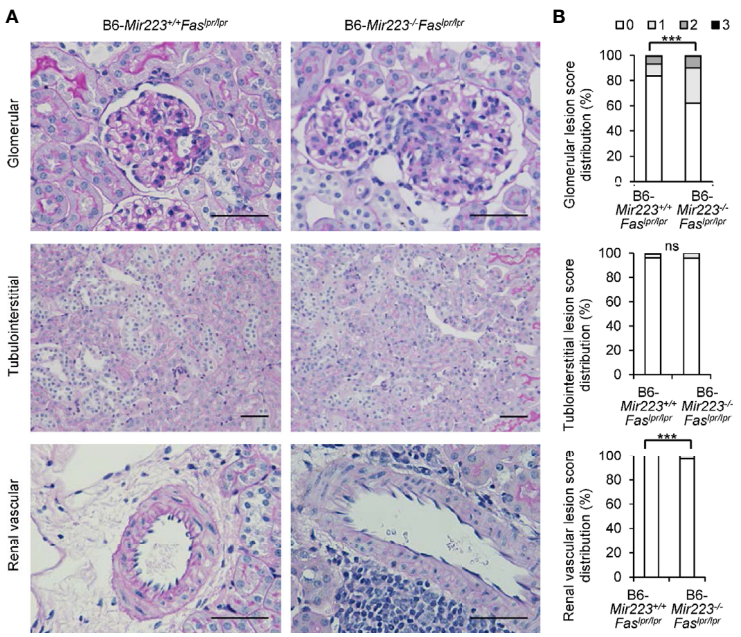


**FIGURE 5** | Exacerbation of lupus nephritis in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice. **(A)** Representative kidney sections from B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ and B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice, stained with periodic acid–Schiff (PAS). Bars = 50 μm. **(B)** Score distribution of glomerular, renal vascular and tubulointerstitial lesions in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ compared to B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (n = 10 per group). Data are presented as mean ± SEM. ***$p < 0.001$, ns, not significant, by Pearson's $\chi 2$ test in **(B)**.

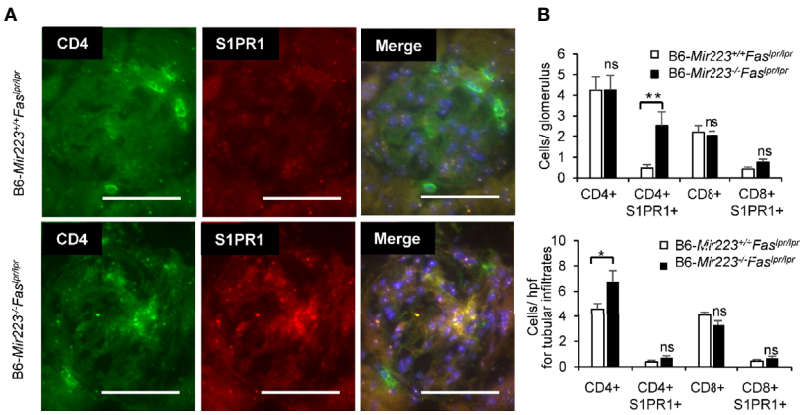


**FIGURE 6** | Glomerulonephritis with infiltration of CD4$^+$S1PR1$^+$ T cells in in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ **(A)** The image of CD4$^+$ cells, CD4$^+$S1PR1$^+$ cells, CD8$^+$ cells and CD8$^+$S1PR1$^+$ cells in glomerular lesion in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ compared to B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (n = 5 per group). Images are representative of five mice per group. Bars = 50 μm. **(B)** The number of CD4$^+$ cells, CD4$^+$S1PR1$^+$ cells, CD8$^+$ cells and CD8$^+$S1PR1$^+$ cells in glomerulus or tubular lesion in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ compared to B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (n = 5 per group). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ns, not significant, by Student's t-test in **(B)**.

and CXCL10 were expressed at high levels at early time points in the spleen and CCL-2, CCL4, CCL5, CXCL10 in nephritic kidneys of *Fas*$^{lpr/lpr}$ mice (38–41). T cell migration from blood into tissue is induced by chemokines CXCL9 and CXCL11 presented on the endothelial surface, which activates surface expression of S1PR1 and S1PR4 on T cells (42). The increased S1P present in inflamed peripheral tissues may further induce T cell retention. Therefore, we next evaluated the chemokine mRNA levels in renal cortex. In both B6 and MRL/lpr mice, *Cxcl10, Cxcl11 and Ccl2* demonstrated higher mRNA levels in *Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ compared with *Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$; however, it did not reach statistical differences (**Supplementary Figures 8D** and **11**). Although *Cxcl10* was one of the predicted target mRNAs of miR-223-3p from miRDB (http://mirdb.org.), the roles of chemokines in infiltration of S1PR1+CD4+ T cells in *Mir223*$^{-/-}$*Fas*$^{lpr/lp}$ may be limited.

Taken together, *Mir223* deficiency can exacerbate the functional and pathologic damage to the kidneys in B6-MRL-Fas$^{lpr}$ mice by facilitating the infiltration of S1PR1$^+$CD4$^+$ T cells in both glomerular and interstitial lesions.

## DISCUSSION

Recent studies have demonstrated that miRNAs play important roles in the pathogenesis of SLE (3). In pristine-induced lupus mice, lupus nephritis is ameliorated by miR-654 mimic injection therapy (43). *Mir155* knockout mice demonstrated the amelioration of autoimmune inflammation (5). miRNAs themselves might become therapeutic modalities in the SLE treatment, as seen in the cancer therapies (44). In the current investigation, we focused on *S1pr1* as a new target of miR-223-3p. In transgenic mice with the persistent expression of human S1PR1 in lymphocytes, the activated T cells demonstrated decreased entry into lymph nodes and increased entry into circulation (45). However, S1PR1 agonists have been shown to prevent T cell migration from lymph nodes into circulation and result in amelioration of the clinical features of SLE in model mice (18, 19, 21–23). After phosphorylation by sphingosine kinases, fingolimod, S1PR1 modulator, functionally antagonizes S1PR1 expressed on lymphocytes by receptor internalization and degradation. This process prevents a functionally normal response to the endogenous S1P gradient, thereby blocking lymphocytes to egress from secondary lymphoid organs to the blood and reducing the circulation of autoreactive lymphocytes (46). Therefore, S1PR1 dysfunction and overexpression in T cells are hypothesized to be linked to the pathogenesis of SLE.

In B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice, the population of splenic S1PR1$^+$CD4$^+$ T cells was significantly increased compared with B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice and thus S1PR1$^+$CD4$^+$ T cells were induced by *Mir223* deficiency. Although *S1pr1* mRNA and protein expression was not altered in isolated splenic CD4$^+$ T cells from B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice compared with B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice, S1PR1$^+$CD4$^+$ T cells were actively infiltrated into the glomeruli in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice. The results suggested that S1PR1$^+$CD4$^+$ T cells induced by *Mir223* deficiency preferentially migrated into spleen and inflamed kidney tissues. We postulate that *Mir223* is a new therapeutic target in the treatment of SLE by modulating the expression of S1PR1.

Since *Mir223* is highly expressed in granulocytes compared to T and B cells (24), we should consider the functional roles of *Mir223* in various immune-mediated cells in the translational research. *Mir223* is essential for innate immune responses and inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (IBD) (47, 48). *Mir223* is overexpressed in fibroblast-like synoviocytes, and synovial fluid of the patients with rheumatoid arthritis and lentivirus-mediated silencing of *Mir223* can suppress collagen-induced arthritis in mice by decreasing macrophage colony-stimulating factor receptor levels in the synovium (49). Of interest, in *Mir223* deficient mice, granulocyte numbers are increased, albeit with an abnormal phenotype, and spontaneously

develop inflammatory lung pathology due to hyperactivity of the neutrophils (24). Indeed, neutrophil numbers in peripheral blood tended to be higher in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice compared to B6-*Mir223*$^{+/+}$*Fas*$^{lpr/lpr}$ mice (**Supplementary Figure 2E**). Neutrophils in SLE have abnormal functions like reducing phagocytosis capabilities and apoptotic pathways and increasing oxidative activity and NETosis (50). These neutrophils infiltrate into kidney tissues affected by lupus nephritis (51). Although *Mir223* is not expressed in kidney cells and there were no renal damages in *Mir223* deficient mice (24), *Mir223* deficient neutrophils were hyperactive and assumed to contribute the glomerular injuries in *Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice. Overlapping abnormality in neutrophils by *Mir223* deficiency and SLE was thought to be one of the causes of exacerbation of lupus nephritis in our B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice.

In addition to the roles in the granulocytes, *Mir223* has been known to be functional particularly in CD4$^+$Th17 cells, which exacerbated the experimental autoimmune uveitis (EAU) by promoting autoreactive Th17 cell responses by inhibiting transcription factor FOXO3 expression (52). CD4$^+$Th17 cells were also shown to be increased in SLE patients and MRL/lpr mice (53, 54) and they infiltrate into the kidney tissues and contribute to tissue damage by producing IL-17 and IFN-$\gamma$ (55). CD4$^+$ T cells also infiltrate in kidney tissues and link to exacerbation of lupus nephritis (23). We initially hypothesized that *Mir223* deficiency may ameliorate lupus nephritis since *MIR223* is overexpressed in CD4$^+$ T cells in the patients with relapsing multiple sclerosis and rheumatoid arthritis (9, 56). However, *Mir223* deficiency exacerbated glomerulonephritis in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice by facilitating the infiltration of S1PR1$^+$CD4$^+$ T cells into kidney tissues.

Although the glomerular deposition of C3 was increased in B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice, there were no differences in the deposition of IgG (**Supplementary Figure 7B**). The complement system is composed of three major arms of activation pathways and plays protective and pathogenic roles in the development of SLE. The classical pathway (CP) contributes to the clearance of immune complexes and apoptotic cells, whereas the alternative pathway (AP) and lectin pathway (LP) in lupus exacerbates renal inflammation (57). Activation of LP is initiated by pattern recognition molecules (PRMs) without antibodies, and they are composed of mannose-binding lectin (MBL), ficolin, collectin-liver 10 (CL-10), and CL-11 (58). MBL-associated serine proteases-1 and -2 (MASP-1 and MASP-2) are the enzymatic constituents of the LP and form a complex with the PRMs. *Masp1* knockout lupus-prone MRL/*lpr* mice (*Masp1/3*–/– MRL/*lpr* mice) lacking both MASP-1 and its splicing isoform MASP-3 demonstrated reduced activation of LP and AP. In this model, there were no significant differences in glomerular IgG but significantly reduced glomerular C3 deposition compared to their wild-type littermates (57). In B6-*Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice, we can speculate that the infiltration of S1PR1$^+$CD4$^+$ T cells may induce the cellular damage of the glomeruli, the release of damage-associated molecular patterns (DAMPs) and subsequent activation of LP (59).

There are some limitations in the current investigation. First, we employed total *Mir223* knockout mice for the investigation. The CD4$^+$ T cell specific *Mir223* knockout mice is required to further confirm the findings observed in this study. In addition, we compared miRNA and mRNA profiling between B6 and MRL/lpr in the initial screening of the candidate miRNAs (**Figure 1**). MRL/MpJ mice are the parent and control strain for MRL/lpr, but we did not compare the expression of *Mir223* and *S1pr1* between these strains. The genetic background B6/lpr and MRL/lpr also influenced the phenotype of the Mir223 knockout mice. However, all other functional gene knockout studies were performed under identical genetic backgrounds such as B6 or MRL/lpr. Second, we did not employ S1PR1 antagonist to confirm the relationship between *S1pr1* and *Mir223*. S1PR1-5 antagonist (FTY720/fingolimod) is approved for multiple sclerosis (60), while selective S1PR1 antagonist (cenerimod) is currently under phase 2 clinical study (NCT-02472795) in the patients with SLE. Cenerimod has been reported to ameliorate systemic and organ-specific pathology and inflammation in MRL/lpr mice (61), and the administration of cenerimod into *Mir223*$^{-/-}$*Fas*$^{lpr/lpr}$ mice should be investigated in future studies.

In conclusion, we presented that the deletion of *Mir223* exacerbated the lupus phenotypes associated with increased population of S1PR1$^+$CD4$^+$ T cells and their enhanced infiltration in inflamed kidney tissues. In addition to the modulation of the function of S1PR1, *Mir223* may be a valid therapeutic modality in the treatment of SLE by targeting S1PR1$^+$CD4$^+$ T cells. We believe that the current investigation provides novel data pertaining to the T cell circulation in SLE.

## DATA AVAILABILITY STATEMENT

All raw and processed data are freely accessible in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE87219.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee, Okayama University Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Care and Use Committee of the Department of Animal Resources, Advanced Science Research Center, Okayama University.

## AUTHOR CONTRIBUTIONS

HA, SW, and JW designed and conceptualized the study. HA, SW, SZ, EK, TM, YM, and JW performed the experiments and were involved in the data acquisition. HA and JW analyzed and interpreted the data. 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.2020.616141/full#supplementary-material

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REVIEW
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# Current Insights and Future Prospects for Targeting IL-17 to Treat Patients With Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by immune cell abnormalities which lead to the production of autoantibodies and the deposition of immune complexes. Interleukin (IL)-17-producing cells play an important role in the pathogenesis of the disease, making them an attractive therapeutic target. Studies in lupus-prone mice and of *ex vivo* cells from patients with SLE humans have shown that IL-17 represents a promising therapeutic target. Here we review molecular mechanisms involved in IL-17 production and Th17 cell differentiation and function and an update on the role of IL-17 in autoimmune diseases and the expected usefulness for targeting IL-17 therapeutically.

**Keywords: T cells, systemic lupus erythematosus (SLE), lupus nephritis, immune responses, interleukin (IL)-17**

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## INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease characterized by the production of autoantibodies, the formation of immune complexes, and immune dysregulation, resulting in damage of multiple organs, including the skin and the kidneys (1, 2). The prognosis for SLE depends on the severity of the disease and the organs that are involved. Lupus nephritis (LN) is the most common and serious complication observed in the majority of patients with SLE. While the etiology of SLE remains largely unknown, genome-wide association studies have identified over 50 gene loci with variants that have been associated with a predisposition to SLE (3–5). These disease-susceptible genes for SLE include variants that have been implicated in aberrant expression of cytokines and abnormalities in innate and adaptive immunity.

Both B cells and T cells are important in the pathogenesis of SLE. Self-reactive B cells that produce autoantibodies are important in the pathogenesis of SLE. Increased plasma memory B cell subsets are associated with disease activity, and therapies targeting B cells have shown some clinical improvement (6). T cells also play a central role in the production of autoantibodies and the subsequent formation of immune complexes. Both B and T cells may act in concert to induce direct damage in multiple organs (7, 8).

CD4+ T helper cells (Th cells) are particularly important in the series of autoimmune responses associated with SLE. Th cells are defined by the cytokines they produce and have been classified into Th1, Th2, Th17, follicular helper T (Tfh) cells, and regulatory T (Treg) cells (7–9).

Th1 cells produce primarily interferon (IFN)-γ, which in turn activates cytotoxic T lymphocytes, macrophages, and natural killer cells. In contrast, Th2 cells produce mainly cytokines, such as interleukin (IL)-4 and activate B cells. The pathogenesis of autoimmune diseases, however, cannot be based on Th1 and Th2 immune responses alone. Th17 cells and Treg cells play important roles in the development of autoimmune-mediated tissue injury.

Th17 cells produce IL-17, IL-21, and IL-22, and they have been shown to be involved in the development of inflammation in various organs. Treg cells are characterized by the expression of FoxP3 and they produce TGF-β and IL-10, which actively terminate immune responses. Interestingly, there is an interrelationship between Th17 and Treg cells that may determine the ultimate outcome of the autoimmune response. Limited numbers and reduced functions of Treg cells have been observed in patients with SLE, and these defects have been associated with increased disease activity (3).

In this review, we discuss the evidence that T cell dysfunctions and IL-17 overproduction are associated with the development of SLE and disease progression in both humans and lupus-prone mice. We also describe recent advances in functional analysis, including analysis of the cell signaling pathways that contribute to increased IL-17 production. It is well understood that the imbalance between Th17 cells and Treg cells, along with IL-17-related cytokine-driven inflammation, plays an important role in autoantibody production and organ damage in SLE. We will also discuss recent advances in IL-17-targeted therapies for autoimmune diseases, including SLE, and their future prospects.

## THE ROLE OF INTERLEUKIN-17 AND INTERLEUKIN-17-RELATED CYTOKINES IN THE PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

The IL-17 family includes at least six (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F) proteins (9). Among these, IL-17A, which is mainly produced by Th17 cells, amplifies the production of inflammatory cytokines and chemokines and stimulates keratinocytes, synoviocytes, fibroblasts, macrophages, and neutrophils (10). Accordingly, it has the potential to promote the recruitment of inflammatory cells, such as monocytes and neutrophils, to the inflamed organ (11, 12). Although Th17 cells produce mainly the cytokine IL-17 (12), IL-17 is also produced by other subsets of T cells, including T cell receptor (TCR)γδ and TCRαβ double negative (DN) T cells ($CD3^+CD4^-CD8^-$), and a number of families of innate lymphoid cells, including ILC3, macrophages, and neutrophils (13–15).

IL-17A is an important cytokine that is involved in the pathogenesis of animal models of autoimmunity and human autoimmune diseases, including SLE (12, 16, 17). It has been demonstrated that patients with SLE not only have higher serum levels of IL-17A, but also have increased numbers of Th17 cells (18–20). It has also been shown that high serum levels of IL-17 at baseline predict poor histopathological outcomes after immunosuppressive therapy (21). Our group has proposed that DN T cells infiltrate the kidneys of patients with LN and are the major source of IL-17 (13). However, a study using lupus-prone mice demonstrated that pharmacological inhibition and genetic ablation of IL-17A did not improve clinical manifestations, including survival rate, glomerulonephritis, and autoantibody production (22). As mentioned above, Th17 cells not only produce IL-17, but also produce multiple pro-inflammatory cytokines, such as IL-21, IL-22, and TNF-α. Thus, the role of IL-17 that has been documented in other studies may not be due to IL-17 alone, but to the additional activity of Th17 cells. Therefore, studies in which Th17 cells, rather than IL-17 production alone, are involved in the pathogenesis of SLE need to formally address this issue.

IL-23 promotes signal transducer and transcriptional activator 3 (STAT3) phosphorylation by Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) by binding to its receptor IL-23R. It also enhances the expression of retinoic acid receptor-associated orphan receptor γt (RORγt), which is involved in the expression of IL-17 and other Th17 cytokines (23). Thus, IL-23 has been shown to be important in the development of various autoimmune diseases in murine models (24–26) and in humans (27) by promoting Th17 cell–mediated tissue inflammation. Our group has shown that the clinical and pathological findings of LN are mitigated in lupus-prone mice with IL-23 receptor deficiency (28) or treated with anti-IL23 antibodies (29). Evidence for the importance of IL-23 in SLE is further supported by the elevated serum IL-23 and IL-23 expression in renal tissues in patients with SLE (21, 30, 31).

Low-density granulocytes, a subpopulation of neutrophils prone to cell death, have been found to contribute to the pathogenesis of SLE through the neutrophil extracellular trap formation (NETosis) process, which includes the release of intracellular material into the surrounding environment (32, 33). IL-17 plays a role in inducing the recruitment of neutrophils and other immune cells by targeting tissues to promote and maintain the inflammatory process. In addition, IL-17 has been demonstrated to induce NETosis in animals prone to lupus (34). Indeed, the cellular debris released by these cells induces activation of the type I IFN pathway by plasmacytoid dendritic cells, which eventually leads to the aberrant activation of T and B cells (35, 36). This consequently perpetuates the inflammatory process characteristic of SLE.

## MOLECULAR MECHANISMS THAT REGULATE INTERLEUKIN-17 IN SYSTEMIC LUPUS ERYTHEMATOSUS

$CD4^+$ T cell dysfunction contributes to the development and progression of organ damage, including LN in lupus-prone mice,

such as MRL/lpr, NZB/NZW, and BXSB mice, and SLE patients (37, 38). Molecules involved in the aberrant expression of IL-17 cytokines and distortion of Th cell differentiation include protein phosphatase 2A (PP2A), calcium/calmodulin kinase IV (CaMK4), CREM, Rho-associated protein kinase (ROCK), and mammalian target rapamycin complex 1 (mTORC1).

## Protein Phosphatase 2A

PP2A is a multifunctional serine/threonine phosphatase that is involved in multiple cellular processes. It is composed of three distinct subunits: the scaffold A subunit (PP2Aa), the regulatory B subunit (PP2Ab), and the catalytic C subunit (PP2Ac).

A previous study carried out by our group showed that transgenic mice that overexpressed PP2Ac in T cells developed glomerulonephritis, which included increased production of IL-17A and IL-17F (39). Consistent with these observations, it has been demonstrated that PP2Ac expression and activity are increased in the T cells of SLE patients, which contributes to a decrease in IL-2 production (40, 41). In addition, Treg cell-specific ablation of the PP2A causes multi-organ lymphoproliferative autoimmune diseases due to defective dephosphorylation of mTORC1 (42).

Recently, our group has shown that PPP2R2D, a regulatory subunit of PP2A, is increased in T cells from SLE patients. Mice lacking this subunit in T cells have less autoimmunity and PPP2R2D negatively regulates IL-2 production in conventional T cells by regulating the chromatin opening of the *IL-2* gene (43).

PP2A is a ubiquitously expressed enzyme. It also has diverse effects on immune cells. Therefore, the use of PP2A inhibitors to treat patients with SLE requires the use of a T cell-targeted delivery system to mitigate off-target effects.

## Rho-Associated Protein Kinase

ROCK is a serine-threonine kinase and its activity is primarily controlled by the binding of activated RhoA (44). ROCK is involved in regulating cell migration, including that of T cells (45). ROCK2 has been suggested to facilitate the activity of interferon regulatory factor 4 (IRF4), which is required for Th17 differentiation and the production of IL-17 and IL-21 (46). PP2Ac in T cells has also been shown to be involved in IL-17 production *via* promotion of the RhoA-ROCK-IRF4 pathway (47). A study showed that ROCK activity levels were significantly higher in SLE patients than in healthy controls and the inhibition of the RhoA-ROCK pathway suppressed the production of IL-17 and IL-21 by Th17 cells (48).

ROCK2 has also been shown to be a major ROCK isoform that is involved in the differentiation of Th17 cells generated under Th17 cell skewing conditions. Therefore, targeting of this pathway can be achieved by both selective and non-selective inhibitors. A better understanding of the functional relevance of the ROCK1-dependent pathway in immune cells and an evaluation of the pattern of ROCK expression in individual SLE patients is required to determine whether ROCK2-selective inhibitors provide a more favorable risk-benefit profile than the broader ROCK inhibitors.

## CREM

CREM is a member of the ATF/CREB-type bZip transcription factor family. It binds to cAMP response elements during cellular processes, including T cell activation. Therefore, CREM plays an important role in the adaptive immune process. Importantly, CREMα functions as a transcriptional regulator of molecules associated with cytokine expression and T cell differentiation in T cells of SLE patients.

Previous studies have demonstrated that mice overexpressing CREMα in T cells have increased IL-17 production and lupus-like disease (49). Mechanistically, CREMα was found to bind to the *IL17* promoter and non-coding conserved areas of the *IL17* locus and enhance its activity at the epigenetic level (50, 51). Consistent with the results obtained in mice, T cells from SLE patients have been found to have increased levels of CREMα and aberrant IL-17A expression (51). In addition, CREMα was found to be essential for expansion of DN T cells due to epigenetic regulation of the *CD8* locus cells in SLE patients and lupus-prone mice (52, 53). In summary, reduced levels of CREMα can suppress the production of IL-17 and reduce the pool of pathogenic DN T cells, which suggests its potential as a disease biomarker and therapeutic target in SLE.

The splice variant of CREM inducible cAMP early repressor (ICER) also has a crucial role in T cell activation, Th cell differentiation, and cytokine production (54). Experiments involving mice have demonstrated that ICER/CREM is required for the development of organ-specific autoimmunity and systemic autoimmunity and ICER is upregulated in CD4+ T cells from SLE patients (55).

Therefore, CREM and CREM-associated molecules may represent potential therapeutic targets for SLE. However, as with PP2A, the CREM family of proteins has an enormous diversity and the development of small molecule compounds that target only specific subunits or splice variants may pose many challenges.

## Calcium/Calmodulin Kinase IV

Calcium/calmodulin-dependent protein kinases (CaMKs) are enzymes that are activated by calcium. CaMK2 and CaMK4, which are multifunctional CaMKs with multiple substrates, play important roles in the immune response, including T cell activation (56, 57) and T cell development (58, 59). CaMK4 is a multifunctional serine/threonine kinase that regulates pro-inflammatory cytokines and cell proliferation-related gene expression by activating a number of transcription factors, including CREB (cAMP response element binding protein) and CREM (60).

CaMK4 is abnormally increased in T cells from SLE patients (61) and lupus-prone mice (62). CaMK4 is rarely expressed in B cells or other immune cells. Among T cells, CaMK4 expression is enhanced in CD4-positive T cells, and it is preferentially induced during Th17 differentiation (63). In line with these findings, genetic or pharmacological inhibition of CaMK4 in MRL/lpr mice resulted in a reduced frequency of IL-17-producing T cells, including CD4+ and DN T cells, a significant reduction in autoantibody production, and improved nephritis (62, 64). As a mechanism to counteract organ damage, we demonstrated that CaMK4 inhibition limits cell infiltration by increasing Treg cells locally in the kidney (65). Inhibition of CaMK4 suppress the CCR6/CCL20 axis which is important for the entry of Th17 cells

to tissues (66). Moreover, we recently discovered that GLUT1-mediated glycolysis is important for the expression of IL-17 induced by CaMK4 (67).

Thus, targeting CaMK4 represents a potential therapeutic strategy for patients with SLE because of its ability to promote differentiation into Th17 cells. However, since CaMK4 is also upregulated in critical organs such as the brain and gonads, research on CD4-targeted therapy using nanolipogels (68), development of CaMK4-specific inhibitors, and verification of their safety must be conducted before further clinical applications are carried out in humans.

## Mammalian Target Rapamycin Complex 1

mTORC1 is a serine-threonine kinase that functions as a regulator of cellular metabolism, including mitochondrial oxidative stress, glycolysis (69), and cell proliferation (70). Rapamycin, an mTORC1 inhibitor, suppressed glomerulonephritis in lupus-prone mice (71) by suppressing the Th17/Treg cell ratio (72). Its molecular signaling mechanism has been suggested to be linked to CaMK4 (63), ROCK (73), and the splicing factor SRSF1 (74). Furthermore, recent studies have shown that rapamycin reverses Th17 cell proliferation in SLE patients (75, 76). Importantly, glutaminolysis has been shown to be essential for mTORC activation, and Th17 cells are more dependent on glutaminolysis than Th1, Th2, and Treg cells (77). Glutaminase 1 inhibitors improve disease activity and there are fewer IL-17A–producing T cells in the kidneys of MRL/*lpr* mice (78).

Activation of the mTOR pathway is important in the development of SLE (79, 80). This allows mTOR to be a therapeutic target for SLE. A single-arm, open-label, phase 1 and 2 study of the mTOR inhibitor sirolimus showed efficacy in patients with active SLE (81). In summary, TORC1 inhibition has also shown several clinical benefits in patients with SLE.

**Figure 1** summarizes evidence that abnormal T-cell signaling leads to overproduction of IL-17 in SLE, which in turn activates immune and other cells, leading to autoantibody production and proinflammatory cytokine production, resulting in organ damage.

# TARGETING INTERLEUKIN-17 THERAPY IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

Several IL-17A blockers, including the anti-IL-17A monoclonal antibodies secukinumab, ixekizumab, and bimekizumab, and the anti-17RA monoclonal antibody brodalumab, are approved for some immune-mediated inflammatory diseases, such as psoriasis (82–84), psoriatic arthritis (85, 86), and ankylosing spondylitis (87, 88). Although a case report described the efficacy of IL-17A inhibitor in a SLE patient (89), clinical trials are warranted to evaluate the long-term efficacy and safety of IL-17 inhibitors in SLE patients.

Several issues should be considered in the development of IL-17-directed therapy for SLE. First, IL-17A blockers are already used in clinical practice for inflammatory diseases, but their

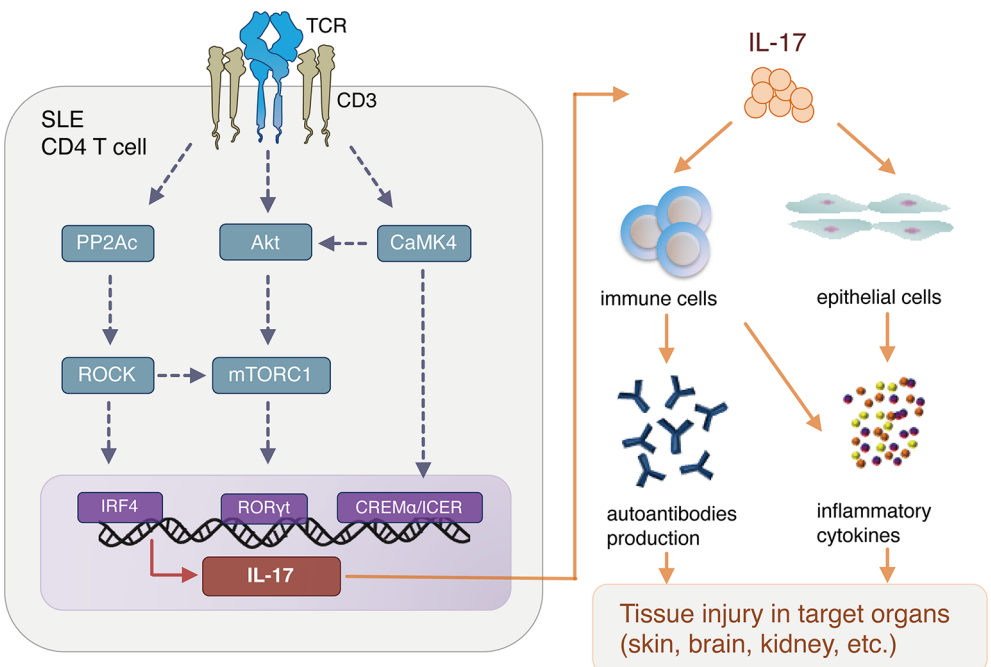


**FIGURE 1** | Aberrant T cell signaling and interleukin (IL)-17 production in SLE pathogenesis. SLE, systemic lupus erythematosus; TCR, T cell receptor; PP2Ac, Protein phosphatase 2A catalytic subunit; CaMK4, Calcium/calmodulin-dependent protein kinase IV; ROCK, Rho-associated protein kinase; CREB, cAMP response element binding protein; IRF4, interferon regulatory factor 4; CREM, cAMP response element modulator; mTORC1, mammalian target of rapamycin complex 1.

long-term safety and efficacy have not been established. Second, anti-IL-17A drugs have been shown to be therapeutically effective in lupus-prone mice, but human studies are needed to determine the exact role of IL-17 in human SLE. Finally, because SLE is a highly heterogeneous autoimmune disease, IL-17 blockade may not be suitable for all patients. The potential beneficial effects of IL-17 blockers may be limited to a subset of SLE patients whose disease is driven by the IL-17 pathway. Therefore, it is important to identify biomarkers that can be used in patient screening to identify those who have the best chance to respond to treatment with IL-17 pathway-directed biologics.

Clinical studies have demonstrated the efficacy and safety of ustekinumab, an anti- IL-12/23 p40 neutralizing monoclonal antibody, in patients with subacute cutaneous lupus (90), psoriasis (91), and psoriatic arthritis (92). More recently, a double-blind phase II study has demonstrated impressive efficacy and safety of ustekinumab when used in patients with active SLE (93). An ancillary study to this trial revealed that persistent reductions in IFN-γ serum protein levels, rather than changes in serum IL-17A, IL-17F, and IL-22 levels, were associated with treatment responses (94).

## CONCLUSION AND FUTURE PERSPECTIVES

In this review, we report recent advances in our understanding of the role of IL-17 and IL-17-related molecules in SLE and their clinical implications. There is sufficient evidence that Th17 and one of their main effector molecules, IL-17, contribute to the development of immunopathology in patients and mice with lupus.

It is true that many biologics have been tried in patients with SLE and the vast majority of them have failed to produce a statistically significant effect admissible by the regulatory agencies even if phase II studies had indicated high promise. Obviously, each biologic accomplishes the expected biologic effect, that is, to neutralize a cytokine or kill a cell, and therefore, the blame should be directed to the design of the clinical trials.

Although this is not the place to argue about clinical trial design in SLE, we believe that the failure of the trials is primarily due to the pathogenetic heterogeneity of the disease (36). Heterogeneity implies that each patient or subgroups of patients share a targeted mechanism. Therefore, a distinct subgroup of patients always responds in each trial. It becomes obvious, that there are only a few logical routes to take to success.

Define *a priori* the subgroup of patients in whom the targeted pathway is driving disease and enroll only those. This represents the exercise of personalized or precision medicine which is long overdue in patients with SLE. Alternatively, administer to all patients more than one biologics simultaneously hoping that a larger number of patients will respond. This approach may be stymied by an increased number of side effects.

It is expected that soon a few more biologics will be approved for SLE including the calcineurin inhibitor voclosporin and the IFN blocker anifrolimumab at which point drugs will be prescribed serially to patients with SLE after each one of them fails. This has been the practice in some ways with patients with rheumatoid arthritis and other autoinflammatory diseases.

## AUTHOR CONTRIBUTIONS

KI, AK, and GT reviewed and edited the manuscript. TK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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# Amino Acid Metabolism in Lupus

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T cell metabolism is central to cell proliferation, survival, differentiation, and aberrations have been linked to the pathophysiology of systemic autoimmune diseases. Besides glycolysis and fatty acid oxidation/synthesis, amino acid metabolism is also crucial in T cell metabolism. It appears that each T cell subset favors a unique metabolic process and that metabolic reprogramming changes cell fate. Here, we review the mechanisms whereby amino acid transport and metabolism affects T cell activation, differentiation and function in T cells in the prototype systemic autoimmune disease systemic lupus erythematosus. New insights in amino acid handling by T cells should guide approaches to correct T cell abnormalities and disease pathology.

**Keywords: cell metabolism, amino acid, T cell, systemic lupus erythematosus, amino acid transporters**

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## INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by autoantibody production, immune complex deposition, tissue inflammation and damage of multiple organs (1). SLE can affect practically all organs, including skin, kidney, and central nerve system (2–4). The etiology of SLE is multifactorial and includes contributions from genetic, environmental, hormonal and epigenetic factors (2). These factors, acting serially or simultaneously, lead to generalize breakdown of tolerance to self-antigens, which results in autoantibody production and tissue inflammation (5). T cells have a vital role in the pathogenesis of SLE. Many subsets of T cells, especially Th1, Th17, regulatory T (Treg) cells, and double-negative ($CD4^-CD8^-$) T cells, are involved through distinct mechanisms in the development of organ inflammation in SLE (6). Since helper T cells can activate B cells to secrete antibodies, which are also involved in the lupus pathogenesis, T cells have earned claim as main therapeutic targets in patients with SLE (7).

Recent studies have shown that the differentiation and function of each T cell subset is controlled by intracellular metabolic processes (8–10). Cell metabolism operates mainly through glycolysis, fatty acid oxidation and amino acid metabolism including glutaminolysis (8–11). Amino acids are classified as essential (leucine, isoleucine, lysine, histidine, valine, threonine, phenylalanine, tryptophan, and methionine), conditionally essential (glutamine, arginine, cysteine, glycine, proline, and tyrosine), or non-essential (alanine, glutamate, serine, asparagine, and aspartate) (12). Essential amino acids cannot be synthesized within the body and must be supplied through dietary intake. Amino acid metabolism is used in many processes that are involved in cell proliferation, growth and cell function. Furthermore, amino acids are also critical for the biosynthesis of nucleotides (13). It has been documented that some amino acids such as leucine, methionine, glutamine, arginine, and alanine, are more essential than other amino acids during T cell activation and expansion or in determining distinct T cell fates (14, 15). The importance of glycolysis, and fatty acid oxidation/synthesis in lupus T cells has been extensively reviewed

elsewhere (8–10, 16–18). Here we summarize amino acid metabolism in mice and people with SLE with a focus on T cells.

## AMINO ACID TRANSPORTERS

Amino acid transporters are important in transporting amino acids from the environment into the cell (19). T-cell receptor (TCR) stimulation triggers dramatic metabolic changes including increased glycolysis, pentose phosphate pathway activity, and glutaminolysis (19, 20). SLC7A5, known as large neutral amino acid transporter 1 (LAT-1), is a transporter dedicated to the transport of essential amino acids (21). SLC3A2, also known as CD98, is a transmembrane protein, which chaperones amino-acid transporters, including SLC7A5 SLC7A6, SLC7A7, SLC7A8, SLC7A10, and SLC7A11 (12), and enables them to execute their function. The LAT-1/CD98 heterodimer transports large hydrophobic amino acids, including the seven essential amino acids leucine, isoleucine, histidine, valine, phenylalanine, tryptophan, and methionine. Notably, the expression of LAT-1 and CD98 in T cells is induced after activation (19). *Slc7a5*$^{-/-}$ CD4$^{+}$ T cells cannot respond to antigen, undergo clonal expansion or effector cell differentiation. Although *Slc7a5*$^{-/-}$ CD4$^{+}$ T cells do not differentiate into Th1 and Th17 cells, differentiation into iTreg is not affected (22). LAT-1 deletion or inhibition blocks the expansion of IL-17 secreting γδ and CD4$^{+}$ T cells in both human cells and imiquimod (a TLR7 agonist)-induced lupus and psoriasis-like animal models (**Figure 1**). The heterodimer comprising CD98 and SLC7A7 transports among other amino acids lysine, arginine, methionine, leucine, alanine, and cysteine (12). Interestingly, whole-exome sequencing in patients with childhood-onset SLE identified a *SLC7A7* mutation to be linked to disease expression (23).

Alanine is also important in T cell activation. It is transported through SLC38A1 in CD4$^{+}$ T cells and TCR stimulation induces its expression (12). Alanine deprivation impairs naïve and memory T cell activations, but it does not affect T cell effector functions (24). Although alanine can be made from pyruvate by a single transamination, extracellular alanine is used mainly for protein synthesis (12, 24).

Glutamine is the most abundant amino acid in the serum (25, 26). T cell stimulation promotes a rapid increase of glutamine uptake and activated T cells need more glutamine than naïve T cells (27). SLC1A5, known as alanine-serine-cysteine transporter 2 (ASCT2), is a transporter of neutral amino acids including glutamine (28). Although *Slc1a5*$^{-/-}$ CD4$^{+}$ T cells do not affect

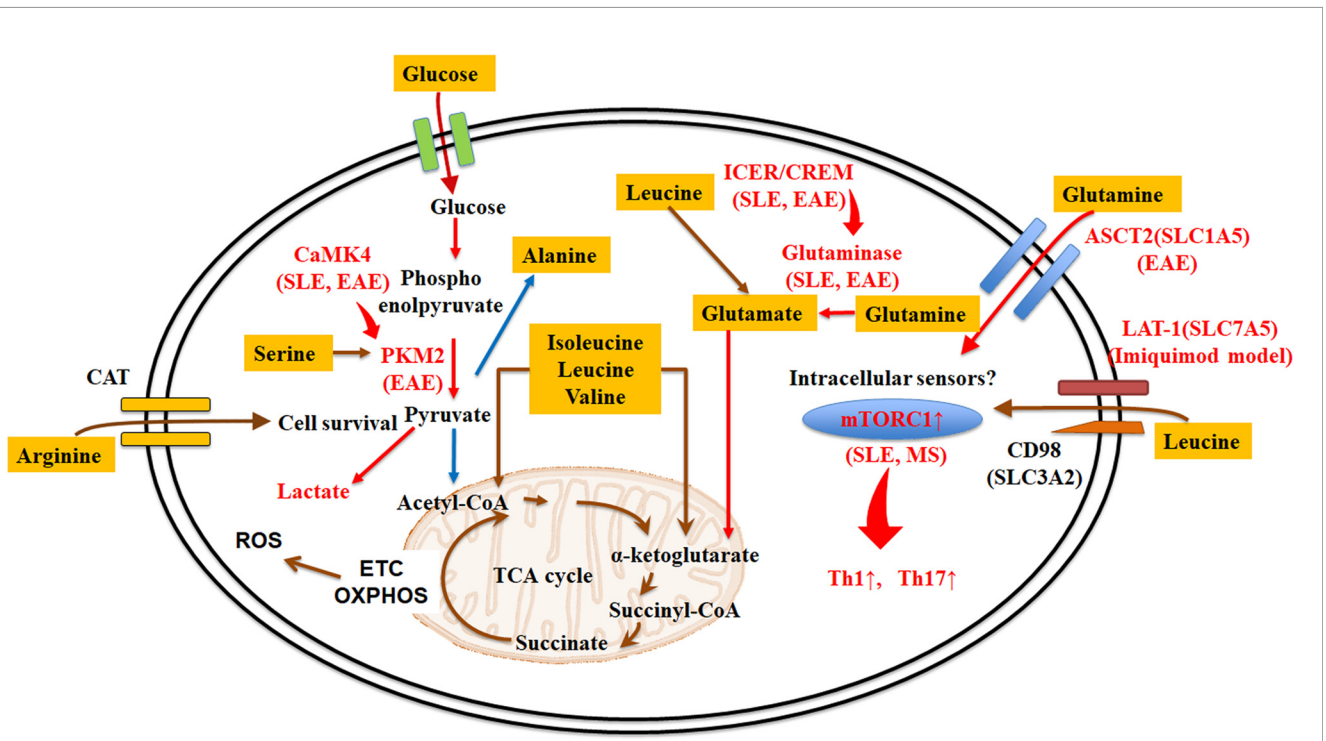


**FIGURE 1** | Amino acid transporters and metabolism in lupus T cells. Amino acid acquisition is crucial for cell function. Amino acid transporters play central roles in acquiring amino acids from the external environment. Some amino acids (e.g. leucine, methionine, glutamine, arginine, and alanine) are more essential than other amino acids in during T cell activation and expansion, or in determining different T cell fates in autoimmune diseases. Red arrows or letters indicate "enhance or active", whereas blue arrows indicate "inhibit or inactivate". ASCT2, alanine-serine-cysteine transporter 2; CaMK4, calcium/calmodulin–dependent protein kinase IV; CAT, cationic amino acid transporters; CREM, cAMP response element modulator; EAE, experimental autoimmune encephalomyelitis; ETC, electron transport chain; ICER, inducible cAMP early repressor; LAT-1, large neutral amino acid transporter 1; mTORC, mammalian target of rapamycin complex; OXPHOS, oxidative phosphorylation; PKM2, pyruvate kinase muscle isozyme 2; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; TCA cycle, tricarboxylic acid cycle.

TCR-mediated activation, deletion of *Slc1a5* impaired Th1 and Th17 cell differentiation (**Figure 1**) (27).

Arginine is transported through cationic amino acid transporters (CAT) (29), which are shared by lysine and ornithine. Elevation of arginine levels induces metabolic changes including a shift from glycolysis to oxidative phosphorylation in activated T cells and promotes the generation of central memory-like cells (30). Arginine and the transporter CAT-1 (SLC7A1) are also requisite for human T cell survival (31).

These findings demonstrate distinct roles for amino acid transporters in TCR/CD3-mediated T cell stimulation, differentiation, and function and indicate that manipulation of these transporters could serve therapeutic approaches for autoimmune diseases including SLE (**Figure 1**). Because several other amino acid transporters have not been studied carefully in T cells, further research is needed.

## AMINO ACID SENSORS

Although multiple mechanisms are involved in sensing amino acids within the intracellular space, it has been well established that the presence or absence of amino acids is sensed by distinct signaling pathways which involve the mechanistic target of rapamycin (mTOR) or the general control nonderepressible 2 (GCN2) (32, 33).

mTOR activity is regulated by amino acid availability, energy levels, and growth factors (34). In mammalian cells mTOR forms two distinct complexes: the mTORC complex 1 (mTORC1) and mTORC2. In fact, mTORC1 senses various stress signals, including the accumulation of amino acids such as leucine, isoleucine, kynurenine, and glutamine (35, 36). Glutamine activates mTORC1 *via* its metabolic product α-ketoglutarate which is generated during glutaminolysis (37). Inhibition of the first enzyme of glutaminolysis, glutaminase 1, reduces the activity of mTORC1 under Th17-polarized conditions (38). mTORC activity is enhanced in Th17 cells and IL-4-producing double negative T cells resulting in the proinflammatory profile recorded in patients with SLE (39). During Th17 cell differentiation, mTOR is required for the induction of hypoxia-inducible factor 1α (HIF1α) which enhances glycolysis (40). In Th1 and Th17 cells, mTORC1 activity, and glycolysis are increased compared with Tregs and Tfh cells (40, 41). Sirolimus, a mTOR inhibitor, was reported to improve disease activity in patients with refractory SLE in a single-arm, open-label, phase I/II trial (42), and other non-randomized controlled studies have reported that sirolimus is efficacious in patients with SLE (**Table 1**) (43). Sirolimus normalized Th17/Treg balance and TCR-induced $Ca^{2+}$ fluxing in patients with SLE (44, 45). Besides the effect on T cells, inhibition of mTOR in plasmacytoid dendritic cells reduced the production of type I interferons (58) and B cell stimulating factor BAFF-mediated B cell activation (59, 60). These results indicate that sirolimus can modify T, B, and plasmacytoid dendritic cell function (46). Further randomized controlled trials are needed to prove the efficacy and record the side effects of sirolimus in patients with SLE (47).

GCN2, a serine/threonine-protein kinase, also senses amino acid starvation by detecting uncharged transfer RNA (33, 61). It plays a vital role in the control of amino acid metabolism as a response to nutrient deprivation. *Gcn2* deficiency significantly inhibits *in vitro* differentiation of Th9 cells but not Th1, Th2, and Treg cells in mouse model, and it ameliorated allergic airway inflammation in mice (62). On the other hand, myeloid cell deletion of *Gcn2* in lupus-prone mice resulted in increased immune cell activation, humoral autoimmunity, renal pathology, and mortality (63). These results suggest that therapeutic inhibition of GCN2 should not be considered to treat SLE.

## GLUTAMINE METABOLISM

Glutaminolysis has a vital role in energy production in proliferating cells including T cells. Because of the indispensable roles of glutaminolysis in the generation of pro-inflammatory effector T cells Th1 and Th17 cells, enzymes involved in glutaminolysis have been studied extensively.

Glutaminase, in charge of converting glutamine to glutamate, promotes Th17 cells through distinct mechanisms (38, 49). Glutaminase expression is controlled by the transcription factor inducible cAMP early repressor (ICER)/cAMP response element modulator (CREM) (38), which is known to be overexpressed in T cells both from patients with SLE or MRL/*lpr* lupus-prone mice (64, 65). The glutaminase 1 inhibitor Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) reduces Th17 cell differentiation and disease activity in animals subjected to experimental autoimmune encephalomyelitis (EAE) (38). BPTES also ameliorates disease activity in MRL/*lpr* mice (50). Glutamate oxaloacetate transaminase 1 (GOT1), which converts glutamate to α-ketoglutarate, an intermediate of the TCA cycle, also contributes to enhance Th17 cell differentiation through epigenetic processes (51). Selective inhibition of GOT1 with aminooxy acetic acid (AOA) treatment or short hairpin RNA (shRNA) silencing markedly decreased Th17 differentiation of murine T cells (51). Systemic AOA treatment or adoptive transfer of *Got1* knockdown Th17-polarized T cells ameliorated EAE (51). Furthermore, inhibition of glutaminolysis with the glutamine analog 6-Diazo-5-oxo-L-norleucine (DON) reduces the frequency of Tfh cells, exogenous antigen-specific germinal center responses, and the production of dsDNA antibody in lupus-prone B6.*Sle1.Sle2.Sle3* mice after T cell-dependent immunization (52).

## BRANCHED-CHAIN AMINO ACID METABOLISM

The branched-chain amino acids (BCAAs) include leucine, isoleucine, and valine. As the most abundant of essential amino acids, BCAAs are not only the substrates for synthesis of nitrogenous compounds, but they also serve as signaling molecules regulating the metabolism of glucose, lipid, and

**TABLE 1** | Tentative therapeutic targets identified in studies of amino acid metabolism in T cells.

| Therapeutic target | Therapy | Effect on T cells | Effects on lupus | References |
|---|---|---|---|---|
| **Amino acid transporters** | | | | |
| LAT-1(SLC7A5)/CD98(SLC3A2) | JPH203 | Cannot respond to antigen, undergo clonal expansion or effector differentiation | Unknown | (12, 19, 21, 22) |
| (Transporter for Leu, Ile, His, Val, Phe, Trp, Met, and Tyr) | | Reduces Th1 and Th17 cell differentiation | | |
| ASCT2(SLC1A5) | V-9302 | Reduces Th1 and Th17 cell differentiation | Unknown | (27, 28) |
| (Transporter for Gln, Ala, Ser, Cys, Asp, and Thy) | GPNA | | | |
| CAT-1 (SLC7A1) | NEM | Requisite for T cell survival | Unknown | (29, 30) |
| (Transporter for Arg, Lys, and Orn) | | | | |
| **Amino acid sensors** | | | | |
| mTOR signaling | Sirolimus* | Inhibits Th17 cell differentiation<br>Promotes Treg cell differentiation | Reduces disease activity (mouse and human) | (39–48) |
| **Amino acid metabolism** | | | | |
| **Glutamine metabolism** | | | | |
| Glutaminase 1 | BPTES | | Reduces disease activity | (38, 49, 50) |
| | CB-839, 968 | Reduces Th17 cell differentiation | Improve kidney disease (mouse) | |
| GOT1 | AOA | Reduces Th17 cell differentiation | Unknown | (51) |
| Glutaminolysis | DON | Reduces the frequency of Tfh cells | Reduces dsDNA antibody production (mouse) | (52) |
| **Cysteine metabolism** | NAC* | Inhibits mTOR activity | Reduces disease activity<br>Improve kidney disease (mouse and human) | (53–57) |

*LAT-1, large neutral amino acid transporter 1; ASCT2, alanine-serine-cysteine transporter 2; CAT, cationic amino acid transporters; mTORC, mammalian target of rapamycin complex; GPNA, L-γ-glutamyl-p-nitroanilide; NEM, N-ethylmaleimide; GOT-1, glutamate oxaloacetate transaminase 1; AOA, (aminooxy)acetic acid; DON, 6-Diazo-5-oxo-L-norleucine; NAC, N-acetyl cysteine; Leu, leucine; Ile, isoleucine; His, histidine; Val, valine; Phe, phenylalanine; Trp, tryptophan; Met, methionine; Tyr, tyrosine, Gln, glutamine; Ala, alanine, Ser, serine; Cys, cysteine; Asp, asparagine, Thr, threonine; Arg, arginine Lys; lysine, Orn, Ornithine. *; Clinical trials of these therapies are ongoing.*

protein synthesis, intestinal health, and immunity through special signaling networks, especially the phosphoinositide 3-kinase/protein kinase B/mTOR (PI3K/AKT/mTOR) signal pathway. The leucine antagonist *N*-acetyl-leucine amide (NALA) inhibits mTORC1 activity and T cells function, impairs IL-2 and IFNγ production in *in vitro* Th1 polarized murine T cells (66). Leucine is also essential for Treg cell function. Leucine promotes mTORC1 activity in Treg cells *via* the small G proteins RagA/B and Rheb1/2 to drive their suppressive activity by inducing the expression of inducible T cell costimulator (ICOS) and CTLA4. Mice bearing RagA-RagB- or Rheb1-Rheb2-deficient Treg cells developed a *Scurfy*-like autoimmune disease and have reduced effector Treg cell accumulation and function (48).

Unlike most other essential amino acids, BCAAs catabolism is initially catalyzed either by transamination by branched-chain amino acid aminotransferases (BCAT) or decarboxylation by branched-chain α-keto acid dehydrogenase enzyme complex (BCKDC). After these reactions BCAA metabolites are further converted to acetyl-CoA and succinyl-CoA and participate in the TCA cycle (67). In $CD4^+$ T cells, BCAT negatively regulates mTOR and glycolysis. Activated T cells from cytosolic branched chain aminotransferase (BCATc)-deficient mice show increased mTORC1 activation compared to T cells from control mice. Furthermore, T cells from *Bcatc*$^{-/-}$ mice display higher rates of glycolysis (68). In another study, the oral administration of a leucine analogue, ERG240, selectively inhibited the activity of BCAT1, reduced the severity of collagen-induced arthritis in mice, and crescentic glomerulonephritis in rats (69).

## SERINE METABOLISM

Serine is used in proliferating cells for protein synthesis as well as the synthesis of other amino acids, such as glycine and cysteine (70). Serine-derived glycine is used in nucleotide synthesis. Moreover, serine is also a precursor for the synthesis of lipids, such as phosphatidylserine and sphingolipids, which have central roles in apoptotic cell clearance and immune cell activation, respectively (71, 72). A key molecule which is associated with serine is the M2 isoform of pyruvate kinase (PKM2) because it ligates and allosterically activates its activity (73). Even in the absence of exogenous serine, PKM2 expression contributes to endogenous serine synthesis and to the maintenance of mTORC1 activity (74).

Upon T cell activation, upregulated enzymes of the serine, glycine, one-carbon (SGOC) metabolic network, increase processing of serine into one-carbon metabolism. Extracellular serine is required for optimal T cell proliferation both *in vitro* and *in vivo*. Shortage of dietary serine impairs pathogen-driven expansion of T cells *in vivo*. Serine supplies glycine and one-carbon units for *de novo* nucleotide biosynthesis in proliferating T cells, and one-carbon units from formate can rescue T cells from serine deprivation (75).

We previously reported that calcium/calmodulin–dependent protein kinase IV (CaMK4) binds to PKM2 and promotes pyruvate kinase activity. Activated PKM2 is requisite for the Th1 and Th17 differentiation (76). Because inhibition of CaMK4 ameliorates pathogenesis of SLE though a Th17 cell manner (77,

78), the serine/PKM2 metabolism axis represents a hub of abnormal T cells in autoimmunity and needs further attention.

Serine also supports mitochondrial metabolism. In Jurkat cells, the catabolic enzyme serine hydroxymethyltransferase (SHMT2) is required for mitochondrial and respiratory activity (79). It has been also shown that SHMT2 promotes inflammatory cytokine signaling, including that of type I interferons, by interacting with the deubiquitylating BRCC36 isopeptidase complex (BRISC) (80). Since it has been recently shown that an inactive form of SHMT2 dimer has the capacity to bind and inhibit BRISC (80), control of the SHMT2-BRISC interaction may represent a new target to control autoimmune diseases.

## GLUTATHIONE/CYSTEINE METABOLISM

Glutathione is made from three amino acids: cysteine, glutamate, and glycine. Glutathione is important in the antioxidant defense, nutrient metabolism, and regulation of cellular events including gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production and protein glutathionylation (81). Glutathione reduces intracellular reactive oxygen species (ROS) levels and inhibits Th17 cell differentiation (49, 82). Glutathione is reported to be decreased in the peripheral blood of patients with SLE (83). Glutathione regulates the elevation of mitochondrial transmembrane potential, which in turn activates mTOR in T cells from patients with SLE (53, 84). To date, N-acetylcysteine (NAC) has been used to correct glutathione levels because NAC is the cell-permeable precursor of cysteine which is the rate-limiting constituent of *de novo* reduced glutathione (53–55). Administration of NAC improves lupus disease activity and ameliorates organ damage mainly by blocking the mTOR pathway in T cells in humans and mice with SLE (56, 57).

Because cysteine contains sulfur, cysteine supports sulfur-dependent metabolism. As discussed above, cysteine is a key amino acid for glutathione function, as it supplies the sulfur necessary for the formation of the disulfide bridge in the glutathione disulfide (13), but its roles extend beyond glutathione synthesis. In humans, naïve T cells express none or very low levels of cystine and cysteine transporters. Thus, early T cells activation does not require cystine and cysteine. However, upon activation, T cells rapidly upregulate the expression of cystine and cysteine transporters and display dependency on exogenous supply of cystine/cysteine for their proliferation (85).

## METABOLISM OF OTHER AMINO ACIDS

Tryptophan, an essential amino acid used for the biosynthesis of crucial compounds, including 5-hydroxytryptamine (5-HT, serotonin) and kynurenine, is important in T cell function. Indoleamine-2,3-dioxygenase 1 (IDO-1) catabolizes tryptophan to kynurenine and T cells require tryptophan for proliferation and activation (13). Accordingly, IDO-1 inhibits T cell activation and Treg cell differentiation of human and murine T cells (86–89). The dysbiotic gut microbiota of lupus-prone mice which is characterized by altered distribution of tryptophan metabolites in the feces of the mice, including an increase in kynurenine levels, has been linked to the production of autoantibodies and autoimmune pathology (90). Low dietary tryptophan prevents disease activity of the lupus-prone mice, whereas high dietary tryptophan has the opposite effect (90).

Methionine can affect the epigenetic reprogramming in $CD4^+$ T cells (91). Activated T cells transport methionine *via* SLC7A5 (92). Methionine serves as the major substrate for the biosynthesis of S-adenosyl-L-methionine (SAM) (91, 93). SAM functions as a substrate for epigenetic modifications. Methionine restriction reduces histone H3K4 methylation at promoter regions of genes associated with Th17 cell proliferation and cytokine production in murine T cells (91).

## CONCLUSIONS

During the last decade great progress has been achieved in the field of immunometabolism. It has now been established that T cell metabolism controls the fate and function of T cells. Amino acids are also crucial in T cell survival, function and differentiation. Besides glycolysis, amino acid metabolism is also involved in the pathogenesis in SLE and by inference to other autoimmune diseases. Although 2-deoxy-d-glucose monotherapy has partial efficacy in improving disease in lupus-prone mice, when combined with metformin, a mitochondrial electron transport chain complex I inhibitor, it leads to normalization of T cell metabolism and reversal of disease activity (94). These results revealed that monotherapy targeting only glycolysis is not sufficient to treat lupus-prone mice. Thus, the focus of research on T cell metabolism in lupus is expanding our understanding of amino acid metabolism.

Although many reports have shown that some metabolic pathways involving amino acids including glutamine, tryptophan, and cysteine can serve as therapeutic targets in lupus-prone mice, the tentative therapeutic targeting of metabolic pathways of other amino acids remains unclear. Sirolimus and NAC are undergoing rigorous clinical trials in patients with SLE (42, 43, 80) and they may end up serving as significant entries in the list of available therapeutic tools for these patients. There are though several challenges to overcome in order to exploit additional amino acid-related treatment targets. Although many studies using mouse models have revealed potential therapeutic targets in amino acid metabolism, further insights are needed from the *ex vivo* study of immune cells from patients with SLE. Such studies should be followed by properly designed clinical trials in patients with SLE and probably other autoimmune diseases. As all drugs display invariably side effects, cell/tissue targeted delivery should be considered (73, 95, 96).

In this brief review we presented evidence that amino acids are important in T cell function and aberrant metabolism may be linked to autoimmunity and related pathology. It appears that their central role in the control of the immune response is underwritten by being

indispensable for the generation of building blocks needed for cell proliferation, the generation of energy by controlling metabolic pathways, the control of epigenetic pathways, the production of phospholipids and the control of oxidative stress.

Amino acids and products of metabolic processes dictate the effector function of T cells and determine whether they will serve as regulators, instigators of inflammation or effectors of cytotoxicity. Alterations of the levels of metabolites within immune cells can be achieved by simply changing their levels in the environment or modulating the activity of transporters and intracellular metabolic enzymes. Drugs altering metabolism or supplementation of amino acids or metabolites or their precursors may prove of great value as modulators of T cell functions in the treatment and well-being of patients with autoimmune disease.

## AUTHOR CONTRIBUTIONS

MK, NY, and GT conceptualized the article, reviewed the literature, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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# Interleukin-22 From Type 3 Innate Lymphoid Cells Aggravates Lupus Nephritis by Promoting Macrophage Infiltration in Lupus-Prone Mice

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Lupus nephritis (LN) is one of the most severe manifestations of systemic lupus erythematosus (SLE). Our previous studies demonstrated increased serum and renal Interleukin (IL)-22 in LN patients and MRL/lpr mice. This study investigated the role of IL-22 and its mechanism in LN. Here, we found that IL-22 was mainly produced by type 3 innate lymphoid cells (ILC3) in kidney of MRL/lpr mice. The systemic illness and local renal lesion were significantly alleviated in IL-22 or IL-22R gene knockout (KO) mice (IL-22 KO or IL-22R KO MRL/lpr mice) than control mice (MRL/lpr mice). IL-22 KO or IL-22R KO MRL/lpr mice had significantly slighter infiltration of macrophage in kidney than MRL/lpr mice. Consistently, by RNA-Seq, the expression of (CC motif) ligand 2 (CCL2) and (CXC motif) ligand 10 (CXCL10) was decreased in kidney of KO mice compared with control mice. By immunoblotting, significantly increased levels of STAT3 phosphorylation were found in the kidney of control mice compared to KO mice. *In vitro*, primary kidney epithelial cells from control mouse stimulated with recombinant IL-22 (rIL-22) expressed higher levels of CCL2, CXCL10, and phosphorylated STAT3. At the same time, when primary kidney epithelial cells were treated with rIL-22, transwell assay demonstrated their supernatant recruited more macrophages. In human kidney epithelial cell line (HK2) cells, when treated with rIL-22, we observed similar results with primary mouse kidney epithelial cells. Moreover, when cells were stimulated with rIL-22 following pre-treatment with STAT3 pathway inhibitor, the expression of CCL2 and CXCL10 were significantly reversed. Our findings demonstrate that IL-22 binding to IL-22R in kidney epithelial cells activated the STAT3 signaling pathway, enhanced the chemokine secretion and then promoted macrophage infiltration to the kidney of MRL/lpr mice, thus aggravated LN in lupus-prone mice. These findings indicate that IL-22 may play a pathogenic role in LN and may provide a promising novel therapeutic target for LN.

**Keywords: systemic lupus erythematosus, lupus nephritis, interleukin-22, interleukin-22 receptor, macrophage**

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease, resulting from auto-immune dysfunction, and presenting with immune complex-mediated lesions in diverse organ (1). Lupus nephritis (LN) is one of the most severe manifestations of SLE (2). Despite the improvement of therapeutic approaches in recent years, 12-month complete renal response rates are only 10%–40% (3), and for all LN patients, the cumulative incidence of end-stage renal disease (ESRD) at 10 years after the diagnosis of LN was 10.1% (4). Therefore, a novel therapeutic target for LN is pressing and timely.

The interleukin (IL)-22 belongs to the IL-10 cytokine family (5). IL-22 is primarily produced by T cell subsets and innate lymphoid cells (ILCs) (6) but preferentially act on non-hematopoietic cells, particularly epithelial cells because IL-22 receptor (IL-22R) is restricted to the epithelial cells of tissues, including the skin, intestine, liver, lung and kidney (7). IL-22 mediates its effects *via* the IL-22-IL-22R complex and subsequent Janus kinase–signal transducer and activators of transcription (JAK–STAT) signaling pathway (5). Accumulated evidence has indicated that IL-22 plays a pathogenic or protective role in different autoimmune diseases (8–10). We previously found that IL-22 was increased in serum and renal tissue of both LN patients and MRL/lpr mice (11), and that elevated urinary IL-22 binding protein(IL-22BP) in LN patients were associated active renal disease (12). Our further experiments demonstrated that MRL/lpr mice treated with anti-IL-22 monoclonal antibody (mAb) had substantial improvement of renal function and less renal injury (11).

Based on observations above, we hypothesized that IL-22 plays a central role in the pathogenesis of LN. We respectively knocked out IL-22 and IL-22R gene of MRL/lpr mice to investigate the role of IL-22 and its mechanism in LN.

## METHODS

### Mice

MRL/lpr female mice were used as the model of lupus. They were obtained from Shanghai Slac Laboratory Animal CO. LTD (Shanghai, China). IL-22 knockout mice deleted of IL-22 exons 1 through 4 (NM_016971) were purchased from Mutant Mouse Resource and Research Centers (MMRRC, USA). IL-22 receptor knockout mice were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). All mice were housed in a specific pathogen free condition in the animal facility at School of Medicine, Zhejiang University, China. IL-22 knockout and IL-22R knockout mice were bred to MRL/lpr mice (designed as control) in our colony and backcrossed for at least 10 generations to generate IL-22$^{-/-}$ MRL/lpr (designed as IL-22 KO) and IL-22R$^{-/-}$ MRL/lpr (designed as IL-22R KO). All animal experiments were performed according to the protocol approved by the Ethics Committee of the Second Affiliated Hospital, College of Medicine, Zhejiang University in compliance with institutional guidelines.

### Patients

Ten LN patients were recruited from March 2017 to December 2018 at the Department of Nephrology and Rheumatology of the Second Affiliated Hospital, College of Medicine, Zhejiang University. All patients fulfilled the American College of Rheumatology (ACR) diagnostic criteria of SLE (13) and was defined by renal biopsy. Three normal renal tissues from para-carcinoma tissues as healthy controls (HCs) were confirmed by light microscope examination. Renal biopsy was handled under ultrasound local isolation. Renal tissue was extracted for immunohistochemical assessment. The study protocol was approved by the Ethics Committee of the Hospital and was conducted in accordance with the 1989 Declaration of Helsinki.

### Primary Mouse Kidney Epithelial Cells

Freshly isolated kidneys were placed in ice-cold DMEM mixed with Hams F12 (1:1 ratio; Life Technologies, Grand Island, NY) on a 60 mm dish. The kidney capsule was removed by peeling with forceps, and the kidney was sliced coronally and homogenized by mincing into 1–2 mm$^3$ pieces. The homogenized kidney tissue pieces were resuspended and mixed in 10 ml of collagenase type IV for 30 min at 37°C to obtain single-cell suspensions. After digestion, the cell suspension was filtered through 70-μm cell strainers. The filtered cell suspensions were centrifuged at 300*g* for 5 min and incubated with ACK lysing buffer (Beyotime Biotechnology, China) to remove red blood cells. Then, the pellet was washed with DMEM/F12 medium with 10% FBS twice and passed through a 40-μm cell strainer. After filtering, cells were generated in DMEM/F12 medium with 10% FBS on a 60 mm dish. Then, medium was replaced with fresh DMEM/F12 medium with 10% FBS 6 h later.

### Cell Culture and IL-22 Treatment *In Vitro*

The HK-2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM/F12 medium with 10% FBS. After FBS starvation in DMEM/F12, cells at 80% confluence were stimulated with recombinant IL-22 (rIL-22) (100 ng/ml) (Peprotech). To inhibit the STAT3 signaling pathways, C188-9 (MCE) was added 24 h before stimulation with IL-22.

### Isolation of Spleen and Kidney Cells

Freshly isolated spleens were placed in ice-cold DMEM (Life Technologies, Grand Island, NY) on a 60 mm dish. Spleens were grinded and passed through a 40-μm cell strainer. The pellet was incubated with ACK lysing buffer (Beyotime Biotechnology) for 2 min at room temperature. The renal single-cell suspensions were obtained according to Chong's protocol (14). Then, the cells were prepared for staining and flow cytometry analysis.

### Flow Cytometry

Single-cell suspensions obtained from the blood, spleen and kidney were stimulated at 37°C for 6 h at 5% CO2 in the presence of 50 ng/ml phorbol myristate acetate (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), and Brefeldin A

Solution (BD Bioscience, USA). After incubation, the cells were blocked with anti-CD16/CD32 antibodies (Biolegend, USA) and then stained with the indicated antibodies for 20 min at 4°C. After fixation and permeabilization with Perm/Wash solution (BD Pharmingen, USA), the cells were stained with allophycocyanin-conjugated anti-IL22 monoclonal antibodies (Biolegend, USA) in the dark at 4°C for 30 min. Then, the cells were washed with Perm/Wash solution once and resuspended. Isotype controls were utilized to enable accurate compensation and to confirm the antibody specificity. The stained cells were analyzed by FCM using ACEA NovoCyte™ cytometer (ACEA Biosciences, USA) and analyzed with NovoExpress software (ACEA Biosciences, USA) and FlowJo software (Tree Star Inc, Ashland, OR, USA). Refer to the antibody table (**Supplemental Table 2**) for a complete list of antibodies.

## Assessment of Proteinuria and Renal Function

Blood urea nitrogen (BUN) was determined by the Quantichrom DIUR 500 kit (BioAssay Systems, Hayward, CA). Serum and urinary creatinine were measured by the QuantiChrom Creatinine Assay Kit (BioAssay Systems). The ratio of albumin to creatinine in urine was measured using an ELISA kit (Exocell).

## Gross Pathology

Skin lesions were scored at age of 24 weeks. We scored the skin lesions by gross pathology using a grade of 0–6 (0 = none; 1 = mild, < 2 cm; 2 = severe, ≥2 cm, scored the snout, ears, and intrascapular separately and added). Lymphadenopathy (cervical, brachial, and inguinal) was graded as 0–4 (0=none; 1=small, one site; 2=moderate, two sites; 3=large, three sites; and 4=very large, four sites or more) and splenomegaly was analyzed by spleen weight upon euthanasia.

## Renal Histopathology

Kidneys were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin (H& E), or periodic acid-Schiff (PAS). To score kidney pathology we evaluated glomerular pathology as previously described (11).

## Immunohistochemistry

Paraffin-embedded Kidney sections (5 μm thick) were deparaffinised and boiled for 10 min in sodium citrate buffer (10 mM, pH 6.0). The sections were depleted of endogenous peroxidase activity by adding methanolic $H_2O_2$ for 10 min and blocked with normal serum for 30 min. After overnight incubation at 4°C with polyclonal antibodies against CD68 (Abcam, USA) or pSTAT3 (CST, USA), the samples were incubated for 30 min with the secondary antibody, biotinylated anti-rabbit IgG (CD68), or biotinylated anti-mouse IgG (pSTAT3), and incubated with streptavidin–peroxidase complex (Vector) for 1 h, followed by incubation with 3,3'-diaminobenzidine (Dako) for 5 min. The sections were counterstained with haematoxylin. The samples were photographed using an Olympus photomicroscope.

## Immunofluorescence

Immunofluorescence was carried out using standard protocol on kidney sections. Briefly, heat mediated antigen retrieval with Tris/EDTA buffer pH 6.0 was performed before commencing with staining protocol. After 30 min in BSA (G5001, Servicebio), slides were incubated overnight with specific primary antibody, rabbit anti-mouse IgG (Abcam, USA) and rat anti-mouse C3 (Santa Cruz, USA) at 4°C overnight, followed by FITC-conjugated secondary antibody (Servicebio, China). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The samples were photographed using NIKON ECLIPSE C1.

## ELISA

We measured serum total IgG, ds-DNA antibody, C3 (Abcam) by ELISA kits according to the manufacturer's protocol. Briefly, standard or sample was added into each well and incubated. The supernatant was removed, and biotin-antibody was added and incubated. After three times wash with PBS, HRP-avidin was added into each well and incubated, followed by incubation with 3,3',5,5' tetramethylbenzidine (TMB) substrate for 20 min in dark. The reaction was terminated by adding stop solution, and the optical density was measured within 30 min using a microplate reader at 450 nm. All samples were assayed in triplicate.

## Quantitative Real-Time PCR

The total RNA was isolated from the cells using TRIzol reagent (Takara) according to the manufacturer's directions, and single-strand cDNA was generated from the total RNA and reverse transcriptase (Toyobo). The SYBR Green master Rox (Roche) was used for the quantitative real-time reverse transcription–PCR analysis according to the manufacturer's protocol. The relative gene quantification was done using the $2^{-\Delta\Delta}$Ct method following normalization to internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Primers used for qPCR were synthesized by Tsingke Biotech Co. Ltd (Beijing, China). Refer to the primer table (**Supplemental Table 1**) for a complete list of primers in this study.

## Transwell Chemotaxis Assay

Primary bone marrow derived macrophages (BMDMs) were obtained according to Chong's protocol (14). $1 \times 10^5$ BMDMs were added to the upper chambers of a 5-μm pore polycarbonate Transwell filter (Corning). The upper and lower chambers were incubated for 30 min at 37°C in macrophage starve medium. The medium in the lower chamber was replaced with supernatant from mouse kidney epithelial cells treated or not with rIL-22. After 24 h, transmigrated cells were fixed and stained using the Crystal Violet Staining Solution (Beyotime, China). For each filter, 10 random images were acquired using OLYMPUS IX53 microscope.

## Immunoblotting

Kidney tissues were homogenized in RIPA buffer (50 mM TriszHCl [pH 7.4], 150 mM NaCl, 1% NP40, 1 mM PMSF,

1 mM NaF, 20 mM Na4P2O7, 2 mMNa3VO4, and 13 protease inhibitor cocktail [Roche Applied Science, Indianapolis, IN]), and equal protein (30 mg) was resolved by PAGE. Proteins from kidneys or HK-2 cells were transferred onto nitrocellulose membrane, and immunoblotting was performed with mouse polyclonal anti-pSTAT3, STAT3, GAPDH, and rabbit polyclonal anti-p38, p-p38 (Cell Signaling Technology) antibodies. Images were captured using the ChemiDocMP Imaging System (Bio-Rad, Hercules, CA).

## RNA-Seq

Total RNA from kidneys was extracted using TRIzol (Takara). Preparation of the library and transcriptomic sequencing were carried out using the Illumina HiSeq ×Ten (Novogene Bioinformatics Technology). Mapping of 100-bp paired-end reads to genes was done using HTSeq software (version 0.6.0), and fragments per kilobase of transcript per million fragments mapped (FPKM) were also analyzed. The sequencing data was submitted to Sequence Read Archive (SRA) of NCBI and the SRA accession is PRJNA648341.

## Statistical Analysis

Data was analyzed using Graph Pad Prism 7, and presented as mean ± standard deviation. For experiments with only two groups, significance was determined by a Student's t test or the Mann-Whitney U-test according to whether the distribution is normal or not. Significance for >2 groups was determined by one-way analysis of variance (ANOVA). Post hoc least significant difference (LSD) tests, in which all groups were tested against a control group as a reference, were performed if the results of the initial analysis of variance were significant. p-values <0.05 were considered significant and marked with one asterisk, while the p-values of <0.01 or <0.001 were marked with two or three asterisks.

# RESULTS

## IL-22 Was Mainly Expressed by ILC3s in Kidney of Lupus-Prone Mice

As we had found increased level of IL-22 in serum and renal tissue from LN patients (11), we determined to explore the expression of IL-22 in MRL/lpr mice. In peripheral blood, the percentage of IL-22$^+$ cells in leukocytes increased significantly in 24-weeks-old MRL/lpr mice compared to 6-weeks-old mice (**Figures 1A, B**). In spleen, total amount and the percentage of IL-22$^+$ cells in leukocytes were both significantly increased in 24-weeks-old MRL/lpr mice (**Figures 1A, B**).

In kidney, total amount and the percentage of IL-22$^+$ cells in leukocytes also increased in 24-weeks-old MRL/lpr mice compared to 6-weeks-old MRL/lpr mice (**Figures 1A, B**). At the same time, we found that the majority (nearly 60%) of IL-22$^+$ cells in kidney of 24 weeks-old mice were IL-22$^+$ innate lymphoid cells (ILCs, Lin$^-$CD127$^+$) (**Figure 1C**). Moreover, the amount of IL-22$^+$ ILCs increased with the development of age-related lupus nephritis (**Figure 1D**), while the absolute number of IL-22$^+$ T cells (IL-22$^+$CD3$^+$) in the kidneys showed no significant difference (**Supplementary Figure 1**). And IL-22$^+$ ILCs were almost all (nearly 90%) from ILC3 (Lin$^-$ CD127$^+$RORγt$^+$) subgroup (**Figure 1C**). Unexpectedly, we found the percentage of CCR6$^+$ IL-22$^+$ ILC3s that can secrete IL-17 cytokine in 24-weeks-old MRL/lpr mice also significantly increased compared to 6-weeks-old mice (**Figures 1C, D**).

## IL-22 Shortened Survival and Promoted Systemic Illness in Lupus-Prone Mice

To further confirm the role of IL-22 in the pathogenesis of LN, we performed experiments on IL-22 KO or IL-22R KO mice. IL-22 KO or IL-22R KO MRL/lpr mice survived longer than control mice (**Figure 2A**). These control mice underwent substantial proteinuria, oliguria and anuria before death (not shown).

We also detected the spleen/weight ratio, skin lesions and lymphadenopathy in 24-weeks-old mice. Gross specimens showed control mice had much severer splenomegaly than IL-22 KO or IL-22R KO mice (**Figure 2B**), and consistently the spleen/weight ratio in MRL/lpr mice was significantly increased compared to IL-22 KO or IL-22R KO mice (**Figure 2E**). The score of skin lesions in control mice was significantly higher than that in IL-22 KO or IL-22R KO mice (**Figures 2C, F**). The score of lymphadenopathy in control mice was significantly increased compared to IL-22 KO mice, and also higher than that in IL-22R KO mice though without significant difference (**Figures 2D, G**).

## IL-22 or IL-22R Deficiency Ameliorated Renal Injury in Lupus-Prone Mice

We next explored the effect of IL-22 KO or IL-22R KO on LN. Proteinuria, renal function and magnitude of renal pathology were examined in 24-weeks-old mice. The ratio of albuminuria/creatinine in IL-22 KO mice was significantly decreased, compared with control mice (**Figure 3A**), and serum BUN levels in both IL-22 KO and IL-22R KO mice were significantly lower than control mice (**Figure 3B**). The serum creatinine levels in KO mice also decreased, though without statistical significance (**Figure 3C**).

The pathological changes in kidneys of lupus-prone mice were analyzed morphologically as shown by H&E staining. There was significantly less renal injury in IL-22 KO or IL-22R KO mice than those in control mice (**Figure 3D**). At 24 weeks of age, more glomerular cellularity, collapse of capillary lumina, and thicker basement membranes were observed in control mice as shown by Periodic Acid-Schiff (PAS) staining (**Figure 3D**). The glomerular pathology score (**Figure 3E**) and the number of cells per glomerulus (**Figure 3F**) were significantly lower in IL-22 KO or IL-22R KO mice than those in control mice. Thus, deleting IL-22 or IL-22R gene ameliorated renal injury in lupus-prone Mice

## IL-22 or IL-22R Deficiency Altered the Levels of Autoantibody and Complement in Lupus-Prone Mice

We examined the effect of IL-22 or IL-22R deficiency on serum anti-dsDNA autoantibody (Ab) production. The levels of

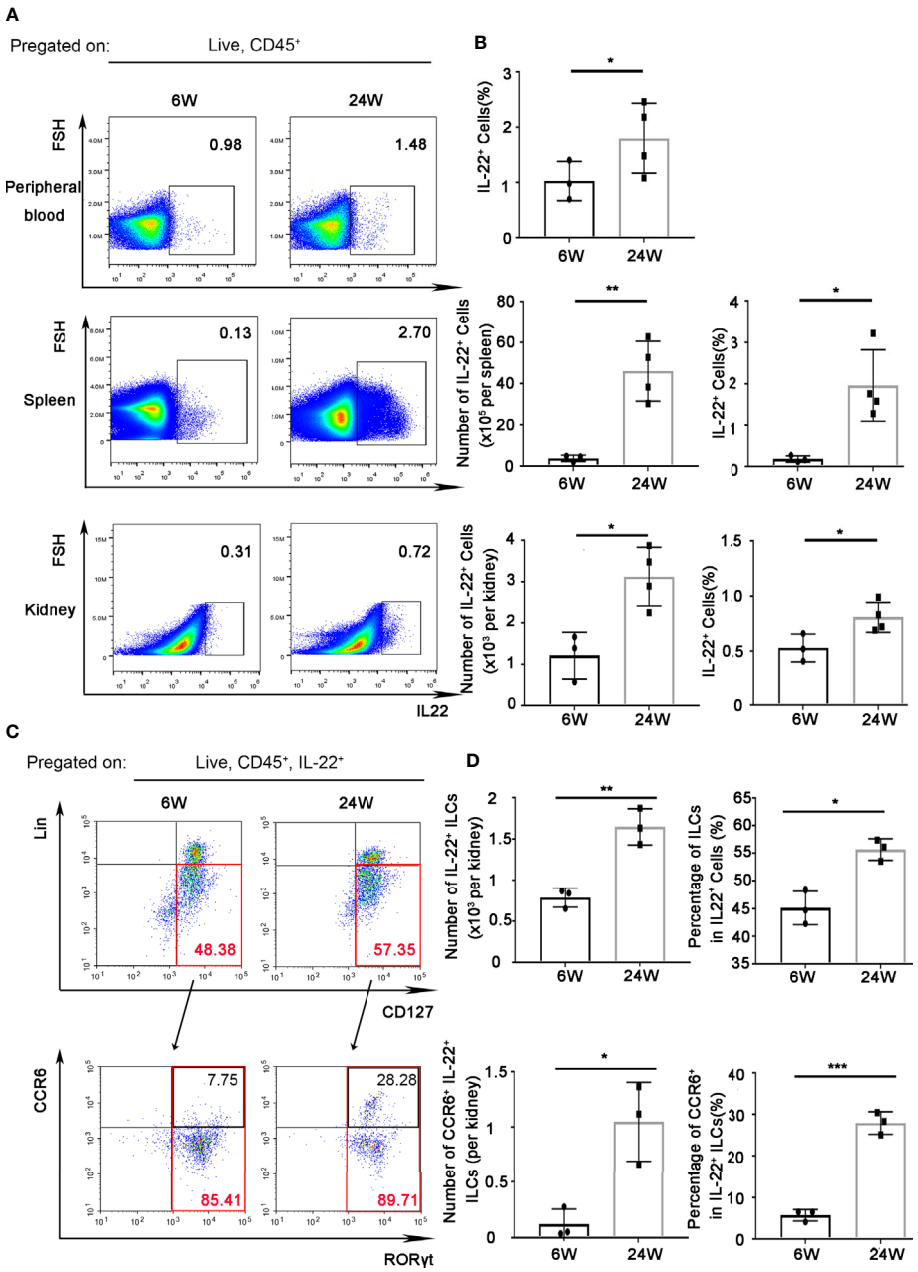


**FIGURE 1** | IL-22 was mainly expressed by ILC3s in kidney of lupus-prone mice. IL-22$^+$ cells in peripheral blood, spleen, kidney from 6-weeks or 24-weeks-old MRL/Fas Mice were analyzed by flow cytometry. **(A)** The gating strategy for detecting IL-22$^+$ cells in peripheral blood, spleen, and kidney. **(B)** The quantitative analysis of the percentage and amount of IL-22$^+$ cells in peripheral blood, spleen, and kidney. **(C, D)** The gating strategy for detecting IL-22$^+$ cell subsets and analysis of IL-22$^+$ ILCs (Lin$^-$CD127$^+$), IL-22$^+$ILC3s (Lin$^-$CD127$^+$RORγt$^+$), and CCR6$^+$ IL-22$^+$ ILC3s in kidney. Data were expressed as mean ± SD, and are representative of three independent experiments. T-test was used for comparison between groups (*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001). ILCs, innate lymphoid cells.

dsDNA Ab were significantly lower in 24-weeks-old IL-22 or IL-22R KO mice than control mice of the same age (**Figure 4A**). We also detected serum total IgG and C3. The levels of serum total IgG were lower in IL-22 KO mice than control mice (**Figure 4B**). Conversely, the levels of serum C3 were significantly higher in IL-22 or IL-22R KO mice than control mice (**Figure 4C**). We also detected the IgG and C3 immune deposition in the glomeruli of 24-weeks-old mice by immunofluorescence (IF). The IgG and C3 immune deposition in the glomeruli of IL-22 or IL-22R KO mice was significantly alleviated (**Figures 4D–F**).

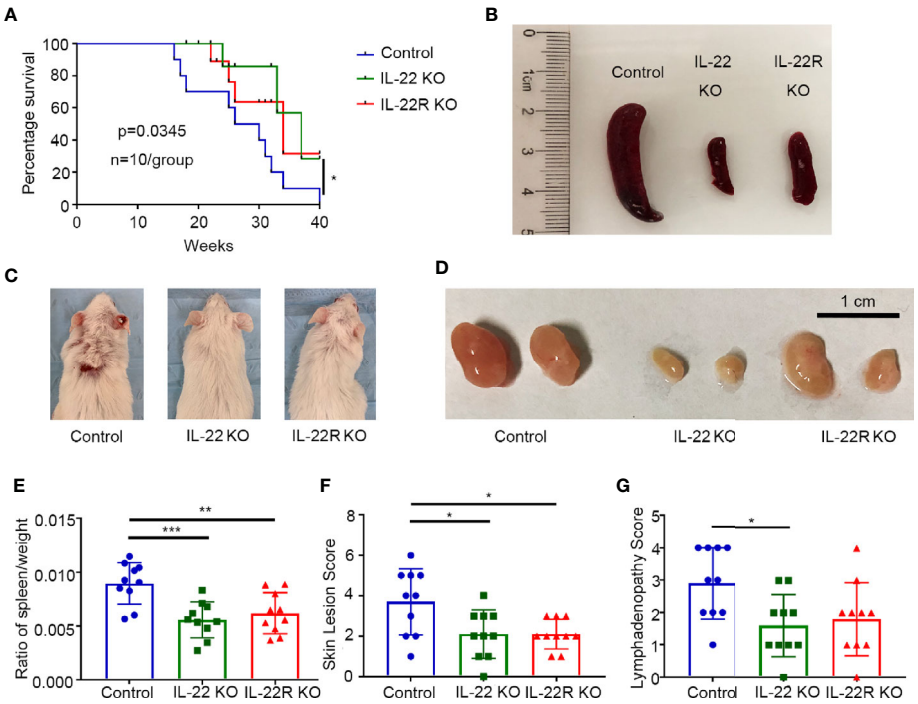


**FIGURE 2** | IL-22 shortened survival and promoted systemic illness in lupus-prone mice. **(A)** Survival curve of control, IL-22 KO, and IL-22R KO mice (n=10/group) over 10 months. **(B–D)** Representative images of spleen, skin, and lymph node from control, IL-22 KO, and IL-22R KO mice (24 weeks old mice). **(E)** The ratio of the spleen/weight, **(F)** skin lesion score and **(G)** lymphadenopathy score were compared between 24-weks-old control, IL-22 KO, and IL22-R KO mice. Skin lesions were scored by gross pathology using a grade of 0–6 (0 = none; 1 = mild, < 2 cm; 2 = severe, ≥2 cm, scored the snout, ears, and intrascapular separately and added). Lymphadenopathy (cervical, brachial, and inguinal) was graded as 0–4 (0=none; 1=small, one site; 2=moderate, two sites; 3=large, three sites; and 4=very large, four sites or more). Data were expressed as mean ± SD, and were representative of three independent experiments. One way ANOVA was used for comparison among all groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

## IL-22 or IL-22R Deficiency Reduced Intrarenal Macrophages by Decreasing the Expression of CCL2 and CXCL10 in Lupus-Prone Mice

By flow cytometry, we detected the expression of these inflammatory cells, including T cells, B cells, neutrophils and macrophages, in the kidney of lupus-prone Mice. The number and the percentege of infiltrating macrophages in the kidneys of IL-22 or IL-22R KO mice was significantly decreased compared with control mice, but there was no significant difference in the number and the percentage of B cells and neutrophils among three groups (**Figures 5A, B**). Though the number of T cells was higher than IL-22 KO mice, the percentage of them showed no difference (**Figure 5B**). Immunohistochemistry also showed less intrarenal macrophages in IL-22 or IL-22R KO mice than control mice (**Figures 5C, D**). These outcomes indicate that IL-22 or IL-22R deficiency reduced macrophages infiltration in lupus-prone mice.

Then, we detected gene expression profile of intrarenal chemokines and chemokine receptors (**Figure 6A**). Lower expression of chemokine (C-X-C motif) ligand 1 (CXCL1) and IFNγ-induced protein-10 (CXCL10) were found in kidney of IL-22 or IL-22R KO compared with control mice (**Figure 6B**). Quantitative reverse transcription PCR (qRT-PCR) further confirmed that deficiency of IL-22 or IL-22R resulted in decreased levels of CXCL1, and monocyte chemo attractant protein-1 (MCP-1, CCL2) and CXCL10 (**Figure 6C**).

*In vitro*, we isolated the mouse kidney epithelial cells from MRL/lpr mice, and then stimulated it with 100 ng/ml recombined IL-22(rIL-22) for 60 min. It was shown that the relative mRNA expression of CCL2, CXCL1 and CXCL10 were significantly increased (**Figure 6D**). And the supernatant from cells stimulated with rIL-22 transferred to the lower chambers for 24 h attracted more macrophages by transwell chemotaxis assay (**Figure 6E**), suggesting that IL-22 may recruit macrophages by upregulating the expression of CCL2, CXCL1, and CXCL10 in kidney epithelial cells.

## IL-22 Promoted Kidney Epithelial Cells to Express CCL2 and CXCL10 by Activating the STAT3 Pathway

By immunoblotting, we detected the signaling pathway activated by IL-22/IL-22R in kidney epithelial cells of lupus-prone Mice. It was shown that the level of phosphorylated STAT3 significantly increased when primary control mouse kidney epithelial cells was treated with rIL-22 (**Figure 7A**). *In vivo*, the phosphorylation of STAT3 was much higher in the kidney of control mice at 24 -weeks age than that of IL-22 KO or IL-22R

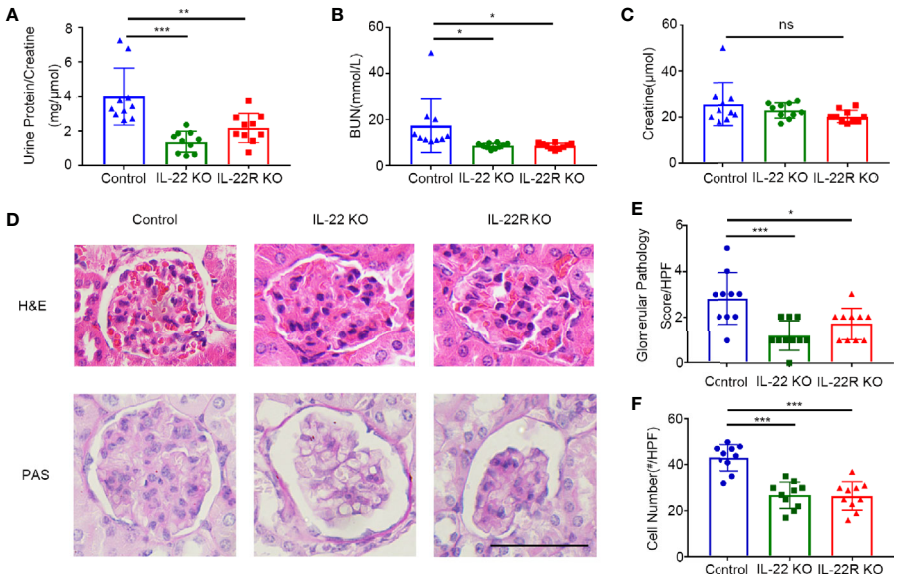


**FIGURE 3** | IL-22 or IL-22R deficiency ameliorated renal injury in lupus-prone mice. Renal function as **(A)** urine protein/creatinine ratio, **(B)** serum BUN, **(C)** serum creatinine of 24-weeks-old control and IL-22 KO, IL-22R KO mice. **(D)** Representative images of kidney biopsies stained in H&E and PAS from control, IL-22 KO, and IL-22R KO mice (24 weeks, scale bar, 50 μm). The glomerular pathology score **(E)** and cell numbers per glomerulus **(F)** were analyzed and counted in the PAS stained slides from 24-weeks-old control, IL-22 KO and IL-22R KO mice. Data were expressed as mean ± SD, and are representative of three independent experiments. One way ANOVA was used for comparison among all groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

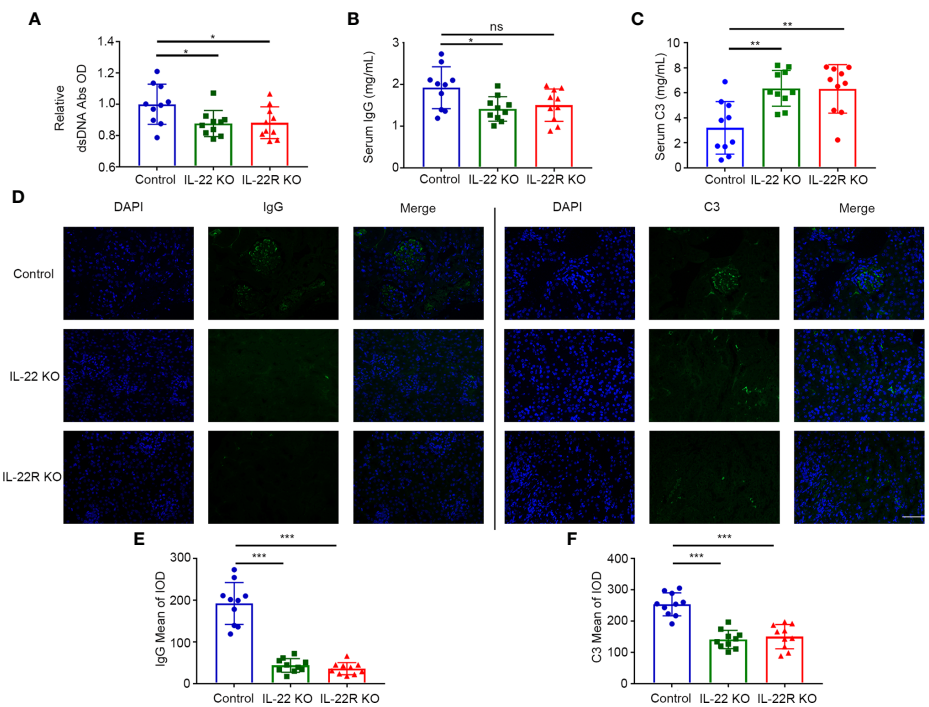


**FIGURE 4** | IL-22 or IL-22R deficiency altered the levels of autoantibody and complement in lupus-prone mice. **(A–C)** The level of serum dsDNA Abs, total IgG and C3 were measured by ELISA in 24-weeks-old control, IL-22 KO and IL-22R KO mice. **(D)** Representative photos of IgG and C3 deposition in the glomeruli of 24-weeks-old control, IL-22 KO and IL-22R (scale bar, 50 μm). **(E, F)** Quantification of the mean IOD of IgG and C3 per glomerular cross-section in all groups of mice. Data were expressed as mean ± SD, and are representative of three independent experiments. One way ANOVA was used for comparison among all groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

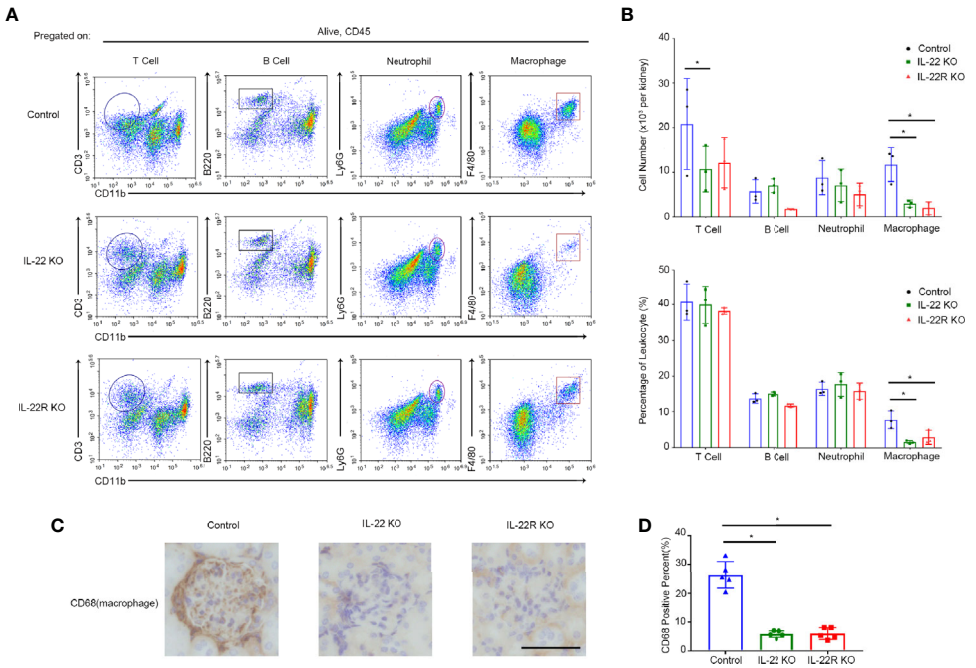


**FIGURE 5** | IL-22 or IL-22R deficiency reduced intrarenal macrophages in lupus-prone mice. **(A)** Kidney cells from 24-weeks-old IL-22 KO, IL-22R KO and control Mice were analyzed by flow cytometry for the percentage of different inflammatory cell subsets, including T cells, B cells, neutrophils and macrophages. **(B)** Quantitative analysis of the amount and the percentage of different cell subsets per kidney in three groups. **(C)** Representative photos of macrophages (marked by CD68) in control IL-22 KO and IL-22R KO mice glomeruli at 24 weeks of age (scale bar, 50 μm). **(D)** Quantitative analysis of the percentage of CD68 positive cells in the kidneys of mice from three groups. Data were expressed as mean ± SD, and are representative of three independent experiments. One way ANOVA was used for comparison among all groups (*$P < 0.05$).

KO mice. But there was no difference in p38 between IL-22 KO or IL-22R KO mice and control mice (**Figure 7B**).

Immunochemistry results indicated increased levels of phosphorylation STAT3 and IL-22 in the kidney of LN patients when compared with healthy controls (**Figure 6C**). In human kidney cell line (HK2) cells, we observed similar results with mouse kidney epithelial cells. Moreover, when cells were treated with rIL-22 for 30 min following 24 h pre-treatment with STAT3 pathway inhibitor C188-9, the relatively mRNA expression of CCL2 and CXCL10 were significantly decreased in HK2 cells (**Figures 6D, E**).

## DISCUSSION

In our study, we found that IL-22 was mainly secreted by ILC3 in MRL/lpr mice, and deleting IL-22 or IL-22R decreased the systemic illness and lupus nephritis severity in lupus-prone mice. We also found that deleting IL-22 or IL-22R downregulated CCL2 and CXCL10 expression, and decreased the filtration of macrophage into the kidney of lupus-prone mice. The phosphorylation of STAT3 was inhibited in the kidney of IL-22 or IL-22R KO mice. *In vitro* studies showed that primary kidney epithelial cells from control mouse stimulated with recombinant IL-22 (rIL-22) expressed higher levels of CCL2, CXCL10, and phosphorylated STAT3, and their supernatant recruited more macrophages. These results revealed that IL-22 may be involved in the pathogenesis of LN through activating STAT3 signaling and promoting CCL2 and CXCL10 expression and the infiltration of macrophage in kidney.

To date, there has been no consensus about which cells has been responsible for the production of IL-22 in SLE. Most studies have demonstrated that IL-22 in peripheral blood of SLE patients was expressed by effector $CD4^+$ T cells (15). In the present study, we found that among $IL\text{-}22^+$ cells, IL-22-producing ILC3s was nearly 60% in renal tissue of MRL/lpr mice, suggesting that IL-22 was mainly produced by ILC3s in LN. ILCs have emerged as important effector cells of the innate immune system and can secrete numerous cytokines, such as IFN-γ, IL-13, IL-22, while using specific mechanisms that directly kill target cells (16). Studies detected abnormal ILCs in a variety of autoimmune diseases, which led to abnormal immune activation and chronic inflammatory diseases (17). In humans and mice models of RA, the circulating ILC2s subset is crucial for the resolution of disease activity (18). As for SLE, one report that circulating ILC1s was increased in patients with SLE compared with HC (19), others report that increased ILC1s/ILC3s in Peripheral blood of SLE patients was correlated with nephritis and disease activity (20), and increased ILC3s may be related to the presence of a type I IFN signature (21). However, no studies so far have assessed ILCs in affected tissues of patients with SLE. We report that IL-22 was produced mainly by ILC3s in renal tissue of LN, suggesting

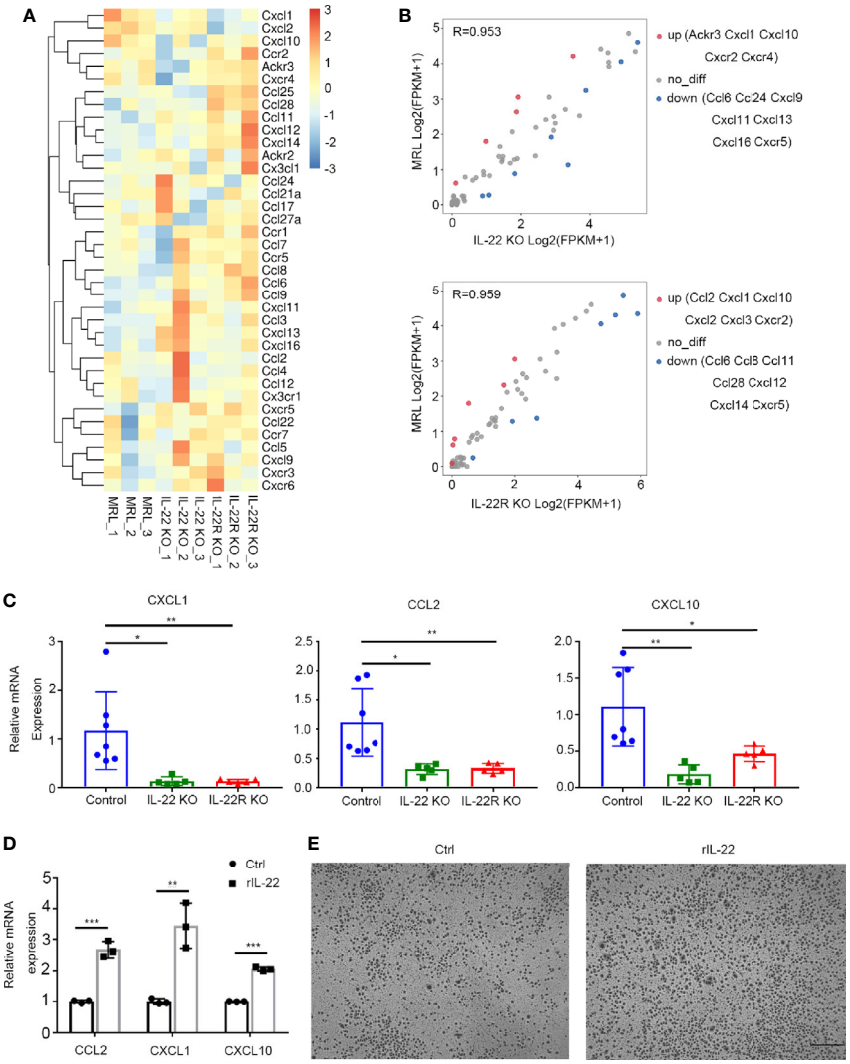


**FIGURE 6** | IL-22 affected the expression of chemokines *in vivo* and *in vitro*. **(A)** Gene expression profile of chemokines and their receptors in kidneys from control, IL-22 KO and IL-22R KO mice. (n = 3 independent experiments). For the gene expression profiles, the colored key shows fold change in gene expression. **(B)** Scatter plot of differential expression of chemokines and their receptors in kidneys from control, IL-22 KO and IL-22R KO mice. (n = 3 independent experiments, P value < 0.05 and fold change> 2). **(C)** Gene expression level of intrarenal chemokine was analyzed by quantitative reverse transcription PCR (qRT-PCR) in kidney of mice at 24 weeks of age. Values are normalized to Actin transcripts and expressed as relative ratio. **(D)** Gene expression level of chemokines in primary kidney epithelial cells from control mice treated with or without rIL-22 (100 ng/ml, 60 min) by qRT-PCR. **(E)** Representative photos of macrophages recruited by supernatant from primary kidney epithelial cells stimulated with or without rIL-22 for 24 h (scale bar, 500 μm). Data were expressed as mean ± SD, and are representative of three independent experiments. One way ANOVA was used for comparison among three groups, T-test was used for comparison between two groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

ILC3s might contribute to renal tissue injury in LN. Additionally, we found the proportion of ILC3s co-expressing IL-22 and CCR6 (IL-17 producing ILC3s) in kidney of MRL/lpr mice was elevated with the development of LN, indicating that ILC3s simultaneously produced IL-17, which is acknowledged as pro-inflammatory cytokine for LN, and promote the development of LN. Therefore, studying local ILCs will add to the understanding of the role of ILCs in SLE immunopathology.

In the knockout experiment, we found that IL-22 KO or IL-22R KO MRL/lpr mice survived longer than control mice, and had less skin lesions, proteinuria and renal function and pathological impairment that is consistent with what we have shown in the past with anti-IL-22 mAb treatment for MRL/lpr mice (11). These results confirmed the pathogenic role of IL-22 in LN. Meanwhile, we found that IL-22 KO or IL-22R KO MRL/lpr mice had milder lymphadenopathy and splenomegaly, which suggesting IL-22 or IL-22R deficiency may also reduce systemic immune response. In LN, deposits of immune complex (ICs) in the glomeruli are regularly found and these activate complements in the kidneys, which results in decreased levels of serum C3 (22), and serum C3 levels and C3b deposition in the kidneys is are a good markers for LN (23). In the present study,

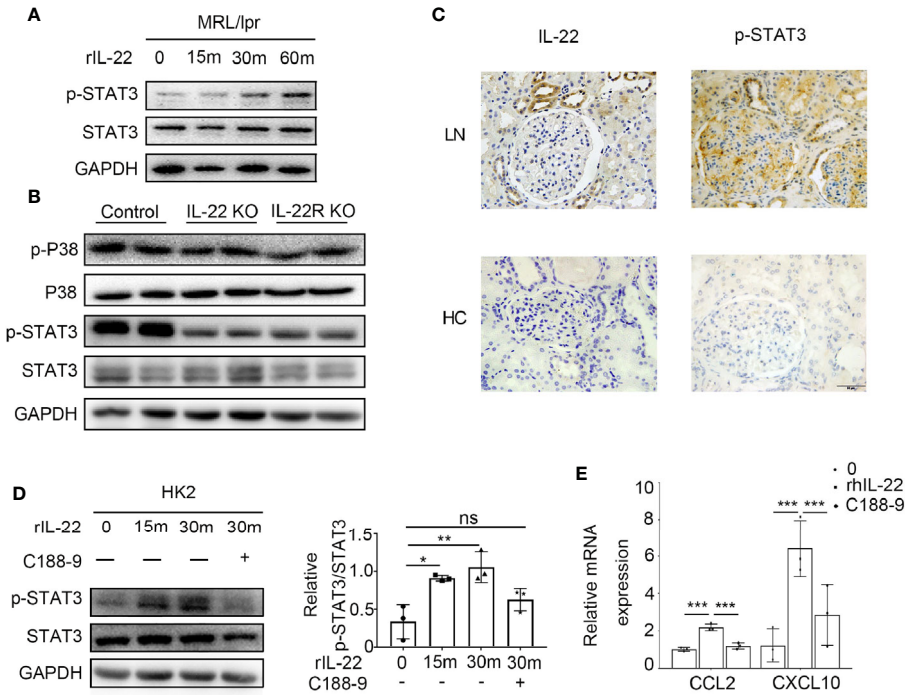


**FIGURE 7** | IL-22 promote the expression of CCL2, CXCL10 by activating the STAT3 pathway. **(A)** The phosphorylation level of STAT3 in primary kidney epithelial cells from control mice treated with or without rIL-22 (100 ng/ml) by western blotting. **(B)** Western blotting showed phosphorylation level of STAT3 and p38 in kidneys from 24-weeks-old control, IL-22 KO and IL-22R KO mice. **(C)** Representative photos of P-STAT3 and IL-22 in lupus nephritis patients and healthy control (HC) by immunochemistry (scale bar, 50 μm). **(D)** The phosphorylation level of STAT3 in HK2 cells treated with rIL-22 (100 ng/ml) by western blotting. Pretreating with or without C188-9 (STAT3 pathway inhibitor), HK-2 were treated with recombinant IL-22 for 0, 15, 30 min. **(E)** Following 24 h pre-treatment with or without C188-9, the relatively mRNA expression of chemokines in HK2 cells after treated with rIL-22 for 0 or 30 min. Values are normalized to GAPDH transcripts and expressed as relative ratio. Data are representative of three independent experiments. T-test was used for comparison between two groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

we found indeed, in lupus-prone mice, IL-22 or IL-22R deficiency decreased production levels of serum ds-DNA autoantibodies and the levels of serum IgG, and increased the levels of serum C3. And decreased depositions of IgG and C3 in kidneys of IL-22 or IL-22R KO mice, suggesting that IL-22 may promote the production of autoantibodies and ICs. Though IL-22 is produced by immune cells, it cannot directly act on immune cells including B cells (7). Therefore, we speculated that IL-22 may upregulate immuno-inflammatory responses, resulting in immune dysregulation, which promote B cells to produce autoantibodies, ICs formation and C3 consumption.

Indeed, in lupus-prone mice, IL-22 or IL-22R deficiency decreased production of ds-DNA autoantibodies and the levels of serum IgG, and increased the levels of serum C3. The amount of renal IgG and C3 depositions in kidneys of IL-22 or IL-22R KO mice was also decreased. The findings pointed to the fact that in the absence of IL-22 or IL-22R, lupus-prone mice do not exhibit some of the readily recognizable features of immune dysregulation characteristic of lupus. Thus, IL-22 KO or IL-22R KO MRL mice may decrease the pathologic glomerular IC and C3 accumulation and in the kidneys. In other words, abrogation of the IL-22/IL-22R signaling may affect the production of Ig and, more importantly, of pathogenic autoantibodies.

A large number of infiltrating inflammatory cells, including T cells, B cells, neutrophils and macrophages are present in renal tissue of LN, following the formation and/or deposition of ICs in the kidney (24). As demonstrated above, control mice had much more cells per glomerulus than IL-22 or IL-22R KO mice. By flow cytometry, we further found fewer infiltrating macrophages into the kidney of IL-22 or IL-22R KO MRL/lpr mice than MRL/lpr mice, which confirmed by immunohistochemical analyses, but there was no significant difference in infiltrating T cells, B cells and neutrophils between IL-22 or IL-22R KO MRL/lpr mice and MRL/lpr mice, suggesting IL-22 may mainly affect the infiltration of Macrophages. Macrophages are believed to contribute to the pathogenesis of LN. In human and murine LN, renal macrophages infiltration is associated with active disease and poor outcomes (25, 26). Our study revealed that IL-22 may contribute to the pathogenesis of LN by promoting the infiltration of macrophages into the kidney.

There is no definitive evidence that IL-22 has capacity to act on hematopoietic cells including macrophages, though one report identified that incubation of DCs with rIL-22 augmented its ability to promote allergic inflammation (27). But binding IL-22 to IL-22R can induce nonhematopoietic cells to produce a variety of inflammatory mediators including

cytokines, chemokines, etc (28). By RNA-Seq, we found that gene expression of local chemokines in the kidney of lupus-prone mice was altered, and qRT-PCR further confirmed down-regulated expression of CXCL1, CCL2, and CXCL10 in the kidney of IL-22 or IL-22R KO MRL/lpr mice compared with MRL/lpr mice. Among these chemokines, CCL2 and CXCL10 are demonstrated to play a critical role in promoting the recruitment of monocytes/macrophages into the kidney in SLE (29–31). Though little is known whether CXCL1 recruit inflammatory cells to the kidney in SLE, recent report showed that CXCL1 signaling can induce the infiltration of monocytes in heart and arteriogenic collaterals (32, 33). Our experiments on primary mouse kidney epithelial cells further confirmed the hypothesis. Our study demonstrated that primary kidney epithelial cells from MRL/lpr mouse stimulated with rIL-22 expressed higher levels of CCL2, CXCL10, and their supernatant recruited more macrophages. These findings indicated that IL-22 may upregulate the expression of CCL2 and CXCL10 and then promote the infiltration of macrophages into the kidney in the pathogenesis of LN.

In the present study, *in vivo*, lower phosphorylation of STAT3 in the kidney of IL-22 or IL-22R KO MRL/lpr mice was found than that in MRL/lpr mice. *In vitro*, with rIL-22 stimulation, levels of phosphorylation of STAT3 in primary kidney epithelial cells and HK2 cells were significantly increased, suggesting that STAT3 signaling pathway may be activated by IL-22-IL-22R in both human and murine kidney cells. Meanwhile, utilizing HK2 cells revealed that following the phosphorylation of STAT3 activated by rIL-22, levels of CCL2 and CXCL10 was highly expressed, and in turn, with inhibition of STAT3 pathway, levels of CCL2 and CXCL10 was decreased. These results phenocopied that of IL-22/IL-22R deficiency mice during LN as above demonstrated, implicating a requirement for STAT3 *in vivo* IL-22-mediating signaling.

In conclusion, we found that IL-22 secreted mainly by local ILC3s acted on kidney cells to enhance STAT3 phosphorylation and over-expression of CCL2 and CXCL10, thus promoting infiltration of macrophages into the kidney and then aggravating LN, and that IL-22 or IL-22R KO delayed the disease development of lupus-prone mice. These data revealed that IL-22 may play a pathogenic role in LN, and therefore, blockade of IL-22 or IL-22R may represent an attractive new strategy for treatment of LN.

## 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 below: NCBI SRA, BioProject PRJNA648341.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the Second Affiliated Hospital, College of Medicine, Zhejiang University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the ethics committee of the Second Affiliated Hospital, College of Medicine, Zhejiang University.

## AUTHOR CONTRIBUTIONS

Research studies were designed by LH, XY, and QW. Experiments were performed and data acquired by LH, JH, LC, and YZ. Data were analyzed by LH, JH, LC, and YZ. LH and XY 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.2021.584414/full#supplementary-material

**Supplementary Figure 1 |** The number of IL-22+ T cells in kidney. The absolute number of IL-22$^+$ T cells (IL-22$^+$CD3$^+$) in the kidneys from 6-weeks or 24-weeks-old MRL/Fas Mice were analyzed by flow cytometry. Data were expressed as mean ± SD, and are representative of three independent experiments. T-test was used for comparison between two groups (*$P$<0.05, **$P$<0.01, ***$P$<0.001).

**Supplementary Figure 2 |** Isotype control for Lin in kidney. The isotype control for Lin in the kidneys from 24W MRL/Fas Mice by flow cytometry.

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ORIGINAL RESEARCH
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# Restoration of NK Cell Cytotoxic Function With Elotuzumab and Daratumumab Promotes Elimination of Circulating Plasma Cells in Patients With SLE

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Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by multiple cellular and molecular dysfunctions of the innate and adaptive immunity. Cytotoxic function of NK cells is compromised in patients with SLE. Herein, we characterized the phenotypic alterations of SLE NK cells in a comprehensive manner to further delineate the mechanisms underlying the cytotoxic dysfunction of SLE NK cells and identify novel potential therapeutic targets. Therefore, we examined PBMC from SLE patients and matched healthy controls by single-cell mass cytometry to assess the phenotype of NK cells. In addition, we evaluated the cell function of NK cells (degranulation and cytokine production) and the killing of B cell subpopulations in a B cell-NK cell *in vitro* co-culture model. We found that SLE NK cells expressed higher levels of CD38 and were not able to adequately upregulate SLAMF1 and SLAMF7 following activation. In addition, ligation of SLAMF7 with elotuzumab or of CD38 with daratumumab on SLE NK cells enhanced degranulation of both healthy and SLE NK cells and primed them to kill circulating plasma cells in an *in vitro* co-culture system. Overall, our data indicated that dysregulated expression of CD38, SLAMF1 and SLAMF7 on SLE NK cells is associated with an altered interplay between SLE NK cells and plasma cells, thus suggesting their contribution to the accumulation of (auto)antibody producing cells. Accordingly, targeting SLAMF7 and CD38 may represent novel therapeutic approaches in SLE by enhancing NK cell function and promoting elimination of circulating plasma cell.

**Keywords: systemic lupus erythematosus (SLE), SLAMF, CD38, elotuzumab, daratumumab, NK cells, CD150/SLAMF1 receptor, CD319/SLAMF7/CS1**

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disease that mainly affects women of childbearing age (1, 2). The pathogenesis remains elusive but includes alterations of the immune system leading to the production of autoreactive cells, autoantibodies and the formation of immune complexes that ultimately damage organs (1, 3). Although important progress was made over the last decades toward the development of new treatments, management of SLE still relies on the use of corticosteroids and immunosuppressive agents that non-specifically target immune cells. Despite the well-established importance of autoreactive B cells and autoantibody production in the pathogenesis of the disease (1), treatments based on B cell depletion have only been moderately successful so far (4). In this context, understanding the role of other immune cells involved in the pathogenesis of SLE and their link with antibody-producing cells is taking a center stage in the development of new therapies. Among the various cellular abnormalities that characterize SLE, Natural Killer (NK) cells' dysfunction has been supported by various studies (5–8). NK cells are innate lymphocytes that play a pivotal role in the immune surveillance (9), through the recognition of healthy cells and the elimination of damaged or infected cells. NK cells from SLE patients are reduced in number in the peripheral blood, show impaired cytokine production upon stimulation, reduced cytotoxicity, and defective antibody-dependent cellular cytotoxicity (5). However, their exact role in the pathogenesis of lupus remains elusive.

In the present study, we used single-cell mass cytometry to perform a comprehensive phenotypic analysis of healthy and SLE NK cells. We sought to identify how these alterations are linked to the altered function of SLE NK cells and might represent therapeutic options to treat SLE.

## MATERIALS AND METHODS

### SLE Patients and Controls

SLE patients (N=44) were diagnosed according to the American College of Rheumatology classification criteria and/or the Systemic Lupus International Collaborating Clinics (SLICC) criteria (10, 11), and were recruited from the Division of Immunology and Allergy at Centre Hospitalier Universitaire Vaudois (CHUV). All patients and controls were included in the Swiss Systemic Lupus Erythematosus Cohort Study (SSCS) (12). Characteristics of the SLE patients included in this study are provided in **Table 1**.

Age-, sex-, and ethnicity-matched healthy individuals were chosen as controls. Disease activity score was measured using the SLE Disease Activity Index (SLEDAI) scoring system. We categorized patients into three groups of disease activity: inactive (SLEDAI 0-3), moderate (SLEDAI 4-10) and active (SLEDAI >10).

### Cell Isolation

Peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation (FICOLL 400, Merck, Switzerland). PBMC were cryopreserved in liquid nitrogen.

**TABLE 1** | Demographic characteristics of SLE patients (N=44) included in the study.

| Characteristic | Value |
|---|---|
| Age, years | |
| Median | 46 |
| Range | 24-73 |
| Sex | |
| Female | 37 |
| Male | 7 |
| Ethnicity | |
| Caucasian | 40 |
| Asian | 3 |
| Hispanic | 1 |
| SLE disease activity | |
| Inactive (0-3) | 21 |
| Moderate (4-10) | 15 |
| Active (>10) | 8 |
| Treatments | |
| Naïve | 7 |
| Hydroxychloroquine only | 11 |
| Other immunomodulatory drugs | 25 |

### Cell Culture

Cells were cultured in RPMI (Gibco; Life Technologies) containing 10% heat-inactivated FBS (Institut de Biotechnologies Jacques Boy), 100 IU/ml penicillin and 100 µg/ml streptomycin (Bio Concept), hereafter referred to as complete RPMI (cRPMI).

### Antibodies

A complete list of mass cytometry, flow cytometry and purified antibodies is provided in the **Supplementary Table 1**.

Some antibodies for the mass cytometry assay were conjugated in our facility (MaxPar® X8 multimetal labeling kit, Fludigm). Briefly, the MaxPar® polymer is loaded with the metal, and then the antibody is partially denatured to allow its conjugation to the polymer. Finally, the metal bound polymer is conjugated to the antibody.

### Mass Cytometry

Cryopreserved PBMC from SLE patients and matched healthy controls were thawed, resuspended in cRPMI, stimulated with cytokines or left unstimulated as mentioned in the figures. Cells were stained for live/dead with cisplatin 50 µg (5min, room temperature (RT)), barcoded with CD45-metal conjugated antibodies (20min, RT) and then pooled. Next, cells were incubated with metal conjugated antibody mix (20min, RT). Cells were washed and fixed with 2.4% paraformaldehyde (10 min; RT). Labeled samples were acquired on a Helios Cytof System (Fluidigm). Flow cytometry standard (FCS) files were normalized to EQ Four Element calibration beads using CyTOF software. FCS files were debarcoded using Cytobank (Beckman Coulter).

### Mass Cytometry Data Analysis

Manual gating of FCS files was performed using FlowJo™ Software version 10.2 (Becton, Dickinson and Company; 2019). Data analysis was performed using R software (version 3.5.1.). Manually gated cell populations were imported into R

environment and single cell expression data were transformed using hyperbolic inverse sine (with cofactor 5) (13). Dimensionality reduction and 2-dimensional visualization were done using the Barnes-Hut implementation of t-stochastic neighboring embedding algorithm (Rtsne package). Unsupervised clustering analysis on cell populations were performed using self-organizing map in combination with consensus clustering (FlowSOM package) in order to define 4 different clusters.

For the analysis of NK cells, we merged two experiments designed with two different panels using CytofMerge (14) with default settings. The CytofMerge methodology is based on the k-nearest neighbor algorithm and a set of common markers in order to impute the value of missing markers by taking the median values of from the k most similar cells.

## NK Cells Cytokine Production and Degranulation

PBMCs were thawed and resuspended in cRPMI. For evaluation of degranulation, NK cells were stimulated with IL-15 (50ng/ml), IL-18 (50ng/ml) or a combination of IL-2 (50ng/ml) and IL-12 (20ng/ml). For evaluation of NK cell activation with monoclonal antibodies cells were resuspended in cRPMI with IL-15 (1ng/ml). Cells were then stimulated with or without cytokines (IL-2 and IL-12, 50ng/ml and 20ng/ml respectively) with the following antibodies: SLAMF1 A12 (5µg/ml), SLAMF7 162.1 (5µg/ml), elotuzumab (0.1µg/ml), daratumumab (1µg/ml), elotuzumab and daratumumab (0.1µg/ml and 1µg/ml respectively) and incubated for either 6 or 18 hours at 37°C. BD GolgiPlug™, BD GolgiStop™ and CD107a-PE were added 6h before readout.

After incubation, cells were stained with Live/Dead Aqua and cell surface antibodies:CD3-BUV737, CD4-PB, CD8-BV605, CD19-FITC, CD56-BUV395. After permeabilization with BD Cytofix/Cytoperm™ kit, cells were stained with IFNγ-AF700, TNFα-APC. Finally, cells were fixed in BD CellFIX™ and stored at 4°C until data acquisition on LSR Fortessa™ (BD Bioscience).

## NK and B Cells Co-Culture

PBMC cells from HC were thawed and sequential positive selection of CD19+ and CD56+ cells was performed (human microbeads, Miltenyi positive selection kits) using the AutoMACS® ProSeparator (Miltenyi Biotec). B cells were stained with CFSE (LifeTech). All cells were resuspended in cRPMI with IL-15 (1ng/ml).

NK cells were incubated for 30minutes at 37°C with the following stimulation conditions: unstimulated, SLAMF1 A12 (5µg/ml), SLAMF7 162.1 (5µg/ml), elotuzumab (0.1µg/ml), daratumumab (1µg/ml), elotuzumab and daratumumab (0.1µg/ml and 1µg/ml respectively). After incubation NK cells were washed and B cells were added (in cRPMI with IL-15) at a 2:1 ratio (NK min 500'000 cells, max 1Mio; B cells min 250'000, max 500'000 cells) and incubated for 5.30hours. After incubation cells were washed and stained with Live/Dead Aqua, CD56-BUV395, CD20-PB, CD21-AF700, CD27-PeCy7, CD38-ECD, SLAMF7-PE. Finally, cells were fixed in CellFIX™ and stored at 4°C until data acquisition on a LSR Fortessa™.

## Depletion Assay

PBMCs were thawed and CD3 negative cells were isolated (EasySep™ Human CD3 Positive Selection Kit II, StemCell Technologies). Cells were resuspended in cRPMI with IL-15 (1ng/ml), and the following stimulation conditions were added: not stimulated, SLAMF1 (5µg/ml) with cytokines (IL-2 (50ng/ml) and IL-12 (20ng/ml)), elotuzumab (0.1µg/ml) with cytokines, daratumumab (1µg/ml) with cytokines and HLA-DR (0.005µg/ml) with cytokines. CD3 negative cells were then incubated for either 6 or 18 hours at 37°C. After incubation, cells were stained extracellularly with Live/Dead Aqua, CD3-BUV737, CD19-FITC, CD20-PB, CD27-AF700, CD38-ECD, CD56-BUV395, SLAMF7-PE. Cells were then washed in annexin buffer (10X Annexin V Buffer, BD Pharmingen) and stained with Annexin V-APC. Cells were stored at 4°C until data acquisition on LSR Fortessa™, for maximum 2h.

## Statistics

Statistical analysis were performed using GraphPad Prism (version 8). Specifications of tests exploited and sample size for each experiment are mentioned in the figure descriptions. In a general manner, Mann-Whitney test was used for comparison between two groups with non-normal distribution (normality was assessed with Shapiro-Wilk test). Kruskal-Wallis test was used for the comparison of multiple groups with non-normal distribution and p-values were adjusted for multiple tests using Dunn's method. One-way ANOVA was used for the comparison of multiple groups with normal distribution and p-values were adjusted for multiple tests using Sidak's method. Two-way ANOVA was exploited for the comparison of multiple groups and p-values were adjusted for multiple tests using Sidak's method. Two-way ANOVA was exploited for multiple comparisons within a group and p-values were adjusted for multiple tests using Tukey's method. A p-value lower than 0.05 was considered significant.

## Study Approval

Informed written consent was obtained from all participants prior to inclusion and the study was approved by the Institutional Review Board (SwissEthics 2017-01434), in compliance with the Declaration of Helsinki.

# RESULTS

## NK Cells Are Reduced in Numbers and Their Function Is Impaired in SLE Patients

As previously reported, SLE patients display a significant decrease in the absolute numbers of NK cells compared to sex-, age- and ethnicity- matched healthy controls (HC; **Figures 1A, D**). Percentage and/or absolute numbers of CD56+CD16+ and CD56hiCD16- NK cell subsets are reduced in SLE patients (**Figures 1B, D**). Decreased NK cell numbers correlate with disease activity, as patients with higher disease activity display a more profound reduction in NK cell numbers compared to patients with inactive disease and HC (**Figure 1C**).

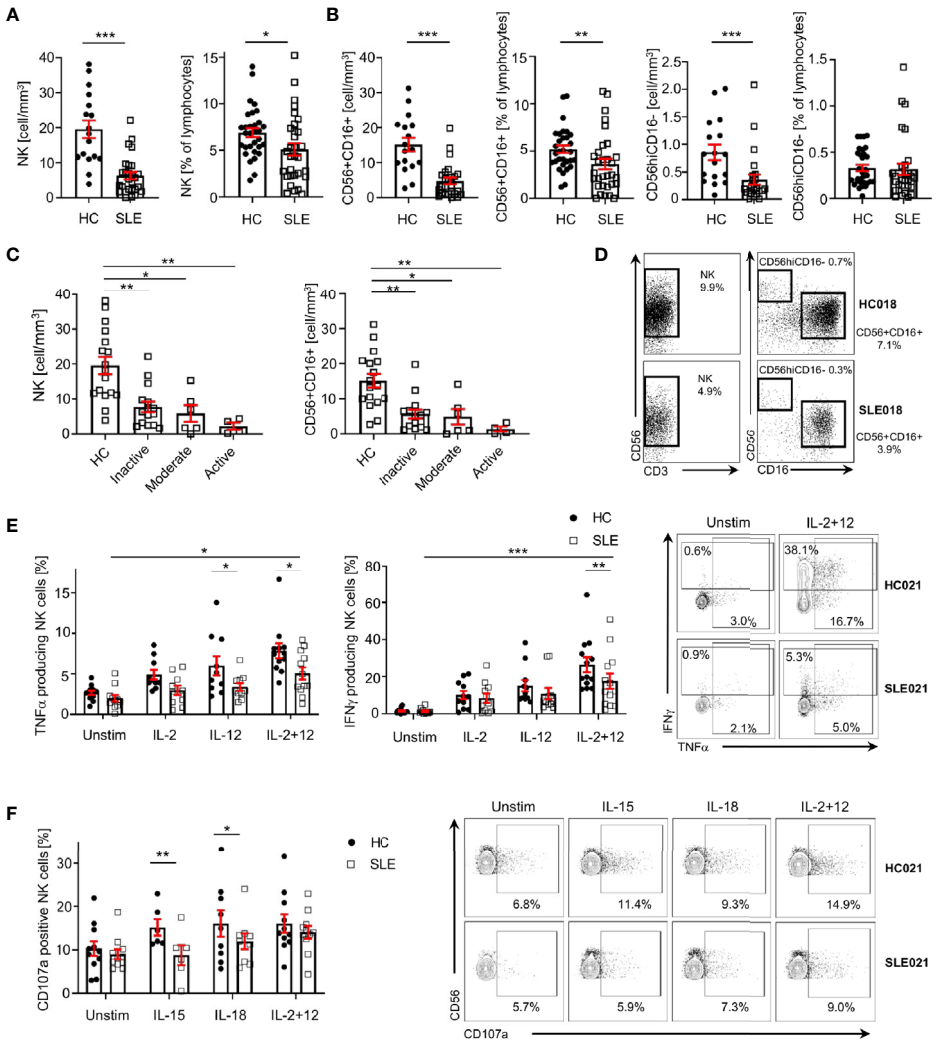


**FIGURE 1** | NK cells are decreased and dysfunctional in patients with SLE. **(A)** Total NK cells and **(B)** CD56+CD16+ and CD56hiCD16- NK subpopulations in SLE patients and HC are shown as absolute number (HC=17, SLE=27; Mann-Whitney Test) and percentage of total lymphocytes (HC=31, SLE=31; Mann-Whitney Test). **(C)** Total NK cells and CD56+CD16+ absolute number according to SLE disease activity (HC=17, Inactive=15, Moderate=6, Active=4; Kruskal-Wallis Test with Dunn's multiple comparison test). **(D)** Representative dot-plot of NK cells (left) and subpopulations (right) staining gated on live CD45+CD14-CD7+CD20-CD19- cells. The percentages of NK, CD56+CD16+ and CD56hiCD16- refer to % of total lymphocyte count. **(E)** Cumulative results and representative dot-plot showing NK cell cytokines production in SLE and HC after overnight stimulation (IFNγ HC=12, SLE=12; TNFα HC=13, SLE=13; mixed-effects analysis and two-way ANOVA with Sidak's multiple comparison test). **(F)** Cumulative results and dot-plot showing NK cell degranulation (CD107a+ cells) after overnight stimulation in SLE and HC (HC=11, SLE=11; two-way ANOVA and Sidak's multiple comparison test). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

To examine the function of NK cells in SLE, we stimulated NK cells with a combination of IL-2 and IL-12, which promoted the production of IFNγ and TNFα by SLE NK cells (**Figure 1E**), although significantly less compared to HC (**Figure 1E**). In response to IL-15 and IL-18, the degranulation of SLE NK cells is impaired compared to HC, as illustrated by the reduced frequency of CD107a+ NK cells in SLE patients (**Figure 1F**). Stimulation with IL-2 and IL-12 provided a strong enough stimulation to activate SLE NK cells degranulation as effectively as in HC (**Figure 1F**). Collectively, our data indicates that SLE NK cells display impaired cytokine production and reduced degranulation in response to activation with different cytokines.

## Phenotypic Alterations of SLE NK Cells

We exploited single-cell mass cytometry to decipher the extracellular phenotype of SLE NK cells. Our panels include lineage markers for T cells, B cells, NK cells, monocytes and dendritic cells. The gating strategy is presented in **Supplementary Figure 1**. Various markers that characterize NK cells subpopulations, as well as markers that have been shown to be aberrantly expressed on other cell subsets in SLE were examined.

These include CD25, CD38, PD-1, activation receptors (NKp46, NKG2D, DNAM-1), inhibitory receptor (NKG2A. KIR2DL, KIR3DL) and receptors belonging to the SLAMF family, including SLAMF1 (CD150), SLAMF2 (CD48), SLAMF3 (CD229), SLAMF4 (CD244, 2B4), SLAMF5 (CD84), SLAMF6 (CD353, NTB-A) and SLAMF7 (CD319, CRACC, CS-1).

Our data indicates that CD38 is expressed at a higher level in total SLE NK cells (CD3-CD14-CD7+CD19-CD56+) (**Figure 2A**; **Supplementary Figure 2A**). This difference is independent of disease activity (**Figure 2B**) and is also observed in CD56+CD16+ and CD56hiCD16- NK subsets (**Figures 2C, D**). Similar results were found for treatment-naïve SLE patients (**Supplementary Figure 2B**), suggesting that this alteration is not drug-related. We applied clustering analysis on pre-gated NK cells and identified four cell clusters (**Figure 2E**) that do not differ in frequency between HC and SLE, indicating that there is no NK subpopulation that is characteristic of SLE patients and could be used as a biomarker. Interestingly, we observed that cluster 2 has a CXCR5 expressing subpopulation and cluster 4 one expressing KIR3DL1, which are only present in SLE patients (**Figure 2E**). Further research is warranted to understand the pathophysiological importance of these subpopulations.

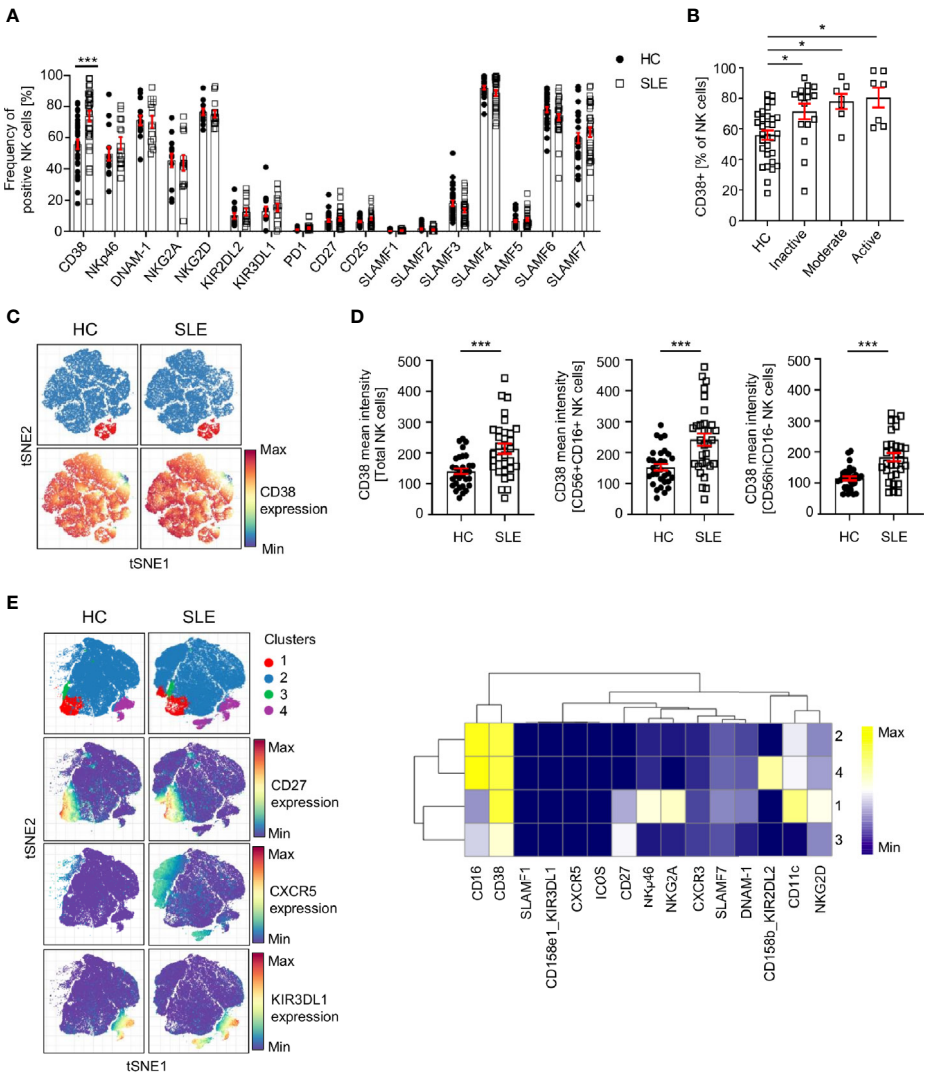


**FIGURE 2** | Analysis of NK cell surface markers in SLE patients and controls by single-cell mass cytometry. **(A)** Percentage of NK cells expressing the indicated cell surface markers in HC and SLE (HC=33, SLE=33; two-way ANOVA and Sidak's multiple comparison test). **(B)** Frequency of CD38+ NK cells in SLE patients according to the SLE disease activity (HC=31, Inactive=16, Moderate=7, Active=7; Kruskal-Wallis Test with Dunn's multiple comparison test). **(C)** Representative t-SNE analysis showing the expression of CD38 on SLE and HC NK cells (down-sample HC=30'000 cells, SLE=30'000 cells; blue: CD56+CD16+ NK cells; red: CD56CD16hi NK cells). **(D)** Cumulative results showing the expression (mean intensity) of CD38 on total NK, CD56+CD16+ and CD56hiCD16- NK cells (HC=31, SLE=31; Mann-Whitney test). **(E)** t-SNE analysis (down-sample HC=10'000 cells, SLE=10'000 cells) and heatmap showing NK cell clusters in HC and SLE patients. Data represent mean ± SEM (*P < 0.05, ***P < 0.001). HC, healthy controls.

## SLE NK Cells Fail to Upregulate SLAMF1 and SLAMF7 in Response to Cytokine Stimulation

Since the response of SLE NK cells to cytokine stimulation is impaired and considering that the function of NK cells relies on their extracellular phenotype (9), we examined the expression of NK cells surface receptors following stimulation with IL-2 and IL-12 for up to 48h in SLE patients and matched HC. We observed a marked upregulation of SLAMF1 and SLAMF7 on NK cells from HC, 11-fold and 9-fold respectively at 48h of cytokines stimulation, compared to unstimulated cells (**Figure 3A**). Interestingly, PD-1 also shows a 5.6-fold increase at 48h of stimulation (**Figure 3A**). Of note, CD38 is not significantly upregulated after NK cells are activated with cytokines (**Figure 3A**). However, NK cells from SLE patients failed to upregulate certain cell surface receptors to the same extent as HC (**Figures 3B, C**; **Supplementary Figure 3**). More specifically, although SLAMF1 expression is also upregulated on SLE NK cells upon cytokine stimulation, the upregulation is less prominent than that observed in HC (**Figures 3B, C**). Similarly, NK cells from SLE patients fail to upregulate SLAMF7 and PD-1 to the same extent as HC (**Figures 3B, C**; **Supplementary Figure 3**). Overall, our data shows that NK cells from SLE patients fail to adequately upregulate SLAMF1 and SLAMF7 in response to cytokine stimulation.

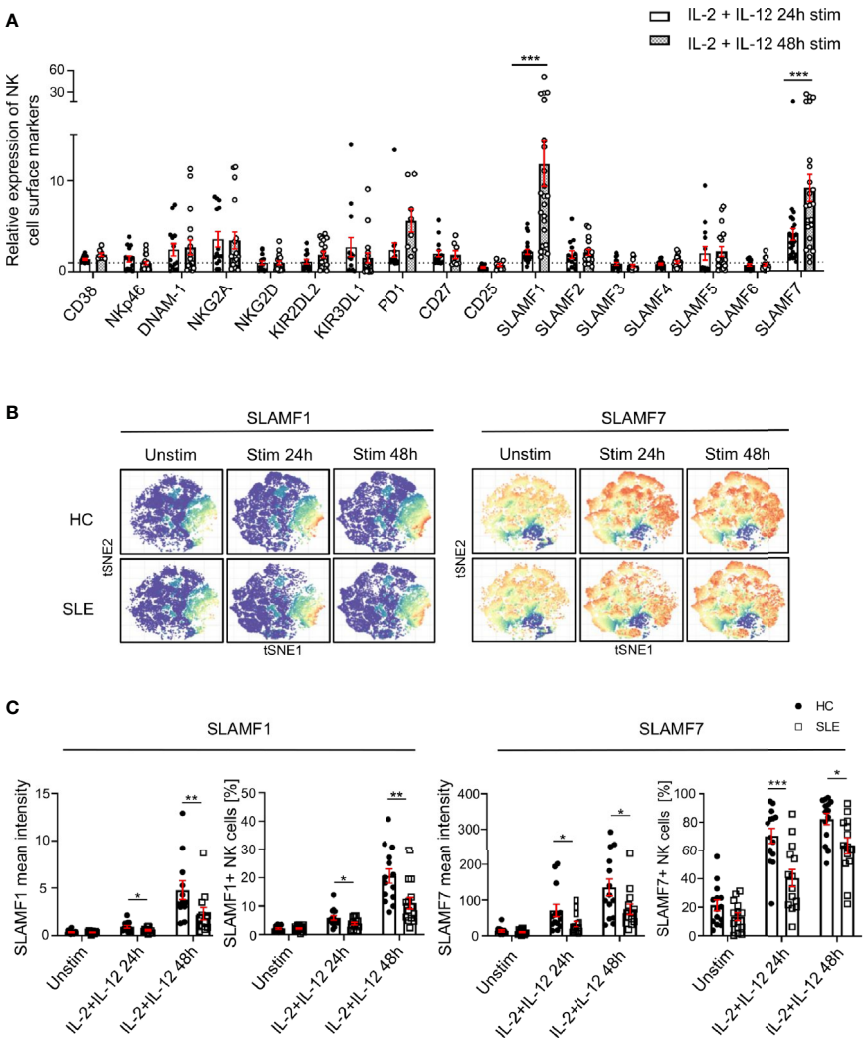


**FIGURE 3** | SLAMF1 and SLAMF7 fail to be properly upregulated on the surface of SLE NK cells after activation with cytokines. **(A)** Expression of surface markers after 24h and 48h of stimulation with cytokines on healthy NK cells, standardized to their level of expression on unstimulated cells (HC=23, SLE=23; Mixed-effects analysis with Sidak's multiple comparison test). **(B)** t-SNE presentation of the expression level of SLAMF1 and SLAMF7 in HC and SLE patients before and after stimulation with cytokines (down-sample HC=12'000 cells, SLE=12'000 cells). **(C)** Comparison of expression of NK cell surface markers after 24h and 48h of stimulation with cytokines between HC and SLE patients as mean intensity (above) and frequency (below) (HC=14, SLE=14; two-way ANOVA and Sidak's multiple comparison). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

## Engagement of CD38 and SLAMF7 With Specific Monoclonal Antibodies (mAb) Enhances the Function of Healthy and SLE NK Cells

We investigated how the engagement of SLAMF1, SLAMF7 and CD38 with mAb influences NK cell function, by examining the production of cytokines, degranulation and cell viability after 6h and 18h. Ligation with elotuzumab, a humanized anti-SLAMF7 mAb approved to treat relapsing multiple myeloma (15), promotes NK cells degranulation and IFNγ production after 18h, whereas no significant NK cells activation was observed at 6h of stimulation in HC (**Figure 4A**). Another clone of anti-SLAMF7 mAb (clone 162.1), which has been shown to enhance the cytotoxic response of SLE CD8+ T cells in response to viral antigen (16), did not produce any significant effect on NK cells degranulation (**Figure 4A**). NK cells stimulation with daratumumab, a mAb that agonizes CD38, strongly enhanced NK cells degranulation, IFNγ and TNFα production (**Figure 4A**). Interestingly, in healthy controls daratumumab effectively promotes NK cell degranulation and production of IFNγ and TNFα after 6h of stimulation, whereas elotuzumab takes longer to activate NK cells (18h) and only promotes degranulation and production of IFNγ, but not TNFα. Stimulation of NK cells with anti-SLAMF1 mAb (clone A12) did not result in any effect on degranulation or cytokine production (**Figure 4A**).

Next, we examined the effect of SLAMF7 ligation with elotuzumab and of CD38 with daratumumab on NK cells from SLE patients. Based on our results from healthy controls, we used anti-SLAMF1 (clone A12) as negative control. We observed that in SLE NK cells both daratumumab and elotuzumab promote degranulation, after 6h and 18h respectively, to the same extent as in HC (**Figures 4B, C**). However, compared to results obtained in healthy controls, daratumumab and elotuzumab do not promote cytokine production by SLE NK cells (**Supplementary Figure 4A**). Furthermore, the magnitude of degranulation at 18h is, for both HC and SLE NK cells, more prominent following ligation with daratumumab (6-fold) compared to elotuzumab (4-fold) (**Figure 4C**). In addition, we examined NK cells viability after stimulation with elotuzumab and daratumumab. Both antibodies lead to a slight increase in mortality of NK cells compared to the control condition (**Supplementary Figure 4B**). Eventually, we examined the effect of elotuzumab and daratumumab on other lymphocyte subsets and observed no effect on the viability or activation of

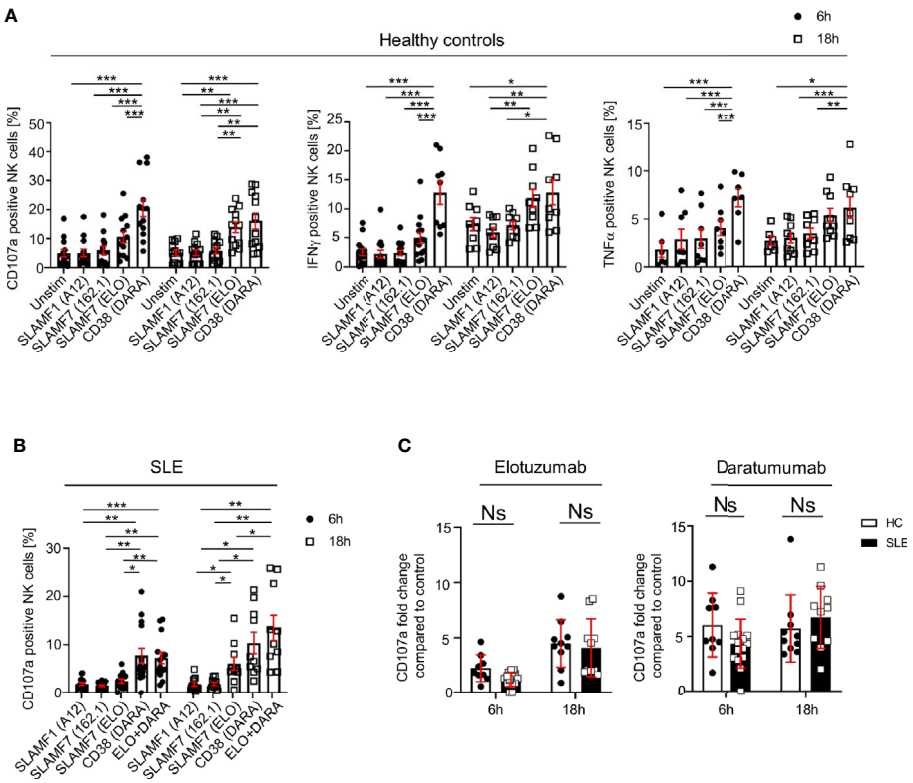


**FIGURE 4** | Engagement of SLAMF7 and CD38 with specific mAb enhances the function of healthy and SLE NK cells. **(A)** Degranulation (CD107a) and production of cytokines (IFNγ and TNFα) in NK cells of healthy controls after stimulation with daratumumab (N=14) and elotuzumab (N=12, Mixed-effect analysis with Tukey's multiple comparison test). **(B)** Degranulation (CD107a+) in NK cells of SLE patients after stimulation with daratumumab (6h N=15, 18h N=10) and elotuzumab (6h N=14, 18h N=10; two-way ANOVA analysis and Sidak's multiple comparison test). **(C)** Fold change of CD107a compared to control after 6h or 18h stimulation with elotuzumab or daratumumab in HC and SLE patients (HC 6h=9, HC 18h=10, SLE 6h=15, SLE 18h=10; mixed-effect analysis with Sidak's multiple comparison). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

CD4+, CD8+ T cells and B cells (**Supplementary Figures 5** and **6**).

Altogether, our data shows that elotuzumab and daratumumab specifically activate SLE NK cells by promoting their cytotoxic activity.

## Expression of CD38, SLAMF1, and SLAMF7 Characterizes SLE Circulating Plasma Cells

The above-mentioned cell surface receptors are important in cell-to-cell contact. Therefore, to understand their relevance for the pathophysiology of SLE, we investigated their expression on other major lymphocyte populations. We manually gated on CD4+, CD8+, DN T cells (CD4- CD8- double negative T cells), B cells and NK cell. We then exploited t-SNE analysis to visualize the expression of CD38, SLAMF1 and SLAMF7 on these cell populations (**Figure 5A**) and quantified their relative expression levels in SLE patients and HC (**Figure 5B**).

Other than NK cells, our data indicates that all three receptors are expressed at higher levels on SLE B cells compared to HC. CD38 expression did not show any difference in its expression between SLE and HC in any other lymphocyte population included in this study (**Figures 5A, B**). The expression of SLAMF1 is significantly higher on B cells and on CD4+ T cells from SLE patients as previously described (17). In addition, our data shows that SLAMF7 is increased on total B cells from SLE patients, despite a low expression level compared to other lymphocytes such as NK cells, CD8+ and DN T cells. We further examined the expression of these receptors on B cell subpopulations. A t-SNE analysis of naïve B cells (CD19+ CD27- IgD+), non-naïve B cells (CD19+ which are not CD27- IgD+) and circulating plasma cells (CD19+ CD20- CD27+ CD38+ IgD-), showed that all three molecules are expressed at a higher level on circulating plasma cells compared to other B cell subpopulations (**Figures 5C, D**). Moreover, the level of expression of all the three receptors is increased in SLE circulating plasma cells compared to HC, suggesting that these molecules could contribute to the dysfunction of SLE B cells.

## Activation of SLE NK Cells With mAb Directed Against CD38 and SLAMF7 Promotes the Killing of Peripheral Blood Plasma Cells

We evaluated whether the activation of NK cells can promote the killing of SLE peripheral blood plasma cells. We generated a NK-B cell *in vitro* co-culture system, in which we pre-stimulated NK cells of HC with elotuzumab or daratumumab, then co-cultured them with autologous B cells and measured the mortality of B cell subsets.

First, our data shows that elotuzumab (18h) and daratumumab (6h) can efficiently kill circulating plasma cells, leading to 2.1 and 2.7 fold more dead cells compared to negative control (SLAMF1 stimulation) respectively (**Figure 6A**). Furthermore, when NK cells are activated with either mAb they kill circulating plasma cells specifically, sparing other B cell subpopulations, such as naïve, activated, resting and tissue like memory cells (**Figure 6A**). Second, we observed that the presence of NK cells is necessary to achieve significant killing of circulating plasma cells, although both mAb alone have a minor impact on circulating plasma cell mortality (**Figure 6B**).

Due to the restrictions in the SLE sample size that we can obtain, we could not repeat this assay in SLE patients. Accordingly, we isolated CD3- cells and stimulated them with mAb. We observed that at 6h, treatment with daratumumab significantly killed circulating plasma cells of SLE patients to the same extent as in the matched HC (**Figure 6C**). In conclusion, our results strongly suggest that these mAb act through the activation of SLE NK cells and effectively kill SLE circulating plasma cells.

# DISCUSSION

We exploited single-cell mass cytometry to decipher the phenotypic alterations that characterize SLE NK cells. Our data identified CD38 as being highly expressed on SLE NK cells compared to HC. Moreover, we observed that SLE NK cells fail to properly upregulate SLAMF1 and SLAMF7 when activated with cytokines; two receptors that play an important role in cell-to-cell interaction. We showed that these three receptors are also highly expressed on SLE peripheral blood plasma cells, a cell population that contributes to the production of autoantibodies in SLE. In addition, we demonstrated that mAb directed against CD38 and SLAMF7 receptors enhance the degranulation of SLE NK cells and selectively promote the killing of peripheral blood plasma cells. Overall, our data suggests that the dysregulation of SLAMF1 and SLAMF7 on the surface of SLE NK cells contribute to their dysfunction and might impair their interaction with plasma cells, resulting in an accumulation of autoantibody producing cells. Additionally, targeting NK cells with activating mAb may represent an attractive direction to eliminate autoantibody-producing cells in SLE.

SLAMF1 and SLAMF7 belong to the signaling lymphocytic activation molecule family receptors. A unique feature of these two SLAMF members is that they act as self-ligand (18). The involvement of SLAMF molecules in SLE pathogenesis has been repeatedly reported (16–24) as well as their importance in NK cells activation and interaction with other cell types (25, 26). SLAMF1 has been reported to be expressed at a higher level on SLE B cells and CD4+ T cells compared to their healthy counterparts and its importance for SLE B cell function was previously reported (26). However, its potential role on SLE NK cells was not previously described. SLAMF7 has been shown to be highly expressed by cytotoxic cells and plasma cells. SLAMF7 displays an altered expression, function and/or regulation on SLE NK cells and CD8+ T cells (16, 19, 22), supporting a role of this molecule in SLE pathogenesis. The importance of SLAMF7 was described in multiple myeloma, where elotuzumab was approved to treat disease relapse (15). The binding of elotuzumab contributes to the elimination of myeloma cells, through various mechanisms including the activation of NK cells cytotoxic response and antibody dependent cell-mediated cytotoxicity (27). A previous

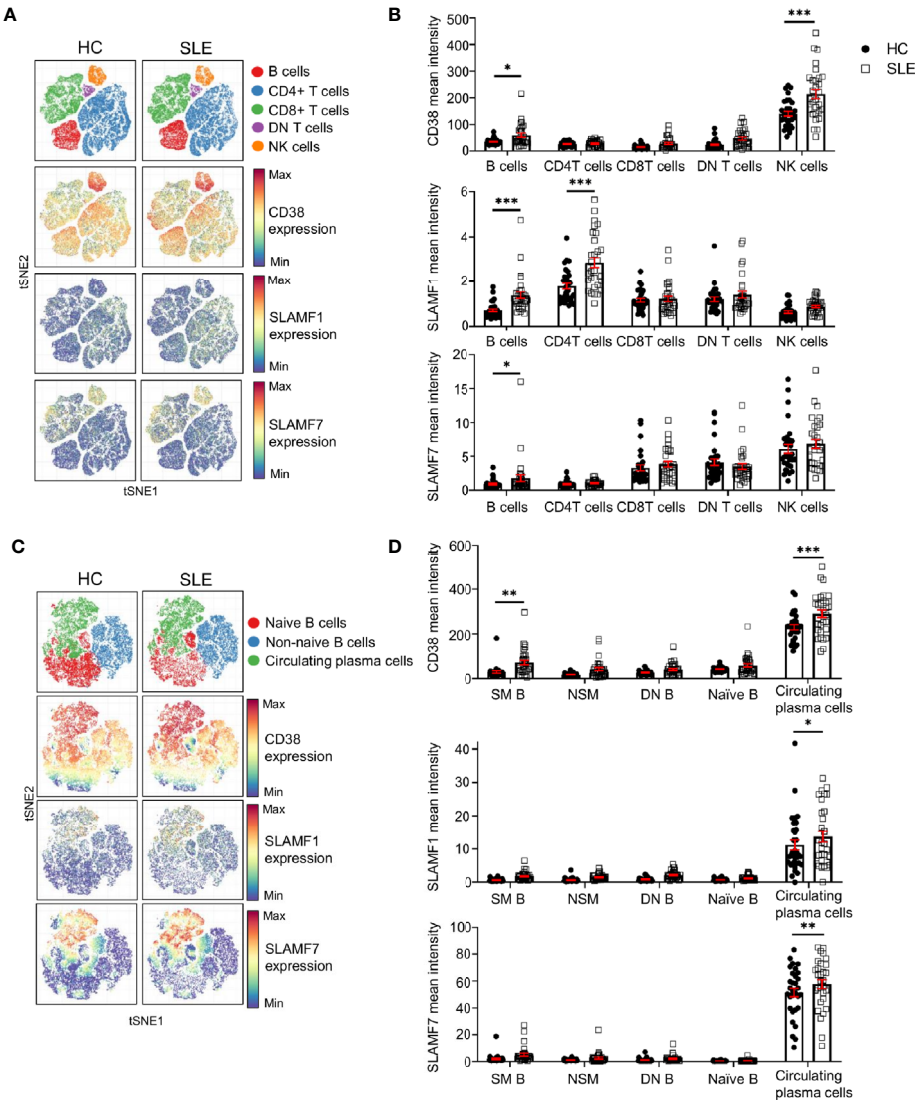


**FIGURE 5** | Expression of CD38, SLAMF1 and SLAMF7 characterizes SLE circulating plasma cells. ***(A)*** t-SNE presentation of the expression level of CD38, SLAMF1 and SLAMF7 in main lymphocyte populations for HC and SLE patients (down-sample HC=100'000 cells and SLE=100'000 cells). **(B)** Comparison of mean expression level of CD38, SLAMF1 and SLAMF7 between HC and SLE patients in main lymphocyte populations (HC and SLE=31; two-way ANOVA with Sidak's multiple comparison test). **(C)** t-SNE presentation of the expression level of CD38, SLAMF1 and SLAMF7 in B cell subpopulations for HC and SLE patients (down-sample N=10'000 cells per subpopulation HC and SLE=26). **(D)** Comparison of the mean expression level of CD38, SLAMF1 and SLAMF7 between HC and SLE patients in B cell sub-populations (HC=31, SLE =31; two-way ANOVA with Sidak's multiple comparison test, SM, switch memory; NSM, non switch memory; DN, double negative). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

study has shown that the ligation of SLAMF7 in SLE promotes the degranulation of CD8+ T cells in response to viral antigens, therefore empowering the antiviral response that is compromised in patients with SLE (16), highlighting the potential therapeutic benefit of targeting SLAMF7.

CD38 is a surface glycoprotein with ectoenzymatic functions and is expressed at high levels on plasma cells. Like SLAMF7, CD38 has been identified as a target for mAb to eliminate myeloma cells in patients with relapsing multiple myeloma with the use of anti-CD38 daratumumab (28). A recent report has shown that daratumumab represents a potential therapeutic approach to eliminate antibody-producing plasma cells in SLE patients (29). Furthermore, it has been shown to ameliorate clinical manifestations and to eliminate antibody producing plasma cells in two patients with refractory SLE (30). A subset of SLE patients who are highly susceptible to infections, exhibit an altered CD8+ T cells cytotoxic response and express a high level of CD38 on their surface (31), thus further underlining the potential benefits of targeting CD38. Our data stresses a preponderant role of NK cells in the process leading to plasma

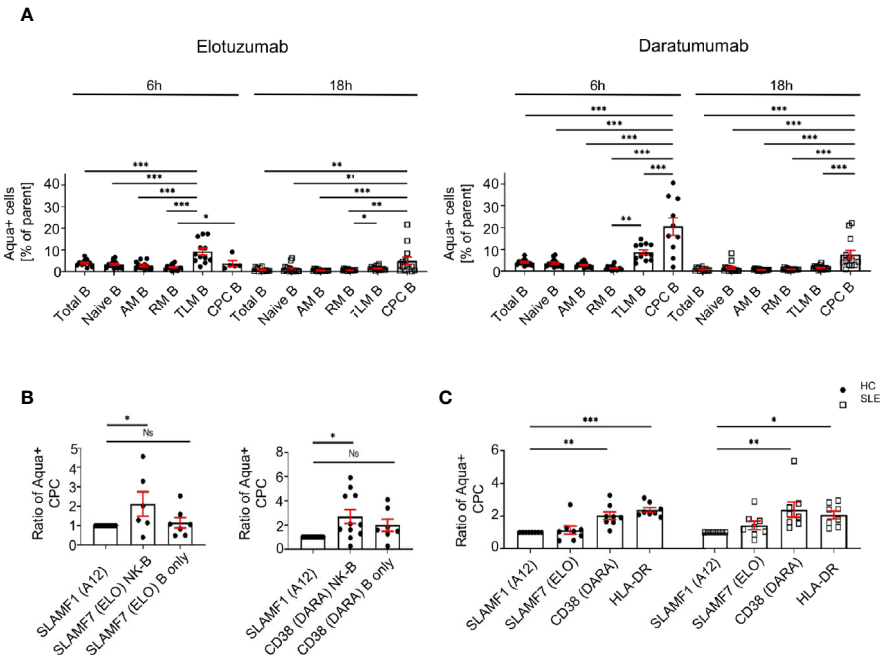


**FIGURE 6** | Activation of SLE NK cells with mAb directed against CD38 and SLAMF7 promotes the killing of peripheral blood plasma cells. **(A)** Frequency of dead cells in a NK-B cell co-culture system after 6h (N=14) and 18h (N=16) following stimulation with SLAMF1, elotuzumab or daratumumab (one-way ANOVA with Tukey's multiple comparison test). **(B)** Fold increase of dead circulating plasma cells following stimulation with daratumumab 6h (N=12) or elotuzumab 18h (N=7) in either B cells alone or B cells co-cultured with pre-stimulated NK cells (one-way ANOVA with Sidak's multiple comparison test) **(C)** Ratio of dead cells over control condition after 6h stimulation with elotuzumab or daratumumab in HC and SLE patients (HC=8, SLE=8; two-way ANOVA, Sidak's multiple comparison test). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC=healthy controls.

cell depletion by daratumumab, as stimulation of isolated NK cells with daratumumab is sufficient to promote the killing of circulating plasma cells in culture, whilst the sole exposure of B cells to the drug isn't.

As previously reported, PD-1 is also upregulated on NK cells in response to inflammatory cytokines (32). This increase is significantly altered in SLE NK cells and likely reflects SLE NK cells compromised activation status. Further investigation is warranted on this aspect.

Our study reveals interesting differences between daratumumab and elotuzumab. Elotuzumab promotes degranulation of NK cells and IFNγ production, but not the production of TNFα by NK cells. Since elevated TNFα levels have been described in SLE patients and may contribute to the pathogenesis of organ damage (33), this property could be of interest if elotuzumab was to be considered as a therapeutic option in SLE. On the other hand, SLE NK cell degranulation and elimination of antibody-producing cells *in vitro* is more robust when NK cells are activated with daratumumab compared to elotuzumab.

Our study has several limitations. First, the use of single-cell mass cytometry limits the identification of cell surface receptors to the antibodies included in our panels. Compared to RNA seq, this method monitors fewer targets but directly identifies cell surface proteins that can be targeted by therapeutic mAb. Second, further experiments are warranted to identify the individual implications of the three cell surface markers evaluated in this study in the interaction between NK cells and circulating plasma cells. So far, this aspect remains unexplored due to the limited number of circulating plasma cells available from the peripheral blood of patients and controls. We are working on plasma cell line culture system that will allow to individually silence each receptor. Finally, examination of secondary lymphoid organ and bone marrow aspirations would allow examination of B cells during their maturation process and long-lived plasma cells. However, these tissues are difficult to obtain.

In conclusion, the failure of SLAMF1 and SLAMF7 regulation on SLE NK cells might contribute to an impaired interaction between NK cells and plasma cells. This might lead to the accumulation of antibody producing plasma cells that characterizes SLE. From this point of view, restoration of NK cell cytotoxicity may contribute to the elimination of SLE plasma cells. Targeting SLAMF7 with elotuzumab and CD38 with daratumumab contributes to the elimination of antibody producing cells *in vitro* and this elimination occurs, at least in part, through the restoration of SLE NK cells degranulation. Because both elotuzumab and daratumumab are safe when used to treat multiple myeloma and appear to be well-tolerated when administrated to SLE patients, their utilization should be evaluated in controlled studies to assess their efficacy to treat SLE.

## 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 Commission cantonale d'Ethique de la Recherche sur l'être humain CER-VD (SwissEthics 2017-01434). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

DC: study design and data analysis. MH, FB, and NF: conducting experiments, data acquisition and analysis. CR and AH: recruitment of SLE patients and healthy controls. CF: responsible of CyTOF facility. MH and MS: bioinformatics analysis. DC and MH: writing and editing of 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.2021.645478/full#supplementary-material

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# Fas/FasL Signaling Regulates CD8 Expression During Exposure to Self-Antigens

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Activation of self-reactive $CD8^+$ T cells induces a peripheral tolerance mechanism that involves loss of CD8 expression. Because genetic deficiency of *Fas* and *Fasl* causes the accumulation of double-negative (DN; $CD3^+$ TCR-$\alpha\beta^+$ $CD4^-$ $CD8^-$) T cells that have been proposed to derive from $CD8^+$ cells, we decided to explore the role of Fas and FasL in self-antigen-induced CD8 downregulation. To this end, we quantified Fas and FasL induction by different stimuli and analyzed the effects of Fas/FasL deficiency during a protective immune response and after exposure to self-antigens. Our data describes how Fas and FasL upregulation differs depending on the setting of CD8 T cell activation and demonstrates that Fas/FasL signaling maintains CD8 expression during repetitive antigen stimulation and following self-antigen encounter. Together, our results reveal an unexpected role of Fas/FasL signaling and offer a new insight into the role of these molecules in the regulation of immune tolerance.

**Keywords: CD8, CD95, Fas, FasL, tolerance, double negative T cell**

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## INTRODUCTION

Fas (CD95) and FasL play essential roles in immune function that include induction of apoptosis and modulation of T cell activation (1). In mice, *Fas* deficiency causes abnormal accumulation of antigen-specific T cells during chronic (but not acute) viral infections and after T cell activation under steady state conditions (2–4). In addition, loss of function (LOF) mutations in the genes that encode Fas and FasL cause ALPS (autoimmune lymphoproliferative syndrome), a lymphoproliferative disease associated with pathological autoimmunity (5). Therefore, Fas and FasL are thought to contribute to the control of lymphoid proliferation and the maintenance of immune tolerance.

It has been proposed that lack of Fas-mediated apoptosis represents the main mechanism behind lymphoid cell accumulation in patients with ALPS (4, 6). However, Fas also plays complex non-apoptotic roles in T cells, where, depending on the context, it can promote or inhibit activation and effector differentiation (7–10). A prominent feature of humans and mice with *Fas* or *Fasl* LOF mutations is the accumulation of an unusual population of $CD3^+$ TCR-$\alpha\beta^+$ T cells that lack CD4 and CD8 (double negative; DN) (11). Because their accumulation is associated with Fas deficiency, DN T cells are thought to represent products of failed T cell apoptosis (1, 4, 12, 13). However, two

lines of evidence argue against this being the only mechanism for DN T cell accumulation: (a) in non-autoimmune mice, T cell-specific deficiency of *Fas* did not cause the accumulation of DN T cells (14); (b) a point mutation that avoided Fas palmitoylation, and therefore its recruitment into lipid rafts, abolished Fas-mediated apoptosis, but did not cause an increase in DN T cells (9). Consequently, the capacity of T cells to undergo Fas-mediated apoptosis and the accumulation of DN T cells do not seem to be mechanistically connected. This aspect of DN T cell biology holds particular relevance considering their possible role in autoimmunity (15, 16), allograft rejection (17), and anti-tumor immunity (18).

A wealth of evidence indicates that DN T cells derive from CD8αβ$^+$ T cells: (a) CD8$^+$ and DN T cells share Vβ usage and CDR3 sequences (19); (b) mice deficient in β2-microglobulin or MHC-I molecules have reduced numbers of DN T cells (20–22); (c) the *Cd8a* locus is hypomethylated in DN T cells, indicating previous transcriptional activity (23, 24); (d) CD8$^+$ T cells lose CD8 when they encounter cognate antigen presented as self (25, 26); (e) DN T cells can upregulate CD8 when they undergo homeostatic proliferation under lymphopenia (27). Importantly, generation of DN T cells is not limited to situations in which Fas/FasL function is compromised, as an increased abundance of DN T cells has been reported in a number of chronic inflammatory conditions that include systemic lupus erythematosus (15), primary Sjögren's syndrome (28), and psoriasis (29). Therefore, regulation of CD8 expression may represent an underestimated mechanism of controlling CD8 T cell function (30–32), particularly in the setting of self-antigen encounter and chronic inflammation, and the accumulation of DN T cells in patients or animals that lack Fas or FasL suggests that signaling through these molecules regulates CD8 expression. In this work, we addressed this question, using a genetic approach, to determine the role of Fas/FasL in the regulation of CD8 expression during protective and tolerance-inducing immune responses.

# MATERIALS AND METHODS

## Mice

B6.MRL-*Fas*$^{lpr}$/J (B6.*lpr*), B6Smn.C3-*Fasl*$^{gld}$/J (B6.*gld*), C57BL/6-Tg(*TcraTcrb*)1100Mjb/J (OT-I), and C57BL/6-Tg(CAG-OVAL) 916Jen/J (Act-mOVA) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). B6.*lpr* OT-I, B6.*gld* OT-I, and B6.*lpr*/*gld* OT-I were generated by breeding. Mice were housed in SPF conditions on a 12 hour light/dark cycle and had *ad libitum* access to food and water. All experiments involving mice were approved by the Animal Care and Use Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (IRE-1725).

## *In Vitro* T Cell Activation

Bone marrow-derived dendritic cells (BM-DCs) were differentiated from WT, B6.*lpr*, or B6.*gld* bone marrow cell suspensions by culturing them in full RPMI (10% FBS) in the presence of GM-CSF (20 ng/mL; Peprotech) during 8 days. BM-DCs (5 x 10$^4$), loaded with the indicated concentration of SIINFEKL, were cultured in U-bottom 96-well plates with 2 x 10$^5$ CD8$^+$ T cells isolated using CD8α$^+$ T Cell Isolation Kit II (Miltenyi Biotec) from OT-I mice in B6 (WT), B6.*lpr*, or B6.*gld* background. For activation with antibodies, 1.25 x 10$^6$ OT-I cells were cultured in 48-well plates coated with anti-CD3 and anti-CD28 (2 μg/mL). For quantification of gene expression by RT-qPCR, live T cells were sorted and lysed in TRIzol. Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Results were normalized using *Actb* and are expressed as ΔCt.

## Adoptive Transfer

OT-I cells were adoptively transferred by i.v. injection. One-day after, 10$^4$ c.f.u. of *Listeria monocytogenes* expressing recombinant OVA (LM-OVA; a generous gift from Dr. Michael J. Bevan, University of Washington) were injected i.v (33). For exposure to self-antigens, OT-I cells were adoptively transferred (i.v.) into Act-mOVA or B6 (control) mice. Transferred cells were analyzed in spleens of recipient mice at the indicated days after injection.

## Flow Cytometry

Labeled antibodies were purchased from Tonbo, Biolegend, and eBiosciences. (**Supplemental Table 1**). For staining, 1 x 10$^6$ cells were incubated with antibodies (1:100), at room temperature, for 30 min. Cells were washed twice and resuspended in PBS + 2% FCS and acquired in a FACS Aria II instrument (BD Biosciences). Data was analyzed using FlowJo software.

## Statistics

Statistical tests were calculated using Microsoft Excel and GraphPad Prism. The statistical test used and P values are indicated in each figure. In general, for comparison between two groups, paired or non-paired Student's t test was used. To compare more than 2 groups, one-way ANOVA was used. P values <0.05 were considered significant.

# RESULTS

## The Kinetics of Fas and FasL Expression Vary According to the Activation Stimulus

Expression of Fas and FasL on naïve CD8 T cells is minimal, but levels of both molecules increase following cell activation (34, 35). To determine the kinetics with which these receptors are induced by activation on CD8$^+$ T cells, we isolated OT-I cells and activated them *in vitro* using plate-bound anti-CD3 and anti-CD28, or in the presence of BM-DCs loaded with the ovalbumin-derived peptide SIINFEKL. As shown in **Figure 1A**, activation of CD8 T cells with BM-DCs induced robust transcription of *Fas* that initiated 24 h after cell activation and reached an ~8 fold increase at 72 h. In contrast, cell activation with anti-CD3 and

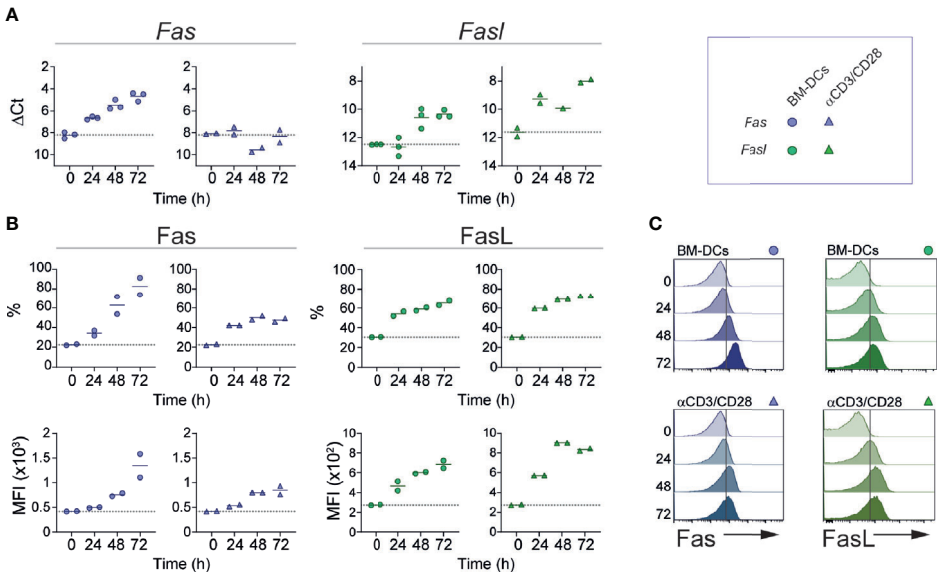


**FIGURE 1** | Fas and FasL expression kinetics. CD8$^+$ OT-I cells were activated *in vitro* using bone marrow-derived dendritic cells (BM-DCs; circles) loaded with SIINFEKL (1 mM) or with plate-bound anti-CD3 and anti-CD28 (triangles). At the indicated time points, cells were lysed for RNA extraction **(A)** or stained for flow cytometry **(B, C)**. **(A)** *Fas* and *Fasl* relative expression (ΔCt). **(B)** Fas and FasL expression is shown as fraction of positive cells (%) and mean fluorescence intensity (MFI) of positive cells. **(C)** Representative histograms of **(B)**. Each symbol **(A, B)** represents one experiment done with pooled cells from 2-3 mice. Solid lines indicate mean; dotted line indicate the expression level of unstimulated cells (0).

anti-CD28 did not induce a detectable increase in *Fas* expression at the mRNA level. Transcription of *Fasl* was promoted by both types of cell activation, but appeared earlier and reached higher levels in cells stimulated by anti-CD3 and anti-CD28. When analyzed at the protein level, expression of surface Fas and FasL reflected the regulation at the mRNA level (**Figures 1B, C**). Fas was induced more strongly by BM-DCs and its levels increased gradually during the observed period of time, whereas FasL was similarly induced by BM-DCs and the combination of anti-CD3 and anti-CD28. These results indicate that in CD8$^+$ T cells, Fas and FasL expression is regulated, at least partially, at the transcriptional level, and is affected by signals present during cell activation.

## Fas-FasL Signaling Maintains CD8 Expression Levels During Cell Re-Stimulation

Genetic deficiency of *Fas* or *Fasl* causes massive accumulation of TCR-αβ DN T cells, suggesting that signaling through these molecules may contribute to CD8 expression (9, 10, 36, 37). Previous work from our group has shown that CD8 expression is lost in CD8 T cells transferred into mice that locally or ubiquitously express the CD8$^+$ T cell cognate antigen as self (25, 26). In that context, several aspects could contribute to CD8 loss. These include factors related to antigen presentation (32, 38) and to the repetitive nature of the antigen encounter (39, 40). To explore this process, and in particular to analyze whether signaling through Fas and/or FasL plays a role in maintaining CD8 expression, we designed an *in vitro* system that would allow us to evaluate the role that repetitive cognate antigen stimulation and signaling through Fas and FasL may exert on CD8 expression during T cell activation. To this end, we took advantage of B6.*lpr* and B6.*gld* mice. B6.*lpr* mice are homozygous for a mutation in *Fas*, caused by the insertion of the ETn retrotransposon that abolishes the expression of the gene (41). B6.*gld* mice have a point mutation near the C-terminal region of FasL that affects its ability to bind Fas (42). Thus, B6.*lpr* cells lack Fas and B6.*gld* cells express a FasL variant that cannot engage Fas. This allowed us to compare the activation of OT-I cells in the presence of WT BM-DCs or BM-DCs lacking Fas (B6.*lpr*) or functional FasL (B6.*gld*). By crossing OT-I mice with B6.*lpr* and B6.*gld* mice, we obtained OT-I cells deficient in Fas and FasL. These cells allowed us to analyze CD8$^+$ T cell activation in cell culture systems devoid of signaling through Fas and FasL (**Figure 2A**). To observe the effects of repetitive antigen encounter, we setup a two-step stimulation system, where we incubated OT-I cells in the presence of cognate antigen-loaded BM-DCs during 48 h and then we replated the OT-I cells with fresh BM-DCs. We analyzed CD8 expression on OT-I cells after the initial stimulation (activation) and after 72 h of re-stimulation.

We considered CD8 expression as percentage of CD8 positive cells within OT-I cells (% CD8$^+$) and as CD8 abundance per cell (CD8 mean fluorescence intensity; MFI). As shown in **Figure 2B**, cells maintained CD8 expression during the initial 48 h activation period, and lack of BM-DC→T cell signaling (through Fas or FasL) did not affect CD8 expression. Likewise, activation of B6.*gld* OT-I cells in the presence of B6.*gld* BM-DCs,

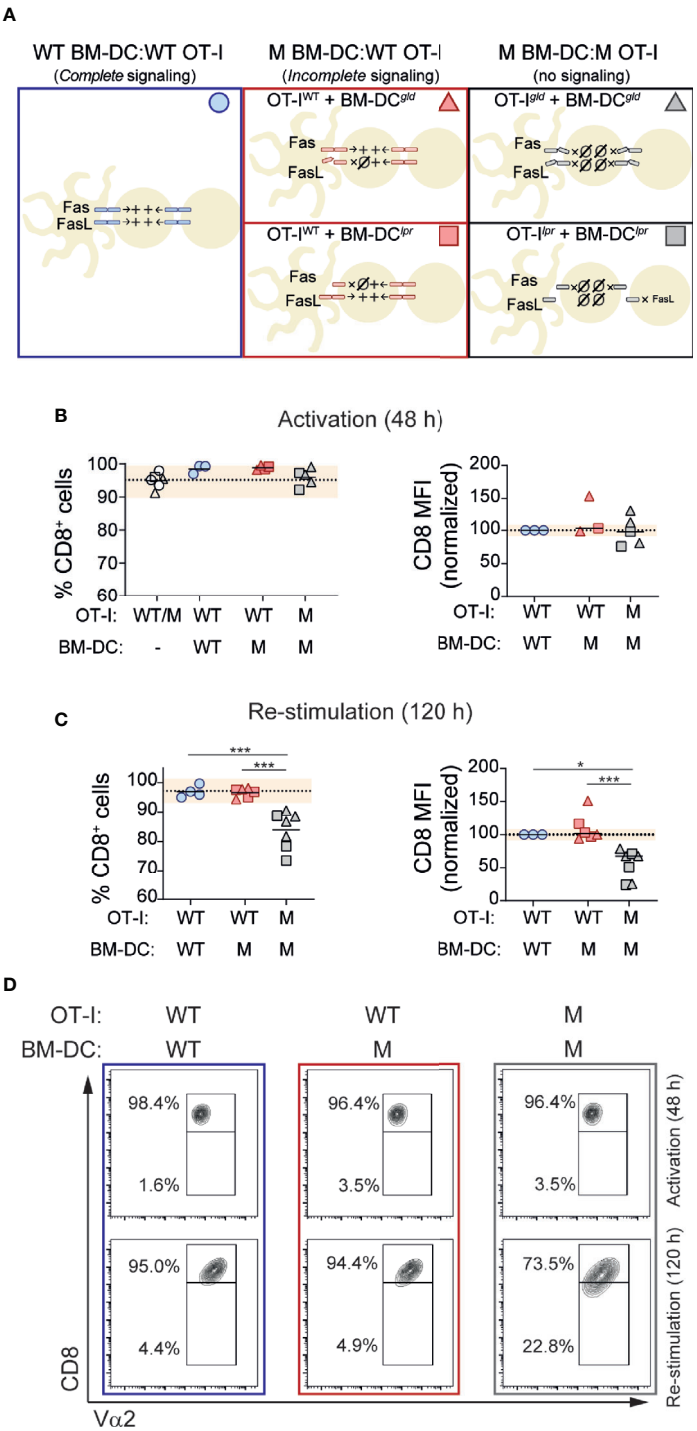


**FIGURE 2** | Fas-FasL signaling maintains CD8 expression during re-stimulation. CD8$^+$ OT-I cells were activated in the presence of BM-DCs loaded with SIINFEKL (1 mM). After 48 hours (activation), CD8 expression levels were quantified by flow cytometry in an aliquot of the cells. The rest were placed in a new plate with fresh BM-DCs (in the presence of SIINFEKL). CD8 expression was reevaluated after 72 hours (120 h re-stimulation). **(A)** Experimental strategy indicating the presence of Fas-FasL signaling in the different cell culture conditions. **(B, C)** CD8 expression after the initial activation (48 h) and after re-stimulation (120 h). Wild-type (WT) or mutant (M; B6.*lpr* or B6.*gld*) OT-I cells were activated in the presence of WT or M BM-DCs, as indicated. Percentage of CD8$^+$ cells (left) or CD8 expression per cell (mean fluorescence intensity; MFI; right) on OT-I cells is shown. Each symbol represents one experiment done with pooled cells from 2-3 mice. Circles represent the conditions where OT-I cells and BM-DCs were WT; triangles when the mutant cells were B6.*gld*; squares when the mutant cells were B6.*lpr*. Dotted lines indicate mean of WT cells; shaded area represents range of WT cells. $^{**}P < 0.01$; $^{***}P < 0.001$ (one-way ANOVA). **(D)** Representative contour plots of OT-I cells after 48 and 120 hours of activation and re-stimulation, respectively.

or B6.*lpr* OT-I cells with B6.*lpr* BM-DCs (no Fas or FasL signaling; **Figure 2A**) had no effect on CD8 levels during the initial 48 h stimulation period (**Figure 2B**).

Re-exposure to cognate antigen had no effects on CD8 expression on WT OT-I cells, activated and re-stimulated by WT or by mutant (M) BM-DCs, indicating that BM-DC→T cell signaling through Fas or FasL does not play an essential role in the modulation of CD8 expression during T cell activation or re-stimulation (**Figure 2C**). In contrast, complete lack of Fas/FasL signaling, was associated with a modest, albeit consistent and statistically significant decrease in CD8 expression, quantified as the fraction of $CD8^+$ cells or as CD8 levels per cell (**Figures 2C, D**).

Because Fas and FasL have been shown to modulate TCR signaling (7, 8), we analyzed the role of cognate peptide concentration during OT-I activation and re-stimulation. CD8 T cell stimulation with higher concentrations of SIINFEKL tended to decrease the expression of CD8 and of the TCR, but only after re-stimulation. This effect was more marked in the absence of Fas and FasL signaling (**Supplemental Figure 1**). This suggests that signaling through Fas and/or FasL signaling may decrease the strength of TCR signaling during repetitive encounters with cognate antigen.

## Loss of Fas/FasL *cis* Signaling Does Not Affect CD8 Loss *In Vivo*

The results from our *in vitro* experiments showed unaltered CD8 expression in the presence of one-way BM-DC→T cells Fas or FasL signaling. To determine whether the absence of Fas or FasL on CD8 T cells affected the behavior of OT-I cells adoptively transferred into mice that ubiquitously express their cognate antigen, we co-transferred WT OT-I (CD45.1) and B6.*gld* or B6.*lpr* OT-I (CD45.2) into CD45.1/2 Act-mOVA mice in a 1:1 ratio (**Figure 3A**). As expected, the presence of OVA was associated with a contraction of transferred antigen-specific cells. However, absence of Fas and FasL affected CD8 T cell contraction in a different manner. Whereas lack of FasL did not affect self-antigen-induced contraction and actually tended to increase the number of OT-I cells at day 7 post-transfer, lack of Fas was associated with a significant decrease in the number of live OT-I cells suggesting that signaling through Fas may in fact promote cell survival in CD8 T cells exposed to self-antigen (**Figure 3B**). When we analyzed CD8 downregulation, our results in this *in vivo* setting were analogous to the ones obtained in our *in vitro* re-stimulation system. B6.*gld* as well as B6.*lpr* OT-I cells downregulated CD8 in a normal manner, not different than the WT OT-I cells they were cotransferred with (**Figures 3C, D**).

These experiments demonstrate that Fas and FasL expression on T cells is dispensable for self-antigen-induced contraction of CD8 T cells. In fact, lack of T cell expression of Fas caused an unexpected drop in the numbers of live OT-I cells transferred into Act-mOVA mice, suggesting that signaling through Fas may directly or indirectly promote T cell survival in this setting. In concordance with the results of our *in vitro* experiments, when Fas or FasL deficiency was limited to T cells, CD8 downregulation induced by self-antigen was not affected.

## Fas/FasL Signaling Maintains Cell Numbers and CD8 Expression During Encounter With Self-Antigens

Collectively, the presented *in vitro* and *in vivo* data indicated that complete absence of Fas/FasL signaling was associated with loss of CD8 expression during repetitive antigen encounter, but that partial interruption of this signaling pathway had modest or no obvious effects. To confirm these observations, we generated double mutant (DM; B6.*lpr*/*gld*) OT-I mice and co-transferred WT and DM OT-I cells (1:1 ratio; **Figure 4A**) into WT recipient mice and infected them with OVA-expressing *Listeria monocytogenes* (LM-OVA). At Day 7, we quantified the abundance of WT and DM cells and analyzed their expression of CD8. As shown in **Figure 4B**, DM cells were modestly more abundant ($P$=0.016) at the peak of clonal expansion (DM : WT ratio 1.45 ± 0.22), consistent with the role of Fas/FasL in re-stimulation-induced cell death (4). However, CD8 expression remained high in WT and DM cells (**Figure 4C**). Thus, in the course of a protective immune response, during pathogen-driven T cell activation, Fas/FasL signaling is not necessary for maintaining CD8 expression, but modestly curbs clonal expansion. To analyze the role of Fas/FasL signaling during encounter with self-antigens, a situation that promotes CD8 downregulation (25, 26), we co-transferred WT and DM OT-I cells into Act-mOVA mice. In this context, lack of Fas and FasL was associated with a marked drop in cell numbers and at Day 7 post-transfer the ratio of DM : WT cells was 0.48 ± 0.03 (**Figure 4D**). Importantly, in concordance with our *in vitro* experiments, the fraction of cells that maintained CD8 expression was significantly lower within DM OT-I cells (WT 44.0% ± 3.5 vs. DM 21.89% ± 1.1, $P$=0.001) (**Figure 4E**). This was probably not associated with increased activation, as CD44 upregulation was similar in WT and DM cells (**Figure 4F**).

Because of the contrasting effects of Fas/FasL signaling during productive and tolerance-inducing immune responses, we analyzed the kinetics of Fas and FasL expression in WT OT-I cells in both scenarios. As shown in **Figure 4G**, expression of Fas increased gradually and peaked at Day 5 in OT-I cells activated in the context of LM-OVA infection. In contrast, Fas expression reached an earlier and higher peak in cells exposed to self-antigens. Analogously, induction of FasL was higher in cells exposed to self-antigens.

# DISCUSSION

Fas and FasL play complex roles in the immune system. Present in a large variety of cells, the regulation of their expression and the consequences of their engagement vary greatly depending on the cell context in which they appear. Here, we have analyzed the effects of complete absence of Fas/FasL signaling in two different settings, during an infection with an intracellular bacterial strain and during the encounter of ubiquitous antigen presented as self. Because in both systems we used cells with the same antigenic specificity, our experimental design eliminated differences in TCR affinity, a factor commonly relevant during the

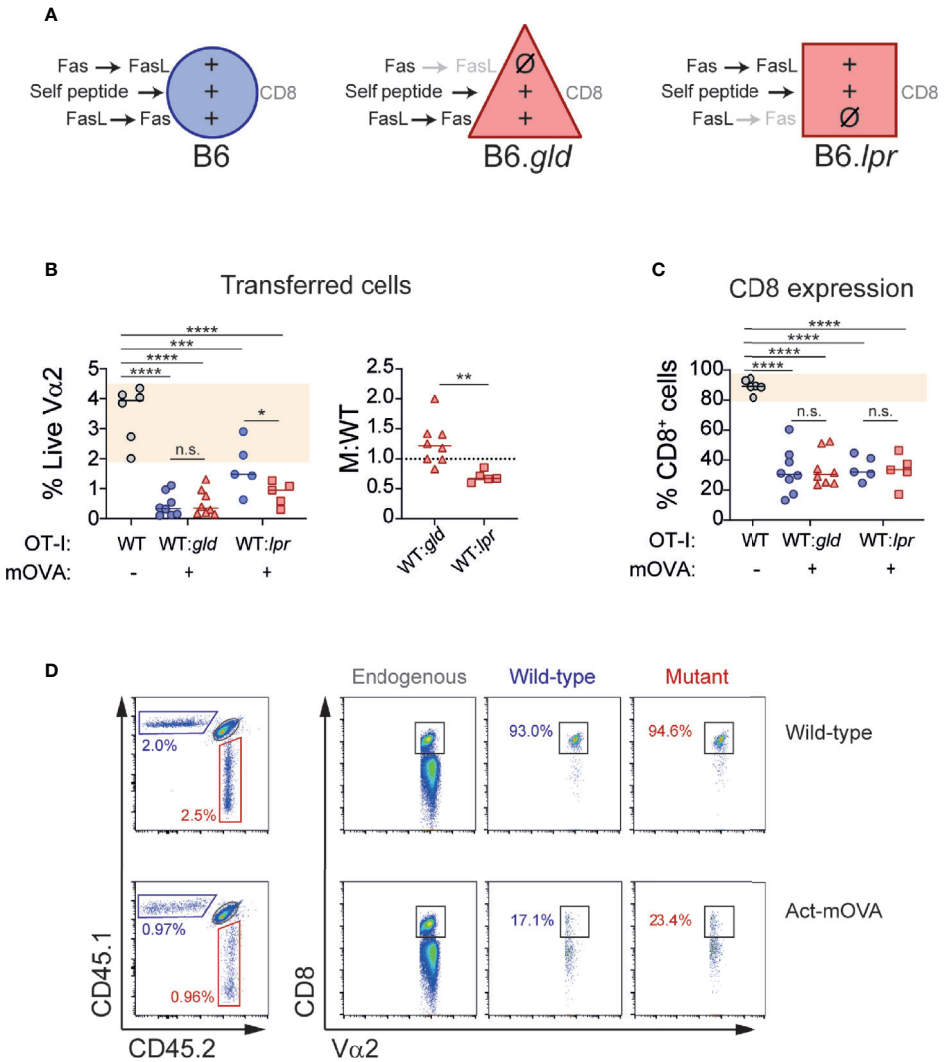


**FIGURE 3** | T cell-specific deficiency of Fas or FasL does not affect CD8 loss during self-antigen exposure. Wild-type CD45.1 (WT) or mutant CD45.2 (B6.*gld* or B6.*lpr*) OT-I cells were adoptively transferred (1:1 ratio) into CD45.1/2 mice that ubiquitously express ovalbumin (Act-mOVA), or into control CD45.1/2 mice. Seven days later, transferred cells and CD8 expression were analyzed. **(A)** Experimental layout, indicating the symbols that represent each cell type (in **B**, **C**) and the signals received by each cell during exposure to self-antigens. **(B)** Abundance of transferred cells, shown as the percentage of CD45.1 (WT) or CD45.2 (B6.*gld* or B6.*lpr*) cells within live Vα2$^+$ T cells (left), or as M:WT ratio (right). **(C)** Fraction of transferred cells positive for CD8. Each symbol **(B, C)** represents one mouse. Solid lines indicate mean. Shaded area indicates range of WT cells. **(C)** Representative dot plots showing transferred cells (left) and CD8 expression (right) in a WT and an Act-mOVA mouse. ***$P$<0.001; ****$P$<0.0001 (one-way ANOVA). **$P$<0.01 (unpaired t test).

comparison of self- and pathogen-derived antigens (T cells usually have lower affinity toward self-antigens than against external antigens), and allowed us to observe the behavior of OT-I cells during a protective and a tolerogenic immune response and to determine whether the absence of Fas/FasL signaling affects CD8 expression in those circumstances.

We observed that the context in which CD8 T cells are primed, affects the expression of both Fas and FasL. *In vitro* experiments showed that CD8$^+$ T cell activation *via* BM-DCs induced higher expression of Fas (but not FasL) than activation of the same cells with plate-bound anti-CD3 and anti-CD28. Although these two systems are different and it is not possible to weigh the influence of TCR affinity, the fact that BM-DCs induced much higher expression of Fas, suggests that signals different to CD28 and CD3 (e.g. DC-derived cytokines and/or surface molecules) may promote transcription of Fas (43). This hypothesis is supported by previous findings that showed that T cell activation with concanavalin A induced higher levels of Fas than activation through CD3 (34). Our *in vivo* experiments further confirmed this and showed that exposure to self-antigen elicits a much stronger and earlier expression of both Fas and FasL than encounter to the same antigen in the context of bacterial infection.

In previous work we have shown that exposure of CD8$^+$ T cells to self-antigen induces an inactivation program that includes the

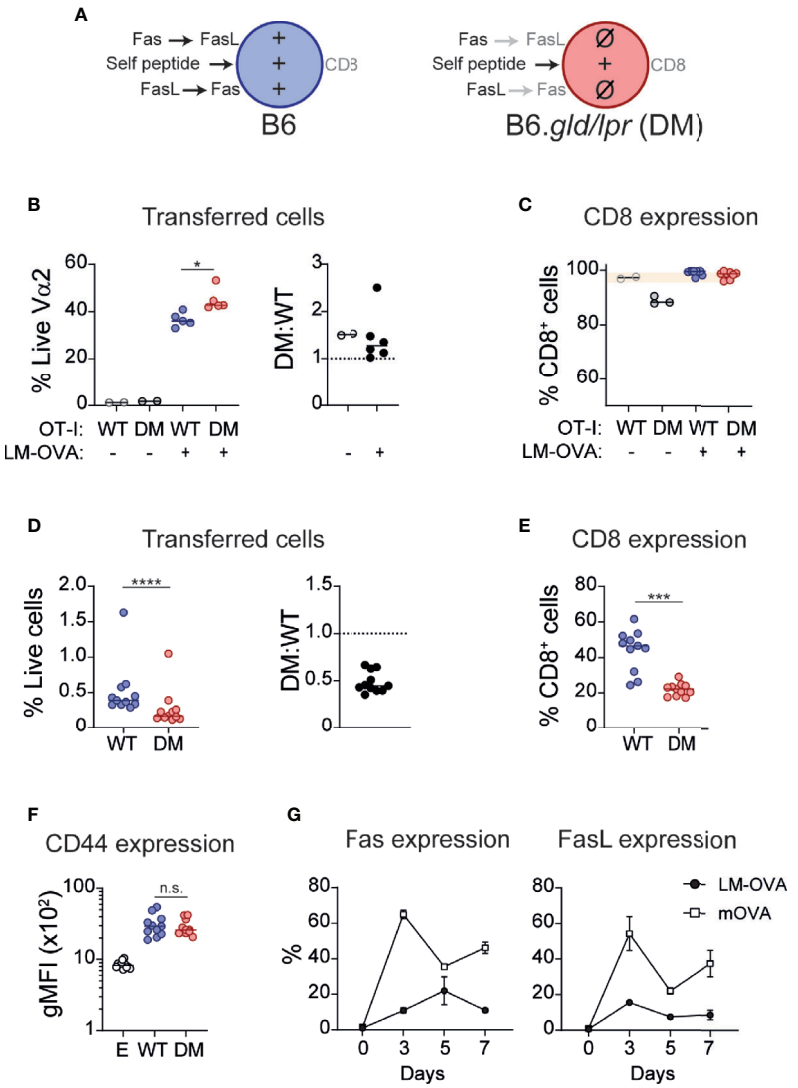


**FIGURE 4** | Fas-FasL signaling maintains CD8 expression during self-antigen encounter. **(A)** Experimental layout, indicating the symbols that represent each cell type (in **B–F**) and the signals received by each cell during exposure to self-antigens. **(B, C)** WT (CD45.1/2) and double mutant (DM; B6.*lpr/gld*; CD45.2) OT-I cells were adoptively transferred (1:1) into CD45.1 mice. The next day, mice were infected with ovalbumin-expressing Listeria monocytogenes (LM-OVA). At Day 7 post-infection, mice were sacrificed and cell abundance and CD8 expression were analyzed. **(B)** Abundance of transferred cells, shown as the percentage of WT or DM cells within live Vα2$^+$ T cells (left), or as DM : WT ratio (right). **(C)** CD8 expression on transferred cells. **(D–F)** WT and DM cells were adoptively transferred (1:1) into mice that ubiquitously express OVA (Act-mOVA) and analyzed 7 days later. **(D)** Abundance of transferred cells. **(E)** CD8 expression on WT and DM cells. **(F)** CD44 expression (gMFI) in endogenous **(E)** and transferred cells. Each symbol represents one mouse. Solid lines indicate mean. * $P<0.05$ (one-way ANOVA). ***$P \leq 0.001$; ****$P<0.0001$ (paired t test). **(G)** Fas (left) and FasL (right) expression kinetics (mean ± SEM) in WT OT-I cells during LM-OVA infection (black circles) or self-antigen exposure (empty squares). n.s., not significant.

downregulation of CD8 expression (25–27). Because we never observed CD8 downregulation during *in vitro* activation and CD8 loss was only observed in tissues locally expressing the cognate antigen (25), we hypothesized that repetitive encounter with antigen –as occurs *in vivo* during self-antigen encounter- may play a role in this process and that signaling through Fas or FasL may regulate CD8 loss. We found that in WT CD8$^+$ T cells, repetitive *in vitro* activation did not affect CD8 levels and, importantly, that absence of Fas or FasL on the APC had no effects. Our interpretation to this observation was that *cis* signaling (Fas and FasL on the same cell) or *trans* signaling (Fas and FasL on different T cells) could avoid CD8 downregulation because the OT-I cells did not require Fas or FasL signals originated from the APC in the presence of *cis* signaling. The importance of *cis* engagement for signaling or as competition for ligands presented in *trans*, has been observed in other T cell co-receptors, for example PD-1, PD-L1 and CD80, HVEM and BTLA, or Notch and Delta (44–49) and also in Fas-FasL during the process of memory development (36). This seems to be the case, because when Fas-deficient OT-I cells were activated in the

presence of Fas-deficient BM-DCs (or FasL deficient T and DCs were used), CD8 levels decreased. These experiments suggested that Fas/FasL signaling, during repetitive antigen encounter, maintains CD8 expression. To determine the role of TCR signaling strength in this process, we activated OT-I cells with varying concentrations of SIINFEKL, in the presence or absence of Fas/FasL signaling. We observed that lack of Fas/FasL signaling increased the magnitude of CD8 and TCR downregulation induced by high antigen concentrations. These data indicate that Fas/FasL may modulate TCR signaling, particularly during repetitive encounter with high affinity antigens. Further, the fact that Fas (and not FasL) deficiency was associated with higher levels of OT-I death during adoptive transfer into Act-mOVA mice suggests that signaling through Fas may modulate TCR signaling in this context thus limiting cell death caused by exposure to persistent antigen.

Exclusive absence of Fas or FasL T cell signaling did not affect CD8 downregulation. In contrast, complete absence of Fas/FasL signaling significantly increased CD8 to DN T cell conversion. This effect could not be attributed to defective apoptosis, because as mentioned earlier, absence of Fas (either alone or combined with FasL deficiency) did not cause an accumulation of OT-I cells. Moreover, in concordance with Hao et al., that demonstrated that FasL blockade is necessary for the accumulation of DN T cells in the presence of T cell-specific *Fas* deficiency (14), we observed that whereas absence of FasL did not promote DN T cell generation when present as an isolated defect, it did robustly in the presence of the concomitant absence of Fas. This suggests that DN T cell expansion in germline mutants is a complex phenomenon where lack of Fas and FasL contribute differently. It also poses the question of whether genetic variants affecting FasL reverse signaling or the crosstalk between Fas and FasL signaling pathways could modify disease expression in patients with ALPS. This may be particularly relevant in cases where specific mutations are associated with heterogeneous phenotypes (50).

Our findings reveal an unexpected role for Fas/FasL signaling during peripheral tolerance: expression of Fas and FasL is robustly induced by self-antigen encounter and *cis* signaling through this receptor pair may protect self-reactive cells from deletion and from CD8 downregulation, perhaps by annulling Fas/FasL signaling from APCs to T cells as has been proposed in other systems (2). In contrast, absence of Fas/FasL had no effects on CD8 expression during a protective immune response induced by a bacteria. Why does absence of Fas and FasL affect so differently the fate of cells in two settings that share the CD8 T cells and the antigen? The fact that Fas and FasL induction differs greatly in these two types of antigen encounter suggests that Fas/FasL signaling may be more relevant for T cells in the context of self-antigen encounter than during responses that induce a strong clonal expansion. Numerous studies have reported that Fas and FasL exert costimulatory effects on T cells (8, 51–54). It is possible that in this system, the absence of Fas and FasL-derived costimulation impairs OT-I proliferation. It also raises the question of whether $CD8^+$ and DN T cells depend differently on Fas/FasL costimulatory properties, as it has been reported that TCR signaling strength is an important modulator of FasL costimulatory and inhibitory effects (55). In patients with ALPS, CD4 and CD8 T cells exhibit abnormal phenotypes reminiscent of terminally differentiated exhausted T cells seen in conditions where T cells are chronically stimulated. This phenotype, also observed in their DN T cells, along with evidence that links the TCR repertoire in $CD8^+$ and DN T cells, suggests that self-antigen encounter drives CD8 to DN T cell conversion in ALPS and emphasizes the importance of Fas in keeping in check self-reactive CD8 T cells (56, 57).

Because in our *in vivo* system we used a unique high affinity antigen, we were not able to consider the role of antigen affinity, which represents a variable that could contribute to the lymphoproliferation observed in mice that lack Fas or FasL in the presence of a diverse repertoire. Together, the evidence indicates that Fas/FasL signaling promotes CD8 expression on self-reactive T cells exposed to self-antigen, but that the degree of clonal deletion is regulated by other factors, perhaps controlled at the level of TCR signaling and therefore regulated by affinity toward the antigen. This hypothesis that would predict that the absence of Fas or FasL would favor the loss of CD8 and the accumulation of low-affinity self-reactive cells. In support of this, we observed that changes in the concentration of SIINFEKL during *in vitro* re-stimulation were inversely correlated with CD8 expression.

Because humans and mice with *Fas* or *FasL* LOF mutations lack a functional molecule in all cells, the net result is lack of signaling through Fas and through FasL. When we used double mutant (B6.*gld*/*lpr*) OT-I cells, we completely blocked all Fas and FasL signaling in T cells. However, it would be important to distinguish between the effects of Fas and FasL engagement. Theoretically, Fas-deficient OT-I cells could be adoptively transferred into FasL deficient recipient mice, to observe the effects of T cell FasL signaling in the absence of Fas signaling (or the inverse experimental setup). The main caveat of these systems is that Fas-deficient cells express FasL and thus induce apoptosis in Fas-bearing cells. Therefore, adoptive transfer of Fas-sufficient T cells into Fas-deficient animals could result in apoptosis of the transferred cells and alter the process of CD8 downregulation. On the other hand, an advantage provided by our system is the opportunity to dissect the individual contribution of Fas/FasL signaling exclusively in the context of the CD8 T cell and avoid the implications of its deficiency in other cell types.

In summary, Fas and FasL expression are differentially induced on CD8 T cells depending on the conditions that prevail during their priming. High expression of Fas and FasL induced during self-antigen presentation could regulate CD8 expression and cell survival and therefore contribute to the regulation of T cell responses to self-peptides.

## 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 animal study was reviewed and approved by the Comité Interno para el Cuidado y Uso de Animales de Laboratorio (CICUAL), Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

## AUTHOR CONTRIBUTIONS

Conceptualization, GF-M, NR-R, FR, and JCC. Methodology, GF-M, NR-R, RMR, IM-S, FR, and JCC. Investigation, GF-M, NR-R, RMR, IM-S, and FR. Writing GF-M and JCC. Funding Acquisition JCC. Supervision, FR and JCC. 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.2021.635862/full#supplementary-material

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# Oxidative Stress in SLE T Cells, Is NRF2 Really the Target to Treat?

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Oxidative stress is a major component of cellular damage in T cells from patients with systemic lupus erythematosus (SLE) resulting amongst others in the generation of pathogenic Th17 cells. The NRF2/Keap1 pathway is the most important antioxidant system protecting cells from damage due to oxidative stress. Activation of NRF2 therefore seems to represent a putative therapeutic target in SLE, which is nevertheless challenged by several findings suggesting tissue and cell specific differences in the effect of NRF2 expression. This review focusses on the current understanding of oxidative stress in SLE T cells and its pathophysiologic and therapeutic implications.

**Keywords: Keap 1, ROS, mTOR, lupus, Tregs, Foxp3**

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## INTRODUCTION

Oxidative stress might be a central factor in the immunopathogenesis of Systemic Lupus Erythematosus (SLE) (1). There are data supporting the hypothesis that excessive reactive oxygen species (ROS) production is, along with many other factors, one of the factors that induce SLE (2). Indeed, ROS production associates with enhanced apoptosis and might delay clearance of apoptotic cells, both of which are hallmarks of SLE (3, 4). Apoptotic cells are a source of autoantigens (e.g., ribonucleoproteins, DNA), which can be recognized by auto-reactive T cells. Furthermore, apoptotic cells release danger-associated molecular patterns (DAMPs), DAMPs are danger signals, which induce inflammatory responses. Therefore, increased auto-antigenic exposure and decreased autoantigen removal might contribute to autoantibody production and autoimmunity. Peripheral blood mononuclear cells (PBMCs), T cells and neutrophils are exposed to increased concentrations of free radicals which leads to aberrant activation of these cells. In addition to this, mitochondrial dysfunction and ROS production of PBMCs themselves contribute to SLE pathogenesis, while mechanistic reasons that cause mitochondrial ROS production have not been identified (5). It is not clear if the chronic inflammation in SLE induces oxidative stress conditions or if ROS production is vice versa a cause of lupus pathology. Nevertheless, there is mounting evidence for a defective redox clearance in SLE. Patients with highly active disease show impaired activity of oxidative stress regulating enzymes, such as superoxide dismutase (SOD) and gluthathione peroxidase (GPX) in serum and saliva while PBMCs of active SLE patients reveal higher intracellular ROS than those of healthy controls (6–9). Increased oxygen intermediates have also been identified in macrophages of lupus mice (10). Therefore, studies even suggest considering assessment of redox status as a serological marker to evaluate disease activity and nephropathy in SLE (11–13).

On the other hand, ROS production has been associated with the prevention of autoimmune diseases. A genetically mediated deficiency of ROS production can contribute to initiation of autoimmunity and can facilitate disease progressing (14). A missense mutation in the p47phox (Neutrophil Cytosolic Factor

1, *NCF1*) subunit of NADPH oxidase (NOX) predisposes to SLE and other autoimmune diseases (15, 16). Mice with a mutation of *Ncf1*, which is associated with low ROS production, develop an accelerated lupus-like disease (16). In addition to this, point mutations in *NCF2* are associated with increased SLE risk (17), since they reduce NOX activity and NCF-2 deficient or NCF-2 haplo-insufficient mice reveal accelerated lupus disease (18).

To sum up these findings, ROS play an ambivalent role in SLE, which depends on source, location, amount and most probably also time-point of their occurrence. As a consequence, balanced ROS levels are important to sustain an immune equilibrium which reduces tissue damage and prevents the development of autoimmunity (19, 20). But how is a redox balance achieved in our cells? To answer this question, we have to study NRF2, the main transcription factor of the anti-oxidative stress response.

## NRF2

Oxidative stress can activate different transcriptional regulators, most importantly NRF2. In steady state conditions, NRF2 is bound in the cytosol by Kelch ECH associating protein 1 (KEAP1), which induces ubiquitinilation and degradation of NRF2 (21). Different cellular stimuli which induce oxidative stress result in conformational changes of KEAP1, which foster the release of NRF2 from KEAP1 followed by NRF2 translocation into the cellular nucleus and activation of genes containing an antioxidant response element (ARE) in their promoter regions (22). NRF2 thereby induces expression of phase II detoxifying enzymes and antioxidant proteins, which preserve cellular homeostasis by reducing chemical or oxidative stress molecules. These proteins include enzymes mediating glutathione (GSH) synthesis, the thioredoxin enzyme system and detoxifying enzymes such as heme oxygenases, or NAD(P)H: quinone oxidoreductase 1 (NQO1). In addition to induction of antioxidant genes, NRF2 also modulates immune responses by regulating transcription of several others including anti-inflammatory and metabolic genes in immune cells (23–26). So, being a critical regulator of cellular oxidative stress responses and inflammatory reactions, it is not surprising that NRF2 is indispensable to prevent cellular damage and subsequent inflammation. NRF2 deficient mice have problems to deal with inflammatory cues and therefore show a more sever phenotype in inflammation-mediated animal models like experimental asthma (27), acute lung injury (28), sepsis (29), T cell-mediated hepatitis (30), or dextran sulfate sodium-induced colitis (31) and arthritis (32). There are also several lines of evidence that NRF2 has a central role in the pathogenesis of SLE. Interestingly, aged female NRF2 deficient mice are prone to develop an autoimmune condition that closely resemble human SLE (33). Furthermore, NRF2 deficiency increased lupus nephritis and Th17 cells in B6/lpr mice (34). NRF2 furthermore suppresses Lupus nephritis by neutralizing ROS and preventing renal damage (35). A recent study revealed that NRF2 activation promotes resolution of chronic inflammation in lupus most probably by repolarization of macrophages and reduction of the IFN signature (36). In contrast, one study revealed a prolonged lifespan, improved autoimmune nephritis, and reduced lymphadenopathy of lupus mice in the absence of Nrf2 (37). However, these effects can be explained by a Nrf2 mediated suppression of the autoimmune accelerating gene lpr, which is used in this mouse model. It is therefore not clear, how this study can be transferred to the human SLE disease. With that regard, Gautam et al. found elevated ROS in SLE specific DCs, which might be due reduced clearance of ROS related to impaired levels of NRF2 (6). In addition, the association of the NRF2 -653G/A polymorphism with lupus nephritis in pediatric-onset SLE has been described (38). This polymorphism was only associated with nephritis, while there was no significant association between NRF2 -653G/A polymorphism and susceptibility to SLE. Another study identified three SNPs and one triplet polymorphism within the promotor and upstream regions of the NRF2 gene, but no association between risk of SLE and these polymorphisms. However, authors state that their analysis is preliminary and only a small number of patients were observed (39). Therefore, until now we can only assume the association between Nrf2 polymorphism and lupus nephritis.

In conclusion, there are several lines of evidence which support the conclusion that NRF2 activation is beneficial in SLE pathogenesis. However, it is not clear whether NRF2 - by induction of the antioxidant machinery - enhances cell survival and reduces apoptosis, which contributes to the control of systemic inflammation and limitation of autoimmune responses or by direct cellular mechanism including transcriptional regulation of inflammatory responses in immune cells.

A very interesting study addressing this question was performed by Suzuki et al., who analyzed NRF2 in the scurfy mouse model. These mice lack functional regulatory T cells ($T_{regs}$) and therefore develop mutiorgan-inflammation and autoimmunity at early ages. Interestingly, while systemic activation of NRF2 by *Keap1* knockdown ameliorated tissue inflammation, NRF2 activation *via* cell lineage-specific *Keap1* disruption (i.e., in T cells, myeloid cells, and dendritic cells) achieved only partial or no improvement in the inflammatory status of scurfy mice (40). This suggests NRF2 activation in multiple cell lineages appears to be required for sufficient anti-inflammatory effects. Our group even observed a pro-inflammatory effect of NRF2 when we specifically deleted NRF2 in $T_{regs}$ (26). This raises the question if it is beneficial to use NRF2 activation in SLE as a therapeutic intervention. It is therefore important to first illuminate the exact role of NRF2 critically and ROS signaling in T cells in SLE.

## NRF2 AND REDOX METABOLISM IN T CELLS

Redox-dependent signaling pathways are of major importance in immune cells and specifically in T cells. They control different functions including T effector cell differentiation and migration, cell cycle progression and inflammation. The role of ROS in T cell signaling has been intensively reviewed elsewhere (41). We will

**Abbreviations:** DAMPs, danger-associated molecular patterns; ETC, electron transport chain; GCLC, Glutamate-Cysteine Ligase Catalytic Subunit; GPX, gluthathione peroxidase; GSH, gluthathione; GvHD, graft-vs-host disease; Keap1, Kelch ECH associating protein 1; MDSC, myeloid derived suppressor cells; mTOR, Mammalian Target of Rapamycin; NCF, Neutrophil Cytosolic Factor; NK, natural killer; NOX, NADPH oxidase; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, Nuclear factor erythroid 2-related factor 2; OXPHOS, oxidative phosphorylation; PBMCs, peripheral blood mononuclear cells; ROS, rective oxygen species; SLE, systemic lupus erythematosus; SOD, superoxid dismutase; TCA, trichloroacetic acid.

therefore only give a short overview about the current knowledge in this field. The production of ROS is enhanced after T cell receptor stimulation and is necessary for clonal expansion. ROS which is produced in the mitochondrial electron transport chain (ETC) is important for the activation of NFAT, a T cell-specific transcription factor that facilitates interleukin-2 (IL-2) expression and thus cell cycle progression, which results in rapid proliferation of the T cells (42). However high ROS concentrations result in GSH depletion, since the murine Glutamate-Cysteine Ligase Catalytic Subunit (GCLC) is not expressed in murine T cells. GSH depletion negatively affects NFAT activity, mTOR signaling and MYC protein expression and thus inhibits T cell proliferation (43). The antioxidant GSH tightly regulates ROS activity, which thus controls cell cycle progression of T cells as well as their metabolic properties. The latter ones influence their differentiation program and as a consequence, the manner of protective immune responses. Thus, ROS influence metabolic programming; too much can be detrimental, but if applied correctly, it is important for protection of the host (19). For metabolic activity, $T_{regs}$ rely on oxidative phosphorylation (OXPHOS), which produces ROS and which are also regulated by GSH. GSH generated by GCLC is indispensable for proper $T_{reg}$ function and lack of GCLC in $T_{regs}$ results in autoimmunity (44).

Giving this critical role of ROS in T cells, it is obvious that NRF2, which regulates cellular redox homeostasis might also critically regulate T cell functions. It is known that NRF2 expression is increased in activated T cells in mice and men (26, 45, 46), however the exact role of NRF2 in T cells remains controversial. Constitutive activation of NRF2 in T cells was protective in a murine acute kidney injury model and involved higher frequencies but not total numbers of intrarenal Tregs, as well as reduced expression of inflammatory cytokines in $CD4^+$ T cells (47). However constitutive ablation of NRF2 in T cells ameliorated graft-vs-host disease (GvHD) (46). Again, a study with human cells indicates that high expression of NRF2 in $CD8^+$ T-cells might be protective against chronic GvHD (48). The role of oxidative stress in $T_{regs}$ is controversely discussed. Mougiakakos et al. have been reported that $T_{regs}$ are more resistant to oxidative stress-induced cell death than conventional T cells (49), while others have found higher toxicity of free oxygen species in $T_{regs}$ to, which was attributed to a diminished NRF2-dependent antioxidant system (50). The weakness of the NRF2-associated antioxidant system facilitates $T_{reg}$ apoptosis but nevertheless causes enhanced immunosuppressive capacity within the tumor microenvironment. Our own study indicates that NRF2 is a negative regulator of $T_{reg}$ function and that Foxp3 specific activation of NRF2 results in a loss of Foxp3 expression and spontaneous accumulation of IFN-γ producing effector T cells and spontaneous inflammation (26).

Consequently, NRF2 has shown pro- and anti-inflammatory potential in T cells in different experimental inflammatory mouse models. In addition to this, there are differences between the role of NRF2 in human and murine T cells. Thus, the exact role of NRF2 in T cells and T cell subsets in autoimmunity is not clear.

## REDOX METABOLISM IN SLE T CELLS

ROS production and cellular metabolism are intimately linked. More interestingly NRF2 is also involved in the regulation of different metabolic pathways. For immune cells, NRF2 increased glucose uptake and mitochondrial function, while reducing ROS in natural killer (NK) T cells and myeloid derived suppressor cells (MDSCs) (25, 51). Immune cells undergo metabolic reprogramming in autoimmune and autoinflammatory diseases. This is also the case in SLE, where T cells are hyper-oxidative (52). T cells from SLE patients differ from activated T cells from healthy persons (53, 54). Activated T cells mostly use glycolysis and pentose phosphate pathway to produce ATP and metabolic intermediates. SLE T cells show a chronically activated phenotype with increased TCA (trichloroacetic acid) cycle activity, they furthermore depend on OXPHOS to fulfill their energetic demands (55). It is suggested that SLE T cells therefore have lower antioxidant capacity with lower NADPH and glutathione levels (1, 56). This is further indicated by the fact that SLE T cells display high oxidative stress (57, 58). Although SLE T cells have an enlarged mitochondrial mass, the ATP generation from OXPHOS is insufficient compared to healthy person and a marked leakage of ROS is present in SLE T cells (58). ROS increase mammalian Target of Rapamycin (mTOR) activity, which is increased in $CD4^+$ T cells in SLE patients (59). So reduced GSH levels induce a redox-dependent mTOR activation. $CD4^+$ T cells from lupus patients show enhanced levels of mTOR activation which has been mechanistically linked with the disease process (60). As a consequence, therapeutic interventions with mTOR inhibitors in SLE patients normalized T cell activation and improved their clinical disease activity (61, 62).

There seem to be T cell subtype specific regulations that also need to be considered. Recently, Cailelli et al. identified $CD4^+$ T cells that are induced by oxidized mitochondrial DNA-activated pDCs in SLE patients and accumulate ROS, secrete succinate and produce IL-10 and IFN-γ. By doing so, they provide superb B cell help (63).

This suggests that ROS accumulation plays a pathogenic role in SLE T cells. As a consequence, enhancing NRF2 activity and thereby reducing intracellular redox metabolism could be beneficial.

## DISCUSSION

Without a doubt redox metabolism critically regulates function, proliferation, and apoptosis of T cells. Furthermore, oxidative stress is a hallmark of SLE pathogenesis and T cells from SLE patients reveal metabolic aberrations that also involve higher ROS production. Therefore NRF2, as main transcriptional regulator of the anti-oxidative stress response and beyond this as regulator of metabolic and anti-inflammatory actions in immune cells, is most likely a central player in abnormal SLE T cell function. In line, 509 unique patent applications that define NRF2 pathway as molecular target and focus on medical conditions such as autoimmunity, liver, kidney, lung and neurodegenerative diseases have been filed since 2017 (64). Furthermore dimethyl fumarate (DMF), which activates Nrf2, is already used clinically to treat inflammatory diseases. DMF was approved for the treatment of patients with moderate and severe psoriasis as well as patients with relapsing remitting multiple sclerosis (65). In addition, there are clinical trials ongoing with DMF for treatment of inflammatory conditions, among others cutaneous lupus erythematosus (66).

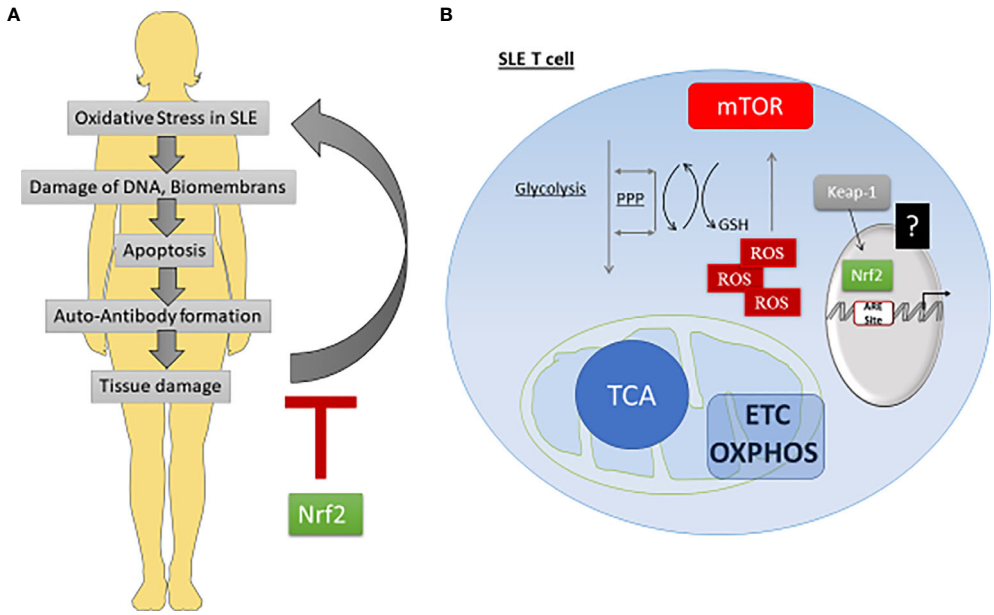


**FIGURE 1** | While systemic NRF2 activation can ameliorate pathogenesis, the exact role in T cells is not clear. **(A)** Oxidative stress is involved in SLE pathogenesis. Due to increased free radicals or a weak antioxidant system, SLE patients reveal high levels of oxidative stress which leads to cell damage and apoptosis. Subsequent release of autoantigens can enhance autoantibody formation and contribute to tissue damage. Tissue damage again can enhance oxidative stress. Several lines of evidence indicate that activated NRF2 can break the vicious circle by inducing anti-oxidative responses. **(B)** SLE T cell are hyper-oxidative. SLE T cells prefer the use of OXPHOS, which reduces NADPH and GSH pools, that are normally filled up during glycolysis and pentose phosphate pathway (PPP). In addition, they reveal a marked leakage of ROS during OXPHOS. High ROS levels induce mTOR activation. It is not clear, if NRF2 is not sufficiently activated or somehow fails to counteract high ROS levels in SLE T cells.

On the other hand, medication currently in use to treat patients with SLE clearly seems to affect Nrf2 signaling. In particular corticosteroids, which are first line treatment drugs downregulate Nrf2 transcriptional activation by direct and indirect means (67–69). Moreover, Cyclophosphamide, which is used in severe cases (lupus nephritis and CNS lupus) has severe side effects like hepatotoxicity and myelosuppression, which can be ameliorated by activation of NRF2 (70, 71). The same is true for methotrexate, which is used in lupus arthritis and downregulates the antoxidative Nrf2 response in the liver (72). On the other hand, mycophenolate mofetile is increasingly used in SLE and preserves the Nrf2 system in the liver as well as in the kidney, which might contribute to the broad therapeutic tolerability of the drug (73, 74). Finally, hydroxychloroquine also preserves the Nrf2 antioxidative system (75). Nevertheless, the described effects have mainly been shown in other systems than T cells and the immune system (liver, kidney and cancer models) and therefore we can only speculate on their effects on immune functions. Although it is well established that NRF2 has anti-inflammatory effects, the cellular mechanism and cell-specific actions are still not fully elucidated. Several controversies exist regarding the ambivalent role of ROS production in autoimmune diseases and the pathogenic effects of ROS are supposed to be dependent on threshold, location, and time. Furthermore, the cellular compartment seems to be of importance. While a global NRF2 activation is beneficial in autoimmune diseases, it is not clear if NRF2 has anti-inflammatory roles specifically in T cells (**Figure 1**). Future experiments might focus on T cells and T cell subtype specific roles of NRF2. Noticeable, SLE T cells display an altered redox metabolism. N-Acetylcysteine reversed glutathione depletion and thereby blocked mTOR activation in T cells and improved disease activity in SLE patients (76). One main problem of SLE patients is their susceptibility to infections. The use of glucocorticoids as immunosuppressive therapy increases this risk. Instead of completely blocking and suppressing the immune system a tight modulation of inflammation while preserving the cells' overall functionality would be much more desirable to treat autoimmune diseases. Therefore, a therapeutic control of NRF2 activity might be a starting point to influence Redox metabolism in SLE T cells, but in-depth analysis of pathways and possible side-effects is absolutely necessary.

## AUTHOR CONTRIBUTIONS

KO and KT agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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