SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLIPID SYNDROME

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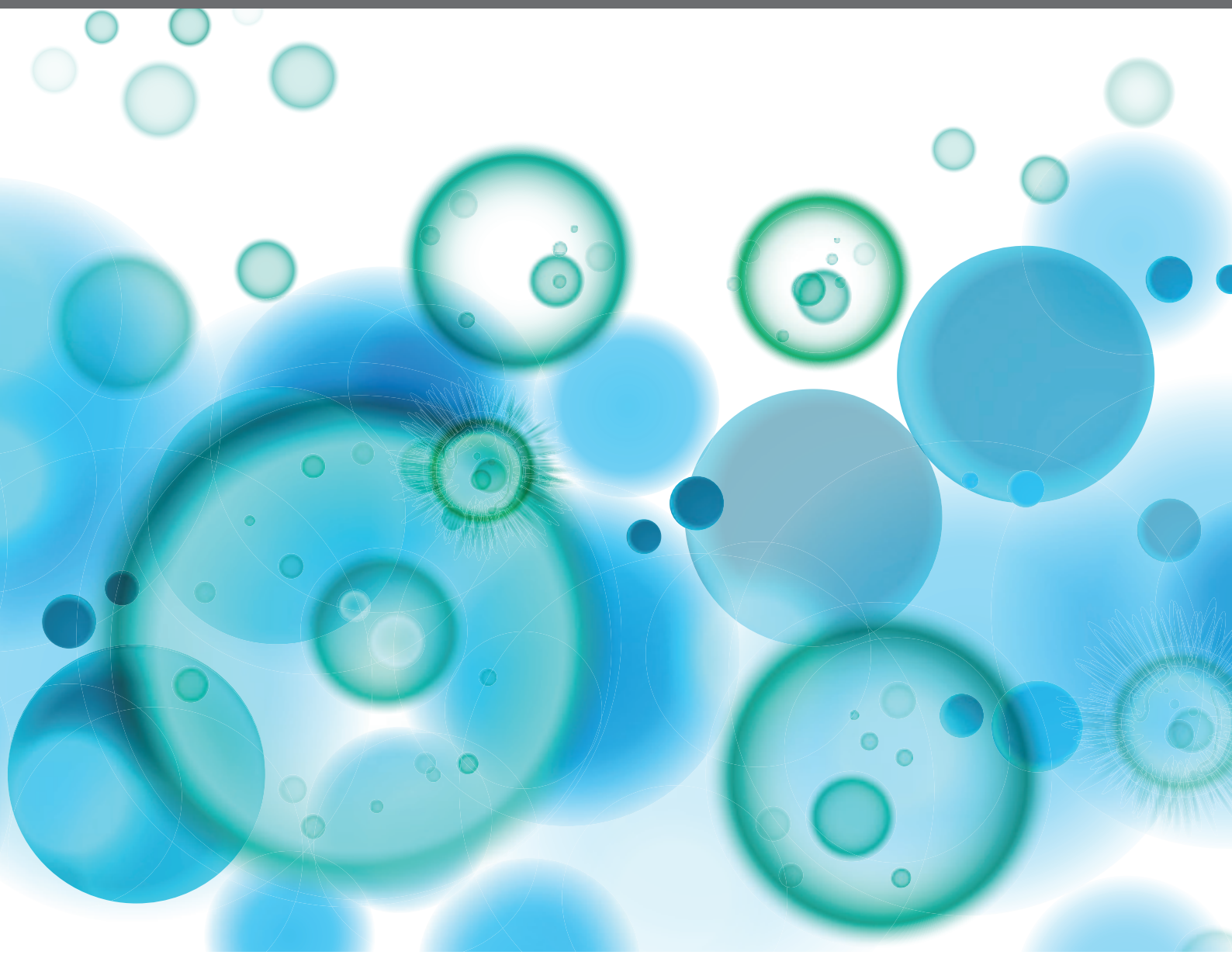

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SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLIPID SYNDROME

Topic Editors:
Pier Luigi Meroni, Istituto Auxologico Italiano (IRCCS), Italy
George C. Tsokos, Harvard Medical School, United States

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EDITORIAL
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Editorial: Systemic Lupus Erythematosus and Antiphospholipid Syndrome

Pier Luigi Meroni[1]* *and George C. Tsokos*[2]

[1] Immunorheumatology Research Laboratory, IRCCS Istituto Auxologico Italiano, Milan, Italy, [2] Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States

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Editorial on the Research Topic

Systemic Lupus Erythematosus and Antiphospholipid Syndrome

Systemic lupus erythematosus (SLE) and anti-phospholipid syndrome (APS) are frequently discussed together and perceived as two closely related diseases (1). Indeed, up to 40% of SLE patients test positive for phospholipid antibodies (aPL) and a significant proportion of patients with primary APS (i.e., with no associated SLE or other autoimmune diseases) have circulating antinuclear (ANA) and dsDNA/chromatin antibodies (2). Patients with primary APS and SLE share lupus susceptibility genes, yet patients with primary APS do not develop complete SLE even after 10 years of follow-up suggesting a more complex link between the two entities (2–4). Indeed, SLE and APS are distinct entities within the spectrum of systemic autoimmune diseases.

In this collection five manuscripts (Caneparo et al.; Han et al.; Knight et al.; Sakata et al.; Weeding and Sawalha) report pathogenic pathways which appear to operate primarily in patients with SLE. The discussed mechanisms (macrophage differentiation via LXRα, association of DNA methylation with SLE triggering and clinical manifestations, IFN-Inducible Protein 16 as an inflammasome regulator in lupus pathogenesis, endonucleotidase in lupus autoimmunity and vascular damage, up-regulation of TLR7-mediated IFNα production) suggest that multiple heterogeneous pathways operate preferentially in patients with SLE rather than in patients with primary APS. For example, the clinical and histological characteristics of renal involvement in patients with APS definitely differentiate the two entities. In particular, a thrombotic vasculopathy involving medium/large and in some cases small vessels is the main pathogenic mechanism in renal APS in contrast with the inflammatory vasculitis which is characteristic of lupus nephritis (Tektonidou; Turrent-Carriles et al.). Furthermore, involvement of the central nervous system (CNS) is frequent in patients with APS and is mainly linked to vascular thrombotic events while a heterogeneous panel of pathogenic mechanisms contribute to the expression of CNS manifestations in patients with SLE including the presence of NMDR antibodies and the activation of microglia by interferon type I (McGlasson et al.). It is obvious that patients need tailored treatment to address the involved pathogenetic mechanisms.

The fact that several distinct, yet intertwined, pathogenic mechanisms covering every aspect of the immune system operate in patients SLE may explain the multifaceted clinical expression of the disease. It is becoming obvious that SLE comprises diverse diseases each characterized by a dominant operating pathogenetic pathway resulting in unique or shared clinical manifestations (Rekvig). Therefore, the classification of patients along the lines of clinical manifestations cannot serve the patient and definitely has not served the multitude of failed clinical trials (5).

The complexity of the pathogenesis of lupus looms even larger in children with SLE in whom hormonal or extensive environmental factors are not yet major contributors but distinct single

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Pierre Miossec,
Claude Bernard University Lyon 1, France

***Correspondence:**
Pier Luigi Meroni
pierluigi.meroni@unimi.it

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gene defects explain the development of SLE. Indeed, as discussed by Lo the list of monogenic SLE patients continues to expand (Lo).

In contrast, the clinical manifestations of patients with APS are easily attributed to thrombophilic events orchestrated by aPL although additional non-thrombotic mechanisms may account for the increased rate of miscarriages (Radic and Pattanaik).

A lot of attention has been paid to aberrant T cell activation pathways in SLE in addition to the tissue damage mediated by immune complex deposition. Several manuscripts in the session of the Journal have actually addressed this issue (Caneparo et al.; Katsuyama et al.; Mizui and Tsokos). SLE "molecular characterization" would be useful for clinicians for a personalized medicine and for better inclusion criteria in clinical trials. In fact, the common biomarkers are not informative enough and we need to enroll more homogenous, along molecular and biochemical lines, populations in the studies and to identify more specific tools for the evaluation of the efficacy of the therapy (5). In contrast, APS is a well-characterized autoantibody-mediated disease but the abnormalities in the cell mediated immune response have been clarified only in part. A manuscript reviewed this issue and discussed the reactivity of T cells against the main antigenic target in APS (i.e., beta2 glycoprotein I), the T-cell epitopes that are recognized and the possible role of T cells in tissue damage (Rauch et al.).

Complement is central in SLE pathogenesis at two levels: luck of the early components C2 and C4 account for the incomplete elimination of autoreactive B cells and lack of C1q for the poor clearance of apoptotic debris whereas excessive activation and generation of the membrane attack complex and the production of C3a and C5a are directly responsible for the execution of tissue damage. APS experimental models support that complement activation takes place in APS as well and it represents a critical step for both aPL-mediated thrombosis and miscarriages. Moreover, there is preliminary evidence for complement activation also in patients. However, the characteristics of complement activation are quite different in SLE and APS further supporting the differences between these two disorders (Tedesco et al.).

We hope that this collection of articles will help readers identify similarities and difference between SLE and APS. More importantly, we hope that they will ask and address critical questions which will advance our understanding of the two entities so that we may serve our patients more effectively.

AUTHOR CONTRIBUTIONS

Both PM and GT (i) had a substantial contribution to the conception or design of the work, or the acquisition, analysis or interpretation of data for the work; (ii) drafted the work or revised it critically for important intellectual content; (ii) provided approval for publication of the content, and (iii) agreed to be accountable for all aspects of the work.

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REVIEW
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β2-Glycoprotein I-Reactive T Cells in Autoimmune Disease

Joyce Rauch[1], David Salem[1], Rebecca Subang[1], Masataka Kuwana[2] and Jerrold S. Levine[3]*

[1] Division of Rheumatology, Department of Medicine, Research Institute of the McGill University Health Centre, Montreal, QC, Canada, [2] Department of Allergy and Rheumatology, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan, [3] Section of Nephrology, Department of Medicine, University of Illinois at Chicago and Section of Nephrology, Department of Medicine, Jesse Brown Veterans Affairs Medical Center, Chicago, IL, United States

Anti-phospholipid syndrome (APS) and systemic lupus erythematosus (SLE) are autoimmune diseases characterized by autoantibody production and autoantibody-related pathology. Anti-phospholipid antibodies (aPL) are found in all patients with APS and in 20–30% of individuals with SLE. aPL recognize a number of autoantigens, but the primary target in both APS and SLE is β2-glycoprotein I (β2GPI). The production of IgG aPL in APS and SLE, as well as the association of aPL with certain MHC class II molecules, has led to investigation of the role of β2GPI-reactive T helper (Th). β2GPI-reactive CD4 Th cells have been associated with the presence of aPL and/or APS in both primary APS and secondary APS associated with SLE, as well as in SLE patients and healthy controls lacking aPL. CD4 T cells reactive with β2GPI have also been associated with atherosclerosis and found within atherosclerotic plaques. In most cases, the epitopes targeted by autoreactive β2GPI-reactive CD4 T cells in APS and SLE appear to arise as a consequence of antigenic processing of β2GPI that is structurally different from the soluble native form. This may arise from molecular interactions (e.g., with phospholipids), post-translational modification (e.g., oxidation or glycation), genetic alteration (e.g., β2GPI variants), or molecular mimicry (e.g., microbiota). A number of T cell epitopes have been characterized, particularly in Domain V, the lipid-binding domain of β2GPI. Possible sources of negatively charged lipid that bind β2GPI include oxidized LDL, activated platelets, microbiota (e.g., gut commensals), and dying (e.g., apoptotic) cells. Apoptotic cells not only bind β2GPI, but also express multiple other cellular autoantigens targeted in both APS and SLE. Dying cells that have bound β2GPI thus provide a rich source of autoantigens that can be recognized by B cells across a wide range of autoantigen specificities. β2GPI-reactive T cells could potentially provide T cell help to autoantigen-specific B cells that have taken up and processed apoptotic (or other dying) cells, and subsequently present β2GPI on their surface in the context of major histocompatibility complex (MHC) class II molecules. Here, we review the literature on β2GPI-reactive T cells, and highlight findings supporting the hypothesis that these T cells drive autoantibody production in both APS and SLE.

Keywords: β2-glycoprotein I, T cells, systemic lupus erythematosus, anti-phospholipid syndrome, autoantibodies, MHC class II haplotypes

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Pier Luigi Meroni, Istituto Auxologico Italiano (IRCCS), Italy

Reviewed by:
Ralf J. Ludwig, Universität zu Lübeck, Germany
Luz Pamela Blanco, National Institutes of Health (NIH), United States
Amedeo Amedei, Università degli Studi di Firenze, Italy

****Correspondence:***
Joyce Rauch
joyce.rauch@mcgill.ca

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease in which individuals develop multiple different autoantibodies, as well as a diversity of organ-related pathologies (1–3). In contrast, anti-phospholipid syndrome (APS) is a more homogeneous syndrome, with a limited number of autoantibodies and pathological outcomes (4, 5). Anti-phospholipid antibodies (aPL) are a key feature in both APS and SLE (4, 5). They are found in all patients with APS and in 20–30% of patients with SLE (6). Among SLE patients, autoantibodies including aPL can be detected up to 10 years before diagnosis (7). Remarkably, SLE autoantibodies targeting a multitude of cellular antigens emerge in a sequential order, with aPL being among the very first (7, 8). While the connection between aPL and autoimmune disease remains strongest for SLE and APS, aPL have also been linked to other autoimmune diseases, such as rheumatoid arthritis (RA) (9). In an inception cohort of patients with connective tissue diseases, the prevalence of aPL was similar for SLE and RA patients at ∼15.7% (9). As in SLE and APS, autoantibodies precede the diagnosis of RA by several years (10). A common feature among APS, SLE, and RA that may help to understand the transition from serologic to pathologic autoimmunity is altered IgG glycosylation (11–14). For example, in RA, IgG glycosylation was similar in patients and controls a decade prior to the diagnosis of RA, but altered substantially ∼3.5 years before disease onset (12). Taken together, these findings suggest a common mechanism for autoantibody generation and progression to organ pathology in autoimmune disease, and one in which aPL may be key, particularly APS and SLE. Although APS and SLE differ in their clinical manifestations, there is significant overlap in individuals affected by both diseases (6). Indeed, APS has been shown to develop in 50–70% of patients with aPL-positive SLE patients after 20 years of follow-up (6).

Both APS and SLE are characterized by the production of high levels of IgG class-switched autoantibodies, consistent with a T helper (Th) cell response. In APS, the autoantibodies primarily recognize phospholipid-binding proteins, such as β2-glycoprotein I (β2GPI) and prothrombin. In SLE, the range of autoantibodies is much broader, and includes aPL as well as autoantibodies targeting non-protein antigens, such as double-stranded DNA (dsDNA). In both APS and SLE, β2GPI is the primary target of the aPL. One of the major gaps in our understanding of SLE is how a T cell response can develop to a non-protein antigen. It has been noted that many of the non-protein autoantigens (e.g., DNA, RNA, phospholipid) targeted in SLE form complexes *in vivo* with protein antigens (1). This has led to speculation that a T cell response to the protein portion of the complex may provide T cell help to the complex's non-protein entity via intermolecular epitope spread. For example, a "hapten-carrier" model has been proposed to explain the production of anti-DNA autoantibodies in SLE (15). In this model, DNA is the "hapten" (i.e., non-immunogenic molecule) and elicits an immune response only when bound to a DNA-binding "carrier protein" (i.e., immunogenic molecule), such as histones, which can activate functional Th cells (15).

Our group has proposed a similar "hapten-carrier" model to address the breadth of the autoantibody response in SLE, in which an apoptotic or other dying cell—in particular, its non-protein determinants (e.g., phospholipid or DNA)—serve as "haptens," while β2GPI serves as the "carrier protein" and promotes the activation of β2GPI-reactive T cells (16). In this regard, the phospholipid-binding property of β2GPI is critical, as it enables β2GPI to bind to the negatively charged surface of apoptotic cells, as well as other negatively charged particles and molecules (17). The ability of β2GPI to interact with dying cells is of particular relevance to this review (18–20). Apoptotic cells have long been proposed as a source of autoantigens in SLE (16, 21–23), and the physical interaction of β2GPI with these cells provides a "carrier protein"-like connection to a large pool of cellular autoantigens. β2GPI-reactive T cells therefore have the potential to promote autoantibody production to a multitude of self-antigens expressed by dying cells (24). Here, we review the literature and present findings supporting the hypothesis that β2GPI-reactive T cell responses stimulate autoantibody production in both APS and SLE.

β2GPI-REACTIVE T CELLS IN APS AND SLE

Overview

Evidence of a role for Th cells in APS comes from the association of aPL with certain MHC class II genes (25), as well as from autoantibody class-switch to IgG. Similarly, Th cells are implicated (26) in the pathophysiology of SLE by virtue of both MHC class II associations (27) and IgG autoantibody production (2), as well as aberrant signaling defects reported in SLE T cells (28). Multiple HLA alleles, including HLA-DR2 and HLA-DR3, are associated with SLE, but the strength of this association and the specific allele(s) identified depend on the ethnic group and clinical presentation studied (29). The lack of consistent MHC class II associations in SLE, and the multitude of autoantigens targeted, make identification of critical Th cell epitopes in this disease a major challenge. Additional evidence of the importance of Th cells in these diseases derives from murine models. Anti-CD4 antibodies prevented disease in a model of SLE with APS (30), and bone marrow cells transferred experimental APS to naive mice only when T cells were present (31).

Interest in β2GPI-reactive Th cells developed in the late 1990's to early 2000's (32–35), about 10 years after the discovery that β2GPI, and not phospholipid, was the antigen recognized by anti-cardiolipin antibodies (anti-CL) (36, 37). Most published studies on human β2GPI-reactive T cells include both primary and secondary APS patients, as well as SLE patients without APS. Hence, it is difficult to discuss findings for β2GPI-reactive T cells in APS patients separately from SLE patients without APS. For this reason, we will discuss β2GPI-reactive T cells in APS and SLE concurrently. In this way, findings (often within the same study) for the different disease groups and subsets can be compared.

Association of β2GPI-Reactive T Cells With Autoantibodies and Disease

In early studies of β2GPI-reactive T cells, patients were usually classified according to aPL reactivity or to the presence vs. absence of APS. Many of these studies evaluated T cell reactivity

using peripheral blood mononuclear cells (PBMCs) from patients and healthy individuals, while others used patient-derived T cell lines or clones. Visvanathan et al. (33) studied the response of PBMCs to native plasma-derived β2GPI using a serum-free system in 24 aPL-positive (anti-CL- or lupus anticoagulant [LA]-positive) individuals, 7 aPL-negative individuals with various autoimmune diseases (including SLE), and 15 healthy controls. Of the 24 aPL-positive individuals, 18 had APS (5 SLE, 13 primary APS) and the remaining 6 autoimmune patients lacked clinical manifestations of APS (only one with SLE). PBMC responses to β2GPI were observed only in the aPL-positive group, and specifically in patients with APS (8 out of 18, 4 with SLE, and 4 with primary APS). Statistically, PBMC responses were associated with a history of APS, but not with IgG anti-β2GPI levels, and were characterized by a selective expansion of CD4 T cells producing IFN-γ, but not IL-4 (Th1-like response) (33).

Hattori et al. (34) also studied the PBMC responses in APS patients (5 SLE, 7 primary APS) and in SLE patients without APS ($n = 13$), as well as in healthy controls ($n = 12$). In contrast to Visvanathan et al. (33), they used dithiothreitol-reduced, not native, β2GPI as the stimulating antigen, and β2GPI-depleted serum in the culture medium. Moreover, patients were analyzed according to anti-β2GPI IgG antibody reactivity. PBMC responses to β2GPI were found in all anti-β2GPI-positive patients (6 primary APS, 4 SLE with APS, 2 SLE without APS), but also in anti-β2GPI-negative individuals (4 SLE without APS, 6 healthy controls). Most (91%) individuals with PBMC responses to β2GPI ("responders") expressed HLA-DR53-associated alleles (DRB1*04, *07, or *09), as compared to 47% of "non-responders." The domain specificity of the CD4 T cell proliferative response to recombinant β2GPI was assessed in six patients positive for anti-β2GPI antibodies (3 primary APS, 2 SLE with APS, 1 SLE without APS), and all recognized an epitope within Domains IV and/or V. Patients with the DRB1*09:01; DQB1*03:03 haplotype also recognized an epitope within Domains III/IV, while T cells from patients not expressing this haplotype recognized only Domains IV/V. Finally, T cells from one primary APS patient recognized Domains I/II as well as Domain IV/V.

To further analyze the epitope specificity and functional capacity of the T cells in these patients, Arai et al. (38) generated CD4 T cell clones from three patients with APS (2 primary APS, 1 SLE with APS). The majority (6 out of 7) of the β2GPI-specific T cell clones recognized a peptide encompassing amino acid residues 276–290 (KVSFFCKNKEKKCSY) in Domain V of β2GPI in the context of the DRB4*01:03 allele. Interestingly, this peptide spans the major phospholipid-binding site of β2GPI. All of the β2GPI-reactive T cell clones produced IFN-γ and had a Th1- or Th0-like cytokine expression profile. While the majority (10 of 12) of the β2GPI-specific T cell clones stimulated autologous peripheral blood B cells to produce anti-β2GPI antibodies *in vitro*, IFN-γ was not involved in B cell activation by these clones. Instead, stimulation was dependent on T cell production of IL-6 and CD40-CD40L interaction. The authors suggest that IL-6 and CD40L could be targeted therapeutically in APS patients resistant to anticoagulation.

T cell receptor (TCR) β chain usage was also analyzed in individuals demonstrating PBMC responses to β2GPI (5 APS patients and 3 healthy controls) (39). Vβ7 and Vβ8 were the most commonly detected TCRβ chains, and T cells expressing these two chains exhibited limited complementarity-determining region 3 (CDR3) sequence diversity. The Vβ7 chain was used by β2GPI-reactive T cells in PBMCs from 5 of 5 patients with APS, and 2 of 3 healthy controls. These findings from a limited group of individuals suggest a preferential usage of TCRβ chains by β2GPI-reactive T cells, whether in APS or healthy individuals.

Ito et al. (35) also investigated T cell responses to β2GPI in PBMCs from 18 patients (1 primary APS, 4 SLE with APS, 10 SLE without APS, and 3 SLE-like without APS) and 10 healthy controls. Instead of full-length β2GPI or its intact domains, they used a peptide library encompassing the full β2GPI sequence to screen PBMCs. Four patients and 2 controls had positive responses, and their T cells were used to generate 7 CD4 T cell lines that did not respond to native plasma-derived β2GPI. A limited number of epitopes were observed. Three of the 4 patient-derived T cell lines recognized peptide 244–264 in Domain V; this same peptide was also recognized by a cell line derived from a healthy control. The other peptides that were recognized were 64–83, 154–174, and 226–246. No association was observed between peptide recognition and a particular HLA class II molecule. Interestingly, however, cytokine production differed significantly between patient- and control-derived T cell lines. Although both produced IL-4 and IFN-γ, patient-derived T cell lines had significantly lower IFN-γ/IL-4 ratios than control lines, primarily due to lower IFN-γ responses in the patient-derived lines. Of note, none of the T cell lines reacted with native β2GPI. Together, these findings indicate that β2GPI-reactive CD4 T cell lines from this group of APS and SLE patients predominantly recognize the 244–264 epitope within Domain V of β2GPI. They do so in the context of various HLA class II molecules, and exhibit Th0- or Th2-like responses. In contrast, T cell lines from healthy controls display a Th0- or Th1-like phenotype.

Important methodological differences exist among the studies summarized to this point. The discovery of β2GPI-reactive T cells among anti-β2GPI- and APS-negative individuals (both SLE patients and healthy controls) by Hattori et al. (34) and Ito et al. (35), but not by Visvanathan et al. (33), may be attributed to such differences. To evaluate PBMC reactivity, Hattori et al. (34) used chemically reduced β2GPI, whereas Ito et al. (35) used a peptide library, and Visvanathan et al. (33) used native β2GPI. The decision of Hattori et al. (34) to use chemically reduced β2GPI was based on their observation that patient PBMCs did not respond to tissue culture medium with 10% human serum containing native β2GPI. While secondary cultures of PBMCs responsive to reduced β2GPI also recognized full-length recombinant β2GPI, they still lacked reactivity to native β2GPI. Similarly, Ito et al. (35) chose to evaluate synthetic β2GPI peptides because their T cell lines did not respond to native β2GPI isolated from human plasma. In contrast, Visvanathan et al. (33), using a serum-free system, showed that PBMCs from APS patients responded both to purified plasma-derived β2GPI and to native β2GPI in whole plasma. A second major difference between the studies was patient selection. Hattori et al. (34) and

Ito et al. (35) selected patients based on a clinical diagnosis of SLE or APS (primary or secondary), while Visvanathan et al. (33) selected patients based on laboratory criteria for aPL (defined in that study as IgG or IgM anti-CL, or LA). Third, the geographical and, likely, the ethnic origin of individuals in the studies by Hattori et al. and Ito et al. differed from that in the study by Visvanathan et al.: Japan (34, 35) and Australia (33), respectively. Finally, Visvanathan et al. (33) evaluated neither the HLA association of the PBMC response nor its epitope specificity. The studies from Hattori et al. (34) and Ito et al. (35), while having many similarities, also exhibit subtle differences. Epitope specificity, although primarily within Domain V for both groups, differed in its precise mapping (35, 38). The pattern of cytokine production also differed; it was Th1-like for healthy controls in both studies (34), but Th1-like vs. Th2-like for patients in studies by Ito et al. (35) and Hattori et al. (34), respectively. The β2GPI T cell epitopes identified in the studies by Ito et al. (35) and Arai et al. (38) are shown in **Table 1**, and compared with epitopes identified in later studies (as discussed below).

Davies et al. (43) directly evaluated whether a PBMC response to native β2GPI was associated with the presence of anti-β2GPI and/or specific MHC class II genotypes in a cohort of Caucasian SLE patients in England. They found a proliferative PBMC response to β2GPI in 15/51 (29%) SLE patients, compared to 7% of controls. Proliferative responses to β2GPI were observed in SLE patients in the presence or absence of anti-β2GPI antibodies; however, some of the anti-β2GPI-negative patients had anti-CL. Patients with anti-β2GPI and/or anti-CL had a significantly higher proliferative response, compared to healthy controls. Despite the fact that certain HLA genotypes were associated with the presence of anti-β2GPI, no association was found between proliferative PBMC responses to β2GPI and any HLA genotypes.

A more recent study comparing PBMC responses to native β2GPI in unselected SLE and primary APS patients found a similar frequency in both groups (32% [12/37] in SLE vs. 25% [3/12] in primary APS) and no response in 23 control subjects (44). Recruitment of both SLE and primary APS patients was consecutive, and 38% of SLE patients had secondary APS. PBMCs proliferating to native β2GPI produced IFN-γ, but not IL-4. Proliferation was statistically associated with all of the following: IgM anti-β2GPI and anti-CL levels, a history of arterial thrombosis, and increased intimal-medial thickness. Interestingly, PBMC proliferation to β2GPI was also associated with a history of anti-nuclear antibodies and anti-dsDNA serum positivity, indicating that β2GPI-reactive T cells can be associated with SLE autoantibodies other than aPL.

Few other studies have addressed the relevance of immune reactivity to β2GPI for autoantibodies other than aPL in human SLE. Arbuckle et al. (7) showed that anti-CL (i.e., β2GPI-reactive antibodies) are among the earliest autoantibodies to appear in individuals who develop SLE, and can appear up to 7.6 years before diagnosis. The same group (8) further showed that, among SLE patients, individuals who developed anti-CL prior to diagnosis of SLE had a more severe and complex clinical outcome than individuals lacking anti-CL. Patients who were anti-CL positive prior to diagnosis presented with a greater number of classification criteria for SLE, compared to other SLE patients (6.1 vs. 4.9 criteria, $P < 0.001$). Disease onset occurred almost 4 years earlier in anti-CL-positive SLE patients, with earlier onset of such clinical manifestations as malar rash, photosensitivity, serositis, neurologic symptoms, and nephropathy. In addition, SLE-specific autoantibodies appeared earlier in aCL-positive individuals. Anti-dsDNA and anti-Sm antibodies appeared ∼1 and 2 years earlier, respectively, and anti-dsDNA antibodies occurred more frequently (79% vs. 55% in anti-CL-negative individuals). Although not evaluated in this cohort of SLE patients, it seems likely that these individuals had β2GPI-reactive T cells, given the association between anti-CL and a T cell response to β2GPI observed in other studies (33, 44).

Taken together, these findings suggest that a cellular immune response to β2GPI exists in patients having both APS and SLE across a wide spectrum of MHC class II genotypes, and is associated with autoantibodies other than those reactive with β2GPI. While the frequency and clinical/serological associations vary among studies, these differences may be attributed to a number of factors, including patient selection and the nature of the antigen (native, reduced, or recombinant β2GPI; or peptide library) used to evaluate T cell reactivity. The presence of β2GPI-reactive T cells in healthy controls also varies among studies, but seems more frequent in studies not using native β2GPI.

The Antigenic Stimulus for β2GPI-Reactive T Cells

Structural Alteration of Self-Antigen

Notably, many β2GPI-reactive T cells derived from PBMCs do not respond to native β2GPI, but respond well to bacterially expressed recombinant β2GPI fragments and to chemically reduced β2GPI. These findings suggest that the generation of β2GPI T cell epitopes requires unfolding or structural modification of β2GPI. Kuwana et al. (45) demonstrated that anionic phospholipid may be involved in the generation of T cell epitopes (often referred to as "cryptic epitopes") not generated through processing of native β2GPI. They showed that dendritic cells or macrophages pulsed with vesicles containing anionic phospholipid and β2GPI, but not β2GPI or phospholipid alone, induced a response in human T cell lines specific for the Domain V epitope (276–290) in an HLA-DRB4*01:03-restricted manner. A later study showed that the same epitope can be generated *in vivo* by monocytes through FcγRI-mediated uptake of negatively charged particles (e.g., phosphatidylserine-containing vesicles) that have bound β2GPI in the presence of IgG anti-β2GPI antibodies (46). β2GPI bound to oxidized LDL or activated platelets also induced β2GPI-specific T cell responses (46). These data suggest that disease-relevant T cell epitopes in β2GPI may arise as a consequence of antigen processing of anionic phospholipid-bound β2GPI.

Buttari and coworkers (47, 48) also demonstrated that modification of β2GPI enhanced T cell activation, but they used alloreactive, rather than β2GPI-specific, T cells. They found that oxidized (47) and glucose-modified (48) β2GPI not only activated immature monocyte-derived dendritic cells from healthy human donors, but also increased allostimulatory ability in a mixed lymphocyte reaction. Dendritic cells activated by these modified

TABLE 1 | β2GPI $CD4^+$ T cell epitopes identified in APS and SLE.

Peptide sequence	Domain	Disease	Source (MHC class II)	Publication
^{1}MISPVLILFSSFLCHVAIAG^{20}	I	APS	Human PBMCs (DRB3*02:02)†	de Moerloose et al. (42)
^{26}PDDLPFSTVVPLKTF^{40}	I	Induced SLE	129S1 (I-A^b)	Salem et al. (40)
^{31}FSTVVPLKTFYEPGE^{45}	I	Induced SLE	BALB/c (I-A^d/I-E^d)	Salem et al. (40)
^{111}NTGFYLNGADSAKCT^{125}	II	PAPS Induced SLE	Human PBMCs (DRB1*04:03) C57BL/6 (I-A^b), 129S1(I-A^b)	Salem et al. (40)
^{154}ECLPQHAMFGNDTITCTTHGN^{174}	III	SAPS	Human PBMCs❖	Ito et al. (35)
^{159}SAGNNSLYRDTAVFECLP^{176}	III	Induced SLE Spontaneous SLE	C57BL/6 (I-A^b), C3H/HeN (I-A^k/I-E^k) MRL/lpr (I-A^k/I-E^k)	Salem et al. (41)
^{165}LYRDTAVFECLPQHAMFG^{182}	III	Induced SLE Spontaneous SLE	C57BL/6 (I-A^b), C3H/HeN (I-A^k/I-E^k) MRL/lpr (I-A^k/I-E^k)	Salem et al. (41)
^{208}PSRPDNGFVNYPAKPTLY^{225}	IV	Induced SLE Spontaneous SLE	C3H/HeN (I-A^k/I-E^k) MRL/lpr (I-A^k/I-E^k)	Salem et al. (41)
^{256}AMPSCKASCKVPVKKATV^{273}	IV/V	Induced SLE	C3H/HeN (I-A^k/I-E^k)	Salem et al. (41)
^{244}SCKLPVKKATVVYQGERVKIQ^{264}	V	SAPS, SLE	Human PBMCs (DRB1*04:03, DRB4*01:03)†	Ito et al. (35)
^{247}VPVKKATVVYQGERV^{261}	V	PAPS	Human PBMCs (DRB1*04:03, DRB4*01:03)	Arai et al. (38)
^{276}KVSFFCKNKEKKCSY^{290}	V	PAPS, SAPS	Human PBMCs (DRB4*01:03)	Arai et al. (38)

APS, anti-phospholipid syndrome; SAPS, secondary APS; SLE, systemic lupus erythematosus. The numbering of amino acids in the studies by Salem et al. (40, 41) and de Moerloose et al. (42) includes the 19-amino acid leader sequence. In studies by Salem et al. (20, 40), murine T cells were derived from spleen.
† Prominent HLA restrictions are noted here, but additional restrictions were found; ❖, HLA restriction was not defined.

forms of β2GPI also primed naïve allogeneic T cells, and induced Th polarization (primarily Th1-like for oxidized β2GPI, and Th2-like for glucose-modified β2GPI) (47, 48). These investigators suggest that oxidized β2GPI leads to dendritic cell maturation via interaction with a toll-like receptor (TLR), while glucose-modified β2GPI utilizes a receptor for advanced glycation end products.

Conformational changes in β2GPI resulting from genetic variants of the protein can also induce stronger T cell responses. For example, the V^{247} polymorphism located on exon 7, which leads to a substitution of leucine (L) for valine (V) at amino acid position 247 in Domain V of β2GPI, is associated with high titers of anti-β2GPI and arterial thrombosis in Mexican patients with primary APS (49). Genotypes for β2GPI can be VL, VV, or LL. Núñez-Álvarez et al. (50) assessed the proliferative response of PBMCs to the VL, VV, and LL isoforms at position 247 of β2GPI in 10 primary APS patients and 10 healthy individuals. PBMCs from primary APS patients had a stronger proliferative response than healthy controls to the VV and VL isoforms of β2GPI, but not to the LL isoform. The strongest response was to the VL form of β2GPI. Proliferation was stronger to chemically reduced vs. native isoforms, particularly for VV. The proliferative response of healthy control PBMCs was much lower than that of primary APS patients, and it did not appear to differentiate between isoforms or reduced/native conditions. Núñez-Álvarez et al. (50) further showed using differential scanning calorimetry that the structures of the V^{247} and the L^{247} isoforms of β2GPI differ, indicating that a single amino acid change at position 247 results in a major conformational change in β2GPI.

Together, these findings suggest that structural changes in β2GPI resulting from molecular interactions (e.g., with phospholipids), post-translational modification (e.g., oxidation or glycation), or genetic alteration (e.g., β2GPI variants) can enhance the presentation of disease-relevant epitopes. It is noteworthy that in a large retrospective multicenter analysis, patients with APS had higher levels of both native β2GPI and oxidized β2GPI than control groups including healthy individuals, autoimmune disease controls (with or without aPL, but lacking APS), and patients with thrombosis but no aPL (51, 52). Krilis and coworkers have proposed that post-translationally modified β2GPI can break immune tolerance, either because the modified form of β2GPI is not represented in the thymus or because intracellular processing of the oxidized form of β2GPI is different from that of the circulating (reduced) protein (51, 52). The latter hypothesis is consistent with the current evidence that some β2GPI-reactive T cells respond to modified, but not, native β2GPI.

Molecular Mimicry

The microbiome may potentially be a source of self-antigens that either trigger or perpetuate an autoreactive T cell response in APS (53) and SLE (54–56). Ruff et al. (53) have proposed that commensal bacteria act as a reservoir of cross-reactive antigen in APS and SLE through a mechanism called "molecular mimicry." Molecular mimicry occurs when B and/or T cells responding to microbial pathogens also recognize (cross-react with) self-antigen. Ruff and coworkers (53) have identified peptides that are potentially cross-reactive with dominant T cell epitopes in APS in *Roseburia intestinalis*, a gram-positive anaerobic commensal particularly abundant in the human gut and stimulatory to lymphocytes from APS patients [unpublished observations in Ruff et al. (53)]. In SLE patients, skin and mucosal commensal orthologs of the human autoantigen Ro60

have recently been shown to activate human Ro60 autoantigen-specific CD4 memory T cell clones, further supporting the notion of human T cell cross-reactivity with commensal antigens (54).

To address experimentally the potential role of commensal bacteria in APS and SLE, Ruff et al. (53) treated (NZW × BXSB)F1 hybrid mice with broad-spectrum antibiotics (vancomycin or ampicillin), and showed that depleting gut microbiota decreased anti-β2GPI antibody levels and prevented thrombotic events in this model (53). SLE-related autoantibodies (anti-dsDNA and anti-RNA) and mortality were also diminished in antibiotic-treated (NZW × BXSB)F1 hybrid mice (55). From a pathophysiologic perspective, it was noted that (NZW × BXSB)F1 hybrid mice had impaired gut barrier function compared to non-autoimmune C57BL/6 mice. Loss of barrier function culminated in bacterial growth within the mesenteric veins, mesenteric lymph nodes, liver, and spleen. Full-length 16S ribosomal DNA sequencing of single colonies from organ cultures of (NZW × BXSB)F1 hybrid mice detected *Enterococcus gallinarum*, a Gram-positive gut commensal of animals and humans. Antibiotic treatment of the mice suppressed translocation of the microbiota, and correlated with reduced levels of T cells (Th17 and T follicular helper cells) and T cell cytokine signatures, as well as autoantibody levels and immunopathology. Of note, Manfredo Vieira et al. (55) also found *E. gallinarum* in liver biopsies from SLE patients, and showed that stimulation of primary non-autoimmune human or murine hepatocytes with *E. gallinarum* induced the production of β2GPI and type I interferon. These investigators (55) propose that translocating commensal bacteria may act in a number of ways to incite or perpetuate autoimmunity, including molecular mimicry, Th cell differentiation skewing, and induction of autoantigens (e.g., β2GPI) in colonized tissues. As Manfredo Vieira et al. (55) did not investigate whether β2GPI-reactive T cells were suppressed after microbiota depletion in their animal model, it is not clear whether antibiotic treatment impacts β2GPI-reactive T cells specifically. However, their findings showing increased β2GPI production in *E. gallinarum*-colonized liver and decreased anti-β2GPI autoantibody production in antibiotic-treated mice support a potential role for microbiota in the APS- and SLE-related manifestations observed in (NZW × BXSB)F1 hybrid mice.

β2GPI-Reactive T Cells in Atherosclerosis

Non-APS-related Atherosclerosis

Relatively little is known about β2GPI-reactive T cells located within tissues, as compared to those found in peripheral blood. Profumo et al. (57) evaluated β2GPI-reactive T cells in patients with carotid atherosclerosis, both those occurring in peripheral blood and those infiltrating advanced carotid atherosclerotic plaques. The study population comprised 35 consecutive patients undergoing endarterectomy for symptomatic carotid artery stenosis or asymptomatic severe or pre-occlusive carotid-artery stenosis (≥70%). Individuals with recent infections, autoimmune diseases, malignancies, and inflammatory diseases prior to enrolment were excluded from the study. Only 1 patient was positive for anti-β2GPI and anti-CL (IgM for both antibodies). Plaque-derived and peripheral blood T cells were analyzed in 5 patients, while only peripheral blood T cells were analyzed in the remaining 30 patients. A proliferative response to native β2GPI was observed in 1 of 5 (20%) plaque-infiltrating T cell isolates, and in 8 of 35 (23%) PBMC samples, compared to no response in PBMCs from 13 healthy controls. β2GPI-reactive T cells in both plaque and PBMCs produced elevated IFN-γ and TNF-α, and were predominantly Th1-polarized. The importance of these findings lies in the occurrence of β2GPI-reactive T cells among atherosclerotic patients, despite the absence of overt autoimmune disease.

APS-Related Atherosclerosis

Benagiano et al. (58) also studied β2GPI-reactive T cells located within atherosclerotic lesions, but included patients with APS. CD4 T cell clones were generated from atherosclerotic lesions of 4 aPL-positive patients with primary APS and 4 aPL-negative individuals, all with arterial occlusive disease of the lower extremities. Thirty-two of 115 (28%) CD4 T cell clones from primary APS patients proliferated in response to native β2GPI, as compared to none of 263 CD4 T cell clones from aPL-negative individuals. CD8 T cell clones from the same lesions did not respond to β2GPI. All β2GPI-reactive plaque-derived T cell clones expressed IFN-γ and TNF-α in response to β2GPI, consistent with a Th1-like phenotype. More than 80% (26 of 32) of the β2GPI-reactive T cell clones recognized an epitope within Domain I, while only 19% (6 of 32) recognized an epitope within Domains IV and/or V. Interestingly, the predominance of Domain I-specific T cell clones in atherosclerotic lesions of primary APS patients differs from the predominant Domain V specificity observed in peripheral T cells of APS patients (34, 35, 38). Of note, the β2GPI-reactive T cell clones induced expression of tissue factor and matrix metalloproteinase-9 by autologous monocytes, and promoted total IgG, IgM, and IgA production in autologous B cells. In addition, plaque-derived β2GPI-reactive T cell clones were able to induce perforin-mediated cytotoxicity in EBV-transformed B cells and Fas/Fas ligand-mediated apoptosis in Jurkat cells, suggesting their ability to cause cellular damage.

A second group (42) also demonstrated an immunodominant T cell epitope within Domain I of β2GPI, in this case in PBMCs of 9 patients with APS (primary or secondary not specified). Five of the 9 patients had a thrombotic event, while the remaining 4 had fetal loss. The patients were all positive for IgG anti-CL and anti-β2GPI. In addition to recognizing recombinant Domain I/II, CD4 T cells responded to reduced, but not native, β2GPI. The authors identified an epitope located in Domain I of β2GPI (within the leader sequence of β2GPI [^{1}MISPVLILFSSFLCHVAIAG^{20}]), and showed that it is recognized in the context of MHC class II haplotype DRB3*02:02.

Benagiano et al. (58) have speculated on the mechanistic role of β2GPI-reactive T cells in atherothrombosis. They hypothesize that endothelial cells and professional antigen-presenting cells within the atherosclerotic plaque may become targets of the cytotoxic and apoptotic activity of β2GPI-reactive T cells, resulting in necrotic cores characteristic of unstable atherosclerotic lesions and leading eventually to atherothrombosis. Conti et al. (44) have shown that PBMC proliferation to β2GPI is associated with a history of arterial

thrombosis and with increased intimal-medial thickness among patients with SLE and primary APS. They suggest that β2GPI-specific T cell reactivity may be associated with subclinical atherosclerosis. Of note, β2GPI has been found in human atherosclerotic plaques (59). Similarly, β2GPI was found in early murine atherosclerotic lesions, and co-localized with macrophages, endothelial cells, and smooth muscle cells in atherosclerosis-prone mice (60). Furthermore, immunization with β2GPI promoted enhanced fatty streak formation in atherosclerosis-prone mice (32, 61), and transfer of β2GPI-reactive T cells promoted early atherosclerosis (60). Finally, Domain I-specific antibodies have been shown to be more strongly associated with thrombosis and obstetric complications than antibodies to other domains of β2GPI (62, 63).

β2GPI-Reactive T Cells in Murine Models of SLE

Our group has developed a murine model of SLE that is induced in healthy non-autoimmune mice by immunization with heterologous β2GPI and a strong pro-inflammatory stimulus (lipopolysaccharide [LPS]). Disease in this model bears striking similarities to human SLE (16). Notably, the specificities and sequential emergence of SLE-associated autoantibodies in this model closely mimic those seen in human SLE (7). The production of autoantibodies culminates in the development of overt glomerulonephritis (16). Furthermore, we have shown that β2GPI-reactive T cells are critical for the development of this model, and they are associated with the development of SLE autoantibodies across a spectrum of MHC class II backgrounds (40, 64). While epitope specificity of the β2GPI-specific T cell response is related to MHC class II haplotype, mice of multiple haplotypes develop SLE-related autoantibodies (40). A common T cell epitope was shared across different MHC class II haplotypes and therefore may be important in the development and spread of the autoimmune response (41). Specifically, peptide 31 (amino acid sequence ^{165}LYRDTAVFECLPQHAMFG^{182}) in Domain III of β2GPI was recognized by T cells in both C57BL/6 (I-A^b) and C3H/HeN (I-A^k/I-E^k) mice immunized with β2GPI and LPS. This epitope was also recognized by T cells from MRL/MpJ-*Tnfrsf6*^{lpr} (MRL/lpr) mice, which develop murine SLE spontaneously. Despite recognizing a common epitope, β2GPI-reactive CD4 T cells from the induced and spontaneous models differ in cytokine production: T cells from the induced model expressed IFN-γ (Th1-like), while T cells from MRL/lpr mice expressed both IL-17 and low levels of IFN-γ (Th17-like) (41). Together, these data demonstrate the sharing of a β2GPI-reactive T cell response by both induced and spontaneous models of SLE, and raise the intriguing possibility that this T cell response mediates epitope spread of autoantibodies in both models.

β2GPI-reactive T cells from the induced SLE model also recognize a Domain II epitope (peptide 23, amino acid sequence ^{111}NTGFYLNGADSAKCT^{125}) that is shared by both murine and human T cells. Unlike peptide 31 in Domain III that is recognized across MHC class II haplotypes in mice, this peptide appears to be recognized only by MHC class II I-A^b-bearing murine T cells (e.g., from C57BL/6 and 129S1). However, human CD4 T cell clones from a patient with primary APS (40) responded to this peptide in the context of a single HLA-DR allele, DRB1*04:03, an allele that has been associated with the presence of aPL (both anti-CL and anti-β2GPI) in a European cohort of SLE patients (65). These findings further point to a potentially similar β2GPI-specific T cell response in SLE and primary APS.

Taken together, these data suggest that β2GPI-specific T cell specificities in murine SLE, both spontaneous and induced, overlap with those found in human SLE and APS. They further indicate that, at least in mice, β2GPI-reactive T cells are associated with the development of multiple and diverse autoantibodies in SLE. **Figure 1** illustrates a possible mechanism by which β2GPI-reactive T cells could promote the development of multiple serological and clinical outcomes. According to this scenario, β2GPI-reactive CD4 T cells provide help to autoantigen-specific B cells that have taken up apoptotic (or other dying) cells and present MHC class II-bound β2GPI peptides on their surface (16, 24). In this manner, B cells specific for various SLE-associated autoantigens (expressed on the surface of dying cells) can receive T cell help, and secrete class-switched autoantibodies against these autoantigens (including anti-β2GPI, anti-CL, and anti-dsDNA). In addition, pro-inflammatory cytokines produced by the β2GPI-reactive T cells can impact other cells and tissues, either locally in a paracrine manner or at a distance in an endocrine manner. Depending on the autoantibodies and cytokines produced, different pathologies could arise (e.g., thrombosis or atherothrombosis with aPL, and glomerulonephritis with anti-dsDNA). Through this mechanism, β2GPI-reactive T cells could be a driving force for autoantibody production and pathology in both APS and SLE.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The nature and role of β2GPI-reactive T cells in APS and SLE remains a field ripe for future investigation. The current literature provides evidence that β2GPI-reactive T cells are critical to the pathogenesis and pathophysiology of APS. While less solid, evidence also exists for a similar role of β2GPI-reactive T cells in SLE. The presence of class-switched IgG aPL in SLE-prone individuals almost a decade before disease onset suggests that β2GPI-reactive T cells are present in these individuals early in the disease process. The association of aPL with earlier disease onset, as well as a more complex and severe clinical course, further supports the potential importance of these T cells in SLE. β2GPI-reactive T cells are also found in both non-autoimmune and autoimmune patients with atherosclerosis, either subclinical (44) or overt (44, 57, 58). This last finding highlights the clinical relevance of β2GPI-reactive T cells in patients other than those with APS and SLE. Experimental findings in murine models of APS (64, 66, 67), atherosclerosis (32, 60), and SLE (40, 41) complement these human data and strengthen the notion that β2GPI-reactive T cells play an important role in the pathogenesis of these diseases.

Despite these advances, many key questions require further investigation. Further comparisons of β2GPI-reactive T cells

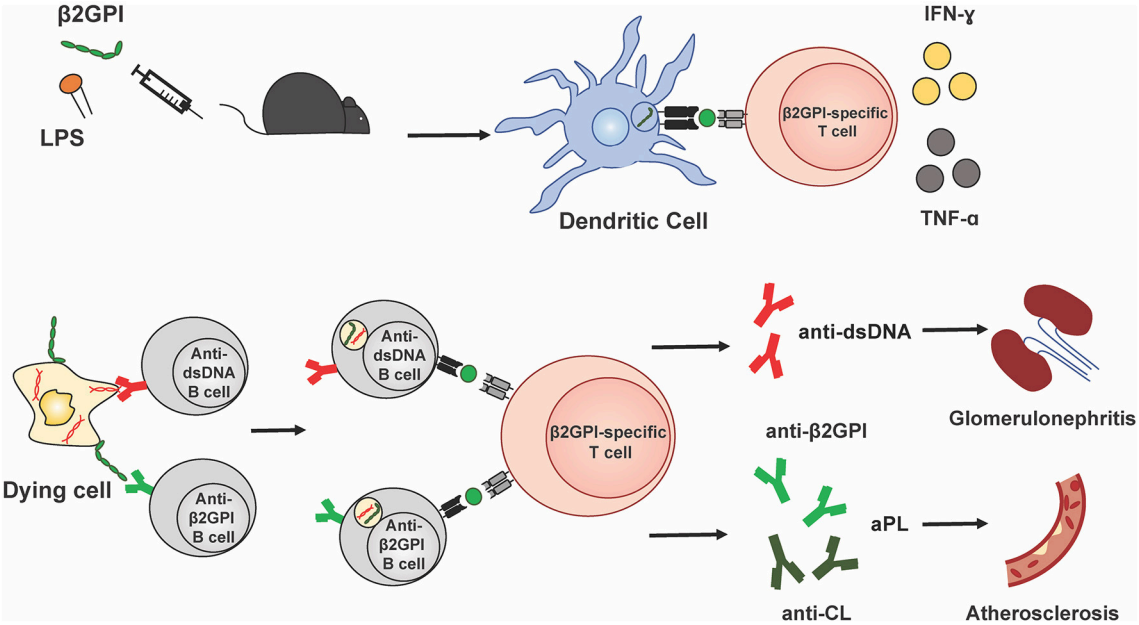

FIGURE 1 | β2GPI-reactive T cells promote autoantibody production and pathology in APS and SLE. This simplified schematic diagram illustrates a possible mechanism by which β2GPI-reactive T cells may promote the development of multiple serological and clinical outcomes. Immunization of mice with β2GPI and lipopolysaccharide (LPS) results in the presentation of β2GPI-derived peptides (green circles) to T cells by dendritic cells and activation and proliferation of β2GPI-reactive CD4 T cells. In the second phase of the response, β2GPI-reactive CD4 T cells could provide help not only to B cells specific for β2GPI, but also to other autoantigen-specific B cells. We propose that autoantigen-specific B cells can recognize their cognate antigen on dying cells and ingest dying cells that have β2GPI bound to their surface. This would result in the presentation of β2GPI peptides in the context of MHC class II on the B cell surface, and allow T cell help from a β2GPI-reactive CD4 T cell. The examples shown here are a B cell specific for the SLE autoantigen dsDNA, and a B cell specific for β2GPI, with β2GPI-reactive T cell help resulting in anti-dsDNA and anti-β2GPI (and/or anti-CL), respectively. In addition, pro-inflammatory cytokines (e.g., IFN-γ and TNF-α) produced by the β2GPI-reactive T cells can impact other cells and tissues, either locally in a paracrine manner or at a distance in an endocrine manner. Depending on the autoantibodies and cytokines produced, different pathologies would arise (e.g., thrombosis or atherosclerosis with aPL, and glomerulonephritis with anti-dsDNA). In this way, β2GPI-reactive T cells could be a driving force for autoantibody production and pathology in both APS and SLE.

from patients with primary vs. secondary APS, as well as from SLE patients with vs. without APS, are needed to determine whether differences in T cell specificity contribute to differences in clinical course between these patient groups. Moreover, careful analyses of the associations between β2GPI-reactive T cells and autoantibodies other than aPL, particularly in SLE patients, are required to establish the role of these T cells in B cell epitope spread. Given the difficulties inherent in human studies, murine models of SLE become critical. For example, determining whether a β2GPI-reactive T cell response is found in spontaneous models of SLE other than MRL/lpr mice would help to establish whether this is a common mechanism for the development of SLE-like autoimmunity. T cell epitopes found to be important in the murine models could then be evaluated in human SLE, and their mechanistic role elucidated by genetic manipulation of the various murine models.

The nature of the antigen recognized by β2GPI-reactive T cells is, of course, critical in any study of these T cells. To date, it has often been difficult to compare studies because of methodological differences in the nature of the antigens used (e.g., native vs. chemically modified) and the lack of epitope mapping in many studies. Despite the practical limitations, studies would include ideally both native and reduced β2GPI, as well as complete epitope mapping using recombinant fragments and peptides encompassing the entire sequence of β2GPI. As ethnicity and HLA restriction likely play an important role, HLA genotyping would be extremely helpful in these studies. Finally, careful comparison of β2GPI-reactive T cells in tissues (e.g., atherosclerotic plaques or nephritic tissue) vs. the peripheral blood of the same individuals would illuminate potential differences in the specificity and function of these T cells.

β2GPI-reactive T cells clearly play a role in APS and SLE, but an improved mechanistic understanding of their contribution to clinical outcomes is needed to render these cells useful diagnostically and/or as therapeutic targets. Equally important, elucidation of the epitope specificity of β2GPI-reactive T cells should provide insight into the nature of the initiating stimulus for these T cells. Finally, an appreciation of whether β2GPI-reactive T cells are involved in promoting epitope spread to non-aPL autoantibodies will further our understanding of how multiple autoantibodies arise in SLE.

AUTHOR CONTRIBUTIONS

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Up-Regulation of TLR7-Mediated IFN-α Production by Plasmacytoid Dendritic Cells in Patients With Systemic Lupus Erythematosus

*Kei Sakata[1,2], Shingo Nakayamada[1], Yusuke Miyazaki[1], Satoshi Kubo[1], Akina Ishii[1,2], Kazuhisa Nakano[1] and Yoshiya Tanaka[1]**

[1] First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan, [2] Mitsubishi Tanabe Pharma, Yokohama, Japan

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George C. Tsokos,
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Keio University, Japan

***Correspondence:**
Yoshiya Tanaka
tanaka@med.uoeh-u.ac.jp

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Objectives: Aberrant and persistent production of interferon-α (IFN-α) by plasmacytoid dendritic cells (pDCs) is known to play a key role in the pathogenesis of systemic lupus erythematosus (SLE). To assess the precise function of pDCs in SLE patients, we investigated the differential regulation of Toll-like receptor 7 (TLR7) and TLR9 responses during IFN-α production by pDCs.

Methods: Peripheral blood mononuclear cells (PBMCs) in SLE patients without hydroxychloroquine treatment, rheumatoid arthritis patients and heathy controls were stimulated with TLR7 and TLR9 agonists. To investigate the priming effect by cytokines, PBMCs from healthy controls were pre-treated with various cytokines and stimulated with TLR7 and TLR9 agonists. The IFN-α production in pDCs was detected by flow cytometry.

Results: TLR7-mediated IFN-α production was up-regulated and correlated positively with disease activity in SLE. Conversely, TLR9-mediated IFN-α production was down-regulated. Differential regulation of TLR7/9 response in SLE was independent of TLR7 and TLR9 expression levels. Furthermore, *in vitro* experiments indicated that TLR7-mediated IFN-α production was up-regulated by pre-treatment with type I IFN, whereas TLR9-mediated IFN-α production was down-regulated by pre-treatment with type II IFN.

Conclusions: Our study indicates the association between up-regulation of TLR7- mediated IFN-α production by pDCs and disease activity and that TLR7 and TLR9 responses were reversely regulated on pDCs in SLE patients. Thus, type I IFN and TLR7-mediated IFN-α production were involved in a vicious cycle, causing hyper production of IFN-α by pDCs during the pathogenic processes of SLE.

Keywords: systemic lupus erythematosus, toll-like receptor 7, plasmacytoid dendritic cells, interferon-α, toll-like receptor 9

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with multiple clinical manifestations that differ from one patient to another (1). Although the cause of SLE remains largely unknown, various factors, including genetic and environmental factors, seem to contribute to the pathogenesis of SLE (2, 3). Thus, SLE is an extremely heterogeneous disease in all aspects, but recent studies have demonstrated characteristic induction of type I interferon-regulated genes (IFN-signature), which is linked to a more severe disease activity with organ failure, in patients with SLE (4–6).

Type I IFN, such as IFN-α, is a pleiotropic immunological mediator that bridges the innate and adaptive immunity. Upon viral infection, type I IFN is mainly produced by plasmacytoid dendritic cells (pDCs) when stimulated through Toll-like receptor 7 (TLR7) and TLR9. In SLE patients, abnormal stimulation of TLR7 and TLR9 by self-nucleic acids seems to contribute persistent production of type I IFN. Type I IFN induces aberrant autoantibody production by stimulation of B cells to differentiate into antibody-producing cells and immunoglobulin isotype class-switch and maturation of antigen presenting cells. Thus, type I IFN production by pDCs upon TLR7/9 stimulation has been implicated as a key player in the pathogenesis of SLE. Indeed, targeting type I IFNs and TLR7/9 has recently become a major treatment strategy in SLE (7–10).

To our knowledge, there is a little information on the function of the pDCs in SLE patients. It is reported that the frequency of circulating pDCs is decreased in SLE patients, because activated pDCs seems to infiltrate to inflamed tissue (11–13). On the other hand, functional analysis of circulating pDCs in SLE demonstrated that dysfunctional IFN-α production upon TLR9 stimulation (14). However, the respective impacts of TLR7 and TLR9 response on IFN-α production in SLE have not been addressed. In particular, attention might be paid to drug development by the analysis of TLR7/9 responses, because hydroxychloroquine (HCQ), a known TLR7/9 inhibitor (15), is a mainstay in the current treatment of SLE. Indeed, IFN-α production upon TLR7/9 stimulation is impaired in pDCs from SLE patients who have been treated with HCQ (16).

The main theme of the present study was investigation of the precise function of pDCs in SLE patients without HCQ treatment. Specifically, we determined the differential regulation of TLR7/9 responses during type I IFN production by pDCs. For this purpose, we assessed the TLR7- and TLR9-mediated IFN-α production by pDCs in SLE patients and compared the finding with those in rheumatoid arthritis (RA) patients and healthy controls. In addition, we analyzed the mechanisms of the differential regulation of TLR7/9 responses in SLE patients.

METHODS

Patients

All cases, who were enrolled in this study, were Asians. SLE patients (n = 68) who fulfilled classification criteria for SLE (17, 18) and who had not been treated with HCQ were enrolled in this study. We also recruited 37 RA patients who fulfilled revised classification criteria for RA (19) and who were not on treatment with biological disease modifying anti-rheumatic drugs (DMARDs), since these drugs are known to influence immunological responses [e.g., anti-TNF Abs are known to induce lupus-like symptoms (20)]. Another control group of 24 healthy subjects free of any autoimmune or infectious disease were recruited to the study (**Table 1**). The clinical activity of SLE was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and the British Isles Lupus Assessment Group (BILAG) activity index. Patients with active SLE (aSLE) represented those with more than 10 points on the SLEDAI score, or classified as A1 or B2 by the BILAG index. All other patients who were not labeled as aSLE were grouped into the inactive SLE group (iSLE). The Human Ethics Review Committee of our university reviewed and approved this study, including the collection of peripheral blood samples from the healthy donors and patients. Each subject provided a signed consent form.

Cell Preparation

Peripheral blood mononuclear cells (PBMCs) isolated using Lympholyte-H (Cedarlane) were cultured in complete medium consisting of RPMI1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin. Primary human pDCs were purified from PBMCs using Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec), and purity was always >90%. PDCs were cultured in complete

TABLE 1 | Demographic and clinical characteristics of the study groups.

	SLE ($n = 68$)	RA ($n = 37$)	Healthy control ($n = 24$)
Age (years)	42.0 (15–80)	61.7 (36–80)	35.8 (26–57)
Females, n (%)	61 (90%)	32 (86%)	23 (96%)
Disease duration, years	11.6 (0–30)	5.7 (0–21)	
SLEDAI score	10.1 (0–35)		
SDAI score		28.1 (6.3–99.5)	
BILAG score, A1 or B2, n (%)	42 (62%)		
Anti-dsDNA antibody (U/mL)	85.6 (0–400)		
Manifestations, n (%)			
Renal	31 (46%)		
Cutaneous	27 (40%)		
Treatment, n (%)			
Hydroxychloroquine	0 (0%)		
Immunosuppressants	36 (53%)		
Corticosteroids	48 (71%)	7 (19%)	
Biological DMARDs		0 (0%)	
Methotrexate		26 (70%)	

Data are number of patients (range) or (percentage).
SLEDAI score, Systemic Lupus Erythematosus Disease Activity Index; SDAI score, Simple Disease Activity Index; DMARDs, disease modifying antirheumatic drugs.

medium containing 0.1 ng/mL of recombinant IL-3 (R&D systems).

TLR Stimulation

PBMCs (1×10^6/well) from patients with SLE or RA, or healthy subjects in 48-well plates or pDCs (2.5×10^4/well) from healthy subjects in 96-well flat-bottom plates were stimulated with TLR7 agonist, loxoribine (1 mmol/L) or R837 (5 μg/mL), or TLR9 agonist, CpG2216 (2 μmol/L) or CpG2006 (2 μmol/L, all from InvivoGen) for 5 h, and brefeldin A (2.5 μg/mL: SIGMA-Aldrich) was added during the final 3 h of stimulation to block cytokine secretion. In the case of pre-treatment experiments, PBMCs or pDCs were treated with IFN-α (IFN-α1: Abcam), IFN-β (Peprotech), IFN-γ (R&D systems), TNF-α (Peprotech), IL-6 (Miltenyi Biotech)/soluble IL-6R (R&D systems) or IL-10 (R&D systems) for 2, 12, and 24 h. Cells were washed three times by complete medium to remove cytokines, thereafter, stimulated with TLR agonist as mentioned above (**Supplementary Figure S1**). Cell number and viability of PBMCs after pre-treatment with each cytokine were calculated under microscope using trypan blue dye.

Flow Cytometry

Cells were stained with FITC-conjugated Lineage cocktail 1 (which includes anti-CD3: clone SK7, anti-CD14: clone MΦP9, anti-CD16: clone 3G8, anti-CD19: clone SJ25C1, anti-CD20: clone L27 and anti-CD56: clone NCAM16.2), V500-conjugated anti-HLA-DR (clone G46-6), PE-Cy7-conjugated anti-CD11c (clone B-ly6), and PerCP-Cy5.5-conjugated anti-CD123 (clone 7G3). After fixation and permeabilization with Fixation/Permeabilization buffer (e-Biosciences), the cells were stained with PE-conjugated anti-IFN-α2b (clone 7N4-1), PE-conjugated anti-IFN-α (clone LT27:295 recognize the majority of the IFN-α subtypes, but not IFN-α2b), and FITC-conjugated anti-IFN-β (clone MMHB-1) for intracellular cytokine or PE-conjugated anti-TLR7 (clone 4G6) and APC-conjugated anti-TLR9 (clone eB72-1665) for intracellular TLR. After intracellular staining, cells were analyzed with FACSVerse (BD Biosciences) and FlowJo software (Tomy Digital Biology). Lin^- $HLA\text{-}DR^+$ $CD11c^-$ $CD123^+$ cells were gated as pDCs (**Supplementary Figure S2**; confirmed by BDCA2 expression), and cytokine positivity in pDCs was determined as an indicator of cytokine production by pDCs. TLR expression levels in pDCs were analyzed before TLR stimulation and were defined as ΔMFI (MFI of anti-TLR Ab – MFI of isotype control), since almost all pDCs were TLR7/9 positive in all donors (**Figure 1C**). All antibodies, except anti-IFN-α (clone LT27:295: Miltenyi Biotec), anti-IFN-β (PBL Assay Science) and anti-TLR7 (ThermoFischer), were purchased from BD biosciences and isotype-matched mouse IgG controls (BD biosciences) were used to evaluate the background.

ELISA for IFN-α in Serum

IFN-α concentration in serum were determined using VeriKine-HS Human Interferon Alpha All Subtype ELISA Kit (PBL Assay Science).

Confocal Microscopy

Primary human pDCs were purified from PBMCs using Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec), and purity was always >90%. PDCs were treated with IFN-α for 2 h. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then treated with Image-iT FX Signal Enhancer (ThermoFischer) for preventing nonspecific staining. Cells were stained with rabbit anti-TLR7 (polyclonal: Novus Biologicals), mouse anti-EEA1 (1G11: Abcam), mouse anti-Rab7 (Rab7-117: Abcam), rat anti-LAMP-1 (1D4B: Abcam) and goat anti-BDCA2 (polyclonal: R&D systems) as primary Abs and subsequently stained with AlexaFluor488-conjugated anti-rat IgG, AlexaFluor594-conjugated anti-rabbit IgG and AlexaFluor647-conjugated anti-goat IgG (all from Invitrogen) as secondary Abs. Cells were spun onto a microscope slide using the Shandon Cytospin 4 (ThermoFischer) and mounted with Fluoromount/Plus (Diagnostic Biosystems). All samples were visualized using FM10i confocal laser scanning microscope (Olympus), and images were captured and analyzed using FV10-ASW viewer (Olympus). PDCs were identified as BDCA2 positive cells. Pearson's coefficient was calculated for analysis of the co-localization of TLR7 and endosomal markers (EEA1, Rab7, and LAMP1).

Statistical Analysis

Comparison between the disease groups was performed with the nonparametric Mann-Whitney's *U*-test. Correlation analysis was performed with the Spearman's correlation coefficients. In the pre-treatment *in vitro* experiments, data are expressed as mean ± S.E.M. of 3–4 experiments. Differences between groups were examined by the student's *t*-test. A *P*-value less than 0.05 was considered statistically significant. All statistical analyses were conducted using the IBM SPSS software ver. 22.

RESULTS

Patients' Characteristics

The characteristics of participating subjects are shown in **Table 1**. All were Asians, and the mean age of SLE was 42.0 years that was matched with healthy donors (mean 35.8 years) but not with RA patients (mean 61.7 years). Most SLE patients were female (90%) that was matched with healthy donors (96%) and with RA patients (86%). The mean duration of illness was 11.6 years in SLE and was 5.7 years in RA. Mean disease activity was 10.1 for SLEDAI in SLE and 28.1 for SDAI in RA. Mean anti-ds DNA antibody titer in SLE was 85.6 U/ml. The proportion of SLE patient with one or more BILAG category A, or two or more BILAG category B was 62%, although concomitant immunosuppressant medication was given.

TLR7-Mediated IFN-α Production Is Up-Regulated in pDCs of SLE Patients

To clarify the function of pDCs in SLE patients, we assessed TLR7- and TLR9-mediated IFN-α production in pDCs in PBMC from patients with SLE or RA, or healthy subjects. Spontaneous cytokine production without TLR stimulation was marginal in any groups. TLR7-mediated IFN-α production was significantly

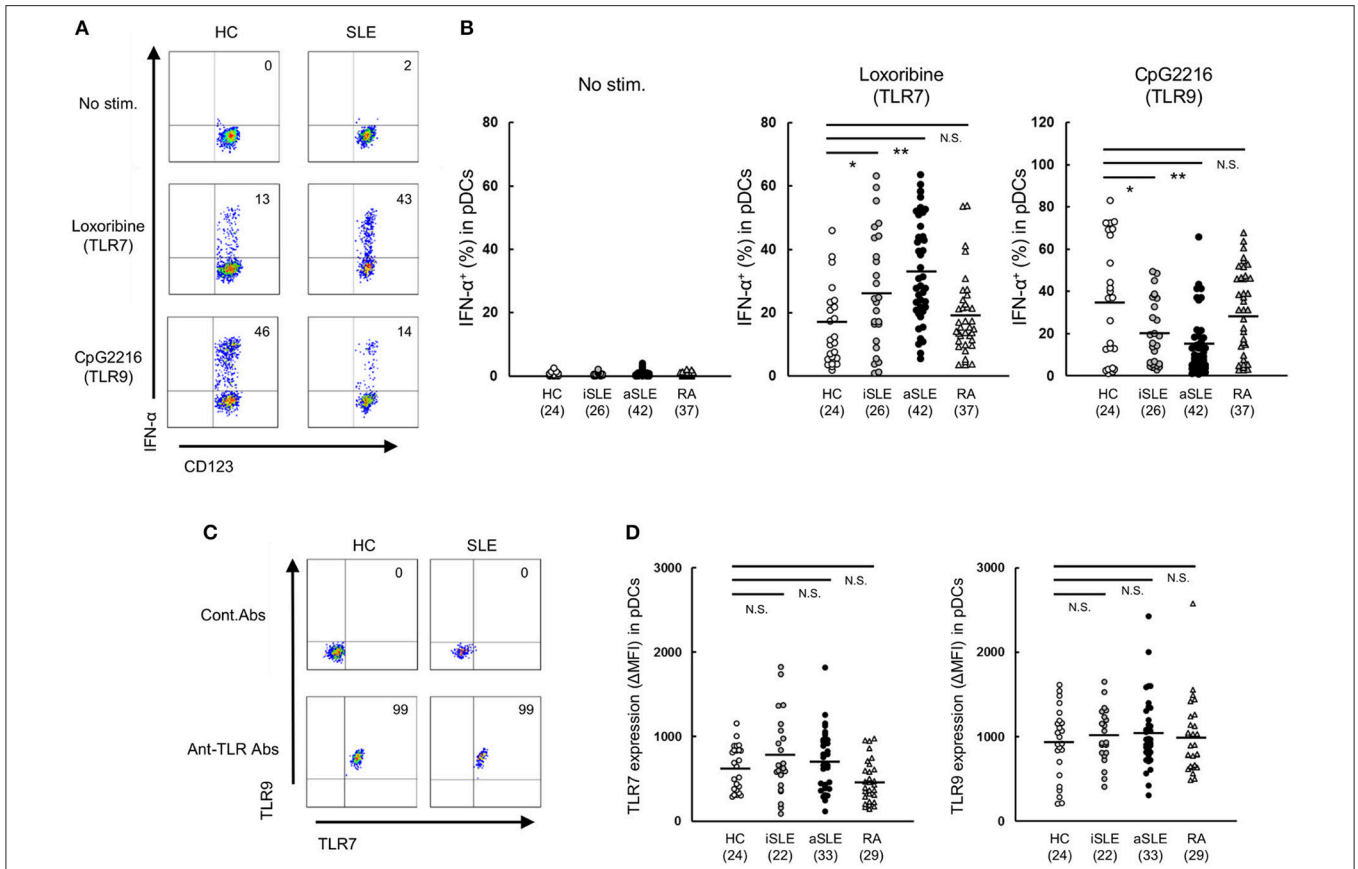

FIGURE 1 | Up-regulation of TLR7-mediated IFN-α production by pDCs in SLE patients. **(A)** Representative flow cytometry plots showing TLR7/9-mediated IFN-α production in Lin$^-$ HLA-DR$^+$ CD11c$^-$ CD123$^+$ pDCs from healthy control subjects and SLE patients. **(B)** TLR7/9-mediated IFN-α production in pDCs of each group. **(C)** Representative flow cytometry plots showing TLR7/9 expression in pDCs of healthy control subjects and SLE patients. **(D)** TLR7/9 expression in pDCs of each group. Horizontal lines represent the mean value of each group. *$p < 0.05$, **$p < 0.01$, compared to the control (Mann-Whitney's *U*-test). Numbers in brackets on the abscissa represent the number of subjects of each group.

up-regulated in both inactive and active SLE, but not in RA patients, compared with healthy subjects. TLR7-mediated IFN-α production in active SLE with glomerulonephritis showed higher tendency than those without glomerulonephritis. There was no significant difference in TLR7-mediated IFN-α production among patients under different treatment (**Supplementary Figures S3A-D**). On the other hand, TLR9-mediated IFN-α production was significantly down-regulated in both inactive and active SLE, compared to healthy subjects (**Figures 1A,B**), consistent with previous reports (14). These differential TLR7/9 responses in pDCs were specific to SLE patients; they were not observed in RA patients.

To investigate whether the differential regulation of TLR7/9 response in SLE patients is dependent on the expression levels of TLR7 and TLR9, we analyzed TLR7/9 expression in pDCs by flow cytometry. The expression levels of TLR7 and TLR9 in pDCs were comparable among the four groups (**Figures 1C,D**). Furthermore, there was no correlation between TLR7 expression level and TLR7-mediated IFN-α production in both SLE patients and healthy subjects. On the other hand, TLR9 expression level correlated with TLR9-mediated IFN-α production in healthy controls, but not in SLE patients (**Supplementary Figure S4**). Taken together, the results indicate that differential regulation of TLR7/9 response in SLE patients did not seem to be dependent on TLR7 and TLR9 expression levels in pDCs.

Further analysis showed that TLR7-mediated IFN-α production was higher in active than inactive SLE. Furthermore, TLR7-mediated IFN-α production correlated positively with SLEDAI score and anti-dsDNA titers, while TLR9-mediated IFN-α production correlated negatively with disease activity (**Figure 2**). These results suggest that TLR7-mediated IFN-α production from pDCs is involved in the pathological processes of SLE.

TLR7-Mediated IFN-α Production Is Up-Regulated by Priming Effect of Type I IFN, While TLR9-Mediated IFN-α Production Is Down-Regulated by Priming Effect of Type II IFN

Next, to investigate the mechanisms of the differential regulation of TLR7/9 response in SLE patients, we analyzed the effects

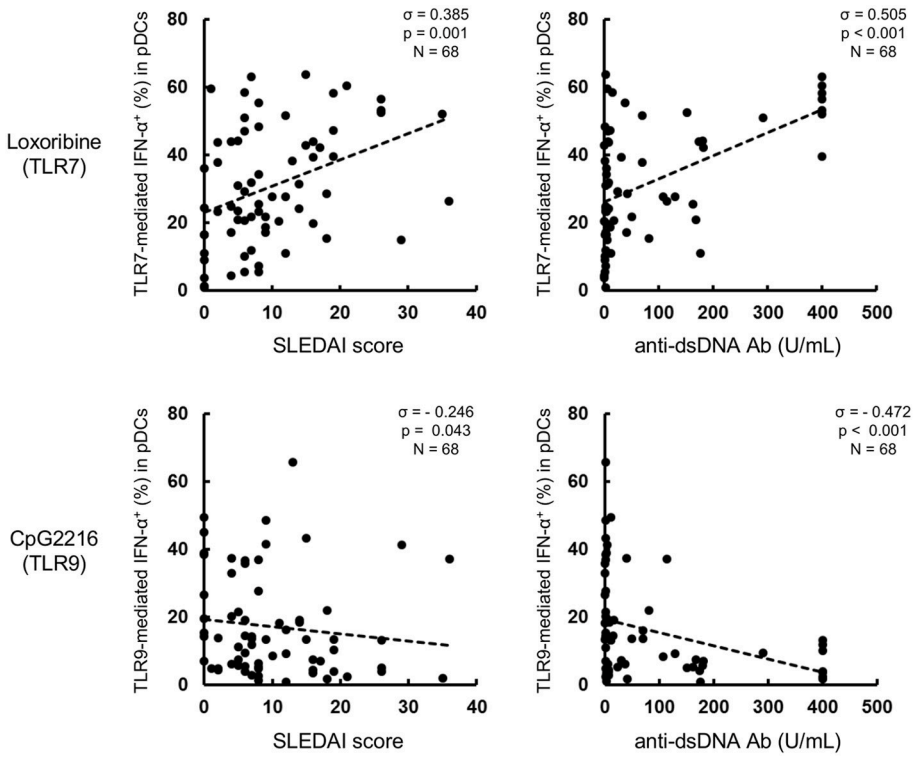

FIGURE 2 | TLR7-mediated IFN-α production correlates with disease activity in SLE patients. Correlation between TLR7/9-mediated IFN-α production and SLEDAI or anti-dsDNA Ab titer. Statistical analysis by the Spearman's correlation coefficient.

of cytokines on TLR7/9 response, since the serum levels of various cytokines (e.g., IFNs) are elevated in SLE patients (21–24). In these experiments (**Figure 1**), PBMCs were washed away from any soluble factor present in the serum, such as cytokines, during the process of their isolation from whole blood, and thereafter stimulated with TLR7 and TLR9 agonists. To imitate the serum condition in SLE, PBMCs from healthy controls were pre-treated with various cytokines for 24 h, washed to remove cytokines, and thereafter stimulated with TLR7 and TLR9 agonists (**Supplementary Figure S1**). Interestingly, TLR7-, but not TLR9-, mediated IFN-α production was significantly up-regulated by pre-treatment with type I IFN, such as IFN-α and IFN-β. TLR7-mediated IFN-α production was also up-regulated by pre-treatment with IFN-γ, although the magnitude of up-regulation was less than with type I IFN. Conversely, TLR9-mediated IFN-α production was significantly down-regulated by pre-treatment with IFN-γ. All subtypes of type I IFN production were regulated by pre-treatment with IFN-α, -β, and -γ (**Supplementary Figure S5A**).

TLR7- and TLR9-mediated IFN-α production was not affected by pre-treatment with other cytokines, such as TNF-α, IL-6, and IL-10 (**Figures 3A,B**). These pre-treatment effects were not due to the effect of survival of pDCs, because no changes were observed in the number and viability of PBMCs, percentage of pDCs in PBMC, and absolute number of pDCs after pre-treatment with any of the above cytokines for 24 h (**Supplementary Figure S5B**). Furthermore, the expression levels of TLR7 and TLR9 in pDCs were not affected by pre-treatment with any cytokines (**Figure 3C**). These results demonstrate that TLR7/9 response in pDCs is regulated by the priming effects of both types I and II IFNs without affecting TLR7/9 expression levels.

Moreover, a dose-dependent priming effect was observed for both IFN-α and IFN-γ; notably, TLR7-mediated IFN-α production was significantly up-regulated by pre-treatment with only 1 U/mL of IFN-α. Conversely, IFN-α production following TLR9 stimulation was down-regulated by pre-treatment with higher concentration (10 ng/mL) of IFN-γ (**Figure 3D**). Furthermore, experiments using R837 and CpG2006, other TLR7 and TLR9 agonists, respectively, confirmed that the specificity of the priming effect of type I IFNs on TLR7 response (**Supplementary Figure S6**). These results were similar to those on the functional differences in pDCs of SLE patients, suggesting that the differential regulation of TLR7/9 response in pDCs in SLE patients is regulated by both type I and type II IFNs. Indeed, there was a positive correlation between TLR7-mediated IFN-α production and IFN-α concentration in serum, and IFN-α concentration in serum and SLEDAI (**Supplementary Figures S7A,B**). However, we couldn't show significant correlation between TLR9-mediated IFN-α production and IFN-γ concentration in serum, because IFN-γ in serum were detected in only few patients (data not shown).

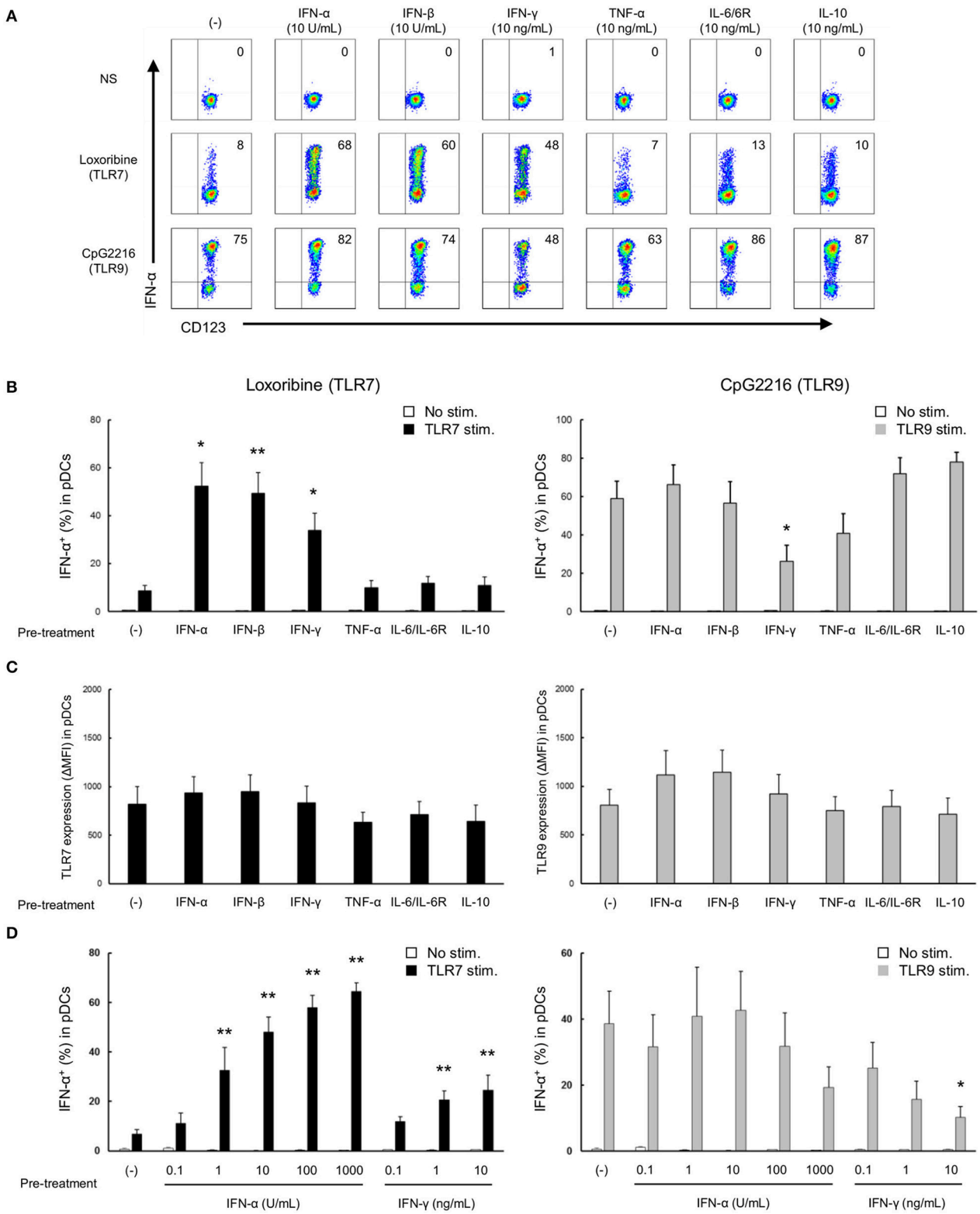

FIGURE 3 | TLR7/9 response of pDCs is regulated by the priming effects of types I and II IFNs in PBMCs of healthy subjects. **(A)** Representative flow cytometry plots showing TLR7/9-mediated IFN-α production in pDCs after pre-treatment with each cytokine. **(B)** TLR7/9-mediated IFN-α production in pDCs after pre-treatment with each cytokine. **(C)** TLR7/9 expression in pDCs after pre-treatment with each cytokine. **(D)** TLR7/9-mediated IFN-α production in pDCs after pre-treatment with different doses of IFN-α and IFN-γ. Data are mean ± S.E.M. of 4 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to pre-treatment with media (Student's *t*-test).

Next, we performed the time course experiment on pre-treatment effects of IFN-α and IFN-γ on TLR responses. TLR7-mediated IFN-α production was up-regulated after pre-treatment with IFN-α within 2 h (**Figure 4**). On the other hand, down-regulation of TLR9-mediated IFN-α production by pre-treatment with IFN-γ required for 24 h (**Figure 4**). Consequently, TLR7 response was quickly up-regulated by IFN-α, but down-regulation of TLR9 response by IFN-γ was required long time.

Type I and Type II IFNs Have Direct Priming Effect on Purified pDCs

We investigated whether type I and type II IFNs have a direct priming effect on purified pDCs. Although IL-3 is generally added in these experiments to maintain survival of cultured purified pDC, it is reported that IL-3 itself up-regulates TLR responses (25). Accordingly, we evaluated the priming effects of IFN-α and IFN-γ on pDCs in the presence of IL-3 (0.1 ng/mL, a concentration that had no effect on TLR7 response (**Supplementary Figure S8**). TLR7-mediated IFN-α production was up-regulated by pre-treatment with IFN-α, and TLR9-mediated IFN-α production was down-regulated by pre-treatment with IFN-γ (**Figures 5A,B**). These results indicate that both type I and type II IFNs have direct priming effects on purified pDCs.

IFN-α Increases TLR7 Trafficking to Lysosome-Related Organelle

Although both TLR7 and TLR9 located in endosome share signaling molecules, downstream signals were bifurcated dependent on endosomal maturation stage; notably, IFN-α production requires TLR trafficking to lysosome-related

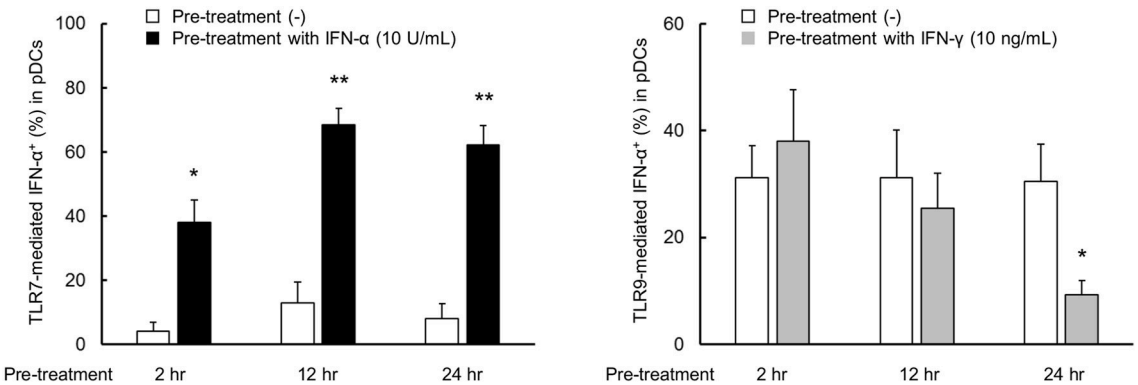

FIGURE 4 | TLR7 response was quickly up-regulated by IFN-α, but down-regulation of TLR9 response by IFN-γ was required long time. PBMCs were pre-treated with IFN-α and IFN-γ for 2, 12, and 24 h, followed by stimulation with TLR7/9 agonist for 5 h. TLR7/9-mediated IFN-α production in pDCs after pre-treatment with each condition. Data are mean ± S.E.M. of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to pre-treatment with media (Student's *t*-test).

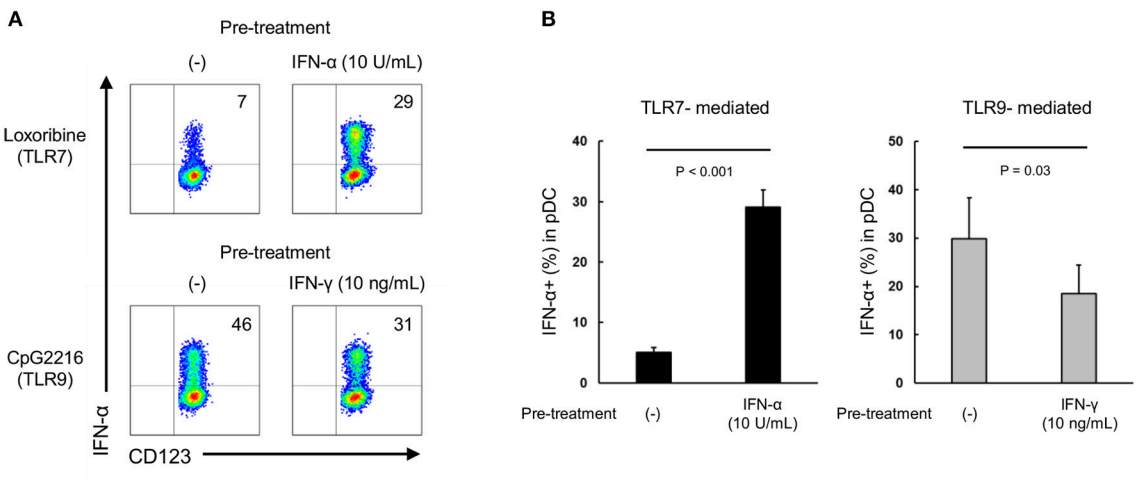

FIGURE 5 | Both IFN-α and IFN-γ have direct priming effect on purified pDCs. **(A)** Representative flow cytometry plots showing TLR7/9-mediated IFN-α production by purified pDCs after pre-treatment with IFN-α and IFN-γ. **(B)** TLR7/9-mediated IFN-α production by purified pDCs after pre-treatment with IFN-α and IFN-γ. Data are mean ± S.E.M. of 3 independent experiments.

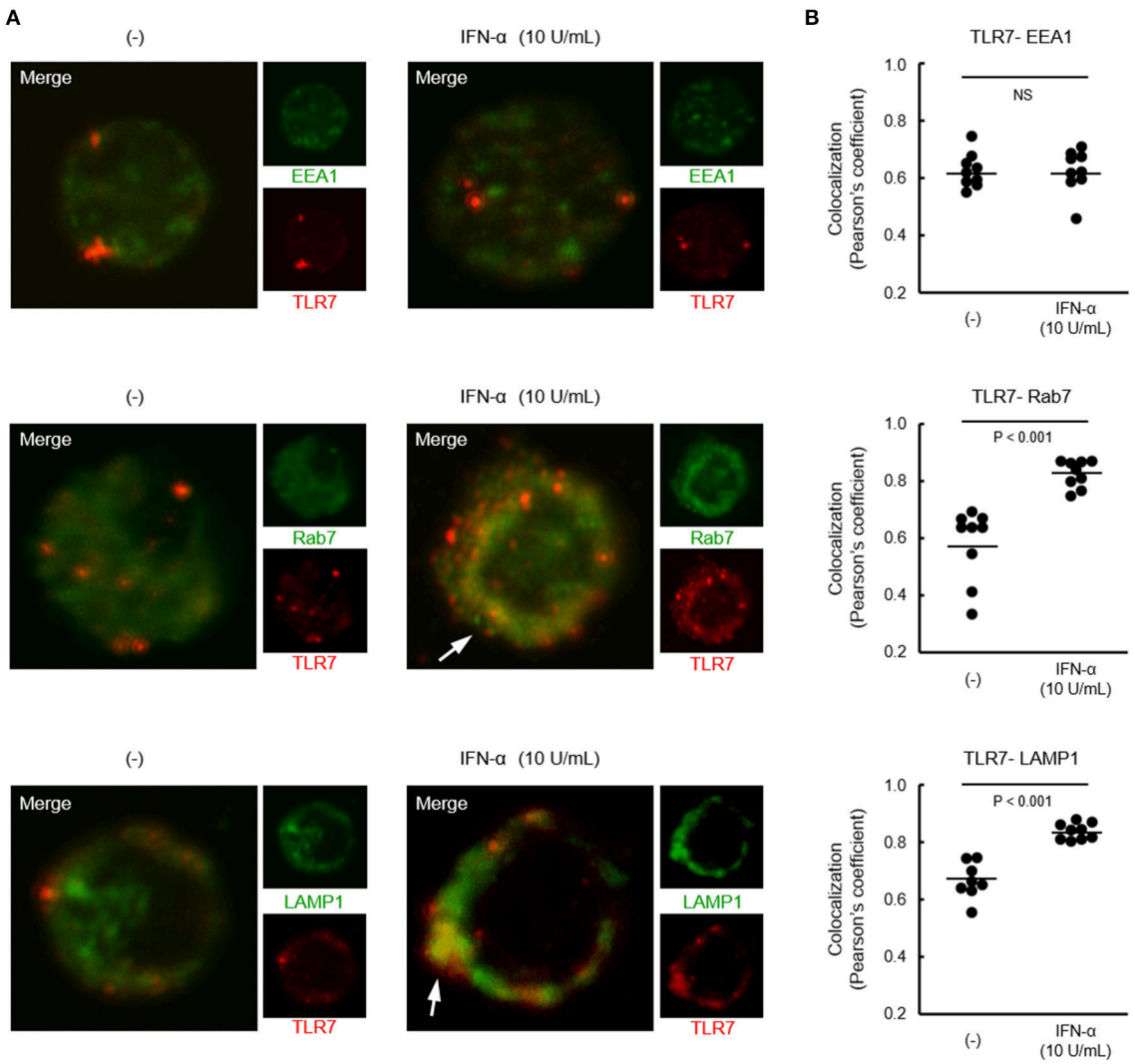

FIGURE 6 | Increased localization of TLR7 in late endosome and lysosome by IFN-α. **(A)** Representative images showing TLR7 (red) and indicated endosomal maturation markers (green) in pDCs pre-treated with or without IFN-α. White arrows indicate robust co-localization of TLR7 with Rab7 and LAMP1. **(B)** Quantification of co-localization between TLR7 and EEA1, Rab7, and LAMP-1. Data shows 10 cells per each condition in one of three independent experiments.

organelle (26). Finally, we investigated the localization of TLR7 in pDCs from healthy controls after treatment with IFN-α. Co-localization of TLR7 with Rab7 (late endosome marker) and LAMP1 (lysosome marker), but not with EEA1 (early endosome marker) was increased by pre-treatment with IFN-α (**Figure 6**). These results demonstrate that increased TLR7 trafficking to lysosome-related organelle by type I IFN may cause the up-regulation of TLR7-mediated IFN-α production in pDCs, without affecting TLR7 expression levels.

DISCUSSION

The main findings of the present study were that TLR7-mediated IFN-α production was up-regulated in SLE and that the level of production correlated positively with disease activity. Conversely, TLR9-mediated IFN-α production was decreased in SLE patients. Thus, TLR7 and TLR9 responses in pDCs were differentially regulated in SLE. The differential regulation of TLR7/9 response was not dependent on the expression levels of TLR7 and TLR9 in pDCs. The results also showed that such differential regulation of TLR7/9 response in pDCs of SLE patients was due to the priming effects of type I and type II IFNs; namely, TLR7-mediated IFN-α production was up-regulated by pre-treatment with type I IFN and TLR9-mediated IFN-α production was down-regulated by pre-treatment with type II IFN.

Functional studies of purified pDCs from patients with SLE have been hindered by technical limitations, because pDCs are rare cells (<1% in PBMC), and especially, circulating pDCs are

reduced in SLE patients (11). Our preliminarily experiments showed that at least 100 mL blood is required to obtain stably, enough and highly purified pDCs from each patient with SLE. It is ethically difficult to collect huge amount of blood from each SLE patient. To overcome these issues, we determined IFN-α positivity in pDCs in PBMC by flow cytometry after stimulation with TLR agonists as an indicator of IFN-α production. By using this method, we found decreased TLR9-mediated IFN-α production from pDCs in SLE patients, consistent with previous report (14). By contrast, we found that TLR7-mediated IFN-α production was significantly up-regulated in SLE patients. Our results also showed that TLR7/9 expression levels in pDCs were similar in SLE patients and healthy controls. Recent studies reported high TLR7/9 expression levels in PBMCs and B cells of SLE patients (22, 27), but the relationship between TLR7/9 expression level and TLR7/9 response remains poorly understood. Zorro et al. (28) demonstrated that increased TLR9 expression had no influence on TLR9 response in B cells from SLE. Our results showed the correlation of TLR9 expression and TLR9 response was observed in the healthy control, but not in SLE patients which probably due to the priming effect of type II IFN. On the other hand, TRL7 expression does not correlate with TLR7 response even in healthy control. In this study, although we showed the priming effects of type I and II IFNs as one of the mechanisms for regulating TLR7/9 responses, TLR7 response in healthy control might be regulated by other mechanisms.

TLR7/9 responses in pDCs are regulated in the presence of certain cytokines, such as IFN-α/β and TNF-α (29–32). Our results showed that both types I and II IFN have priming effects on pDCs, and that the TLR7/9 response is regulated by these cytokines without affecting TLR7/9 expression levels, even after the removal of these cytokines. On the other hand, TNF-α and IL-10 had no priming effect on pDCs, although TLR7/9-mediated IFN-α production was inhibited in the presence of these cytokines (30, 32). IFN signature is probably induced by type II IFN in addition to type I IFN, and both types are elevated in sera of SLE patients (21, 24). Interestingly, no differential regulation of TLR7/9 response was observed in RA patients, in whom IFNs play negligible pathogenic role (6). Considered together, our results suggest that differential regulation of TLR7/9 response in pDCs of SLE patients is mediated through the priming effects of types I and II IFNs. We confirmed the positive correlation between TLR7-mediated IFN-α production and IFN-α concentration in serum from patients with SLE.

It is noteworthy that TLR7-mediated IFN-α production was quickly up-regulated after pre-treatment with low concentration of IFN-α (10 U/mL) within 2 h, by the modulation of TLR7 trafficking to lysosome-related organelle, without affecting TLR7 expression levels. More recently, it is reported that increased TLR7 co-localization with Rab7 and LAMP1 in pDCs from patients with SLE (33), probably due to the priming effect of type I IFN. Conversely, down-regulation of TLR9 response was required long time (approximately 24 h) after pre-treatment with high concentration of IFN-γ (10 ng/mL). Although we investigated the effect of IFN-γ on TLR9 trafficking, co-localization of TLR9 with any endosome markers were not influenced by treatment with IFN-γ (data not shown). TLR signaling is regulated by multilayer control mechanisms, including cellular trafficking, cooperation with coreceptors, cleavage and interaction of signaling molecules with negative regulators (34). Thus, further analysis is required for unveiling the precise mechanisms of the differential regulation of TLR7/9 responses in patients with SLE.

Finally, (1) TLR7-mediated IFN-α production was positively correlated with SLE disease activity, (2) TLR7-mediated IFN-α production was positively correlated with IFN-α concentration in serum and (3) IFN-α concentration in serum was positively correlated with SLE disease activity. Furthermore, TLR7-mediated IFN-α production were up-regulated by type I IFN itself. Taken together, these results suggest that TLR7-mediated IFN-α production from pDCs play a pivotal role in the pathogenesis of SLE. In contrast, TLR9-mediated IFN-α production was negatively correlated with SLE disease activity. While the pathological role of TLR7 in human SLE and lupus nephritis in murine models is relatively accepted, the role of TLR9 remains controversial. Several murine studies have highlighted the importance of TLR7, and that TLR9 surprisingly provides protection, in lupus pathogenesis (35, 36). In this study, TLR9-mediated IFN-α production was down-regulated, but still detected in SLE patients. Although the precise role of TLR9 in SLE remains unclear, TLR7 might be more important than TLR9 in the pathogenesis of SLE. In this study, synthetic TLR agonists were used instead of physiological immune complex such as dsDNA-IC or RNP-IC. Since main trigger of TLR7/9 responses in SLE is immune complex with self-nucleic acids, we had attempted to assess the pDC response by RNA-IC stimulation. Unfortunately, we could not detect IFN-α positive pDCs after 5 h stimulation with RNA-IC. It may be due to limitation of the culture period, because more longer culture caused breakdown of gating strategy, especially pDC marker such as CD123 or BDCA2 were down regulated. Consequently, we could not technically distinguish pDCs among PBMCs after longer culture. In addition, our study was limited on SLE in Asia, particularly in Japanese, which may be more interferonopathy than other races. Further investigation will be necessary for a better understanding of the function of pDCs in SLE patients.

In summary, our results suggest that type I IFN and TLR7-mediated IFN-α production establish a vicious cycle, causing aberrant and persistent production of type I IFN in the pathogenic process of SLE. Although SLE is a complex autoimmune disease with extremely heterogeneous clinical manifestations as well as pathogenesis, TLR7 seems to play a pivotal role in the pathogenesis of SLE. Furthermore, TLR7 and TLR9 responses were reversely or differentially regulated on pDCs in SLE, implying that for the pharmaceutical application, TLR7, but not TLR9, should be targeted when targeted therapies are developed in patients with SLE.

AUTHOR CONTRIBUTIONS

KS and SN designed the research. KS conducted experiments, analyzed data and wrote the manuscript. YM, SK, AI and KN helped to conduct experiments. YT created the research concept and supervised the research and the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01957/full#supplementary-material

Supplementary Figure S1 | Procedures for evaluating TLR7/9 responses in patients samples and *in vitro* pre-treatment experiment. HC, healthy control subjects.

Supplementary Figure S2 | Gating strategy for pDC from PBMC.

Supplementary Figure S3 | TLR7/9-mediated IFN-α production in pDCs of each group with or without lupus nephritis **(A)** and with receiving different medications among whole SLE **(B)**, active SLE **(C)**, and active SLE with lupus nephritis **(D)**. Horizontal lines represent the mean value of each group. $*p < 0.05$, $**p < 0.01$, compared to the control (Mann-Whitney's *U*-test).

Supplementary Figure S4 | Relationship between TLR7/9-mediated IFN-α production and TLR7 or TLR9 expression levels in pDCs of healthy control subjects (HC) and SLE patients. Statistical analysis with the Spearman's correlation coefficient.

Supplementary Figure S5 | Pre-treatment effects of types I and II IFNs on TLR7/9 responses in pDCs. **(A)** All subtypes of type I IFN production were regulated by pre-treatment with IFN-α, -β, and -γ. **(B)** The effects of pre-treatment of types I and II IFNs are not due to the effect of survival of pDCs. Number and viability of PBMCs, percentage of pDCs among PBMC and absolute number of pDCs after pre-treatment with each cytokine for 24 h. $*p < 0.05$, $**p < 0.01$, compared to pre-treatment with media (Student's *t*-test).

Supplementary Figure S6 | TLR7 specificity in the priming effect of type I IFN. The percentages of IFN-α producing pDCs stimulated with R837 and CpG2006 after pre-treatment with each cytokine for 24 h. $*p < 0.05$, $**p < 0.01$, compared to pre-treatment with media (Student's *t*-test).

Supplementary Figure S7 | Relationship between TLR7-mediated IFN-α production **(A)** and IFN-α levels in serum **(B)** of SLE patients. Statistical analysis with the Spearman's correlation coefficient.

Supplementary Figure S8 | TLR7/9-mediated IFN-α production were not affected by IL-3 at the concentration of 0.1 ng/mL. Percentages of IFN-α-producing pDCs stimulated with TLR7 agonist, loxoribine, and TLR9 agonist, CpG2216, for 5 h before and after pre-treatment with IL-3 (0.1 ng/mL) for 24 h. $*p < 0.05$, $**p < 0.01$, compared to pre-treatment with media (Student's *t*-test).

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Pathogenic Role of Complement in Antiphospholipid Syndrome and Therapeutic Implications

Francesco Tedesco[1], Maria Orietta Borghi[1,2], Maria Gerosa[2], Cecilia Beatrice Chighizola[1,2], Paolo Macor[3], Paola Adele Lonati[1], Alessandro Gulino[4], Beatrice Belmonte[4] and Pier Luigi Meroni[1]*

[1] Immunorheumatology Research Laboratory, Istituto Auxologico Italiano, Milan, Italy, [2] Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy, [3] Department of Life Sciences, University of Trieste, Trieste, Italy, [4] Tumor Immunology Unit, Human Pathology Section, Department of Health Sciences, University of Palermo, Palermo, Italy

Antiphospholipid syndrome (APS) is an acquired autoimmune disease characterized by thromboembolic events, pregnancy morbidity, and the presence of antiphospholipid (aPL) antibodies. There is sound evidence that aPL act as pathogenic autoantibodies being responsible for vascular clots and miscarriages. However, the exact mechanisms involved in the clinical manifestations of the syndrome are still a matter of investigation. In particular, while vascular thrombosis is apparently not associated with inflammation, the pathogenesis of miscarriages can be explained only in part by the aPL-mediated hypercoagulable state and additional non-thrombotic effects, including placental inflammation, have been described. Despite this difference, evidence obtained from animal models and studies in APS patients support the conclusion that complement activation is a common denominator in both vascular and obstetric APS. Tissue-bound aPL rather than circulating aPL-beta2 glycoprotein I immune complexes seem to be responsible for the activation of the classical and the alternative complement pathways. The critical role of complement is supported by the finding that complement-deficient animals are protected from the pathogenic effect of passively infused aPL and similar results have been obtained blocking complement activation. Moreover, elevated levels of complement activation products in the absence of abnormalities in regulatory molecules have been found in the plasma of APS patients, strongly suggesting that the activation of complement cascade is the result of aPL binding to the target antigen rather than of a defective regulation. Placental complement deposits represent a further marker of complement activation both in animals and in patients, and there is also some suggestive evidence that complement activation products are deposited in the affected vessels. The aim of this review is to analyze the state of the art of complement involvement in the pathogenesis of APS in order to provide insights into the role of this system as predictive biomarker for the clinical manifestations and as therapeutic target.

Keywords: complement, antiphospholipid syndrome, anti-beta2 glycoprotein I antibodies, thrombosis, miscarriages, animal models, inflammation, therapy

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****Correspondence:***
Francesco Tedesco
tedesco@units.it

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INTRODUCTION

In recent years, major efforts have been made to define the molecular mechanisms responsible for the clinical manifestations of antiphospholipid syndrome (APS) including vascular thrombosis and adverse pregnancy outcomes (1–3). Blood clots can occur in both venous and arterial vessels with preferential localization in the brain and coronary arteries, although other vascular districts can

also be involved. Vascular thrombosis mediated by beta2 glycoprotein I (β2GPI)-dependent antiphospholipid antibodies (aPL) represents the main pathogenic mechanism that is responsible for the major clinical manifestations of the syndrome and it has been suggested to be the cause also for other non-classification clinical events (4).

Pregnancy morbidity manifests as unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, eclampsia, or severe preeclampsia, particularly early, severe preeclampsia (5). Although it is clear that the specific antigenic reactivity of aPL and their targeting to the placenta are critical to produce their effect, pathogenic mechanisms that damage the fetal–maternal unit and cause abnormal placental development are incompletely understood (6). Indeed, the *in vivo* antigenic targets of lupus anticoagulant (LA), the strongest risk factor for adverse pregnancy outcomes in APS patients, are not known (7). While blood clotting represents the main clinical manifestation of vascular APS, non-thrombotic mechanisms have been suggested to be a more important cause of defective placentation characteristic of the syndrome (1). Moreover, although most patients display both manifestations, isolated vascular or obstetric variants can also be found and there is some discussion as to whether vascular and obstetric APS are the same disease (8).

Despite the fact that not all the animal models of aPL-mediated fetal loss display inflammatory signature at the placental level, inflammation has been suggested to play a role in APS miscarriages (9). Analysis of human placental tissues has not clarified this issue, since an inflammatory infiltrate was reported in the decidua only in some but not all studies. No sign of inflammation was observed in the vessel wall of human tissues at variance with the findings in obstetric APS. However, endothelial perturbation with the expression of a pro-thrombotic and pro-inflammatory phenotype was reported in APS models (10).

Complement (C) activation has been shown to be critical in APS models, since its blockade protects animals from both aPL-mediated clotting and fetal loss (11). In line with the data obtained in animal models, C was suggested to be involved in vascular APS following the observation of increased plasma levels of activation products and reduced C3 and C4 levels or CH50 activity in some patients (12–18).

Similar findings were reported in obstetric APS and C deposits were detected at placental level in some but not all studies (19–21). Moreover, the beneficial effect of eculizumab, a human monoclonal antibody that prevents C activation by neutralizing C5, observed in individual cases further supports the role of C activation in human APS (22).

COMPLEMENT AND VASCULAR APS

Anecdotal reports revealed the involvement of C in vascular APS many years before the sound evidence of C contribution to both fetal loss and thrombosis models. Low serum levels of C4 were reported in a patient with thrombosis, miscarriages, and aPL, and C4 null alleles and low C3/C4 were found to be associated with aPL in systemic lupus erythematosus (SLE) patients (12, 14). Moreover, the suggestion that C is involved in APS was strongly supported by the demonstration of increased plasma levels of soluble C5b-9 in nearly 40% of a small series of APS patients with stroke (13).

Despite these early reports, no further studies on C activation in vascular APS have been carried out for a long time. Oku et al. reported reduced serum levels of C3, C4, and decreased CH50 activity in a small series of primary APS (PAPS). C consumption was associated with increased levels of the activation products C3a, C4a but not C5a in the absence of reduction of regulatory proteins factor H and I suggesting an enhanced turnover rather than a defective C regulation (15). However, no clear relationship was found between this finding and clinical or serological APS parameters (15). Similar increase in plasma levels of the C activation products Bb and C3a were reported by other groups in large series of patients with both vascular and obstetric PAPS (16, 18). The plasma levels of C3a were also found to be higher in another small series of persistently LA positive patients with no correlation with thrombosis (17). Moreover, we recently observed a significant increase in platelet- and red blood cell-bound C4d in PAPS in comparison to SLE and healthy controls further supporting the occurrence of C activation in the syndrome (23). C consumption and release of C activation products have been reported in a case of catastrophic APS (CAPS) (24).

The mechanism responsible for C activation has not been clarified. In one study, circulating immune complexes (CIC) found in a high proportion of PAPS patients have been suggested to trigger the classical pathway (15). The prevalence of CIC in APS was much lower in other studies suggesting that additional mechanisms may be responsible for C activation (16, 25). However, the issue is still a matter of research due to technical problems in detecting CIC.

C activation in the fluid phase can be associated with C deposition at tissue level, but few reports have been published documenting localization of tissue-bound C in APS patients. Immunoglobulin (Ig), C1q, and C3 deposits were described in the heart valve leaflets from patients with aPL-related valvulopathy (26), and Ig, C1q, and C3 have also been found in kidney biopsies from some but not all patients with APS nephropathy (27). Altogether, these findings suggest that aPL-mediated C activation can take place in the tissues of patients affected by this syndrome. We recently reported the case of a PAPS patient with arterial popliteal thrombosis who underwent arterial surgical bypass. Deposits of C1q, C4, C3, and C5b-9 co-localizing with β2GPI and IgG were found in the affected artery wall together with increased plasma levels of C5a and C5b-9. Interestingly, a short treatment with eculizumab resulted in a substantial decrease in the C5a and C5b-9 levels. Overall, these findings strongly suggest that C activation takes place in vascular APS and that C deposition at the anatomical site of thrombosis plays a key role in aPL-mediated clotting (28).

THE ROLE OF COMPLEMENT IN MODELS OF VASCULAR APS

Animal models of vascular APS have been instrumental in establishing the pathogenic role of antibodies to β2GPI in the formation of thrombi and the mechanism of their action. The model

has been reproduced in various animal species including mice, hamsters, and rats using different experimental approaches.

APL-treated mice have been used extensively to monitor the development of thrombi in the femoral vein after a pinch injury (10). A somewhat similar approach was adopted in hamsters and mice that received monoclonal or patients' antibodies to β2GPI, respectively, to induce clot formation in the carotid artery (hamster) or cremaster muscle microcirculation (mice) injured either by a photochemical reaction (29) or following laser exposure (30). Both approaches rely on mechanical or chemical vascular damage to initiate the coagulation process that is further enhanced by the administration of aPL resulting in enlargement of the blood clot. We followed a different strategy establishing a rat model that, in our view, reflects more closely the situation in the clinic (31). The model consisted of priming the animals with an amount of LPS, that does not induce thrombosis, followed by administration of aPL. Formation of thrombi in the mesenteric microvessels containing arterioles, capillaries, and postcapillary venules was monitored by optical imaging. This approach allowed us to firmly establish that aPL was totally ineffective in naïve animals and that the pro-coagulant effect of these antibodies required a second hit provided by LPS that was not needed for their proabortive activity.

Despite the different experimental approaches in the mouse and rat models of vascular thrombosis, blockade of C activation with a neutralizing antibody to C5 was shown to prevent thrombus enlargement in mice (32) or clot formation in rats (31) suggesting the important contribution of C to aPL-induced promotion of coagulation. The finding that aPL fail to exert a pro-coagulant effect in C3- and C5-deficient mice is consistent with the conclusion that C plays a critical role in mediating the damaging effect of the antibodies (32). However, there is a major difference in the mechanisms of C-mediated pregnancy loss and vascular thrombosis. While C5a has been shown to play a major role in causing adverse pregnancy outcome induced by aPL (33), blood clot formation is apparently dependent on the action of the terminal complex C5b-9, as suggested by the failure of aPL to promote thrombosis in C6-deficient rats and mice (31, 34). Deposition of C9 at sites of localization of IgG on the endothelium of the mesenteric microvessels of rats treated with aPL is a clear indication that C activation proceeds till the assembly of the membrane attack complex. We and others have provided evidence that the terminal complex either in a sublytic or cytolytically inactive form can stimulate endothelial cell to express on their surface tissue factor that triggers the extrinsic pathway of coagulation (35, 36).

COMPLEMENT AND OBSTETRIC APS

Studies in humans support the role of C in aPL-associated pregnancy complications. Mild hypocomplementemia and low C3, C4 levels were reported in some studies including aPL-positive patients with no other associated systemic autoimmune diseases (16, 37–39). Although this finding is suggestive for C involvement in aPL-mediated miscarriages, C activity was not reduced in all pregnant women and a clear relationship with pregnancy complications was not supported by statistical analysis. Interpretation of C levels in pregnant women is difficult because they reflect both increased synthesis stimulated by estrogens and consumption (40). To obtain a correct information on the C levels in aPL-positive pregnant women, the data should be compared with those found in normal pregnant controls, but this comparison was made only in one study (38).

More recently, increased plasma levels of the activation products Bb and C5b-9 were reported in women with aPL and adverse pregnancy outcome suggesting the contribution of C activated through the alternative pathway to the pathogenesis of this clinical condition (41). The activation products are considered a more sensitive marker of C activation, and may contribute to promote leukocyte recruitment/activation and release of pro-inflammatory and anti-angiogenic mediators responsible for placental damage. Deposition of C4d and to some extent of C3d in term placentas was reported in aPL women in two studies further suggesting the contribution of C activation to placental impairment mediated by aPL (19, 21).

COMPLEMENT DEPOSITION ON HUMAN PLACENTAS FROM APS PATIENTS

While the finding of C activation products in the circulation of patients with obstetric APS is suggestive of C involvement in the pathogenesis of adverse pregnancy outcome, there is no doubt that the detection of these products in placenta provides more direct evidence for C contribution to tissue damage. Search for C deposits should of course be restricted to placentas from patients with PAPS to avoid confounding results that may derive from the analysis of tissue from patients with secondary APS associated with C-mediated disorders such as SLE. C localization was investigated in term placentas from patients with aPL antibodies by Shamonki and colleagues (19), who focused their analysis on the deposits of C4d, C3b, and C5b-9 complex. They reported the presence of these C activation products in the cytoplasm of villous trophoblast and on extravillous trophoblast, but it is unclear whether there was a preferential cytoplasmic localization also in these cells. Histologic examination of placentas revealed pathological lesions including decidual vasculopathy, increased syncytial knots, and villous infarcts, that were correlated with increased C4d staining of villous trophoblast. Surprisingly, the presence of C5b-9 in the cytoplasm of villous trophoblast was significantly lower suggesting that this complex may not contribute to tissue damage. In addition, the degree of C3b and C5b-9 deposition in extravillous trophoblast of placentas from aPL-positive patients was not significantly different from that found in control placentas raising the question of the relevance of these observations to the pathogenic role of C in aPL-mediated alterations in maternal decidua.

Our group has conducted a prospective study on 13 pregnancies in 11 patients with PAPS, who were under treatment with low molecular weight heparin (100 IU/kg/day s.c.) and low dose aspirin (100 mg/day). The majority of these patients (10 out 13) had medium to high titers of anti-β2GPI and positive LA. The pregnancies resulted in eight live births at gestational ages ranging between 30 and 38 weeks, one abortion and four fetal loss

after 10 weeks' gestation (**Table 1**). The study was approved by the Istituto Auxologico Italiano Ethics Committee (22-07-2010) and patients gave their written informed consent.

Decidual vasculopathy and intervillous thrombi were the most common histologic findings observed in PAPS placentas while inflammation was less frequent and was seen in both PAPS and control placentas, as were also villitis and villous infarcts equally detected in both groups of placentas. Deposits of C components and C activation products were found in all PAPS placentas examined with some variation in the degree of C deposition. C1q, C4, and C3 were detected on the decidual endothelium vessels at sites of IgG and IgM deposition while C5b-9 showed a prevalent subendothelial distribution (**Figure 1**). Analysis of the villi revealed the presence of IgG, IgM, C1q, C4, C3, and C5b-9 on the surface of syncytiotrophoblast with additional distribution of IgM and C5b-9 on intervillous fibrin deposits and of IgG and C3 on the endothelium of villous vessels (**Figure 2**). Interestingly, C activation occurred in placental tissue of APS patients despite heparin treatment. This was a surprising finding because heparin was shown by Girardi and colleagues (42) to inhibit C activation and to prevent pregnancy loss in a mouse model of obstetric APS. C localization in decidua and villi of control placentas was negligible with the only exception of C1q detected on decidual endothelium and on extravillous trophoblast confirming previous observation that C1q is constitutively expressed in these cells in physiological pregnancy (43–45). Altogether, these findings support the conclusion that C activation in APS placenta is activated by aPL antibodies and justify a possible pathogenic role of C activation in fetal loss documented in this study and in murine models of APS. However, the presence of C deposits in placentas from patients who have had live births is more difficult to interpret. It is possible that the damaging effect of C activation on placental tissue that affects pregnancy outcome depends on the extent and distribution of C deposits in APS placenta. Binding of C activation products to restricted placental areas may cause tissue alterations that marginally affect the regular progression of pregnancy.

THE ROLE OF COMPLEMENT IN ANIMAL MODELS OF PREGNANCY

The initial observation by Branch and colleagues (46) that passive infusion of serum IgG from patients with aPL induced an increased rate of fetal resorptions in pregnant mice was the first evidence that suggested a role of the antibodies in the pathogenesis of fetal loss. These findings and similar data obtained by other groups (47, 48) led to the conclusion that obstetric APS is a clinical disorder mediated by antibodies that are preferentially directed against β2GPI (4). The high degree of protein sequence homology between human and animal β2GPI explains the ability of human antibodies to cause fetal demise in mice. The β2GPI molecule has been found to be localized in the placenta of pregnant mice in the absence of antibodies with a prevalent distribution on syncytio and extravillous trophoblasts, and decidual endothelial cells (49). *In vitro* experiments have shown that aPL interacting with the target molecule expressed on trophoblast impair several functions of these cells including proliferation, syncitia formation, invasion into maternal deciduas, as well as secretion of chorionic gonadotrophin and growth factors (1, 50). However, the *in vivo* relevance of these observations to placental dysfunction is unclear as the administration of aPL to C3-deficient pregnant mice has no adverse impact on the progression of pregnancy while resulting in an increased rate of fetal loss and growth retardation in wild-type animals (51). These data argue for a major role of C activated by aPL in inducing adverse pregnancy outcome, a conclusion which is also supported by the ability of the C3 convertase inhibitor Crry to prevent aPL-mediated fetal loss (51).

Further analysis of the critical step of the C sequence involved in this pathological process points to C5 as the key component based on the finding that aPL failed to increase fetal resorption rate in C5-deficient mice and in animals treated with anti-C5 antibodies (33). Similar results were obtained in C4 and factor B-deficient mice suggesting that C activation is triggered by aPL through the classical pathway and is further amplified through

TABLE 1 | Clinical characteristics of the PAPS patients examined for placental C deposits.

Patients	Diagnosis	LA	aCL IgG/IgM	anti-β2GPI IgG/IgM	Outcome	Therapy
BAC 1	PAPS	Pos	High/high	High/high	Fetal loss < 10 weeks	LMWH/ASA
BAC 2	PAPS	Pos	High/high	High/high	Fetal loss > 10 weeks (twins)	LMWH/ASA
BAC 3	PAPS	Pos	High/high	High/high	Live baby 35 weeks	LMWH/ASA/ivIg/CS
BA	PAPS	Pos	High/high	High/high	Fetal loss > 10 weeks	LMWH/ASA
TD	PAPS	Neg	Med/low	Med/neg	Live baby 38 weeks	LMWH/ASA
SA	PAPS	Pos	nd	nd	Fetal loss > 10 weeks	ASA[a]
SE	PAPS	Pos	High/high	High/med	Live baby 30 weeks	LMWH/ASA
PA	PAPS	Pos	High/high	nd	Fetal loss > 10 weeks	None[b]
FO	PAPS	Neg	High/neg	High/neg	Live baby 38 weeks	LMWH/ASA
BO	PAPS	Pos	Neg/neg	Neg/neg	Live baby 35 weeks	LMWH/ASA
PU	PAPS	Pos	High/neg	High/med	Live baby 38 weeks	LMWH/ASA
AC	PAPS	Neg	High/neg	High/med	Live baby 33 weeks	LMWH/ASA
BL	PAPS	Pos	High/med	High/med	Live baby 31 weeks	LMWH/ASA

PAPS, primary antiphospholipid syndrome (5); C, complement; LA, lupus anticoagulant; aCL, anti-cardiolipin antibodies; anti-β_2GPI, anti-beta2 glycoprotein I antibodies; LMWH, low molecular weight heparin; ASA, aspirin; ivIg, intravenous immunoglobulins; CS, corticosteroids; nd, not detected.

[a]*The patient was classified as aPL-positive asymptomatic carrier, and her first pregnancy was treated with ASA only.*

[b]*The patient was not treated with the standard therapy because the positivity for aPL was found after the abortion.*

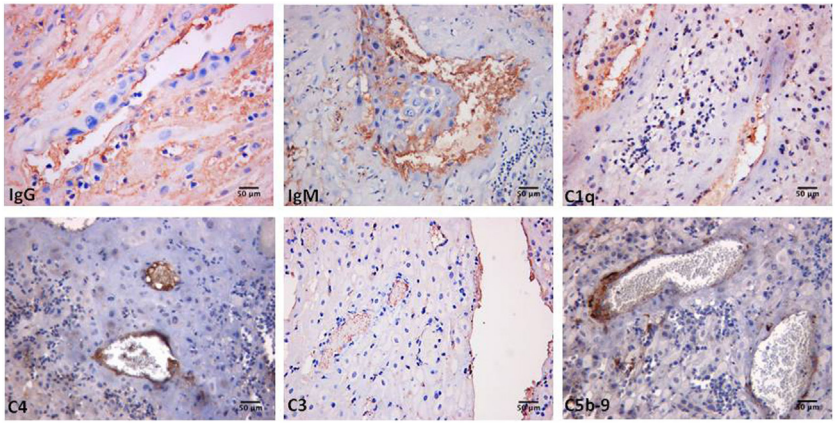

FIGURE 1 | Immunoperoxidase staining of a representative placental decidua from a primary antiphospholipid syndrome patient showing deposition of immunoglobulin (Ig) and various C components (20× magnification).

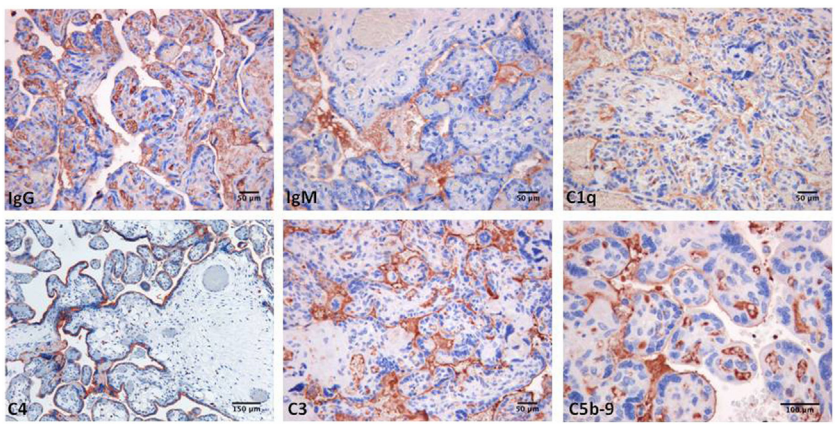

FIGURE 2 | Immunoperoxidase staining of representative placental villi from a primary antiphospholipid syndrome patient showing deposition of immunoglobulin (Ig) and various C components (20× magnification).

the alternative pathway (52). As activation of C5 results in the release of the small pro-inflammatory peptide C5a and the large fragment C5b that initiates the assembly of the terminal complex C5b-9, experiments conducted to clarify their relative contribution to fetal damage have led to the identification of C5a as the main mediator of fetal injury. The effect of C5a has been attributed to its ability to interact with C5aR expressed on PMN and to stimulate the release of TNF-α that induces apoptosis of cytotrophoblasts and promotes inflammation (53, 54). C5a was also found to induce expression of tissue factor in PMN that contributes to favor decidual inflammation and in turn increased fetal loss (55). The terminal complex does not seem to play a role in aPL-mediated fetal injury as C6-deficient mice had a similar rate of pregnancy loss as wild-type mice.

THERAPEUTIC PERSPECTIVES

A wealth of experimental and clinical data have clearly shown that C is implicated in the pathogenesis of the clinical manifestations of APS and have led to the suggestion that this syndrome should be considered a C-dependent disorder (31, 54). Despite the large body of evidence mainly obtained from animal models supporting this conclusion, antibodies or other reagents neutralizing C have not been regarded as first-line treatment to prevent adverse pregnancy outcome or thrombus formation. One possible explanation is that anticoagulants and low-dose aspirin have been used successfully to prevent vascular thrombosis and pregnancy abnormalities and there is a general consensus on their use in the primary treatment of APS patients. However, it is worth mentioning that this type of therapy is not always effective and does not always prevent recurrences of obstetric and vascular complications particularly in patients with triple positivity for LA, anti-cardiolipin, and anti-β2GPI antibodies (56). There are some suggestions that the overall therapeutic efficacy of anticoagulants can be increased by a combined treatment with other drugs, although specific clinical trials are still lacking (22).

Several case reports have been published describing patients with CAPS who have benefited from eculizumab administration

with a substantial amelioration of their clinical manifestations and successful kidney transplantations have also been published (22, 57). Similar beneficial results were obtained blocking C activation in APS patients with multiple arterial thrombosis refractory to standard therapy (58). It must be pointed out that chronic administration of eculizumab would be an expensive therapy to prevent thrombus formation due to the high cost of treatment to avoid an unpredictable event. This therapeutic measure can satisfactorily be restricted to situations in which blood clots are more likely to occur. We have shown that eculizumab administered to an APS patient prior to femoro-popliteal bypass surgery aimed at removing arterial occlusion was effective in preventing re-thrombosis (28). As surgical intervention represents the second hit required for thrombus formation in APS patients with circulating aPL, eculizumab would be expected to have a beneficial effect in patients undergoing vascular surgery. One clinical trial is ongoing to assess the efficacy of eculizumab in the prevention of APS-associated thrombotic microangiopathy following renal transplantation (Clinical Trial.gov #: NCT01029587).

Pregnancy is more frequently susceptible to complications in APS patients as this condition provides by itself a second hit in addition to anti-β2GPI antibodies. Fortunately, a large proportion of APS pregnant women responds to the combination of heparin and low dose aspirin, but approximately 20% are resistant to the standard therapy (22). These patients and in particular those with a history of recurrent abortions may preferentially respond to treatment with C5-neutralizing antibody. Recent data have excluded any adverse effect of eculizumab on fetus as only a negligible amount of antibody administered to pregnant women crosses the placenta leaving the C activity of the newborn largely unaffected (58, 59). We propose an alternative option based on the use of a human anti-β2GPI monoclonal antibody that targets the antigen highly expressed on syncytiotrophast, extravillous trophoblast, and decidual endothelial cells and is functionally ineffective being unable to activate C (60).

CONCLUSION

Experimental and clinical data accumulated over recent years support the conclusion that the C system is a key factor in the pathogenesis of the clinical manifestations of both vascular and obstetric APS. In fact, all the *in vivo* models and the reports from human studies clearly showed C activation; on the other hand, *in vitro* experimental models demonstrated that aPL may directly affect tissue targets or coagulation factors. Further studies are warranted to establish if C activation products should be considered useful markers to monitor disease severity and also if C neutralization should be included as first-line therapeutic option in APS patients with CAPS or scheduled for organ transplant or different vascular surgical procedures.

AUTHOR CONTRIBUTIONS

FT and PLM contributed to conception of the work and wrote the first draft of the manuscript. AG and BB provided the immunohistochemistry pictures. All authors contributed to manuscript revision, read and approved the submitted version.

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Ectonucleotidase-Mediated Suppression of Lupus Autoimmunity and Vascular Dysfunction

Jason S. Knight[1], Levi F. Mazza[1], Srilakshmi Yalavarthi[1], Gautam Sule[1], Ramadan A. Ali[1], Jeffrey B. Hodgin[2], Yogendra Kanthi[3,4] and David J. Pinsky[4,5]*

[1] Division of Rheumatology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, United States, [2] Department of Pathology, University of Michigan, Ann Arbor, MI, United States, [3] Division of Cardiology, Ann Arbor Veterans Administration Healthcare System, Ann Arbor, MI, United States, [4] Division of Cardiovascular Medicine, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, United States, [5] Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, United States

Objectives: CD39 and CD73 are surface enzymes that jut into the extracellular space where they mediate the step-wise phosphohydrolysis of the autocrine and paracrine danger signals ATP and ADP into anti-inflammatory adenosine. Given the role of vascular and immune cells' "purinergic halo" in maintaining homeostasis, we hypothesized that the ectonucleotidases CD39 and CD73 might play a protective role in lupus.

Methods: Lupus was modeled by intraperitoneal administration of pristane to three groups of mice: wild-type (WT), CD39$^{-/-}$, and CD73$^{-/-}$. After 36 weeks, autoantibodies, endothelial function, kidney disease, splenocyte activation/polarization, and neutrophil activation were characterized.

Results: As compared with WT mice, CD39$^{-/-}$ mice developed exaggerated splenomegaly in response to pristane, while both groups of ectonucleotidase-deficient mice demonstrated heightened anti-ribonucleoprotein production. The administration of pristane to WT mice triggered only subtle dysfunction of the arterial endothelium; however, both CD39$^{-/-}$ and CD73$^{-/-}$ mice demonstrated striking endothelial dysfunction following induction of lupus, which could be reversed by superoxide dismutase. Activated B cells and plasma cells were expanded in CD73$^{-/-}$ mice, while deficiency of either ectonucleotidase led to expansion of T_H17 cells. CD39$^{-/-}$ and CD73$^{-/-}$ mice demonstrated exaggerated neutrophil extracellular trap release, while CD73$^{-/-}$ mice additionally had higher levels of plasma cell-free DNA.

Conclusion: These data are the first to link ectonucleotidases with lupus autoimmunity and vascular disease. New therapeutic strategies may harness purinergic nucleotide dissipation or signaling to limit the damage inflicted upon organs and blood vessels by lupus.

Keywords: systemic lupus erythematosus, ectonucleotidases, CD73, CD39, T_H17 cells, endothelial dysfunction, neutrophil extracellular traps

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George C. Tsokos, Harvard Medical School, United States

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Fabio Malavasi, Università degli Studi di Torino, Italy
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****Correspondence:***
Jason S. Knight
jsknight@umich.edu

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INTRODUCTION

Systemic lupus erythematosus (commonly referred to as "lupus") is the prototypical systemic autoimmune disease. In the United States, the prevalence of lupus approaches 1 in 500, with a disproportionate impact on women of childbearing age and minorities. The immunopathology of lupus is complex, with derangements present in both lymphocyte- and myeloid-lineage cells.

Beyond the well-recognized damage inflicted by lupus upon organs such as kidneys, skin, and joints, lupus cardiovascular disease has emerged as a leading cause of morbidity and mortality. Indeed, young women with lupus carry a 50-fold increased risk of cardiovascular events when compared with their unaffected peers (1).

Although the proximal trigger for lupus is unknown, there is some evidence that environmental factors are contributory. In mice, the intraperitoneal administration of pristane (a naturally occurring hydrocarbon) recruits inflammatory macrophages into the peritoneal cavity, where they robustly produce type I interferons (2, 3). Mediated at least in part by these interferons, female mice take on features of lupus over the ensuing 6–9 months, including anti-ribonucleoprotein (anti-RNP) antibody production, splenic immune cell derangements, and immune complex glomerulonephritis (2, 3). Mechanistically, the pristane model of lupus depends upon both the type I interferon receptor and toll-like receptor 7 for autoantibody formation and other aspects of the lupus phenotype (4, 5). In recent years, the pristane model has been used to assess wide-ranging concepts in lupus pathogenesis including the roles of leptin (6), selectin-mediated leukocyte adhesion (7), and the inflammasome (8).

Leukocytes and endothelial cells are regulated by a dynamic halo of ATP, ADP, AMP, and adenosine. Purine nucleotides are liberated in large quantities from dying cells at sites of hypoxic, ischemic, or inflammatory stress (9). ATP and ADP then engage cell-surface receptors to launch proinflammatory and prothrombotic cascades (9). By contrast, adenosine (the extracellular concentration of which can rise by orders of magnitude during acute inflammation) has potent antithrombotic, anti-inflammatory, and immunosuppressive properties mediated by surface G protein-coupled receptors (10).

To regulate the local concentrations of purine nucleotides and adenosine, the ectonucleotidases CD39 and CD73 extend into the extracellular space from the surfaces of leukocytes and endothelial cells (**Figure 1A**). CD39 is a membrane-spanning enzyme with an ectodomain that cleaves the terminal phosphate group from ATP to form ADP, and then from ADP to form AMP (11). From there, CD73 (a GPI-anchored protein) clips the final phosphate group from AMP to generate adenosine (11). The endothelium is a key site of ectonucleotidase expression, with well-recognized upregulation in response to stressors such as hypoxia, thereby limiting leukocyte activity and efflux (12–14). Leukocytes (including lymphocytes and neutrophils) also express ectonucleotidases, not only to autoregulate activation, adhesion, and transit but also to manipulate neighboring cells (15, 16). Indeed, specialized immune cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells mediate their effects in part through local ectonucleotidase-generated adenosine (15, 16). To the best of our knowledge, the only connection between ectonucleotidases and lupus reported to date is the observation that some lupus patients lack adequate T-cell expression of CD39 and CD73, hinting at a defect in regulatory T-cell function (17, 18). The studies described here seek to provide new insight into how a pathway that functions as an endogenous guardian against inflammation may be exploited to counteract lupus.

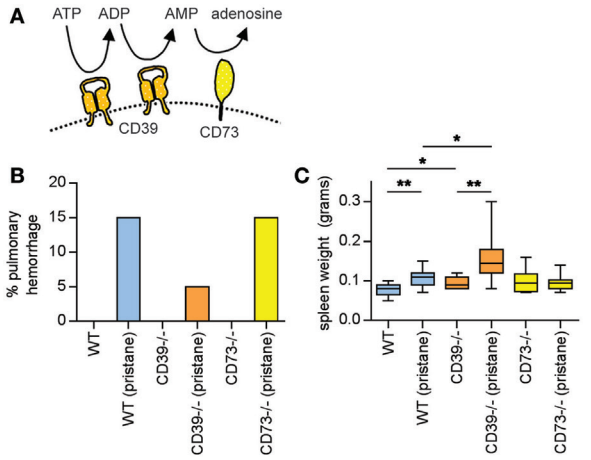

FIGURE 1 | Pulmonary hemorrhage and splenomegaly in pristane-treated mice. **(A)** Schematic of CD39 and CD73, which together mediate the step-wise phosphohydrolysis of extracellular ATP into adenosine. **(B)** Some mice developed clinically overt pulmonary hemorrhage within the first month after pristane administration; these mice required euthanasia. $N = 10$ saline-treated mice and 20 pristane-treated mice per genotype; no comparisons were statistically significant. **(C)** Mice were administered either saline or pristane, as indicated. 36 weeks later, spleen size was measured. $N = 10$ per control group and 17–19 per pristane group; $^{*}p < 0.05$ and $^{**}p < 0.01$. Box-and-whisker plots denote minimum, 25th percentile, median, 75th percentile, and maximum.

MATERIALS AND METHODS

Animal Housing and Treatments

Mice were housed in a specific pathogen-free barrier facility, and fed standard chow. Female C57BL/6 mice were purchased from The Jackson Laboratory. $CD39^{-/-}$ mice have been described by our group previously (19). $CD73^{-/-}$ mice in the C57BL/6 background were originally obtained from Dr. Linda Thompson and have been used by our group previously (20). Pristane was purchased from Sigma. At 8–10 weeks of age, female mice were administered a single intraperitoneal dose of 500 µl pristane or 500 µl normal saline. Unless otherwise indicated, studies were performed on mice euthanized at 36 weeks. This study was carried out in accordance with the recommendations of the National Research Council, Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee.

Complete Blood Counts

Peripheral leukocyte and platelet counts were determined with an automated Hemavet 950 counter (Drew Scientific).

ELISAs

Kits for mouse anti-nRNP IgG (5415) and mouse total IgG (6320) were purchased from Alpha Diagnostic International and performed according to the manufacturer's instructions.

Kidney Scoring

At the time of euthanasia, kidneys were gently perfused with heparinized saline. A portion of the cortex was frozen in Tissue-Tek

OCT (Sakura Finetek) for immunofluorescence staining. Staining for kidney IgG and C3 was performed on frozen sections as described (21, 22). Another portion of the cortex was fixed in formalin and embedded in paraffin. Formalin-fixed sections were stained by periodic acid-Schiff and then scored in a blinded manner as previously described (21, 22). In brief, a semiquantitative scoring system: 0, no involvement; 0.5, minimal involvement of <10%; 1, mild involvement (10–30% section); 2, moderate involvement (31–60% of section); and 3, severe involvement. This system was used to assess 13 different parameters of activity and chronicity (mesangial hypercellularity, mesangial deposits, mesangial sclerosis, endocapillary cellular infiltrate, subepithelial and subendothelial deposits, capillary thrombi, capillary sclerosis, cellular or organized crescents, synechiae, tubular atrophy, and interstitial fibrosis). For glomerular indices, 30 glomeruli were examined per mouse, and a cumulative score was determined for each parameter.

Flow Cytometry

A single-cell suspension of splenocytes was analyzed with the following anti-mouse antibodies (all from BioLegend): CD138, B220, CD80, CD19, CD44, and CD62L. Mouse Th1/Th17 Phenotyping Kit was from BD Pharmingen and performed according to the manufacturer's instructions. Staining was typically for 30 min at 4°C. After washing, cells were fixed in 2% paraformaldehyde before analysis with a CyAn ADP Analyzer (Beckman Coulter). Further data analysis was done using FlowJo analysis software.

Endothelial Function

Studies were performed as previously reported by our group (21, 22). After euthanasia with pentobarbital, thoracic aortas were excised, cleaned, and cut into 2-mm length rings. The endothelium was left intact and rings were mounted in a myograph system (Danish Myo Technology A/S). Rings were bathed with warmed and aerated (95% O_2/5% CO_2) physiological salt solution. Aortic rings were set at 7 mN passive tension and equilibrated for 1 h. Cumulative doses of phenylephrine (10^{-9} to 10^{-6} M) were then added to the bath to establish a concentration-response curve. After washing, a phenylephrine concentration corresponding to 80% of the maximum was added, and contraction was allowed to reach a stable plateau. To examine endothelium-dependent relaxation, acetylcholine (10^{-9} to 10^{-6} M) was added cumulatively to the bath. Finally, a normal vascular smooth muscle response was confirmed by washing out phenylephrine and acetylcholine, and then repeating the experiment with phenylephrine contraction followed by cumulative addition of sodium nitroprusside (10^{-9} to 10^{-5} mol/L). In some experiments, one aortic ring from each mouse was treated as usual, while a second ring was incubated with superoxide dismutase (SOD) 1.2 kU/ml during the equilibration phase of the experiment.

Blood Pressure

Non-invasive blood pressure was measured by tail cuff as described (23). Briefly, using the IITC Life Science blood pressure measurement system, conscious and restrained mice were acclimated for 3 days in a temperature controlled environment (model 306 warming chamber). The tail vein was occluded with an integrated sensor-cuff (model I-B60-1/4) and return of pulsation (RTP) detected by the RTP-computerized blood pressure monitor (model 6M 229 6 channel mouse system). Repeated measures were averaged for determination and report of systolic blood pressure and heart rate.

Quantitative PCR

At the time of tissue harvest, aortas were snap frozen in liquid nitrogen and stored at −80°C. Later, the aortas were mechanically homogenized in TriPure Isolation Reagent (Roche). RNA was prepared by the Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. RNA integrity number was >7 for all included samples. cDNA was synthesized using MMLV RT (Invitrogen) and 100 ng of RNA using a MyCycler thermocyler (Bio-Rad). Quantitative PCR was with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions, and carried out using an ABI PRISM 7900HT (Applied Biosystems). Primers for the housekeeping gene beta-actin were purchased from Qiagen (QuantiTect Primer Assays, which have proprietary primer sequences). Primer sequences for endothelial nitric oxide synthase (eNOS) were 5′-GACCCTCACCGCTACAACAT-3′ and 5′-TTTGGCCAGCTGGTAACTGT-3′; primer sequences for inducible NOS (iNOS) were 5′-TGGTGGTGACAAGCACATTT-3′ and 5′-GCCAAACACAGCATACCTGAA-3′. Ct values were normalized to the housekeeping gene to determine ΔCt. ΔΔCt values were then determined by comparing each ΔCt to the average ΔCt for the wild-type (WT) control group. Data were presented as relative fold change by the formula $2^{\Delta\Delta Ct}$.

Measurement of Cell-Free DNA

Cell-free DNA was quantified in plasma using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) according to the manufacturer's instructions.

Neutrophil Purification and NETosis Assay

Bone marrow neutrophils were isolated as previously described (21, 22). Briefly, total bone marrow cells were spun on a discontinuous Percoll gradient (52–69–78%) at 1,500 × *g* for 30 min. Cells from the 69–78% interface were collected. These cells were >95% Ly-6G-positive by flow cytometry and had typical nuclear morphology by microscopy. To assess *in vitro* NETosis, a protocol similar to what we have described previously was followed (21, 22). Culture was for 4 h at 37°C in RPMI media supplemented with 2% bovine serum albumin and 10 mM HEPES buffer. Stimulation with phorbol-12-myristate-13-acetate (100 nM, Sigma) was also for 4 h. For immunofluorescence, cells were fixed with 4% paraformaldehyde. DNA was stained with Hoechst 33342 (Invitrogen), while protein staining was with rabbit polyclonal antibody to citrullinated histone H3 (Abcam), followed by FITC-conjugated anti-rabbit IgG (SouthernBiotech). Images were collected with an Olympus IX70 microscope and a CoolSNAP HQ2 monochrome camera (Photometrics) with Metamorph Premier software (Molecular Devices). Neutrophil extracellular traps (NETs) (decondensed areas of extracellular DNA co-staining with one of the aforementioned protein markers) were quantified by two blinded

observers, and digitally recorded to prevent multiple counts; the percentage of NETs was calculated after counting 10 400× fields per sample.

Statistical Analysis

Data analysis was with GraphPad Prism software version 6. For continuous variables, normally distributed data were analyzed by unpaired two-tailed *t* testing, while skewed data were assessed by Mann–Whitney test. For dichotomous variables, analysis was by Chi square. For endothelial function experiments, curve fit was by the least squares method, and comparisons were by two-way ANOVA. Statistical significance was defined as $p < 0.05$.

RESULTS

The one-time intraperitoneal administration of the natural hydrocarbon pristane promotes features of lupus, which emerge over 6–9 months (2, 3). Here, pristane was administered to three groups of mice at 8–10 weeks of age, all in the C57BL/6 background: WT, CD39$^{-/-}$, and CD73$^{-/-}$. Twenty mice were treated with pristane and 10 with saline for each genotype.

A feature of pristane administration to C57BL/6 mice is the induction of diffuse alveolar hemorrhage, which becomes clinically apparent in 15–20% of mice approximately 4 weeks after treatment (24, 25). Mechanistically, roles have been suggested for both B cells (24) and macrophages (25) in the pulmonary-hemorrhage phenotype. Here, three WT mice (15%), one CD39$^{-/-}$ mouse (5%), and three CD73$^{-/-}$ mice (15%) developed clinically overt pulmonary hemorrhage in response to pristane (and required euthanasia), all within 4 weeks of pristane administration (**Figure 1B**). No saline-treated mouse developed a similar phenotype (**Figure 1B**). All remaining mice survived to 36 weeks, at which time they were euthanized.

CD39 Deficiency Potentiates Splenomegaly in Response to Pristane

At 36 weeks, pristane promoted splenomegaly in WT mice, which was further potentiated by CD39 deficiency (**Figure 1C**).

Ectonucleotidase Deficiency Potentiates Autoimmunity in Pristane-Treated Mice

Pristane administration induces the production of autoantibodies, especially to ribonucleoproteins (RNPs) (2, 3). Here, serum anti-RNP antibody levels were measured 36 weeks after pristane administration. While WT mice demonstrated a strong trend toward induction of anti-RNP antibodies with pristane administration ($p = 0.054$), this induction was further potentiated by ectonucleotidase deficiency (**Figure 2A**). Anti-RNP antibodies were not detected at a significant level in any of the saline-treated groups (**Figure 2A**). We also measured serum total IgG. In WT mice, pristane triggered higher levels of IgG when compared with saline-treated controls (**Figure 2B**). A trend for increased IgG was also observed in the ectonucleotidase-deficient mice, although this did not reach statistical significance (which may have been at least partially attributable to a higher "baseline" in the ectonucleotidase-deficient controls) (**Figure 2B**). In summary, these data indicate that ectonucleotidase deficiency potentiates autoantibody formation, but not total IgG levels, in response to pristane.

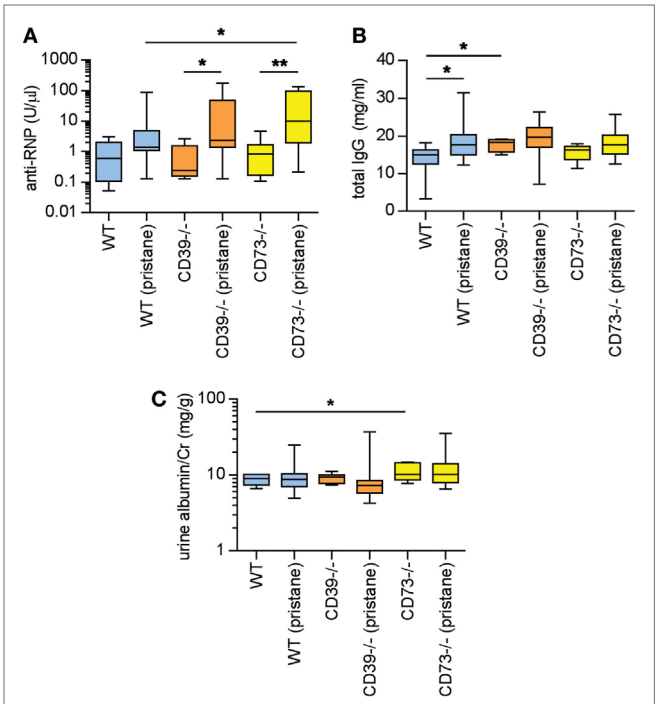

FIGURE 2 | Ectonucleotidase deficiency potentiates autoimmunity in pristane-treated mice. Mice were administered either saline or pristane, as indicated. 36 weeks later, various endpoints were tested. **(A)** Anti-ribonucleoprotein (Anti-RNP) IgG was measured in serum. **(B)** Total IgG was measured in serum. **(C)** Spot albumin/Cr (albumin/creatinine) ratios in urine. $N = 10$ per control group and 17–19 per pristane group. $^{*}p < 0.05$ and $^{**}p < 0.01$.

C57BL/6 Mice Do Not Develop Significant Proteinuria in Response to Pristane

In previous studies of pristane administration to C57BL/6 mice, the kidneys have demonstrated a relatively mild phenotype of increased mesangial cellularity, which is compatible with World Health Organization Class II lupus nephritis in patients (2, 26). Immune complex and complement deposition have also been appreciated in pristane-treated C57BL/6 mice, albeit in the setting of minimal proteinuria (2, 26). Here, the albuminuria detected at 36 weeks was at a level that would be considered microalbuminuria (i.e., albumin/creatinine <300 mg/g) (**Figure 2C**); the only statistically significant difference between groups was that saline-treated CD73$^{-/-}$ mice demonstrated higher levels of microalbuminuria than saline-treated WT mice. Beyond albuminuria, kidney glomeruli were scored for IgG and C3 deposition at 36 weeks. Pristane administration did enhance both IgG and C3 deposition when compared with saline-treated controls (Figures S1A–C in Supplementary Material); however, ectonucleotidase deficiency did not potentiate either IgG or C3 deposition. Kidney histopathology was also assessed across a variety of inflammatory parameters (see Materials and Methods). As compared with

saline-treated controls, WT mice demonstrated an increase in mesangial hypercellularity upon pristane administration (Figures S1D,E in Supplementary Material); there were no other statistically significant differences between the groups. In summary, there is evidence that pristane administration induces modest glomerular immune complex deposition and mesangial hypercellularity, albeit without significant proteinuria, and without potentiation by ectonucleotidase deficiency.

Ectonucleotidase Deficiency Expands B- and T-Cell Populations

As above, there is evidence of exacerbated autoimmunity (splenomegaly, increased anti-RNP antibodies) when ectonucleotidase-deficient mice are treated with pristane. To understand this mechanistically, we first characterized splenic B cell populations. As compared with WT mice, CD73$^{-/-}$ mice demonstrated expansion of plasma cells (**Figures 3A,B**) and CD80^{+} activated B cells (**Figures 3C,D**); a similar expansion was not seen in CD39$^{-/-}$ mice. Moreover, as compared with pristane-treated WT mice, there was an increase in splenic follicular and marginal zone B cells in pristane-treated CD73$^{-/-}$ mice (Figure S2 in Supplementary Material). We also characterized splenic T cells. As compared with pristane-treated WT mice, there was an expansion of effector/memory CD4^{+} T cells (CD44^{hi} CD62L^{lo}) in pristane-treated CD73$^{-/-}$ mice (**Figure 4A**); interestingly, this increase was also noted in the saline-treated CD73$^{-/-}$ controls. While there was no difference in IFNγ-producing T_H1 cells (**Figure 4B**), IL-17A-producing T_H17 cells were significantly increased in both CD39$^{-/-}$ and CD73$^{-/-}$ mice (**Figure 4C**). The T_H17 expansion was robust—present at baseline (i.e., the saline groups) and then further potentiated by pristane (**Figure 4C**). In summary, these data demonstrate that ectonucleotidase deficiency promotes expansion of T_H17 cells, while plasma cells and activated B cells are expanded only in the CD73-deficient mice.

Ectonucleotidases Protect Against Endothelial Dysfunction in Pristane-Treated Mice

Dysfunction of the arterial endothelium (as defined by impaired flow-mediated dilation) is a harbinger of atherosclerosis in lupus

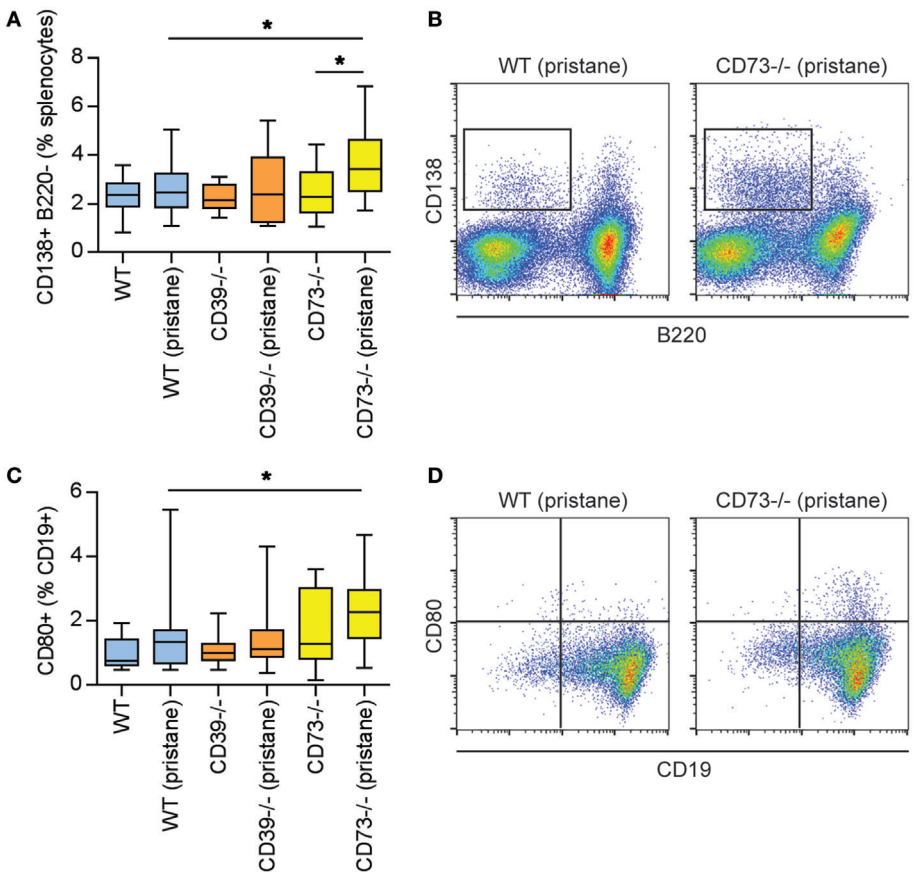

FIGURE 3 | Modulation of plasma cells and B cells by ectonucleotidase deficiency in pristane-treated mice. Mice were administered either saline or pristane, as indicated. 36 weeks later, splenocytes were analyzed by flow cytometry. **(A)** CD138^{+} B220^{-} plasma cells, presented as the percentage of total splenocytes. **(B)** Representative data as presented in panel **(A)**. **(C)** CD80^{+} activated B cells, presented as the percentage of CD19^{+} B cells. **(D)** Representative data as presented in panel **(C)**. $N = 10$ per control group and 17–19 per pristane group; $^{*}p < 0.05$.

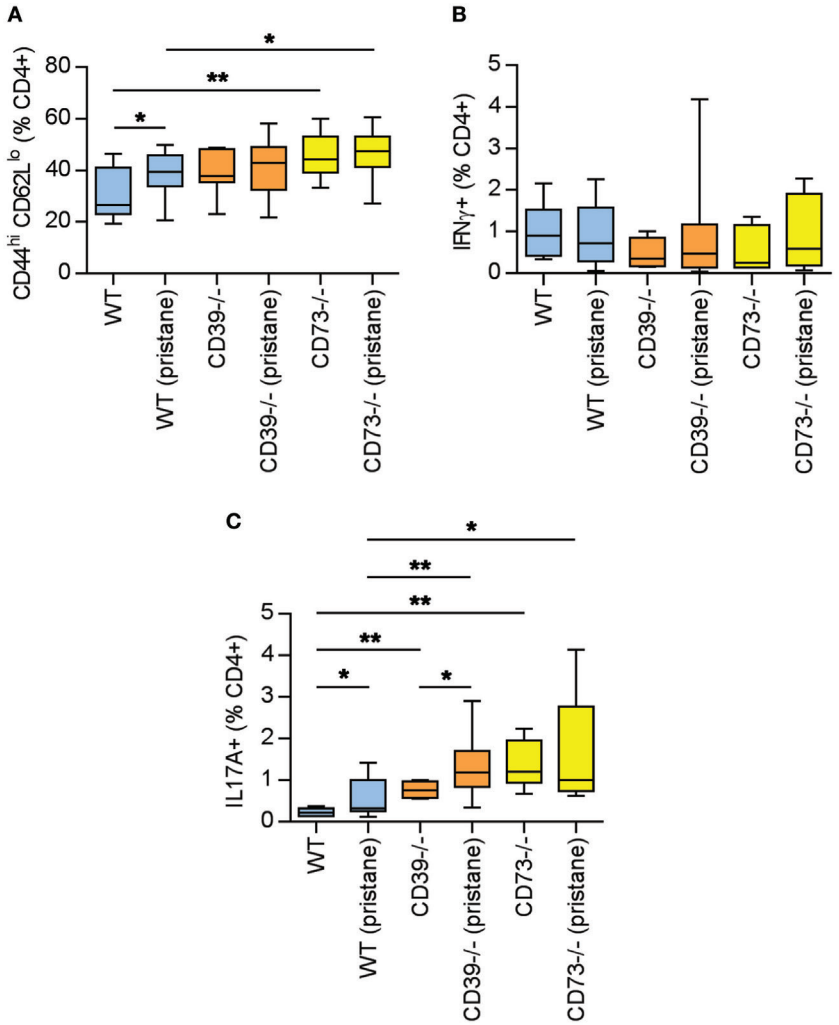

FIGURE 4 | Potentiation of T cell activation and T_H17 polarization by ectonucleotidase deficiency. Mice were administered either saline or pristane, as indicated. 36 weeks later, splenocytes were analyzed by flow cytometry. **(A)** $CD44^{hi}$ $CD62L^{lo}$ effector/memory T cells, presented as the percentage of $CD4^+$ T cells. **(B)** Percentage of $CD4^+$ T cells expressing interferon gamma. **(C)** Percentage of $CD4^+$ T cells expressing interleukin 17A. For panel **(A)**, $n = 10$ per control group and 17–19 per pristane group. For panels **(B,C)**, $n = 5$ per control group and 8–10 per pristane group; $^*p < 0.05$ and $^{**}p < 0.01$.

patients (27). Endothelial dysfunction is driven by oxidative stress, under which nitric oxide synthase becomes "uncoupled" and produces vasoconstrictive superoxide anion rather than vasodilatory nitric oxide (27). In both $CD39^{-/-}$ and $CD73^{-/-}$ mice (but not WT mice), pristane administration induced significant dysfunction of the arterial endothelium, when compared with their respective control groups (**Figures 5A–C**). Interestingly, $CD39^{-/-}$ mice demonstrated a trend toward more robust baseline aorta relaxation, when compared with the other two genotypes (**Figures 5A–C**). The endothelial dysfunction of $CD39^{-/-}$ and $CD73^{-/-}$ mice was not explained by alterations in either blood pressure or heart rate (Figure S3 in Supplementary Material) and could be mitigated by *ex vivo* administration of SOD to aortic rings (to neutralize reactive oxygen species) (**Figures 5D,E**). We also assessed transcription of the genes for both eNOS and iNOS in aortas and found that eNOS transcription was upregulated in $CD39^{-/-}$ and $CD73^{-/-}$ mice when compared with WT mice, but was not further modified by pristane administration (Figure S4A in Supplementary Material). Transcription of iNOS was not significantly regulated by any of the conditions (Figure S4B in Supplementary Material). In summary, these data demonstrate that ectonucleotidases protect against dysfunction of the arterial endothelium in response to pristane, and that pristane-induced dysfunction can be rescued by administration of SOD.

Ectonucleotidase Deficiency Potentiates Neutrophil Activation

Neutrophils are being increasingly recognized as pathogenic agents in lupus (28, 29). For example, a "neutrophil signature" in blood heralds the onset of lupus nephritis in lupus patients (30, 31). NETs released by lupus neutrophils are at least one driver

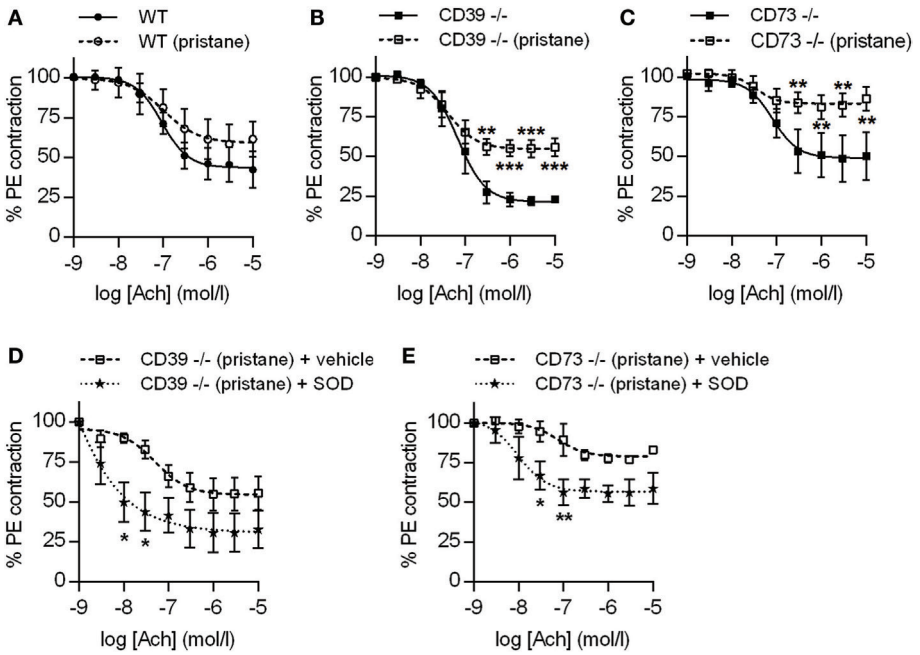

FIGURE 5 | Ectonucleotidase deficiency potentiates endothelial dysfunction in pristane-treated mice. Mice were administered either saline or pristane, as indicated. 36 weeks later, "aortic rings" were harvested for *ex vivo* determination of endothelial function by measuring the contractile force remaining in pre-contracted (by phenylephrine/PE) aortic rings in response to progressively increasing concentrations of acetylcholine. A "deeper" curve indicates a healthier endothelium, while a "flatter" curve denotes endothelial dysfunction. **(A–C)** $N = 5$ control mice and 7 pristane mice per graph. **(D,E)** Two aortic rings were isolated from each mouse ($n = 5$), and one was treated with superoxide dismutase (SOD). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

of type I interferon production (which counteracts endothelial homeostasis) (32, 33), while hyperactive neutrophils are likely an important stressor of the lupus endothelium (21, 22, 34). As compared with WT mice, $CD73^{-/-}$ mice demonstrated an increased neutrophil-to-lymphocyte ratio, especially in response to pristane (**Figure 6A**); the increased ratio was related to an increase in absolute neutrophil count, more so than a decrease in the lymphocyte count (Figure S5 in Supplementary Material). $CD73^{-/-}$ mice also demonstrated elevated levels of cell-free DNA (a surrogate for NETs) in serum (**Figure 6B**). *Ex vivo*, NET release was accelerated by deficiency of either CD39 or CD73, both at baseline (i.e., in the saline groups) and in response to pristane (**Figures 6C,D**). In summary, these data reveal that ectonucleotidases mitigate the release of NETs by neutrophils, both at baseline and in the context of lupus.

DISCUSSION

The literature already hints at an intersection between adenosine signaling and lupus. One study has suggested that lupus-prone $Fas^{lpr/lpr}$ mice are protected from nephritis by an adenosine-receptor agonist (35). In some lupus patients, there is increased adenosine deaminase activity (and presumably lower adenosine content) in blood (36, 37). Perhaps to compensate for this, adenosine-receptor density may be increased on lupus lymphocytes (38). Moreover, these pathways are potentially amenable to pharmacologic manipulation in patients. For example, adenosine signaling is indirectly amplified by a number of medications with relevance to rheumatology including methotrexate, dipyridamole, and phosphodiesterase 4 inhibitors.

Here, we have demonstrated for the first time a role for the ectonucleotidases CD39 and CD73 in protecting against lupus. Deletion of CD39 and CD73 leads to higher levels of anti-RNP antibodies in response to pristane, with CD73 deletion in particular promoting expansion of splenic B cell and T cell populations that likely contribute to autoantibody production. Within the T cell compartment, it is notable that some of these changes (expansion of effector/memory T cells and T_H17 cells) were present independent of pristane administration, which would fit with a general predisposition toward inflammation and autoimmunity conferred by loss of ectonucleotidase activity (11, 39, 40). For example, protective roles for ectonucleotidases have been suggested in rheumatoid arthritis (41, 42), juvenile idiopathic arthritis (43), inflammatory bowel disease (44, 45), autoimmune hepatitis (46), and atherosclerosis (47, 48). This is the first study to explore these pathways in lupus.

The classic markers of Tregs are CD25 and the forkhead transcription factor FoxP3. Recently, it has also been recognized that both CD39 and CD73 are surface markers of Tregs, generating adenosine to induce anergy in effector T cells *via* the A_{2A} receptor (15, 49, 50). In contrast to the protective role of Tregs, effector T_H17 cells have a well-established role in promoting autoimmunity in various diseases including multiple sclerosis, rheumatoid arthritis, and lupus. Indeed, lupus mice and patients have increased frequency of circulating T_H17 cells, which correlate with disease activity (51–53). Furthermore, the induction of lupus by pristane

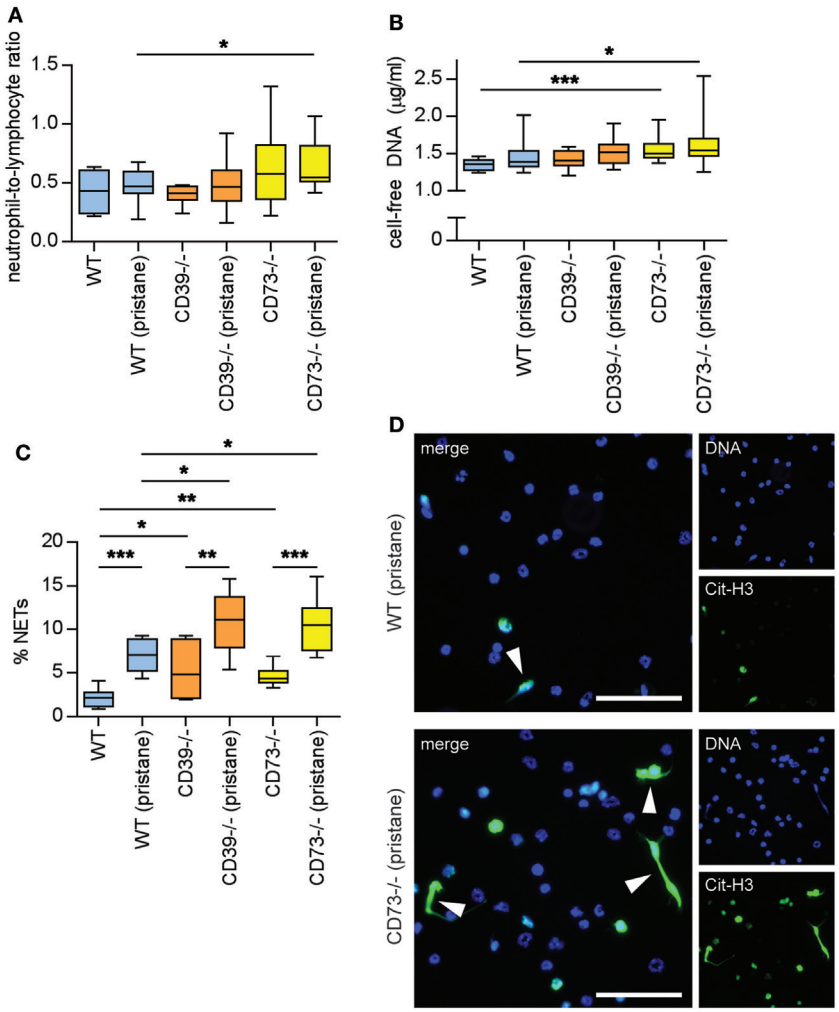

FIGURE 6 | Ectonucleotidase deficiency potentiates neutrophil activation. Mice were administered either saline or pristane, as indicated. 36 weeks later, various endpoints were assessed. **(A)** Neutrophil-to-lymphocyte ratio in peripheral blood. **(B)** Cell-free DNA in mouse serum. **(C)** Mature neutrophils were purified from mouse bone marrow and cultured for 4 h on polylysine-coated glass coverslips. Spontaneous neutrophil extracellular trap (NET) release was assessed by immunofluorescence microscopy. **(D)** Representative photomicrographs from the data presented in panel C. DNA is stained blue and citrullinated histone H3 (Cit-H3) green. NETs are identified as extracellular areas of blue and green overlap (arrowheads). Scale bar = 50 μm. For panels **(A,B)**, $n = 10$ per control group and 17–19 per pristane group. For panel **(C)**, $n = 6$–9 per group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

is significantly mitigated by deletion of the IL-17 gene (54). Our data now demonstrate that deficiency of either CD39 or CD73 leads to polarization toward T_H17 cells, which we hypothesize to be at least partially attributable to a defect in immunosuppressive Treg function in ectonucleotidase-deficient mice (15).

While this study focused on ectonucleotidases, follow-up studies should further characterize the purine species that they regulate and downstream signaling events. While the conversion of ATP to AMP can be countered by extracellular kinase activity, the conversion of AMP to adenosine can only be reversed upon intracellular transport of adenosine. This places CD73 at a crucial checkpoint in the conversion of extracellular, proinflammatory ATP into anti-inflammatory adenosine. Indeed, in animal models, CD73 has been shown to protect against LPS-induced neutrophil trafficking into lungs (55), and permeability of hypoxic endothelium to neutrophils (12–14). In our hands, CD73 deficiency, when compared with CD39 deficiency, was the greater potentiator of both autoimmune-exacerbating and inflammatory neutrophil phenotypes in the pristane model, thereby hinting that adenosine (and its downstream signaling pathways) in particular warrants further study.

It should be emphasized that our work is performed in a mouse model of lupus and has potential limitations when extrapolated to human lupus. For example, a series of families identified with CD73 null mutations did not have a clinical autoimmune phenotype, but were instead predisposed to severe calcification of lower extremity arteries and joint capsules (56). To the best of our knowledge, CD73 mutations have never been described in lupus patients. There is growing interest in the role of purinergic signaling in immune function, and as a therapeutic target. Recent

work has described CD38/CD203a as an alternative mechanism of ATP catabolism by both human T cells (perhaps especially Tregs) and cancer cells (57, 58). Indeed, antagonism of CD38 is currently being explored as a therapeutic approach to boost the anticancer immune response (59, 60). Interestingly, in mice, CD38 deficiency has in some cases been shown to attenuate autoimmune/inflammatory disease (61), again emphasizing differences between these pathways in mouse and human. Now that our study has highlighted the potential importance of purinergic signaling in lupus, future studies should consider parallel/compensatory pathways such as CD38/CD203a, as well as adenosine deaminase (62, 63), in both mice and humans.

Adenosine receptors vary in both affinities for adenosine and tissue distribution (10). For example, neutrophils express all four adenosine receptors (64). The A_1 receptor (which has a high affinity for adenosine) promotes neutrophil chemotaxis, while the other three receptors (which may only become activated when the environment is flooded with excess adenosine) tend to silence neutrophils (10, 64). In particular, the A_{2A} and A_3 receptors are expressed at high levels on neutrophils and are recognized as suppressors of neutrophil effector functions (64–66). One study demonstrated that agonism of the A_{2A} receptor is protective against nephritis in a different model of lupus (35). However, the impact of adenosine signaling on lupus vascular disease and neutrophil activity, and the role of adenosine receptors beyond the A_{2A} receptor, are heretofore unexplored. Our data now for the first time link ectonucleotidases to the release of NETs by neutrophils. At baseline (i.e., the saline condition) both $CD39^{-/-}$ and $CD73^{-/-}$ mice demonstrated exaggerated NET release, which was further potentiated by pristane administration. We speculate that ectonucleotidases play a role in generating a local "halo" of adenosine that suppresses NET release.

In the 1950s, mortality attributable to lupus was more than 50%, with that startling number driven especially by renal failure. With advances in immunosuppressive therapy and transplant medicine, nephritis is now a rare cause of death. In its place, cardiovascular disease has emerged as a leading cause of mortality in lupus (27). While NETs were originally described as key players in host defense, recent work has pointed to a multifaceted (and generally deleterious) intersection with the vasculature. Proteases in NETs kill endothelial cells. NETs stimulate interferon production, which reduces the numbers and function of restorative endothelial progenitors (21, 67). Furthermore, in lupus-relevant mouse models, inhibition of NETosis mitigates both arterial and venous thrombosis (21, 34, 68). Here, we posit that neutrophil hyperactivity (in the absence of nucleotide dissipation) is an important mediator of the pristane-dependent endothelial dysfunction observed in ectonucleotidase-deficient mice, although confirmation of this will require further study.

In summary, we have revealed a previously unrecognized role for ectonucleotidases in protection against lupus. In particular, these data are the first to link ectonucleotidases with lupus autoimmunity and vascular disease. Future therapeutic strategies may harness purinergic signaling to limit the damage inflicted by lupus upon organs and blood vessels.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Research Council, Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LM, SY, GS, RA, JH, and YK conducted experiments and analyzed data. JK, YK, and DP designed the study. All the authors participated in writing the manuscript and gave approval before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01322/full#supplementary-material.

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REVIEW
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Neurological Disease in Lupus: Toward a Personalized Medicine Approach

*Sarah McGlasson[1,2,3], Stewart Wiseman[2], Joanna Wardlaw[2], Neeraj Dhaun[4] and David P. J. Hunt[1,2,3]**

[1] MRC Institute of Genetics and Molecular Medicine, Edinburgh, United Kingdom, [2] The UK Dementia Research Institute, University of Edinburgh, Edinburgh, United Kingdom, [3] The Anne Rowling Clinic, University of Edinburgh, Edinburgh, United Kingdom, [4] Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom

The brain and nervous system are important targets for immune-mediated damage in systemic lupus erythematosus (SLE), resulting in a complex spectrum of neurological syndromes. Defining nervous system disease in lupus poses significant challenges. Among the difficulties to be addressed are a diversity of clinical manifestations and a lack of understanding of their mechanistic basis. However, despite these challenges, progress has been made in the identification of pathways which contribute to neurological disease in SLE. Understanding the molecular pathogenesis of neurological disease in lupus will inform both classification and approaches to clinical trials.

Keywords: neurolupus, personalized medicine, lupus erythematosus, systemic, targeted therapy, interferon type I

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***Correspondence:**
David P. J. Hunt
david.hunt@igmm.ed.ac.uk

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INTRODUCTION

Systemic lupus erythematosus (SLE, lupus) is a multiorgan autoimmune disease, initially described on the basis of its cutaneous manifestations (1). During the nineteenth century, the true multisystem nature of the disease was recognized with the initial descriptions of severe brain involvement (2, 3). The first dedicated clinical studies of neurological dysfunction in lupus were reported in 1945 by David Daly (4). His observations were astute, noting a high degree of heterogeneity in the neurological manifestations, and a prominent contribution of neurovascular disease. Over the following decades, the effects of lupus on all levels of the nervous system have been recognized.

The diversity of neurological disease in lupus stimulated calls for a classification system to facilitate its clinical and scientific study (5). In 1999, the American College of Rheumatology (ACR) developed criteria for case definitions for neurolupus (6). These broadly distinguish between complications which affect the central nervous system and peripheral nervous system (**Table 1** and **Figure 1**). While minor modifications have been proposed to these criteria, they have remained largely unchanged for almost two decades (7, 8). Neurological events have also been incorporated into diagnostic criteria for lupus, as well as outcome metrics such as the SLICC/ACR Damage index (9, 10).

The development of the ACR neurolupus definitions helped stimulate the epidemiological study of neurological disease in lupus, and has demonstrated that nervous system involvement is a major negative determinant of quality of life (11–13). However, such studies have highlighted one of the major problems in the field—the issue of establishing a causal association between a neurological syndrome and lupus (14). For example, the ACR criteria include terms such as *headache* and *mood disorder* which are highly prevalent in the general population and observed at similar frequency in

healthy, matched controls, as well as patients with other chronic inflammatory diseases (15). As such they are less likely to be caused directly by lupus. When "minor events" such as headache and anxiety disorders are included in population studies, then 40% of patients had at least one neuropsychiatric event (12). Exclusion of minor symptomatology leads to much improved specificity of the criteria (15). Neurological manifestations can occur at any stage of disease. Longitudinal studies of newly diagnosed patients show that neurological events attributable to lupus can occur around the time of diagnosis in approximately 5–10% of cases (16). Prospective studies show that major neurological events develop in about 5% of patients with SLE, followed over 3 years (17). Magnetic resonance imaging evidence (MRI) of brain changes indicating microvascular disease can develop early in disease course and in young patients (18, 19).

Much of the difficulty in classification stems from a comparative lack of understanding as to how neurological disease develops in people with lupus. It is notable that the ACR definitions focus largely on neurological syndromes, rather than pathophysiological mechanisms. This is in major contrast to renal lupus, where pathophysiological classification influences treatment and prognosis (**Figure 2**) (20). With the development of increasingly targeted treatments, an understanding of the molecular pathogenesis of brain disease is ever more important if it is to inform clinical trial design and, ultimately an individualized therapeutic approach.

TABLE 1 | Clinical syndromes seen in people with systemic lupus erythematosus.

	Syndrome	Implicated mechanisms and potential therapeutic targets
CNS	Large and small vessel disease	• Large vessel atheromatous disease (57) • Accelerated cerebral small vessel disease (18) • Antiphospholipid antibodies (49)
	Seizures	• Unknown (69)
	Myelopathy	• Antibody-mediated [aquaporin-4, myelin oligodendrocyte glycoprotein (MOG)] (21, 147, 148) • Vascular
	Meningitis	• Unknown (78)
	Movement disorder	• Unknown (84)
	Demyelinating syndrome	• Not clearly associated with SLE (89)
	Headache	• Not clearly associated with SLE (90)
	Psychiatric disease	• Cytokine dysregulation (107) • Antibody-mediated (NMDA-R, Ribosomal-P) (97)
	Cognitive dysfunction	• Cytokine dysregulation (38) • Small vessel disease (18, 61)
PNS	Peripheral neuropathy	• Vasculitis (124) • Antibody-mediated (ganglioside) (149)
	Cranial neuropathy	• Vasculitis • Antibody-mediated (aquaporin-4/MOG) (132, 150)
	Myasthenia Gravis	• Antibody-mediated (anti-AChR) (151)

PATHOPHYSIOLOGY OF NEUROLOGICAL DISEASE IN LUPUS

Genetics

Genome-wide association studies of large cohorts of lupus patients have identified an increasing number of associations with pathways involved in both the innate and adaptive immune systems (24). However, to date there has been little dedicated genetic study of neurolupus. An evaluation of *TREX1*, a 3′–5′ exonuclease associated with SLE (25, 26), revealed a common risk haplotype in lupus patients with brain manifestations, particularly seizures (27, 28). While these mechanistic insights are of interest, testing of *TREX1* is unlikely to be of clinical utility (27, 29) given the relatively high frequency of variants in the general population (30).

Cytokines

There is dysregulation of multiple cytokine pathways in patients with SLE (31), and recent work has focused on the extent to which these pathways might contribute to brain damage. Approximately 80% of individuals with lupus have aberrant activation of their type I interferon pathway, identified by either a transcriptomic

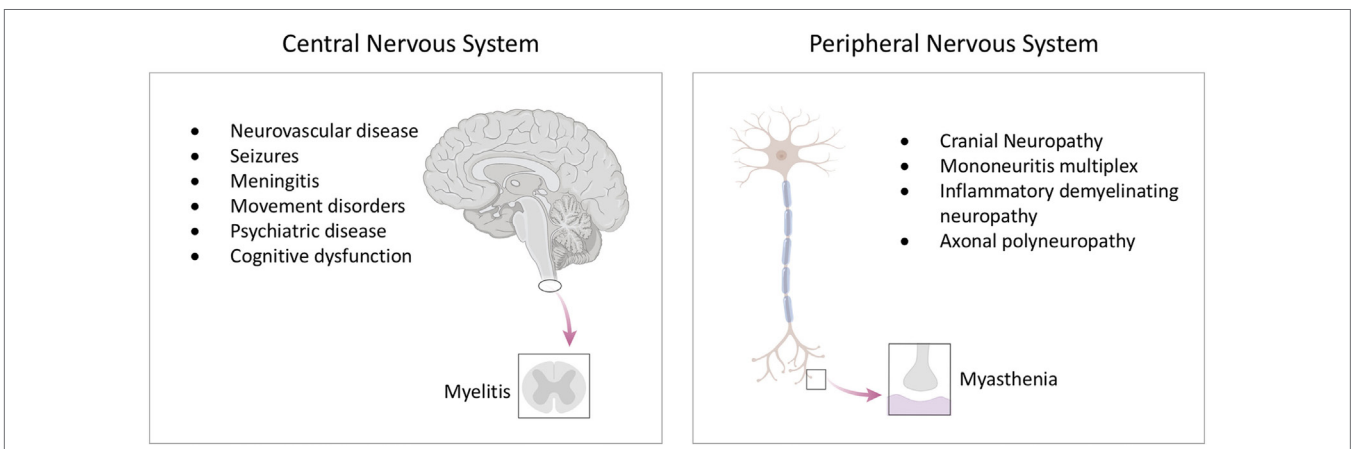

FIGURE 1 | The spectrum of neurological disease in lupus. Lupus can affect all levels of the nervous system, including the brain and spinal cord, as well as the peripheral nervous system. See text for detailed descriptions of individual syndromes.

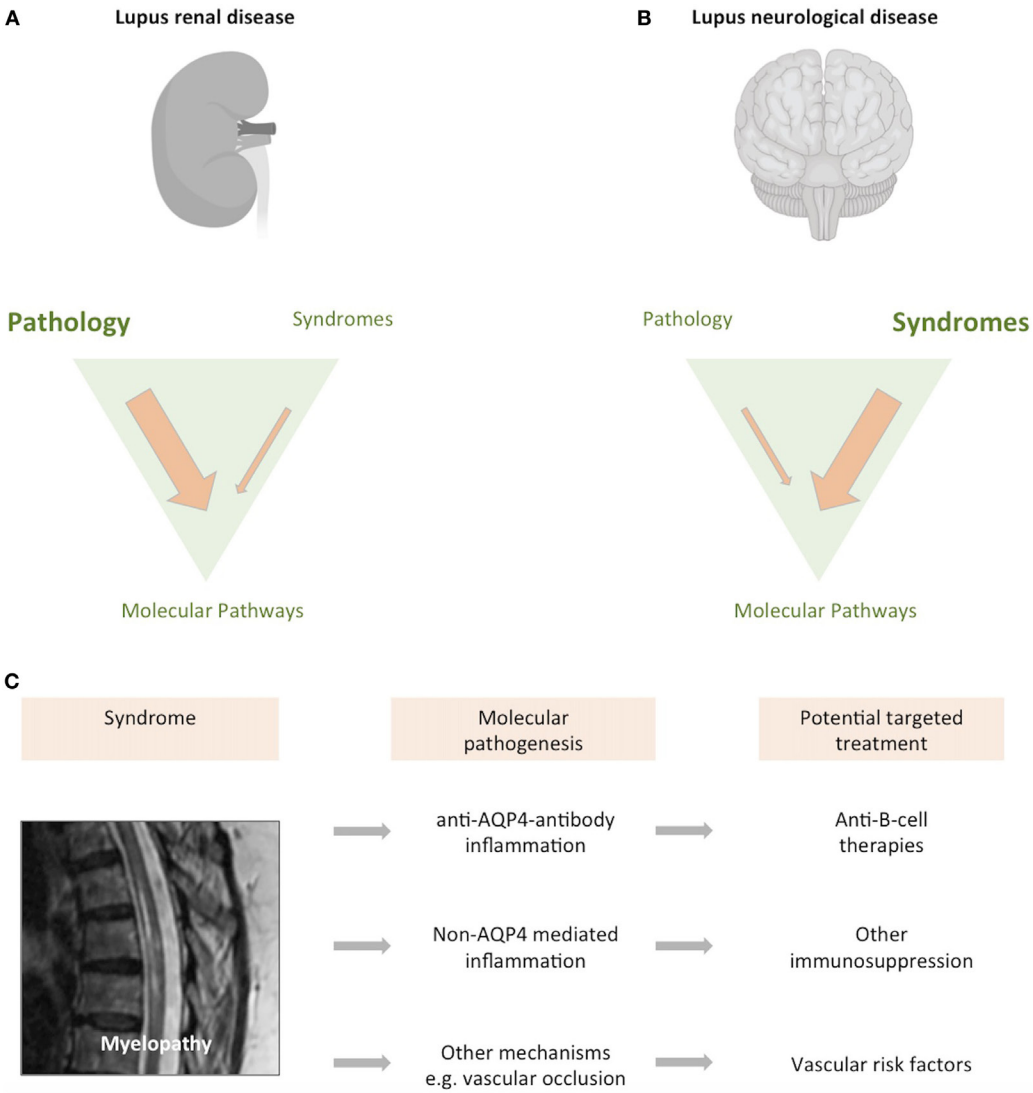

FIGURE 2 | Classifying neurological disease in lupus. Both the brain and kidney can be severely affected in up to 10% of patients with lupus. **(A)** While lupus nephritis can present with different clinical syndromes, it is largely defined by a pathological classification of the renal biopsy. Image created with Biorender. **(B)** In contrast, neurological forms of lupus are usually classified according to neurological syndrome, and pathological material is rarely obtained. **(C)** We have an increasingly precise understanding of the syndrome previously described as "lupus myelopathy," T2-weighted magnetic resonance imaging of long inflammatory lesion in person with lupus shown, with high signal from within the thoracic spinal cord. A proportion of such spinal cord presentations are driven by antibodies directed against aquaporin-4, a glial water channel (21). Other cases are caused by spinal cord inflammation without these antibodies, while some cases are associated with spinal cord ischemia (22, 23). Each of these causes may require consideration of differing therapeutic approaches. As such, what was previously considered a single disease entity can be caused by differing pathogenic mechanisms, with implications for treatment and clinical trial design.

signature, or ultrasensitive detection of the interferon-alpha proteins (32, 33). Detailed longitudinal studies have shown that activation of this pathway influences lupus disease phenotype (33).

The ability of type I interferon proteins to cause brain damage and affect mood is well documented in clinical trials of recombinant type I interferon proteins (34–36). Activation of the type I interferon response in the post-mortem brains of lupus patients has been shown (37), and multiple cell types within the brain, including endothelial cells, microglia, and neurons, respond to type I interferon activation.

Many other cytokines are dysregulated in SLE, with potential neurotoxic effects. For example, IL-6 has been associated with cognitive dysfunction in these patients and causes brain disease in brain-targeted overexpression experiments (38, 39). Type II interferons, interleukins (IL-2, IL-12, IL-18, IL-23), and TNF cytokine families are all dysregulated in lupus and their roles in brain disease are being evaluated (40).

Inflammatory Cells

Although B cells and T cells undoubtedly play an important role in the pathogenesis of SLE, neuropathological analyses in individuals with lupus show little in the way of immune cell infiltration within the brain (41). This contrasts with other neuroinflammatory diseases such as multiple sclerosis (MS) where abundant B

and T cells are found within inflammatory brain lesions (42). There has been an increasing focus on how brain-resident immune cells, such as microglia, might mediate brain disease. Recent elegant studies have shown that microglia are sensitive to elevated circulating cytokines such as type I interferon, and the resulting activation can lead to activation of a number of effector pathways within these cells, including the ability to engulf and "prune" synaptic connections (37, 43). These studies show how dysregulated cytokines can cause structural brain damage by manipulating the normal physiological processes of brain-resident immune cells.

Antibodies

Antibodies are a major mediator of organ damage in SLE, and antibodies directed against multiple brain antigens are frequently produced (44). The extent to which such antibodies cause neurological disease remains to be fully determined. In some cases, for example, antibodies directed against the astrocytic water channel aquaporin-4 (AQP4), there is evidence to support a causal relationship with spinal cord and optic nerve inflammation (21, 45). Antibodies against neuronal cell surface proteins such as the NMDA-receptor (NMDA-R) have also been described in lupus, but a causal association with neurological symptomatology is less clear, despite their ability to mediate brain disease in animal models. Although anti-NMDA-R antibodies can cause a very distinct clinical phenotype of autoimmune encephalitis (46), this syndrome is rarely seen in SLE, and the degree to which lower titers of such antibodies can cause neuropsychiatric dysfunction outside this clinical picture is unclear (47). Interestingly, more classic lupus-associated antibodies directed against nucleic acids, can also cross-react with NMDA-R epitopes and cause neurological dysfunction in rodent models (48). In patients with SLE who have co-existing antiphospholipid syndrome there is a role for antiphospholipid antibodies in the mediation of thrombotic events including intracranial thromboembolism (49). Therefore, a broad spectrum of antibodies is implicated in the pathogenesis of neurolupus, though neurological expertise may be needed in their interpretation.

Pathology and Imaging

Brain biopsies are performed rarely in people with lupus. Consequently, much of our understanding of the pathological basis of neurolupus comes from post-mortem studies, which introduce a bias toward severe disease. The first dedicated studies identified prominent cerebral small vessel disease as a major neuropathological feature in most cases (50). Importantly, this is not a small vessel vasculitis, but rather a noninflammatory microangiopathy associated with microinfarction (50). Pathological changes of small blood vessels include necrosis of the vessel wall, endothelial cell proliferation, and hypertrophy (41, 50, 51). Subsequent studies have confirmed these findings (52, 53). Paired pathology-imaging studies show that these cerebral small vessel lesions seen on brain pathology correspond to "white matter hyperintensities" identifiable on MRI of the brain (54). These MRI abnormalities are seen in the majority of people with lupus, even with mild neurological symptomatology (**Figure 3A**) (18). Sophisticated MRI imaging techniques such as diffusion imaging and quantitative tractography can map the brain's white matter tracts and have identified evidence of microstructural damage in SLE (**Figure 3C**), although robust association between such changes and neurological dysfunction remains unclear (38).

CLINICAL APPROACH IN NEUROLUPUS

The European League against Rheumatism recommendations for management of neurolupus emphasizes the importance of careful evaluation of new neurological events in individuals with SLE (55). It is important to remember that neurological symptoms may not be caused by lupus, and may simply represent highly prevalent neurological disease such as migraine or tension headache. Furthermore neurological symptoms may be caused directly or indirectly by drug therapies (14, 56). As such investigation of these symptoms should involve a detailed history, careful examination and further investigation where indicated, including MRI scan, cerebrospinal fluid analysis, and neurophysiology (56). Multidisciplinary discussion with a neurologist with an interest in neuroinflammatory disease and SLE can help.

Recognized Clinical Syndromes

The recognized clinical neurological syndromes associated with lupus are based loosely on the framework of the ACR criteria.

Stroke

The earliest descriptions of lupus brain disease emphasized a prominent role for neurovascular disease (4). Subsequent studies have shown that stroke occurs more frequently in people with SLE than in the general population, with ischemic stroke developing in up to 20% of lupus patients (57–61). This observation of an increased stroke risk has been confirmed in large prospective registry based studies (59) and meta-analyses (61). Recognized risk factors, such as hypertension, smoking, and hypercholesterolemia may play an important role in this increased risk (60), but do not fully account for the excess of cases, implicating an additional inflammatory etiology (62). As such, addressing the modifiable stroke risk factors of smoking, diet, and blood pressure, is an important priority for lupus patients. Patients with lupus who present with stroke should be carefully evaluated for the antiphospholipid syndrome, given that this may direct a different strategy based on anti-coagulation rather than anti-platelet therapies. Intracranial vasculitis causing stroke—either ischemic or subarachnoid hemorrhage—is rare in SLE, but can sometimes occur and may be identified by abnormal angiographic appearances or biopsy (63–65), highlighting the heterogeneity of underlying mechanisms which drive neurovascular disease in lupus.

Small Vessel Disease (SVD)

Cerebral SVD is a disorder of the brain's perforating arterioles with typical MRI brain imaging features which include white matter hyperintensities (WMH, **Figure 3A**). Such appearances can occasionally cause diagnostic confusion with MS, although improved imaging should aid the distinction. Accelerated

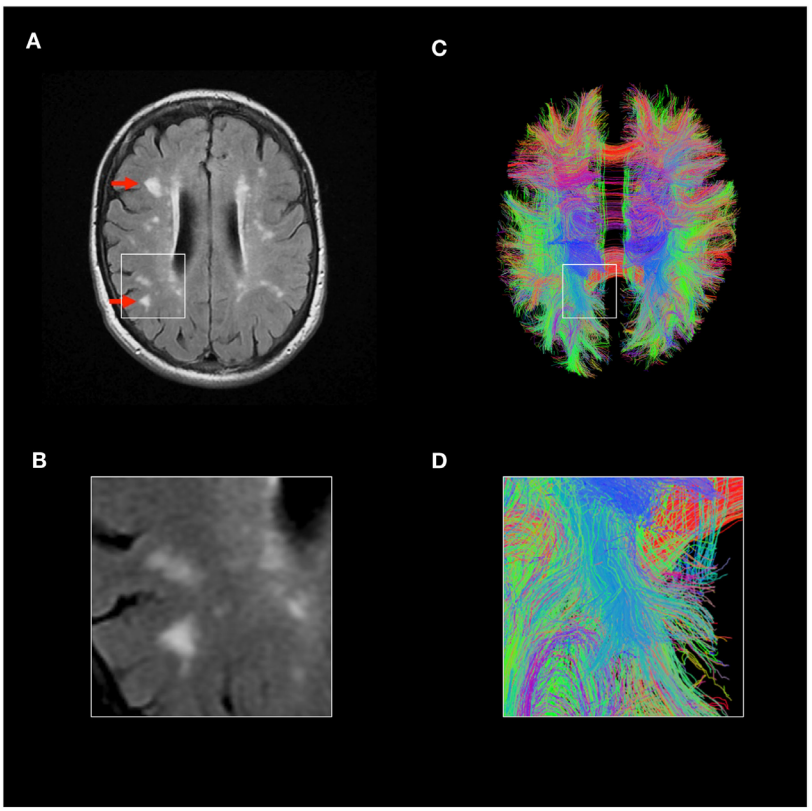

FIGURE 3 | Magnetic resonance imaging (MRI) imaging in lupus brain disease. **(A,B)** Fluid-attenuated inversion recovery MRI scan of a representative individual with lupus, showing accelerated cerebral small vessel disease, highlighted red arrows. **(C,D)** Advanced MRI techniques such as diffusion tensor imaging and tractography can allow identification of individual white matter tracts and parameters such as mean diffusivity can identify microstructural disease. Tractography images of lupus patient shown, each line represents individual white matter tract. Credit: Mark Bastin, Joanna Wardlaw, and Stewart Wiseman.

cerebral SVD is a major cause of dementia in the general population, although the neurological significance of these findings in lupus remains to be determined (18). Quantified MRI brain studies of individuals with lupus show significantly accelerated cerebral SVD, suggesting that this is the most frequently observed radiological–pathological brain abnormality in lupus (41, 54, 66), seen even in patients with mild and inactive disease (18). It is likely that inflammatory mediators such as cytokines play a direct role (67), though the precise factors—and whether they might be more accurately targeted—remain to be determined.

Seizures

Seizures can occur in approximately 5% of individuals with lupus. These are often generalized, though can also be of focal onset (68, 69). It remains unclear as to whether such events represent a form of autoimmune epilepsy, or a lowered seizure threshold. Seizures can also occur in the context of underlying disorders, such as infection, macrophage activation syndrome (MAS) (70), or posterior reversible encephalopathy syndrome (PRES) (71), highlighting the need for appropriate investigation of seizures depending on the clinical context. There is no clear association between seizures and autoantibody formation, including the potentially epileptogenic anti-NMDA-R antibody (68). While recurrence rate of seizures appears to relatively low (69), large-scale epidemiological analyses of large databases confirm higher rates of epilepsy in people with lupus (72). Seizures should be carefully evaluated with a neurologist for underlying cause and use of anticonvulsant agents discussed in those at high risk of seizure recurrence. If anticonvulsant medication is used, particular attention may need to be paid to issues such as drug interactions and teratogenicity.

Myelopathy

Spinal cord disease is an uncommon but serious neurological complication in people with lupus. Over the past decade, the identification of pathogenic antibodies against glial antigens such as the AQP4 water channel has demonstrated that "lupus myelitis" can, in part, be explained by concomitant neuromyelitis optica spectrum disorder (NMOSD) (73). These autoantibodies, together with anti-myelin oligodendrocyte glycoprotein (MOG) antibodies, should be tested in spinal cord presentations, especially in the context of "longitudinally extensive transverse myelitis" where inflammation extends over at least three vertebral segments (74). The presence of AQP4 antibodies is associated with a risk of relapse and immunosuppression is typically used to prevent further events. The B-cell depleting monoclonal antibody

rituximab is increasingly used as a first- or second-line agent (21, 74, 75). Antibodies against AQP4 can be generated in people with lupus without an opticospinal inflammatory event. These antibodies can be associated with other neurological syndromes such as intractable hiccups and vomiting due to lesions in the *area postrema*, highlighting the broadening spectrum of AQP4-associated neurological disease, both with and without lupus (45, 76). Spinal cord disease in SLE is heterogeneous and short transverse myelitis and ischemic transverse myelitis can also occur (22, 77). Our increased understanding of the pathogenesis of spinal cord disease in lupus highlights that a myelopathic presentation can be caused by multiple different etiologies (77), with diverse treatment options (23), requiring careful evaluation (**Figure 2C**).

Meningitis

Meningitis, as described in the ACR case definitions, specifically refers to an autoimmune aseptic meningitis. This can occur in lupus patients in isolation, but can also accompany other events such transverse myelitis (78). It is rare. Given that many individuals with lupus are immunosuppressed, a critical differential diagnosis is one of infectious meningitis caused by typical or opportunistic pathogens. A broad spectrum of pathogens including *Cryptococcus neoformans* and *Listeria monocytogenes* can cause meningitis in lupus patients and microbiological advice should be sought (78). The clinical presentation of opportunistic organisms may vary, for example, fungal meningitis or listeriosis may present with raised intracranial pressure and cranial neuropathies rather than meningism and fever (78). Aseptic meningitis has also been described as a consequence of drugs used to treat lupus, including NSAIDs (79).

Movement Disorders

Chorea, a hyperkinetic movement disorder, has been reported in lupus patients (80), although reversible forms of parkinsonism, a hypokinetic movement disorder, has also been described, particularly in young-onset disease (81, 82). Myoclonus has also been described (83). The etiology of these movement disorders is poorly understood and neuroimaging studies do not usually identify evidence of a localizing lesion (84). Both ischemic and antibody-mediated causes have been postulated, though not convincingly demonstrated.

Demyelinating Syndrome

An association between lupus and MS-like brain changes have been suggested, and sometimes termed "lupoid sclerosis" (85). However, many such studies pre-date high quality MRI brain imaging which has greatly facilitated accurate diagnosis of MS. Much of this confusion stems from the superficial similarities between the presence of small white matter lesions on the MRI brain scans of patients with both MS and lupus. Advances in our understanding of the pathogenesis of MS in the past decades highlight that these lesions are distinct from those observed in lupus (86). Lesions in MS can usually be distinguished from those of lupus with MRI brain imaging. For example, lesions in lupus rarely enhance and correlate at a pathological level with small vessel injury (54), rather than the lymphocytic infiltration and demyelination seen in MS lesions (42, 86). Active MS lesions often display incomplete ring enhancement, and typically occur in a more periventricular distribution. True co-existence of lupus and MS is uncommon (19, 87), and there is no convincing evidence that lupus can cause an MS-like syndrome (87). In patients with both lupus and convincing clinical and paraclinical evidence of MS (88), a more plausible explanation is that, as is sometimes seen autoimmunity, the two diseases co-exist in a single individual (89). This presents a specific management challenge of identifying immunotherapies that might offer efficacy against both diseases.

Headache

Headache is a highly prevalent disorder in people with SLE (90), but there is no convincing evidence that this incidence is higher than that seen in the general population (91). Thus the entity of "lupus headache" is controversial (92). Headache in individuals with lupus should be approached in the same way as in the general population, noting the broader differential diagnosis of any new acute headache to include a higher risk of infectious and neurovascular etiologies (64).

Psychiatric Disease

The term "lupus psychosis" has been used to describe single or repeated episodes of thought disorders such as hallucinations and delusions occurring in people with SLE (93, 94). Like many neuropsychiatric symptoms, the biology of psychosis remains poorly understood, although the possibility of an autoimmune contribution is the subject of intense current research interest (47, 95). Individuals with lupus are exposed to a number of biological substances which can cause psychosis, in particular corticosteroids and circulating antibodies directed against the NMDA-R (47). An association has also been identified between psychosis in lupus and anti-ribosomal-P antibodies (96), which can react against neuronal cell surface antigens (97). However, while antibodies directed against dsDNA, NMDA-R, and ribosomal-P may exhibit some neurotoxic effects in adoptive transfer experiments, their role in mediating psychiatric symptomatology and other brain symptoms in humans is not clear (98). A proportion of psychotic events in lupus are temporally related to corticosteroid use, although such observations are likely to be confounded by increases in systemic disease activity which might precede increased steroid dose (99–101). Differentiation of steroid-induced psychosis from lupus-associated psychosis is particularly challenging (100).

Depression and anxiety are common in the general population and observed more frequently in chronic disease states. It is, therefore, not surprising that about 15% of patients diagnosed with lupus develop mood disorders and 5% an anxiety disorder (12, 102). However, the use of both interviews and validated scales to quantify affective disorders suggest that the prevalence of mood and anxiety disorders may be significantly higher, around 20–40% (103–106). It has been established in clinical trials of therapeutic cytokines that inflammatory factors, such as type I interferon proteins, can induce depressive illness in humans (36, 107). Therefore, the degree to which lupus-related inflammatory factors contribute to the high burden of psychiatric disorders in this condition remains unresolved.

Cognitive Dysfunction

Longitudinal cognitive assessment in people with SLE show that cognition can vary over time (108, 109), though true dementia is not common (110). There is no clear association with lupus activity (111). Screening tools are of use to identify cognitive dysfunction in the clinic and should prompt more detailed neuropsychological testing if abnormal (112). However, cognitive changes can be transient and their substrate poorly defined. While some correlation with MRI abnormalities has been identified, this is not a robust association (113). Associations with elevated cytokines such as IL-6 have also been identified, but again a causal relationship is unclear (38). Evaluation of cognitive symptoms in people with lupus requires careful clinical evaluation, paying attention to additional factors such as depression and medication which can contribute to cognitive dysfunction. Neither corticosteroids (114) nor NMDA-R antagonists (115) have been shown to improve cognitive functioning in SLE, though cognitive rehabilitation approaches have shown some promise (116).

Rare Entities

Posterior reversible encephalopathy syndrome is a clinical–radiological syndrome of headache, seizures, and encephalopathy associated with white matter changes which occur mainly toward the posterior regions of the brain (117). Despite its name, the neurological damage caused by PRES is not necessarily reversible and can occur throughout the brain. A number of cases of PRES in people with SLE have been reported (71), but this syndrome can be confounded by associations with immunosuppressive medications and uncontrolled hypertension, and, therefore, the precise etiological factors are not fully understood (71). PRES-like appearances on neuroimaging can be mimicked by venous sinus thrombosis, which is an important differential diagnosis.

Another rare manifestation of lupus is the macrophage activation syndrome which can occur with prominent neurological involvement including seizures and encephalopathy (70). This is an important differential diagnosis of the acutely unwell lupus patient with multisystem involvement and requires prompt identification and treatment.

Inflammatory Neuromuscular Disease

Neuromuscular disease is an important cause of morbidity in SLE. The ACR neurolupus case definitions consider cranial nerve, peripheral nerve, and neuromuscular junction disease together, stopping at the motor end-plate and excluding muscle disease, which is classified separately. Muscle disease is, therefore, not reviewed in depth here, although it should be noted that a spectrum of inflammatory muscle disease can occur in about 10% of patients with SLE, including myositis and vasculitis, sometimes requiring biopsy confirmation (118–120).

Peripheral Neuropathy

Peripheral neuropathy can occur in approximately 8% of patients with lupus, presenting mainly as a symmetrical polyneuropathy (121, 122). Mononeuritis multiplex can also occur occasionally in lupus and is associated with small vessel vasculitic change on nerve biopsy, often developing during periods of high lupus activity (123, 124). Prospective studies, based on electrophysiological studies rather than symptoms, suggest that the commonest electrophysiological pattern is that of a sensorimotor axonal neuropathy (122). Among lupus-associated neuropathies, the identification of demyelinating inflammatory neuropathies is of particular importance, given the demonstrated response of such neuropathies to intravenous immunoglobulin (125). Identification of inflammatory demyelination on nerve conduction studies should provoke examination of the CSF and a search for paraproteinemic comorbidities (126). Very rarely, Guillain–Barré Syndrome—an acute inflammatory neuropathy—has been observed (127) as has myasthenia gravis.

Cranial Neuropathy

Optic neuropathies, manifesting as either optic neuritis or ischemic optic neuropathy, have been observed in SLE (128–132). Given the association of NMOSD with lupus, evaluation of anti-AQP4/MOG antibodies is important and may potentially guide treatment (74, 133). Cranial neuropathies affecting all cranial nerves have been reported in lupus (134–137), either as single events or as a cranial mononeuritis multiplex (137, 138).

Functional Disorders

Functional symptoms are real but are not caused by underlying neurological disease. Functional neurological disorder is a common cause of neurological symptoms, in both general medicine and neurology clinics, and can, therefore, frequently co-exist with inflammatory diseases such as lupus (139). Incorrectly attributing functional symptoms to an inflammatory cause can lead to an inappropriate escalation in immunotherapy or unnecessary investigation. A specialist neurological opinion can help to identify positive findings of functional neurological disease. The incidence of functional symptomatology in lupus and other inflammatory diseases is unknown and merits further study (139).

Treatment of Neurolupus

While efforts have been made to guide best practice in the diagnosis and management of neurolupus, there is only a weak evidence base on which to develop such recommendations (55). There have been a handful of clinical studies for the treatment of lupus-associated neurological disease, none which provide high quality evidence. A small randomized trial of cyclophosphamide suggested potential benefit, but interpretation of these data are limited by small sample size and methodological issues (140, 141). There have also been observational studies of azathioprine (142) and rituximab (143), but the high degree of variability of clinical symptomatology and a lack of standardized neurological outcome measures makes these results difficult to interpret. Furthermore, meaningful metrics of neurological disease are rarely captured in large lupus clinical trials, and patients with neurological disease are often excluded from such studies (144).

FUTURE DIRECTIONS

Systemic lupus erythematosus is a strong candidate for a "personalized immunotherapy" approach, since individual patients may have different molecular pathways driving their

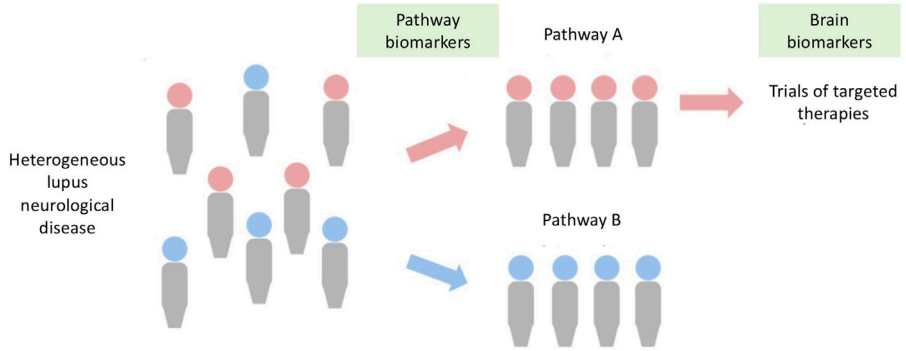

FIGURE 4 | A stratified medicine approach for neurolupus. Brain disease in lupus is clinically heterogeneous (left), but may be driven by certain molecular pathways (e.g., type I interferon pathway, pathogenic autoantibodies), allowing stratification of populations. Improvements in biomarkers will allow identification of aberrant pathways in patients, such that they can be directed to clinical trials targeted at the specific pathway. Central to the success of such a strategy is the development of brain biomarkers (e.g., magnetic resonance imaging scans, markers of brain damage) to supplement clinical assessment.

disease. Longitudinal studies of lupus patients, together with their peripheral transcriptomic responses, support this approach to developing targeted therapies. These analyses show that targetable pathways—or combinations of pathways—can drive different aspects of lupus (33). For example, activation of the type I interferon response is an important determinant of organ-specific disease and is implicated in aspects of brain disease. Similarly, B-cell pathways play an important role in neurological syndromes caused by pathogenic autoantibodies. Thus, with the advent of more accurate biomarkers to identify aberrant immunological pathways, heterogeneous populations could be divided into those who are predicted to respond to targeted therapies, acting as a basis for rational trial design (**Figure 4**) (32, 37, 145). If this approach is to provide a logical framework for developing therapies, then we need to incorporate such a molecular understanding into clinical classification.

At present, the classification system for neurological disease in lupus is largely based on neurological syndromes and does not incorporate a pathophysiological understanding of the disease (**Figure 2**). The need to move from a syndromic toward a mechanistic classification is perhaps best exemplified by spinal cord disease in lupus (**Figure 2C**). The 1999 ACR case definitions refer to a broad syndrome of "lupus myelopathy." However, as we describe above, our understanding of the pathogenesis of spinal cord disease in lupus has advanced, together with the discovery of strong biomarkers and improved imaging. It is clear that "lupus myelopathy" can be caused by at least three different pathophysiological processes. These include antibodies against AQP4, antibody-independent inflammation, and spinal vascular disease. It is likely that each of these different mechanisms may require a different therapeutic approach. Furthermore, some syndromes, such as "lupus headache," may not exist at all. As such the classification system used in neurolupus requires substantial revision, reflecting the transition to a molecular understanding of disease.

A critical step in the future success of neurolupus clinical trials will be improving the quantification of neurological outcomes. There is a particular need to develop validated imaging and laboratory biomarkers of neurological disease in lupus which can supplement complex clinical assessment. MRI brain scans are invariably abnormal in lupus, and change over time. As such, imaging biomarkers may play a role as our ability to quantify macrostructural and microstructural damage (**Figure 3**). Serum and CSF biomarkers of "brain damage," such as ultrasensitive detection of neurofilament protein, have been developed as a surrogate marker for clinical trials in neuroinflammatory and neurodegenerative diseases (146). Thus the rapid progress in our understanding of both pathophysiology and biomarkers of neurolupus is providing a much-needed roadmap to advance the field.

SUMMARY

Neurological disease is an area of major unmet need for people with lupus, providing a complex conceptual and practical challenge. An improved molecular understanding of how lupus can damage the brain and nervous system is providing opportunities to pursue stratified medicine approaches. Advancing the field will require our tools for classifying and measuring neurological disease in lupus to be reevaluated.

AUTHOR CONTRIBUTIONS

DH and SM drafted the original manuscript. Further revisions were made by SW, ND, and JW. SW and JW provided additional images.

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Insights Gained From the Study of Pediatric Systemic Lupus Erythematosus

Mindy S. Lo[1,2]*

[1] Division of Immunology, Boston Children's Hospital, Boston, MA, United States, [2] Department of Pediatrics, Harvard Medical School, Boston, MA, United States

The pathophysiology of systemic lupus erythematosus (SLE) has been intensely studied but remains incompletely defined. Currently, multiple mechanisms are known to contribute to the development of SLE. These include inadequate clearance of apoptotic debris, aberrant presentation of self nucleic antigens, loss of tolerance, and inappropriate activation of T and B cells. Genetic, hormonal, and environmental influences are also known to play a role. The study of lupus in children, in whom there is presumed to be greater genetic influence, has led to new understandings that are applicable to SLE pathophysiology as a whole. In particular, characterization of inherited disorders associated with excessive type I interferon production has elucidated specific mechanisms by which interferon is induced in SLE. In this review, we discuss several monogenic forms of lupus presenting in childhood and also review recent insights gained from cytokine and autoantibody profiling of pediatric SLE.

Keywords: systemic lupus erythematosus, pediatric lupus, monogenic lupus, complement deficiency, DNASE1L3, TREX1, interferonopathy, rasopathy

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Sergio Iván Valdés-Ferrer, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico

***Correspondence:**
Mindy S. Lo
mindy.lo@childrens.harvard.edu

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INTRODUCTION

Systemic lupus erythematosus (SLE) is typically thought of as an autoimmune disease that affects women of childbearing age. However, 10–20% of patients have onset of disease in adolescence or younger. Referred to variably as childhood-onset or pediatric SLE (pSLE), these patients represent a subset with distinct characteristics. Clinically, children with pSLE typically have more severe disease and organ damage. From a pathophysiologic perspective, early onset of disease may also hint at a stronger genetic contribution. Over the years, identification of rare gene variants causing lupus-like phenotypes, so-called monogenic lupus, have in turn offered insights into lupus pathogenesis as a whole.

This review will summarize recent insights into the genetic origins of SLE that have been demonstrated by the study of pSLE patients. Recent work on molecular profiling and biomarker development in pSLE will also be reviewed here.

CLINICAL ASPECTS OF pSLE

There are limited data on precise incidence and prevalence of SLE in children, in part, because age definitions for "childhood-onset" vary. One U.S. study estimated a prevalence of 9.73 per 100,000 children, with an incidence rate of 2.22 cases/100,000/year (1). Non-White children show higher prevalence of disease (1). Non-Caucasian children also have higher rates of renal involvement and younger age of onset (2). African-American and Hispanic children with pSLE have higher rates of

end-stage renal disease and death according to a survey of U.S. hospital admissions data (3). A large Canadian cohort study of pSLE patients followed over time also found that Afro-Caribbean children had higher early disease damage and a higher trajectory of damage accrual (4). These results are largely similar to demographic associations described in adults with SLE (5, 6).

In contrast to the similarities in racial and ethnic patterns, female sex predominance is less significant in children as compared to adults. SLE has been found in multiple studies to disproportionately affect women at a ratio of ~9 to 1, especially among patients of peak child-bearing age (7). This pattern is strong evidence for the importance of a hormonal role in the pathogenesis of SLE. In children, estimated female:male ratios range from 3.6–5.3 to 1 (1, 8, 9). The sex predominance becomes less and less pronounced with younger age of onset, and children with prepubertal development of SLE show essentially no sex bias (10). With the hormonal influence presumably removed, pSLE patients represent a unique opportunity to study the genetic contributions to lupus pathogenesis.

MONOGENIC LUPUS

Complement

The classic example of single gene mutation leading to a lupus-like phenotype (so-called "monogenic lupus") is that of complement deficiency. Hypocomplementemia was recognized as a common laboratory abnormality of SLE relatively early on, thought to be related to consumption and/or tissue deposition. Subsequently, however, the first familial cases of SLE in children due to C1 deficiency were described in the 1970s (11). Lupus-like presentations have now been associated with inherited deficiencies in many classical pathway complement components, including C1q, C1r, C1s, C2, C3, C4A, and C4B (12–15). Characteristically, lupus in these patients develops at an early age and many have severe cutaneous involvement (16). Extrapolating from these observations, it has also been noted that SLE patients as a whole are more likely to have lower copy numbers of *C4A* and *C4B* genes as compared to healthy populations, and this is especially striking in earlier onset disease (17, 18).

In the absence of normal complement regulation, inadequate clearance of apoptotic debris may encourage presentation of self-antigen. Aberrant apoptosis and clearance is now thought to be an important mechanism in lupus pathogenesis. Complement proteins facilitate the appropriate clearance of immune complexes that can lead to tissue damage in SLE and may also regulate the production of inflammatory cytokines by immune cells (16). These hypotheses, and the clinical presentations of complement deficiency, are reviewed in detail elsewhere (16, 19, 20).

Circulating autoantibodies against complement proteins such as C1q and C3b can be found deposited in the kidneys of lupus nephritis patients, provoking inflammation and mediating tissue damage (21, 22). The titer of anti-C1q antibodies correlates with disease activity in children with lupus nephritis (23). However, it is not clear if the depletion of C1q by these autoantibodies also contributes to immunopathogenesis of SLE.

The use of fresh frozen plasma (FFP) to replete complement components may be effective for patients with inherited complement deficiency (24, 25). One recent case series describes three children with C1q deficiency and severe SLE. In all three patients, treatment with FFP allowed rapid recovery and the ability to discontinue steroids (26). Whether repletion of complement is useful for patients without inherited deficiency remains to be seen. In a recent intriguing report, an adolescent girl with SLE and severe hypocomplementemia but no identified genetic deficiency was noted to have effective but transient responses to B cell depletion with rituximab (27). The authors then administered FFP together with ofatumumab to facilitate complement-mediated B cell lysis, resulting in more profound and longer lasting B cell depletion. Her complement levels later recovered as she went into remission (27).

DNase1L3

The importance of normal clearance of cellular debris is demonstrated by another example of Mendelian inheritance in SLE. Linkage analysis of six consanguineous families with apparent autosomal recessive pSLE revealed a loss-of-function mutation in *DNASE1L3* (28). The children described in this study had very young age of onset and high disease activity with variable degree of renal involvement. Serologically, the patients all had hypocomplementemia, while most also had positive anti-dsDNA and antineutrophil cytoplasmic antibodies (28). Subsequently, DNASE1L3 mutations have been described in another family with childhood-onset SLE, as well as a family with three siblings affected by hypocomplementemic urticarial vasculitis (29, 30). This finding of monogenic SLE due to *DNASE1L3* deficiency followed previous observations of decreased DNase1 activity in adult SLE patients without Mendelian inheritance of disease (31). Heterozygous *DNASE1* mutations had also been described previously in SLE but definitive link to pathogenicity was still unclear (32).

DNase1 and DNase1L3 are related endonucleases that degrade extracellular DNA. Mice deficient in either DNase1 or DNase1L3 expression develop features similar to other mouse models of lupus (33, 34). Interestingly, the distinction between the two enzymes appears to be related to an additional C-terminal peptide on DNase1L3 that facilitates its ability to digest microparticle-bound DNA from apoptotic cells (35). Circulating microparticles from apoptotic cells in SLE patients are known to activate plasmacytoid and myeloid dendritic cells, resulting in the production of interferon α (IFN-α) (36). Overproduction of type I IFN is now understood to be a key feature of SLE [reviewed in detail by Eloranta and Ronnblom (37)]. Intracellular DNase1L3 may have other functions yet to be determined; for example, inhibition of DNase1L3 appears to inhibit inflammasome-mediated production of IL-1β (38).

DNaseII

More recently, inherited deficiency of DNaseII has also been associated with an SLE-like phenotype. Rodero and colleagues described three children with loss-of-function mutations in *DNASE2*, resulting in neonatal onset of disease involving severe cytopenias, hepatosplenomegaly, and cholestatic hepatitis (39). All three later developed proteinuria with features of membranous glomerulonephritis; one child also developed deforming arthritis. In contrast to DNase1 and DNase1L3, DNaseII digests intracellular rather than

extracellular DNA. Specifically, DNaseII recruitment to lysozymes is necessary for the cleavage of CpG DNA and the appropriate activation of TLR9 in response to infection (40). At the same time, DNaseII is important for the clearance of DNA from apoptotic cells within macrophage phagosomes; deficiency of this pathway leads to overproduction of IFN-β and TNF-α (41, 42).

TREX1/DNaseIII

Appropriate clearance of cytosolic DNA is also necessary to prevent the development of autoimmunity. TREX1, also known as DNaseIII, is a 3′–5′ exonuclease that digests cytosolic DNA that would otherwise be immunostimulatory, inducing type I IFN production as part of antiviral immunity. The precise nucleic acid antigen that is responsible for triggering autoimmunity in the setting of TREX1 deficiency is not known but has been hypothesized to include endogenous retroelements as well as oxidized or otherwise damaged self DNA and RNA (43–47).

TREX1 has been linked to SLE due to the identification of two related disorders. Familial chilblain lupus (FCL) is an autosomal dominant condition characterized by vasculitic skin lesions and variable presence of autoantibodies (48). Aicardi–Goutieres syndrome (AGS) is another inherited disorder characterized by infantile neurological disease, hypergammaglobulinemia, chilblain lesions, and cerebrospinal fluid (CSF) lymphocytosis. Patients are noted to have high serum and CSF levels of IFN-α. Both FCL and AGS have been associated with defects in *TREX1*, among other genes (49, 50). The overlap between these conditions is further emphasized by the report of two siblings with homozygous *TREX1* mutations, one of whom has only chilblain lesions while the other has cerebral vasculitis reminiscent of AGS (51). Another report of a 4-year-old girl with classic features of SLE and central nervous system vasculitis was found by whole exome sequencing to have a homozygous mutation in *TREX1*, implying that TREX1 might play a broader role in the pathogenesis of non-Mendelian SLE (52). Further, heterozygous mutations in *TREX1* have been described at a higher rate in SLE patients as compared to healthy controls, and one particular *TREX1* haplotype has been associated with neurological manifestations in SLE (53, 54).

Taken together, inadequate clearance of extracellular, endosomal, and cytosolic DNA have all been associated with lupus-like autoimmunity. In these cases, self-DNA is inappropriately stimulates the activation of intracellular nucleic acid sensing pathways, resulting in the excessive production of type I IFN. Mutations in multiple other genes related to processing and sensing of intracellular nucleic acid have also been described to cause AGS and other monogenic autoimmune/autoinflammatory conditions, collectively termed "interferonopathies" (55). Notably, C1q deficiency is also characterized by excessive type I IFN, and clinical manifestations bear resemblance to other interferonopathies (56, 57). As overproduction of type I IFN is also a feature of non-Mendelian SLE, these monogenic disorders give insight into specific mechanisms by which IFN is induced in SLE, and how this influences the development of autoimmunity.

There may also be broader implications beyond lupus. In one cohort of 187 pediatric patients with a variety of autoimmune conditions without molecular genetic diagnosis, 69% had a positive IFN score (IS), as measured by overexpression of type I IFN-induced genes (58). As expected from prior studies, 82% of children with SLE and 75% of children with dermatomyositis had a positive IS. However, positive IS was also seen in conditions not typically characterized by type I IFN overproduction, including 29% of children with systemic juvenile idiopathic arthritis and 38% of children with non-interferonopathy autoinflammatory conditions (58). These findings raise the question of whether there may be subtypes of these conditions for which type I IFN has a pathophysiologic role, and whether these patients might be candidates for therapies that target IFN signaling.

Protein Kinase C delta (PKCδ)

More recently, whole exome sequencing was used to identify mutation in *PRKCD* as the genetic defect underlying a family of siblings with early onset SLE and lupus nephritis (59). PKCδ, the serine/threonine kinase encoded by *PRKCD* is a component of multiple signal transduction cascades in different cell types. In B cells, PKCδ activation is downstream of signaling through both the B cell receptor and the BAFF receptor. PKCδ regulates BAFF-mediated survival and exerts a pro-apoptotic effect, promoting negative selection. Accordingly, deficiency of PKCδ leads to dysregulated B cell proliferation and loss of B cell tolerance (60). The described children with *PRKCD* mutation showed increased numbers of immature and transitional B cells with fewer switched and unswitched memory B cells (59). *In vitro*, B cells from these children demonstrated hyperproliferative response to stimulation and resistance to calcium flux-induced apoptosis (59). Because of these B cell abnormalities, rituximab was given with excellent response to two young siblings with SLE due to homozygous *PRKCD* mutation; these children had previously had disease that was refractory to other more standard treatments (61). Although *PRKCD* polymorphisms have not yet been studied at a population level in SLE, interestingly the heterozygous mother of these two siblings later developed SLE while pregnant with her third child (61). This finding raises the possibility that less severe defects in the PKCδ signaling pathway may have a broader role in the development of SLE in adults.

Ras

There are multiple case reports of pSLE developing in patients with Noonan syndrome, an autosomal dominant disorder characterized by dysmorphic facial features, short stature, and cardiac and chest wall defects (62). Noonan syndrome and several related Noonan-like disorders are caused by mutations affecting genes in the Ras/MAPK signaling pathway. Examples of genes associated with these so-called "RASopathies" include *PTPN11*, *KRAS*, *NRAS*, *SOS1*, *SHOC2*, and *SHP2*, among others (63). The Ras/MAPK pathway is shared by multiple cellular processes, including proliferation, differentiation, and apoptosis. The coexistence of two relatively rare disorders within the same individual has raised questions about the role of Ras/MAPK signaling in SLE, as have two recent descriptions of children with SLE-like disease due to somatic gain-of-function (GOF) mutations in Ras pathway genes (64, 65). In the first case, a 4-year-old boy was diagnosed initially with Rosai-Dorfman disease with lymphadenopathy, hepatosplenomegaly, and pancytopenia. At age 7, he developed features of SLE with pericarditis, arthritis, and autoantibodies,

and was eventually found to have somatic GOF mutation in *KRAS* (65). In the second case, a 3-year-old boy with chilblain lupus, pancytopenia, and autoantibodies was ultimately diagnosed with myelodysplastic syndrome due to somatic GOF mutation in *NRAS* (64). The contribution of Ras/MAPK signaling to SLE pathogenesis is further supported by a report that SHP2 activity is increased in one mouse model of lupus; the disease was ameliorated by treatment with a SHP2 inhibitor (66).

It remains unclear at this point how much continued characterization of monogenic lupus will contribute to our understanding of SLE physiology or treatment as a whole. The French GENetic and Immunologic Abnormalities in SLE (GENIAL/LUMUGENE) study is a longitudinal cohort describing the genetic and laboratory features of children with SLE. Initial findings were recently reported (67). The authors divide the cohort into three groups: (1) syndromic SLE, in which patients show clinical characteristics such as growth failure or intracranial calcifications suggestive of interferonopathies or other congenital disorder; (2) familial SLE, in which patients have either familial consanguinity or a first-degree relative with SLE; (3) all other early-onset SLE. Among the 64 patients described, 10 were considered syndromic, 12 familial, and 42 other. While the syndromic patients had younger age of onset than the other two groups, the authors were unable to find any other distinguishing physical or clinical characteristics, including response to therapy (67). More detailed immune profiling was not done in these patients, and as more targeted therapies become available, identification of specific pathway defects in familial cases may have more bearing on treatment.

MOLECULAR PROFILING

Both pediatric and adult-onset SLE are characterized by clinical heterogeneity, presumably accompanied by pathophysiologic differences. A recent study used whole blood gene expression profiling from samples collected longitudinally to stratify pSLE patients into several groups (68). Expression data were categorized into distinct modules such as IFN response, plasmablast, neutrophil, erythropoiesis, and other gene signatures. The neutrophil, myeloid, and inflammation modules correlated with presence of lupus nephritis. Increased expression of the plasmablast module correlated with increased disease activity (68). Overall, differential expression of these modules was used to stratify pSLE patients into seven groups. As these stratifications did not necessarily correlate with distinct clinical features, the authors argue that molecular profiling, rather than clinical profiling, should be considered in the design of clinical trials for targeted therapies (68).

Immune cell and cytokine profiling using mass cytometry is another approach that has been used recently in pSLE. In a group of 10 clinically heterogeneous pSLE patients naïve to therapy, O'Gorman and colleagues found a shared signature of activated $CD14^{hi}$ monocytes, characterized by increased monocyte chemoattractant protein (MCP-1), MIP1β, and IL-1RA production (69). Strikingly, the activated $CD14^{hi}$ monocyte signature was seen in all 10 pSLE patients but none of the healthy controls, emphasizing the role of these cells as a common pathogenic factor in clinically variable SLE. The MCP-1/MIP1β/IL-1RA signature correlates strongly with disease activity and is at least partially dependent on type I IFN, although the authors did not find a type I IFN signature in all of the studied patients (69). Prior studies have suggested that IP-10, an IFN-γ-induced cytokine, may be a useful marker for disease activity. In a Chinese cohort of 46 pSLE patients, cytokine profiling revealed that IP-10 level performed better than anti-dsDNA, C3, or C4 in predicting active disease (70).

Autoantibody profiling has also been pursued in hopes of developing better biomarkers of disease activity. One study used an autoantigen array of over 140 antigens to study a cohort of new-onset pediatric SLE patients (71). The authors identified anti-BAFF antibodies in the majority of these patients and found that titer of these antibodies associated with disease activity level. The authors also identified autoantibodies associated with proliferative lupus nephritis. These include known antibodies such as anti-dsDNA and anti-C1q antibodies, but also antibodies against alpha-actinin, fibrinogen, collagens IV and X, aggrecan, and multiple histone proteins (71). While the anti-dsDNA and anti-C1q antibodies are known to correlate not just with nephritis but with flares of renal disease, the pathogenicity of these other antibodies is as yet undetermined.

CONCLUSION

Pediatric SLE, while phenotypically often similar to adult-onset disease, may also present with more unusual or more severe features. In some cases, such as the neurologic disease associated with *TREX1* deficiency, it has been these differences that have highlighted the presence of an underlying pathogenic mechanism. The study of monogenic disease in children has opened new areas of investigation applicable to SLE as a whole, and it is very likely that more examples of this will be found in future. Molecular and immune profiling of pSLE patients has also generated insights into biomarker development and targets for therapy.

AUTHOR CONTRIBUTIONS

ML drafted the manuscript in its entirety.

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Antiphospholipid Syndrome Nephropathy: From Pathogenesis to Treatment

*Maria G. Tektonidou**

Joint Rheumatology Academic Program, First Department of Propaedeutic Internal Medicine, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

Kidney damage is a well-recognized complication of the antiphospholipid syndrome (APS), either primary or systemic lupus erythematosus (SLE)-associated APS. Kidney involvement in APS involves a variety of manifestations, such as renal artery thrombosis or stenosis, renal vein thrombosis, allograft loss due to thrombosis after kidney transplantation, and injury to the renal microvasculature, also known as APS nephropathy. Biopsy in patients with APS nephropathy includes acute thrombotic microangiopathy lesions and chronic intrarenal vascular lesions such as interlobular fibrous intimal hyperplasia, arterial and arteriolar recanalizing thrombosis, fibrous arterial occlusion, and focal cortical atrophy. The most frequent clinical features are hypertension, microscopic hematuria, proteinuria (ranging from mild to nephritic levels), and renal insufficiency. It is uncertain whether antiphospholipid antibodies or other factors are implicated in the development of APS nephropathy, and whether it is driven mainly by thrombotic or by inflammatory processes. Experimental models and clinical studies of thrombotic microangiopathy lesions implicate activation of the complement cascade, tissue factor, and the mTORC pathway. Currently, the management of APS nephropathy relies on expert opinion, and consensus is lacking. There is limited evidence about the effect of anticoagulants, and their use remains controversial. Treatment approaches in patients with APS nephropathy lesions may include the use of heparin based on its role on complement activation pathway inhibition or the use of intravenous immunoglobulin and/or plasma exchange. Targeted therapies may also be considered based on potential APS nephropathy pathogenetic mechanisms such as B-cell directed therapies, complement inhibition, tissue factor inhibition, mTOR pathway inhibition, or anti-interferon antibodies, but prospective multicenter studies are needed to address their role.

Keywords: antiphospholipid antibodies, antiphospholipid syndrome, nephropathy, pathogenesis, treatment

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Pier Luigi Meroni, Istituto Auxologico Italiano (IRCCS), Italy

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Yong-Gil Kim, University of Ulsan College of Medicine, South Korea

***Correspondence:**
Maria G. Tektonidou
mtektonidou@gmail.com

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INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by thrombotic episodes in the arterial or venous circulation, in the presence of antiphospholipid antibodies (aPL), namely lupus anticoagulant (LA), anticardiolipin antibodies, and anti-β2glycoprotein-I antibodies (anti-β2GPI) (1). APS can be either primary or secondary when it occurs in the context of other underlying autoimmune disorder, mainly systemic lupus erythematosus (SLE). aPL positivity may occur in 0–5% of healthy individuals and in approximately 30–40% of patients with SLE; one third of SLE patients with positive aPL develop thrombosis during their follow-up.

Antiphospholipid syndrome can affect any part of kidney vasculature such as renal arteries and veins, intrarenal arteries and arterioles, and glomerular capillaries (2). In addition to thrombotic manifestations from the large renal vessels that are part of the updated Sapporo criteria for APS, characteristic microvascular nephropathy lesions are included in the non-criteria manifestations of APS (1, 2).

LUPUS NEPHRITIS AND aPL

A number of studies have shown that aPL positivity is a poor prognostic factor in lupus nephritis (3, 4). In a study of 111 SLE patients with nephritis and a mean follow-up of 14 years, the presence of positive aPL ($p = 0.02$) was identified as independent predictor of chronic renal function deterioration (5). Natejumnong et al. showed that patients with SLE nephritis and LA positivity had higher systolic blood pressure (133.7 versus 121.9 mmHg, $p = 0.005$), serum creatinine (233.0 versus 94.9 μmol/L), and 24-h urine protein excretion (2.6 versus 1.4 g, $p = 0.02$), features associated with worse renal prognosis (6). However, in other lupus nephritis studies, aPL did not correlate with long-term kidney function (7, 8) or even showed a protective effect against renal damage (9).

In addition, several studies demonstrated an association between aPL and a variety of intrarenal vascular lesions in kidney biopsies of patients with lupus nephritis (10). Thrombotic microangiopathy in glomeruli and/or renal arterioles was the most common lesion, characterized by fibrin thrombi without inflammatory cells or immune complexes (11, 12). Several studies from the early 1990s have examined the impact of aPL-associated intrarenal vascular lesions on long-term outcomes of SLE patients (10–14). In 2013, Song et al. reported that thrombotic microangiopathy in patients with lupus nephritis was an independent predictor of poor renal outcome (HR: 2.772, 95% CI 1.009–7.617, $p = 0.048$) (15). Wu et al. showed that thrombotic microangiopathy lesions in lupus nephritis was associated with worse renal prognosis compared to other vascular lesions and suggested that vasculopathy be included in ISN/RPS classification system in order to increase its predictive value for renal outcomes (16).

KIDNEY TRANSPLANTATION AND aPL

There is growing evidence that patients with positive aPL and/or APS requiring renal transplantation have increased risk of early graft loss due to post-transplant thrombosis of graft vessels or thrombotic microangiopathy (17, 18). In a large single-center cohort of 1,359 kidney transplantations, the prevalence of aPL was 3%, and LA-positive patients had high rates of allograft aPL-associated vascular lesions and poor transplantation outcomes during the first year after transplantation (18). However, other studies could not confirm an association between aPL and allograft survival after kidney transplantation (19). A very recent study of 446 kidney transplant recipients showed that the risk of GFR decline within the first year post-transplant was elevated in patients with positive aPL, even without thrombotic events prior to transplantation (20).

Perioperative anticoagulation therapy with low molecular weight or unfractionated heparin has been shown to protect from graft failure in patients with positive aPL or APS, However, allograft loss due to thrombosis can develop despite this treatment (17). Anticoagulation treatment can also increase the risk of bleeding complications. Treatment with eculizumab, which blocks the complement cascade at the C5 level, improved post-transplant renal outcomes in case reports of aPL-positive patients with recurrent thrombotic microangiopathy after kidney transplantation (21, 22).

RENAL ARTERY THROMBOSIS OR STENOSIS

Although rare, renal artery thrombosis is a well-recognized clinical manifestation of renal involvement in APS (2). The pathophysiology of renal artery thrombosis implicates either an *in situ* thrombosis or an embolic event in renal artery vasculature. Patients typically present with sudden-onset or uncontrolled systemic hypertension, or the diffuse abdominal or flank pain in the cases of renal infarct (23). The above manifestations in a patient with the diagnosis of APS should raise the suspicion for renal artery thrombosis, and APS should be considered in all cases of well-documented renal artery thrombosis of unknown origin. Renal angiography has the highest diagnostic accuracy, while both contrast-enhanced CT or MRI angiography are less invasive methods with a similar diagnostic performance (24).

Renal artery stenosis without evidence of thrombosis has also been described in the context of APS, representing a significant cause of hypertension in this group of patients. Sangle et al. examined 77 patients with aPL and uncontrolled hypertension, 91 patients attending hypertension clinics, and 92 normotensive healthy controls; renal artery stenosis was diagnosed by magnetic resonance renal angiography in 26, 8, and 3% of each group, respectively (25). A more recent study using ultasonography, for the diagnosis of renal stenosis, showed elevated intrarenal vascular resistance in 14% of APS patients versus none of the aPL carriers ($p = 0.00007$) (26).

Both thrombosis and atherosclerosis have been suggested as major underlying mechanisms for the stenotic lesions (27). In a retrospective study of 23 APS patients, high-intensity anticoagulation (INR $\geq$ 3) seemed to decrease the rates of renal artery re-stenosis and had a favorable impact on blood pressure control and renal function during their follow-up (28).

RENAL VEIN THROMBOSIS

Either unilateral or bilateral renal vein thrombosis can occur in patients with APS, resulting in acute kidney injury or chronic kidney disease (29, 30). Patients usually present with nephrotic syndrome or less frequently with flank pain and macroscopic hematuria in the acute onset of thrombosis. This diagnosis should be suspected in patients with APS who suddenly develop neprhotic-range proteinuria. Doppler ultrasonography is the examination of choice and may reveal edematous kidney with decreased echogenicity, disruption of parenchymal architecture, and/or thrombus in renal veins.

APS NEPHROPATHY

Since the early 1990s, thrombotic microangiopathy has been detected in renal biopsies of patients with primary APS (10–13). In

addition to thrombotic microangiopathy, Amigo et al. described a number of chronic renal vascular lesions as a part of kidney involvement in APS and in the absence of overt lupus nephritis (31).

Antiphospholipid syndrome nephropathy, a renal small-vessel vasculopathy characterized by acute thrombosis and/or chronic arterial and arteriolar lesions, was first defined as a distinct histological and clinical entity in 1999. After examining 16 renal biopsies of primary APS patients, Nochy et al. suggested that at least one of the following lesions should be detected for the diagnosis of APS nephropathy: thrombotic microangiopathy (acute lesion), interlobular fibrous intimal hyperplasia, arterial and arteriolar recanalizing thrombi, fibrous arterial occlusion, and focal cortical atrophy (32) (**Figure 1**). The same French group later observed the same histological lesions in patients with SLE-associated APS, over and above lupus nephritis lesions (33). APS nephropathy has been associated with LA, arterial thrombosis, and fetal loss. It was also associated with an higher risk of hypertension, elevated serum creatinine levels, and kidney interstitial fibrosis, all recognized as predictors of worse renal outcomes.

Tektonidou et al. showed that in lupus nephritis biopsy samples, APS nephropathy lesions were much more prevalent in aPL-positive patients (39.5% versus only 4.3% of those with negative aPL) (34). Furthermore, APS nephropathy was found in two-thirds of those meeting APS criteria among those aPL-positive patients with SLE. A strong association with APS nephropathy was also noted in patients with arterial thrombosis and livedo reticularis. APS nephropathy was characterized by a higher frequency of hypertension and elevated creatinine levels on biopsy, but did not predict the risk of decline in kidney function, end-stage renal disease or death at the end of follow-up. The rate of APS-related clinical manifestations, such as arterial thrombosis, was higher in SLE patients with versus without APS nephropathy during a long-term follow-up.

Some years later, Tektonidou et al. examined three different APS groups for acute and chronic APS nephropathy lesions: primary APS, SLE-associated APS, and for the first time, catastrophic APS (35). Thrombotic microangiopathy, the acute lesion, was prominent in catastrophic APS while the prevalence of chronic lesions was similar among all APS groups. In all three APS groups, hypertension, proteinuria (mild to nephrotic syndrome), microscopic hematuria, and renal insufficiency (usually mild) were the main clinical features of APS nephropathy.

Further studies confirmed the above findings. However, the impact of APS nephropathy on long-term renal outcomes varied among different studies. In a single cohort from Thailand, APS nephropathy lesions were present in 34% of 150 patients with biopsy-proven lupus nephritis. APS nephropathy was correlated with indices of disease activity and chronicityhypertension, renal failure, severe proteinuria, class III and IV histology, and end-stage renal disease (36). In another study, APS nephropathy was present in 10% of kidney biopsy specimens from 162 Mexican patients with lupus nephritis and was associated with anticardiolipin antibodies and elevated rates of rapidly progressive

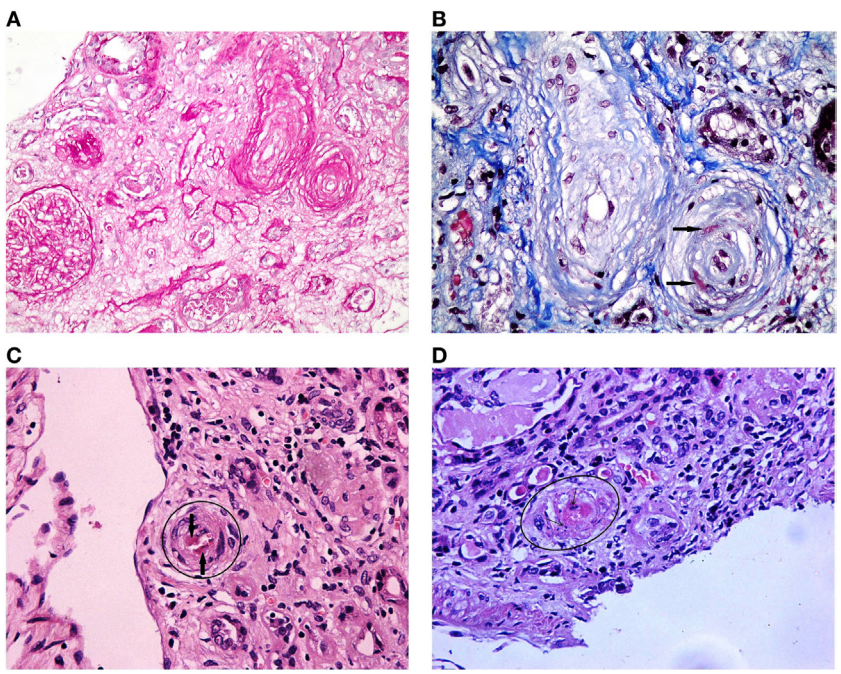

FIGURE 1 | Antiphospholipid syndrome nephropathy histologic lesions. **(A)** Luminal narrowing due to circumferential myointimal thickening of the wall of one arteriole and one interlobular artery. Glomerulus exhibiting ischemic features with wrinkling of the glomerular capillary basement membranes (PAS 200×). **(B)** An interlobular artery and an arteriole showing luminal narrowing due to pale mucoid intimal thickening and myointimal cellular proliferation. Additionally, the arteriole reveals fibrin insudation within the wall (black arrows) (Masson trichrome 400×). **(C)** Arteriole showing luminal thrombus (HE 400×). **(D)** Arteriole showing TMA with platelet-fibrin thrombus occluding the lumen and nuclear debris in the arterial wall (HE 400×).

glomerulonephritis, nephrotic syndrome, and death during follow-up (37). In a Spanish cohort of 77 SLE patients with biopsy-proven renal involvement, a strong correlation was found between APS nephropathy and aPL ($p = 0.003$), especially the combination of LA and IgG anticardiolipin antibodies (OR: 3.61, $p = 0.002$). The levels of serum creatinine were higher in APS nephropathy patients ($p = 0.038$), however, no significant difference in complete or partial remission, not response, and chronic renal damage was observed between the two groups (38).

In 2011, all published studies examining the association between aPL and APS nephropathy were identified and graded in the context of a "Task Force on Non-criteria APS Manifestations" (39). The task force group reported a higher frequency of APS nephropathy in patients with positive aPL ($p < 0.001$) compared to those without aPL (Evidence Level II) and in primary APS compared to SLE-APS, and to SLE with positive aPL but without APS. The specificity, positive predictive value, and negative predictive value of APS nephropathy for the detection of APS were 96, 85 and 87, respectively. Some years later, another task force group evaluated the relevance of non-criteria clinical manifestations of APS according to the GRADE system to support their inclusion in the APS classification criteria (40). The overall quality of evidence was "very low" or "low" for most of non-criteria manifestations, but "moderate" for APS nephropathy.

Renal pathologists should be aware of this histological entity, and clinicians should include APS in the differential diagnosis of small-vessel nephropathy. Because thrombotic microangiopathy is a non-specific lesion, other conditions associated with its presence should be ruled out such as thrombotic thrombocytopenic purpura, atypical hemolytic uremic syndrome, HELLP syndrome, malignant hypertension, systemic sclerosis, preeclampsia or eclampsia, and medications (cyclosporine, chemotherapy).

GLOMERULAR LESIONS IN PRIMARY APS

Fakhuri et al. found APS nephropathy lesions in 20 of 29 biopsies of primary APS patients, and they reported a variety of glomerular lesions such as membranous nephropathy, minimal change disease/focal segmental glomerulosclerosis, mesangial C3 nephropathy, and pauci-immune crescentic glomerulonephritis (41). Additional case reports described histologic lesions of proliferative glomerulonephritis in primary APS patients with biopsy-proven renal involvement, with no evidence of thrombotic microangiopathy or any other APS-related renal vascular lesions (42, 43).

An Italian multicenter cohort study of 160 primary APS patients examined the kidney biopsy findings of 10 patients with evidence of renal involvement. Four patients had findings consistent with APS nephropathy, four had membranous, and two had proliferative glomerulonephritis. Patients with renal involvement were older ($p = 0.0269$), had positive LA test ($p = 0.0068$), and low complement levels ($p < 0.05$) (44).

PATHOPHYSIOLOGY OF APS NEPHROPATHY

The significant association between the presence of APS nephropathy and aPL suggests a pathogenetic role of aPL in the development of this nephropathy. However, it is unknown if additional disease-related factors contribute to the pathogenesis of APS nephropathy. It is also unclear whether this is a purely thrombotic or inflammatory process. Complement cascade activation has been recognized as an important mechanism for aPL-mediated thrombosis in murine models (45, 46). Experimental studies also showed that absence of complement regulatory proteins on glomerular cells is associated with thrombotic microangiopathy (47). In clinical studies, C4d is a common finding in thrombotic microngiopathy (48). C4d staining and microthrombi were found to co-exist in biopsy samples of patients with SLE and positive aPL (49, 50).

In addition, complement-mediated tissue factor seems to be involved in the pathogenesis of thrombotic microangiopathy in APS (51). Seshan et al. showed that mouse aPL and human aPL of IgG isotype can induce glomerular histologic lesions characteristic of thrombotic microangiopathy in mice. They also found an increased deposition of fibrin, tissue factor, and C3 in glomeruli of mice treated with mouse and human aPL supporting their role in thrombotic microngiopathy pathogenesis (52).

Antiphospholipid syndrome nephropathy is also characterized by a number of chronic lesions with fibrous intimal hyperplasia being the most common. A recent study in patients with APS showed activation of the mTORC pathway in the renovascular endothelium, leading to intimal hyperplasia. Vascular activation of mTORC was also demonstrated in autopsy specimens of a catastrophic APS case series (53).

TREATMENT OF APS NEPHROPATHY

Currently, a consensus on the treatment of APS nephropathy is lacking. Patients with APS nephropathy histologic lesions who fulfill the clinical and laboratory criteria for APS (1) should receive the standard anticoagulant treatment for APS. However, whether anticoagulation or other treatment is indicated in patients with APS nephropathy lesions in the absence of definite APS criteria is not well established. In patients with co-existent lupus nephritis, the use of hydroxychloroquine and immunosuppressive treatment is recommended (54). Since hypertension and proteinuria are predominant manifestations of APS nephropathy, the standard of care includes inhibitors of the angiotensin system (55).

The role of anticoagulation in renal prognosis has not been well examined due to the limited number of patients on anticoagulation in the previous studies of APS nephropathy. Prospective studies are lacking. A successful use of anticoagulants was reported in some case reports or case series but with no or short follow-up data (56). The use of novel oral anticoagulant medications has not been examined in patients with APS nephropathy. Other treatment approaches may include the use of heparin based on its effect on the classical complement activation pathway inhibition or the use of intravenous immunoglobulin and/or plasma exchange given their efficacy on severe/refractory cases and catastrophic APS (56, 57). Targeted therapies may also be considered such as B-cell directed therapies, complement inhibition, tissue factor inhibition, or mTOR pathway inhibition, but large prospective multicenter studies are needed to address their role.

Experimental APS murine models have shown that the use of BAFF blocking agents delays the development of disease and

prolongs survival (58). In humans, case reports showed a successful use of anti-CD20 treatment in patients with APS nephropathy and/or other non-criteria manifestations for APS (severe thrombocytopenia, hemolytic anemia, and skin ulcers) (59). In a 12-month, unblinded study evaluating the potential usefulness of rituximab for non-criteria manifestations of APS, two patients with APS nephropathy had partial responses with two doses of 1,000 mg rituximab on days 1 and 15 (60). Belimumab, a BAFF antagonist, has been used in two cases with primary APS, one with recurrent alveolar hemorrhage and one with recurrent skin ulcers. Both patients had clinical improvement and were able to discontinue corticosteroids (61).

Regarding T-cell-directed therapies, an experimental study of CTLA4-Ig in the NZW/BXSB mice that develop APS with small coronary thrombosis, showed promise in preventing the development of myocardial infarcts (62). There is no available evidence about the use of co-stimulation blockade Abatacept (CTLA4-Ig), in patients with APS.

Regarding complement inhibition in APS, animal studies demonstrated the ability of C5 inhibitors to prevent blood clots in animals receiving intravascular infusion of antibodies to β2GPI (50). Seshan et al. showed that genetic deletion of C5aR prevents thrombotic microangiopathy and renal failure in aPL (FB1)-treated mice (52). Eculizumab, a recombinant humanized monoclonal antibody that binds to C5 inhibiting its cleavage to C5a and C5b, has been successfully used in "thrombotic microangiopathy" group of disorders (characterized by thrombocytopenia and microangiopathic hemolytic anemia), including paroxysmal nocturnal hemoglobinuria, hemolytic uremic syndrome, and catastrophic APS. Case reports have described its effect in refractory catastrophic APS cases (63), post kidney transplant thrombotic microangiopathy (22), and patients with lupus nephritis and thrombotic microangiopathy (64).

The role of statin use in APS has also been extensively discussed. Experimental models showed that low tissue factor expression prevents glomerular injury in mouse aPL- and human aPL-treated mice. Pravastatin, which down-regulates tissue factor, prevents glomerular injury in both mouse aPL- and human aPL-treated mice (52).

Inhibition of mTOR pathway in kidney transplant recipients with APS nephropathy decreased vascular proliferation on renal biopsy and prevented vascular lesion recurrence. Canaud et al. showed that among the 10 patients who received mTOR pathway inhibition treatment, 7 patients (70%) had a functioning allograft 10 years after transplantation versus 3 (11%) of 27 untreated patients, and the effect of treatment was independent of anticoagulation treatment (53).

Two recent studies showed a type I interferon signature in primary APS (65, 66). The first study showed an impaired ability of endothelial progenitors from patients with APS to differentiate into endothelial cells, reversed by a type I IFN receptor-neutralizing antibody (65). The above findings could support a novel therapeutic approach, that of anti-interferon antibodies, to reverse vascular damage in APS.

CONCLUSION

Manifestations associated with renal vasculature involvement in the presence of persistently positive aPL and/or APS include renal artery or vein thrombosis, thrombotic microangiopathy lesions in lupus nephritis biopsies, allograft thrombosis after kidney transplantation, and a small-vessel nephropathy characterized as APS nephropathy with variable outcomes. Better understanding of the pathogenetic mechanisms of APS nephropathy may lead to more precise and targeted treatments.

AUTHOR CONTRIBUTIONS

MT drafted the manuscript.

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REVIEW
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The Absent in Melanoma 2-Like Receptor IFN-Inducible Protein 16 as an Inflammasome Regulator in Systemic Lupus Erythematosus: The Dark Side of Sensing Microbes

*Valeria Caneparo[1,2,3], Santo Landolfo[1], Marisa Gariglio[2,3] and Marco De Andrea[1,2,3]**

[1] Viral Pathogenesis Unit, Department of Public Health and Pediatric Sciences, Turin Medical School, Turin, Italy, [2] Virology Unit, Interdisciplinary Research Center of Autoimmune Diseases (IRCAD), Department of Translational Medicine, Novara Medical School, Novara, Italy, [3] Intrinsic Immunity Unit, CAAD – Center for Translational Research on Autoimmune and Allergic Disease, University of Piemonte Orientale, Novara, Italy

Absent in melanoma 2 (AIM2)-like receptors (ALRs) are a newly characterized class of pathogen recognition receptors (PRRs) involved in cytosolic and nuclear pathogen DNA recognition. In recent years, two ALR family members, the interferon (IFN)-inducible protein 16 (IFI16) and AIM2, have been linked to the pathogenesis of various autoimmune diseases, among which systemic lupus erythematosus (SLE) has recently gained increasing attention. SLE patients are indeed often characterized by constitutively high serum IFN levels and increased expression of IFN-stimulated genes due to an abnormal response to pathogens and/or incorrect self-DNA recognition process. Consistently, we and others have shown that IFI16 is overexpressed in a wide range of autoimmune diseases where it triggers production of specific autoantibodies. In addition, evidence from mouse models supports a model whereby ALRs are required for IFN-mediated host response to both exogenous and endogenous DNA. Following interaction with cytoplasmic or nuclear nucleic acids, ALRs can form a functional inflammasome through association with the adaptor ASC [apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)] and with procaspase-1. Importantly, inflammasome-mediated upregulation of IL-1β and IL-18 production positively correlates with SLE disease severity. Therefore, targeting ALR sensors and their downstream pathways represents a promising alternative therapeutic approach for SLE and other systemic autoimmune diseases.

Keywords: IFN-inducible protein 16, absent in melanoma 2 (AIM2)-like receptor, inflammasome, interferon, systemic lupus erythematosus

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***Correspondence:**
Marco De Andrea
marco.deandrea@unito.it

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INTRODUCTION

Inflammation is an innate immune response largely mediated by phagocytic cells whose goal is to protect the body from invading bacteria and viruses (1, 2). Pattern recognition receptors (PRRs) constitute a large family of molecules expressed on the cell surface and in the cytoplasm of various cell types, such as macrophages and antigen presenting cells (APC), able to interact with evolutionarily conserved pathogenic structures [i.e., pathogen-associated molecular patterns (PAMPs)],

thus giving rise to multimeric protein complexes termed inflammasomes, which are then responsible for mediating a caspase-1-dependent inflammatory response (3–6). These so-called "canonical inflammasomes," which can be triggered by a wide variety of ligands, consist of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs) (7–10). More recently, "non-canonical inflammasomes," containing caspase-11 in mice and caspase-4/5 in humans, have also been described (11, 12).

Chronic inflammatory responses, which could last for weeks or even years, are characterized by episodes of tissue injury and healing resulting in severe tissue damage, which can eventually lead to the development of autoinflammatory/autoimmune diseases such as systemic lupus erythematosus (SLE) (13–15). This latter is an autoimmune disease characterized by a wide range of clinical and serological manifestations accompanied by a polyclonal autoimmune response against various nuclear autoantigens (16). Although genetic and environmental factors such as infections are known to be involved in the pathogenesis of SLE, the clear etiology of this disease still remains to be fully established (17).

Despite this gap in knowledge, it is now clear that ALRs, especially the IFN-inducible protein 16 (IFI16, **Figure 1**), along with other inflammasome-induced inflammatory responses, contribute to the development of SLE. In this review, we will summarize recent advances on the role of the inflammasome and ALRs in SLE, which could ultimately provide the rationale for the design and development of novel therapeutic tools for the treatment of patients affected by SLE or other systemic autoimmune diseases.

THE INFLAMMASOME

The canonical inflammasome is a multimodular complex that, upon induced oligomerization, stimulates the activation of caspase-1, an enzyme primarily responsible for the release of the pro-inflammatory cytokines IL-1β and IL-18 (6). Strongly associated with the activation of the inflammasome, pyroptosis is a caspase-1-dependent type of inflammatory cell death mainly seen during intracellular infections (18). Inflammasomes specific for intracellular PAMPs involve different classes of cytoplasmic PRRs. Classically, the NLR, such as NLRP3, and the retinoic acid inducible gene I (RIG-I)-like receptor (RLR) families (**Figure 2**).

NLRP3 holds a C-terminal leucine-rich repeat domain, a central nucleotide-binding and oligomerization domain (NOD or NACHT), and an N-terminal pyrin domain (PYD). The NLR-associated PYD interacts with the PYD of the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (ASC). ASC is then able to engage caspase-1 through its CARD domain causing the oligomerization of several caspase-1 molecules that, in turn, cleave and activate each other (8). RIG-I is made of two N-terminal CARDs, a central RNA helicase domain and a C-terminal regulatory domain (CTD). As for ASC, the RIG-I CARD is a sticky domain responsible for recruiting adaptor proteins and triggering downstream pathways (19). Whereas the RNA helicase domain contains a conserved Asp–Glu–Ala-Asp motif, also known as DEAD box, and exerts ATPase activity, the CTD is responsible for binding dsRNA PAMPs (20). Following dsRNA binding and associated conformational changes, RIG-I interacts with mitochondrial outer membrane proteins called mitochondrial antiviral signaling (MAVS) through CARD–CARD interactions (21). Depending on the adaptors involved, RIG-I–MAVS interaction then results in either type I IFN (IFN-I) or pro-inflammatory cytokines production (22).

Interestingly, recent studies have shown that there also exist non-canonical inflammasomes, which, through recruitment of caspase-4/5 in human or caspase-11 in mouse, can induce caspase-1-dependent maturation and secretion of IL-1β and IL-18 (23–25). In particular, non-canonical inflammasomes appear to promote pyroptosis in a TLR4- and caspase-1-independent fashion in response to cytoplasmic Gram-negative bacteria infection (26). Although the innate immune response mediated by caspase-4/5 resembles, at least in term of outcomes, that driven by caspase-1, studies on macrophage-mediated inflammatory responses have revealed that they are indeed two quite different processes (12, 27, 28). In human cells, in fact, the CARD motif allows pro-caspase-4/5 to directly interact with lipopolysaccharide (LPS) through the lipid A moiety leading to pro-caspase-4/5 oligomerization and induction of pyroptosis coupled with secretion of IL-1β and IL-18. Adding complexity to this scenario, recent evidence has shown that murine caspase-11 activation triggers an NLRP3–ASC–caspase-1-dependent signaling pathway, also known as "non-canonical NLRP3 inflammasome activation pathway," which is different from the aforementioned "canonical NLRP3 inflammasome activation pathway" (29). However, even though it appears that caspase-4/5 and -11 can directly detect intracellular LPS derived from Gram-negative bacteria (24, 30), the exact mechanism of the non-canonical inflammasome activation is not totally understood.

Recently, a new family of inflammasome-associated PRRs has been described, including AIM2 and IFI16, grouped as ALRs. ALRs can assemble inflammasomes that respond to DNA molecules in both the cytosol and nucleus (31–33). AIM2 and IFI16 display an N-terminal PYD and one (AIM2) or two (IFI16, **Figure 1**) phylogenetically conserved hematopoietic interferon (IFN)-inducible nuclear protein with a 200-amino-acid repeat (HIN200) domains at the C-terminus, thus the other name PYHIN (or PYHIN200) previously given to these proteins. Interestingly, the HIN200 domain, which consists of two oligonucleotide/oligosaccharide-binding (OB) folds (34), appears to be the major DNA recognition site (35, 36). However, due to the lack of a dedicated ATP-dependent oligomerization domain, it appears that ALRs require a longer stretch of double-stranded DNA (dsDNA) compared with that required by NLRs to bind effectively and promote oligomerization (37, 38). Notably, since DNA is a common genetic material, pathological stimulation of these nucleic acid-recognizing inflammasomes by self-DNA can lead to autoinflammatory/autoimmune diseases as well (**Figure 2**). In this regard, aberrant immune responses involving ALRs have long been involved in the pathogenesis of SLE, Sjogren's syndrome (SjS), psoriasis, and systemic sclerosis (SSc) (39–45).

INFLAMMASOME AND AUTOIMMUNITY

Although adaptive and innate responses are often opposite to each other in the immunological spectrum, they are essentially

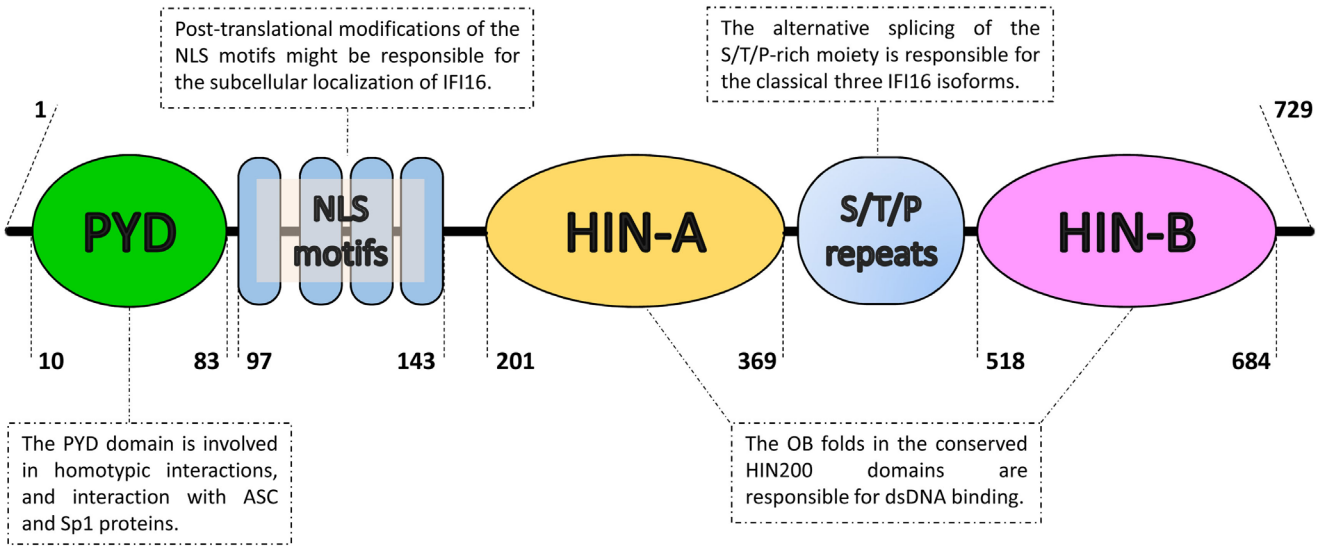

FIGURE 1 | Domain organization of the IFN-inducible protein 16 (IFI16). From the N- to the C-terminal (left to right), IFI16 comprises a pyrin domain (PYD) involved in protein–protein interactions, a linker region containing four nuclear localization signal motifs (NLS) and two hematopoietic interferon-inducible nuclear protein with 200-amino-acid repeats (HIN200) domains, which is an hallmark of the absent in melanoma 2-like receptors/PYHIN proteins. The HIN200 domains include two tandem b-barrels, known as oligonucleotide–oligosaccharide-binding (OB) fold, which allow DNA docking in a non-sequence-specific manner. They are separated by serine/threonine/proline-rich (S/T/P) repeats, which are regulated by alternative mRNA splicing. The numbers represent the amino acid positions based on NCBI Reference Sequence NP_005522.2.

integrated in a complex system (i.e., the human body) as innate immune dysregulation (i.e., the classical driver of autoinflammatory diseases) induces autoreactive T and B cell responses (i.e., autoimmunity) (13, 46). Indeed, classical autoinflammatory diseases, such as inflammatory bowel disease (IBD), are also characterized by the presence of autoantibodies, whereas classical autoimmune conditions, such as SLE, can also display organ-specific inflammation, as in the case of lupus nephritis (LN) (47, 48).

An additional feature encompassing the full inflammatory spectrum is inflammasome activation, which is usually essential for host defense against microbes. However, recent studies have also found this activation to be responsible for or simply be associated with the pathogenesis of several diseases featuring autoinflammatory/autoimmune traits such as type 1 and type 2 diabetes, IBD, multiple sclerosis (MS), rheumatoid arthritis (RA), and SLE (49–55).

Genetic polymorphisms (SNPs) associated with autoimmune diseases have been identified in components of both NLR and ALR inflammasomes, including NLRP1, NLRP3, CARD8, IFI16, and AIM2 (52, 56–61). Several studies, most of which related to ethnicity, have highlighted an association between SNPs in inflammasome end products and autoimmune diseases such as SLE, RA, and MS (62–64). Furthermore, inflammasomes have been directly involved in autoimmunity. For example, NLRP1 is overexpressed in T and Langerhans cells in the leading edge of vitiligo skin, leading to increased IL-1β production and activation of the Th17 axis (65). Furthermore, NLRP3 expression and NLRP3-mediated IL-1β secretion are increased in RA patients (66), and NLRP3 is involved in the pathogenesis of experimental autoimmune encephalomyelitis (51). Moreover, APC with an activated NLRP3 inflammasome can trigger CD4 T cell-mediated upregulation of the chemokine receptor CCR2, which is elevated in the peripheral blood of MS patients during relapse (67). With regard to ALR family members, AIM2 is directly activated by cytoplasmic DNA (68), and a strong correlation between AIM2 overexpression and disease severity has been described in both SLE patients and mouse models (61, 69). Finally, SLE is characterized by AIM2 inflammasome-mediated production of IL-1β, triggered by accumulation of cytosolic self-DNA and IFI16-induced IFN-I release (40).

Systemic lupus erythematosus is a systemic autoimmune disease characterized by a polyclonal autoimmune response against various nuclear autoantigens (16). Although genetic and environmental factors, such as infections, have been linked to the pathogenesis of SLE, the exact etiology of this disease is still unknown (17). SLE is characterized by hyperactive autoreactive immune cells and production of many autoantibodies, immune complex (IC) formation, organ inflammation and damage. More than 200 different autoantibodies including those against single-stranded DNA and dsDNA, Ro/La antigens, and ribonuclear protein have been described in lupus patients (70, 71). Among these, the so-called antinuclear antibodies (ANAs) and anti-dsDNA antibodies, which seem to play an important role in the pathogenesis of LN, represent valuable diagnostic and prognostic markers of disease (72, 73).

Along with elevated production of autoantibodies, 50–75% of SLE adults and up to 90% of SLE children display increased IFN-I production and enhanced expression of IFN-inducible genes, which is therefore regarded as a gene expression signature of SLE (74). Notably, a few studies have shown that patients with

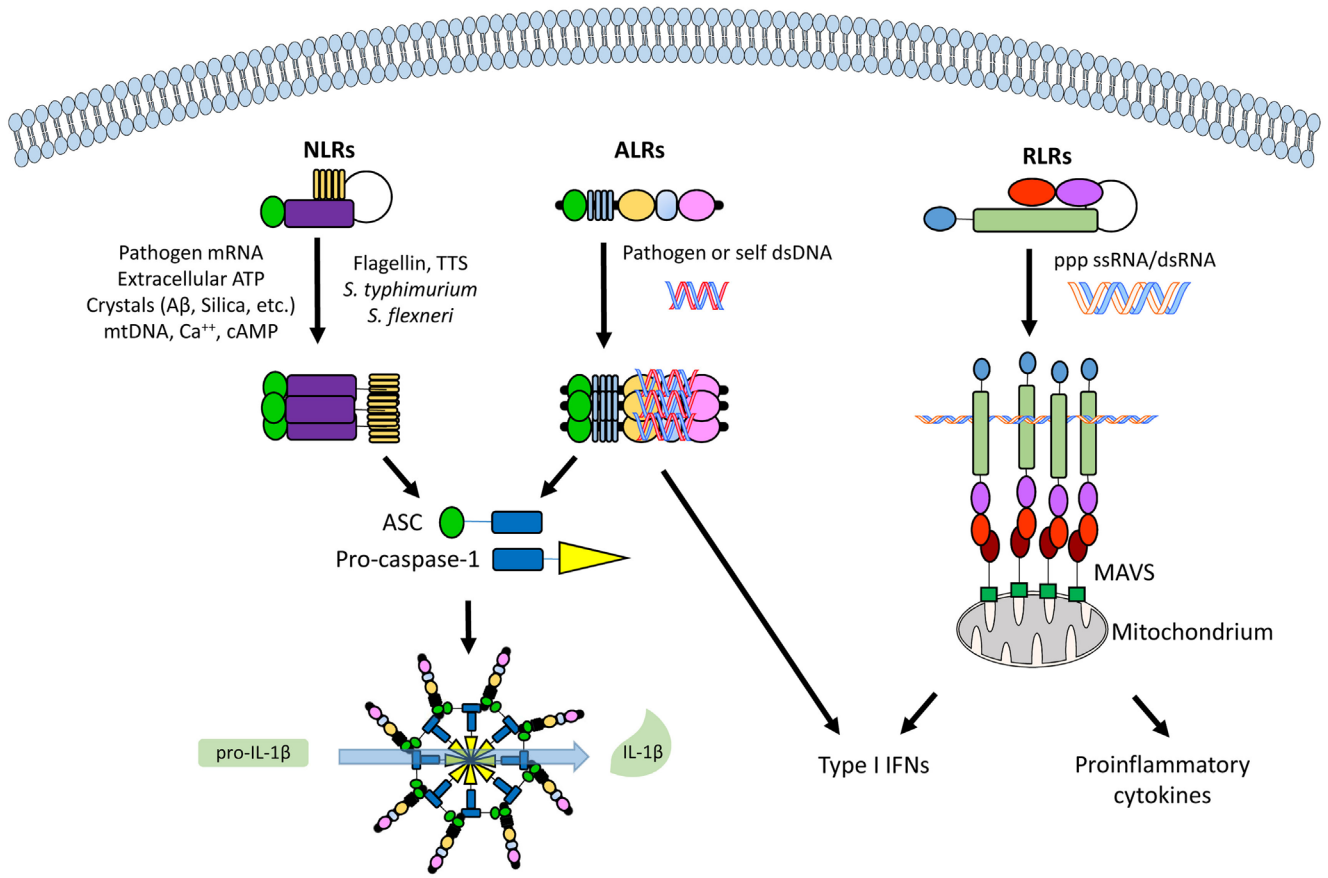

FIGURE 2 | An overview of the different classes of cytoplasmic and nuclear pathogen recognition receptors (PRRs) and their involvement with inflammasome activation. From left to right, a nucleotide-binding oligomerization domain-like receptor (NLR, e.g., NLRP3), an absent in melanoma 2 (AIM2)-like receptor (ALR, e.g., IFI16), and a retinoic acid inducible gene I (RIG-I)-like receptor (RLR, e.g., RIG-I), with their commonest ligands. See text for details. Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD); MAVS, mitochondrial antiviral signaling protein.

enhanced IFN-I signature can be considered a distinct subset of SLE patients. In this context, an association between IFN signature and some clinical manifestations, such as nephritis and CNS disease, has been reported (75).

Recent studies have defined the role of autologous dsDNA in SLE pathogenesis [reviewed in Ref. (72)]. Briefly, in physiological conditions, dsDNA is localized in the nucleus and mitochondria; however, once it relocalizes into the cytoplasm and endosomes, it is rapidly degraded by DNases. In SLE patients, impaired dsDNA degradation activity coupled with defective clearance of both apoptotic cell bodies and neutrophil extracellular traps (NETs) results in self-dsDNA accumulation (76, 77). In the meantime, self-dsDNA released by apoptotic cells in the germinal center is processed by follicular dendritic cells as non-self-antigen and presented to autoreactive B cells, which as a result will survive and expand instead of being eliminated (78). Afterward, self-dsDNA together with autoantibodies triggers the formation of ICs that in turn will mediate tissue damage, stimulate pro-inflammatory cytokine production and an array of IFN-inducible genes (i.e., IFN signature). Noteworthy, self-dsDNA is mainly sensed by plasmacytoid dendritic cells (pDCs) by means of different DNA sensors, which ultimately lead to elevated IFN-I production and inflammasome activation (70).

Type I IFNs are endowed of several immune functions ranging from dendritic cell differentiation and maturation to T cells activation and induction of antibody production by B cells. IFN-I pleiotropic activities underscore the critical function of these molecules in the pathogenesis of autoimmune diseases, in particular SLE (70, 75). In parallel, inflammasome activation leads to the release of inflammatory cytokines including IL-1β and IL-18, which contributes to the maintenance of the inflammatory state followed by cell death.

However, the association between SLE and IL-1β production is highly debated. Animal models of SLE (MRL/lpr mice) have shown that IL-1β gene expression, and protein secretion is increased in the glomerular macrophages and mesangial cells of LN (79), whereas polymorphisms studies on SLE patients have led to conflicting results (80).

Altogether, these observations stress the relevant role of IFN-I alongside the other inflammatory cytokines in fine-tuning both the innate and adaptive immune responses. One can therefore easily understand how slight perturbations of the signaling

pathways can lead to the dysregulation of the immune response that inevitably brings to the development of the autoimmune response.

ROLE OF AUTOLOGOUS dsDNA IN SLE

The major source of autologous dsDNA, which, as mentioned earlier, plays a pivotal role in SLE pathogenesis, is represented by cells dying by necrosis, apoptosis or NETosis, with the latter being a type of cell death mediated by NETs, extrusions of intracellular material to the surrounding extracellular medium to concentrate antibacterial substances and entrap invading microorganisms (81, 82). Intriguingly, also pyroptosis, that is the type of cell death induced by the inflammasome in response to both infectious and non-infectious stimuli, has been linked to SLE initiation (83).

Apoptosis, also known as programmed cell death, is an essential mechanism of tissue homeostasis during development and aging, characterized by cell shrinkage, cytoskeleton remodeling, chromatin condensation, nuclear breakup, plasma membrane blebbing and formation of typical apoptotic bodies (84). Under normal physiologic conditions, apoptotic cells directly undergo phagocytosis by specialized cells (i.e., professional phagocytes) and are degraded within the lysosomes with no signs of inflammation or immune response. In physiological conditions, cellular membranes are well preserved and readily cleared by engulfing phagocytes (85). Unless properly cleared, the apoptotic cells undergo secondary necrosis characterized by cell membrane leakage with consequent release of intracellular contents, including autologous dsDNA (86). Notably, release of intracellular material, which ultimately contributes to the development of autoimmune diseases, can also be triggered by primary necrosis due to exogenous factors, as demonstrated both in animal models and human infections (87, 88).

NETosis, a type of cell death first associated with neutrophils, causes the extrusion of nuclear DNA, histones and granular antimicrobial proteins entrapped leading to formation of NETs (81, 89). Yet, mounting evidence has shown that other cell types, including eosinophils and mast cells, can undergo cell death through a similar mechanism. Therefore, NETosis appears not be limited to neutrophils and should therefore be regarded as a new type of cell death that generally causes the release of extracellular traps (90). Physiologically, monocyte-derived phagocytes clear NETs efficiently thanks to C1q- and DNase I-mediated extracellular preprocessing of NETs. After ingestion by phagocytes, NETs are degraded in the lysosomes. Remarkably, this entire process is immunologically silent since the uptake of NETs by macrophages does not seem to stimulate pro-inflammatory cytokine secretion (91). On the other hand, impaired clearance of NETs by phagocytes can lead to the accumulation of several autoantigens including self-dsDNA (92), thereby increasing the chance of anti-dsDNA antibody formation, although a study on an animal model of SLE showed a protective role of NETs (93).

A particular type of NETosis, mitochondrial NETosis, causes the release of mitochondrial DNA (mtDNA) from neutrophils following the mitochondrial production of ROS. Since mitochondria share several features with bacteria, including a circular genome carrying unmethylated CpG dinucleotide repeats, mtDNA is similarly immunogenic and may promote inflammation through surface and endoplasmic TLR9 binding. Moreover, IL-1β production can also be driven by cytosolic release of mtDNA, dominantly acting on NLRP3/AIM2 inflammasomes (94). Interestingly, NETs from low-density granulocyte of SLE patients are highly enriched in mtDNA compared with NETs from healthy controls neutrophils (95), whereas abnormal extrusion of oxidized mtDNA from SLE patient neutrophils may triggers a pathogenic interferogenic response (96). Finally, mtDNA and autoantibodies against it are present in elevate levels in SLE and in particular in LN, where levels correlate with activity index better than anti-dsDNA (97).

Altogether, these findings indicate that cell death-originating self-dsDNA plays a crucial role in SLE pathogenesis.

ENVIRONMENTAL FACTORS TRIGGERING IFN-I PRODUCTION AND INFLAMMASOME ACTIVATION IN SLE

We have beforehand described that DNA from dying cells, as well as DNA from microbial pathogens, is strong immune stimulants that can accumulate in the cytosol and activate the production of various immune system modulators, including IFN-I. This pathway is critically dependent on a protein known as stimulator of interferon genes (STING) (98), which indirectly responds to DNA through the cyclic dinucleotide 2′,3′-cGAMP, produced upon the stimulation of the enzyme cyclic GMP-AMP synthase (cGAS) (99). In turn, the 2′,3′-cGAMP-related activation of STING induces a conformational change which is thought to mediate the phosphorylation and activation of interferon regulatory factor 3 (IRF3), a transcription factor for various gene targets, including but not limited to IFN-I (100).

It is becoming increasingly clear how several environmental factors that can promote IFN-I production are also able to induce an SLE syndrome as well as cause a flare of this disease. One of these agents is represented by ultraviolet B (UVB) light, which has been shown to trigger SLE flares and induce severe systemic manifestations including cutaneous reactions (101). Interestingly, all UVB light-induced exacerbations are associated with enhanced levels of IFN-I and -III along with pro-inflammatory cytokines (102, 103). In this regard, UVB light can promote redistribution of nuclear antigens on the cell surface and keratinocyte apoptosis (104). Furthermore, additional inflammatory cells, recruited by type III IFN into the skin, are likely responsible for priming activated pDCs to produce higher levels of IFN-I. Consistently, UV irradiation of keratinocytes has been shown to activate the STING/IRF regulatory axis in response to cytosolic DNA due to the loss of the STING negative regulator Unc51-like kinase 1 (105).

Systemic lupus erythematosus onset along with disease flare is also frequently associated with infections. Although many viruses and bacteria have been implicated in SLE pathogenesis (88, 106, 107), no specific etiologic pathogen has thus far been identified. Inflammation, as part of the innate immune response, is triggered when PAMPs are recognized by PRRs, which can be either associated with the cell membrane or located within the cell in the cytosol or nucleus. There is a growing number of

identified PRRs, including toll-like receptors (TLRs) and various intracellular nucleic acid receptors. The signaling pathway leading to IFN-I production or inflammasome activation strictly relies on the PRR repertoire of the responding cell type and the subcellular localization of the immunostimulatory nucleic acid. TLR3, TLR7/8, and TLR9, present in immune cells (i.e., pDCs and monocytes), sense dsRNA, ssRNA, and DNA containing CpG motifs (108, 109). Another group of PRRs (i.e., the RLRs) include the cytosolic RNA receptor RIG-I and the melanoma differentiation factor 5, and are responsible of detecting dsRNA and ssRNA molecules in the cytoplasm of cells infected with RNA viruses (20, 110–112). In addition, several DNA sensors located in both the cytosol and the nucleus have been described. These include cGAS (113), DNA-dependent activator of IFN-regulatory factors (DAI) (114), AIM2 (115), IFI16 (116, 117), NLRs (118), and DEAD/H-box helicase 41 (DDX41) (119). Binding of these DNA sensors to their ligands activates signaling pathways, including TLR9-, STING-, and inflammasome-dependent pathways, which not only induce production of IFN-I but also promote inflammatory gene expression and inflammasome-associated cell death (i.e., pyroptosis). In physiological conditions, these intracellular sensors and related pathways are tightly regulated to impede the development of autoimmunity (120), which would otherwise take place due to uncontrolled recognition of self-nucleic acids (121, 122).

Upon PAMP recognition, the intracellular receptors assemble cytoplasmic platforms known as myddosomes and inflammasomes, which are supramolecular organizing centers regulating the inflammatory and immunoregulatory response following microbial detection. Specifically, TLRs initiate a toll/interleukin-1 receptor domain-containing adapter protein (TIRAP)-dependent assembly of the myddosome, which consists of the adaptor MYD88 and several serine/threonine kinases of the IL-1 receptor-associated kinase family (123). As stated previously, the canonical inflammasome contains a DNA sensor protein, the adaptor protein ASC and procaspase-1. Upon inflammasome assembling, activation of caspase-1 converts the immature IL-1β and IL-18 into mature secreted forms (124). Importantly, different NLR family members, such as NLRC4, NLRP1, and NLRP3 and the two ALR family members AIM2 and IFI16 have been shown to be differentially stimulated in a ligand-specific fashion.

Recently, it has been demonstrated that the canonical inflammasome pathway can be by-passed by the non-canonical one, which as stated previously consists of a complex formed by procaspase-11 and bacterial LPS activated in mouse macrophages. Consistently, caspase-4 and caspase-5, the human counterpart of mouse caspase-11, can interact directly with intracellular LPS and activate the non-canonical inflammasome in human myeloid cells (12, 23).

Two important features distinguish myddosomes from inflammasomes: (1) inflammasomes do not trigger gene activation at the transcriptional level, but rather induce inflammation by promoting the release of preexisting immature cytokines; (2) inflammasomes activating PRRs are localized in the host cytosol, which is rarely attacked by non-pathogenic bacteria. Therefore, inflammasomes are generally assembled when intracellular PRRs interact with pathogenic bacteria in the cytosol. By contrast, TLRs, which are localized on the cell surface, cannot distinguish whether PAMPs originated from pathogenic or non-pathogenic microorganisms.

Thus, taken together, these findings suggest a scenario where the redundancy of PAMPs sensing immune receptors may easily lead to dysregulation of the immune response when not regulated properly.

AIM2-LIKE RECEPTORS: INFLAMMASOME ACTIVATORS AND IFN-I PRODUCTION REGULATORS

The PYHIN (or PYHIN200) family encodes evolutionary related human (i.e., IFI16, IFIX, MNDA, and AIM2) and murine (i.e., Ifi202a, Ifi202b, Ifi203, Ifi204, Ifi205/D3, and Ifi206) proteins (116–118). Increasing evidence has shown that these proteins may act as regulators of a wide range of cell functions, such as differentiation, proliferation, senescence, apoptosis, and inflammasome assembly (117, 125–130). Recently, two members of the human family, IFI16 and AIM2, have been implicated in the recognition of pathogen DNA and classified into the ALR group, still maintaining their peculiarity. In normal conditions, expression of the nuclear phosphoprotein IFI16 is limited to vascular endothelial cells, keratinocytes, and hematopoietic cells (131). Following activation by pathogen DNA, IFI16 translocates into the cytoplasm, triggers type I IFN production, cytokines, and eventually cell death (**Figure 3**). By contrast, AIM2, upon binding DNA in the cytosol, stimulates inflammasome activation in the absence of type I IFN production.

From a structural point of view, IFI16 harbors an N-terminal PYD and two C-terminal HIN200 domains (see **Figure 1** for details). While AIM2 uses its PYD to interact with the inflammasome component ASC, which also contains a PYD (31, 33, 68), the direct interaction between IFI16 and ASC is still matter of debate. Nevertheless, IFI16 has been reported to induce ASC-dependent inflammasome activation during infection with some nuclear DNA viruses (32, 132, 133). Following viral DNA recognition in the nucleus, the IFI16-ASC-procaspase-1 inflammasome formation is induced. The complex is then released in the cytoplasm, where processing of pro-IL-1β into active IL-1β occurs.

Moreover, IFI16 is also an inducer of IFN-β in response to intracellular DNA. RNA interference-mediated depletion of IFI16 or its presumed mouse ortholog p204 has revealed that both proteins are required for a functional IFN response to transfected dsDNA or infection with HSV-1 in various cell types, including human and mouse monocytic cell lines (134), mouse corneal epithelial cells (135), human primary and immortalized fibroblasts (136, 137), human primary macrophages (138), neutrophils (139), and dendritic cells (140). In this regard, IFI16 has been shown to interact with STING, leading to phosphorylation and nuclear translocation of IRF3 *via* the IFI16–STING–TBK signaling axis, resulting in IFN-β production during HSV-1 infection (137). Moreover, IRF3 activation has been also demonstrated following direct cooperation between IFI16 and cGAS, by a mechanism in which cGAS promotes IFI16 stability in response to incoming nuclear HSV DNA, rather than through the production of 2′,3′-cGAMP (141) (**Figure 3**).

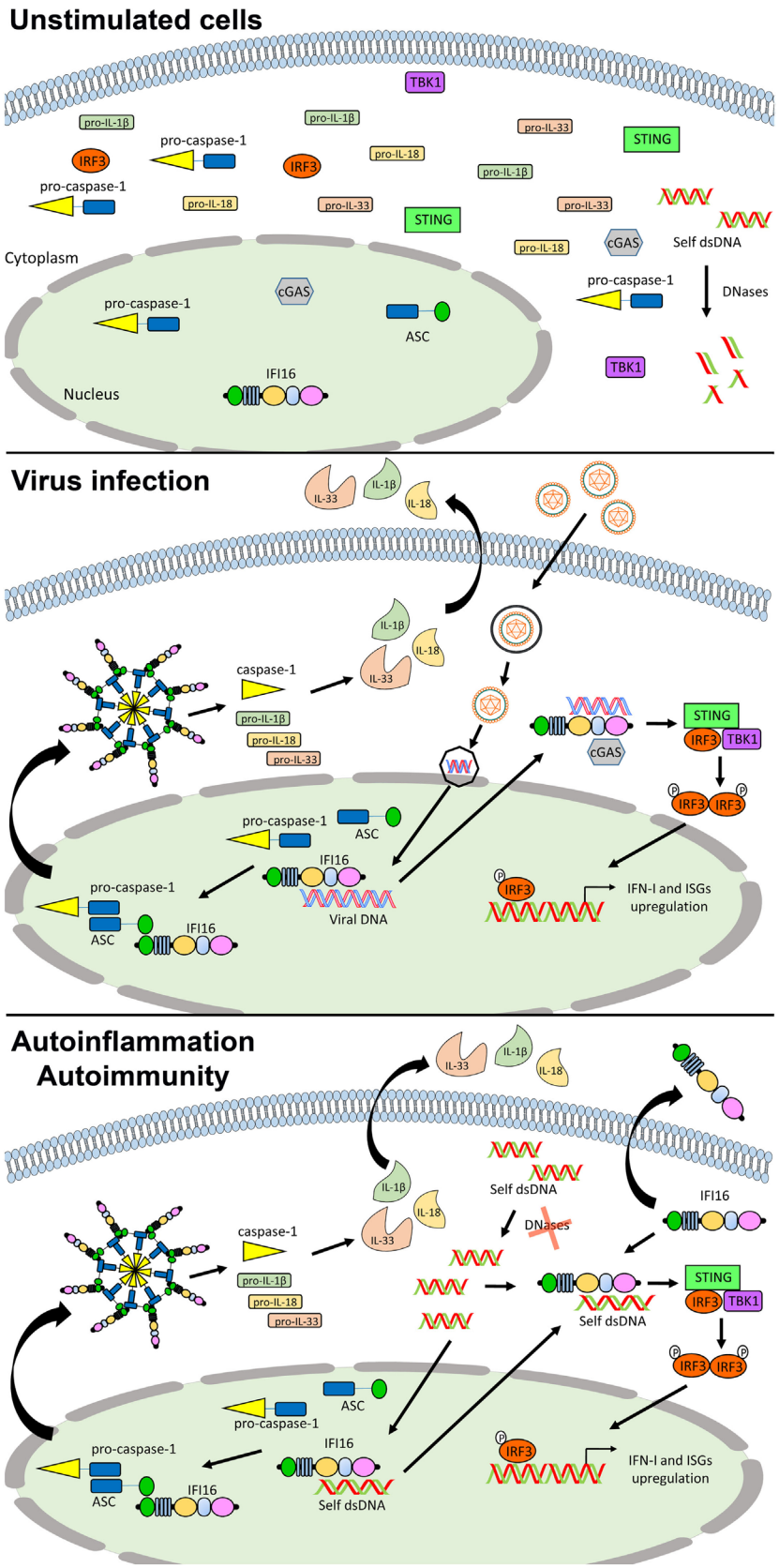

FIGURE 3 | Continued

FIGURE 3 | Role of IFN-inducible protein 16 (IFI16) as inflammasome regulator in viral infections and autoimmunity. In unstimulated cells, IFI16 is mainly nuclear (upper panel). Following viral DNA recognition and binding, IFI16 can induce the activation of the canonical inflammasome through the recruitment of ASC and pro-caspase 1, and the production of type I IFN (IFN-I) through the STING–IRF axis (middle panel). In the course of autoimmune (e.g., systemic lupus erythematosus) and autoinflammatory conditions, following the recognition of self-DNA, IFI16 might be responsible for the production of pro-inflammatory cytokines and IFN-I through the same pathways. Moreover, its aberrant expression can also lead to the extracellular leakage causing amplification of the inflammatory signals and production of protective autoantibodies (lower panel). See text for details. Abbreviations: cGAS, cyclic GMP-AMP synthase; IRF3, interferon regulatory factor 3; ISG, interferon stimulated genes; STING, stimulator of interferon genes; TBK1, TANK-binding kinase.

As aforementioned, IFI16 is unique among DNA sensors as it shuttles between the nucleus and the cytoplasm and is predominantly nuclear at steady state. Furthermore, IFI16 is able to sense DNA in both compartments depending on the localization of its DNA ligands (134, 137, 138). Thus, the ability of IFI16 to detect DNA viruses, such as HSV-1 in the nucleus, appears to contradict the long-held assumption that foreign DNA is sensed merely because of its cytosolic localization. Interestingly, the conserved HIN200 domains of the IFI16 protein are responsible for the interaction with oligonucleotide/oligosaccharide moieties (142). To what extent IFI16/p204 is involved in the sensing of DNA during infection with viruses or intracellular bacteria *in vivo*, and what domains are indispensable for recognition, awaits the generation of mice lacking this receptor. However, the structural studies elucidating the DNA binding of IFI16 have improved our understanding on non-self-DNA sensing and IFI16 localization. Few more issues concerning the nuclear/cytosolic interaction of STING and IFI16 and activation of inflammasome remain unanswered, mainly related to the different cellular and infection models analyzed so far.

IFN-inducible protein 16 has also been described to play a role in the DNA damage response (143, 144) and promote apoptosis and senescence (145–148). Recent reports have implicated IFI16 in autoimmunity, pointing to a role of PYHIN proteins in the pathogenesis of human autoimmune disease. Since the IFN system is largely regarded as playing a key role in autoimmune disorders including SLE, SSc, and SjS (75, 149, 150), it is possible to hypothesize that also PYHIN may play a causative role in autoimmunity thanks to its ability to induce apoptosis and trigger an inflammatory response (**Figure 3**). It follows that during systemic autoimmune conditions of tissue injury and apoptosis the exposure of autoantigens leads inevitably to breaking of tolerance and dysregulation of the immune response. Under physiological conditions, dead cells and tissue debris are normally cleared by the monocyte/macrophage system. However, under conditions that hamper clearance of apoptotic bodies by phagocytes, chronic exposure of autoantigens, including PYHIN proteins, may lead to the development of autoimmunity. Consistent with this scenario, various autoantigens and corresponding autoantibodies have been identified in the sera of patients affected by different systemic autoimmune diseases, such as SLE, SjS, and SSc.

NOVEL FUNCTIONS FOR IFI16 TO TRIGGER INFLAMMATION

We have previously demonstrated that IFI16 is a key component for the tight regulation of cellular and viral promoters, through physical interaction with the nuclear transcription factor Sp1 and regulation of NF-κB pathway (151, 152). As inducer of pro-inflammatory molecules (e.g., ICAM-1, RANTES, and CCL20) and apoptosis in primary endothelial cells, IFI16 might be active during the initial phases of the inflammatory processes paving the way to the onset of autoimmunity (145). In addition, IFI16 has been shown to translocate in the cytoplasm of normal keratinocytes following UVB-induced cell injury and be subsequently released in the extracellular milieu (104). *In vivo*, the expression of IFI16 is significantly increased in all layers of the epidermis from patients affected by SLE or SSc, whereas in the epidermis from healthy control subjects IFI16 expression is only found in the basal layer. In the same setting, the dermal inflammatory infiltrate has been found positive for IFI16 staining indicating that IFI16 is aberrantly expressed also in lymphocytes, fibroblasts, and endothelial cells. Similarly, we and others have also recently demonstrated that IFI16 is aberrantly expressed in the intestinal mucosa of patients affected by IBD, where dysregulation of host–microbial interactions has been shown to play a major pathogenic role (153, 154). In addition, we and others have evaluated the etiopathogenic role of PYHIN proteins in the development of SLE in human pathology as well as in mouse models (**Table 1**). In this regard, we have found that IFI16 overexpression in primary human umbilical vein endothelial cells (HUVECs) efficiently inhibits tube morphogenesis *in vitro*, triggers production of pro-inflammatory molecules and leads to cell death by apoptosis, suggesting that IFI16 might induce inflammation along with other detrimental cellular pathways primarily involved in autoimmunity (145, 155).

In another context, IFI16 has been shown to restrict human cytomegalovirus (HCMV) and papillomavirus replication through different mechanisms (152, 156). Interestingly, IFI16 has been observed entrapped in HCMV virions undergoing cell egression (116). Consistent with our results, Singh et al. have demonstrated that IFI16 is aberrantly expressed in the cytoplasm of KSHV latently infected cells, wrapped up in exosomes and then released extracellularly (133). However, since IFI16 was originally identified as a molecule localized in intracellular compartments, in particular the nucleus, all studies on IFI16 were subsequently limited to determine the biological and physiological activity of this protein exclusively within the cellular compartment, thus disregarding a possible role of extracellular IFI16 as pro-inflammatory trigger. To fill this gap, we sought to determine the effects of extracellular IFI16 protein on HUVECs. Surprisingly, we observed a cytokine-stimulating activity of extracellular IFI16 (rIFI16) on primary endothelial cells, which led to the production and secretion of pro-inflammatory cytokines such as IL-6, IL-8, CCL2, CCL5, and CCL20. Moreover, we found that rIFI16 protein, alone or in synergy with LPS, acted by propagating "danger signals" through a MyD88-dependent TLR pathway (126).

TABLE 1 | Summary of IFN-inducible protein 16 (IFI16) correlations with systemic lupus erythematosus (SLE) and other autoimmune diseases.

Disease	Observation	Reference
Systemic lupus erythematosus	First description of anti-IFI16 antibodies in the sera SLE patients	(159)
	Presence of anti-IFI16 antibodies detected by SEREX in the sera of SLE patients	(43)
	Increased expression of IFI16 in the skin of SLE patients and detection of anti-IFI16 antibodies by ELISA	(41)
	Increased IFI16 mRNA levels in leukocytes from SLE patients	(55)
	IFI16 overexpression and redistribution in the skin of SLE patients	(104)
	High significant levels of circulating IFI16 protein in the sera of SLE patients	(155)
	High serum titers of anti-IFI16 antibodies inversely correlated with proteinuria and C3 hypocomplementemia	(158)
Sjögren's syndrome	Presence of anti-IFI16 antibodies detected by SEREX in the sera of Sjogren's syndrome (SjS) patients	(43)
	Significant levels of circulating IFI16 protein in the sera of SjS patients	(155)
	De novo expression of IFI16 in ductal and acinar epithelial cells in salivary glands	(39)
	High serum titers of IFI16 antibodies against an epitope outside the N-terminus of the protein	(160)
Systemic sclerosis	Presence of anti-IFI16 antibodies detected by SEREX in the sera of systemic sclerosis (SSc) patients	(43)
	Increased expression of IFI16 in the skin of SSc patients and detection of anti-IFI16 antibodies by ELISA	(41)
	Anti-IFI16 antibodies associated with the limited cutaneous form of the disease in patients negative for the classical serological markers	(161)
	Significant levels of circulating IFI16 protein in the sera of SSc patients	(155)
Rheumatoid arthritis (RA)	Presence of anti-IFI16 antibodies detected by SEREX in the sera of RA patients	(43)
	High levels of circulating IFI16 protein in the sera of RA patients	(155)
	Increased levels of both anti-IFI16 antibodies and circulating IFI16 in the sera of RA patients, IFI16 protein correlating with RA-related pulmonary disease	(157)
Inflammatory bowel disease	*De novo* overexpression of IFI16 in colonic epithelial cells of inflammatory bowel disease (IBD) patients	(153, 154)
	Detection of anti-IFI16 antibodies by ELISA in the sera of IBD patients	(153)
Psoriasis	IFI16 upregulation in psoriatic skin lesions, with cytoplasmic localization	(44)
	IFI16 upregulation in keratinocytes is induced by psoriasis-related cytokines, including IFN-γ, TNF-α, IL-17, and IL-22	(45)

Altogether, these results unveil a novel function of extracellular IFI16 at the endothelial interface, which might explain the ability of this protein to induce endothelial cell activation and injury during systemic inflammation.

In summary, IFI16 can promote inflammation by (1) acting as regulator of transcription factors to activate expression of genes encoding pro-inflammatory cytokines; (2) activating type I IFN production following translocation into the cytoplasm; and (3) binding to cells such as endothelial cells and keratinocytes, once released in the extracellular milieu, to activate production of pro-inflammatory chemokines and cytokines. Concomitantly, IFI16 leakage into the extracellular milieu leads to tolerance breaking and autoantibody production.

ANTI-IFI16 ANTIBODIES AND THEIR RELATION TO SLE CHARACTERISTICS

We and others have previously reported the presence of anti-IFI16 antibodies in sera of patients suffering from various autoimmune diseases such as SLE, SjS, AR, SSc, and IBD (39, 41, 153, 157–161) (**Table 1**). Among these latter, SLE stands out as the disease where IFI16 autoantibodies have been more thoroughly characterized. This aspect is of paramount importance in view of the prognostic and diagnostic relevance of other SLE autoantibodies such as ANAs and autoantibodies against Ro/SSA and La/SSB ribonucleoproteins (162). However, not all autoantibodies seem to play a causative role in autoimmunity as autoantibodies against chromatin molecules, such as HMGB1, exert a protective effect in animal models of autoimmune disease (163). Thus, new criteria for autoantibodies classification based on both their functionality and ability to trigger or dampen immunologic disturbances are clearly needed.

With regard to IFI16, it is conceivable to hypothesize that the previously described over- or aberrant expression and mislocalization of this nuclear protein, earlier in the cytoplasm and later on in the extracellular milieu, might lead to loss of tolerance and development of anti-IFI16 antibodies, as demonstrated in skin lesions from SLE patients and in keratinocytes cultured *in vitro* under conditions of UVB light-induced cell injury (104).

Although the occurrence of anti-IFI16 antibodies in SLE patients has long been known, their associations with clinical and serological parameters of SLE are still under debate. To address this aspect, we have recently set out to determine the prevalence of anti-IFI16 autoantibodies in a population of SLE patients from northern Italy (158). Specifically, in a cross-sectional study, we compared anti-IFI16 antibody levels of SLE patients at various stages of disease with those of patients with non-SLE primary glomerulonephritis (GN) or healthy individuals. Remarkably, we measured significantly higher anti-IFI16 titers in SLE patients compared with both disease and control groups, and, according to cutoff levels, we were able to estimate that more than 60% of the SLE patients tested positive for anti-IFI16 autoantibodies compared with just 24% of patients with primary non-SLE GN and 5% of healthy individuals. Of note, in this SLE cohort, univariate analysis showed that autoantibodies to IFI16 were inversely associated with proteinuria, whereas multivariate analysis confirmed a reduced risk of proteinuria for anti-IFI16-positive patients despite renal function. Furthermore, an inverse association between anti-IFI16 and C3 hypocomplementemia was also observed.

In this regard, the association of anti-IFI16 antibodies with reduced C3 hypocomplementemia was independent of the disease activity parameters SLEDAI and anti-dsDNA. The described inverse associations between anti-IFI16 positivity, proteinuria, and C3 hypocomplementemia, together with the observation that nephritis does not occur in other systemic autoimmune diseases characterized by high titers of anti-IFI16 antibodies such as SjS and SSc, imply that ultimately these antibodies do not play a relevant role in the pathogenesis of renal inflammation in SLE, but rather most likely prevent complement consumption. Thus, based on these findings, it is likely that the occurrence of IFI16 autoantibodies might protect from the detrimental activity of the free circulating IFI16 protein, exerting beneficial functional effects rather than pathogenic ones.

Consistent with the data obtained in SLE patients, in previous studies, we found a significant prevalence of anti-IFI16 antibodies in SSc, which was more evident in the more benign limited cutaneous form of this disease (42). More recently, we have shown that enhanced titers of anti-IFI16 in IBD patients undergoing infliximab therapy correlates with a more favorable outcome of the disease (153), which can be partly explained by the protective role exerted by these antibodies against the progression of the autoimmune process.

CONCLUSION AND PERSPECTIVES

In the last decade, we have greatly expanded our knowledge of the relationship between aberrant innate immune response and development of autoinflammatory/autoimmune diseases such as SLE. Specifically, we now know that multiple inflammasome-induced inflammatory responses correlate with the development of SLE. In this regard, the ALR family member IFI16 has been found aberrantly expressed in various target tissues of a range of autoimmune diseases, including SLE skin, SjS salivary glands, and IBD colonic epithelium. With this scenario in mind, the occurrence of anti-IFI16 antibodies is likely due to the response of the immune system to IFI16 protein release through one of the aforementioned cell death mechanisms. Alternatively, the presence of anti-IFI16 autoantibodies could be the result of IFI16 translocation from the nucleus to the cytoplasm and, eventually, being secreted into the extracellular milieu where it is recognized by the immune system. In addition, the observation that IFI16 enhances the inflammation response against microbial patterns, such as bacterial LPS, is highly suggestive of a role of ALRs also in non-canonical inflammasome-mediated signaling.

Overall, understanding the role of ALRs in SLE pathogenesis and chronic inflammation would contribute to the development of novel therapeutic options, which may not only be limited to the treatment of patients affected by systemic autoimmune disease but also to cure conditions in which prolonged inflammatory flares progressively lead to organ-specific disorders (e.g., cancer).

AUTHOR CONTRIBUTIONS

VC, SL, MG, and MDA have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Aberrant T Cell Signaling and Subsets in Systemic Lupus Erythematosus

*Takayuki Katsuyama, George C. Tsokos and Vaishali R. Moulton**

Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States

Systemic lupus erythematosus (SLE) is a chronic multi-organ debilitating autoimmune disease, which mainly afflicts women in the reproductive years. A complex interaction of genetics, environmental factors and hormones result in the breakdown of immune tolerance to "self" leading to damage and destruction of multiple organs, such as the skin, joints, kidneys, heart and brain. Both innate and adaptive immune systems are critically involved in the misguided immune response against self-antigens. Dendritic cells, neutrophils, and innate lymphoid cells are important in initiating antigen presentation and propagating inflammation at lymphoid and peripheral tissue sites. Autoantibodies produced by B lymphocytes and immune complex deposition in vital organs contribute to tissue damage. T lymphocytes are increasingly being recognized as key contributors to disease pathogenesis. CD4 T follicular helper cells enable autoantibody production, inflammatory Th17 subsets promote inflammation, while defects in regulatory T cells lead to unchecked immune responses. A better understanding of the molecular defects including signaling events and gene regulation underlying the dysfunctional T cells in SLE is necessary to pave the path for better management, therapy, and perhaps prevention of this complex disease. In this review, we focus on the aberrations in T cell signaling in SLE and highlight therapeutic advances in this field.

Keywords: SLE, autoimmunity, signaling, T cells, Autoimmune disease

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***Correspondence:**
Vaishali R. Moulton
vmoulton@bidmc.harvard.edu

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease involving multiple organs leading to tissue damage and diverse clinical manifestations. Although the etiology of SLE is still unclear, a number of recent studies have advanced our understanding of disease pathogenesis. Clinical heterogeneity of SLE suggests that there are number of players in the immune system that contribute to the pathogenesis of SLE. B cells obviously are important in autoimmune diseases through the production of antibodies by plasma cells and presenting antigens to T cells. However, there is an increasing recognition and validation of the critical role of T cells in SLE pathogenesis (1–5). Historically, the T helper (Th)1/Th2 balance was considered to be important in the pathogenesis of SLE (6, 7). However, recent understanding of the detailed mechanisms of T cell differentiation and subsets have elucidated the more important and complicated role of T cells in the pathogenesis of this autoimmune disease. Many studies have shown abnormal cytokine production and aberrant cell signaling in SLE T cells, which dictate not only the abnormalities in T cell differentiation but also the excessive activation of B cells. It is expected that these abnormal signaling molecules can serve as therapeutic targets for the treatment of patients with SLE. In this review, we focus on signaling molecules and pathways in T cells from SLE patients and lupus-prone mice, and highlight those that can be exploited therapeutically (**Figure 1**).

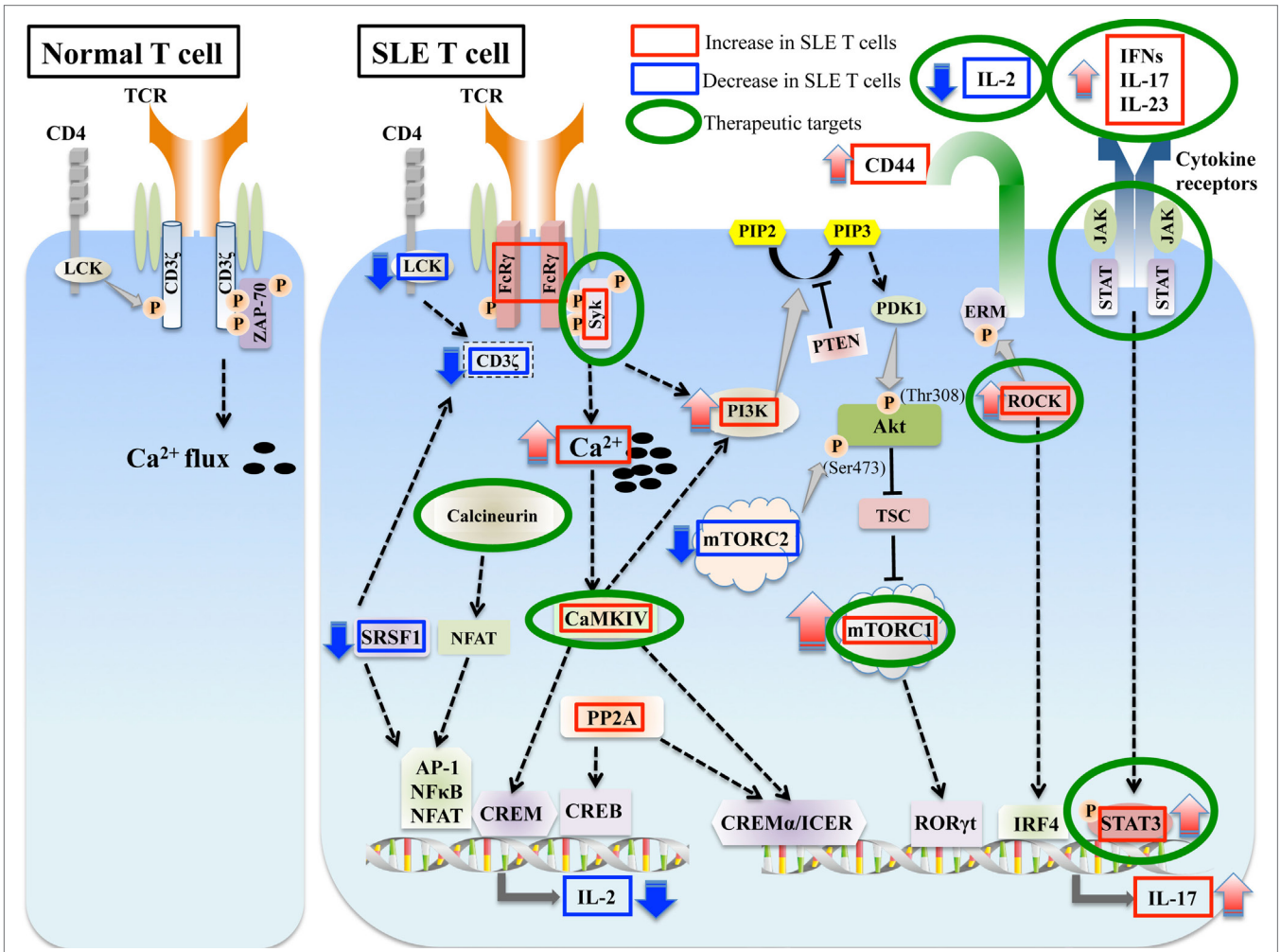

FIGURE 1 | Aberrant signaling in systemic lupus erythematosus (SLE) T cells. SLE T cells are characterized by multiple aberrant signaling pathways, such as decreased CD3ζ, activated PI3K-Akt-mTORC1 pathway, Rho associated protein kinase (ROCK), calcium/calmodulin kinase IV (CaMKIV), and protein phosphatase 2A (PP2A). These are associated with abnormalities in T cell differentiation and production of proinflammatory cytokines such as IL-17 and decreased production of vital cytokines such as IL-2. Molecules aberrantly increased or decreased in SLE are indicated in red and blue boxes, respectively, and molecules that are potential therapeutic targets are in green circles.

T CELL RECEPTOR (TCR)

The TCR is a heterodimer, consisting of the TCRα and TCRβ chains in most cells, which recognizes antigenic peptides presented by the major histocompatibility complex (MHC) on antigen presenting cells. The TCR is assembled with a complex of CD3 proteins (CD3δ, ε, γ, and ζ). CD3δ, ε, and γ are members of the immunoglobulin superfamily and genetically related to each other, whereas CD3ζ subunit is genetically and structurally unrelated to the other CD3 subunits (8–10). CD3ζ contains three immunoreceptor tyrosine-based activation motif (ITAM) domains, and the phosphorylation of ITAM residues is a key step in the complex process of TCR signaling. Following TCR recognition and engagement of the MHC—antigen complex, the Src kinase lymphocyte-specific protein tyrosine kinase (Lck) phosphorylates ITAMs of CD3ζ. Phosphorylated CD3 ITAMs recruit the spleen tyrosine kinase (Syk) family kinase ζ-associated protein kinase 70 (ZAP-70) *via* Src-homology 2 domain, and Lck phosphorylates the bound ZAP-70, resulting in the activation of ZAP-70 (11). Activated ZAP-70 phosphorylates tyrosine residues on the adaptor proteins linker for activation of T cells (LAT) and SLP-76, which bind and activate phospholipase Cγ (PLC-γ). Activated PLC-γ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol 1,4,5-trisphosphate and diacylglycerol, resulting in the calcium flux and the activation of protein kinase C (PKC) and Ras-mitogen-activated protein kinase pathway through the recruitment of Ras guanine releasing protein 1 (12, 13).

The expression levels of CD3ζ chain are significantly decreased in T cells from SLE patients (14–16), and this defect coupled with a rewiring of the TCR complex, contributes to the aberrant signaling phenotype of SLE T cells. In association with the reduced levels of CD3ζ protein in SLE T cells, the TCR–CD3 complex bears a substitution by the homologous Fc receptor common gamma subunit

chain (FcRγ), which is not normally expressed in resting T cells. Although FcRγ was identified as a component of the high affinity IgE receptor (FcεRI), it is now recognized as a common subunit of other Fc receptors (17, 18). FcRγ is upregulated upon activation in effector T cells (19–22). CD3ζ and FcRγ are structurally and functionally homologous (23). FcRγ recruits Syk instead of ZAP-70, which is normally recruited by CD3ζ. FcRγ–Syk interaction is significantly stronger than CD3ζ–ZAP-70 interaction, resulting in the higher calcium influx into T cells (14, 21). Reconstitution of CD3ζ in SLE T cells restores the aberrant signaling and calcium flux (24). Interestingly, CD3ζ-deficient mice spontaneously develop multi-organ tissue inflammation (25). Therefore, the reduced expression levels of CD3ζ are important in the aberrant T cell signaling phenotype, and understanding the mechanisms leading to its downregulation would help target those factors to correct the T cell signaling defect. A number of mechanisms for the downregulation of CD3ζ mRNA and protein in T cells from SLE patients have been elucidated. In addition to abnormalities in transcription (14, 26), aberrant alternative splicing (27–29) and stability (30, 31) of CD3ζ mRNA contribute to the decreased expression levels of CD3ζ protein in T cells from SLE patients. Serine/arginine-rich splicing factor 1 (SRSF1), also known as splicing factor 2/alternative splicing factor controls the alternative splicing (32) and contributes to the transcriptional activation (33) of CD3ζ, to promote normal expression of CD3ζ protein. Decreased SRSF1 expression in T cells from SLE patients correlates with worse SLE disease activity (34), and with reduced CD3ζ levels. Recently, it was reported that hypermethylation marks are present within the CD3ζ gene promoter in SLE patients (35). These findings suggest that CD3ζ hypermethylation may contribute to the downregulation of CD3ζ in T cells from SLE patients.

The serine/threonine protein phosphatase 2A (PP2A) is a ubiquitous serine-threonine phosphatase and composed of three distinct subunits; the scaffold A subunit ($PP2A_A$), the regulatory B subunit ($PP2A_B$), and the catalytic C subunit ($PP2A_C$) (36). PP2A controls the expression of CD3ζ and FcRγ at the transcription level through the dephosphorylation of Elf-1 (37). In T cells from SLE patients, increased PP2Ac activity results in aberrant TCR signaling leading to abnormal T cell function.

PROXIMAL TCR SIGNALING

TCR-CD3 engagement with antigens induces the phosphorylation of ITAM residues by Lck, a member of the Src kinase family. The expression levels of Lck are decreased in T cells from SLE patients (38–41). A potential mechanism for the reduced Lck expression is its degradation due to increased ubiquitination. Lipid rafts, microdomains in the plasma membrane enriched in cholesterol, sphingomyelin, and glycosphingolipids, play important role in TCR signaling (42, 43). Lck localizes to lipid rafts, and accumulation of lipid rafts induces the increased phosphorylation and signal transduction (44, 45). Freshly isolated SLE T cells express higher levels of ganglioside M1 and cholesterol, a component of raft domain, and aggregated lipid rafts (46–48). Atorvastatin, which reduces cholesterol synthesis, restores Lck expression and lipid raft-associated aberrant signaling *in vitro* in T cells from patients with SLE (49). Atorvastatin also reduces the production of IL-10 and IL-6 by activated T cells (49).

Phosphorylation of ITAM residues of the TCR-CD3 complex molecules following antigen recognition by the TCR leads to the recruitment and activation of downstream signaling molecules such as adaptor proteins and enzymes. As described above, phosphorylated ITAMs of CD3ζ serve as a recruitment site for tyrosine kinase ZAP-70, a member of the Syk kinase family (50). It is unclear whether ZAP-70 expression levels T cells from SLE patients are comparable to those in T cells from healthy individuals (51) or decreased (52).

In addition to its role in T cell signaling, Syk is also an important molecule downstream of the B cell receptor. Expression levels of Syk and phospho (p)-Syk in B cells from active SLE patients are increased compared with controls (53). Therefore, Syk inhibitors are promising therapeutics. Fostamatinib, also known as R788 is a small molecule pro-drug of the biologically active R406 (54, 55), which selectively inhibits Syk. Inhibition of Syk by fostamatinib prevents disease development including skin and renal involvement in MRL/lpr and BAK/BAX lupus-prone mice, and the discontinuation of the treatment results in extended suppression of renal disease for at least 4 weeks (56). The administration of fostamatinib after the development of disease also improves kidney damage in New Zealand black/white (NZB/NZW) lupus-prone mice (57). Further studies are required to assess the efficacy of Syk inhibitors in patients with SLE.

Enhanced early T cell signaling events and heightened calcium responses lead to increased activation of calcineurin. Calcineurin dephosphorylate inactive cytoplasmic nuclear factor of activated T cells (NFAT) and dephosphorylated NFAT translocates to the nucleus. Increased recruitment of NFATc2 is observed in the nuclei of activated T cells from SLE patients after CD3 stimulation compared with those from controls, and it binds and activates the promoters of CD154 (CD40L) and IL2 genes (58). CD40-CD40L signaling is also important for the differentiation of Th17 cells (59). Expression of NFATc1 is elevated in lupus-prone MRL/lpr mice (60). Dipyridamole, an inhibitor of the calcineurin-NFAT pathway, reduces CD154 expression and improves nephritis in MRL/lpr mice (60). Calcineurin inhibitors cyclosporine and tacrolimus are widely used for the treatment of SLE. They are known to be effective in the treatment of lupus nephritis as both remission induction and maintenance therapy (61).

CD44-ROCK-ERM AXIS

CD44 is a cell surface glycoprotein involved in T cell activation, adhesion, and migration (62). Recent genome wide association studies (GWAS) have identified CD44 as a gene associated with SLE on meta-analysis of two SLE GWAS datasets by OASIS, a novel linkage disequilibrium clustering method (63). It was also reported that the expression levels of CD44 are increased in T cells from SLE patients (48, 64). The CD44 gene includes 10 variable (v) exons and there are numerous splice variants of CD44. CD44v3 and CD44v6 are expressed on T cells following activation (65, 66). The expression levels of CD44v3 and CD44v6 are increased and correlate with disease activity in patients with SLE (64).

The ezrin/radixin/moesin (ERM) proteins are important in linking plasma membrane proteins with actin filaments, and the interaction between ERM proteins and the intracellular domain of CD44 is associated with cell adhesion and migration function (67). T cells predominantly express ezrin and moesin (68). Moesin-deficient mice, which exhibit significantly lower levels of pERM (69), develop systemic autoimmune phenotype including glomerulonephritis (70), and exhibit reduced $CD8^+CD44^+$ $CD122^+Ly49^+$ regulatory T (Treg) cells and defects in the signal transducer and activator of transcription (STAT) 5 activation by IL-15, which is known to regulate the development of CD8 Treg cells. The levels of ERM phosphorylation are increased in SLE T lymphocytes, and forced expression of constitutively active ezrin enhances the adhesion and migration in normal T cells, suggesting that phosphorylated ERM is responsible for increased adhesion and migration of SLE T cells (48).

Rho associated protein kinase (ROCK) is a serine/threonine kinase that phosphorylates ERM. The ROCKs play important roles in migration, activation, and differentiation of T cells (71). ROCKs are a family of two serine-threonine kinases, ROCK1 and ROCK2, which exhibit a high degree of identity in their kinase domains (72). ROCKs regulate the activity of cytoskeletal components including ERM and cell migration. ROCK activity is important for chemokine-mediated polarization and transendothelial migration of T cells (73). Recently, it was reported that ROCK also regulates the interstitial T cell migration (74). In addition to its role in T cell migration, ROCK2 plays an important role in the differentiation of Th17 cells by activation of interferon regulatory factor 4 (IRF4) and controls the production of IL-17 and IL-21 (75, 76). ROCK2 signaling is also required for the induction of T follicular helper cells (Tfh cells) (77). Peripheral blood mononuclear cells (PBMC) from patients with SLE express significantly higher levels of ROCK activity as compared with healthy controls (78, 79).

In accordance with these results, ROCK inhibitors are candidates to be used for the treatment of patients with SLE. KD025 is a selective ROCK2 inhibitor (80), whereas Y-27632 (81), and Simvastatin are broad non-isoform selective ROCK inhibitors (82). Oral administration of KD025 to healthy subjects in a randomized phase I clinical trial, decreased the production of IL-17 and IL-21 from human T cells (76). KD025 also reduced the number of Tfh cells and autoantibody production in MRL/lpr mice (77). Y-27632 decreased serum levels of IL-6, IL-1β, and TNF-α and increased serum levels of IL-10 and Treg cell proportions in spleen cells from MRL/lpr mice, whereas the improvement of clinical manifestations was not shown in the paper (83). Rozo et al. demonstrated that each Y-27632, KD025 or simvastatin inhibits the increased ROCK activity in Th17 cells from SLE patients. These agents also decreased the production of IL-17 and IL-21 from SLE T cells or Th17 cells (79).

Fasudil, a pan ROCK inhibitor, has been approved for clinical use in Japan and China for the improvement of cerebral vasospasm after surgery for subarachnoid hemorrhage (71, 84). Fasudil decreases the production of IL-17 and IL-21 and improve disease including production of autoantibody and proteinuria in MRL/lpr mice (75), and NZB/W F1 mice (85). These results indicate that ROCK signaling is a promising therapeutic target for patients with SLE.

PHOSPHOINOSITIDE-3 KINASES (PI3Ks) AND PHOSPHATASE AND TENSIN HOMOLOG DELETED ON CHROMOSOME 10 (PTEN)

Class I PI3Ks, family members of lipid kinases, are classified as class IA and IB by activation mode. Class IA PI3Ks are activated by receptor tyrosine kinases including the TCR and costimulators, whereas Class IB PI3Ks are activated by G protein-coupled receptors such as chemokine receptors (86–88). Class I PI3Ks are composed of catalytic subunits p110 and regulatory subunits p85 or p87. There are three catalytic isoforms of Class IA PI3Ks (p110α, p110β, and p110δ), whereas only p110γ is a PI3K Class IB catalytic subunit. Compared with the ubiquitous expression of p110α and p110β, p110δ and p110γ are selectively expressed in lymphocytes (89). Class I PI3Ks phosphorylate PIP2 to form phosphatidylinositol-3,4,5-triphosphate (PIP3). Both Class IA and IB PI3Ks are expressed in leukocytes and play important roles in homeostasis, differentiation and function of T cells (88, 90, 91). PIP3 recruits phosphoinositide-dependent kinase 1 and activates Akt.

Phosphoinositide-3 kinase plays an important role in T cell differentiation (92). Transgenic mice expressing an active form of PI3K in T cells, $p65^{PI3K}$ Tg mice, develop lupus-like autoimmune phenotypes including kidney disease (93). Cleaved CD95 (Fas) ligand (CD95L/FasL) is increased in serum from patients with SLE and promotes cell migration through a c-yes/Ca^{2+}/PI3K signal (94). Class I PI3K signaling is activated in lymphocytes of MRL/lpr mice, and treatment with AS605240, a PI3Kγ selective inhibitor, reduces the severity of glomerulonephritis and prolongs lifespan in these lupus-prone mice, indicating an important role of PI3K signaling in SLE pathogenesis (95). Activation of PI3Kp110δ is enhanced in T cells from SLE patients, and the activation of PI3K pathway is associated with the defect of activation-induced cell death (AICD) in SLE T cells (96). PI3Kδ inhibition by GS-9289, a selective inhibitor of p110δ subunit, prolongs life span and reduces kidney damage in MRL/lpr mice (97), and general PI3K inhibition by Ly294002 rescues the AICD defect in T cells from SLE patients (96), suggesting that PI3K inhibitors may be potentially important drugs to treat patients with SLE.

Phosphatase and tensin homolog deleted on chromosome 10 dephosphorylates PIP3 and regulates the PI3K/Akt pathway (98). PTEN was originally reported as a tumor suppressor gene in 1997 (99–101), and T-cell-specific PTEN deficient mice exhibit increases in thymic cells and develop T-cell-derived lymphomas (102, 103). Treg-specific PTEN deficient mice show autoimmune phenotypes by loss of Treg function and stability (104, 105). On the other hand, the role of PTEN in Th17 cell differentiation is controversial. Overexpression of PTEN inhibits STAT3 activation and Th17 differentiation, and ameliorates the development of collagen-induced arthritis (106). By contrast, Th17-specific PTEN deficient mice exhibit impaired *in vitro* Th17 cell differentiation and mitigated symptoms of experimental autoimmune encephalomyelitis (107). PTEN deficiency increases the production of IL-2 and phosphorylation of STAT5, but reduces STAT3 phosphorylation, suggesting that further studies are required to

determine the exact role of PTEN in T cell differentiation and the activation of STAT signals.

There is limited evidence demonstrating how PTEN is associated with the pathogenesis of SLE. Overexpression of miR-148a-3p, which is increased in the glomeruli of patients with lupus nephritis, induces mesangial cell proliferation in glomeruli and reduces the expression level of PTEN (108). Also, SLE B cells exhibit decreased expression levels of PTEN, which inversely correlates with disease activity (109), whereas there is no clear evidence available to elucidate the role of PTEN in SLE T cells.

MECHANISTIC TARGET OF RAPAMYCIN (mTOR) PATHWAY

Mechanistic target of rapamycin, a ubiquitous serine-threonine kinase, integrates environmental cues from a variety of pathways to regulate various cellular processes including cellular survival, proliferation and differentiation, and cellular metabolism (110, 111). mTOR is a component of two distinct complexes, mTOR complex (C)1 and mTORC2. The components of mTORC1 are mTOR, regulatory protein associated with mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8) and inhibitory subunits proline-rich Akt substrate of 40 kDa and DEP domain containing mTOR-interacting protein (DEPTOR). mTORC2 also contains mTOR, mLST8, DEPTOR, whereas it is composed of rapamycin insensitive companion of mTOR (Rictor), instead of Raptor, and inhibitory subunits mammalian stress-activated protein kinase interacting protein 1 and Protor (protein observed with Rictor) 1/2 (112). mTORC1 phosphorylates two key effectors for protein synthesis; p70S6 kinase 1 (S6K1) and EIF4E binding protein, whereas mTORC2 phosphorylates serum- and glucocorticoid-induced kinase 1, Akt (Ser473), and PKC.

Mechanistic target of rapamycin plays an important role in cellular metabolism (113). mTORC1 increases the translation of the transcription factor hypoxia-inducible factor 1α, which induces glycolytic genes (114). Glycolysis is elevated in CD4+ T cells from lupus-prone (B6.*Sle1.Sle2.Sle3* mice and B6.*lpr* mice) and SLE patients (115, 116). mTORC1 also regulates both general autophagy and mitophagy, which are important in maintaining mitochondrial function (117). T cells from SLE patients exhibit increased mitochondrial mass and mitochondria dysfunction, characterized by elevated mitochondrial transmembrane potential (118, 119). Increased mitochondrial metabolism in SLE T cells can contribute to aberrant T cell function (111). Along these lines, normalization of $CD4^+$ T cell metabolism by mitochondrial metabolism inhibitor metformin and the glucose metabolism inhibitor 2-Deoxy-D-glucose reduced IFNγ production from $CD4^+$ T cells *in vitro* and suppressed autoimmunity and nephritis in B6.*Sle1.Sle2.Sle3* mice and NZB/W F1 mice (115).

Recent studies have proven the important role of mTOR in the polarization of T cells. Th1 and Th17 differentiation is selectively regulated by mTORC1 signaling (120), and the inhibition of mTOR *in vivo* reduces the proportion of Th1 cells and Th17 cells in the lamina propria and mesenteric lymph nodes (121). It is also reported that both mTORC1 and mTORC2 are essential for Tfh cell differentiation and germinal cell reaction under steady state and after antigen immunization and viral infection (122).

The role of mTOR in Treg differentiation is complicated. mTORC1 signaling is constitutively active in Treg cells and its disruption in Treg cells leads to profound loss of Treg suppressive activity, although mTORC1 does not directly impact the expression of Foxp3 (123). On the other hand, both mTORC1 and mTORC2 suppress induced-Treg generation *in vitro* (120, 124). PP2A activation induces the inhibition of the mTORC1 pathway but has no effect on the mTORC2 pathway, and Treg cell-specific ablation of the PP2A results in a severe systemic autoimmune disorder through Treg dysfunction (125).

Recently, it has been recognized that activation of the mTOR pathway plays an important role in the pathogenesis of autoimmune diseases including SLE (119). mTORC1 activity is increased in the livers of MRL/lpr mice (126). In SLE T cells, mTORC1 activity is increased while mTORC2 is reduced compared with T cells from healthy donors (127). Tuberous sclerosis complex (TSC), an autosomal dominant disorder, affects multiple organ systems resulting from mutations in either of TSC 1 or TSC2 genes, which negatively regulate mTORC1 activation (128). Singh et al. reported a fatal lupus patient complicated with TSC, suggesting that mTORC1 activation led to the development of unusually severe SLE (129). Therefore, mTOR has become a therapeutic target in SLE. Rapamycin, the best-known inhibitor of mTOR, has been approved by the FDA to preserve renal allografts (111). Recent studies have uncovered the effect of rapamycin on SLE T cells *in vitro*. Increased IL-17 expression in CD4+ T cells from SLE patients is suppressed and Treg cells are expanded by rapamycin (127, 130). SLE Treg cells exhibit increased mTORC1 and mTORC2, and IL21 stimulates mTORC1 and mTORC2 and blocks the differentiation of Treg cells (131). Rapamycin reduces both the activation of STAT3 and the number of IL-17 producing cells in patients with SLE (132), and decreases the severity of lupus nephritis and prolongs survival in MRL/lpr mice (133). There are reports of studies with small numbers of patients with SLE showing the efficacy of oral administration of rapamycin (22, 134). Importantly, the deficiency of the CD3ζ chain and upregulation of FcεRIγ chain and Syk in T cells from SLE patients *in vitro* are reversed by rapamycin treatment (22).

N-acetylcysteine (NAC), a precursor of glutathione, is another inhibitor of mTOR. A randomized double blind placebo-controlled study to assess the efficacy and the safety of NAC in SLE patients (135), demonstrated that 2.4 and 4.8 g daily NAC reduced disease activity and mTOR activity, reversed the expansion of CD3+ CD4-CD8- double negative (DN) T cells, and stimulated Foxp3 expression in $CD4^+CD25^+$ T cells. There are other reports showing the efficacy of NAC in SLE patients with lupus nephritis (136, 137).

Overall, mTOR inhibitors are accepted as a novel class of drugs that can target both cellular signaling and metabolism. To establish the efficacy of mTOR inhibitors in SLE patients and identify patients who respond to treatment, further studies with larger number of patients are necessary. Recently, results of a large prospective open-label, phase 1/2 trial of rapamycin (Sirolimus) in patients with active SLE were reported (138). During the course of 12 months of treatment, disease activity scores reduced in 16 (55%) of 29 patients treated with Sirolimus. Sirolimus treatment expanded Tregs and $CD8^+$ memory T cell populations and

inhibited IL-4 and IL-17 production by $CD4^+$ and DN T cells. Although this study is a single-arm study and placebo-controlled clinical trials with increased number of patients are required, the trial suggests that mTOR blockade may be a promising therapeutic target in the treatment of SLE.

CYTOKINE SIGNALING

Cytokines play critical roles in the proliferation, activation, differentiation, and function of T cells. The Janus kinase (JAK)–STAT signaling pathway following cytokine-receptor activation is one of the most important pathways used by multiple cytokines. In humans, seven STAT family members have been identified (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) (139). Different cytokines can activate specific STATs, and STATs regulate transcription of various genes including master regulators of differentiated T cell subsets. STAT1/STAT4 activate Tbet, the transcription factor which drives Th1 cell differentiation, STAT6 induces GATA3 in Th2 differentiation, STAT3 activates RORγt which activates IL-17 and Th17 differentiation, STAT3 induces Bcl6 transcription factor of Tfh cells, and STAT5 activates Foxp3 which drives Treg differentiation (140). STAT proteins are, therefore, essential for the establishment of lineage-specific enhancer landscapes of differentiating T cells (141). A number of studies have shown that STAT signaling plays a critical role in autoimmune diseases including SLE (142).

STAT1 and Interferons

A number of studies have revealed that IFNs play important roles in lupus pathogenesis (143, 144). The phosphorylation of STAT1, which is activated by all types of IFNs, is increased in MRL/lpr mice (145, 146). Consistent with these results, it was observed that the expression levels of STAT1 are increased in leukocytes from SLE patients (147–149). The expression levels of miR-145, a suppressor of STAT1, are decreased in T cells from SLE patients, and increased levels of STAT1 in human SLE T cells are associated with lupus nephritis (150). Recently, it was reported that the levels of STAT1 protein were increased in CD4 T cells from SLE patients and positively correlated with disease activity (151), and high STAT1 phosphorylation responses were observed in activated Tregs, which were decreased in peripheral blood from SLE patients. These results suggest that STAT1 can be a therapeutic target in SLE. However, the involvement of STAT1 in SLE is complex because STAT1 deficient lupus-prone mice exhibit interstitial kidney inflammation associated with Th17 cells, by shunting to STAT3/4 activation (152).

IL-23—STAT3—IL-17 Axis

Th17 cells produce the IL-17 cytokines IL-17A and IL-17F. Increased numbers of Th17 cells and increased levels of IL-17 have been found in patients with SLE and in lupus-prone mice (153–155). IL-17-producing cells have been found in kidney biopsies of patients with lupus nephritis (156) and in kidneys and spleen of MRL/lpr lupus-prone mice (157), and levels of IL-17 correlate with SLE disease activity (153). DN T cells are a key source of IL-17 in MRL/lpr mice (156, 157), and more importantly they are present in the kidney tissue of patients with lupus nephritis (156).

Recent studies have uncovered aberrant mechanisms associated with Th17 differentiation and IL-17 production in SLE T cells. IL-23, a member of the IL-12 family, is important for the maintenance of Th17 cells. Serum levels of IL-23 are increased in patients with SLE with high disease activity (158). IL-23 induces the activation of STAT3 (159–161). STAT3 directly binds the promoters of *IL-17A* and *IL-17F* (162), and T cell-specific deletion of STAT3 reduces IL-17 expression and impairs RORγt expression (163). STAT3 is upregulated and activated in both lupus-prone mice (164, 165) and T cells from patients with SLE (166, 167).

In addition to its role in Th17 differentiation, STAT3 is also important for the development of follicular helper T cells (Tfh cells), which induce the differentiation of germinal center B cells into memory and antibody-secreting cells (168). Tfh cells are expanded in both patients with SLE and lupus-prone mice (169). STAT3 also plays a role in the production of other cytokines including IL-10, which promotes B-cell proliferation and antibody production, and is elevated in the serum and kidneys of patients with SLE (167, 170–172). STAT3 was shown to promote IL-10 expression through trans-activation and chromatin remodeling of the *IL-10* locus in T cells from patients with SLE (167).

Therefore, STAT3 inhibitors could be promising therapeutic candidates to treat patients with SLE. Indeed, administration of a STAT3 inhibitor to MRL/lpr mice delays the onset of lupus nephritis in Ref. (173).

Janus kinase inhibitors are also promising therapeutic agents. JAK2 inhibitor AG490 suppressed the production of anti-histone/dsDNA antibodies in short-term culture (174). Tofacitinib is an oral JAK inhibitor, which inhibits JAK1, JAK3 (to a less extent), and JAK2, and has been approved for the treatment of rheumatoid arthritis. Tofacitinib improves disease activity of lupus-prone mice including nephritis, skin inflammation, and autoantibody production (175, 176). Baricitinib, another JAK inhibitor, is also under investigation for the treatment of SLE (177).

There are some reports indicating that IL-23 contributes to organ inflammation independent of its contribution to Th17 differentiation. IL-23 is important in the development of T cell-dependent colitis (178), yet IL-23-dependent colitis does not require IL-17 secretion by T cells, because $CD4^+$ $CD45RB^{hi}$ T cells cannot induce colitis in *Il23a*$^{-/-}$ Rag1$^{-/-}$ recipients even though intestinal IL-17 is unaffected by the absence of IL-23 (179). Furthermore, although IL-23 is not essential for the expression of Foxp3, IL-23 can have an indirect effect on Treg cell generation. IL-23 receptor deficiency in lupus-prone mice results in decreased production of anti-dsDNA antibodies and proliferation of DN T cells (180, 181). Interestingly, IL-23 not only promotes IL-17 production but also decreases the production of IL-2 by impairing the *Il2* gene enhancer NFκBp65 in mice (181). Also, IL-23 stimulation expands DN T cells from SLE patients *in vitro* (182). A phase IIa trial of Ustekinumab, targeting the p40 subunit common to IL-12 and IL-23, is underway in patients with SLE ((183). Inhibition of IL-23 signaling by an anti-IL-23p19 antibody ameliorates nephritis in MRL/lpr mice (184). Tildrakizumab (MK-3222), a monoclonal antibody targeting the p19 subunit, is under investigation for treatment of moderate-to-severe chronic plaque psoriasis (185, 186). Another monoclonal antibody targeting the p19 subunit, MEDI2070 (also known as AMG 139),

improved clinical activity of Crohn's disease in a phase IIa trial (187), although no data are available yet in patients with SLE.

There are other factors related to Th17 differentiation in SLE T cells. PP2A controls various signaling pathways, and CD4 T cells from transgenic mice that overexpress the catalytic subunit of PP2A in T cells produce increased amounts of IL-17 (188). The cAMP response element modulator (CREM) family of transcription factors also plays an important role in the differentiation of Th17 cells and IL-17 production. The suppressor isoform CREMα, which is increased in SLE T cells, reduces CpG-DNA methylation of the *IL-17A* locus, and controls IL-17A expression (189). Inducible cAMP early repressor (ICER), a transcriptional repressor isoform of CREM, is important for Th17 cell differentiation. ICER binds to the *IL-17A* promoter and enhances accumulation of the canonical IL-17 transcription factor RORγt (190). Calcium/calmodulin kinase IV (CaMKIV) is activated in T cells from SLE patients and MRL/lpr mice (191–193), and promotes the differentiation of Th17 cells and IL-17 production by activating the Akt/mTOR pathway (130). In MRL/lpr mice, genetic deletion of CaMKIV prolongs survival, and CaMKIV inhibitor KN-93 leads the suppression of nephritis and skin disease (192, 193). Moreover, as described above, ROCK is also associated with Th17 differentiation and production of IL-17 through the activation of IRF4 (75, 76).

Secukinumab and Ixekizumab are monoclonal antibodies targeting IL-17A while Brodalumab targets the IL-17A receptor, thus inhibiting the IL-17 signaling pathway. Although the evidence is clear for the efficacy and safety of these agents in the treatment of psoriasis and ankylosing spondylitis (194), there are no data showing efficacy of inhibition of IL-17 in SLE patients so far. Despite the overwhelming evidence that IL-17 contributes to lupus pathology, IL-17A deficiency in lupus-prone MRL/lpr mice or IL-17A neutralization in NZB/NZW mice did not affect the course of nephritis (195). Further work is needed to dissect the role of this signaling pathway in lupus pathogenesis in order to target it effectively.

STAT5 and IL-2

IL-2 is a key cytokine important in the proliferation, activation, and differentiation of T cells (196). Importantly, IL-2 plays a vital role in the homeostasis of Treg cells. Mice and humans deficient in IL-2, IL-2Rα (CD25), or IL-2Rβ (CD122) develop systemic autoimmunity due to impaired Treg cells (197–203). Also, IL-2 negatively regulates IL-17 production *in vivo* and *in vitro* (204, 205). In addition, IL-2 inhibits the differentiation of Tfh cells through the activation of Akt-mTORC1 signaling, and instead promotes the differentiation of Th1 cells (206). IL-2 also plays a critical role in the induction of AICD, a key process responsible for the deletion of autoreactive cells (207, 208).

It has been known for a long time that the insufficient production of IL-2 from T cells is one of the most important characteristic features of both SLE patients and lupus-prone mice (209–211). The molecular mechanisms of the decreased IL-2 production from SLE T cells have not completely been elucidated, whereas a number of studies have identified several mechanisms. Various transcription factors binding to the IL-2 promoter affect the expression of IL-2. NF-κB and activator protein 1 (c-fos/c-jun heterodimer) are downregulated in T cells from SLE patients, and linked to decreased IL-2 transcription (212–214). PP2A, a ubiquitous phosphatase, is increased in SLE T cells. PP2A dephosphorylates cyclic AMP-responsive element-binding protein 1, which can directly bind to the *IL-2* promoter and reduce IL-2 production (215). CaMKIV plays a role in the shortage of IL-2 in SLE T cells as well. CaMKIV is increased in SLE T cells, and phosphorylates CREM to suppress IL-2 transcription (191). As described above, it was recently reported that PTEN deficiency increases the production of IL-2 and phosphorylation of STAT5 (107), suggesting a novel mechanism of the IL-2 deficiency in SLE T cells, whereas the role of PTEN in SLE T cells remains unclear. SRSF1 is a multifunctional protein, which contributes to the transcriptional activation of IL-2. SRSF1 levels are decreased in T cells from SLE patients, and overexpression of SRSF1 into SLE T cells, rescues IL-2 production (34). It was demonstrated that increased expression of miR-200a-3p is associated with the decreased production of IL-2 through zinc finger E-box binding homeobox–C-terminal binding protein 2 in MRL/lpr mice (216).

Although the molecules that contribute to the decreased production of IL-2 can serve as therapeutic targets for the treatment of patients with SLE, strategies to restore IL-2 levels have been exploited. Recently, the safety and efficacy of low-dose IL-2 therapy for patients with graft-versus-host disease (217, 218), type 1 diabetes (219), and cryoglobulinemia associated with HCV infection have been reported (220). There are uncontrolled reports indicating the efficacy of low-dose IL-2 therapy in patients with SLE (221–223). Treatment of MRL/lpr lupus-prone mice with an IL-2-expressing recombinant adeno-associated virus resulted in reduced pathology, decreased DN cell numbers and increased Treg cell numbers (224). Subcutaneous injection of low-dose IL-2 on five consecutive days in a small number of patients with SLE, achieved decreases in SLE Disease Activity Index (SLEDAI) and increased peripheral Treg cells (221, 225). An uncontrolled study of 37 consenting patients with SLE claims that subcutaneous administration of recombinant IL-2 every other day for 2 weeks decreased SLEDAI, Th17, Tfh, and DN T cells, and increased Treg cell numbers (222). Further studies are required to overcome the challenges of maintaining IL-2 levels due to a very short half-life of the cytokine. It is important to note that not only the production of IL-2 by T cells from patients with SLE impaired, but also the response to exogenous IL-2 is impaired in CD4 T cells compared with healthy controls (226). These results suggest that we should also consider strategies to restore IL-2 sensitivity of T cells during low-dose IL-2 therapy. Indeed, the engagement of SLAMF3 in T cells from normal subjects and patients with SLE increased their IL-2-initiated signaling strength (227).

Transforming Growth Factor-β (TGF-β) Signaling

Transforming growth factor-β has three different isoforms (TGF-β1, 2, and 3), and regulates cell growth and differentiation. TGF-β signaling is essential for the differentiation of Treg cells. TGF-β signaling induces the expression of Foxp3 (228), and T cell-specific loss of TGF-β results in the defect in the differentiation of thymic Treg cells in mice (229). In addition, TGF-β also acts as a direct regulator against autoreactive T cells in part through the regulation of GM-CSF production (230, 231). Moreover, TGF-β

TABLE 1 | Signaling molecules as potential therapeutic targets for systemic lupus erythematosus (SLE).

Molecule	SLE patients	Mice	Targeting studies *in vitro*/*ex vivo*	Pre-clinical	Clinical
CD3ζ	Decreased	CD3ζ ko mice develop multi-organ inflammatory disease	Overexpression in SLE T cells restores Ca^{2+} flux and p-Tyr and IL-2 production		
Calcium/calmodulin kinase IV (CaMKIV)	Activated	Higher activity in T cells from MRL/lpr mice	Inhibition in SLE T cells decreases IL-17 production	Genetic depletion and inhibition with KN-93 are effective in MRL/lpr mice	
Spleen tyrosine kinase (Syk)	Increased	Syk is expressed in the skin lesion of MRL/lpr mice	Inhibition with R406 in SLE T cells	Syk inhibitor is effective in MRL/lpr, New Zealand black/white, and BAK/BAX mice	
Ezrin/radixin/moesin (ERM)	Increased phosphorylation	Moesin-deficient mice develop autoimmune phenotypes	Forced expression of active ezrin enhanced the adhesion and migration in T cells		
Rho associated protein kinase (ROCK)	Higher activity in peripheral blood mononuclear cells from SLE patients	Higher activity in T cells from MRL/lpr mice	Inhibition with ROCK inhibitor in SLE T cells	ROCK inhibitor reduces autoantibodies and proinflammatory cytokine production in MRL/lpr mice	
Calcineurin-nuclear factor of activated T cells (NFAT)	Increased nuclear recruitment/activation of NFATc2	Elevated NFATc1 in MRL/lpr mice			Calcineurin inhibitors widely used
Phosphoinositide-3 kinase (PI3K)	PI3Kp110δ is activated	Activated in T cells from MRL/lpr mice	PI3Kδ inhibitor restores activation-induced cell death in SLE T cells	p110δ inhibitor is effective in MRL/lpr mice	
Mechanistic target of rapamycin (mTOR)	mTORC1 activity is increased, and mTORC2 is decreased	mTORC1 is activated in the livers of MRL/lpr mice		Rapamycin is effective in MRL/lpr mice	Rapamycin is effective, and clinical trial is ongoing

also contributes to the differentiation of Th17 cells (232), whereas Th17 cells also can be generated without TGF-β signaling but with IL-6, IL-1β, and IL-23 (233).

The role of TGF-β in SLE patients remains unclear. It was reported that serum levels of TGF-β are decreased in active SLE patients (234, 235). On the other hand, some reports demonstrated that TGF-β1 production is increased from SLE PBMC (236). Impaired response of peripheral blood cells to TGF-β1 in patients with active SLE has been reported (237). $CD4^+CD25^-Lag3^+$ Treg cells expressing early growth response gene (Egr)2 and Egr3 exhibit immune suppressive capacity by secreting TGF-β3, and mice with T cell-specific deletion of Egr2/3 mice develop lupus-like disease (238, 239). Further studies are required to uncover the role of TGF-β in the pathogenesis of lupus.

CONCLUSION

A great effort has been made to delineate specific abnormalities in immune cells from SLE patients, and a dramatic expansion has been achieved in our understanding of cellular and molecular phenotypes in the pathogenesis of SLE. Here, we have reviewed the important features of aberrant signaling pathways in SLE T cells. T cells have a vital role in the immune response, whereas other immune cells such as B cells, dendritic cells, macrophages, and neutrophils cannot be ignored in the development of autoimmune diseases. Abnormal activation of the TCR and PI3K-Akt-mTOR signaling pathways and various molecules including PP2A, CaMKIV, CD44, ROCK, mTOR, and SRSF1 affect the function and the differentiation of T cells. Moreover, aberrant cytokine production and the activation of JAK–STAT pathways are also involved in the differentiation of pathogenic effector T cells and impaired Treg cells. In addition to the aberrant pathways described above, alterations in metabolism of immune cells have been recently recognized in patients with autoimmune diseases (113, 117).

Clinical manifestations including symptoms, severities, and clinical response are extremely variable in SLE patients, indicating that no single mediator or pathway can account for the complex pathogenesis. For example, decreased expression levels of CD3ζ are found in many but not all SLE patients (240). The more we understand and elucidate cellular and molecular aberrations in SLE, the more we realize the complexities of the pathogenesis of SLE. However, each aberration has the possibility to be a promising therapeutic target (**Table 1**). In addition, the analysis of various molecular phenotypes may contribute to patient stratification leading the development of more personalized strategies in SLE treatment.

AUTHOR CONTRIBUTIONS

TK, VM, and GT conceptualized the article, reviewed the literature, and wrote the manuscript.

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REVIEW
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Renal Involvement in Antiphospholipid Syndrome

*Alonso Turrent-Carriles[1], Juan Pablo Herrera-Félix[2], Mary-Carmen Amigo[1]**

[1] *Internal Medicine Rheumatology Service, Centro Médico ABC, Mexico City, Mexico,* [2] *Nephrology Department, Centro Medico ABC, Mexico City, Mexico*

Antiphospholipid syndrome is a complex autoimmune disease, characterized by the presence of vascular thrombosis, obstetric, hematologic, cutaneous, and cardiac manifestations. Renal disease in patients with antiphospholipid syndrome was not recognized in the first descriptions of the disease, but later on, the renal manifestations of the syndrome have been investigated widely. Renal manifestations of antiphospholipid syndrome conform a wide spectrum of diverse renal syndromes. Hypertension is one of the most frequent, but less commonly recognized renal alteration. It can be difficult to control as its origin is renovascular. Renal vascular thrombosis can be arterial or venous. Other alterations are renal infarction and vascular thrombosis in arterial territories. Venous thrombosis can be present in primary and secondary antiphospholipid syndrome; it presents with worsening of previous proteinuria or *de novo* nephrotic syndrome, hypertension and renal failure. Antiphospholipid syndrome nephropathy is a vascular disease that affects glomerular tuft, interstitial vessels, and peritubular vessels; histopathology characterizes the renal lesions as acute or chronic, the classic finding is thrombotic microangiopathy, that leads to fibrosis, tubule thyroidization, focal cortical atrophy, and glomerular sclerosis. Antiphospholipid syndrome nephropathy can also complicate patients with systemic lupus erythematosus, and there is vast information supporting the worse renal prognosis in this group of patients with the classic histopathologic lesions. Treatment consists of anticoagulation, as for other thrombotic manifestations of antiphospholipid syndrome. There is some evidence of glomerulonephritis as an isolated lesion in patients with antiphospholipid syndrome. The most frequently reported glomerulonephritis is membranous; with some reports suggesting that immunosuppressive treatment may be effective. Patients with end stage renal disease commonly are positive for antiphospholipid antibodies, but it is not clear what is the role of aPL in this setting. Patients with vascular access may have complications in the presence of antibodies so that anticoagulation is recommended. Patients ongoing renal transplant with persistent antiphospholipid antibody positivity may have early and late graft failure.

Keywords: antiphospholipid syndrome, systemic lupus erythematosus, renal disease in antiphospholipid antibody syndrome, antiphospholipid antibody syndrome nephropathy, renal thrombotic microangiopathy

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****Correspondence:***
Mary-Carmen Amigo
marycarmenamigo@gmail.com

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INTRODUCTION

Antiphospholipid antibody syndrome (APS) is a complex autoimmune systemic disease, characterized by the presence of circulating antibodies directed against anionic phospholipids, and the proteins bound to them (aPL) in the serum of patients with thrombosis or pregnancy complications. There are classic manifestations of APS, including thrombosis involving arterial and venous territories and

obstetric morbidity, that are considered as classification criteria (1). Moreover, there are many other manifestations of APS, the "non-criteria" manifestations that include livedo reticularis, hematologic manifestations (thrombocytopenia and hemolytic anemia), cardiac valve disease, and renal involvement.

Renal involvement was not mentioned in the first description of APS (2). Kidney compromise in APS represents a vast and complex myriad of syndromes that are a consequence of the vascular dysfunction and the coagulation dysregulation characteristic of the syndrome. Kidney disease associates with aPL is not an inflammatory condition in contrast with lupus nephritis. Recently, many groups are interested in this frequent complication of APS (3–5).

All the vessels, veins, and arteries, from the renal arteries to the glomerular tuft capillaries can be involved. **Table 1** shows the renal syndromes that are related to APS.

The real prevalence of renal involvement in APS is very difficult to establish, mainly due to the limitation of histopathology research, biopsy contraindications, and its association with lupus (SLE). Retrospective series have mentioned a prevalence of 9–10% (6), but in series where APS renal disease has been intentionally studied the prevalence ranges from 10–40% (7–9).

Hypertension

Hypertension is a fairly common health problem in the adult population. Depending on the definitions used for classifying patients with high blood pressure (JNC8 or ACC/AHA 2017), 32–46% of adults has hypertension (7, 8). According to the last ACC/AHA definitions, a normal blood pressure is <120/<80 mmHg, elevated blood pressure 120–129/<80 mmHg, stage 1 hypertension 130–139/80–89 mmHg, and stage 2 hypertension >140/>90 mmHg (10, 11).

Since the initial descriptions of APS, hypertension was one of the frequent signs related to the disease. Hughes in 1983, described patients with livedo reticularis in association with elevated blood pressure, suggesting a renovascular etiology. In 1986, he described a group of patients with APS and hypertension, which ranged from mild elevation to malignant hypertension (2, 12).

The etiology of the elevated blood pressure within this group of patients is thought to be renovascular in origin, since there are case reports (13, 14), and series where intrarenal vascular lesions demonstrated in biopsies, were the only physiopathologic explanation. In a large series of renal biopsies in patients with APS, Nochy et al. found systemic hypertension on 93% of their patients, this being the most common clinical manifestation of APS nephropathy (APSN). Hypertension was severe in 31% of the patients and malignant in 12% (15). Taking into account the high prevalence of hypertension in APSN, elevation of blood pressure is considered one of the most important signs that suggest renal activity.

Some studies have suggested that the presence of aPL is related directly to hypertension. Rollino et al. compared healthy controls with matched hypertensive patients and patients with renal artery stenosis, finding that 8% of the patients with essential hypertension had aPL vs. none of the healthy controls (16). Frostegard et al. analyzed the presence of anti-B2GP1 in patients with hypertension vs. matched controls. They found that the presence of IgG aPL correlated with elevated levels of insulin, insulin-like growth factor binding protein-1, and more insulin resistance, suggesting that patients with anti-B2GP1 have more or are prone to develop more atherosclerotic lesions and higher blood pressure (17).

Hypertension in patients with APS may be very difficult to treat, taking into account that its exact nature is only partially understood. Nowadays, the best treatment for these patients is anticoagulant therapy and optimal blood pressure control with antihypertensive drugs. With this combination, the progression to end stage renal disease (ESRD) could be delayed or even prevented (18). Treatment with prednisone has also anecdotically been reported with good results.

TABLE 1 | Renal involvement in antiphospholipid antibody syndrome (APS).

a) Hypertension
b) Renal artery stenosis, thrombosis, and infarction
c) Renal vein thrombosis
d) Intrarenal vasculopathy [APS nephropathy (APSN)]
e) Glomerular disease
f) APS in kidney transplant
g) APS in end stage renal disease and hemodialysis
h) APSN in patients with systemic lupus erythematosus
i) APSN in catastrophic APS

Renal Artery Thrombosis, Stenosis, and Renal Infarction

Renal artery disease is an infrequent but well recognized manifestation with important consequences in APS patients. Since 1990 when Ostuni et al. reported for the first time the occurrence of renal artery thrombosis and hypertension in a young patient with anti-phospholipid antibodies (19), many similar cases have been published (20–23). Renal artery disease can present as renal infarction, ischemic acute renal failure, slowly progressive chronic renal failure, or renovascular disease. The most common clinical picture in this group of patients is the new onset of severe hypertension or given the case worsening of a previously documented and controlled hypertension. Other clinical features are lumbar pain, localized around the renal area, hematuria, or renal failure.

Sangle et al., in an elegant study, reported two different patterns of arterial disease in APS patients. With magnetic resonance angiography, they visualized the renal arteries of 77 APS patients with resistant hypertension and compared them with the arteries of young hypertensive patients and healthy controls. In the APS group, 20 (28%) of the patients had renal artery lesions, compared with 8% in young hypertensive patients and 3% of healthy controls. Moreover, they reported two different kinds of renal artery lesions. The first and more common one was characterized by a smooth, well delineated, non-critical stenosis localized distal to the ostium of the renal artery. The second was similar to the common atherosclerotic lesions presented in other metabolic and chronic diseases. They were located proximal to the ostium and may involve the aorta (24). In APS, there is accelerated atherosclerosis and could be associated with renal lesions (25). Vasoconstrictive mechanisms observed in APS are related to high endothelin-1 levels (26).

Different imaging studies have been useful to visualize the arterial alterations, including renal ultrasound with Doppler, abdominal CT, gadolinium magnetic resonance angiography,

renal angiography, and renal scintigraphy have proved their usefulness. Ultrasound may be the first screening method, followed by CT or magnetic resonance. An algorithm approach has been proposed (27).

In patients presenting with APS, renal artery disease, and hypertension, treatment based on anticoagulant therapy requires a concomitant strict blood pressure control. On a retrospective study, Sangle et al. analyzed blood pressure response of patients receiving anticoagulant therapy. Patients with higher INR (>3.0) had better blood pressure control, maintained renal function, and arterial lesions reversed in some patients (28). Blood pressure and renal function maintenance during anticoagulant therapy suggest a thrombotic etiology for the arterial lesions presented in most patients with APS. Some studies have demonstrated successful thrombolysis and balloon angioplasty; however, anticoagulation was used in all patients.

Renal infarction, another manifestation of the APS is caused either by *in situ* thrombosis affecting renal arteries, infrarenal aorta, smaller diameter intraparenchymal vessels, or due to embolic disease from a pre-existing upstream arterial lesion or altered cardiac valves (29–31).

Renal infarcts are characterized clinically by intense lumbar pain that may be unilateral or bilateral, accompanied by hypertension and acute renal injury. Renal infarction may be the initial clinical manifestation of APS (32, 33).

The histological features are glomerular ischemia, tubular atrophy, and interstitial fibrosis. Histological changes consistent with thrombotic microangiopathy (TMA) are not present in this subgroup (29, 34).

Subclinical cases have also been reported as an incidental finding when on CT scans performed with other purpose, images revealed an old silent infarct. It is suggested to perform antiphospholipid testing only in young patients with no other identified cause of subclinical renal infarction.

Patients with SLE that are stable with treatment, mainly hydroxychloroquine, at the moment of pharmacologic withdrawal may have this complication (35).

Renal Vein Thrombosis

Patients with primary APS or more frequently patients with SLE and APS may present renal vein thrombosis. Thrombosis may involve the inferior vena cava or the main and minor renal veins.

Asherson et al. published the first reported cases, describing two patients with SLE and proliferative nephritis with nephrotic syndrome and positive lupus anticoagulant (36). Glueck et al. reported three cases of renal vein thrombosis in patients with SLE and positive lupus anticoagulant (37). These studies associated renal vein thrombosis with the presence of lupus anticoagulant.

The most common clinical manifestation of renal vein thrombosis is nephrotic range proteinuria, and occasionally, renal failure when thrombosis is bilateral.

Sudden onset or acute worsening of nephrotic range proteinuria should make the clinician suspect this complication. Doppler of renal vasculature enhanced CT or MRI are the studies recommended to confirm or rule out this complication (31, 38, 39). Other causes of renal vein thrombosis like pregnancy, oral contraceptive use, extrinsic compression, trauma, and other nephrotic syndrome causes, should also be evaluated (30).

Intrarenal Vascular Lesions: APSN

The APSN is considered a vascular nephropathy that can present acutely or chronically. Patients with primary and secondary APS have shown the classic histopathologic lesions of APSN.

D'Agati et al. published the first reports in 1990, who described three patients, two with primary APS and one with SLE who had acute TMA on renal biopsy (40). Becquemont and coworkers reported one case of renal microangiopathy associated with anticardiolipin antibodies (41).

Amigo et al. described the correlation between the clinical characteristics and the pathologic findings in five patients with primary APS and renal involvement. All the patients had hypertension, three had mild renal impairment, and two had ESRD requiring renal substitution therapy (hemodialysis). Renal biopsies were consistent with TMA, with acute and chronic vascular lesions (**Figures 1**–**3**). Subendothelial fibrosis and arteriolar luminal narrowing were also found (42).

In 1999 Nochy et al. studied retrospectively 16 patients with primary APS and renal involvement, all of them had a previous renal biopsy (15). The following histologic lesions are the ones described and supported the actual definition of APSN:

- Arteriosclerosis characterized by fibrous intimal thickening with luminal reduction of arcuate and interlobular arteries, associated with arteriolar hyaline and arteriolosclerosis.
- Fibrous intimal hyperplasia (FIH) whose characteristics are thickened intima and intense myofibroblastic intimal cellular proliferation in interlobular arteries and their branches. The media shows proliferative changes with hypertrophic myocytes or atrophic and fibrous changes.
- Fibrocellular and arteriolar occlusion in small diameter interstitial arteries.
- TMA that commonly affects preglomerular arterioles, small interlobular arteries, and glomerular capillaries. The histologic pattern is non-inflammatory with occlusion of vessel lumen

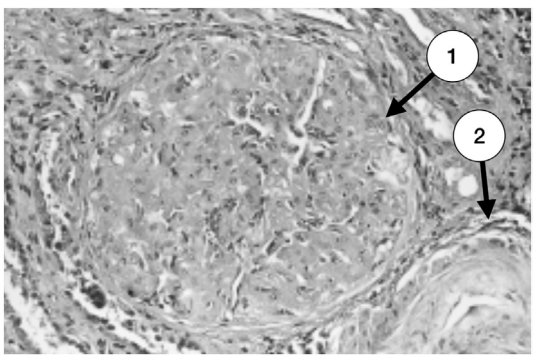

FIGURE 1 | Glomerulus with severe and advanced thrombotic microangiopathy. Capillary lumina are occluded by mesangiolysis and heterogeneous subendothelial deposits (1). An arteriole with a great deal of lucent subendothelial deposits is partially seen at the right inferior corner (2) (with permission of the publisher).

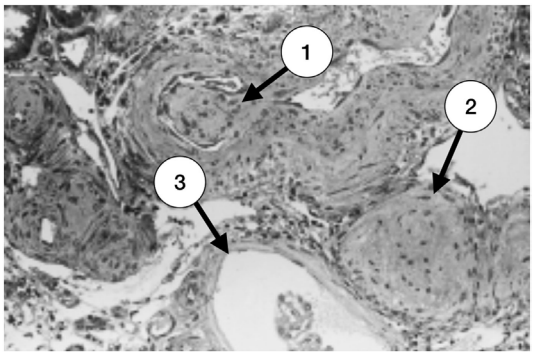

FIGURE 2 | Late stage of thrombotic microangiopathy in a small arteriole. There is fibrotic medial hyperplasia and the lumen is irregular. A fibrotic intraluminal "cushion" caused by a mural thrombus organization is shown in (1). There are also fibrohyperplastic arterioles and two glomeruli; one is ischemic (2) and the other, fibrotic (3) (with permission of the publisher).

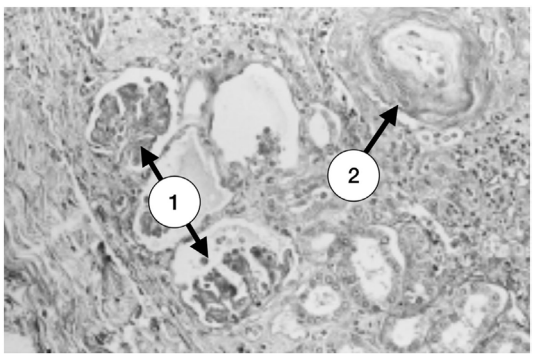

FIGURE 3 | A small focus of superficial ischemic cortical atrophy. There are several ischemic glomeruli with tuft retraction and Bowman's space enlargement (1). At the right upper corner a small vessel with a great amount of a subendothelial clear material and marked narrowing of the lumen is seen (2) (with permission of the publisher).

by red blood cell fragments, leukocytes, and eosinophilic fibrinoid material. When analyzed by immunofluorescence fibrin is the only material of the thrombi and immunoglobulins are absent.

- Vasculitis is typically absent.
- Focal cortical atrophy (FCA) involves superficial cortex under the renal capsule, disposed of as foci or triangles, with depression of the contour of the renal capsule. All of the elements of the renal parenchyma can be affected, creating lesions that are typical of APSN. The glomeruli can appear sclerotic or pseudocystic and voluminous. The immunofluorescence reveals fibrin and sometimes C3 and IgM deposits in the vessels showing thickened cellular intima. Renin can be found in the juxtaglomerular apparatus and the wall of the interlobular arteries.
- Tubular thyroidization is characterized by zones with tubular atrophy containing eosinophilic casts that resemble thyroid tissue. These zones are frequently found in the deep cortex or medulla.

Classically, acute vascular lesions are secondary to TMA, and the other histologic patterns are chronic. The typical histological features of APSN are the combination of TMA with chronic lesions. Other ultrastructural changes are typical of APSN including mainly glomerular basement membrane wrinkling and reduplication (42, 43).

It is important to recognize that none of the histological patterns is pathognomonic of APSN, since the lesions can be present in malignant hypertension, scleroderma renal crisis, HIV, cyclosporine use, chemotherapy, preeclampsia, and thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS) (7, 44). The distinction between APSN and TTP/HUS can be made by the presence of schistocytes on blood smear, severe thrombocytopenia, negative aPL, and lack of microcirculatory thrombosis, which are characteristics of TTP/HUS and not of APSN (45).

The clinical features of APSN are hypertension (generally severe), acute or chronic renal injury, proteinuria (mild to nephrotic), and hematuria (31).

Treatment of APSN includes antihypertensive agents aiming strict control of blood pressure, oral anticoagulation with vitamin K inhibitors, and there are some small series addressing the benefit of immunosuppressive therapy in APSN (46, 47). Korkmaz et al. reported benefit treating patients with steroids, azathioprine, and cyclophosphamide with good response (46). A phase II trial with rituximab (RITAPS) showed efficacy in two cases with APSN (48), but further information regarding this topic is needed. The use of C5a inhibition with eculizumab may be an option in patients with TMA, but more information is needed to support its recommendation.

The prognosis of APSN is variable with a high prevalence of chronic hypertension reported in most series. Proteinuria, nephrotic syndrome, chronic renal failure, or ESRD may also occur.

Catastrophic APS (CAPS) is a very rare (<1%) and extremely severe variant of APS. It is characterized by multiple systems and thrombotic organ involvement that occurs in a very short period (days to weeks). Renal involvement is a common feature in CAPS, the most frequent finding is TMA, but other chronic lesions of APSN can also be found (49). The treatment of CAPS includes high dose steroids, anticoagulation, IV Immunoglobulin, and plasma exchange. In patients with CAPS associated with SLE, cyclophosphamide may be effective. Moreover, eculizumab has been succesfully used in few cases.

Glomerular Involvement in APS

Besides the classic APSN that typically consists of vasculopathy and intrarenal thrombosis, there is enough evidence that other clinical and histopathologic patterns can be present in patients with APS.

Fakhouri et al. in 2003 retrospectively studied the pathologic patterns of 29 renal biopsies of patients with primary APS and no evidence of another autoimmune disease. In this study, 20 biopsies had findings characteristic of classic APSN, and the other nine biopsies had different patterns that included three membranous glomerulonephritis, two with mesangial C3 nephropathy, two with minimal change disease, one with focal segmental glomerulosclerosis, and one biopsy had mixed changes consistent with

pauci-immune vasculitis and focal segmental glomerulosclerosis. Seven cases had a subacute or chronic clinical course, and two of them presented acute renal failure. All cases had relevant proteinuria and five patients presented nephrotic syndrome (50).

Sinico et al., studied retrospectively 160 APS patients demonstrating renal involvement in 14 (8.7%) patients. Ten patients underwent renal biopsy, four of them had membranous glomerulonephritis, two had diffuse proliferative glomerulonephritis, and the other four had classic pathologic findings consistent with APSN. Patients with membranous glomerulonephritis had lower levels of complement. None of the patients developed SLE on follow up (6).

Membranous glomerulonephritis is the most frequently reported glomerular disease in APS in different series and case reports (6, 37, 40, 50–55). Quereda et al. analyzed the frequency of aPL in different non SLE nephropathies, finding aPL in 20% of the patients with membranous nephropathy, 2 of them fulfilling classification criteria for APS (56).

Even though glomerulonephritis is infrequent in patients with APS, there is enough evidence and information that this kind of renal disease is related with APS, and they should be taken in account when analyzing renal biopsies from patients with APS. There are no studies regarding the treatment in this group of patients, probably the best treatment is a combination of immunosuppressive drugs with anticoagulation, but studies are needed to support the recommendation.

APSN in Patients With SLE

Patients with SLE can have persistent positivity to aPL, with a prevalence of 15–60% depending on the series. However, only 30% of them have APS. Patients with aPL in SLE commonly have a history of thrombosis, obstetric morbidity, and hematologic alterations.

Considering APSN as a renal dysfunction caused primarily by capillary thrombosis, FIH, FCA, or TMA, Kant and Glueck reported higher glomerular capillary thrombotic lesions initially in SLE patients with positive aPL compared with patients with negative aPL (37, 57). The prevalence of APSN in SLE varies between 11% to more than 50%, but most series are retrospective, and the pathologists used different criteria to define the presence of APSN on SLE renal biopsies (7, 9, 58, 59).

Vascular thrombotic lesions that are typical of APSN can be isolated or associated with the classic lesions of lupus nephritis. The clinical manifestations in patients with APSN in SLE are hypertension, nephrotic syndrome, and renal dysfunction.

Most studies have reported poor renal prognosis in patients with SLE and coexisting APSN. However, Naiker et al. reported a high prevalence of aPL in patients with SLE nephritis but did not found worse renal prognosis (60).

One of the most relevant studies addressing prognosis, analyzed prospectively 111 patients with SLE nephritis followed for 14 years. 26% of the patients were aPL positive, and those patients had a poor renal outcome, higher creatinine levels, and higher chronicity index on biopsy (52).

Bhandari et al., in a cohort study, found a relevant association of positive aPL and a higher prevalence of crescentic, sclerotic, and glomerular necrosis in renal biopsies of SLE patients, supporting the worse prognosis conferred by aPL (61).

Tektonidou et al. studied the natural history of APSN performing repeated renal biopsies. They found the progression from acute capillary thrombosis to chronic obstructive and fibrotic lesions. TMA was followed by chronic lesions, such as FIH, FCA, or sclerotic lesions. Since the evolution to chronic lesions conferred a worse prognosis, it is extremely important to recognize the acute histologic findings in an early period (7).

A recent study by Barrera-Vargas et al. compared renal function outcome between SLE patients with TMA associated with lupus nephritis and patients with isolated lupus nephritis. The authors did not find an association with positive aPL. However, patients with TMA had worse renal prognosis (62).

As patients with SLE and APSN tend to have a worse prognosis, it is crucial to document the presence of APSN in a kidney biopsy. A renal biopsy must be done with great caution because these patients have an increased risk of bleeding after the procedure (63). When SLE nephritis predominates, immunosuppressive therapy with mycophenolate or cyclophosphamide must be used, and when APSN is found, anticoagulant therapy must be added.

ESRD and Renal Transplantation

Progression to ESRD is an uncommon course in patients with APS. Erkan et al. in a prospective study that included 39 patients with APS found that only 1 patient developed ESRD during the 10 years follow up (64). Other studies have investigated this relationship (35). Sinico et al., studied retrospectively 160 APS patients, and only 1 developed ESRD (6). Amigo et al. studied 20 consecutive primary APS patients finding acute and chronic TMA lesions in renal biopsies of 5 patients. Two of these five patients presented ESRD (42). This poor renal outcome is uncommon in children with APS (65).

Patients with ESRD independently of its cause have a higher frequency of aPL positivity compared with the general population. Different studies have assessed these findings (18, 66–71), but the patient characteristics and antibody assays were different in each study.

The proposed mechanisms to explain aPL positivity in patients with ESRD are: uremia as an altered immunogenic state predisposing to autoimmunity (72), antibody induction by dialysis membrane incompatibility (71), blood trauma generated in hemodialysis circuits (73), and, induction by microbial products like endotoxins present in dialyzate (71). However, there is no explanation why only a few patients using the same membranes, different length of time on dialysis or using the same dialyzate develop antibody production. The type of vascular access may have a role as suggested by the incidence of a higher prevalence of aPL in patients who use a AV graft (22%) vs. AV fistula (6%); even further, vascular access failure was increased significatively in patients with AV grafts and higher aPL titers (74). The presence of these antibodies has not been associated with demographic features, length of time on dialysis, sex, drugs, or chronic B and C hepatitis. aPL generated have been found to be β2-gycoprotein-1 independent, and their clinical relevance are still unclear (75).

Some authors have not found a relevant clinical relationship or the pathogenic role of antibodies in ESRD (70, 76), but others have found a worse outcome and prognosis in patients with positive aPL (77–79).

Patients with antiphospholipid antibodies that undergo renal transplantation are at risk of thrombosis at any site and graft failure (79–81). McIntyre et al. reported that transplant patients that had positive aPL before the transplant presented a higher rate of graft failure (79). When compared patients who had an early kidney graft failure vs. patients with functioning grafts, the number of patients who had positive aPL were more prevalent in the graft failure group. The histopathologic pattern in patients with APS and graft failure is characterized by thrombotic features and graft infarction (82, 83). Treatment with anticoagulation is not completely preventive for graft loss (79). One report presented good outcomes in patients receiving a renal graft using preoperative immunosuppressive therapy and anticoagulation (84). Therapy with mTOR inhibitors can also be an option that can be used in this group of patients (85).

PATHOPHYSIOLOGY OF APS

Clinical studies have shown a strong association of aPL with thrombosis and obstetric morbidity.

Thrombotic APS

The mechanisms by which aPL cause thrombosis are not completely understood. The underlying pathogenic mechanisms came from animal studies demonstrating that aPL activate endothelial cells, platelets, monocytes, neutrophils, fibroblasts, and trophoblasts. Cellular activation is key in thrombotic APS.

Cellular Activation

In platelets, aPL induce expression of thromboxane B2 and fibrinogen receptor glycoprotein IIb/IIIa, resulting in platelet aggregation (86).

In APS there are signs of endothelial activation. aPL can activate endothelial cells to express tissue factor (TF) and adhesion molecules (87, 88). A possible surrogate for endotelial activation is the finding of endothelium-derived microparticles in the circulation of patients with APS (89).

APS patients have increased monocyte TF expression and increased levels of monocyte-derived microparticles, a possible important source of TF (90). TF is the major initiator of coagulation *in vivo*, thus, may be one of the most important contributors to thrombosis.

Neutrophils have recently received attention in APS as they are activated by aPL and release neutrophil extracelular traps (NETs). NETs are composed of chromatin and antimicrobial proteins coming from neutrophils in response to both inflammation and infection. NETs activate platelets and the coagulation cascade and can serve as scaffolding upon which a thrombus can assemble (91). APS patients have elevated levels of low-density granulocytes, a subpopulation of granulocytes that release NETs in exaggerated fashion (92). Moreover, APS patients have impaired ability to degrade NETs (93).

Cell Receptors and Signaling Pathways

It has been demonstrated that cell surface receptors that interact with aPL and/or B2GP1 include annexin A2, ApoER2, and TLRs. The intracelular signaling pathways p38MAPK, and subsequent nuclear translocation and activation of NFkB in endothelial cells and monocytes mediate thrombosis in APS (94). On platelets, the main receptors that bind B2GP1/aB2GP1 complexes that induce activation include ApoER2 and glycoprotein Iba. The main signaling pathway is p38MAPK with minor roles of the ERK-1, ERK-2, and phosphatydlinositol 3-kinase/Akt (95).

Recently, it demonstrated the activation of the mammalian target of rapamycin complex pathway in the vascular endothelium of intrarenal vessels from patients with APSN and in the vessels of autopsy specimens from patients with CAPS (96).

Complement Activation

Complement activation has a pathogenic role in thrombotic APS (97). Complement activation amplifies coagulation and inhibits fibrinolysis, through C5a, inducing expression of TF and plasminogen activator inhibitor 1 (98).

Coagulation Pathways

aPL affect hemostasis at multiple levels. In addition to cellular activation, upregulation of coagulation and inactivation of fibrinolysis are well known mechanisms of thrombosis in APS. Upregulation of TF (99), resistance to activated protein C (100) and complement activation (98) are important mechanisms in aPL-induced thrombosis.

Nitric Oxide

Nitric oxide (NO) is a key signaling molecule for the maintenance of normal vascular funtion. Oxidative stress dysregulate the eNOS system which produces superoxide species contributing to vascular dysfunction. In patients with APS, decreased bioavailable NO and increased oxidative stress have been demostrated (101).

Obstetric APS

The pathogenesis of obstetric APS remains uncertain. Intraplacental thrombosis was thought to be the main pathogenic mechanism of fetal loss. However, placental thrombosis or infarction was not observed in all the cases. There is evidence that aPL impair trophoblastic invasion and human chorionic gonadotrophin production leading to miscarriages, fetal loss, and placental insufficiency (102). Mechanisms relevant to obstetric complications include activation of the complement system with secondary inflammation (103, 104), defective placentation due to interference of anti-B2GP1 with trophoblasts growth and differentiation, and displacement of annexin A5 by aPL-B2GP1 complexes (105).

CONCLUSION

The kidney is a major target organ in APS. APSN occurs in primary, secondary, and CAPS. It is characterized by vascular compromise involving large, medium, and small vessels including capillaries. Because clinical features are diverse and not pathognomonic, all physicians treating APS or related diseases need to be aware of these complications. Early recognition and treatment are essential to prevent a poor outcome. The recommended treatment is anticoagulation and tight blood pressure

control. In patients who are difficult to treat refractory disease, IGIV, rituximab, or eculizumab could be considered.

AUTHOR CONTRIBUTIONS

AT-C, JPH-F, and M-CA contributed to the design of this review. AT-C wrote the first draft of the manuscript. AT-C, JPH-F, M-CA wrote sections of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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REVIEW
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Cellular and Molecular Mechanisms of Anti-Phospholipid Syndrome

Marko Radic[1] and Debendra Pattanaik[2]*

[1] *Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, United States,* [2] *Division of Rheumatology, Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, United States*

The primary anti-phospholipid syndrome (APS) is characterized by the production of antibodies that bind the phospholipid-binding protein β2 glycoprotein I (β2GPI) or that directly recognize negatively charged membrane phospholipids in a manner that may contribute to arterial or venous thrombosis. Clinically, the binding of antibodies to β2GPI could contribute to pathogenesis by formation of immune complexes or modification of coagulation steps that operate along cell surfaces. However, additional events are likely to play a role in pathogenesis, including platelet and endothelial cell activation. Recent studies focus on neutrophil release of chromatin in the form of neutrophil extracellular traps as an important disease contributor. Jointly, the participation of both the innate and adaptive arms of the immune system in aspects of the APS make the complete understanding of crucial steps in pathogenesis extremely difficult. Only coordinated and comprehensive analyses, carried out in different clinical and research settings, are likely to advance the understanding of this complex disease condition.

Keywords: anti-phospholipid syndrome, systemic lupus erythematosus, neutrophil extracellular traps, autoantibodies, beta2 glycoprotein I, phospholipids, coagulation protein disorders, thrombosis

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****Correspondence:***
Marko Radic
mradic@uthsc.edu

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INTRODUCTION

Anti-phospholipid syndrome (APS) and systemic lupus erythematosus (SLE) are two autoimmune disorders that have puzzled researchers for decades (1–3). The two disorders have a range of shared clinical manifestations and can occur together in the same individual, often after a period of exclusive APS or SLE manifestations. Therefore, it is possible to consider them as different points of departure along a continuum of potential clinical manifestations. According to that view, secondary APS may arise as consequence of a worsening overall disease presentation. Antibodies to phospholipids (PL) and DNA are emblematic of the two disorders. Here, we highlight similarities and differences between the two disorders (**Figure 1**) in order to argue that discoveries across related research fields will help advance understanding of the unifying factors in their pathogenesis and help explain their notable overlap in presentation. Below, we raise important and as yet unanswered questions that address the relation between external stimuli or insults to the immune system, the diverse and often unique immune responses to these stimuli, the characteristics of the resulting antigen specificities, and the initial break in tolerance mechanisms. Importantly, we summarize how autoantibody binding shapes the observed pathology of the disorders and how it informs the search for new therapies.

A striking feature of APS and SLE is the nature of the defining antigens. Both DNA and PL are among the most abundant and pervasive antigens in the body and both are highly negatively charged. It is not surprising that charge interactions play an important role in DNA/PL recognition and that antibodies with positively charged residues in the complementarity determining regions are positively

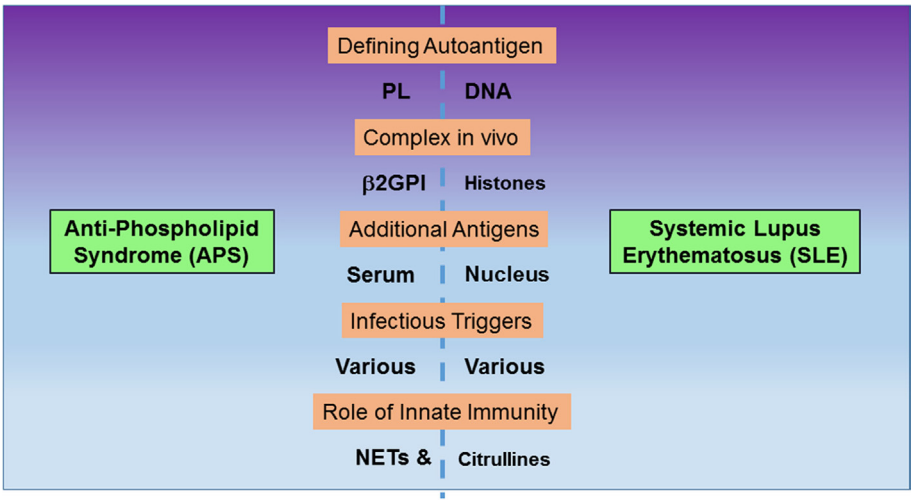

FIGURE 1 | Comparison of features between anti-phospholipid syndrome (APS) and systemic lupus erythematosus (SLE). The two autoimmune disorders exhibit autoantibodies to negatively charged, non-protein antigens, phospholipids (PL), and DNA. However, autoantibodies also recognize complexes between PL and β2GPI or DNA and histones, respectively. Additional autoantibody targets include other serum proteins in APS and nuclear proteins in SLE. Both disorders are potentially triggered by infections, and innate immunity contributes to pathogenesis, as neutrophil extracellular traps (NETs) form integral components of thrombi *in vivo* and citrullinated histones are prominent anti-citrullinated protein autoantibodies.

selected to recognize both autoantigens (4, 5). In fact, the similar charge distribution is, in part, one reason for the observed cross-reactivity between anti-PL and anti-DNA antibodies (6). Both DNA and the negatively charged PL are usually shielded from the humoral immune system by the cell membrane but become externalized during cell death on the surface of apoptotic cells (6, 7). In other forms of cell death, such as necrosis or NETosis, a recently defined neutrophil death (8) that involves the dispersal of chromatin in the form of neutrophil extracellular traps (NETs), DNA and negatively charged PL are also likely to be externalized and to become accessible to antibodies. Therefore, it is reasonable to conclude that cell death contributes antigens that stimulate the anti-self response in APS and SLE (9).

Additional features of both autoantigens include the fact that they exist as multi-molecular complexes *in vivo*. As is the case for most charged macromolecules in the body, both DNA and PL are neutralized by basic proteins that carry countercharges, such as the positively charged histones for DNA and β2 glycoprotein I (β2GPI) for PL. Interestingly, DNA and PL are also recognized by other abundant serum proteins including C-reactive protein, serum amyloid protein, collectins, and pentraxins (9). These proteins contribute to scavenge and clear apoptotic cell debris and possibly the remnants of other forms of cell death. More recently, β2GPI was observed to bind microvesicles and thus potentially participate in the signal transduction mediated by these subcellular particles (10). By several pathways, β2GPI contributes to the physiological clearance of dead cells (11) and it may serve to restore homeostasis following an insult to the body in the form of an infection or other tissue injury.

Depending on the precise molecular interactions, antibody binding to β2GPI could either assist in the clearance of dead cells or derail the normal course of apoptotic cell removal. Obstructive binding of antibodies to β2GPI, therefore, could delay clearance of cell debris and increase the risk of apoptotic cell dispersal. In that way, anti-β2GPI could promote the broader autoantibody reactivity to autoantigens displayed on apoptotic cells, such as DNA and chromatin. The binding of anti-β2GPI to β2GPI may interfere with apoptotic cell recognition and clearance, thus favoring the generation of autoantibody specificities that are indicative of lupus or related autoimmune diseases. Because APS shares certain vascular manifestations not only with Wegener's granulomatosis and polyarteritis nodosa but also other vasculitis conditions (12), a deeper insight into the autoreactivity in APS may shed light on the mechanisms shared by this broad constellation of autoimmune disorders. Detection of anti-PL prior to diagnosis in subsequent patients with SLE is associated with more severe SLE manifestations, including renal disease, thrombocytopenia, and thrombosis (13). Experimental support for the initiating role of anti-β2GPI antibodies in a broader autoimmune response derives from mice immunized with human β2GPI in lipopolysaccharide (LPS) adjuvant, which exhibit delayed clearance of apoptotic cells and, over time, an increase in autoantibody binding to nuclear autoantigens (14). Importantly, T cell recognition of β2GPI peptides may contribute to epitope spread in mice and humans that may include typical SLE autoantigens (15).

An intriguing open question is whether infections induce anti-DNA and anti-PL antibodies. This may be the case because microbes and the host may share cross-reactive antigens; APS was initially discovered due to a false-positive test for syphilis (16). Alternatively, the infectious process may induce the exposure of self-molecules on the cell surface. In the latter case, posttranslational modifications (PTM) that characterize the innate response to infections may determine the reactivity profile of the induced autoantibodies. Such is indeed the case, as autoantibodies frequently target the specific PTM that arise during an immune response to infections. One notable example is the induction of

autoantibodies to self-antigens that contain citrulline residues (17). Citrullines are produced by peptidylarginine deiminases (PADs) that convert certain arginine residues in proteins to citrulline residues (18) and become activated in granulocytes that are exposed to infectious or inflammatory stimuli (19). In fact, citrullinated histones are integral components of NETs. Notably, autoantibodies to citrullinated self-proteins are diagnostic for a range of autoimmune disorders, including SLE (20), and NETs appear to play a key role in the formation of thrombi (21–23). Additional PTM may result from infections and affect the binding of APS antibodies to β2GPI, as circulating levels of oxidized β2GPI correlate with the appearance of anti-β2GPI IgG (24).

An additional mechanism may link β2GPI to the pathogenesis of thrombotic events in APS. This may result from the direct binding of β2GPI to endothelial cells and the activation of inflammatory receptors on these cells (25). The direct binding of β2GPI to endothelial cells, a process that is aided by TLR4, directly activates endothelia. Similarly, Laplante et al. (26) showed in a carotid artery injury model that anti-β2GPI activation of endothelial cells is dependent on TLR4. The binding of β2GPI to TLR4 is enhanced by LPS and may reflect a possible scavenging of LPS. Conversely, anti-β2GPI antibodies enhance the production of pro-thrombotic and pro-inflammatory responses in blood vessels, a mechanism that, in part, is driven by activation of the nuclear factor κB (NF-κB) and AP1 signaling pathways (27). In the following sections, we focus on APS and leave a more detailed comparison to SLE for a separate venue.

THE FUNDAMENTALS OF APS

Anti-phospholipid syndrome is characterized by vascular thromboembolism, miscarriages, and other pregnancy comorbidities (1). The presence of anti-PL, which include anti-cardiolipin (anti-CL) anti-β2GPI antibodies, and lupus anticoagulant (LA), are the *sine qua non* for the diagnosis of APS (28). Vascular thrombosis, which can affect venous, arterial, or small blood vessels, is identified by histopathologic or imaging analysis. These antibodies are essential for the diagnosis and likely to play a pathogenic role in various disease manifestations (29). Thrombotic events in APS are rarely accompanied by histological evidence of vessel wall inflammation, yet many APS patients have underlying systemic autoimmune disease (30). APS pathogenesis clearly involves inflammatory pathways in endothelial cells, monocytes, and neutrophils and a variety of intercellular interactions promotes disease progression.

Anti-phospholipid syndrome-associated manifestations may include thrombocytopenia, livedo reticularis, skin ulcers, cardiac valve and kidney damage, pulmonary hemorrhage, and certain neurological manifestations (31). Patients experiencing these manifestations generally do not improve with anticoagulation therapy, suggesting that additional pathophysiologic processes may cause these outcomes of thromboembolism.

Initially, anti-PL antibodies were thought to bind directly to PL but later it was found that anti-PL may recognize negatively charged PL indirectly *via* PL-binding plasma proteins (32, 33). Anti-PL antibodies are quite heterogeneous and react with PL, PL-binding proteins, and their complexes (33). β2GPI is the main binding cofactor of these antibodies (34) and detection of anti-β2GPI has the greatest clinical significance (33). The analysis of antibody binding to β2GPI must take into account that β2GPI consists of five independently folded domains, including domain V, which resembles a "hook" and interacts with the PLs in the cell bilayer, and, at the opposite end, domain I, which is recognized by most clinically relevant antibodies in APS (35).

Depending on the redox state of the extracellular milieu, domains I and V expose different epitope surfaces for antibody binding. A tight interaction between domains I and V, which defines the circular form of β2GPI *in vivo*, shields various epitopes on domain I. The dissociation between the two domains gives rise to the linear, fishhook-like structure of β2GPI in which the domain I epitopes are exposed (36). Cysteine residues at positions 288 and 326 of domain V, which either remain as free thiols or form a disulfide bond, control the conversion between the two alternative *in vivo* conformations. In the plasma of healthy individuals, β_2GPI occurs in the free thiol form, which folds into a ring configuration and blocks antibody access to the principal domain I epitopes (37). Oxidative stress unfolds the ring conformation of β2GPI, exposing the normally shielded antigenic determinants of domain I, which form epitopes for pathogenic antibodies (36, 38). This form inserts with domain V into the cell bilayer of anionic PL. Raimondo et al. determined a strong positive correlation between IgG anti-domain I and the proportion of oxidized β2GPI, but not with IgM or IgA anti-domain I (24). This observation suggests that either anti-domain I IgG stabilizes the extended, oxidized form of β2GPI or that chronic inflammatory conditions lead to an abundance of oxidized β2GPI that stimulates the production of anti-domain I IgG.

Other potential antigen targets include phosphatidylserine, tissue plasminogen activator, plasmin, thrombin, prothrombin, antithrombin III, activated protein C, and annexin V (33). The diversity of potential antigens argues for the existence of "seronegative" APS and some investigators have disputed the primary significance of anti-β2GPI antibodies (39). Indeed, some cofactor independent antibodies can induce thrombus formation in a mouse model (40). Overall, autoantibodies in APS, as the disorder itself, are thought to arise due to a pernicious interaction between environmental factors and increased genetic predisposition to the disease (41).

There is no general agreement on the mechanisms that contribute to thrombotic complications in APS (42). Inconsistencies that prevent a consensus from emerging are: a. the differences between patient populations used to isolate the autoantibodies, b. the specificity of the antibodies used, and c. the experimental model in which the antibodies are tested (43). Anti-PL antibodies increase the risk of thrombosis through different mechanisms that go beyond a simple dysregulation of coagulation pathways (44). It is likely that mechanisms other than simple vascular thrombosis contribute to various APS manifestation. The fact that thrombotic events occur sporadically in spite of persistently high level of anti-PL antibodies suggests that factors in addition to anti-PL antibodies are required for thrombosis to arise (45).

GENETIC FACTORS PREDISPOSING TO APS

A genetic basis for anti-PL antibodies was suspected by Harvey and Shulman from their finding of familial clustering of false-positive

tests for syphilis (46). Anti-CL antibodies occur more frequently in first-degree relatives of SLE or primary APS patients than in unrelated control individuals, indicating that a genetic susceptibility favors the expression of anti-PL. Extended kinships with elevated expression of anti-PL were analyzed with regard to APS clinical presentation and provided evidence for a familial form of APS (47, 48). In another study, Goel et al. examined possible modes of genetic inheritance and noted the potential involvement of candidate genes. Their study, which involved 30 family members of APS patients, failed to confirm the contribution of several candidate genes to the disorder (49).

The combination of HLA-DQw7 (HLA-DQB1*0301) with HLA-DR4 or HLA-DR5 was significantly elevated in patients with SLE and LA as compared to 139 race-matched controls (50). Patients also expressed other HLA-DQB1 alleles from which the authors deduced a shared amino acid sequence, TRAELDT, which they proposed to constitute a potential autoantibody epitope (50). In another study, DR4 and DRw53 occurred with increased frequency in patients with primary APS (51), and a study of 577 European SLE patients presenting with anti-CL antibodies found a positive association with DPB1*1501 (*P* value: 0.005, OR 7.4), and DPB1*2301 (*P* value: 0.009, OR 3.3). Anti β_2GPI antibody was positively associated with DPB1*0301 (*P* value: 0.01, OR 1.9), and DPB1*1901 (*P* value: 0.004, OR 8.1). The authors conclude that the genetic risk of anti-PL antibodies—along with other clinical manifestations of APS—may be increased in SLE patients who are positive for certain HLA-DPB1 alleles (52). In Japanese patients with APS secondary to SLE, DRB1*09 has been linked to anti-CL (53). In Caucasians and Mexican Americans, HLA-DQ8 (DQB1*0302) and related HLA-DR4 haplotypes may predispose to anti-β2GPI, whereas British patients with primary APS show an association between anti-β2GPI and the HLA-DRB1*1302 and DQB1*0604/0605 (50, 54–56). Furthermore, C4A or C4B null alleles may associate with the presence of anti-CL antibodies in African-American populations (57). Notably, a polymorphism in domain V of β2GPI is observed more frequently in APS patients with anti-β2GPI antibodies than in matched controls (58, 59). Genetic polymorphisms have also been linked to thrombosis in APS patients. These polymorphisms range from variants of tissue factor (TF) pathway inhibitor, type-I plasminogen activator inhibitor, tumor necrosis factor α (TNFα), annexin A5, p-selectin, p-selectin glycoprotein ligand-1 (PSGL-1), platelet Fc receptor IIa, platelet glycoproteins GP Ia/IIa and GP IIb/IIIa, thrombomodulin, factor XIII, methylenetetrahydrofolate reductase, toll-like receptor 4, and CD40 (33). In view of the many diverse genetic factors that predispose to APS, a picture of a delicate balance of steps in the coagulation pathway emerges, in which a disequilibrium at any one point may tilt the equation toward thrombosis.

GENETIC ANALYSIS IN MODEL SYSTEMS

The first evidence that genetics contributes to pathogenic anti-PL in APS came from studies in mice. The spontaneous production of pathogenic IgG anti-CL that depend on β2GP for binding to CL occurs in NZW x BXSB F1 (W/B F1) male mice (60). W/B F1 mice develop autoantibodies to negatively charged PLs, including phosphatidylserine and phosphatidylinositol, and generate circulating immune complexes, which ultimately result in glomerulonephritis. The pathogenic anti-CL antibodies preferentially use certain V_H and V_κ genes, whereas non-pathogenic anti-CL antibodies use more heterogeneous V genes (61). Microsatellite markers have enabled genetic analysis of BXSB alleles that affect production of anti-CL and anti-platelet antibodies, cytopenia, and coronary artery disease in W/B F1 male offspring (62). Disease was dependent on two dominant alleles that acted as complementary genes and localized to separate chromosomes. Anti-platelet antibodies and thrombocytopenia were genetically and mechanistically linked but anti-CL and myocardial infarction depended on independent genetic contributions, suggesting that genetics of APS is complex (62). In another mouse model, the MRL-lpr/lpr mice, the specificity of a monoclonal anti-CL was shown to depend on stochastic events, including somatic mutations in the V_H gene, indicating that failure in peripheral tolerance mechanisms followed by antigen-driven selection and clonal expansion contribute to this autoreactivity (61).

Papalardo et al. demonstrated that pathogenic anti-PL and clinical manifestations of APS depend, in part, on particular MHC-II alleles (63). Wild-type mice, or mice that expressed human DR4, DQ6, or DQ8 genes, but not MHC-II knockout mice, produced thrombogenic anti-PL and TF after immunization with human β2GPI. In addition, in wild-type C57BL/6J mice, anti-CL antibodies were not β2GPI dependent and instead showed diminished binding to CL in the presence of the β2GPI cofactor (64). This study suggested the importance of certain MHC class II haplotypes in determining the levels of anti-PL antibodies and their pathogenic capacity.

INFECTIONS AS APS TRIGGERS

Infections are potential inducing factors for the production of autoantibodies in APS (65). Various infectious agents have been linked to the pathogenesis of APS but a definitive proof is still lacking. BALB/c mice infected with *Haemophilus influenzae*, *Neisseria gonorrhoeae* or immunized with tetanus toxoid developed antibodies to the TLRVYK peptide and anti-β2GPI reactivity (66). Moreover, naïve mice developed features of classic APS after infusion of these antibodies. The hexapeptide TLRVYK is a component of proteins expressed by these microbes and is also recognized by a pathogenic monoclonal anti-β2GPI antibody, suggesting the role of molecular mimicry as the potential cause of development of APS. A literature review revealed that, in people, the development of APS may be linked with HIV, HTLV, HBV, HCV, parvovirus B19, and varicella zoster virus infections (67). Infectious agents may induce autoantibodies through various mechanisms. Possible mechanisms include molecular mimicry, increased secretion of cytokines and chemokines, selective activation or depletion of lymphocyte populations, and exposure of cryptic epitopes due to the induction of cell death (68, 69).

Certain infectious agents may also directly affect the immunogenicity of β2GPI. Patients with APS exhibit a significant increase in oxidized β2GPI (70). Infectious agents could generate conditions that favor reactive oxygen and nitrogen species that may

enhance β2GPI oxidation and autoantibody production (71). Medications, such as chlorpromazine, amoxicillin, quinine, chlorothiazide, and propranolol, in addition to oral contraceptives, alpha-interferon and infliximab, may promote the expression of anti-PL antibodies (72). The preferred interpretation of these results is that medications may bind to self-antigens and create new binding determinants, so-called neo-antigens, which may induce autoantibody production (73).

ENDOTHELIAL AND PLATELET CONTRIBUTIONS

Cell activation is a key element in the increased thrombotic response (42). Some authors suggested endothelial cells are critical in APS-associated thrombosis (74), whereas others proposed a paradigm shift, which favored a central role of platelets (75). It is also possible that endothelial cells, directly or indirectly, promote the release of pro-thrombotic microparticles (76). This promises to be an exciting area of research in the near future.

INNATE IMMUNITY AND NETs

The cellular immune response to infections may be directly responsible for generating conditions that are favorable for the initiation of APS. Although lymphocytes, monocytes, and platelets receive much deserved attention for their role in the pathogenesis of APS, neutrophils contribute in a unique and relevant manner to the development of APS (77). Neutrophils are by far the most abundant leukocyte in the blood and they rapidly respond to inflammatory stimuli (78). Circulating neutrophils attach to activated endothelia, which express adhesion molecules, and invade tissues that harbor infectious organisms or exhibit other signs of inflammation. The neutrophils have alternative mechanisms to combat microbes, including phagocytosis and granule discharge (79). An intriguing antibacterial mechanism is the release of NETs. NETs consist of nuclear chromatin that escapes from the confines of the nucleus and disperses as an amorphous lattice from the cell. The NET fibers attach to various components of neutrophil granules that help to enhance the bactericidal properties of the lattice (80).

Neutrophil extracellular traps are important in the context of APS because APS patient neutrophils are prone to spontaneous NET release (22), and thrombi incorporate NET-derived materials (21–23, 81). *In vitro*, neutrophils respond to incubation with anti-β2GPI antibodies by an intensified NET release (22). In animal models, inhibitors of NET release show promise in reducing thrombus formation, and mice deficient for PAD4, the enzyme that deiminates histones and promotes DNA unraveling in NETs, are resistant to pro-thrombotic stimuli (82). A recent study identified PSGL-1, a neutrophil protein that mediates adhesion to endothelia, as an important regulator of the pro-thrombotic functions of neutrophils, and small molecules that target this protein may hold the key to new therapies for APS (83). Clearly, neutrophil biology in the context of APS warrants further attention and is likely to reveal new and exciting implications for APS pathogenesis.

MECHANISMS OF ANTIBODY-MEDIATED THROMBOSIS

The pathogenic mechanisms that contribute to thrombus formation have been examined using both *in vitro* and *in vivo* models of APS. Anti-PL antibodies increase thrombus formation in the venous and arterial circulation (84–86). Infusion of autoantibodies from APS patients to mice with injured blood vessels potentiates thrombus formation in a way that suggests a pathogenic role for APS antibodies. Anti-β2GPI IgG autoantibodies, but not IgG depleted of anti-β2GPI reactivity, or normal human IgG, increase thrombus size in a dose-dependent manner (87). Administration of human anti-PL IgG along with LPS causes micro thrombosis in rat model (88). In contrast, infusion of anti-PL antibodies alone into the experimental animal models does not result in spontaneous thrombotic complications, thus suggesting the requirement for priming with a small vascular injury or injection of a low dose of LPS. This is in line with the "Two Hit Hypothesis" (89) that was proposed to account for the clinical observation that, despite the continued presence of anti-PL, thrombotic events are rare. According to the two-hit hypothesis, the anti-PL antibody induces a thrombophilic state, but requires a second condition (e.g., an infection) for clotting to take place. Infusion of purified anti-PL antibodies with or without dimeric β2GPI alters endothelial adhesion molecule expression and leads to a perturbation of vascular function associated with TLR 2 and TLR4 signaling and the upregulation of nitric oxide and TF expression (86, 90–93). As microbes and microbial products signal through TLRs, so it is possible that an infection and anti-PL signaling through the TLRs can additively increase the risk of thrombosis. Thus, infections or inflammation may increase the expression of the anti-PL target or enhance the exposure of previously hidden epitopes (37). None the less, the "two hit hypothesis" does not conform well with the obstetric manifestations of APS, where the anti-PL is the single factor that leads to the increased risk of venous thromboembolism during pregnancy (94), although pregnancy itself may be viewed as the "second hit."

A recent systematic review and meta-analysis found that LA and anti-CL antibodies are associated with an increased risk of venous thromboembolism [OR = 6.14 (CI 2.74; 13.8) and OR = 1.46 (CI 1.06; 2.03), respectively] (95). All three antibodies show a significant association: ORs for LA, anti-CL, and anti-β2GPI were 3.58 (CI 1.29–9.92), 2.65 (CI 1.75–4.00), and 3.12 (CI 1.51–6.44), respectively, with arterial thrombosis (95). Anti-β2GPI antibodies with LA activity are considered the main culprits for the thromboembolic complications in APS (96). A subgroup of anti-β2GPI antibodies that bind the epitope comprising Gly40-Arg43 (G40-R43) in domain I were shown to act as LA and correlate strongly with thrombosis (34, 97).

Subjects positive for LA, high titers of anti-CL, and anti-β2GPI antibodies (called "triple positives"), more than any other anti-PL profile, have high risks for thrombosis and pregnancy morbidity (98). The risk of recurrent thrombosis in triple-positive patients was around 30% over a 6-year follow-up period. Triple-positive anti-PL patients usually have high titers of antibodies to the major β2GPI epitope on domain I (99). Thus, anti-domain I β2GPI autoantibodies, which frequently present in triple anti-PL-positive

patients, confer LA activity, associate with the highest risk of thrombosis (100), predispose to both thrombosis and pregnancy loss (100), and promote thrombosis in mouse models (101). Clearly, a detailed profile of anti-β2GPI antibody specificity and avidity may be useful as a risk stratification resource in the clinic (30).

PREGNANCY LOSS

Intraplacental thrombosis leading to poor vascular supply to placenta was thought to be the major pathogenic mechanism but is certainly not the universal mechanism of fetal loss in APS. Other anti-PL antibody-induced pro-inflammatory, complement-mediated pathways, and defective placentation might be playing a role (94). Passive transfer of anti-PL antibodies causes fetal loss due to placental thrombosis and also inhibits trophoblast and decidual cell function *in vitro* and in animal models (102). Anti-PL antibodies, in particular anti-β2GPI antibodies, may compete with the anticoagulant annexin A5 for binding to trophoblast and endothelial cells, thus increasing the risk of placental thrombosis (103). However, the *in vitro* studies may be challenged by the fact that microscopic analysis of tissues from miscarried fetuses or placentas of women with APS rarely show thrombosis (104). This could be related to the timing of the examination of the placental samples, as many of the events may occur early in the pregnancy, and later only residual damage may remain (94).

Complement products, TNFα and CC chemokines, along with other pro-inflammatory mediators, contribute to anti-PL-induced fetal loss in animal models (105). Injection of human anti-PL IgG into naïve mice following embryo implantation caused placental inflammatory changes. Human IgG and mouse complement deposited along the decidua, and a transient increase in blood TNFα coincided with neutrophil infiltration into the tissues (106–108). Studies of animal and human placenta indicate that complement activation by anti-PL may be major contributor to the recurrent pregnancy loss in APS (107). The complement system contributes to fetal loss in the mouse model as either complement inhibition or deficiency of complement components protects the mouse from fetal loss (109).

Complement activation by anti-PL antibodies, which bind decidua and placenta preferentially, may involve the classical and, perhaps, lectin pathways. In the process, potent anaphylatoxins (C3a and C5a) may be generated, leading to the recruitment of inflammatory cells. Further activation of the alternative pathway creates a localized pro-inflammatory amplification loop, which enhances C3a activation and deposition and generates additional anaphylatoxins, thus attracting additional inflammatory cells to the placenta (110). Inflammatory tissue injury is probably mediated by TNF-α, which increases in murine decidua after exposure to anti-PL (108). Additionally, the therapeutic effect of heparin can be traced to inhibition of complement rather than inhibition of coagulation (111). Treatment with unfractionated or low molecular weight heparins protects against pregnancy loss induced by anti-PL antibodies, whereas use of plain anticoagulants, such as hirudin or fondaparinux that have no anti-complement effects, do not protect from pregnancy loss (110).

Nonetheless, investigations have not gathered conclusive evidence to support the pathogenic roles of inflammation and complement deposition in obstetric complications (112). There was no evidence of inflammation in placenta in a mouse model of anti-PL antibody-induced fetal loss following IV administration of human anti-PL IgG before implantation (113). Data from *in vivo* animal models may be inconclusive because of the fact that observations cannot be continuous during the pregnancy and depend on the time chosen for the infusion of the putative pathogenic autoantibodies (94).

Additional mechanisms may be involved in anti-PL-induced fetal loss. Binding of β2GPI-dependent antibodies to human trophoblasts inhibits cell proliferation and syncitia formation, decreases production of chorionic gonadotrophin, perturbs secretion of growth factors, and induces apoptosis (114). Moreover, β2GPI-dependent antibodies may impair the expression of cell adhesion molecules, such as integrins and cadherins, in trophoblastic and decidual cells that perturb function at the maternal side of the placenta (115). Defects in endometrial differentiation, including the impaired expression of complement decay-accelerating factor (also known as CD55), arise and are evident on endometrial biopsies. Such alterations may compromise implantation, if they occur at or before conception. After conception, endometrial defects are likely to predispose to complement-mediated pregnancy failure (116).

Anti-PL greatly increase the risk of preeclampsia. A recent study concluded that anti-PL act, in part, by compromising the mitochondria in the syncytiotrophoblast and increasing the amount of mitochondrial DNA released *via* placental vesicles (117). The vesicles may increase the risk of preeclampsia because the mitochondrial DNA, which is recognized as a DAMP by TLR-9, may activate endothelial cells. If this concept is confirmed, then pharmaceutical intervention aimed at reducing placental vesicles and the signaling by mitochondrial DNA through TLR-9 may have the potential to lessen the adverse consequences of anti-PL in pregnancy (117).

IMMUNE SIGNALING PATHWAYS

It is not clear how binding of anti-PL antibodies to endothelial cells may lead to cell activation, as no clear cellular activation pathway has been identified. Candidate interactions include the binding of the anti-PL-β2GPI complex to TLR 2 or 4, the binding of annexin A2, or mediation of the low density lipoprotein receptor-related protein 8, followed by activation of a signal transduction pathway inside the cells. In each case, a more pro-thrombotic cell phenotype may be the outcome (42). Activation of individual or sets of receptors are possible (118). A recent study has shown that antibody uptake is essential for anti-PL antibody-induced cellular signaling (119). MyD88 and TRAF6-dependent signaling, as well as NF-κB and p38 mitogen-activated protein kinase signaling, may be involved downstream from anti-PL binding to β2GPI on the cell surface (114). However, it is not clear whether clinical manifestations differ depending on which cell signaling pathways are engaged, or whether different anti-PL subpopulations have different effects on cell activation (94).

Activation of the mechanistic target of rapamycin (mTOR) pathway plays a role in endothelial proliferation and intimal hyperplasia in anti-PL-positive patients, which leads to multiple potential

outcomes, including micro thrombosis, peripheral ischemia, skin ulcers, diffuse alveolar hemorrhage, or anti-PL nephropathy. IgG antibodies from APS patients, when incubated with vascular endothelial cells, stimulate the mammalian/mTOR through the phosphatidylinositol 3-kinase–AKT pathway (120) leading to cell proliferation. The authors showed that sirolimus, a mTOR complex inhibitor reduced endothelial cell proliferation and vascular lesions among patients with APS nephropathy, who required transplantation, as compared with patients with anti-PL antibodies, who did not receive sirolimus. Furthermore, *in vitro* studies have shown that treatment of anti-β_2GPI/β_2GPI or APS-IgG/β_2GPI complex could markedly induce mTOR activation as well as expression of TF and IL-8 in THP-1 cells (a human monocytic cell line) or primary monocytes. The mTOR inhibitor rapamycin (100 nM) could attenuate the elevated expression of TF and IL-8 (121).

MEDICATIONS AND POTENTIAL THERAPIES

A necessary step in anti-PL-mediated thrombosis and fetal loss seems to be the activation of complement, as discussed above. The activation of the classical complement pathway in APS-associated thrombosis is evident from mouse studies (88, 122). Activation of complement by anti-PL autoantibodies generates C5a, which attracts and activates neutrophils and enhances expression of TF (123). Conversely, mice treated with APS patient IgG had higher titers of anti-CL antibodies and anti-β2GPI leading to thrombosis; subsequently, they developed larger thrombi and higher soluble TF activity than controls. The recombinant C5 activation inhibitor rEV576 (coversin) reduced thrombus formation and suppressed TF activity from cells treated with IgG-APS (124, 125). The murine studies are in agreement with human studies. In a study of 186 patients, levels of fragments Bb and C3a were significantly increased compared to normal controls (126). APS patients who suffered from venous thromboembolism had significantly increased complement activation compared to normal controls, which Rivaroxaban effectively reduced (127). Mildly reduced complement levels (C3, C5), perhaps indicating complement consumption, occur in some APS patients (128), although this may not be a consistent feature of the syndrome (94). Supporting the role of complement, case studies indicate the benefits of C5-inhibitor eculizumab in preventing APS-associated thrombotic microangiopathy, a complication of renal transplantation, as well as for treatment of patients with acute catastrophic APS (129, 130).

Additional approaches have involved synthetic peptides (**Figure 2**). TIFI is a 20 amino acid synthetic peptide that shares similarity with the β2GPI PL-binding site. Administration of the peptide prevents anti-PL-mediated thrombosis *in vivo*, and, as expected, TIFI inhibits the binding of β2GPI to human endothelial cells *in vitro* (131). Infusions of TIFI protected pregnant mice from human anti-PL-induced fetal loss (132), thus providing evidence for the detrimental effect of β2GPI–anti-β2GPI complexes binding to trophoblasts in anti-PL-induced fetal loss (133). Similarly, the recombinant DI domain of β2GPI, the major anti-PL antibody target in APS, could inhibit experimental thrombus development in mice infused with APS patient IgG (134). The observation that β2GPI binds avidly to the ApoER2 A1 domain, the main LDL binding domain 1 (92), was the impetus to construct and test the recombinant dimer of A1 as an effective inhibitor of the prothrombotic functions of anti-β2GPI antibodies in mice (135). The successful deployment of each of these three recombinant protein domains (and their variants) raises the possibility that biologic therapies based on these peptide structures (**Figure 2**) may be developed in the near future.

Because neutrophils likely exert a unique and important function in APS pathogenesis, a range of approaches that limit neutrophil activation and NET release may move into the spotlight as targeted treatments for patients with APS. For example, *N*-acetyl cysteine, an effective scavenger of ROS that reduces the release of NETs *in vitro* and inhibits mTOR in T cells, has shown promise in SLE trials (136). Similarly, inhibitors of myeloperoxidase, a granule component in neutrophils that may catalyze reactions leading to NET release, have been used in patients with vasculitis and may be considered candidates for trials in APS (137). Moreover, the specific TLR4 inhibitor, TAK-242, which acts upstream of mTOR to reduce NET release and inhibit ROS production in neutrophils, has shown potential as treatment for APS (121).

In sum, we propose that APS therapy is at the doorstep of its most exciting stage. Numerous pathogenic mechanisms have been proposed and experimentally supported, and diagnostic and prognostic measures of APS activity have improved to the point that a broad range of potential therapies have appeared

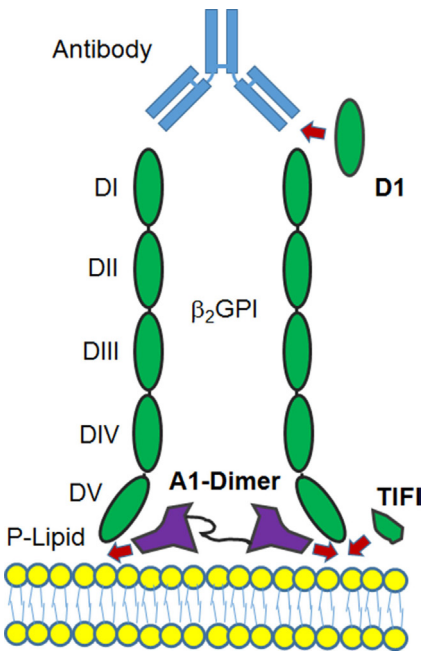

FIGURE 2 | Diagram of potential sites where binding of β2GPI to a phospholipid membrane may be disrupted. Recombinant versions of the D1, a portion of the DV domain (TIFI) or a dimer of the ApoER2 domain A1 are shown to indicate where the formation of the β2GPI-anti-β2GPI complex at the cell surface may be inhibited for therapeutic benefit. For details, see main text.

on the horizon and could soon advance through regulatory tests toward a safe and effective use in the clinics.

AUTHOR CONTRIBUTIONS

MR provided initial planning and wrote sections of the manuscript, edited the text, and gave final approval. DP participated in the planning and writing of sections of the manuscript, edited the text, and gave final approval.

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REVIEW
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Deoxyribonucleic Acid Methylation in Systemic Lupus Erythematosus: Implications for Future Clinical Practice

*Emma Weeding[1] and Amr H. Sawalha[2,3]**

[1] Department of Internal Medicine, University of Michigan, Ann Arbor, MI, United States, [2] Division of Rheumatology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, United States, [3] Center for Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI, United States

Differential deoxyribonucleic acid (DNA) methylation has emerged as a critical feature of systemic lupus erythematosus (SLE). Genome-wide DNA methylation studies have revealed methylation patterns characteristic of SLE—in particular, robust hypomethylation of interferon-regulated genes is a prominent finding in all cells of the immune system studied to date. These patterns reliably distinguish individuals with SLE from healthy controls and from individuals with other autoimmune diseases. For example, hypomethylation within *IFI44L* is both highly sensitive and highly specific for SLE, superior to currently available biomarkers. Furthermore, methylation status of other genetic loci has been associated with clinically relevant features of SLE including disease severity and organ-specific manifestations. Finally, DNA methylation studies have provided important insights into the pathophysiology of SLE. Most recently, there is a growing body of evidence that the transcription factor enhancer of zeste homolog 2 (EZH2) plays an important role in triggering SLE disease activity *via* epigenetic mechanisms, and that EZH2 blockade may be a future treatment option in SLE. In this short review, we discuss the DNA methylation patterns associated with SLE, their relationship to clinically significant features of SLE, and their implications in the development of novel diagnostic and therapeutic approaches to this complex disease.

Keywords: autoimmunity, biomarker, EZH2, IFI44L, lupus, methylation, T cells, therapeutic

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***Correspondence:**
Amr H. Sawalha
asawalha@umich.edu

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a highly heterogeneous autoimmune disease that can affect virtually any organ system in the body, resulting in protean clinical and serological manifestations which range from mild to life-threatening. The disease is broadly characterized by the production of antinuclear autoantibodies, resulting in the formation and deposition of immune complexes, which

Abbreviations: *BST2*, bone marrow stromal cell antigen 2; CD, cluster of differentiation; *CHST12*, carbohydrate sulfotransferase 12; DNA, deoxyribonucleic acid; EZH2, enhancer of zeste homolog 2; HLA, human leukocyte antigen; *HZF*, ring finger protein 39; *IFI44L*, interferon-induced protein 44 like; *IFIT*, interferon-induced proteins with tetratricopeptide repeats; *IRF7*, interferon regulatory factor 7; *MMP9*, matrix metallopeptidase 9; *MX1*, MX dynamin like GTPase 1; *PDGFRA*, platelet-derived growth factor receptor A; *RHOJ*, ras homolog family member J; RNA, ribonucleic acid; *RUNX3*, Runt Related Transcription Factor 3; SLE, systemic lupus erythematosus; *STAT1*, signal transducer and activator of transcription 1; TGFB, transforming growth factor beta; *TRIM22*, tripartite motif containing 22; *USP18*, ubiquitin-specific peptidase 18; VEGF, vascular endothelial growth factor; *VTRNA2-1*, vault RNA 2-1.

in turn leads to inflammation and damage of affected tissue. As with many other autoimmune diseases, the pathogenesis of SLE is complex and incompletely understood. A genetic component to the disease has long been presumed—first-degree relatives of individuals with SLE have up to a 30-fold higher risk of developing the disease as compared to the general population (1), which clearly suggests some degree of heritability. Indeed, extensive investigation has revealed dozens of genetic risk loci for SLE. Yet, these loci account for less than 20% of disease susceptibility (2). In the vast majority of patients, any one genetic polymorphism in isolation does not confer clinical disease. Rather, SLE arises due to some combination of genetic risk factors and various environmental factors, such as exposure to infections, chemicals, radiation, sex hormones, or other alterations to an individual's immunologic substrate.

The study of epigenetics has emerged as an important approach in investigating the contributions of both heritable and environmental factors, as well as the interplay between them, in the pathogenesis of autoimmune disease. Epigenetic mechanisms regulate gene expression in a tissue-specific manner by controlling the accessibility of deoxyribonucleic acid (DNA) to the transcription complex without modifying the underlying nucleotide sequence. Epigenetic changes can be either inherited or induced, and can be highly dynamic over the course of a cell's lifespan. The potentially reversible nature of epigenetic events makes them attractive candidates as biomarkers of disease activity and as targets for therapeutic strategies.

Deoxyribonucleic acid methylation is one of the most well-studied epigenetic mechanisms in humans. Methylation most commonly occurs at the C5 position of cytosine residues in CG dinucleotides and classically results in gene silencing. Conversely, demethylation is generally associated with increased chromatin accessibility and thus active gene expression. Aberrant DNA methylation has emerged as an important epigenetic feature of SLE, and the study of these abnormal methylation patterns has revealed numerous possibilities for a deepened understanding of this complex disease.

In this short review, we discuss the putative role of differential DNA methylation in the pathogenesis and pathophysiology of SLE, with a focus on how these patterns influence clinically relevant features, such as disease severity and heterogeneity, and their implications in the development of novel diagnostic and therapeutic strategies.

DNA METHYLATION IN THE PATHOPHYSIOLOGY OF SLE

In one of the earliest studies of epigenetic patterns in SLE, Richardson et al. examined the total percentage of methylated cytosine residues in T cells isolated from participants with SLE (3). This revealed significant global DNA hypomethylation of T cells in individuals with SLE when compared to healthy age-matched controls. In a similar vein, the treatment of $CD4^+$ T cells by DNA methylation inhibitors, such as procainamide or hydralazine, has been shown to induce hypomethylation and autoreactivity *in vitro* (4). Furthermore, injecting these hypomethylated T cells into syngeneic mice can induce lupus-like autoimmunity *in vivo* (5). While these studies provide a critical foundation in understanding the epigenetic patterns in SLE, the ultimate result of autoreactivity induced *via* hypomethylation is almost a forgone conclusion in that the relationship between DNA methylation inhibitors and autoimmune disease in humans has already been established in clinical practice. Indeed, treatment of humans with procainamide or hydralazine can cause a lupus variant known as drug-induced lupus erythematosus, which has similar clinical manifestations as SLE, and usually resolves within months after discontinuation of the culprit medication.

A seminal work by Javierre et al. was the first to examine genome-wide DNA methylation patterns in SLE and thereby associate epigenetic changes with specific genetic loci (6). Specifically, DNA methylation status was compared in total white blood cells obtained from monozygotic twins who were discordant in SLE status. Global hypomethylation was again observed in SLE participants when compared to their healthy twins or matched controls. Furthermore, sequence-specific demethylation was found in genes associated with several cellular processes which are likely relevant to SLE pathophysiology, including immune response, cell activation, cell proliferation, and cytokine production.

Subsequent studies have examined the DNA methylation patterns of specific cells in the immune system, particularly in T cells, given the earlier evidence of T cell hypomethylation in SLE as described above. In the first study to investigate genome-wide DNA methylation changes in $CD4^+$ T cells, the methylation status of over 25,000 CG sites (corresponding to the promoter regions of nearly 15,000 genes) was compared in SLE participants versus healthy controls (7). A total of 341 CG sites were found to be differentially methylated in SLE, with the majority of these being hypomethylated as expected. Hypomethylated genes included *MMP9* and *PDGFRA*, both of which are involved in the development of connective tissue, as well as *CD9*, which has been shown to provide potent costimulatory signals promoting the activation of T cells (8). Hypermethylated genes were primarily involved in metabolic pathways, particularly folate biosynthesis, which is essential in maintaining DNA integrity and stability. Hypermethylation was also noted in *RUNX3*, which encodes a transcription factor that is required for T-cell maturation.

A follow-up study surveyed DNA methylation status across over 485,000 CG sites in naïve $CD4^+$ T cells, with the intent to identify methylation changes preceding T cell differentiation and activation, and thereby revealing early epigenetic events which potentially predispose individuals to clinical manifestations of disease (9). A total of 86 differentially methylated CG sites in 47 genes were identified in SLE participants as compared to controls. Most notably, the majority of hypomethylated genes in naïve T cells found in this study are regulated by type I interferons, including *IFIT1*, *IFIT3*, *MX1*, *STAT1*, *IFI44L*, *USP18*, *TRIM22*, and *BST2*. Additionally, gene expression analysis was performed in the same naïve $CD4^+$ T cells. This revealed that despite being hypomethylated, none of these interferon-regulated genes were overexpressed in naïve $CD4^+$ T cells. Conversely, most of them were significantly overexpressed in total $CD4^+$ T cells from participants with SLE. In summary, these results suggest that naïve $CD4^+$ T cells undergo epigenetic priming toward a rapid response

to type I interferons, resulting in T cell differentiation and activation, and presumably, increased disease activity.

Subsequent studies in memory T cells, regulatory T cells, and neutrophils (including low-density granulocytes) have revealed similar patterns of global hypomethylation, particularly in interferon-regulated genes (10, 11). Most recently, DNA methylation patterns in a T cell subset, specifically $CD4^+CD28^+KIR^+CD11a^{hi+}$ T cells, were examined (12). This previously undescribed subset of T cells has been found to be present in patients with SLE, with the size of the subset correlating to disease severity (13). Differential DNA methylation analysis yet again revealed global hypomethylation in this T cell subset. Moreover, this hypomethylation, in combination with increased chromatin accessibility, resulted in increased expression of pro-inflammatory genes, such as cytokine genes, adhesion molecules, Fc-gamma receptor genes, toll-like receptor genes, human leukocyte antigen molecules, and metalloproteinases. These results further emphasize the important role that this demethylated T cell subset may play in SLE pathophysiology, and suggest that blocking these downstream pro-inflammatory effects might provide novel therapeutic avenues in SLE.

DNA METHYLATION AS A BIOMARKER

Even for the experienced physician, SLE can be difficult to diagnose. Owing to the significant heterogeneity of the disease, patients can present with any variety of symptoms at disease onset, some of which may be vague or non-specific. There is no single test for SLE—the diagnosis is ultimately made through clinical judgment by interpreting a patient's symptomatology in the context of serological, radiographic, and/or histological evidence of disease. Several laboratory markers of SLE are already used in clinical practice, including antinuclear antibodies (ANAs), anti-double stranded DNA (anti-dsDNA) antibody, and anti-Smith (anti-Sm) antibody. Interpretation of autoantibody titers has significant limitations, however. While effectively all patients with SLE test positive for at least one ANA, nearly one-quarter of the general population is also ANA positive (14). Conversely, anti-dsDNA and anti-Sm antibodies are highly specific for SLE, but are only detectable in roughly half of patients (15, 16).

Zhao et al. sought to investigate whether DNA methylation status could act as a more robust biomarker in SLE (17). Specifically, they examined the methylation status of two CG sites located within the *IFI44L* promoter (an interferon-regulated gene found to be hypomethylated in SLE) in DNA from peripheral blood obtained from participants with SLE. They found that a given threshold of hypomethylation at either CG site had a sensitivity and specificity of greater than 90% for SLE versus healthy controls. As discussed above, this is superior to currently available biomarkers such as ANAs or anti-dsDNA antibody. Differential methylation at these CG sites also distinguished SLE from rheumatoid arthritis and primary Sjögren's syndrome, two autoimmune diseases which can have clinical overlap with SLE. Although *IFI44L* is also hypomethylated in naive $CD4^+$ T cells from individuals with primary Sjögren's syndrome as compared to healthy controls (18), the degree of hypomethylation was found to be significantly higher among those with SLE per the work of Zhao and colleagues. Further validation is required to determine the role of *IFI44L* methylation status in distinguishing these two autoimmune diseases from one another. Nevertheless, the results of this study provide strong evidence that an assay for DNA methylation status in whole blood could be a powerful tool in the diagnosis of SLE. To this point, a summary of the DNA methylation patterns associated with various clinical features of SLE is provided in **Table 1**. These patterns are discussed in more detail below.

Systemic lupus erythematosus can be reviewed as a relapsing-remitting disease, with the majority of patients experiencing intermittent flares of disease activity alternating with relative quiescence. Given the evidence of epigenetic T cell priming toward a robust interferon-mediated response, the question arises of whether methylation status might correlate with disease flares. To address this question, Coit et al. performed genome-wide DNA methylation analysis on naïve $CD4^+$ T cells from participants with SLE with varying levels of disease activity as measured by SLE Disease Activity Index (SLEDAI) scores (19). They identified over 5,000 CG sites that either negatively or positively correlated with disease activity, and more broadly, discovered that higher disease activity is associated with progressive hypomethylation of genes involved in Th2, Th17, and follicular helper T cell response. Progressive hypermethylation was noted in inhibitory pathways

TABLE 1 | Summary of differential deoxyribonucleic acid (DNA) methylation patterns in naïve $CD4^+$ T cells associated with systemic lupus erythematosus (SLE), disease severity, and organ-specific manifestations.

SLE versus healthy controls
• Individuals with SLE exhibit robust DNA methylation changes, primarily hypomethylation, among genes associated with interferon-signaling pathways   ▪ Hypomethylation within the *IFI44L* promoter was found to be 94% sensitive and 97% specific for SLE in one study (17) • Hypomethylation of interferon-regulated genes is independent of disease activity
SLE disease activity
• Hypomethylated sites associated with increased disease activity include non-Th1 cytokine genes and human leukocyte antigen class II genes • Hypermethylated sites associated with increased disease activity are involved in inhibitory pathways, most notably the transforming growth factor beta signaling pathway • Binding sites for the repressive transcription factor enhancer of zeste homolog 2 are enriched among the above hypermethylated loci, and depleted among hypomethylated loci
Cutaneous SLE
• There is consistent hypomethylation of interferon-regulated genes regardless of cutaneous manifestation (or lack thereof) • Unique differentially methylated regions are associated with malar rash, discoid rash, or lack of either • Both cutaneous manifestations are uniquely differentially methylated in pathways associated with cell proliferation, apoptosis, and antigen processing and presentation
Renal involvement
• Individuals with renal involvement exhibit more robust hypomethylation both globally and specifically within interferon-regulated genes compared to those without renal involvement   ▪ The type I interferon master regulator gene *IRF7* is only hypomethylated in those with lupus nephritis • Hypomethylation within CHST12 is 86% sensitive and 64% specific for lupus nephritis (23)

such as the transforming growth factor beta signaling pathway. Gene expression analysis was performed and demonstrated that these epigenetic events do indeed precede gene expression. Overall, these results suggest that not only are naïve $CD4^+$ T cells epigenetically predisposed toward an effector T cell response in SLE, but also that these epigenetic changes and putative downstream effects are associated with increased disease activity. The clinical relevance of this conclusion is that determination of DNA methylation status might provide prognostic information in predicting SLE flares, and may thus be useful in tailoring selection of medical therapy and subsequent monitoring of a patient's response to treatment.

In current clinical practice, selection of therapy for SLE is based not only on overall disease activity but also on a given patient's particular manifestations of disease. Understanding the methylation patterns of particular manifestations of SLE may provide additional prognostic information and help guide future development of targeted therapies. As an example, cutaneous manifestations such as malar rash or discoid rash are common in patients with SLE. Methylation patterns specific to each of these kinds of rash have been found. Specifically, Renauer et al. compared genome-wide DNA methylation profiles in naïve $CD4^+$ T cells from participants with SLE who had a history of malar rash, discoid rash, or neither cutaneous manifestation (20). Between these three groups, they identified several hundred differentially methylated sites, the majority of which were specific to each cutaneous manifestation (or lack thereof). For those with a history of malar rash, the most extensively hypomethylated region was located in the promoter region of precursor microRNA miR-886 (*VTRNA2-1*). Independent studies have shown that hypomethylation of this region modulates signaling pathways which determine cell survival versus apoptosis (21). For those with a history of discoid rash, hypomethylation within *RHOJ* and *HZF* were found, both of which are also involved in determining cell survival versus apoptosis. These results correlate with older findings that the epidermis of patients with cutaneous SLE is in part characterized by an accumulation of apoptotic cells (22).

Another organ commonly affected in SLE is the kidneys. It is estimated that just over half of all patients with SLE have renal involvement, which can range in severity from mild and near-quiescent to fulminant and life-threatening. Early recognition of renal impairment is critical, as treatment is more likely to be successful when it is started as quickly as possible, and conversely, delayed diagnosis is associated with a significantly increased risk of renal failure and death (23). In an effort to determine how DNA methylation patterns might correlate with renal disease in SLE, one study examined genome-wide DNA methylation in naïve $CD4^+$ T cells from SLE patients with and without renal involvement (24). The authors discovered 191 differentially methylated CG sites (corresponding to 121 genes) associated with the presence or absence of renal involvement. Genes which were more hypomethylated in SLE participants with renal disease included *IRF7*, which is a well-known genetic risk locus for SLE. Indeed the majority of hypomethylated sites were located in interferon-regulated regions, as expected. Notably, the degree of hypomethylation in these regions was significantly more robust in SLE participants with a history of renal disease, independent of overall disease activity. Genes which were more hypermethylated in SLE participants with renal disease included *CD47*, which has been shown to regulate T cell production of vascular endothelial growth factor (25), and *CD247*, which encodes the T-cell receptor zeta chain, and in turn, plays a key role in antigen receptor-mediated signaling and has been shown to be downregulated in SLE T cells (26). Finally, the authors identified a single CG site, CG10152449 in *CHST12*, for which hypomethylation had a sensitivity of 86% and specificity of 64% in detecting renal disease in SLE participants. No comparable biomarker currently exists in clinical practice.

DNA METHYLATION AND FUTURE THERAPIES

Arguably, the ultimate goal in the study of the epigenetics of human disease is not only to identify biological pathways which drive pathophysiology but also to use our new-found understanding of these pathways to develop novel treatment strategies. One of the most promising future treatment options revolves around a transcription regulator known as enhancer of zeste homolog 2 (EZH2). EZH2 is a histone-lysine *N*-methyltransferase enzyme which promotes transcriptional regulation by way of histone methylation as part of the polycomb repressive complex 2. Similar to DNA methylation, posttranslational modifications of histone proteins are epigenetic events which contribute to the pathophysiology of SLE and other autoimmune disorders by regulating gene expression (27). EZH2 trimethylates lysine 27 in histone H3, resulting in H3K27me3 and transcriptional repression. It can also recruit DNA methyltransferases DNMT1, DNMT3A, and DNMT3B (28, 29). When phosphorylated, EZH2 acts as a transcriptional activator at least in part by suppressing H3K27me3, thus disrupting gene silencing (30, 31).

In the aforementioned study by Coit et al., which examined the relationship between epigenetic changes and disease activity, methylation sites which correlated with disease activity were found to be either enriched (at hypermethylated loci) or depleted (at hypomethylated loci) in binding sites for EZH2, suggesting that it might play an important role in inducing a pro-inflammatory epigenetic shift (19). EZH2 expression in T cells is inhibited by glucose restriction *via* increased expression of microRNAs miR-26a and miR-101 (32). Increased glycolysis has been noted in $CD4^+$ T cells from individuals with SLE and in mouse models of lupus, and furthermore, treatment of this abnormally enhanced glycolysis in mice resulted in a shift of immunophenotype toward that of healthy controls (33). As such, it was hypothesized that decreased levels of the above microRNAs, indicating enhanced glycolysis and subsequently increased EZH2 activity, would correlate with increased disease activity in SLE (**Figure 1**). This association was indeed found when comparing SLEDAI scores to levels of miR-26a expression (19).

Most recently, a follow-up study examined expression levels of EZH2 in $CD4^+$ T cells, as well as the effects on DNA methylation associated with EZH2 overexpression, in participants with SLE versus healthy controls (34). First, this study confirmed previous findings that T cell production of EZH2 is downregulated by

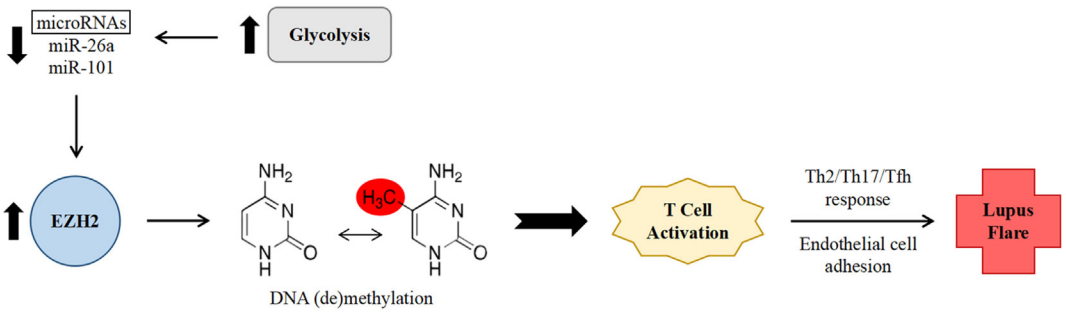

FIGURE 1 | Proposed mechanism of increased systemic lupus erythematosus (SLE) disease activity *via* enhancer of zeste homolog 2 (EZH2)-mediated epigenetic remodeling within CD4+ T cells. Abnormally enhanced glycolysis in SLE results in decreased levels of the microRNAs miR-26a and miR-101. Decreased microRNA levels leads to lessened downregulation of the expression of transcription factor EZH2. EZH2 in turn promotes deoxyribonucleic acid methylation changes, leading to T cell activation, a non-Th1 effector T cell response, and increased adhesion to endothelial cells, thereby promoting SLE disease activity.

miR-26a and miR-101. Notably, both of these microRNAs were present at reduced levels in SLE CD4+ T cells. Next, overexpression of EZH2 was induced in CD4+ T cells from healthy controls, and the resulting genome-wide DNA methylation patterns were assessed. This revealed several hundred differentially methylated CG loci, most notably in regions associated with cell adhesion and leukocyte migration. Indeed, CD4+ T cells from both the EZH2-overexpression group and the SLE group showed increased adhesion to human dermal microvascular endothelial cells. Finally, blocking EZH2 effectively reduced the capacity of these T cells to adhere to endothelial cells, providing proof of principle that EZH2 blockade may be a future therapy for SLE. Though no EZH2 inhibitor is yet widely available in clinical settings, one such agent, tazemetostat, is currently being investigated in clinical trials as a treatment for certain cancers.

CONCLUSION

Differential DNA methylation has emerged as a critical feature of SLE. Characterization of these methylation patterns has provided important insights into the pathophysiology of this complex disease. Furthermore, assessing an individual's methylation status shows promise as a future clinical tool and may aid not only in the diagnosis of SLE itself but also act as a prognostic indicator to help predict disease flares and facilitate detection of organ-specific manifestations. Finally, the study of differential DNA methylation and its downstream functional effects on the immunologic environment has now revealed an encouraging potential future treatment option for SLE, namely EZH2 blockade.

AUTHOR CONTRIBUTIONS

EW and AS drafted and critically revised the manuscript.

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Targeting Regulatory T Cells to Treat Patients With Systemic Lupus Erythematosus

Masayuki Mizui[1]* *and George C. Tsokos*[2]*

[1] Department of Nephrology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan, [2] Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, United States

Regulatory T cells (Tregs) are central in integration and maintenance of immune homeostasis. Since breakdown of self-tolerance is a major culprit in the pathogenesis of systemic lupus erythematosus (SLE), restoration of the immune tolerance through the manipulation of Tregs can be exploited to treat patients with SLE. New information has revealed that Tregs besides their role in suppressing the immune response are important in tissue protection and regeneration. Expansion of Tregs with low-dose IL-2 represents an approach to control the autoimmune response. Moreover, control of Treg metabolism can be exploited to restore or improve their function. Here, we summarize the function and diversity of Tregs and recent strategies to improve their function in patients with SLE.

Keywords: systemic lupus erythematosus, regulatory T cells, tissue Treg, low-dose IL-2 treatment, immunometabolism

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***Correspondence:**
Masayuki Mizui
mmizui@kid.med.osaka-u.ac.jp;
George C. Tsokos
gtsokos@bidmc.harvard.edu

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INTRODUCTION

Breakdown of self-tolerance is critical in the development of systemic lupus erythematosus (SLE) (1). Innate and adaptive immune responses against self-antigen induce the production of autoantibodies and the deposition of immune-complexes in tissues leads to the activation of complement, accumulation of neutrophils and monocytes, and self-reactive lymphocytes (2). The variety of clinical manifestations may reflect the multiple and heterogeneous pathways that account for the expression of disease (3). Chronic inflammation caused by the immune response against self-antigens leads to the development of irreversible damage in tissues including the kidney. Efforts to resolve or contain the inflammatory response include curtailing autoantibody production and the levels of type I interferon and various chemoattractants (4, 5). Belimumab, the soluble B-lymphocyte stimulator (BAFF) blocking antibody, which has been approved by FDA to treat patients with SLE has marginal clinical efficacy and only in patients with moderate disease. However, *post hoc* analysis and long-term follow-up studies have revealed that belimumab does not induce a rapid clinical benefit and the clinical efficacy seems to be limited (6). Recent advances in our understanding of regulatory T cell (Treg) physiology and metabolism have fueled new therapeutic strategies which involve the improvement of Treg function for the treatment of SLE and other autoimmune diseases as well as transplant rejection and cancer (7, 8). Low-dose IL-2 supplementation was first shown to expand Tregs and improve clinical manifestations in patients with chronic graft-versus-host disease (cGVHD) and hepatitis C virus-associated vasculitis (9, 10). Low-dose IL-2 therapy has been claimed in case reports and non-controlled studies to improve Treg numbers and clinical manifestations in patients with SLE (11, 12). Ongoing clinical trials (NCT03312335: Charact-IL-2, NCT 01988506: TRANSREG) will test the therapeutic value of low-dose IL-2 in patients with SLE. Therefore, a better understanding of the molecular

events which account for the poor function of Tregs in patients with SLE along with their poor response to IL-2 is needed to optimize therapeutic approaches. Further, it should be clarified how Tregs may contribute to containing tissue inflammation or repair organ damage. Tregs can modulate the function of the immune system as well as the function of non-lymphoid organs through the acquisition of tissue-defined gene expression.

PLEIOTROPIC EFFECTS OF Tregs

Treg Subsets and Tissue Tregs

Self-tolerance is accomplished with the deletion of self-reactive lymphocytes during development. However, self-reactive T cells escape negative selection in the thymus and persist in the periphery (13), where Tregs are important gatekeepers in preventing aberrant activation of self-reactive lymphocytes. Tregs develop in the thymus (tTreg) through strong T cell receptor (TCR) signaling just below the threshold for negative selection. Therefore, Tregs recognize self-antigens for their differentiation. Tregs are also induced from naïve $CD4^+$ T cells (pTreg) (14). In the human, peripheral blood Tregs may be present in resting ($Foxp3^+CD45RA^+CD25^+CD127^-$) or activated/memory ($Foxp3^{++}CD45RA^-CD25^{++}CD127^-$) phenotype (**Table 1**) (15). A subset of memory Tregs is known as T helper-like Tregs which can be further classified, depending on the kind of environmental stimuli, as T helper-like Tregs type1 (TH1), TH2, TH17-like, and T-follicular regular populations which co-express T-bet, GATA3/IRF4, RORγt, and Bcl6, respectively (16, 17). These T helper-like memory Tregs share chemokine receptors with individual T helper cells and are thought to be distributed into the appropriate site of each class of the immune response (13). Besides the conventional Tregs, $CD4^+Foxp3^-$ type 1 T regulatory (Tr1) cells expressing IL-10 were recently identified and shown to display strong immunosuppressive activity and to be involved in the maintenance of tolerance (18–20).

In tissues, Tregs are more abundant, percentage-wise, than in the peripheral blood and most of tissue-resident Tregs have an activated/memory phenotype (21). Moreover, gene expression patterns in tissue-resident Tregs depend distinctly on the hosting tissue. For example, an intestinal pTreg subset expresses RORγt and can produce IL-17 (22) and visceral adipose tissue (VAT) Tregs express PPARγ to regulate insulin sensitivity (23, 24). VAT Tregs also highly express IL-33 receptor (ST2), a receptor for alarmin that induces T_H2 responses, which is required for Treg accumulation into VAT (25). Tregs seem to be formed in the thymus by the time of birth but they diverge dependent on the tissue environment. Perinatally generated tTregs that are Aire-dependent translocate into tissues and persist to maintain self-tolerance (26). In addition, some strains of gut microbiota can induce Tregs in intestine (27). Therefore, colonic Tregs originate from both tTreg and pTreg but VAT and muscle Tregs are reported to be of thymic origin (tTreg) (28). The biology and characteristics of human tissue-resident Tregs have been reviewed (29) and are summarized in **Table 1** (30–34).

Immunosuppressive Aspects of Tregs

The suppressive action of Tregs on effector T cells (Teffs) is well established. First, Tregs inhibit Teff expansion by consuming local IL-2 because they express higher levels of CD25 (35). Second, Tregs inhibit Teffs in a contact-dependent manner. Tregs downregulate the expression of costimulatory ligands CD80/86 on antigen-presenting cells through trans-endocytosis. This is accomplished by CTLA4 which is expressed on Tregs and binds to CD80/86 with higher affinity than CD28 (36). Furthermore, Tregs can deprive energy factors from Teff cells. CTLA4-mediated signals induce indoleamine 2,3-dioxygenase in antigen-presenting cells resulting in the starvation of Teff cells (37, 38). A subset of Treg expresses an ectonucleotidase CD39 which catalyzes the degradation of proinflammatory molecule adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) and dampens Teff activation and proliferation (39). In the resting state, Tregs localize in clusters of IL-2-producing T cells that are activated by self-antigen within secondary lymphoid tissues (35). Many other different mechanisms of suppression have been documented and are summarized in **Table 2** (38, 40). Recently, Tregs were shown to suppress autophagy in antigen-presenting cells and thus limit the production of autoantigens (41).

Tregs in Wound Repair and Tissue Regeneration

Neutrophils and myeloid mononuclear cells such as monocytes infiltrate injured tissues in early phases. Monocytes differentiate into M1-type macrophages which are involved in the clearance of apoptotic and necrotic cells and debris and subsequently to M2-type macrophages which become involved in matrix

TABLE 1 | Genes and phenotypes of regulatory T cells detected in humans.

Tissue	Phenotype	Foxp3	CD45RA	CD25	CTLA4	GITR	ICOS	CD127	Others genes	References
Blood	Resting/naive	+	+	+	−	−	−	−		(15)
	Activated/memory	++	−	++	+	+	+	−	T-bet, GATA-3, RORγt, Bcl-6	(13, 16, 17)
	Type 1 T regulatory	−	−	−	n.d.	n.d.	+	−	IL-10, CD49b, LAG3, AhR	(18–20)
Skin	Memory	+	−	++	+	+	+	−	IL-17	(46)
Lung	Activated	+	−	+	+	−	+	−		(30)
Colon	Effector	++	−	++	+	+	n.d.	−	IL-17, RORγt, CD49d, CD103	(22, 31)
Visceral adipose tissue	Activated	+	−/+	+	n.d.	n.d.	n.d.	−	ST2, OX40	(25, 32, 34)
Joint	Activated	+	−	++	n.d.	n.d.	n.d.	−	IL-17, CD161	(33)

n.d., not determined.

TABLE 2 | Modes of action of Tregs.

Target	Modes of actions	Reference
Effector T cells	IL-2 consumption to inhibit clonal expansion	
	Suppressive cytokine secretion (TGFβ, IL-10, IL-35)	(38)
	Hydrolysis of adenosine phosphates *via* CD39, CD73	(39)
	Direct cell killing *via* perforin/granzyme	(40)
Dendritic cells/antigen-presenting cells	Blocking CD80/86 through CTLA4	(36)
	Inhibition of autophagy through CTLA4	(41)
	Indoleamine 2,3-dioxygenase induction	(37)
Hair follicle stem cells	Promoting proliferation and differentiation	(48)
Muscle progenitor cells	Amphiregulin-mediated differentiation	(43)
Adipocytes	Maintain insulin tolerance through IL-10	(23)

remodeling and promotion of angiogenesis and tissue regeneration (42). Lymphocytes, including $CD4^+$ and $CD8^+$ T cells, are also recruited to the sites of inflammation and have been thought to promote tissue injury. Recent reports though have demonstrated that $CD4^+Foxp3^+$ Tregs accumulate in the skeletal muscle after injury on time to switch from proinflammatory to the proregenerative (43). The Treg population persists at high numbers even 1 month after the injury. Notably, these Tregs express high levels of amphiregulin (Areg), an epithelial growth factor (EGF) family protein, and promote muscle regeneration through the EGF receptor (EGFR) signaling axis (43). Tregs expressing Areg can protect lungs from infection-induced damage (44). In addition, Tregs also express EGFR and the Areg-EGFR axis is critical for the local Treg function. Areg is produced not only by Tregs but by Th2 cells and other myeloid cells including mast cells and control the immune response by regulating Treg cell function (45). Involvement of Areg-EGFR signals in Treg-mediated tissue regeneration is also observed in skin injury by promoting wound healing (46, 47). Furthermore, the same group recently demonstrated that skin Tregs preferentially reside close to hair follicle stem cells (HFSCs) and help HFSC-mediated hair regeneration (48). More recently, Tregs were demonstrated to promote directly myelin regeneration in the central nervous system indecently of immunomodulation (49).

IL-2, Tregs, AND SLE

IL-2 Deficiency and Impaired Treg Function in SLE

While IL-2 is critical for the differentiation and function of Tregs, it is a well-known fact that IL-2 production by conventional T cells (Tconv) is impaired in SLE (50). IL-2 gene is silenced through transcriptional regulator, cyclic AMP response element modulator alpha (CREMα), which is overexpressed by SLE Tconv cells. Repression of IL-2 also caused by enhancement of calcium/calmodulin-dependent kinase IV (CaMK4) (51) and decrease of serine/arginine-rich splicing factor 1 (52, 53). The absence of IL-2 probably favors differentiation and expansion of IFNγ-producing T_H1 cells and IL-17-producing T_H17 cells, accumulating in organs such as the skin and the kidney (54, 55). Regulatory T cell numbers decrease in lupus-prone mice as they age and the disease progresses (56). In humans, several studies have analyzed the frequency of Tregs in SLE and reported conflicted results (57). The reported discrepancies may be due to the applied gating strategies in flow cytometry. Some studies gated Tregs based only the expression of $Foxp3^+$ $CD25^+$ cells a population which contains non-Treg activated T cells. Recent studies using less ambiguous gating strategies reported that $CD45RA^+CD25^+$ naïve Treg and $CD45RA^-CD25^{++}$ activated Tregs in SLE patients are comparable to those in healthy individuals, although the frequency of $CD45RA^-CD25^+$ activated T cells showed linear relationship with SLEDAI (58). In addition, $Foxp3^+$ T cells in the kidney and skin are comparable to those seen in tissues obtained from several control diseases. Considering that IL-2 production by T cells from SLE patients is impaired, it appears that this deficiency does not influence the numbers of Tregs in SLE. However, recent studies described that CD25 expression levels on the surface of Tregs were decreased in SLE patients (59). The reduction of CD25 expression in Tregs from patients with SLE correlated with the production of IL-2 by memory T cells indicating that deficiency of IL-2 in SLE patients reflects CD25 reduction in Tregs. Because IL-2 receptor-dependent activation of transcription factor STAT5 is essential for the suppressive function of Tregs, decreased expression of CD25 may affect the function of Tregs in SLE patients.

IL-2 Therapy in Lupus-Prone Mice

The first report of IL-2 treatment for lupus-prone mice presented in 1990 prior to the discovery of Tregs (60). An IL-2-encoding vaccinia virus was used to deliver IL-2 *in vivo* in MRL*lpr* mice. Treated mice survived longer and had reduced lymphadenopathy and kidney pathology. As $TCR\alpha\beta^+CD4^-CD8^-$ (double-negative, DN) T cells are the most likely culprit of lymphoadenopathy in MRL*lpr* mice, these DN T cells were significantly decreased after treatment with IL-2. Several methods for delivering IL-2 have been tried and confirmed these findings (61–63). Although DN T cells are also expanded in patients with SLE, their origin of is still unclear. DN T cells from lupus-prone mice and patients with SLE produce IL-17 (63, 64), indicating involvement of DN T cells in the pathogenesis of SLE. By IL-2 supplementation, Treg number is increased substantially in lymphoid and peripheral organs in NZB/NZW F1 mice and MRL/*lpr* mice and DN Tcells are significantly decreased in MRL/lpr mice (56, 63). However, Treg-specific expansion following the administration of IL-2/anti-IL-2 antibody complexes did not lead to the reduction of DN T cells (63), suggesting that an effect of IL-2 on non-Treg population might contribute to the inhibition of DN T cell expansion.

IL-2 Therapy for Patients With SLE

Deficiency of IL-2 production in patients with SLE might contribute to detrimental perturbation in immune systems. Therefore, it is conceivable that low-dose IL-2 treatment can restore

these pathogenic processes (65). Humrich and colleagues first reported a patient with SLE who achieved clinical improvement following treatment with low-dose IL-2. Specifically, 1.5 to 3×10^6 IU IL-2 (aldesleukin) was injected subcutaneously on five consecutive days for four cycles with 9–16 days of separation. Skin eruption, myositis and arthritis were improved within 10 days and serum anti-dsDNA antibody titer was decreased after for cycles of treatment. $CD4^+CD25^+Foxp3^+CD127^{lo}$ Tregs were upregulated temporarily at around 40% among $CD4^+$ T cells (11). Subsequently, they conducted a combined phase I/IIa clinical trial to address the safety, tolerability, efficacy, and immune response of low-dose IL-2 therapy in patients with active and refractory SLE (PRO-IMMUN, EudraCT-number: 2013-001599-40, Germany) (66). In this study, they demonstrated that Tregs from SLE patients showed decreased number of $CD25^{high}$ population and that IL-2 production was deficient in SLE $CD4^+$ T cells. After five patients were treated with daily subcutaneous injection of IL-2 at 1.5×10^6 IU for 5 days, they confirmed that low-dose IL-2 therapy induced substantial increases of the numbers of Tregs without major side effects. As the primary endpoint (immune response rate) has been completed, phase II trial is now ongoing. The latest clinical trial of low-dose IL-2 in 38 SLE patients in China (NCT02084238) demonstrated that IL-2 treatment significantly decreased SLEDAI after 12 weeks (12). Subcutaneous 1×10^6 IU of IL-2 was administered alternate-day for seven times at three cycles. More than 80% of patients achieved composite endpoint of SLE response index with 4-point drop in SLEDAI (SRI(4)), with increased Tregs, decreased T_H17, Tfh, and DN T cells. Unfortunately, the study was not controlled and various observations including the rapid disappearance of DNA antibodies remain unexplained. Another clinical study involving the induction of Tregs by low-dose IL-2 in SLE and other autoimmune and inflammatory diseases (Charact-IL-2 and TRANSREG) is now in progress (67). Since all studies are non-controlled ones, controlled prospective study is necessary. Taken together, low-dose IL-2 treatment in SLE patients could alleviate clinical severity by altering the balance of T-cell subsets.

Efficacy and Safety of Low-Dose IL-2 Therapy

Further analysis using mass cytometry of low-dose IL-2 treatment in cGVHD patients revealed that $CD4^+CD25^+Foxp3^+Helios^+$ Tregs and $CD56^{bright}CD16^-$ NK cells were selectively expanded (68). $Helios^+$ Tregs were shown to be fully demethylated at the Treg-specific demethylated region and was recognized as a subset with enhanced suppressive potential (69). Ki67 expression was increased 1 week after starting IL-2 but declined to baseline after 12 weeks. It is notable that even 48 weeks after daily treatment with low-dose IL-2, phosphorylation of STAT5 and increased expression of Foxp3, CTLA-4, CD25, and Bcl-2 were sustained (68). A recent study reported that inflammation-experienced memory Tregs exert enhanced suppressive function which was lost over time to obviate general immunosuppression (70).

Long-term treatment with low-dose IL-2 has been tested in mice. Recombinant adeno-associated vector (rAAV) encoding IL-2 was injected intraperitoneally at various viral titers. This approach enabled sustained higher IL-2 concentrations for more than 20 weeks compared to controls and substantially prevented diabetes in NOD mice (71). Although mice injected with high viral titers (10^{12} rAAV IL-2) died within 2 weeks, mice injected with lower titer (10^9–10^{11} rAAV-IL-2) lived normal life spans with unaffected vaccine-mediated antibody responses, infection-induced immune responses, and notably, not-enhanced tumor growth (71). Interestingly, low-dose recombinant IL-2 administration could protect mice from food allergy and the immune tolerance was sustained for more than 7 months after the last dose of IL-2 (72). These results indicate that Tregs can maintain their specific inhibitory function during long-term exposure to IL-2 and long thereafter.

Manipulation of IL-2

Although low-dose IL-2 can substantially expand Tregs, frequent injection is required for the induction of significant increase because of its short half-life in human serum (5–7 min). To overcome this disadvantage, modified IL-2 such as polyethylene glycol-modified IL-2 (PEG-IL-2), which prolongs the half-life of IL-2, has been constructed. PEG-IL-2 has been developed in the 1990s and undergone phase I/II clinical trials in cancer patients (73) and was recently revisited and tried in mice with asthma (74). Bell et al. recently developed monovalent or bivalent IL-2-fused with non-FcR binding IgG1 molecules which had a prolonged half-life *in vivo* and caused prolonged activation and proliferation of Tregs after a single ultra-low dose (75). IL-2/anti-IL-2 complexes can also prolong the half-life of IL-2. In mice, IL-2/anti-IL-2 complexes have been well established: IL-2/JES6-1A12 specifically binds to CD25 and IL-2/S4B6 selectively binds to CD122. IL-2/JES6-1A12 and IL-2/S4B6 induce specific expansion of Tregs and cytotoxic lymphocytes, respectively. IL-2/JES6-1A12 administration was shown to expand efficiently both peripheral and tissue Tregs (43, 76). When human IL-2/anti-IL-2 complexes are fully developed, they will be useful for the specific expansion of target cells and will probably require less frequent injections (77). Biologic nanoparticles have attracted attention over the years for targeted therapy. For example, nanoscale liposomal polymeric gels (nanolipogels) are biologically compatible and slowly biodegradable agents. Fahmy and colleagues recently developed nanolipogels encapsulated recombinant IL-2 and TGFβ, and anti-CD4-labeled nanolipogels with IL-2 and TGFβ successfully expand Tregs *in vitro* and *in vivo* (78). Use of IL-2-nanoparticles tagged with an antibody recognizing specific tissues will result in bore specific delivery and lower toxicity.

TARGETING METABOLISM TO INCREASE Treg STABILITY

Mechanistic Target of Rapamycin (mTOR)

Dynamic changes of cellular metabolism are necessary for efficient immune cell activation, growth, proliferation, and differentiation. In the quiescent state, T cells use mitochondrial tricarboxylic acid cycle to generate ATP and sustain homeostasis. When stimulated, cell metabolism shifts to anabolic pathways

to produce building blocks needed to promote and sustain cell proliferation. Therefore, glycolytic metabolism is induced in activated T cells. The hosphatidylinositol-3-kinase (PI3K)–Akt–mTOR pathway plays a critical role in the regulation of glycolysis. Generally, resting Tregs utilize a distinct metabolic program based on mitochondrial oxidation of lipids (β-oxidation). When Tregs proliferate, glycolysis is also observed but their suppressive function is reduced. Conversely, Foxp3 inhibits the PI3K-Akt-mTOR pathway and glycolysis (79). mTOR consists of two multiprotein complexes (mTORC1 and mTORC2) and acts as a critical regulator of cell growth, metabolism, differentiation and survival. In mice with Treg-specific depletion of the regulatory-associated protein of mTOR, a component of mTORC1, Tregs lose their suppressive function resulting into severe autoimmunity (80). Inhibition of mTORC2 by mTORC1 has been shown to be important for Treg function and generation. On the other hand, mTORC1 inhibits *de novo* Treg differentiation and proliferation (81). Furthermore, uncontrolled activation of mTORC1 leads to the development of autoimmunity with deficiency of suppressive function of Tregs (82). Several studies with mice deficient in the mTOR regulatory systems showed functional impairment of Tregs that leads to systemic autoimmunity (82–85). Human Tregs have been reported to be expanded efficiently in the presence of the mTORC1 inhibitor rapamycin (86). In SLE, activated mTOR in T cells accounts for several abnormalities including the downregulation of CD3ζ, the expansion of T_H17 and $CD3^+CD4^-CD8^-$ DN T cells and the contraction of Tregs (87, 88). Administration of rapamycin has been reported to improve clinical outcomes in lupus-prone mice (89) and patients with SLE (90). Moreover, rapamycin can block the production of antiphospholipid antibody in lupus-prone mice (91) and enhance renal allograft survival of antiphospholipid syndrome patients (92). Therefore, rapamycin is a promising candidate for the treatment of patients with SLE because it normalizes various T cell functions including that of Tregs. Interestingly, inhibition of both mitochondrial electron transport by metformin and glucose metabolism by 2-deoxy-D-glucose (2DG) ameliorated disease in lupus-prone mice and cGVHD (93). Metformin can also inhibit mTORC1 by activating AMPK. Activated T cells, Tfh, and germinal center B cell and anti-dsDNA antibody titer were decreased, indicating that metabolic control can prevent aberrant activation of immune cells in autoimmunity (93).

Calcium/Calmodulin-Dependent Kinase IV (CaMK4)

CaMK is a serine/threonine kinase family protein which becomes activated when intracellular calcium binds to calmodulin to generate the calcium/calmodulin complex. CaMK4 translocates

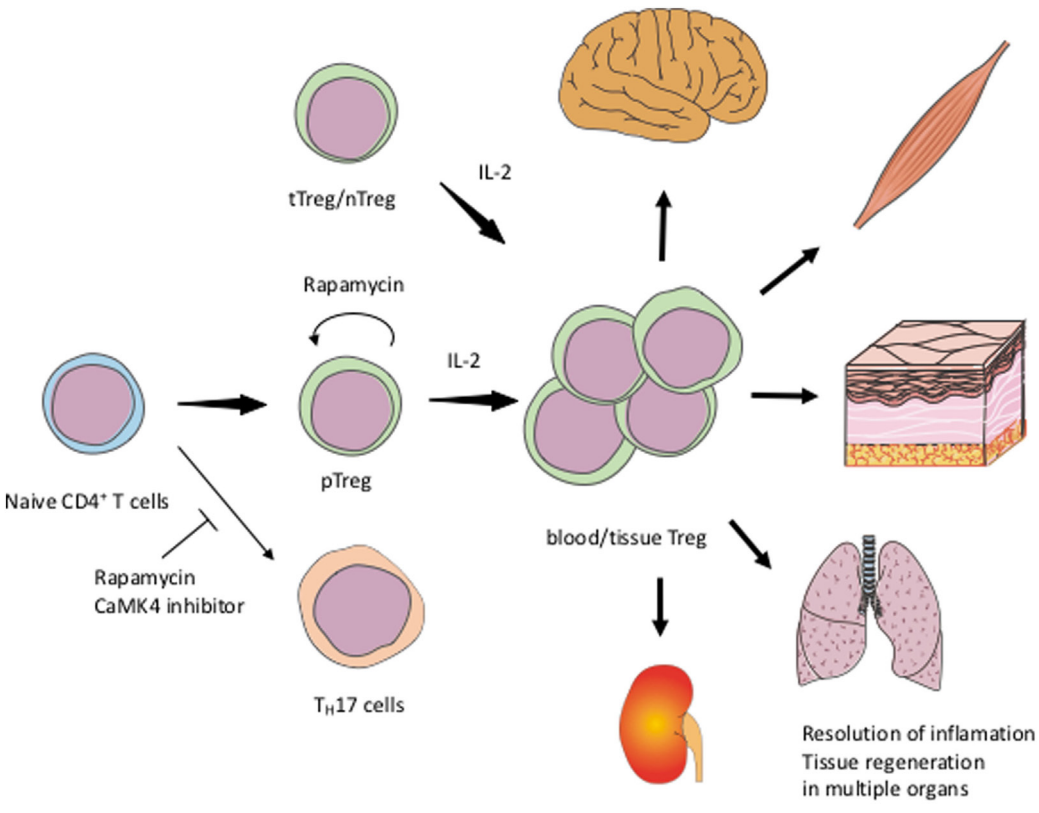

FIGURE 1 | Schematic view of exploiting Tregs for the treatment of SLEIL-2 expands both tTreg/nTreg and pTreg including tissue Treg. Rapamycin and CaMK4 inhibitor facilitate the differentiation of Treg and inhibit T_H17. Rapamycin can also stabilize the Treg function. tTreg, thymic regulatory T-cells; nTreg, naïve Treg; pTreg, peripheral Treg; T_H17, IL-17-producing helper T-cells.

in the nucleus and regulates the activation of several transcription factors including CREB and CREM (94). CaMK4 expression of SLE T cells is upregulated and CaMK4-deficient lupus mice show amelioration of autoimmunity with decreased T_H17 and increased Treg cell numbers. Therefore, CaMK4 is involved in the pathogenesis of SLE by altering the balance between T_H17 and Treg cells. Both T_H17 and iTreg cells need TGFβ for their development. T cells expressing both Foxp3 and RORγt are generated intermediately and can differentiate to T_H17 or iTreg dependent on the milieu. Therefore, T_H17 and iTreg cells display plasticity which allows them to exchange phenotype. We recently reported that CaMK4 regulates T_H17 cell differentiation by activating Akt–mTOR pathway as well as by enhancing CREMα-mediated IL-17 transcription (51). CaMK4 is preferentially expressed by T_H17 cells and its deficiency mitigated the differentiation of naïve $CD4^+$ T cells into T_H17 cells. Because the mTOR–TORC1 pathway is essential for T_H17 differentiation, CaMK4–Akt–mTOR axis might be critical for effective T_H17 development. Moreover, administration of the CaMK4 inhibitor KN93 sufficiently expanded Tregs *in vivo* and alleviated disease in lupus-prone mice. Lastly, Otomo et al showed that anti-CD4-tagged nanoparticles loaded with KN93 selectively delivered the drug to $CD4^+$ lymphocytes and mitigated disease in lupus-prone mice and in mice induced to develop experimental autoimmune encephalomyelitis (95).

Treg INFUSION THERAPY

Several studies using animal models have demonstrated that adaptive transfer of natural or *ex vivo*-expanded Tregs can inhibit GVHD (96, 97) and solid organ transplant rejection (98). Treg infusion for human GVHD has been reported to be effective and currently a number of clinical trials involving the infusion of Tregs in patients receiving hematopoietic stem cell, kidney, and liver transplants are in progress (99). Since the number of Tregs which can be isolated from the peripheral blood or umbilical cord blood is limited, various strategies to expand Tregs *in vitro* have been considered including anti-CD3/CD28-coated beads in the presence of IL-2 and/or TGF-β and in the presence or absence of rapamycin (NCT02129881, NCT01624077). A recent study confirmed besides the efficacy, the safety, and feasibility of the injection of isolated or *ex vivo*-expanded Tregs in patients receiving transplant organs (100). Treg cell therapy is also ongoing in patients with type 1 diabetes (T1D) based on the evidence that deficiency of Tregs is important in the pathogenesis of the disease. A phase I trial (NCT01210664) designed to assess safety of adoptive Treg immunotherapy in patients with T1D has been completed. Specifically, T1D patients received *ex vivo*-expanded autologous $CD4^+CD127^{low/-}CD25^+$ polyclonal Tregs (0.05×10^8 to 26×10^8 cells) using anti-CD3/CD28 beads plus IL-2. Twenty-five percent of transferred Tregs were detected even after one year without any adverse effect (101). A study involving the infusion of expanded autologous Tregs in a similar manner for the treatment of SLE has now been launched (NCT02428309). Since adaptive transfer of Treg in the setting of lupus-prone mice was reported effective in the suppression of glomerulonephritis and prolonging survival (102, 103), clinical efficacy is also expected from the trial in patients with SLE.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Sustained production of autoantibody and intermittent release of self-antigen can induce chronic activation of innate and adaptive immune responses in SLE. Chronic inflammatory conditions induce aberration of the immune system homeostasis. Further studies are necessary to elucidate the detailed mechanisms by which Tregs suppress autoimmune-associated tissue inflammation and regeneration.

Low-dose IL-2 for the treatment of patients with SLE appears to be a promising, selective therapeutic strategy to expand Tregs numerically and functionally. Formulations of IL-2 to expand its half-life in the blood and to decrease the number of required injections are needed. Rapamycin and CaMK4 inhibitors would also be candidate drugs to enhance Treg function (**Figure 1**). IL-2/Sirolimus/Tacrolimus combination therapy was tried in patients undergoing hematopoietic stem cell transplantation with promising results (104). In mice, combination therapy of IL-2 and rapamycin effectively expanded Tregs and prevented acute rejection of skin grafts (105). Clinical trials of Treg-targeted treatments are currently in progress and it is expected to demonstrate that expansion or supplementation of Tregs can be added to the treatment choices physicians have in their disposal to treat patients with SLE.

AUTHOR CONTRIBUTIONS

MM and GT wrote and edited the review.

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Systemic Lupus Erythematosus: Definitions, Contexts, Conflicts, Enigmas

*Ole Petter Rekvig**

Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway

Systemic lupus erythematosus (SLE) is an inadequately defined syndrome. Etiology and pathogenesis remain largely unknown. SLE is on the other hand a seminal syndrome that has challenged immunologists, biologists, genetics, and clinicians to solve its nature. The syndrome is characterized by multiple, etiologically unlinked manifestations. Unexpectedly, they seem to occur in different stochastically linked clusters, although single gene defects may promote a smaller spectrum of symptoms/criteria typical for SLE. There is no known inner coherence of parameters (criteria) making up the disease. These parameters are, nevertheless, implemented in The American College of Rheumatology (ACR) and The Systemic Lupus Collaborating Clinics (SLICC) criteria to classify SLE. Still, SLE is an abstraction since the ACR or SLICC criteria allow us to define hundreds of different clinical SLE phenotypes. This is a major point of the present discussion and uses "The anti-dsDNA antibody" as an example related to the problematic search for biomarkers for SLE. The following discussion will show how problematic this is: the disease is defined through non-coherent classification criteria, its complexity is recognized and accepted, its pathogenesis is plural and poorly understood. Therapy is focused on dominant symptoms or organ manifestations, and not on the syndrome itself. From basic scientific evidences, we can add substantial amount of data that are not sufficiently considered in clinical medicine, which may change the paradigms linked to what "The Anti-DNA antibody" is—and is not—in context of the imperfectly defined syndrome SLE.

Keywords: systemic lupus erythematosus, syndrome, anti-dsDNA antibodies, criteria, definitions, enigma

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***Correspondence:**
Ole Petter Rekvig
olepr@fagmed.uit.no

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INTRODUCTION

This study represents an open-minded approach to try to understand the nature of the syndrome Systemic lupus erythematosus (SLE), how it is defined, and how to comprehend its pathogenesis and biomarkers. The core of this approach is that it seems difficult for relevant basic and clinical scientists to agree to conformed definitions of the syndrome.

Systemic lupus erythematosus is an historically old disease described already in antiquity. The disease is a scientifically challenging (1, 2), problematic (3–6), inspiring (7, 8) and seminal (9–11), clinical syndrome (12). The syndrome is real in its existence—although hidden behind obstacles, cumbersome for patients and clinicians, and rebellious for scientists. It has inspired medical and basic biological scientists that focus on molecular biology, basic immunology, immunopathology, clinical science, genetics, and epidemiology. Scientists belonging to all these

disciplines attempt to describe the nature of the syndrome SLE but also of individual parameters that constitute criteria characterizing the syndrome.

From a wider perspective, studies of anti-dsDNA antibodies in SLE have significantly enriched our knowledge about more general aspects of the immune system itself. For example, studies of SLE have promoted a better insight into how the immune system controls discrimination between anti-self and anti-non-self responses. This includes the role of the innate immune system in autoimmunity (13–16), the regulation of B cell and T cell tolerance and deletion (17–19) and receptor editing in B cells (20–24). Yet, the nature and origin of the anti-dsDNA antibody itself remain largely enigmatic. We are today not able to explain why these antibodies appear in something called SLE. On the other hand, problems to define SLE has been a concern for empirical and system sciences (25) and has been applied to nearby all aspects of the disease (7–9, 26–39).

Systemic lupus erythematosus and its biomarkers have been and are still investigated by an interdisciplinary scientific field that combine elements from empirical, basic, and clinical sciences. Historical descriptions represent an origin for empirical arguments to describe SLE as a serious disease with cutaneous manifestations (40–43). Herbernus of Tours (916 AD) was among the first to use the term "lupus" to characterize this disease [see Ref. (43)]. Further descriptions were in the nearer past expanded by the pioneering studies of Osler and Kaposi who extended our insight into the disseminated nature of lupus erythematosus; the involvement of other organs than the skin [see, e.g., Ref. (40, 44)].

The different empirical, clinical and experimental approaches to understand what SLE is, does not imply that individual aspects are implemented in systemic multidisciplinary approaches. This statement is formulated simply because results from basic sciences relevant for SLE are only halfheartedly implemented in clinical contexts. This is discussed in detail subsequently with a focus on what SLE and "The anti-dsDNA antibody" are—and what they are not. In my opinion, there is a lack of critical cross-talks between the different fields of science that are applied to, or relevant for SLE. Many approaches deals with cohort studies based on classification criteria [see, e.g., Ref. (33, 45–47)]. Per definition, patients that fulfill a minimum of classification criteria are implemented in a cohort. This means that the patients compared to each other are phenotypically different (see **Figure 1** for principle problems). This is a problematic situation.

Without implementing principles evolving from system analyses, which mean to unify interdisciplinary fields that attempt to study complex natural (48) or, e.g., medical syndromes (49), we may be left with SLE as we know it today, an enigmatic and a controversial, unclassified syndrome. Without a systematic approach, this will preclude a holistic view on the complexity of SLE as a functional or formal unit.

Unfortunately, instead of being concerned with complete systems, there is today a clear trend toward studies of SLE by individual disciplines unlinked from each other. As a paradox, many disciplines are engaged in the search to understand SLE, although the different disciplines hardly communicate with each other. Thus, we miss an organized approach to prioritize

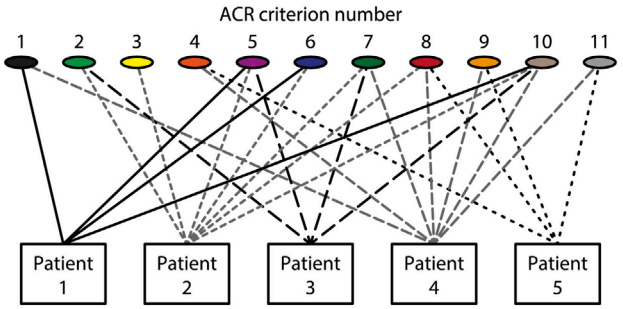

FIGURE 1 | Patients classified to have systemic lupus erythematosus (SLE) by the The American College of Rheumatology (ACR) classification criteria—diversity of the clinical phenotypes. On top of the figure, each of the 11 ACR criteria is presented symbolically (see **Table 1** for details on the ACR criteria). Five patients are demonstrated. The patients share some criteria, but diverge with respect to others. This chaotic figure demonstrates that the use of criteria is dubious to investigate pathogenesis of the syndrome and to search for biomarkers to characterize the syndrome SLE. How can we determine common features or biomarkers, when SLE presents so many different phenotypes? The patients in this figure are fictive but they reflect problems with the ACR in real life.

a holistic perspective by not taking all aspects of the syndrome SLE into account. This can only be achieved by concentrating on the interactions between its different elements. System science (25, 49) in our context provides a framework in which assessment of data generated by experts in different fields can be combined, and confronted with each other in order to determine what we agree on, what must be done, and what the best strategy forward must be. The following discussion is an attempt to underscore the need for profound cross-talks between scientific disciplines.

DEFINING SLE: DO WE MAKE SIMPLIFICATIONS BY IGNORING PROBLEMS THAT DO NOT FIT INTO THE SYNDROME AS AN ENTITY?

Contemporary problematic situations have precipitated the trivial view that SLE is a multiorgan disease with poor understanding of its pathogenesis (2, 9). How can we think of SLE as a well-defined syndrome when it presents with hundreds of different phenotypes (defined by combined classification criteria)? And how can we search for biomarkers for such a poorly defined syndrome? Werner Heisenberg, who was a principal scientist in the German nuclear energy project during World War II, and a Nobel Prize laureate, formed the following anti-positivistic idiom that, I think, can be applied to all kinds of complex scientific problems. This includes also definition and understanding of syndromes like SLE: "The positivists have a simple solution: the world must be divided into that which we can say clearly and the rest, which we had better pass over in silence. But can anyone conceive of a more pointless philosophy, seeing that what we can say clearly amounts to next to nothing? If we omitted all that is unclear, we would probably be left with completely

uninteresting and trivial tautologies" (50). And in this context, it is also relevant to cite Ludvik Fleck, a polish microbiologist and philosopher. He developed a system of the historical philosophy and sociology of science: "For the current state of knowledge remains vague when history is not considered, just as history remains vague without substantive knowledge of the current state" (51). Here, Fleck points to, and reminds us of an important element of system science; the implementation of empirical and historical knowledge.

The two paradigms cited earlier will function as backdrops for the problems discussed subsequently related to try to understand what SLE is. Obviously, there are needs to develop new hypotheses and to test them critically. To do so, we then have to consider one objective in sight; how do we define a hypothesis that may enable us to understand the substance of a syndrome like SLE? Here we have to ask the central questions: When are data proving something beyond subjective interpretations and simplifications, and when do we accept tautologies in order to simplify our research and paradigms related to SLE? These questions are closely associated with the term hypothesis. What is a hypothesis, and what purpose will the hypothesis serve; An approach to search for truth (the ideal context)? Or to confirm contemporary or historically simplified models (the historical or subjective context)?

THE SYNDROME SLE: HISTORICAL AND CONTEMPORARY CONTEXTS

Systemic lupus erythematosus has been studied intensively since the last century (since 1942, about 65,000 SLE-related articles appear on PubMed with the search term "systemic lupus erythematosus"). This enormous amount of data and paradigms has not provided us with profound consensus on its nature, etiology or pathophysiology [see, e.g., Ref. (52, 53)]. Therefore, unclear or contradicting data and results in the past force many of us to choose solutions as if they are real although they are based on tautologies that may simplify our interpretations—leaving its significant historical context in silence (40–43). Thus, it may still be difficult to define SLE, as it was in historical, and yet in contemporary times. It seems that in antiquity, the disease was characterized by serious cutaneous affections while in modern times, more and more parameters and criteria are added to the list making up the SLE phenotypes. This makes it difficult to comprehend the nature of the disease. Here, I will discuss what we understand of SLE in terms of its wide definition as a syndrome, and if it is a possible task to use such a definition to determine biomarkers that characterize it, or point to it.

SLE: A MULTI-ORGAN DISEASE OR A DISEASE LINKING ANTI-dsDNA ANTIBODIES AND EXPOSED CHROMATIN TO NEPHRITIS AND DERMATITIS?

Systemic lupus erythematosus is described as a multiorgan, though mysterious disease (2, 5, 6, 9, 54). The different organ and laboratory manifestations are confusing since they have no inner pathogenic coherence, but can appear in a non-concurrent way. Still it is defined as a syndrome. Then, how can we think of SLE as a well-defined syndrome when it presents with quite different phenotypes [see, e.g., Ref. (55)], and how can we search for biomarkers in such a diffuse and non-stringent situation? We do not see radical solutions in the near future as to how to explain its nature. Rather, we make simplifications, worryingly in line with what Heisenberg stated, in trying to understand the disease, and to find its biomarkers. Today we classify SLE by sets of criteria, like The American College of Rheumatology (ACR) (6) and The Systemic Lupus Collaborating Clinics (SLICC) (5) criteria, and search for biomarkers in situations where minimum requirements of criteria are fulfilled irrespective of which of the criteria are present. In this situation, the current state of knowledge remains vague, and does not take into account descriptions back in antiquity as being a serious cutaneous disease probably involving the kidneys as the malignant element. This is deduced from the fact that lupus-associated kidney and skin affections may have a common or similar pathogenic origin(s) (7, 34, 56, 57). The same problems relate to treatment; we classify a disease phenotypically as a syndrome, but we treat the most serious organ manifestations, not the syndrome as a whole [see a discussion in Ref. (58)]. Are we in fact disseminating the core of the classical disease into a myriad of parameters, biomarkers, symptoms and statistics (manuscript in progress)? Regardless, it seems that the classification systems for SLE are established, and used in diverse contexts; diagnostics, search for biomarkers, but also for single manifestations, and for therapeutics. There are obvious needs to develop new hypotheses to describe SLE!

For example, one approach would be to analyze expression levels of factors indispensable for chromatin metabolism *in vivo*. Recently, a familiar form of SLE was described. This was linked to a null mutation of the gene that encodes the secreted deoxyribonuclease DNase 1L3 (59). Sisirak et al. nicely confirmed the link between experimental DNase 1L3 deficiency in mice and a consequent autoimmunity to dsDNA and nephritis (60). Thus, the clinical version of the DNase 1L3 deficiency (59) is directly copied by the DNase 1L3 deficiency in experimental mice. This is an important approach to describe functional defects leading to pathogenic autoimmunity caused by single gene defects. More such murine models are expected to appear in the near future where deficiencies of single genes that appear central for chromatin metabolism may result in a lupus-like phenotype. If, like in the DNase 1L3 deficient mice, the clinical phenotype is characterized by anti-dsDNA antibodies and nephritis, this would give a hint to the need for re-classification of the human SLE into a "hot" SLE with an anti-dsDNA-antibody-driven chromatin-mediated nephritis phenotype. This would leave non-nephritis/non dermatitis behind as lupus phenotypes. Such studies are awaited.

HOW DO WE DEVELOP TESTABLE HYPOTHESES AIMED TO DESCRIBE SLE?

Kuhn (61) argued that a "paradigm determines the kinds of experiments scientists perform, the types of questions they

ask, and the problems they consider important" [cited in Ref. (62)]. Thus, according to Kuhn, a paradigm may form the bases for different hypotheses. Unfortunately, these may promote evolution of incommensurable models to explain the nature of the disease or the study-object. This can be anticipated as far as different hypotheses raised to solve a problem release different experimental models that may result in divergent interpretations, simply because different hypotheses are tested by different analytical approaches. These yield different analytical results and consequently, different models may appear (see subsequently for details). On the other hand, a hypothesis is formed to describe a process that may be real (as relevant for SLE), or to describe a phenomenon that lacks scientific evidence for its very existence, like the scientific history of the assumption and subsequent prove for the existence of the Higgs boson (63). In fact, the history of SLE is paralleling the history of the Higgs boson—do we lack formal evidence for the existence of the syndrome called SLE, or is SLE still formally an abstraction?

In a biological context, rather than in, e.g., theological or philosophical contexts, it is required that we can test a hypothesis by scientific methods that materialize its real biological and explainable existence. A critical hypothesis is therefore the basis to help us solve complicated system-related biological aberrations like those encountered in SLE.

One fundamental hypothesis could be formulated with the aim to study why manifestations like the classification criteria in SLE appear in various clusters, like criteria in the ACR (**Table 1**) and SLICC (**Table 2**) classification systems do. This may result in one of two possible answers; there is no causal or biological link between them; or the clusters are based on biological processes that form a causal reason for this linkage. We are far from knowing the truth about SLE, its nature, and its heterogenic phenotypes.

TABLE 1 | 1997 American College of Rheumatology SLE Classification Criteria[a] (6).

- Malar rash: butterfly shaped rash across cheeks and nose
- Discoid (skin) rash: raised red patches
- Photosensitivity: skin rash as result of unusual reaction to sunlight
- Mouth or nose ulcers: usually painless
- Arthritis (non-erosive) in two or more joints, along with tenderness, swelling, or effusion. With non-erosive arthritis, the bones around joints don't get destroyed
- Cardio-pulmonary involvement: inflammation of the lining around the heart (pericarditis) and/or lungs (pleuritis)
- Neurologic disorder: seizures and/or psychosis
- Renal (kidney) disorder: excessive protein in the urine, or cellular casts in the urine
- Hematologic (blood) disorder: hemolytic anemia, low white blood cell count, or low platelet count
- Immunologic disorder: antibodies to double stranded DNA, antibodies to Sm, or antibodies to cardiolipin
- Antinuclear antibodies (ANAs): a positive test in the absence of drugs known to induce it

[a]*Requirements: Any combination of four or more of 11 criteria, well documented at any time during a patient's history, makes it likely that the patient has SLE (specificity and sensitivity are 95 and 75%, respectively).*

TABLE 2 | The Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for Systemic lupus erythematosus[a] (5).

CLINICAL CRITERIA
Acute cutaneous lupus or subacute cutaneous lupus
• **Acute cutaneous lupus:** lupus malar rash (do not count if malar discoid), bullous lupus, toxic epidermal necrolysis variant of SLE, maculopapular lupus rash, photosensitive lupus rash (in the absence of dermatomyositis) • **Subacute cutaneous lupus:** nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with postinflammatory dyspigmentation or telangiectasias
Chronic cutaneous lupus
• Classic discoid rash localized (above the neck) or generalized (above and below the neck), hypertrophic (verrucous) lupus, lupus panniculitis (profundus), mucosal lupus, lupus erythematosus tumidus, chillblains lupus, discoid lupus/lichen planus overlap
Oral ulcers or nasal ulcers
Oral: palate, buccal, tongue Nasal ulcers In the absence of other causes, such as vasculitis, Behcet's disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis, and acidic foods
Nonscarring alopecia
Diffuse thinning or hair fragility with visible broken hairs, in the absence of other causes such as alopecia areata, drugs, iron deficiency, and androgenic alopecia
Synovitis involving 2 or more joints
▪ Characterized by swelling or effusion ▪ OR tenderness in 2 or more joints and at least 30 min of morning stiffness
Serositis
▪ Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub ▪ Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day OR pericardial effusion OR pericardial rub OR pericarditis by electrocardiography ▪ In the absence of other causes, such as infection, uremia, and Dressler's pericarditis
Renal
▪ Urine protein–to-creatinine ratio (or 24-h urine protein) representing 500 mg protein/24 h OR red blood cell casts
Neurologic
▪ Seizures, psychosis, mononeuritis multiplex (in the absence of other known causes such as primary vasculitis), myelitis, peripheral or cranial neuropathy (in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus), acute confusional state (in the absence of other causes, including toxic/metabolic, uremia, drugs)
HEMOLYTIC ANEMIA
Leukopenia (<4,000/mm^3) or lymphopenia (<1,000/mm^3)
▪ Leukopenia at least once: In the absence of other known causes such as Felty's syndrome, drugs, and portal hypertension ▪ Lymphopenia at least once: in the absence of other known causes such as corticosteroids, drugs, and infection
Thrombocytopenia (<100,000/mm^3)
▪ At least once in the absence of other known causes such as drugs, portal hypertension, and thrombotic thrombocytopenic purpura

(*Continued*)

Immunologic criteria

(1) ANA level above laboratory reference range
(2) Anti-dsDNA antibody level above laboratory reference range (or 2-fold the reference range if tested by ELISA)
(3) Anti-Sm: presence of antibody to Sm nuclear antigen
(4) Antiphospholipid antibody positivity, as determined by
- Positive test for lupus anticoagulant
- False-positive test result for rapid plasma reagin
- Medium- or high-titer anticardiolipin antibody level (IgA, IgG, or IgM)
- Positive test result for anti–2-glycoprotein I (IgA, IgG, or IgM)

(5) Low complement (C3, C4, or CH50)
(6) Direct Coombs' test (in the absence of hemolytic anemia)

[a]*Requirements: ≥4 criteria (at least 1 clinical and 1 laboratory criteria) or biopsyproven lupus nephritis with positive ANA or anti-DNA.*

THE CURRENT DEFINITION OF THE SYNDROME SLE AND PROBLEMS LINKED TO IT

Systemic lupus erythematosus is a syndrome without a clear definition of what it is, and it is unclear whether the use of the term syndrome should at all be used in the context of SLE. The word syndrome descends from the Greek word σύνδρομον, that concisely translate into the word "concurrence" in the sense of the simultaneous occurrence of symptoms, events or parameters that are timely appearing together through a common etiology or cause (64). This strict definition of a syndrome as a condition with simultaneously appearing events is not implemented when we discuss SLE as a syndrome. In fact, the proposed ACR SLE classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have SLE if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observations (**Table 1**). Thus, the manifestations do not have to appear timely together, they may appear also in an accumulated fashion one by one, and then later make up a syndrome that fulfill a term analogous to the idiom syndrome [see, e.g., Ref. (6) and discussion in Ref. (1)]. But this is not harmonizing with the classical use of the term "syndrome" (=concurrence). We are therefore facing two principally different problems: (i) the definition of the syndrome SLE and (ii) its lack of a unifying or inciting pathophysiological explanation. So we have to make a distinction between a disease with secondary manifestations, and a syndrome with (non-) concurrent manifestations that are linked in a yet not understood way—if biologically linked at all. This may be further problematized by the following perceptions using anti-dsDNA antibodies as biomarkers regarded as specific for SLE.

Systemic lupus erythematosus is per definition composed of divergent organ manifestations and laboratory aberrancies This may open for a heterogenic population of SLE patients included in cohort studies [see, e.g., Ref. (5, 9, 11, 22, 53)]. There is no common denominator for the large varieties of this disease's phenotypes (see the principle **Figure 1**). In ACR criteria (**Table 1**), 4 out of these 11 criteria must be fulfilled to classify a disease as SLE. This means, by random combinations of 4/11 criteria, we have to accept that SLE by these criteria may theoretically present 330 different clinical phenotypes! The more recent SLICC SLE classification system (**Table 2**) has not helped us here. Therefore, SLE has many phenotypes that by reciprocal comparisons are quite different, is characterized by different distinct organ/laboratory manifestations, and present quite different clinical pictures. How then can we search for biomarkers correlating with the syndrome SLE as it is defined today?

THE ANTI-dsDNA ANTIBODY AS BIOMARKER IN SLE: POOR DEFINITION OF THE PARTNERS

Antibodies to dsDNA are claimed to be associated with, and to serve as biomarkers for SLE (8, 65, 66). However, "The anti-dsDNA antibody" is not an unambiguous parameter (7, 8, 52), and SLE is not an unambiguously defined disease. How then can "The anti-dsDNA antibody" serve as a biomarker for the syndrome SLE?

Theories, models or algorithms are defined as incommensurable if they derive from contrasting experimental or theoretical contexts although aimed to describe the very same problem. Their basic parameters may not be sufficient to permit scientists to directly compare the models or to cite empirical evidence favoring one theory over another (51). Incommensurable models inevitably promote scientists to be confused about terms, contexts and consequences, as is the case for SLE. With respect to SLE and to, e.g., lupus nephritis, divergent pathophysiological models may preclude consensus on pathogenesis [see, e.g., Ref. (61)]. To harmonize models that are divergent in order to reach *de facto* consensus may be a *sine qua non* in development of causal therapies.

In this context, we have to accept that facts simply are the case, subsequently interpreted as that—objective, physically distinguishable traced cases. They are discovered through proper observations from experimentally testable realities. Fleck would here submit that facts are invented or interpreted—not discovered. One can add to the problem of "conclusive facts or data" the following paradox in serious science: If experiments are performed to prove a hypothesis, then data can be interpreted as if the model simply reflects the exact fact. A problem in this context is the traditional impediment to generate experiments aimed to actively prove the opposite; namely that the hypothesis is wrong. This situation envisages how the same study-object promotes incommensurable models because those that will prove the validity of a hypothesis describes a model that differs from alternative models that are based on experiments instigated by other hypotheses.

How then can we succeed when the efforts are aimed to explain a syndrome with so many clinical phenotypes, and how can we search for biomarkers like "The anti-dsDNA antibody" in this landscape? Do we here see the contour of serious problems linked to cohort studies were the study-objects (here SLE patients) are classified by internationally well-accepted criteria (like ACR or SLICC)? In this context, it is clear that the cohort is basically

heterologous and not suitable for causal and penetrating studies of SLE. Can a search for biomarkers help us here?

SLE AND ANTI-dsDNA ANTIBODIES: DO THE LATTER REFLECT THE FIRST?

In order to discuss the nature and role of anti-dsDNA antibodies as biomarkers or pathogenic factors in clinical medicine, it may be wise to shortly describe the history of the antibody.

ANTI-dsDNA ANTIBODIES—A SHORT HISTORY

In the history of immunity, hardly any naturally produced autoantibody has attracted so many basic- and clinical-oriented scientists as the antibody against mammalian B helical DNA. During the last 40 years, more than 2,200 articles are published (PubMed search term: anti-dsDNA antibodies). The anti-DNA antibodies were first described in 1938 and 1939 in bacterial infectious contexts (67–69), and further studied and their presence confirmed 15 years later (70). In 1957, anti-DNA antibodies were described in a strict autoimmune context; in SLE (71–74).

In 2015 (7) and in 2016 (8), manuscripts were published with apparently opposite views regarding the clinical and biological impact of anti-dsDNA antibodies. In the first, their impact as biomarkers for SLE were questioned and critically discussed (7, 66). In the other, they were after a sound and critical survey of the literature defined as quintessential biomarkers for SLE (8). The clear statement denoting anti-dsDNA antibodies as strong biomarkers for SLE (8) is, however, difficult to comprehend in light of the definition of SLE, its many different phenotypes, and the wide range of biological properties and unique specificities of "The anti-DNA antibody" [a term used in the classification criteria for SLE (5, 6)]. On the other hand, the view that hesitates to accept anti-dsDNA antibodies as biomarkers for SLE (7) is hard to understand in light of the enormous efforts and data to prove exactly that. The ACR criterion 10 says: "Anti-DNA: antibody to native DNA in abnormal titer" (6), while in the SLICC criteria anti-dsDNA antibodies is described as valid if the Anti-dsDNA antibody level is above laboratory reference range (or twofold the reference range if tested by ELISA) (5).

There are several practical and theoretical tribulations linked to these definitions. The anti-dsDNA antibody is a poorly defined term that does not take into account that anti-dsDNA antibodies comprise a large array of unique specificities, quite different and unique origins, some based on spontaneous autoimmunity, some linked to cancers, others linked to external factors like drugs and infections. Furthermore, according to the classification criteria, the antibody may be weak (although above a threshold value), transient, sustained at high or low titers in different patients suffering from, e.g., SLE, cancers or infectious diseases (see the principles in **Figure 2**), and they may differ in avidity and cross-reactivity as long as they bind dsDNA. Still, they are accepted as a SLE classification criterion. This will be discussed in detail subsequently.

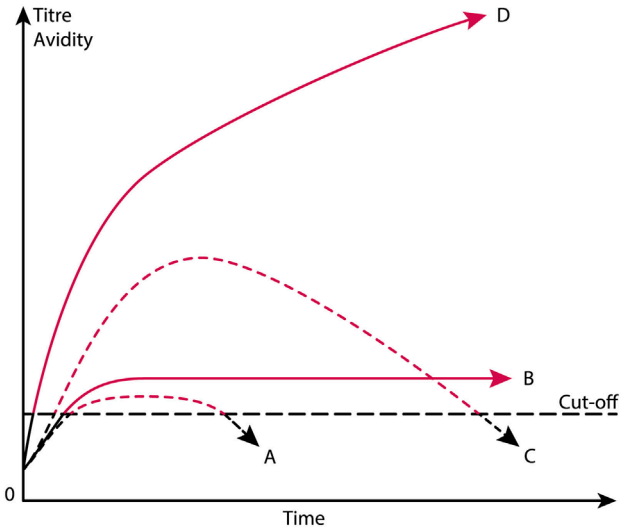

FIGURE 2 | Theoretical anti-dsDNA antibody profiles in context of systemic lupus erythematosus (SLE) classification criteria. The American College of Rheumatology (ACR) and SLICC SLE classification criteria include anti-dsDNA antibodies as a criterium. As a criterium the antibodies are poorly defined. For example, a short-lived stimulus by an infectious agent may induce transient antibodies at low titers **(A)**. If the infectious stimulus prevails, the anti-dsDNA antibody may prevail at low titers, even though above the assay cutoff level **(B)**. The anti-dsDNA antibody production in **(C)** is transient, although at high titers, as a consequence of a strong, transient stimulus either of autologous or, e.g., infectious origin. In **(D)**, the immune response is characterized by sustained production of anti-dsDNA antibodies at high to very high titers. The red parts of each profile represent autoantibody levels above the antibody cut-off levels as defined by ACR or SLICC criteria. The curves are fictive and constructed empirically in order to demonstrate the variability of anti-dsDNA antibody profiles, all of which fulfill requirements in the ACR and SLICC classification criteria for SLE. See text for details.

ANTI-dsDNA ANTIBODIES—STATUS OF THEIR DEFINITION AND CLINICAL IMPACT

Dogmas say that anti-dsDNA antibodies are real—they exist (although they may be induced by non-dsDNA immunogens), they occur in SLE, and they represent a classification criterion for SLE. These dogmas can, however, be analyzed in light of the philosophical view cited earlier by Heisenberg and thus be transformed and applied to our present problem. Our way to describe SLE practically represents a brave circumvention of problems or facts that do not fit into the simple solution (in the context expressed by the anti-positivists); namely that "the anti-dsDNA antibody" is not linked to SLE. Anti-dsDNA antibodies are detected in many other conditions, but the antibody may still be a pathogenic factor in SLE, provided DNA is exposed *in vivo* (7, 8, 66). The antibodies may recognize all nucleic acid structures presented in the chromatin, both in its resting state and in structures related to activation of chromatin (75, 76). These structures include DNA sequences, ssDNA, dsDNA, B dsDNA, Z dsDNA, elongated, or bent dsDNA [(77–82), reviewed in Ref. (7)]. It has never been determined if the manifold of anti-dsDNA antibodies

are all pathogenic. This problem has a strong impact on choice of DNA used as targets in clinical assays if we know which of the structures are important in a clinical context.

Already after the 1938/1939 and the 1957 observations on anti-dsDNA antibodies, a growing conflict ascended when scientists tried to describe the origins of the antibodies [infectious immunity versus true autoimmunity see, e.g., Ref. (7, 27) for discussions] and their clinical impact [in diagnostic and pathogenic contexts (5, 8, 54, 66)]. In particular, the antibodies have after 1957 been surrounded by myths, conflicts and enigmas up to contemporary times, due to the problem to determine (i) their biological origin—chromatin/dsDNA or crossreactive non-dsDNA structures; (ii) if they represent one antibody population or a heterogenic mixture of DNA-reactive antibodies with different precise specificities for unique DNA structures, and (iii) if targeted and inciting DNA structures originate from different species like viruses, prokaryotes, or eukaryotes. These aspects will be discussed in detail subsequently.

In this discussion, we need to challenge the canonical impact of "The anti-dsDNA antibody" as biomarker for the autoimmune syndrome SLE. "The anti-dsDNA antibody" exists and is described in context of bacterial and viral infections (27, 67–69, 83–93), different cancer forms (94–107) and in autoimmune syndromes like autoimmune hepatitis (108), Sjøgren syndrome (109), SLE (33), or primary antiphospholipid syndrome (110) and other disorders. The term "anti-dsDNA antibody" must therefore be changed to "anti-dsDNA antibodies," also in light of the different DNA structure-specificities that characterize the diverse anti-dsDNA antibodies. In a blinded study, it was found that assessment of anti-dsDNA antibodies by different assays was not reliable as a diagnostic tool in unselected patients with rheumatic symptoms (111, 112). Furthermore, anti-dsDNA antibodies had low positive predictive value for the SLE diagnosis [discussed in Ref. (52)]. For non-SLE patients, anti-dsDNA antibodies seemed to represent a poor predictor for SLE within an observation period of 5 years (111, 112).

Thus, as mentioned previously it can be stated that the ability to produce anti-dsDNA antibodies is not restricted to SLE. For example, normal mice respond to nucleosome-peptide immunization by producing anti-nucleosome antibodies, anti-ssDNA and anti-dsDNA antibodies, some of which may have pathogenic effects *in vivo* (4, 83, 86–88, 113–117).

THE ANTI-dsDNA ANTIBODY—LACK OF CONSENSUS STRUCTURE FOR DNA USED AS TARGET ANTIGEN IN CLINICAL ASSAYS

Anti-dsDNA antibody assay principles and nature of the assay targets are not recommended or specified in the ACR or SLICC criteria. Thus, we have not developed classification criteria for anti-dsDNA antibodies used in clinical analyses. We need here to define stringent structural criteria for the anti-dsDNA antibody assay targets. These must be combined with consensus on specific antibody profiles (transient versus persistent, **Figure 2**) and structural specificities (dsDNA, ssDNA, viral, plasmid, or elongated or bent mammalian dsDNA). Notably, the nature of target nucleic acids used in assays differ from laboratory to laboratory, which may result in detection of quite different nucleic acid structures and hence of different antibody specificities, unknown origins, or of different pathogenic impacts. For example, some of these antibodies are easy to induce experimentally, while some derive from processes yet not understood, as in SLE (7, 80, 118, 119), or exert differences in specificities for, e.g., B dsDNA versus Z dsDNA (80). It is not even settled whether autologous or heterologous instigating stimuli impose antibodies with similar or identical specificities as to those produced in SLE, although experimental data may indicate that [(87, 115, 117, 120, 121), reviewed in Ref. (7, 8)].

ANTI-dsDNA ANTIBODIES—ORIGINS AND CLINICAL CONTEXTS IN A BROADER SENSE

Anti-DNA antibodies can, as stated earlier be induced in different contexts and clinical situations. Some of the antibodies are clinical epiphenomena (e.g., due to low avidity, or because the targets for the antibodies are hidden or not exposed *in vivo*). Others may serve as quasi biomarker for SLE, but occur in many other disorders as well (7, 52, 66, 122). Some may serve as pathogenic factors mostly in kidneys (3, 7, 57, 123–128) or in skin (56, 123, 129).

In the next section, models and evidences will be presented and discussed that may make distance to the idiom that anti-dsDNA antibodies are biomarkers for SLE. As a devil's advocate[1], I will turn this statement up-side-down and argue that SLE is not consistently defined, and anti-dsDNA antibodies are not confined to SLE, but to many quite different conditions. Some of these conditions will be described subsequently and serve to demonstrate that anti-dsDNA antibodies are not unique for SLE. The main statement is that the anti-dsDNA antibodies are produced transiently or permanently, and all are accepted as ACR/SLICC criteria (**Figure 2**). The antibodies may be specific for different species DNA (e.g., from mammalia, fungi, bacteria, and viruses), and are produced in context of diverse malignancies. They present surprisingly different specificities for shapes exposed by the whole universe of dsDNA structures as they appear in relaxed and activated chromatin (7, 75). Still they are accepted as criteria for SLE!

BACTERIAL INFECTIOUS-RELATED IMMUNE RESPONSES TO NUCLEIC ACIDS—THE HAPTEN-CARRIER MODEL

Antibodies to nuclei acids have been known for 80 years in bacterial infectious contexts, while known for 60 years in

[1]Devil´s advocate (originally a catholic paradigm): someone who pretends, in an argument or discussion, to be against an idea or plan that a lot of people support, in order to make people discuss and consider it in more detail (Cambridge Dictionary).

an autoimmune context. Thus, anti-DNA antibodies were described distinctively earlier than their discovery in SLE in 1957. These findings implied that DNA stimulated the immune system during bacterial infections, and in context of SLE. Still, we do not know the molecular and cellular processes that promote the production of the antibodies in SLE, while we understand more about mechanisms that impose anti-DNA antibodies in context of infections [discussed in Ref. (7, 8, 27, 130)]. The different infectious models provide central concepts to describe autoimmunity to dsDNA, and to non-dsDNA proteins contained within chromatin (for details, see subsequently).

The infectious paradigm to explain incitement of anti-dsDNA autoimmunity, changed dramatically in mid 1990s by the pioneering and important studies of Pisetsky et al. (27, 83, 85). They successfully induced immune responses to bacterial, but also to autologous mammalian dsDNA. They did so by coupling bacterial DNA to the immunogenic carrier molecule methylated bovine serum albumin (mBSA), often used to induce antibodies to different natural and synthetic DNA structures [see, e.g., Ref. (77, 78, 131–133), reviewed in Ref. (7)]. This approach was in general known as the "hapten-carrier" paradigm. It says that nucleic acids or chromatin fragments serve as a B cell-specific non-immunogenic hapten-like structure. The carrier protein was immunogenic and presented to cognate T helper cells by the nucleic acid-specific B cells [(85, 87, 88, 131, 134), see examples of hapten-carrier models in **Figures 3** and **4**]. This type of cognate B cell and T cell interaction resulted in humoral responses against DNA structures recognized by the B cell.

Notably, bacterial DNA, in contrast to mammalian DNA, contains immune-stimulatory structures characterized by CpG motifs or immune-stimulatory sequences [ISS, discussed in Ref. (136, 137)]. These are characterized by the presence of two 5′ purines, an un-methylated CpG motif and two 3′ pyrimidines (138). The presence of a CpG motif in bacterial DNA stimulates the secretion of proinflammatory cytokines and therefore functions similarly to an adjuvant (136, 139, 140).

Pisetsky et al. observed that immune responses to bacterial DNA–mBSA complexes presented a remarkable dichotomy pattern. While responses to bacterial DNA in normal, non-autoimmune mice were dominated by antibodies specific for bacterial DNA (**Figure 3**, left panel) (141), the same immunization regime in young lupus-prone mice resulted in accelerated appearance of antibodies against mammalian dsDNA—i.e., for autologous DNA typical for the enigmatic antibodies appearing in SLE (**Figure 3**, right panel) (83, 142). This means that bacterial infections may promote production of lupus-like anti-dsDNA antibodies on certain genetic backgrounds. Since SLE is disposed for infections (143–146) the operational mechanism to produce anti-dsDNA antibodies in SLE may therefore well be linked to the infectivity state of the patients, at least for some of them (145, 147). From this, there is no doubt that bacterial infections have the potential to promote production of anti-bacterial and anti-mammalian dsDNA antibodies [discussed in Ref. (7, 8)]. Still, the model described by Pisetsky et al. is the most adequate explanation to describe at least initiation of immune responses to dsDNA in a natural *in vivo* situation related to SLE.

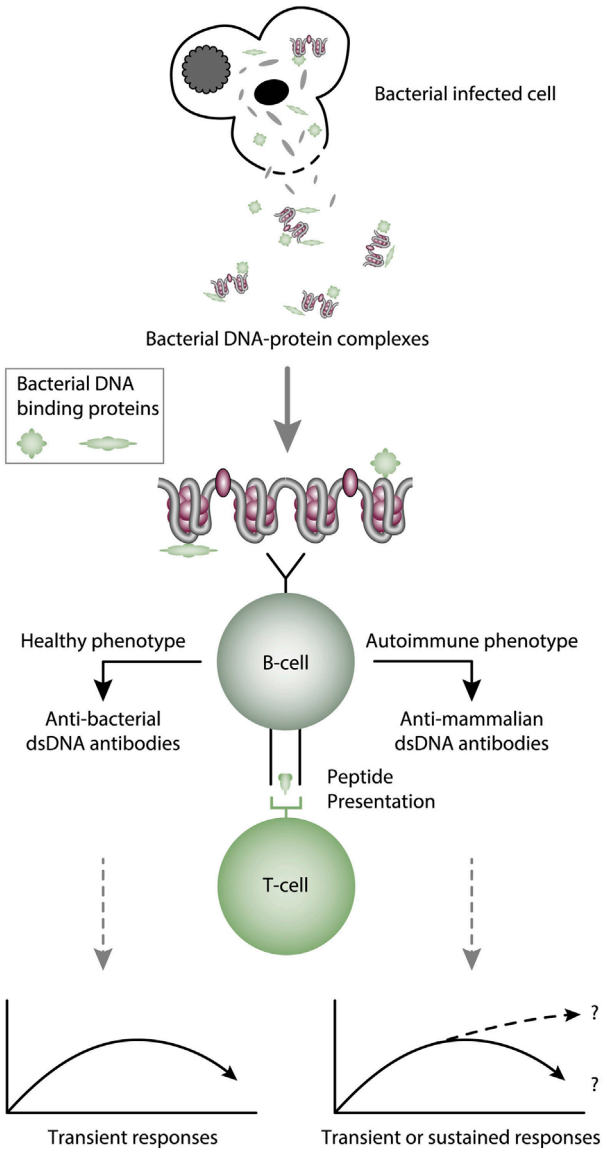

FIGURE 3 | Cognate interaction of DNA-specific B cells and bacterial-derived peptide-specific T cells. This example describes a classical hapten-carrier-like model to explain production of anti-dsDNA antibodies in non-SLE (left panel) and in SLE conditions (right panel). In this model, chromatin-associated dsDNA functions as a non-immunogenic hapten that is recognized by the B cell antigen receptor, while heterologous, bacterial DNA-binding protein-derived peptides function as carrier proteins that activate peptide-specific T helper cells. In this scenario, T cell tolerance for nucleosomes is maintained intact, and the immune response is transient and is limited to the duration of the bacterial infection. According to Pisetsky et al. (83, 135), the immune response is dichotomous in the sense that in a normal immunogenic context, the antibodies recognize bacterial DNA (left part of the panel), while in an autoimmune (SLE-like) context antibodies are also produced that recognize mammalian dsDNA (right part of the panel). These processes may be operational *in vivo* in experimental and native contexts.

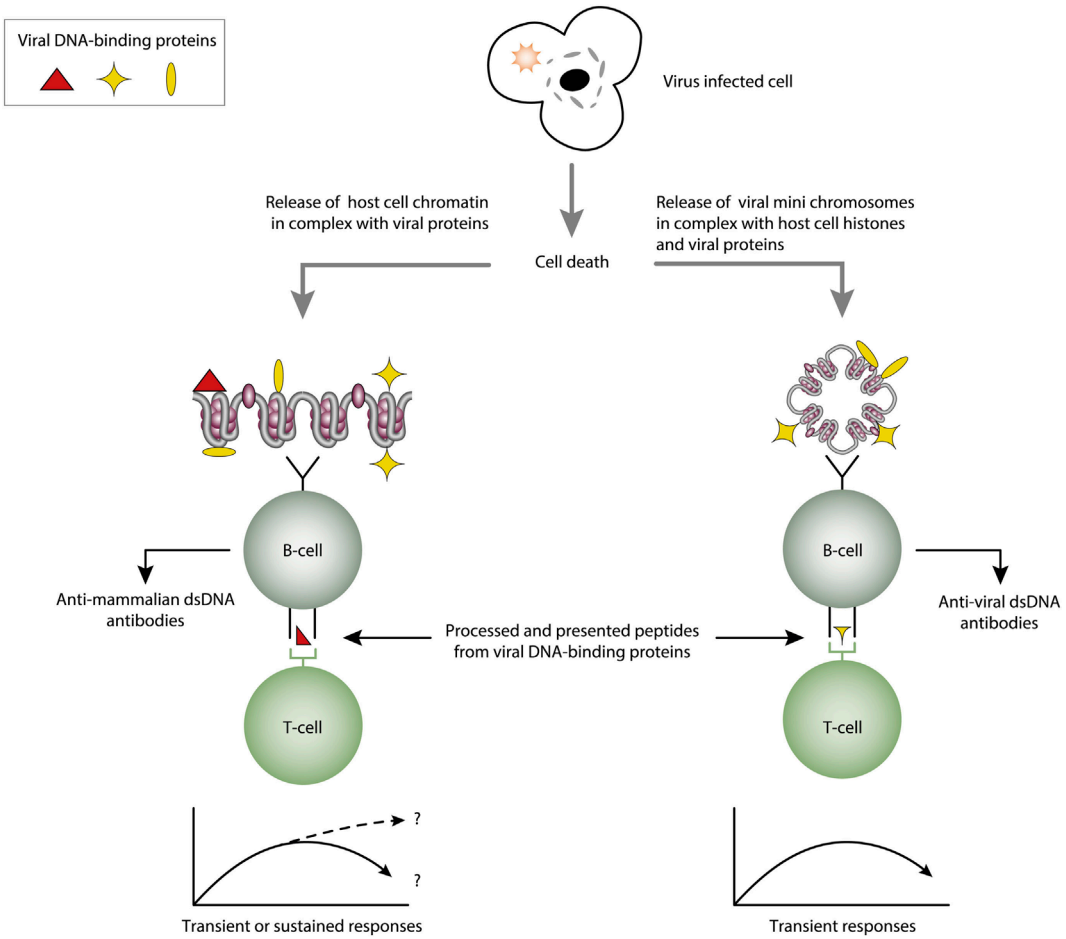

FIGURE 4 | Cognate interaction of DNA-specific B cells and DNA-binding and virus-derived peptide-specific T cells. The figure presents two variants of a classical hapten-carrier-like model. In this model, chromatin from virus-infected cells are released in complex with DNA-binding viral proteins (left panel). Cognate interaction of mammalian dsDNA-specific B cells and virus peptide-specific T cells result in production of antimammalian dsDNA-specific antibodies. This process may be operational in genetically normal individuals (115, 117). In the right panel, the virus-infected cells also release viral mini chromosomes in complex with viral proteins. B cells recognize viral DNA, and present processed DNA-bound virus-encoded peptides to non-tolerant T cells. In this situation, antivirus DNA antibodies are produced. The antibody profiles depend on the time-line and kinetics of the virus infection. From this, lupus-like anti-dsDNA antibodies may appear in non-lupus individuals, thus questioning the validity of pure detection of anti-dsDNA antibodies as a SLE classification criterion.

Marion et al. observed and described at the same time another infectious-related mechanism that could instigate production of anti-dsDNA antibodies. They did the important observation that mammalian dsDNA in complex with the DNA-binding peptide Fus 1 derived from Tryanozoma cruzii had the capacity to induce antibodies to mammalian dsDNA (87, 148). Furthermore, they demonstrated that these induced antibodies were nephritogenic, as the immunized, normal non-autoimmune mice developed a kidney disease that was very similar to lupus nephritis. The studies described earlier represent significant seminal experiments that have helped to understand processes that may explain also spontaneous production of lupus-like anti-dsDNA antibodies. These data may also demonstrate that anti-mammalian dsDNA antibodies is not an integrated part of SLE since they can be observed in conditions other than SLE.

VIRUS INFECTIOUS-RELATED IMMUNE RESPONSES TO NUCLEIC ACIDS—THE HAPTEN-CARRIER MODEL

Viruses have been discussed as pathogenic factors in SLE for decennials (88, 149–153). This discussion refers to two possible roles of the viruses. One is that viruses may promote autoimmunity during productive infection, where the viral transcriptional factor is expressed and bind viral and host cell DNA/chromatin (115, 154–156). In dying cells, chromatin-viral transcription factor complexes are released and presented to the immune system in context of a hapten-carrier analog [see, e.g., Ref. (88, 115)]. The other role of viruses is linked to sustained productive infections, a situation that may promote sustained autoimmunity [discussed in Ref. (88, 157)]. Inspired by the Pisetsky observations, we

observed a similar dichotomous response when we immunized mice with linearized polyomavirus dsDNA in complex with a carrier-protein. Normal mice produced antibodies to viral dsDNA (158), while applying the same immunization regime on young lupus-prone mice, they produced antibodies to both viral and mammalian dsDNA (159, 160); i.e., results that were in agreement with the data published by Pisetsky et al. (83, 135, 161).

POLYOMAVIRUS T ANTIGEN: A NATURAL CARRIER PROTEIN FOR dsDNA AND CHROMATIN IN AN AUTOIMMUNE CONTEXT

Since polyomaviruses obviously had the potential to induce the production of anti-dsDNA antibodies in experimental animals, this pointed at virus-encoded proteins as potential carrier molecules rendering DNA immunogenic. Indeed, in the permissive host, polyomavirus large T antigen is required for viral transcription and replication, and binds both viral and host DNA/chromatin (117, 134), and it was shown to be expressed in SLE patients (117). Thus, virus-encoded dsDNA-binding proteins could represent a non-self DNA-bound protein that served as the T cell determinant that could provide help for mammalian dsDNA-specific B cells (**Figure 4**, left part) and for viral dsDNA-specific B cells (**Figure 4**, right part) provided they processed and presented T antigen derived peptides.

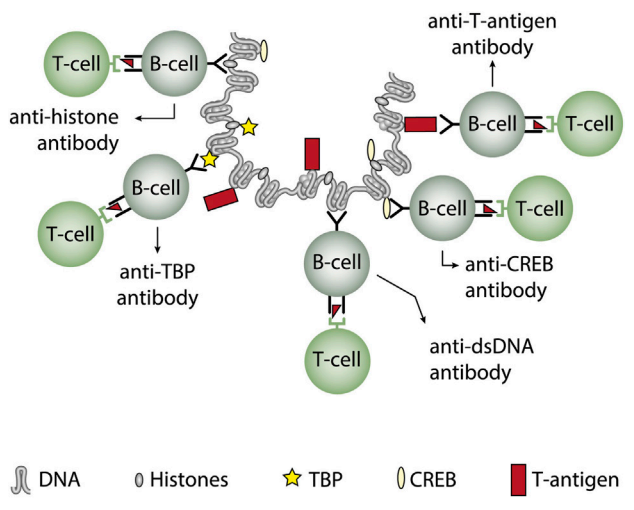

FIGURE 5 | Induction of anti-dsDNA antibodies by *in vivo* expression of a single viral dsDNA-binding protein. Injection of normal mice with plasmids encoding wild type polyomavirus DNA-binding T antigen in context of eukaryotic promoters induced production of antibodies to T antigen and significant production of antibodies to mammalian dsDNA, histones, and to certain transcription factors like TATA-binding protein (TBP) and cAMP-responsive element-binding protein (CREB). All autologous chromatin-derived ligands physically linked to T antigen can therefore be rendered immunogenic to autoimmune B cells that present peptides derived from T antigen. Therefore, concerted production of autoantibodies specific for chromatin antigens, including dsDNA and histones, is not depending on a systemic lupus erythematosus background, but may appear also in quite healthy individuals.

In two experimental systems, these presumptions were verified. In one, we demonstrated that injection into normal mice with plasmids encoding wild type DNA-binding T antigen under control of eukaryotic promoters produced antibodies to T antigen. These antibodies were kinetically linked to significant production of antibodies to dsDNA, histones, and to certain transcription factors like TATA-binding protein and CREB, deduced to be produced according to the idea of the model: all autologous ligands physically linked to T antigen could theoretically be rendered immunogenic provided the presence of a (functional) repertoire of autoimmune B cells [see **Figure 5** for a theoretical model based on experiments and descriptive observations (115, 117), discussed in Ref. (1)]. In this model, a diversified repertoire of chromatin-specific B cells processed and presented a single chromatin-bound viral protein. The validity of the model was further proven by the following observations. Injection of plasmids expressing irrelevant non-DNA-binding proteins like luciferase, plasmids containing T antigen sequences but lacking a promoter, or plasmids encoding and expressing a truncated T antigen without the property to bind DNA did not result in such antibodies (115). In a similar experimental system, Dong et al. (162) demonstrated that the proto-oncogene p53 can bind T antigen. When injecting *in vitro* formed complexes of p53 and T antigen, the mice responded to the immunization regime by producing autoantibodies to p53 and to T antigen, thus indicating that T antigen may render non-immunogenic autoantigens immunogenic upon complex formation. in a non-SLE condition. In other similar experiments, it has been demonstrated that immunization of normal mice with the C-terminal DNA-binding domain of the human papillomavirus E2 protein (163), and the *in vivo* expression of the Epstein–Barr virus nuclear antigen-1 (EBNA-1) in normal mice (86) both instigated the production of anti-dsDNA antibodies.

What have these experiments learned us? The infectious models are relevant to explain evolution of anti-dsDNA antibodies in SLE as well as in non-SLE conditions. Still, true spontaneous autoimmunity—i.e., driven by true autoimmune T helper cells—to dsDNA and chromatin is poorly understood, but the results discussed earlier clearly demonstrate that anti-dsDNA antibodies is a reflection of immune responses to dsDNA and chromatin in many different situations, and they can therefore principally not be a biomarker for SLE. Notably, we are today not able to distinguish between anti-dsDNA antibodies produced as true autoantibodies in SLE from anti-dsDNA antibodies produced in other contexts.

MOLECULAR MIMICRY

Molecular mimicry is an alternative approach to study origin and impact of anti-dsDNA antibodies. For example, several distinct anti-dsDNA antibodies cross-react with non-nucleic acid structures like, e.g., phospholipids (164, 165), α-actinin (166–168), peptides like DWEYSVWLSN (169), entactin (113), the platelet integrin GPIIIa49-66 (170), and others. Which of the cross-reacting structures are initiating this dual immune response *in vivo* is not known. This open for the idea that the B cell recognition of dsDNA is a "by-standing" specificity which in fact has no

meaning for the "real" immune response [see **Figure 6**, and e.g., early discussions in Ref. (93, 164), and also (171, 172)]. In this respect, it is of interest to read the manuscript of Wang et al. (173). In that review, they highlight the biological roles and structures of different reported proteins that mimic DNA. Their analytical approach might be used to discover other proteins that have this peculiar characteristic.

I do not intend in this study to discuss in depth origin and impact of anti-dsDNA antibodies that cross-react with non-DNA structures. Whether such antibodies at all should be named "anti-dsDNA antibodies" has not reached consensus, not even been principally discussed. Since this study concerns about how we define SLE, and how we define anti-dsDNA antibodies as biomarkers for SLE, it may be relevant to define three intricate difficulties related to molecular mimicry; (i) who are the initiators of the dual-specificity antibodies *in vivo*, (ii) will both specificities prevail, as the affinity maturation of their B cell heavy and light chain variable-regions prevail, and (iii) which of the structures will in the end be the *in situ* target for the antibodies. How does this translate into the understanding of avidity in context of anti-dsDNA antibodies and SLE? In a situation where an immune response is instigated by an antigen mimicking dsDNA, the primary humoral immune response will most likely produce cross-reactive antibodies. However, secondary immune responses instigated by the DNA-mimicking ligand will most probably affinity maturate toward that ligand and the antibodies may gain higher affinity toward the inducer. At the same time, the paired self-specific branch of clones may die out because they somatically mutate away for the real immunogen if not the two antigens are structurally identical. The whole story is, notably, that the immune response is instigate by the non-self antigen that engage relevant B cell clones. However, as the self antigens targeted by the crossreactive antibodies are not immunogenic, they are assumed to prevail non-immunogenic, simply because there is no reason to assume that a cross-reacting antibody may render autoantigens immunogenic. Therefore, the autoimmune branch of a cross-reaction will not influence on affinity maturation, they will turn away from the non-self branch, and they will die out.

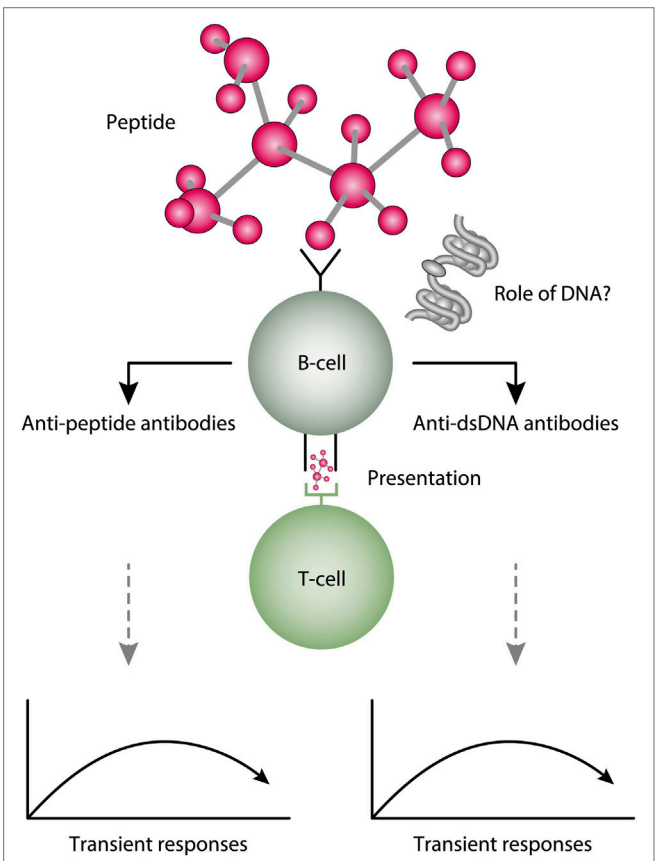

FIGURE 6 | A theoretical model to explain peptide-induced anti-dsDNA and anti-peptide antibodies. Some peptides have the property to act as inducers of anti-dsDNA antibodies. There are problems with this cross-stimulating model, since it is not obvious that the peptide-induced immune response will affinity maturate toward dsDNA. Rather, somatic hypermutations in the variable heavy chain complementary determining regions (VH CDR) may shift the dual specificity toward a focused specificity for the peptide. Whether chromatin (indicated in the figure) may drive the peptide-induced anti-dsDNA antibody further is unlikely from two reasons. For the first, the initial response is controlled by peptide-specific, and not by chromatin-specific T cells. Second, if chromatin was not involved in early phases of the responses, it is no reason to believe it is rendered immunogenic in later phases of the responses.

I therefore hesitate in this study to discuss origin and impact of anti-dsDNA antibodies that cross-react with non-DNA structures. In a native situation we have to accept that we cannot say which of the cross-reacting antigens are the inducer that may prevail the immune response *in vivo*, and which one may be targeted by the antibodies. This is in the end a matter of antibody avidity and antigen availability with relevance to ask if anti-dsDNA antibodies in a deeper sense can act as a biomarker for SLE. However, the molecular mimicry model for instigating anti-dsDNA antibodies definitively show that these antibodies are not strictly linked to SLE. Most of the information from immunization experiments, and from theoretical and clinical information discussed earlier, is in disagreement with the notion that anti-dsDNA antibodies are, or can act as biomarkers for SLE. They appear in so many non-autoimmune and autoimmune situations.

CLOSING REMARKS

Systemic lupus erythematosus is an intriguing and engaging condition. The present human SLE paradigm evolved from being a skin disease in antiquity into a complicated syndrome involving many organs and biological processes. Although an object for many different scientific approaches, still SLE presents itself as an enigma and an abstraction difficult to comprehend in a physical and intellectual context. The studies described earlier provide insight deriving from clinical and experimental information. These have helped significantly to understand processes linked to production of anti-dsDNA antibodies in non-autoimmune and autoimmune SLE-like contexts. The data also demonstrate that anti-mammalian dsDNA antibodies are definitively not an integrated part of the syndrome SLE. They can be observed in other conditions. SLE has been described as a serious skin disease, thereof the antique name in association with a clinical morphology bringing the idea of a wolf bit, thereby "lupus erythematodes." In modern medicine, this simple comprehensive has been left behind, and modern

classification criteria have been introduced. This implies that the "lupus erythematodes" has evolved from a serious cutaneous disease, and that the seriousness implied nephritis. Nephritis was, however, not a major criterion in the antique times. In later times, lupus erythematodes has transformed from a strange, oligosymptomatic disease into a syndrome with classification criteria leaving the modern "SLE" quite different to former times disease definition. We have learned much from these paradigm shifts, about molecular biology, molecular pathology, clinical and epidemiological science, and basic and clinical immunology. In the case of our understanding of SLE, I do not think we have learnt much. It is stated that "The anti-dsDNA antibody" is a classification criterion for SLE. From all described earlier, the pure appearance of "The anti-dsDNA antibody" is closer to be an epiphenomenon in clinical medicine, rather than to be a pathogenic factor or biomarker, which, however indisputably also is associated with SLE. One potentially important question raised earlier is the following: "SLE and Anti-dsDNA antibodies: Do the latter reflect the first?" The answer to this central question is given from what is described earlier.

ETHICS STATEMENT

The present manuscript is a review on murine and human SLE. All data are taken from original studies approved by relevant ethical committees.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Liver X Receptor Agonist Therapy Prevents Diffuse Alveolar Hemorrhage in Murine Lupus by Repolarizing Macrophages

*Shuhong Han[1], Haoyang Zhuang[1], Stepan Shumyak[1], Jingfan Wu[1], Chao Xie[2], Hui Li[2], Li-Jun Yang[2] and Westley H. Reeves[1]**

[1] Division of Rheumatology & Clinical Immunology, Department of Medicine, University of Florida, Gainesville, FL, United States, [2] Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL, United States

The generation of CD138+ phagocytic macrophages with an alternative (M2) phenotype that clear apoptotic cells from tissues is defective in lupus. Liver X receptor-alpha (LXRα) is an oxysterol-regulated transcription factor that promotes reverse cholesterol transport and alternative (M2) macrophage activation. Conversely, hypoxia-inducible factor 1-α (HIF1α) promotes classical (M1) macrophage activation. The objective of this study was to see if lupus can be treated by enhancing the generation of M2-like macrophages using LXR agonists. Peritoneal macrophages from pristane-treated mice had an M1 phenotype, high HIFα-regulated phosphofructokinase and TNFα expression (quantitative PCR, flow cytometry), and low expression of the LXRα-regulated gene ATP binding cassette subfamily A member 1 (*Abca1*) and *Il10* vs. mice treated with mineral oil, a control inflammatory oil that does not cause lupus. Glycolytic metabolism (extracellular flux assays) and *Hif1a* expression were higher in pristane-treated mice (M1-like) whereas oxidative metabolism and LXRα expression were higher in mineral oil-treated mice (M2-like). Similarly, lupus patients' monocytes exhibited low LXRα/ABCA1 and high HIF1α vs. controls. The LXR agonist T0901317 inhibited type I interferon and increased ABCA1 in lupus patients' monocytes and in murine peritoneal macrophages. *In vivo*, T0901317 induced M2-like macrophage polarization and protected mice from diffuse alveolar hemorrhage (DAH), an often fatal complication of lupus. We conclude that end-organ damage (DAH) in murine lupus can be prevented using an LXR agonist to correct a macrophage differentiation abnormality characteristic of lupus. LXR agonists also decrease inflammatory cytokine production by human lupus monocytes, suggesting that these agents may be have a role in the pharmacotherapy of lupus.

Keywords: lupus, diffuse alveolar hemorrhage, therapy, inflammation, macrophage polarization, liver X receptors, hypoxia-inducible factor 1-α

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****Correspondence:***
Westley H. Reeves
whreeves@ufl.edu

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INTRODUCTION

Mice with pristane-induced lupus develop an autoimmune syndrome closely resembling systemic lupus erythematosus (SLE) with lupus-specific autoantibodies, nephritis, arthritis, diffuse alveolar hemorrhage (DAH), and hematological manifestations (1). Pristane-induced lupus in C57BL/6 (B6) mice is the only model of lupus-associated DAH (2, 3), an often fatal complication seen in ~3% of SLE patients (4). DAH in pristane-induced lupus is associated with antineutrophil cytoplasmic antibody negative pulmonary capillaritis and is mediated by macrophages (Mϕ) (3).

Pristane-treated mice develop lupus in the setting of non-resolving inflammation (5), which may result in part from impaired clearance of dead cells (6). $CD11b^+F4/80^+Ly6C^{hi}$ inflammatory Mϕ ($Ly6C^{hi}$ Mϕ) accumulate in the peritoneum after pristane injection (6, 7). In contrast, peritoneal exudate cells (PEC) from mice treated with mineral oil (MO), an inflammatory hydrocarbon that does not cause lupus, are progressively enriched in a subset of anti-inflammatory $CD11b^+F4/80^+CD138^+$ Mϕ reminiscent of alternatively activated (M2) Mϕ (6). $CD138^+$ Mϕ are highly phagocytic for apoptotic cells and their deficiency in pristane-treated mice may promote non-resolving inflammation resulting in end-organ damage.

Although an over-simplification (8, 9), bone marrow (BM)-derived Mϕ are classified as classically activated (M1) or alternatively activated (M2). Murine M1 Mϕ express high levels of Ly6C, CD80/CD86, CD274 (PD-L1), and CCR2 and produce TNFα, IL-1β, and IL-12. In contrast, M2 Mϕ express Fizz1 (*Retnlb*), Ym1 (*Chil3*), Arginase 1 (*Arg1*), CD206 (*Mrc1*), CD273 (PD-L2, *Pdcd1lg2*), scavenger receptors, CX_3CR1, and low levels of Ly6C and produce TGFβ and IL-10 (10). Phosphorylation of the transcription factor CREB promotes M2 Mϕ polarization (11). $CD138^+$ Mϕ from MO-treated mice express M2 activation markers and have high levels of p-CREB (6). The present study addresses the role of two additional transcription factors, liver X receptor-alpha (LXRα) and hypoxia inducible factor 1-alpha (HIF1α), in lupus.

Liver X receptor-alpha, an oxysterol-regulated transcription factor activated via the endosome/lysosome associated Lamtor1-mTORC1 pathway, helps determine whether or not M0 Mϕ polarize to M2 (12, 13). Oxysterols derived from the phagocytosis of apoptotic cells activate the LXR pathway in Mϕ, upregulating genes involved in the recognition of dead cells (*Mertk*) and cholesterol efflux (e.g., ATP binding cassette A1, *Abca1*) and downregulating proinflammatory gene expression (14). Along with their dependence on LXRα, M2 Mϕ rely on oxidative phosphorylation and fatty acid oxidation to fuel mitochondrial oxidative metabolism whereas M1 Mϕ rely on glycolysis (15, 16). M1 polarization is promoted by HIF1α, a key regulator of glycolytic metabolism (15, 17, 18), which upregulates glycolytic enzymes, proinflammatory cytokines, and expression of the M1 marker CD274 (17). We show that an imbalance between LXRα and HIF1α activity is involved in the pathogenesis of end-organ damage (DAH) in lupus. Therapy with an LXR agonist corrected this imbalance and prevented DAH.

MATERIALS AND METHODS

Mice

B6 mice (Jackson) maintained under specific pathogen free conditions were injected with pristane (Sigma-Aldrich, 0.5 ml i.p.), mineral oil (MO; C.B. Fleet Co.), PBS, or left untreated. PEC were collected 14 days later. Some mice were treated with pristane on d0 plus either LXR agonist T0901317 (200 μg in DMSO per mouse i.p. daily) or DMSO alone. Mice received T0901317 on d1–d14 or on d1–d3, d3–d14, or d7–d14 only. On d14, lungs were evaluated for DAH by gross inspection of the excised lungs followed by microscopic confirmation as described previously (3). This study was carried out in accordance with the recommendations of the Animal Welfare Act and US Government Principles for the Utilization and Care of Vertebrate Animals and was approved by the UF IACUC.

Patients and Healthy Donors

For flow cytometry and isolation of peripheral blood mononuclear cells (PBMCs), heparinized blood was obtained from 22 SLE patients meeting ACR criteria who were seen consecutively in the UF Autoimmune Disease Clinic (19) and 24 matched healthy donors with no autoimmune disease. For RNA isolation, blood was collected in PAXgene tubes (BD Biosciences). SLE activity was assessed using the SLEDAI (20). This study was carried out in accordance with the recommendations of the International Committee of Medical Journal Editors and was approved by the UF IRB. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Quantitative PCR

Quantitative PCR (Q-PCR) was performed as described (21) using RNA extracted from 10^6 mouse PEC (TRIzol, Invitrogen). RNA was isolated from human blood with the QIAamp RNA Blood Mini Kit (Qiagen). cDNA was synthesized using the Superscript II First-Strand Synthesis kit (Invitrogen). SYBR Green Q-PCR analysis was performed using an Opticon II thermocycler (Bio-Rad). Gene expression was normalized to 18 S RNA, and the expression level was calculated using the $2^{-\Delta\,\Delta Ct}$ method. Primer sequences are in **Table 1**.

Culture of Adherent Peripheral PBMC-Derived Monocytes

Peripheral blood mononuclear cells from lupus patients and healthy donors were isolated from heparinized blood by density gradient centrifugation (Ficoll-Hypaque, GE Healthcare Bio-Sciences). PBMCs were incubated at 37°C for 1 h in AIM-V medium (Invitrogen), and non-adherent cells were removed. Adherent cells (90–95% $CD14^+$) were lysed with RLT lysis buffer (Qiagen) for RNA isolation. Monocytes were cultured with LXRα agonist GW3965 (1 μM, Sigma-Aldrich), for 24 h in AIM-V medium before isolating RNA. Gene expression was measured by Q-PCR. In some experiments, monocytes were treated with IFNα (1,000 U/ml) (R&D Systems) for 1 h, followed by addition of LXR agonists (GW3965 or T0901317, 1 μM in DMSO), or DMSO alone, and then cultured for 24 h. Some cells were lysed for RNA isolation. The remaining cells were analyzed by flow cytometry. About 10–50,000 events per sample were acquired using an LSRII flow cytometer (BD-Biosciences) and analyzed with Flowjo software (Tree Star Inc.).

Flow Cytometry and Sorting of Mouse Mϕ

Flow cytometry was performed as described (21) using anti-mouse CD16/32 (Fc Block; BD Biosciences) before staining with primary antibody or isotype controls. Cells were surface-stained, then fixed/permeabilized (Fix-Perm buffer, eBioscience) before intracellular staining. Antibodies are listed in **Table 2**. Uptake of low-density lipoproteins was assessed by incubating PEC

TABLE 1 | Primer sequences.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
18 S	AGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Human		
NR1H3 (LXRα)	ACTCGAAGATGGGGTTGATG	GGAGGTACAACCCTGGGAGT
ABCA1	AACAAGCCATGTTCCCTCAG	GACGCAAACACAAAAGTGGA
MX1	CACGAGAGGCAGCGGGATCG	CCTTGCCTCTCCACTTATCTTC
LY6E	AGGCTGCTTTGGTTTGTGAC	AGCAGGAGAAGCACATCAGC
HIF1A	TCCATGTGACCATGAGGAAA	TCTTCCTCGGCTAGTTAGGG
PFKL	CTCCTCGCCCACCAGAAG	CTGTGTGTCCATGGGAGATG
HK2	TCTATGCCATCCCTGAGGAC	AAACCCAGTGGGAGCTTCTT
Mouse		
Nr1h3	TGGAGAACTCAAAGATGGGG	TGAGAGCATCACCTTCCTCA
Abca1	GCTGCAGGAATCCAGAGAAT	CATGCACAAGGTCCTGAGAA
Hif1a	TCCATGTGACCATGAGGAAA	GGCTTGTTAGGGTGCACTTC
Mx1	GATCCGACTTCACTTCCAGATGG	CATCTCAGTGGTAGTCCAACCC
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Tnfa	CATCTTCTCAAAAT TCGAGTGACAA	TGGGAGTAGAC AAGGTACAACCC
Chil3	TGTACCAGCTGGGAAGAAAC	GAGAGCAAGAAACAAGCATGG
G6pd	CCCCCACAGTCTATGAAGCA	TGGTTCGACAGTTGATTGGA
Pfkl	GGGCTGATTGGCTATTCATT	TGATGATGTTCAGCCGAGAG
Hk2	GGGTTTCACCTTCTCCTTCC	TTCAGCAAGGTGACCACATC

TABLE 2 | Antibodies used for flow cytometry.

Specificity (clone)	Fluorochrome	Source
Mouse CD273 (TY25)	Phycoerythrin	Biolegend
Mouse CDE274 (10F.9G2)	Phycoerythrin	Biolegend
Mouse CD138 (281-2)	Phycoerythrin; Allophycocyanin	Biolegend
Mouse CD11b (M1/70)	Brilliant violet-421	Biolegend
Mouse Ly6C (HK1.4)	Allophycocyanin-Cy7	Biolegend
Mouse Ly6G (1A8)	Phycoerythrin	BD Bioscience
Mouse CD80 (16-10A1)	PerCP-Cy5.5	Biolegend
Mouse CD86 (GL-1)	Allophycocyanin-Cy7	Biolegend
Mouse CD36 (HM36)	Phycoerythrin	Biolegend
Mouse TNFα (MP6-XT22)*	Allophycocyanin	Biolegend
Mouse/human ABCA1 (5A1-1422.22)[a]	Allophycocyanin	Novus Biologicals
Human CD14 (MφP9)	PerCP	BD Bioscience
Human CD16 (3G8)	Fluorescein isothiocyanate	BD Bioscience
Human CD64 (10.1)	Phycoerythrin	eBioscience
Human PFKL (polyclonal)	Fluorescein isothiocyanate	Aviva Systems Biology

[a]*Intracellular staining.*

with BODIPY-labeled LDL (10 μg/ml, Invitrogen) (16). Data were acquired and analyzed as above. $CD11b^+Ly6C^{hi}$ LyG^- and $CD11b^+CD138^+$ $Ly6G^-$cells were sorted using a FACSaria cell sorter and 40,000 cells/subset were lysed immediately for RNA extraction.

Extracellular Flux Analysis

For real-time analysis of mitochondrial oxygen consumption rate (OCR) and extracellular aerobic acidification rate (ECAR), peritoneal adherent cells and FACS-sorted $Ly6C^{hi}$ Mϕ and $CD138^+$ Mϕ were analyzed with an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience) (16). Briefly, peritoneal cells were collected by lavage from mice treated with pristane or MO for 14 days and stained with antibodies against CD11b, Ly6G, Ly6C, and CD138 (**Table 2**). $CD11b^+Ly6G^-Ly6C^{hi}$ Mϕ and $CD11b^+Ly6G^-CD138^+$ Mϕ were sorted using a FACSAira II Cell Sorter (BD Biosciences). A total of 5×10^4 peritoneal cells, $Ly6C^{hi}$ Mϕ, or $CD138^+$ Mϕ were resuspended in AIM-V medium (Thermo Fisher) and placed into 96-well XF cell culture microplates (Seahorse Bioscience). Two hours later, the cells were washed three times with warm XF assay medium and cultured in XF assay medium. Three or more consecutive measurements were obtained under basal conditions and after sequential addition of 1 μM oligomycin, 0.75 μM FCCP (fluoro-carbonyl cyanide phenylhydrazone), and 250 nM rotenone plus 250 nM antimycin A (Sigma-Aldrich).

Statistical Analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software). Differences between groups were analyzed by two-sided unpaired Student's *t*-test unless otherwise indicated in the figure legend. Before comparing the means, we tested for equality of variance using the F-test. If the variances did not differ, we used Student's *t*-test. If there was statistically significant evidence that the variances differed, we used Welch's *t*-test. Data were expressed as mean ± SD. Correlation was analyzed using the Pearson correlation coefficient. $p < 0.05$ was considered significant. All experiments in mice were repeated at least twice.

RESULTS

Diffuse alveolar hemorrhage in pristane-induced lupus is prevented by peritoneal Mϕ (but not neutrophil) depletion (3). In contrast, MO-treated mice do not develop DAH despite their high numbers of peritoneal Mϕ. We have shown recently that pristane treatment favors classical (M1) Mϕ activation whereas MO favors the generation of pro-resolving alternatively activated (M2) Mϕ (6). We examined transcriptional activation in peritoneal Mϕ from pristane- vs. MO-treated mice.

Pristane Treatment Increases Hif1a

M1 Mϕ are highly dependent on glycolytic metabolism, which is regulated by HIF1α (15, 17, 22). In B6 mice, expression of both *Hif1a* and the proinflammatory cytokine *Tnfa* was higher in PEC from pristane- vs. MO-treated mice (**Figure 1A**). Expression of *Hif1a* and *Tnfa* correlated. As PEC from pristane- (but not MO-) treated mice contain many $Ly6C^{hi}CD11b^+F4/80^+$ cells (7), we determined *Hif1a* expression in flow-sorted $Ly6C^{hi}CD11b^+$ PEC from pristane- and MO-treated mice. $Ly6C^{hi}$ Mϕ from pristane-treated mice exhibited higher levels of *Hif1a* than $Ly6C^{hi}$ Mϕ from MO-treated mice (**Figure 1B**), suggesting that glycolysis might be more active in Mϕ from pristane- vs. MO-treated mice. The increased ECAR and decreased OCR of PEC from pristane- vs. MO-treated mice in extracellular flux assays supported that hypothesis (**Figures 1C,D**). Consistent with the correlation between *Tnfa* and *Hif1a* in PEC (**Figure 1A**), higher *Hif1a* expression in the $Ly6C^{hi}$ Mϕ subset from pristane-treated mice

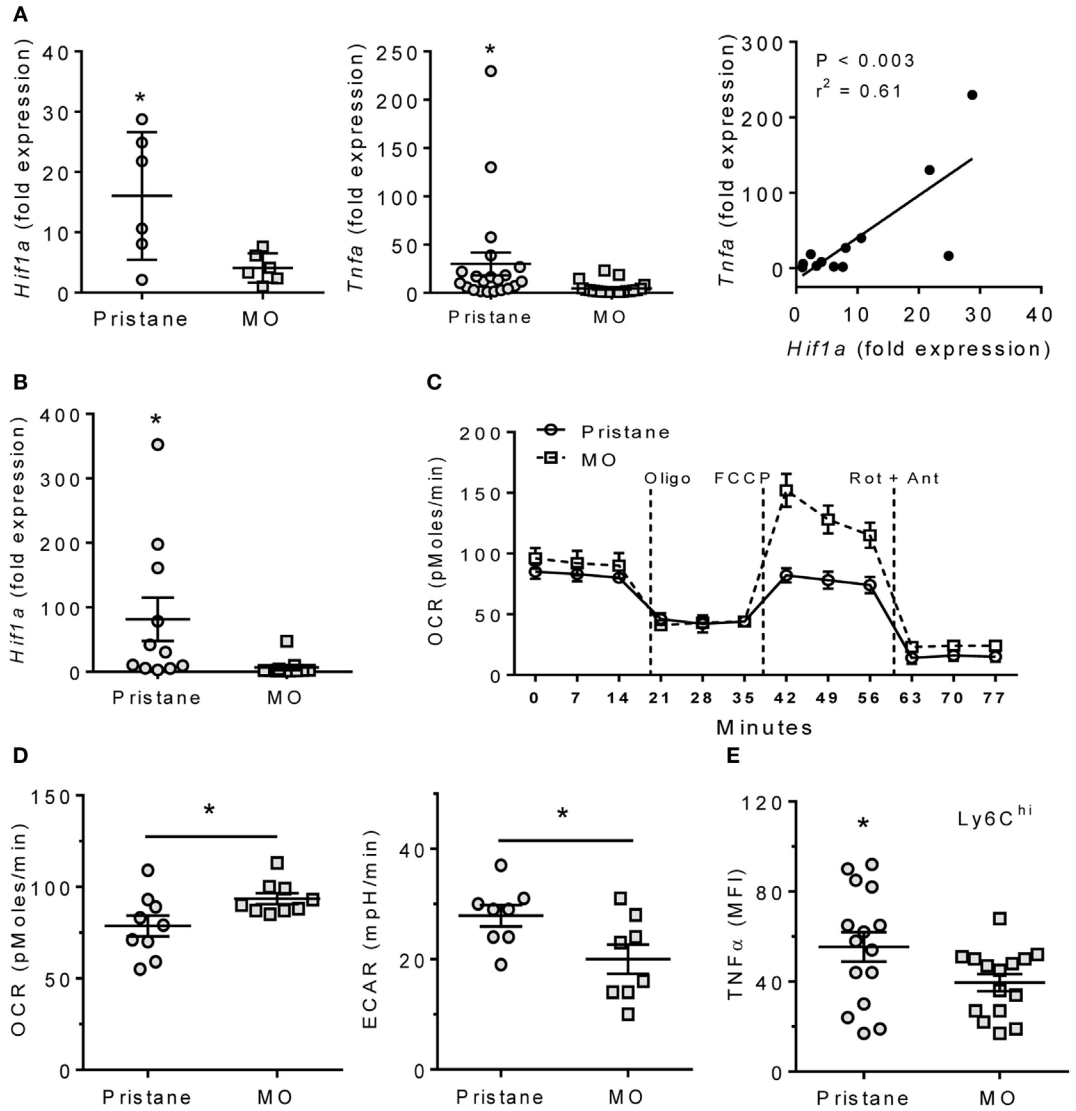

FIGURE 1 | Pristane increases HIF1α, TNFα, and glycolysis. B6 mice were injected i.p. with pristane and MO. Peritoneal cells were collected at d14 and RNA was extracted. **(A)** Expression of *Hif1a* and *Tnfa* mRNA relative to 18 S (Q-PCR). *$P < 0.05$ vs. control (unpaired Welch's *t*-test). **(B)** Peritoneal CD11b$^+$Ly6C^{high} cells were flow-sorted from pristane- and MO-treated mice, and *Hif1a* expression was measured by Q-PCR. *$P = 0.05$ vs. control (unpaired Welch's *t*-test). **(C)** Extracellular flux analysis of adherent peritoneal cells from pristane- and MO-treated mice (14 days after treatment). After 1 h incubation, oxygen consumption rate (OCR) was determined with sequential addition of 1 μg/ml oligomycin (Oligo), 400 nM FCCP, and 1 μM rotenone + 1 μM antimycin A (Rot + Ant). **(D)** Effects of pristane and MO on basal OCR (left) and extracellular acidification rate (ECAR, right) (XF96 Analyzer). Experimental treatments were performed with five technical replicates and three biological replicates. *$P < 0.05$ vs. control (unpaired Student's *t*-test). **(E)** Intracellular TNFα staining of CD11b$^+$Ly6C^{high} cells from pristane vs. MO treated mice. *$P < 0.05$ vs. control (unpaired Student's *t*-test).

also was associated with higher intracellular staining for TNFα (**Figures 1B,E**).

MO Treatment Increases LXR Activity

Peritoneal exudate cells from MO-treated mice are enriched in M2 Mϕ (6). As alternatively activated Mϕ which depend on mitochondrial oxidative metabolism (15), the increased OCR and decreased ECAR of MO- vs. pristane-treated Mϕ in extracellular flux assays (**Figures 1C,D**) suggested an M2-like phenotype. We therefore examined the activity of LXRα, a transcription factor that regulates M2 polarization (13). Expression of *Nr1h3* (encoding LXRα), increased slightly in PEC from MO-treated vs. pristane-treated mice, but it was not statistically significant. However, expression of the LXRα-regulated gene *Abca1* was substantially higher in PEC from MO-treated mice (**Figure 2A**). Expression levels of *Abca1* and *Nr1h3* correlated. Treatment of PEC from wild-type mice with the LXR agonist GW3695 induced *Abca1* but had only a modest effect on *Nr1h3* expression (**Figure 2B**). Anti-inflammatory CD138$^+$ Mϕ expand in PEC from MO- vs. pristane-treated mice (6). Sorted CD11b$^+$CD138$^+$ Mϕ from MO-treated mice expressed higher levels of *Abca1* than those from pristane-treated mice and modestly higher levels of *Nr1h3*

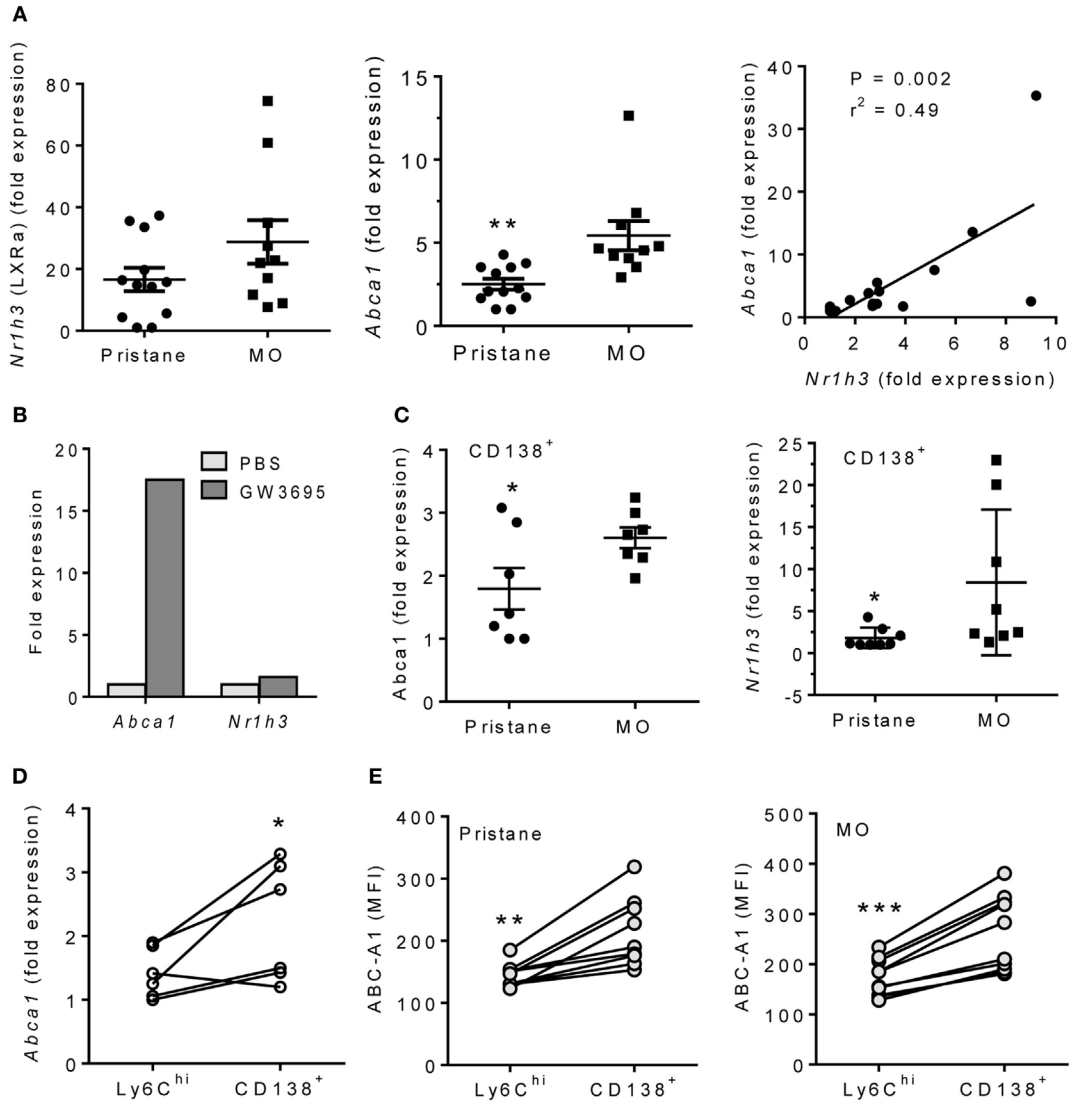

FIGURE 2 | Pristane decreases LXRα activity in PEC. B6 mice were injected i.p. with pristane or MO. PEC were collected at d14 and RNA was isolated. **(A)** Q-PCR for *Nr1h3* and *Abca1* expression relative to 18 S. $^{**}P < 0.01$, Welch's *t*-test. **(B)** PEC from wild-type mice were stimulated with 1 μM GW3965 for 24 h. *Nr1h3* and *Abca1* expression levels were determined by Q-PCR (representative of three experiments). **(C)** CD138$^+$CD11b$^+$ cells from pristane- and MO-treated mice were flow sorted, and mRNA was analyzed by Q-PCR. Left, *Abca1*; right, *Nr1h3*. $^{**}P < 0.05$, Student's *t*-test (left) and Welch's *t*-test (right). **(D)** Peritoneal CD11b$^+$Ly6C^{hi} and CD11b$^+$CD138$^+$ cells from MO-treated mice were flow sorted, and *Abca1* expression was analyzed (Q-PCR). $^*P < 0.05$ (paired Student's *t*-test). **(E)** Peritoneal cells from pristane- and MO-treated mice were stained with antibodies against CD11b, CD138, Ly6C, and Abca1. Mean Fluorescence Intensity (MFI) of Abca1 staining (flow cytometry) was compared between CD11b$^+$Ly6C^{hi} and CD11b$^+$CD138$^+$ subsets. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control (paired Student's *t*-test).

(**Figure 2C**). *Abca1* expression was higher in sorted CD138$^+$ Mϕ than in Ly6C^{hi} Mϕ from the same mouse (**Figure 2D**). Intracellular Abca1 protein also was higher in CD138$^+$ vs. Ly6C^{hi} Mϕ from both pristane- and MO-treated mice (**Figure 2E**).

Phenotypes of CD138$^+$ Mϕ from Pristane vs. MO Treated Mice

Although MO-treatment favors the development of CD138$^+$ (pro-resolving) rather than Ly6C^{hi} Mϕ (6, 7), surface staining unexpectedly revealed that the phenotypes of CD138$^+$ Mϕ from pristane- and MO-treated mice were not identical (**Figure 3A**). CD138 staining and staining for the M2 Mϕ marker CD273 were higher in MO- than pristane-treated mice. Conversely, staining for the M1 marker CD274, Ly6C, and CD86 was higher in CD138$^+$ Mϕ from pristane- vs. MO-treated mice (**Figure 3A**). By Q-PCR (**Figure 3B**, CD138$^+$ Mϕ from MO-treated mice expressed more *Il10* and *Chil3* (Ym1) and less *Hif1a*, *Pfkl* (phosphofructokinase, HIF1α-regulated), and *Tnfa* than CD138$^+$ Mϕ from pristane-treated mice. In addition, sorted CD138$^+$ Mϕ from MO-treated mice exhibited a higher OCR than CD138$^+$ Mϕ from pristane-treated mice (**Figure 3C**, left). In both pristane- and MO-treated mice, the OCR was higher in CD138$^+$ Mϕ than in Ly6C^{hi} Mϕ (**Figure 3C**, middle and right). A similar pattern (higher in CD138$^+$ vs. Ly6C^{hi} Mϕ) was seen after staining PEC

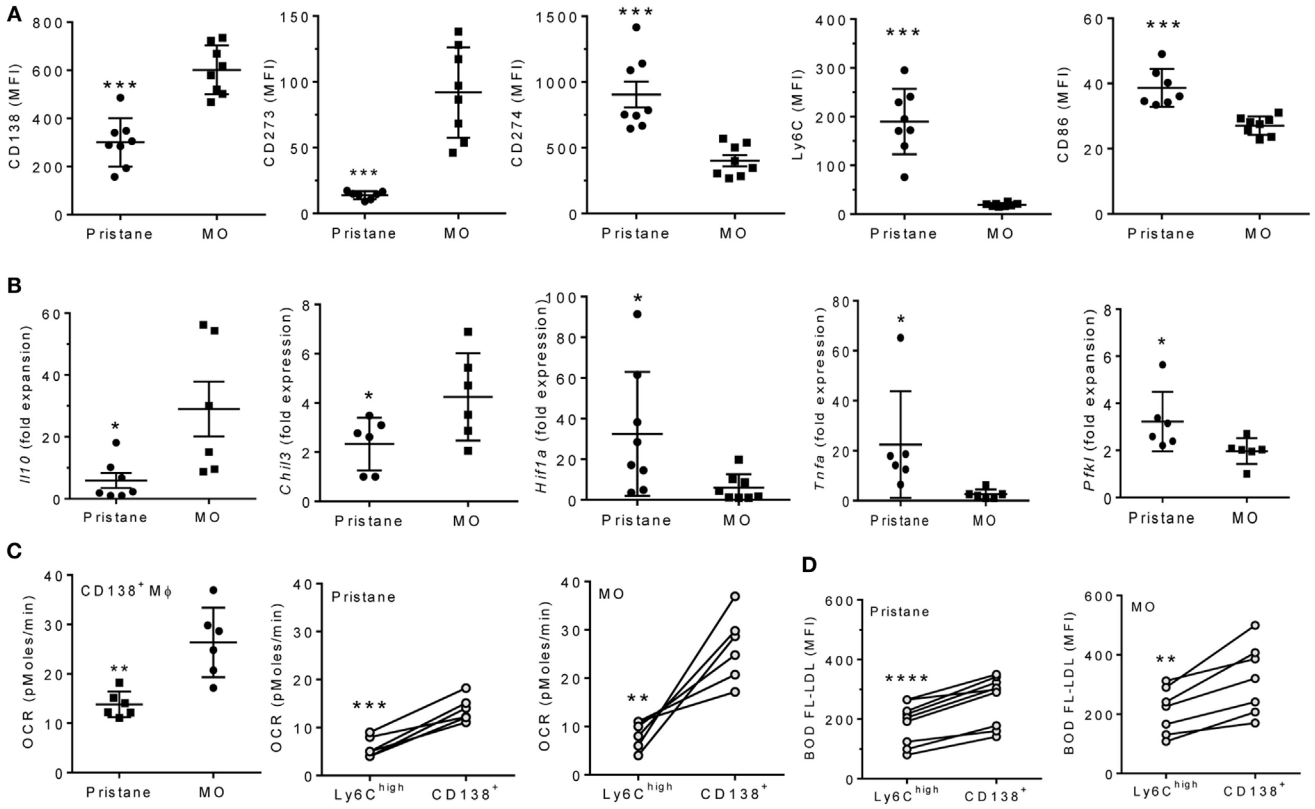

FIGURE 3 | CD138⁺ Mϕ in pristane-treated mice are M1-like. B6 mice were injected i.p. with pristane or mineral oil (MO). Peritoneal exudate cells were collected at d14. **(A)** PEC were stained with antibodies against CD11b, CD138, CD273, CD274, Ly6C, and CD86. CD11b⁺CD138⁺ cells were gated to analyze staining of the other markers.***$P < 0.001$ by unpaired Student's *t*-test (panels 1 and 5) or Welch's *t*-test (panels 2, 3, and 4). **(B)** CD11b⁺CD138⁺ cells were flow sorted, and expression levels of *Il10*, *Chil3*, *Tnfa*, *Hif1a*, and *Pfkl* were determined relative to 18 S (Q-PCR). *$P < 0.05$ by Student's *t*-test (panels 2 and 5) or Welch's *t*-test (panels 1, 3, and 4). **(C)** Peritoneal CD11b⁺Ly6C^{hi} and CD11b⁺CD138⁺ cells were flow sorted from pristane- and MO-treated mice. OCR was measured (XF96 Analyzer). Left, CD138⁺ Mϕ from pristane- vs. MO-treated mice; middle and right, Ly6C^{hi} vs. CD138⁺ Mϕ from individual pristane- and MO-treated mice. Experimental treatments were performed with six technical replicates. **$P < 0.01$, ***$P < 0.001$ by Welch's unpaired *t*-test (left) or Student's paired *t*-test (middle and right). **(D)** BODIPY-labeled LDL (10 μg/ml) was added to PEC from pristane- and MO-treated mice for 2 h and cells were then stained with anti-CD11b, Ly6C, and CD138. Mean fluorescence intensity (MFI) of BODIPY-LDL was analyzed. Comparison of Ly6C^{hi} vs. CD138⁺ Mϕ from individual pristane- (left) and MO- (right) treated mice **$P < 0.01$; ****$P < 0.0001$ vs. control, paired Student's *t*-test.

from pristane vs. MO-treated mice with BODFL-LDL to assess uptake of exogenous LDL (**Figure 3D**). Overall, CD138⁺ Mϕ from MO-treated mice were more M2-like than the CD138⁺ Mϕ subset from pristane-treated mice and in comparison with the Ly6C^{hi} subset, CD138⁺ Mϕ were more M2-like.

Inverse Relationship of HIF-1α and LXRα Expression in Lupus Mice

Although CD138⁺ Mϕ from lupus (pristane-treated) mice were more "inflammatory" than those from MO-treated controls, *Hif1a* expression was still higher in peritoneal M1-like Ly6C^{hi} than in M2-like CD138⁺ Mϕ from pristane-treated mice (**Figure 4A**). *Hif1a* mRNA levels correlated inversely with *Abca1* in pristane-treated mice (**Figure 4B**). Expression of the HIF-1α regulated genes *Pfkl* (23, 24) and *G6pd* (glucose-6-phosphate dehydrogenase) (25) (but not *Hk2*) was higher in pristane- vs. MO-treated mice (**Figure 4C**). To see if LXR activation downregulates *Hif1a*, peritoneal Mϕ from pristane-treated mice were treated for 24 h with the LXR agonist GW3965, which decreased expression of *Hif1a* as well as *Pfkl*, but not hexokinase-2 (*Hk2*) (**Figure 4D**). As expected, expression of the LXR-regulated *Abca1* gene increased after GW3965 treatment. These data suggested that treatment with LXR agonists might normalize HIF-1α activity in Mϕ from pristane-treated mice. We therefore examined the possibility of treating DAH using LXR agonists to induce Mϕ repolarization.

LXR Agonist Therapy Prevents DAH

LXR agonists include naturally occurring oxysterols and synthetic ligands, such as GW3965 and T0901317 (26). *In vitro* treatment with GW3965 or T0901317 increased OCR in RAW-264.7 cells (**Figure 5A**) and adherent peritoneal Mϕ from pristane-treated mice (**Figure 5B**), suggesting that LXR activation promotes alternative activation.

We treated B6 mice with pristane (d0) plus daily injections of either T0901317 or vehicle and assessed DAH at d14. Daily T0901317 treatment for 14 days completely protected the

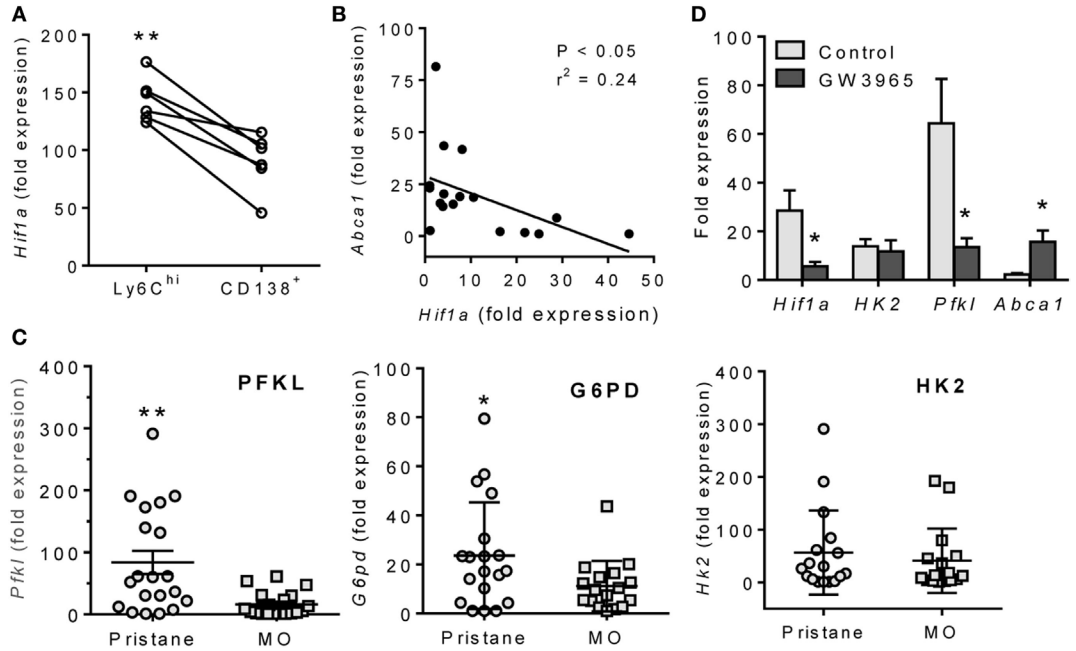

FIGURE 4 | Inverse relationship of LXRα and HIF-1α. **(A)** Peritoneal Ly6C^{hi} and CD138^{+} Mϕ were flow sorted from pristane-treated mice, and *Hif1a* mRNA expression was measured relative to 18 S (Q-PCR). $^{**}P < 0.01$, Student's paired *t*-test. **(B)** Inverse relationship of *Hif1a* and *Abca1* mRNA levels in PEC from pristane-treated mice. **(C)** PEC were collected 14 days after pristane- or MO-treatment and expression of HIF1α-regulated genes (*Pfkl*, *G6pd*, and *Hk2*) was measured (Q-PCR) ($^{*}P < 0.05$, unpaired Welch's *t*-test). **(D)** Adherent peritoneal cells from pristane-treated mice were incubated with GW3965 or DMSO for 24 h, and expression levels of *Hif1a*, *Hk2*, *Pfkl*, and *Abca1* mRNA were measured relative to 18 S (Q-PCR). ($^{*}P < 0.05$, $^{**}P < 0.01$, unpaired Welch's *t*-test).

mice from lung hemorrhage (**Figure 5C**). Mice treated from d1–d3 or d–d14 may exhibit partial protection, but this did not reach statistical significance. Treatment from d7–d14 had no effect. As expected, intracellular Abca1 staining was higher in CD11b^{+}CD138^{+} Mϕ from T0901317-treated mice than in controls (**Figure 5D**). T0901317 also decreased surface CD11b and intracellular TNFα staining in CD11b^{+}CD138^{+} Mϕ (**Figure 5E**).

Expression of HIF-1α and LXRα in SLE Patients

The altered expression of LXRα and HIF-1α in mice with pristane-lupus prompted us to look for similar changes in circulating monocytes from SLE patients. *NR1H3* and *ABCA1* expression levels were lower in adherent PBMCs from 22 consecutively seen SLE patients vs. 24 healthy controls (**Figure 6A**). As in pristane-induced lupus, *NR1H3* and *ABCA1* expression correlated in humans (**Figure 6A**). GW3965 treatment induced *ABCA1* and *NR1H3* expression in adherent PBMCs from healthy controls (**Figure 6B**). As in mice, *HIF1A* and *PFKL* expression levels were higher in adherent PBMCs from SLE patients vs. healthy controls (**Figures 6C,D**).

Systemic lupus erythematosus is associated with overproduction of IFNα/β (27). In the 22 consecutive SLE patients, CD64 fluorescence intensity on CD14^{+} cells, a marker of IFNα /β stimulation (28), was inversely associated with *ABCA1* expression (Q-PCR) (**Figure 6E**). CD64 surface staining also correlated inversely with ABCA1 intracellular staining intensity (flow cytometry) (**Figure 6F**). SLE patients with a SLEDAI ≥ 3 had low ABCA1 and high CD64 staining, whereas healthy controls exhibited the opposite pattern (**Figure 6G**).

To further examine the effects of LXRα activation on proinflammatory cytokines, we treated adherent PBMCs from healthy donors with IFNα or IFNα + GW3965 (**Figure 7**). GW3965 reduced expression of the IFN-I inducible genes *MX1* and *LY6E* (**Figure 7A**) and reduced fluorescence intensity of the IFN-I inducible surface markers CD64 and CD16 on CD14^{+} peripheral blood monocytes (**Figure 7B**), suggesting that LXR activation may downregulate the expression of interferon-regulated genes (interferon signature).

DISCUSSION

CD138^{+} Mϕ, which are highly phagocytic for apoptotic cells and promote the resolution of inflammation, are deficient in mice with pristane-induced lupus (6). This deficiency impairs the clearance of dead cells, a defect also seen in monocyte-derived Mϕ from SLE patients (29). We explored the possibility of treating lupus by enhancing the generation of these phagocytic CD138^{+} Mϕ. Consistent with their M2-like phenotype (6), CD138^{+} Mϕ from MO-treated mice had a metabolic profile consistent with alternatively activated Mϕ and expressed high levels LXRα, a transcription factor implicated in generating M2 Mϕ (13). In contrast, CD138^{+} Mϕ from pristane-treated mice were M1-like, expressing low levels of LXRα and high levels of HIF1α, a

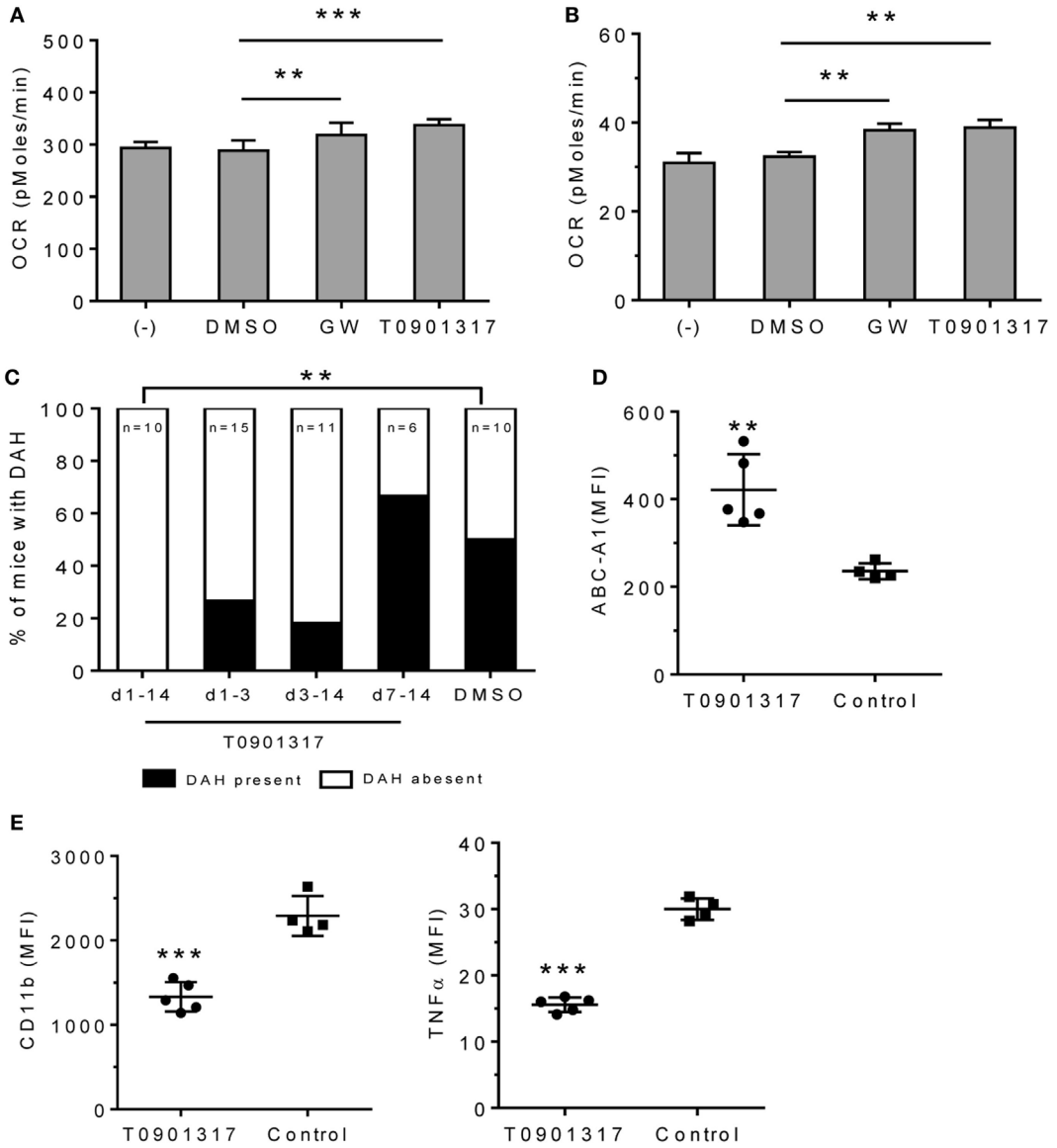

FIGURE 5 | Effect of LXRα agonist on pristane-induced lung hemorrhage. **(A)** *In vitro* treatment of RAW-264.7 cells with GW3965 (GW, 1 μM), T0901317 (1 μM), or DMSO for 24 h. Oxygen consumption rate (OCR) was measured (XF96 Analyzer). Experimental treatments were performed with six technical replicates. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control (Student's unpaired *t*-test). **(B)** Adherent peritoneal Mϕ from pristane-treated B6 mice were incubated for 24-h with GW3965, T0901317, or DMSO followed by measurement of OCR. Experimental treatments were performed with six technical replicates. $^{**}P < 0.01$ vs. control (Student's unpaired *t*-test). **(C–E)**, B6 mice were injected once with pristane and treated i.p. with T0901317 (200 μg/mouse/day) or DMSO ($n = 10$) starting on the day of pristane treatment. One group received T0901317 daily from d1–d14 ($n = 10$), another from d1–d3 ($n = 15$), another from d3–d14 ($n = 11$), and another from d7–d14 ($n = 6$). **(C)** Frequency of lung hemorrhage in the four groups. 5/10 control mice and 0/10 mice treated with T0901317 (d1–d14) developed DAH ($^{**}P < 0.01$, χ^2). **(D, E)** Flow cytometry of $CD11b^+CD138^+$ Mϕ from mice treated with pristane plus T0901317 (d1–d14) vs. DMSO (Control). MFI, mean fluorescence intensity. **(D)** Intracellular staining for Abca1 in $CD11b^+CD138^+$ cells. $^{**}P < 0.01$ vs. control (Welch's unpaired *t*-test). **(E)**, Surface staining for CD11b and intracellular staining for TNFα. $CD11b^+CD138^+$ cells were gated to analyze the expression level (MFI) of CD11b and TNFα. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. control (Student's unpaired *t*-test).

transcription factor that promotes glycolytic metabolism and the generation of M1 Mϕ (17, 22). Treatment of mice with pristane-induced lupus using an LXR agonist enhanced the expression of M2 Mϕ markers and prevented DAH, a severe inflammatory lung disease associated with pulmonary vasculitis that occurs in 3% of SLE patients (4, 30). Like PECs from pristane-treated mice, peripheral blood monocytes from SLE patients exhibited high HIF1α and low LXRα activity and LXR agonist treatment attenuated the interferon signature in these cells. The data suggest that abnormal Mϕ polarization contributes to the pathogenesis of SLE and that correcting the imbalance between M1- and M2-like Mϕ polarization may be a useful therapeutic strategy.

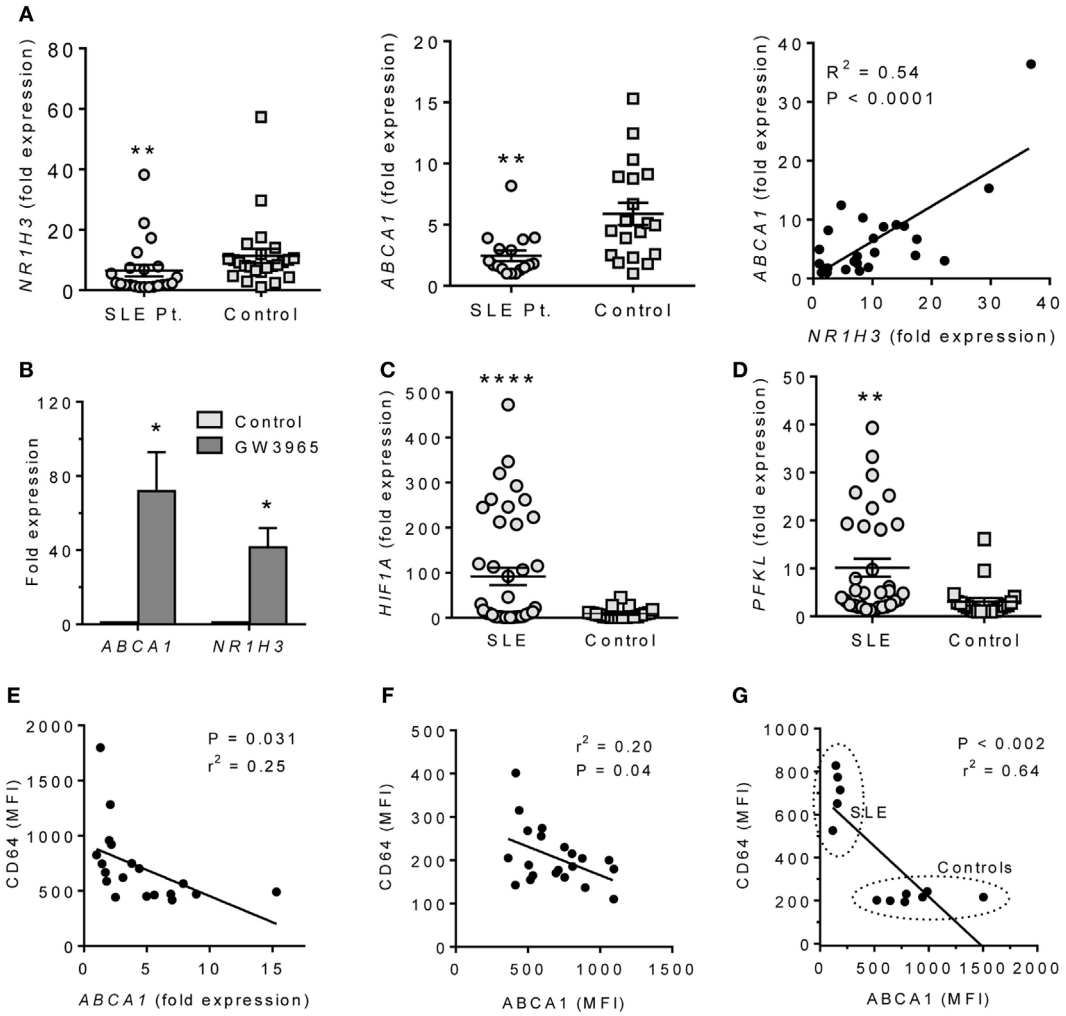

FIGURE 6 | ABCA1 and HIF1α expression in monocytes from SLE patients. **(A)** Expression of *NR1H3* and *ABCA1* in adherent PBMC (Q-PCR) and bivariate analysis of *ABCA1* vs. *NR1H3* (right). Left $^{**}P < 0.01$ (Student's unpaired *t*-test); Middle, $^{**}P < 0.01$ vs. control (Welch's unpaired *t*-test). **(B)** Adherent PBMCs were treated with 1 μM GW3965 or vehicle alone (Control) for 24 h. *ABCA1* and *NR1H3* expression levels were measured by Q-PCR. $^{*}P < 0.05$ (Welch's unpaired *t*-test). **(C)** Expression of *HIF1A* in adherent PBMCs from SLE patients vs. healthy controls (Q-PCR). $^{*}P < 0.0001$ (Welch's unpaired *t*-test). **(D)** *PFKL* expression on adherent PBMCs from SLE and healthy controls (Q-PCR). $^{*}P < 0.01$ (Welch's unpaired *t*-test). **(E)** Flow cytometry of the IFN-regulated protein CD64 staining (MFI, flow cytometry) vs. *ABCA1* mRNA expression (Q-PCR) in monocytes from unselected SLE patients. **(F)** Flow cytometry of CD64 (surface staining) vs. ABCA1 (intracellular staining) in monocytes from unselected SLE patients. **(G)** CD64 vs. ABCA1 staining in PBMCs from five patients with active SLE and seven healthy controls.

M1–M2 Mϕ Imbalance in Pristane-Induced Lupus

We reported recently that a novel subset of CD138^{+} Mϕ with an M2 phenotype is highly phagocytic for apoptotic cells and promotes the resolution of inflammation. This subset is deficient in pristane-treated mice in comparison with MO-treated controls (6). In contrast, the M1-like Ly6C^{hi} Mϕ subset expands in pristane-treated mice. M1 Mϕ rely on glycolysis (high ECAR) whereas M2 Mϕ rely on fatty acid oxidation (high OCR) (15, 16). Mϕ from MO-treated mice had higher OCR, whereas ECAR was higher in pristane-treated mice (**Figure 1**), consistent with expansion of the M1 subset in pristane-induced lupus. Unexpectedly, CD138^{+} Mϕ from MO-treated mice had a higher OCR and expressed higher levels of M2 Mϕ markers [CD273, *Chil3* (Ym1), and IL-10] than those from pristane-treated mice, which preferentially expressed the M1 markers CD274, CD86, and TNFα (**Figure 3**). Thus, either the phenotype of CD138^{+} Mϕ subset exhibits some plasticity or there is more than one subset of CD138^{+} Mϕ. Our recent studies suggest the presence of an additional subset of proinflammatory CD138^{+} monocyte/Mϕ in pristane-treated B6 mice (S Han, unpublished data). Since HIF1α and LXRα regulate the gene expression programs of M1 and M2 Mϕ, respectively, we examined the activity of these transcription factors in pristane- vs. MO-treated mice.

High HIF1α Activity in Lupus

Hypoxia-inducible factor 1-α and HIF1α-regulated genes were expressed at higher levels in both murine and human lupus

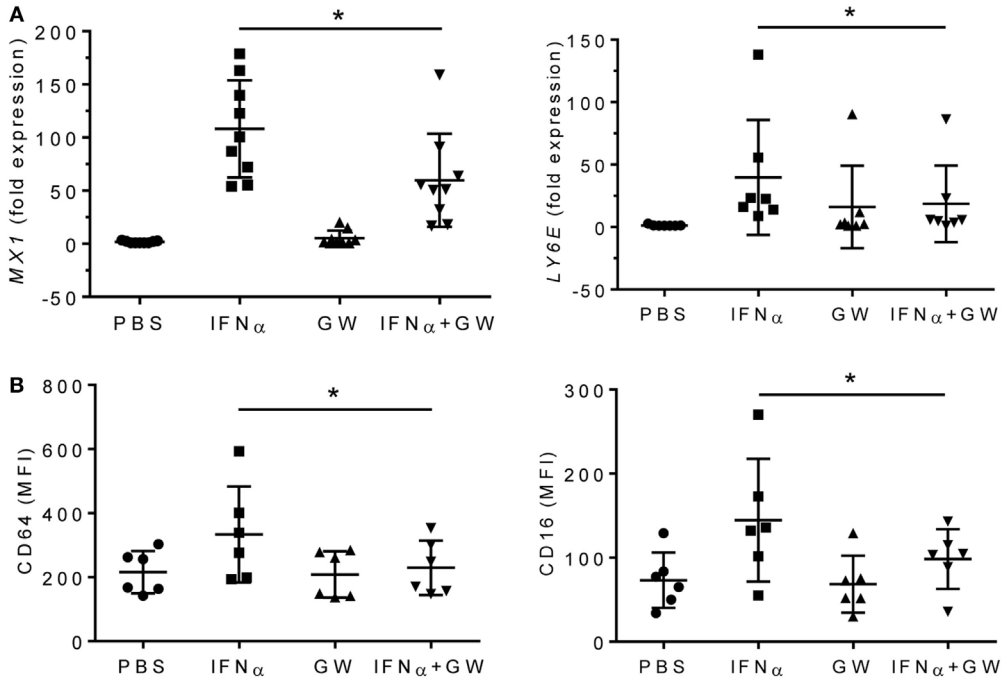

FIGURE 7 | LXR agonist attenuates the type I interferon signature. Adherent PMBCs from healthy donors were incubated for 24 h with IFNα (1,000 U/ml), GW3965 (GW, 1 μM), or both. **(A)**, mRNA levels of *MX1* and *LY6E* were measured by Q-PCR. **(B)** CD64 and CD16 staining (mean fluorescence intensity, MFI) was determined by flow cytometry. *$P < 0.05$ vs. control (unpaired Student's *t*-test).

(**Figures 4** and **6**). HIF1α is a hypoxia-induced regulator of glycolytic enzymes (e.g., HK2, PFKL, and G6PD) (17), and an inducer of M1 activation and the production of TNFα and other proinflammatory cytokines (18, 31). Heterodimers of HIF1α with the constitutively expressed aryl hydrocarbon receptor nuclear translocator bind and transactivate target genes containing hypoxia response elements (17). The transcriptional program induced by HIF1α is important for Mϕ and neutrophil function in infected (hypoxic) tissues (32). HIF targets include genes involved in aerobic glycolysis as well as inflammation (17, 33). The M1 marker CD274 (PD-L1) is HIF1α regulated and was expressed at higher levels in Mϕ from pristane- vs. MO-treated mice (**Figure 3A**).

Impaired LXRα Activity in Lupus

In contrast to HIF1α, LXRα promotes M2 Mϕ development (13, 34). *Hif1a* mRNA expression correlated positively with *Tnfa* (**Figure 1A**) and inversely with the LXR-regulated gene *Abca1* (**Figure 4B**). Transcription factors of the LXR family form heterodimers with the retinoid X receptor, are activated by oxysterols (e.g., 25-hydroxycholesterol) (12), and regulate the transport of cholesterol transport to the liver and its biliary excretion (26, 35). Following uptake of apoptotic cells, oxysterols from the cell membranes activate the LXR pathway, upregulating the apoptotic cell receptor *MerTK* (14) and genes involved in cholesterol efflux (e.g., *ABCA1*). LXR activation downregulates innate immunity and inflammation by suppressing TLR signaling in Mϕ (12, 36). This may be one reason that phagocytosis of apoptotic cells is usually anti-inflammatory. Mice doubly deficient in LXRα and LXRβ exhibit proinflammatory signaling in response to apoptotic cells and develop lupus-like disease (14).

LXR activation is critical for M2 Mϕ polarization, expression of M2 signature genes, and downregulation of inflammation in activated Mϕ (34). In both pristane-induced lupus and SLE patients, expression of the LXR-regulated gene ABCA1 was impaired at both the RNA and protein level (**Figures 2A** and **6A**). Lupus and control Mϕ did not exhibit substantially different *Nr1h3* gene expression, suggesting that the low Abca1 levels in lupus mice reflect impaired activation of LXR protein rather than low *Nr3h1* mRNA levels. However, our studies did not address the issue of whether the observed differences in Mϕ function specifically reflect the expression level of ABCA1 gene/protein or if the expression of other LXR-regulated genes plays a role. In mice, low LXRα was associated with high levels of TNFα and IFN-I regulated genes and low IL-10, especially in $CD138^+$ Mϕ. In human monocytes, LXR agonists inhibited the induction of *MX1* and other type I IFN-stimulated genes by IFNα (**Figure 7**). Inhibition of *Hif1a* and *Pfkl* gene expression by LXR agonists (**Figure 4C**) further suggests that LXR may cross-regulate the HIF pathway, providing a potential mechanism for switching from M1 to M2 polarization.

LXR Agonist Treatment Prevents DAH in Lupus

Our data suggested that HIF1α inhibitors or LXR agonists might benefit lupus patients by promoting M2 Mϕ polarization.

Selective HIF1α inhibitors are not readily available, although there is interest in targeting the HIF1α activation pathway for cancer therapy (33, 37). Synthetic LXR agonists protect mice from atherosclerosis, myocardial ischemia-perfusion injury, and other conditions (26, 38). Unfortunately, their clinical use is complicated by hepatic steatosis, degradation of hepatic LDL receptors via the LXR-IDOL (inducible degrader of the LDL receptor) pathway, and/or unexplained neurological side effects (26, 38). However, the development of safer LXR agonists for clinical use is ongoing.

We gave pristane-treated mice the LXR agonist T0901317 to see if it could prevent DAH, an often fatal complication of SLE (2, 3). Daily LXR agonist treatment protected mice from DAH and promoted M2 repolarization of $CD138^+$ Mϕ (**Figure 5**), suggesting that M1 Mϕ play a role in SLE-associated DAH. As DAH is similar in pristane-induced and human lupus (3), LXR agonists also might be useful in patients with DAH. We speculate that LXR agonists also might have a role in treating other Mϕ-mediated clinical manifestations of lupus. In lupus nephritis patients, glomerular and tubular Mϕ are among the best early correlates of proteinuria, declining creatinine, and poor renal outcome (39, 40). Mϕ also promote lupus nephritis in NZB/W mice (41, 42). Thus, lupus nephritis is a potential target for future testing of LXR-agonist therapy.

Low LXR expression also may be involved in accelerated atherosclerosis in SLE (43). Non-resolving inflammation in the vessel wall mediated by infiltrating Mϕ plays a central role in atherosclerosis and LXRs reciprocally regulate inflammation and lipid metabolism (34, 44). Similar to pristane-induced lupus (6), chronic inflammation in atherosclerotic plaques is associated with decreased non-inflammatory clearance of apoptotic cells by Mϕ (45). Thus, the LXR pathway may have far-reaching effects on the pathogenesis of organ damage in SLE.

Impaired Mϕ-mediated uptake of apoptotic cells is strongly associated with both human and murine lupus (6, 21, 29, 46). LXR signaling upregulates the clearance of apoptotic cells and its absence promotes autoimmunity (14). The present study provides the first evidence that LXR activity is abnormally low in monocytes/Mϕ from SLE patients whereas activity of HIF1α, a transcription factor that promotes inflammation and M1 polarization, is increased. The data support the clinical relevance of defective M1-M2 polarization, impaired apoptotic cell clearance, and non-resolving inflammation seen in pristane-induced lupus (6) and indicate that LXR agonist therapy aimed at repolarizing Mϕ can prevent disease, suggesting that a similar response may be achievable in SLE patients. LXR agonists modulated type I interferon production (**Figure 7**) and there is evidence for interplay between LXR signaling and Type I/Type II interferon production (47–49). However, LXR agonists are likely to have additional, interferon-independent, effects in lupus, since Type I interferon does not play a major role in the pathogenesis of DAH (3). It will be of interest to elucidate how signaling pathways downstream of LXR modulate the inflammatory response in lupus patients. Finally, the results identify imbalanced HIF1α and LXRα activity as a potential biomarker for assessing chronic inflammation in SLE patients and the response to anti-inflammatory therapy.

AUTHOR CONTRIBUTIONS

SH: Acquired the data and assisted in the analysis and interpretation and preparation of the manuscript. HZ: Acquired the data and assisted in the analysis and interpretation. SS: Assisted with data acquisition and analysis. JW: Assisted with data acquisition and analysis. CX: Assisted with data acquisition and analysis. HL: Assisted with data acquisition and analysis. LY: Assisted with data interpretation and preparation of the manuscript. WR: Responsible for the overall design of the study, analysis and interpretation of the data, and manuscript preparation.

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