NEW BIOMARKERS FOR THE DIAGNOSIS AND TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

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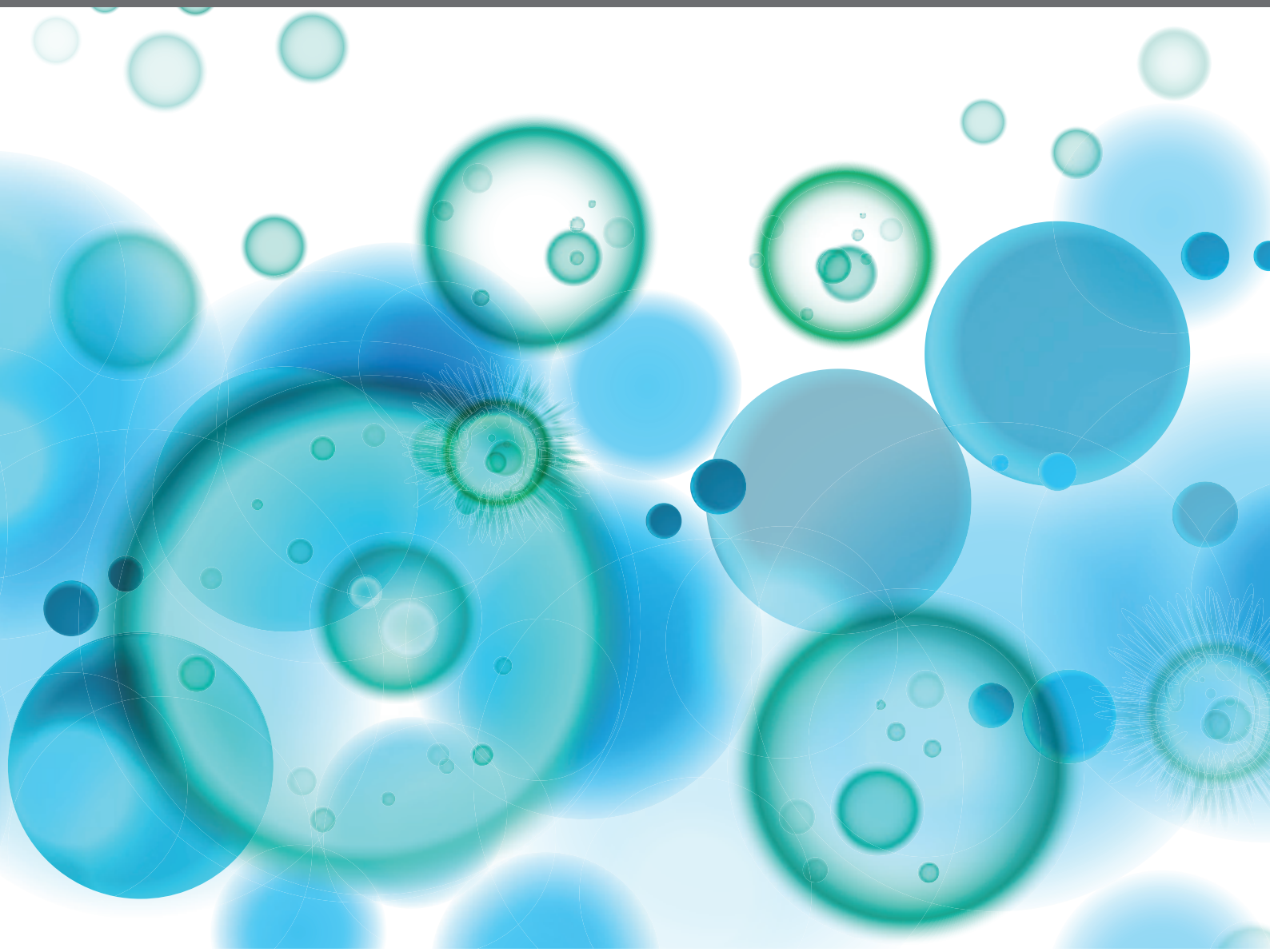

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NEW BIOMARKERS FOR THE DIAGNOSIS AND TREATMENT OF SYSTEMIC LUPUS ERYTHEMATOSU

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Editorial: New biomarkers for the diagnosis and treatment of systemic lupus erythematosus

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Editorial on the Research Topic
New biomarkers for the diagnosis and treatment of systemic lupus erythematosus

Systemic lupus erythematosus is an autoimmune disease of unknown etiology that primarily affects females of child-bearing age with various morbidities (1). Mortality of SLE still exceeds 10% over 5 years (2, 3). While current treatments are partially effective, they carry significant side effects (4), with infections due to toxicity of immunosuppressant medications being a major cause of death (5, 6). This includes belimumab, the 1st drug approved by the FDA for SLE treatment in 56 years (7), and more recently anifrolumab, both of which also predispose to infections (8). Therefore, a significant unmet need exists to identify biomarkers that can be targeted for safe and effective therapeutic intervention in SLE. A research topic centered around new biomarkers for the Diagnosis and Treatment of SLE included 15 publications with a wide range of focus and experimental design. This Editorial addresses the challenges of integrating a series of newly reported single biomarkers, composite biomarkers based multi-omics approaches, and biomarkers based on machine learning with the complex systems biology of SLE. These newly reported biomarkers are shown in Table 1.

S100 calcium-binding protein A8 protein (S100A8) levels as biomarkers for systemic lupus erythematosus (SLE) were quantified in serum, urine, and saliva samples from 249 patients with SLE and 52 age- and sex-matched healthy controls (HCs) and a receiver operating characteristic curve was used to analyze whether they may be used as biomarkers for diagnosis and prediction of flares (17). For SLE diagnosis, the area under the receiver operating characteristic curve (AUC) was 0.831 for serum S100A8 (95% CI, 0.765–0.897),

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TABLE 1 New biomarkers for the diagnosis and treatment of SLE.

Biomarker	Source	Outcome	Impact	Reference
Bacilli, *Lactobacillales*	Gut	SLE	Risk	(9)
Bacillales, *Coprobacter*, *Lachnospira*	Gut	SLE	Protection	(9)
IL-6	Hippocampus	NPSLE	Diagnosis	(10)
Lactoceramide	Plasma	CVD	Diagnosis	(11)
Anti-DNA	Serum	LN	Flare	(12)
ABCB1, IFI27, PLSCR1	PBMC	SLE	Diagnosis	(13)
P3H1, PHACTR4, RGS12	Serum	LN	Diagnosis	(14)
ALCAM, VCAM-1 and PF4	Urine	LN	Flare	(15)
S100A8	Kidney	SLEDAI, LN	Diagnosis	(16)
S100A8	Blood, Urine, Saliva	SLEDAI, LN	Flare	(17)
Adiponectin, MCP-1, sVCAM-1,PF4	Urine	LN	Flare	(18)
CD11c, T-bet, and CD21high B cells	Blood	LN	Protection	(19)

LN, lupus nephritis.

0.751 for urine S100A8 (95% CI, 0.648–0.854), and 0.729 for salivary S100A8 (95% CI, 0.646–0.812). Pearson's correlation analysis showed that S100A8 in serum, urine, and saliva was significantly associated with the SLEDAI ($r = 0.267$, $p < 0.001$; $r = 0.274$, $p < 0.001$; and $r = 0.629$, $p < 0.001$, respectively). Among the clinical manifestations, nephritis was the only organ involvement that was associated with increased concentration of S100A8 in serum, urine, and saliva in comparison to SLE patients without LN (17). An independent study demonstrated that enhanced glomerular S100A8 staining in class IV LN patients over controls (16). S100A8 has been identified as a differentially expressed gene (DEG) with overexpression in kidneys of LN patients (16).

Mass spectroscopy of circulating immune complexes identified 300 proteins in the serum of SLE patients, several of which were found to be highly associated with LN in two independent patient-control cohorts (14). Prolyl 3-hydroxylase 1 (P3H1), phosphatase and actin regulator 4 (PHACTR4), and regulator of G-protein signaling 12 (RGS12) discriminated LN AUC values of 0.82, 0.99, and 0.90, respectively.

Serial kidney biopsies for initial diagnosis and subsequent monitoring of lupus nephritis (LN) remain challenging, thus non-invasive biomarkers are needed. Urinary ALCAM, PF4, and VCAM-1 were identified as potential biomarkers for predicting kidney disease activity in childhood-onset SLE with ALCAM (AUC 0.83) being the single most predictive (15). Herpes virus entry mediator (HVEM) demonstrated comparable diagnostic ability to creatinine normalization when distinguishing active lupus nephritis from inactive SLE patients using the candidate biomarker ALCAM (20). In a 3-stage study including a total of 321 LN patients, a combination of four biomarkers, adiponectin, MCP-1, sVCAM-1 and PF4, were found to have the greatest predictive value for the detection of proliferative, active LN (18). Patients with LN exhibit a profound depletion of atypical age-associated B-cell (ABC) like $CD11c^{+}T\text{-}bet^{+}CD21^{hi}$ B cells in comparison with healthy individuals and SLE patients without LN (19). Selected from 284 DEGs identified in two independent SLE cohorts in the Gene Expression Omnibus (GEO) database, machine learning validated ABCB1, IFI27, and PLSCR1 as top predictors of SLE in a Chinese validation cohort of patients over ethnically matched controls (13). Expression of each these genes was correlated with the expansion of pro-inflammatory lineages of the adaptive and innate immune systems (13). Separately, a comprehensive analysis of gut microbiome genome databases newly identified *Bacilli* and *Lactobacillales* as promoters of SLE and *Bacillales*, *Coprobacter* and *Lachnospira* as protectors from SLE (9).

Among plasma sphingolipids, lactoceramide has been identified as a potential predictor of cardiovascular disease (CVD) in African-American patients with SLE (11). Tan et al. provide an extensive review of biomarker development for SLE (21). The biomarkers are divided by their molecular nature: i) autoantibodies; ii) serum proteins (cytokines, chemokines, complement components, soluble receptors and transporters); iii) microRNAs and long non-coding RNA (LncRNAs); and relevance for organ involvement, such as nephritis, neuropsychiatric lupus, and cutaneous lupus. The review does not discuss cellular biomarkers, such as Tregs, memory B or T cells, or metabolites. Indeed, a comprehensive review of all biomarkers implicated in lupus pathogenesis and patients care remains daunting. In contrast, Ole Petter Rekvig focuses on the role of DNA structure in triggering anti-DNA antibodies and its relationship to lupus nephritis (12). Mitochondrial N-formyl methionine (fMet) is newly implicated in promoting neutrophil-mediated inflammation in systemic sclerosis (22).

Neuropsychiatric SLE (NPSLE) can be diagnosed in the majority of patients with appropriate screening instruments (23). NPSLE and particularly depression has been associated with elevated levels of type 1 interferons, TNFs, and IL-6 in the cerebrospinal fluid (CSF) of SLE patients (24). Accumulation of senescent cells in the hippocampus has been linked to major depression (25). Apparently, depression in lupus-prone MRL/lpr mice is associated with the accumulation of senescent cells in the

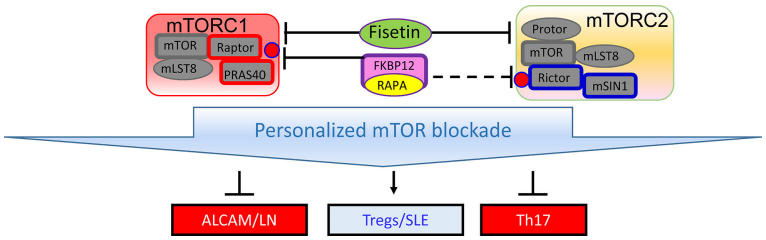

FIGURE 1
Schematic diagram of mTOR complexes 1 (mTORC1) and 2 (mTORC2) in SLE. Similar to rapamycin, fisetin inhibits both mTOR complexes (34), which may be involved in expanding Tregs (35) and thus improving depression and other organ involvement in SLE (10, 35).

cornu ammonis 3 (CA3) region of hippocampus (10). Importantly, oral fisetin, a senolytic drug, reduced the number of senescent neural cells and IL-6 mRNA in the hippocampus and improved depressive behavior in the MRL/lpr mice (10).

Given the diversity of hypotheses, methodologies, experimental models and study design, integration of the newly reported biomarkers with the systems biology of SLE present multiple challenges. Such challenges can be easily attributed to a general lack of understanding of lupus pathogenesis. However, several key facts need to be considered when integrating such interesting but diverse outcome. A hallmark of SLE is the production of antinuclear autoantibodies (ANA) (26). Although ANAs are directed to an ever growing number of nucleic acid and nucleoprotein targets and their titers can greatly vary due to the course of disease and impact of therapies, their detection remains a key criterion of diagnostic workup (27). Thus, biomarkers may be mechanistically connected, either upstream or downstream, to the generation or handling of autoantibodies and cell-mediated autoreactivity. T (28) and B cells of the adaptive immune system (29, 30) and IFN-producing dendritic cells are essential for lupus pathogenesis (31). Therefore, integrating biomarkers into the signaling networks that connect the adaptive and innate arms of a dysfunctional immune system is critical for appreciating their significance for controlling pathogenesis, predicting flares or serving as target for treatment in SLE. Notably, only few of these studies involved cellular biomarkers (13, 19) that can be connected to central pathways of lupus pathogenesis. Nevertheless, certain easily detectable biomarkers may also serve other purposes, such as sensing organ damage and obviating the need for invasive procedures, i.e., renal biopsy in LN (15, 17–19). Fisetin was found to control depression by preventing the senescence of neuronal cells in the hippocampus of MRl/lpr mice (10). Fisetin is known to exert its antiaging effect by blocking the mechanistic target of rapamycin (mTOR) (32, 33) (Figure 1), which serves a sensor of cellular stress and central regulator of pro-inflammatory lineage development in the immune system (36). Importantly, T cells of SLE patients (37–41) and mice exhibit activation of the mechanistic target of rapamycin (mTOR) (42, 43). Th17 and IL-4 and IL-17-producing DN T cells are expanded, while CD8 EMT cells (44, 45) and Tregs are deficient in SLE patients due to cell type-specific skewing of autophagy that can be corrected with therapeutic efficacy by mTOR blockade (45, 46). Rapamycin blocks nephritis in SLE (47–49). Rapamycin also blocks the production of vascular cell adhesion molecule-1 (VCAM-1) by vascular endothelial cells (50). Of note, VCAM1 was identified as a sensitive biomarker of active LN by two independent studies published under this Research Topic (15, 18). Therefore, it's possible that mTOR blockade with sirolimus or fisetin would block LN *via* reducing the expression and urinary excretion of VCAM1 and other adhesion molecules.

In conclusion, while a single marker may not adequately capture all the finesses of LN, SLE-CVD, NPSLE or other subcategories of SLE, it is conceivable that the creation of easily accessible screening assays measuring one or more factors will provide fast and reliable information about the development of organ-specific symptoms, the onset of flares, and the prediction of different therapeutic intervention among diverse SLE patients. Before we can reach such goal, it is however important that new markers are tested across different patient groups. For example, it will be of interest to know if S100A8, PF4 and (s)VCAM-1 are similarly upregulated in SLE patients with NPSLE or CVD, or if this phenotype is specific for LN. Future studies are clearly warranted to substantiate the importance of these biomarkers for the diagnosis and treatment of SLE.

Author contributions

AP conceived and wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Erythropoietin in Lupus: Unanticipated Immune Modulating Effects of a Kidney Hormone

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Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease with variable clinical presentation, typically characterized by a relapsing-remitting course. SLE has a multifactorial pathogenesis including genetic, environmental, and hormonal factors that lead to loss of tolerance against self-antigens and autoantibody production. Mortality in SLE patients remains significantly higher than in the general population, in part because of the limited efficacy of available treatments and the associated toxicities. Therefore, novel targeted therapies are urgently needed to improve the outcomes of affected individuals. Erythropoietin (EPO), a kidney-produced hormone that promotes red blood cell production in response to hypoxia, has lately been shown to also possess non-erythropoietic properties, including immunomodulatory effects. In various models of autoimmune diseases, EPO limits cell apoptosis and favors cell clearance, while reducing proinflammatory cytokines and promoting the induction of regulatory T cells. Notably, EPO has been shown to reduce autoimmune response and decrease disease severity in mouse models of SLE. Herein, we review EPO's non-erythropoietic effects, with a special focus on immune modulating effects in SLE and its potential clinical utility.

Keywords: erythropoietin, SLE, immunology, lupus, T cell, Treg

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder with multiorgan involvement. Interactions amongst genetic, hormonal and environmental factors lead to immune dysregulation and loss of tolerance to self-antigens, with consequent autoantibody production, inflammation, and tissue damage (1). SLE is characterized by a relapsing-remitting course with a wide spectrum of clinical presentations, including—but not limited to—cutaneous, articular, hematologic, pulmonary, neurological and renal complications. In particular, the prevalence of neurological manifestations, both of the central and peripheral nervous system, ranges between 14 and 95% and is associated with worse outcomes and higher mortality rates (2). The pathogenesis of neural disease in SLE remains unclear, but it likely involves a direct role of autoantibodies, inflammatory cytokines and chemokines, and brain blood barrier dysfunction (3). Renal disease affects between 28 and 74% of SLE patients and is also associated with increased mortality (4–6). Despite treatment, a substantial percentage of SLE patients still develops end stage kidney disease (ESKD) and disease may recur after kidney transplantation (7, 8).

Available therapeutic options for SLE have limited efficacy and are burdened by significant toxicities. Therefore, new, hypothesis-driven therapies are needed to improve the outcomes of individuals with SLE.

SLE Pathogenesis

Our understanding of SLE pathogenesis is still incomplete, but the following mechanisms are thought to play a major role. Defective clearance of debris from apoptotic cells exposes nuclear antigens, which initially triggers an innate inflammatory response via activation of toll-like receptors (TLR) and then bolsters T cell and B cell responses against autoantigens (9, 10). B cells present autoantigens to T cells, produce autoantibodies, and promote local inflammation. The autoantibodies bind to self-antigens and form immune complexes in various organs, further fueling the autoinflammatory response through the activation of complement and the recruitment of FcyR- and TLR-expressing innate immune cells. In turn, these cells release proinflammatory cytokines and chemokines, sustaining leukocyte infiltration and activation and formation of lymphoid aggregates, leading to organ damage (10, 11).

Dendritic Cells and Macrophages

Multiple abnormalities in dendritic cells (DCs) have been identified in SLE patients. In particular, plasmacytoid DCs (pDCs), responsible for secretion of high levels of type I interferon (IFN) via TLR7 and TLR9 stimulation, are increased in patients with SLE (12). Sustained production of type I IFN by pDCs in response to immune complexes represents a hallmark of SLE (13). Importantly, massive pDCs infiltrates are found in renal and skin lesions of SLE patients (14). This, together with the observation that ablation of these cells in lupus-prone mice reduces autoantibody production and lupus nephritis disease severity (15), supports the role of pDCs in the pathogenesis of SLE. Increase in pDCs is paralleled by a decline in conventional DCs (cDCs) in peripheral blood of SLE patients (16). These DCs are involved in maintaining self-tolerance and their reduction leads to an imbalance in DC subsets that favors a proinflammatory environment (17, 18).

Impaired clearance of apoptotic cell debris is a central pathogenic mechanism in the development of SLE. Defective clearance of apoptotic cell debris promotes release of autoantigens and autoreactive B cell stimulation, which leads to loss of tolerance and generation of autoantibodies. Consequent immune complex formation and deposition results in organ damage (19). As macrophages are a key cell subset in the clearance of apoptotic debris, it is not surprising that defective macrophage activity contributes to the pathogenesis and correlates with disease severity (20). Macrophage infiltrates in the kidney represent a strong prognostic biomarker for progression of lupus nephritis and correlates with the disease activity index (21).

B Cells

The role of B cells in the development of SLE has recently raised interest, not only for their ability to produce autoantibodies that lead to organ damage, but also for complex interactions with other cell types. Immune phenotypic studies showed abnormalities in the proportion of different B cell subsets in SLE individuals. In particular, B cell lymphopenia with reduced numbers of naïve B cells and an increase in circulating class-switched memory B cells, plasma blasts and plasma cells is observed and correlates with disease activity (22).

Under the influence of genetic susceptibility and environmental factors, B cells in SLE patients show increased activation, as documented by active B cell receptor (BCR) signaling with increased phosphorylation of PI3K and AKT-1 and abnormal phosphatase activity (23), increased production of cytokines IL-6 and IL-10, constitutive expression of costimulatory molecules that affect T cell function and antigen presenting cells (APCs) (24), and loss of tolerance.

T Cells

Murine and human data converge to indicate that SLE is associated with defective and/or decreased numbers of regulatory T cells (Treg), which normally act to control conventional T cells (Tconv) and promote self-tolerance (25). Tconv in SLE individuals also display abnormalities that are likely the result of primary defects and the consequence of the proinflammatory environment. T cell abnormalities include altered activation signaling pathways, increased expression of pro-migratory markers, and upregulation of co-stimulatory CD40 ligand, contributing to B-cell activation. T cells from SLE patients also show an altered cytokine profile, including decreased transforming growth factor beta (TGF-β) and IL-2, and increased IL-6 and IL-17 expression, which may contribute to the imbalance in T cell subsets (26, 27).

In particular, increased IL-17 and decreased IL-2 levels account for the higher Th17/Th1 ratio reported in SLE compared to healthy controls (28). Altered IL-2 production is also associated with Treg dysfunction and further promotes expression of IL-17, with a decreased Treg/Th17 ratio, which is detectable not only during flares, but also when the disease is in remission (29, 30). SLE patients show increased Th17 cells in peripheral blood and in kidney and skin lesion infiltrates, as well (31). SLE patients also display an imbalanced Th1/Th2 ratio, which is thought to play a major role in disease pathogenesis. Plasma levels of IL-10, a main driver of Th2 differentiation, are significantly increased and correlate with disease activity (32).

EPO and EPO Receptors

Erythropoietin is a glycoprotein initially discovered for its role in stimulating red blood cell production. More recently, evidence has accumulated indicating that EPO also displays non-erythropoietic properties. Interstitial fibroblasts in the kidney produce a basal level of EPO which binds to receptors on erythroid progenitor cells in the bone marrow to maintain a steady red blood cell mass (33, 34). Tissue hypoxia increases EPO production by stabilizing the Hypoxia Inducible Factor (HIF) transcriptional complex and activating EPO gene transcription (35).

Studies have identified two distinct EPO receptors. One is a homodimer receptor consisting of two EPO receptor (EPOR) monomers. Activation of this homodimer on erythroid progenitor cells triggers downstream signaling via JAK2 and subsequently STAT5, MAPK and PI3K pathways (36) which maintains erythropoiesis. The other receptor is a heterodimer,

consisting of an EPOR monomer subunit and the β-common receptor CD131. EPOR-CD131 requires a higher concentration of EPO for activation and has been implicated in the non-erythropoietic, "tissue-protective" effects of EPO, due to its downstream effects that mediate suppression of proinflammatory cytokines and inhibition of apoptosis (37–39).

EPO Derivatives

Current FDA-approved indications for EPO include treatment of anemia associated with chronic kidney disease (CKD) or chemotherapy (40, 41). The increased risk of thrombosis and stroke associated with EPO administration (39, 40, 42–44) prompted researchers to design asialoerythropoietin, a desialytated version of recombinant EPO notable for its shorter half-life which allowed for its neuroprotective effects with limited effects on erythrocyte mass (45).

An alternative approach was to develop molecules that selectively bind the EPOR-CD131 heterodimer and are therefore devoid of erythrogenic effects associated with the activation of the EPOR homodimer. This gave rise to carbamylated EPO (produced by carbamylation of lysine residues) and ARA290 (an 11-amino acid peptide that mimics EPO's helix B region), which have also been shown to maintain EPO's tissue-protective but not hematopoietic effects (39, 46, 47).

EPO's Non-erythropoietic Effects

Over the last few decades, many non-erythropoietic effects of EPO have been identified in multiple organs. In the nervous system, EPOR expression has been detected in neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells. Importantly, animal studies have shown that EPO has neuroprotective effects via neurogenesis, angiogenesis and anti-apoptotic, anti-oxidative, and anti-inflammatory mechanisms (48). Although one clinical trial of EPO in the treatment of acute ischemic stroke found that EPO administration within 6 h of symptoms was associated with increased mortality (49), another trial suggested that EPO administration post-acute ischemic stroke in non-tPA (tissue plasminogen activator) candidates was associated with improved long-term neurological outcomes (50). EPO showed promising neuroprotective effects also in animal models of autoimmune optic neuritis (51), setting the basis for a clinical trial in humans (NCT01962571) (52). Further ophthalmological effects have been noted, including protection against retinal degeneration (53–55).

In the cardiovascular system, both endothelial cells and cardiomyocytes express EPORs. In experimental studies, EPO protects against cardiac ischemic injury by decreasing apoptosis and inflammation, and by promoting neovascularization (56). However, clinical trials of EPO administration after myocardial infarction (MI) have reported mixed results (57, 58) and a meta-analysis on 1,336 patients showed no improvement in infarct size, left ventricular function, or mortality when EPO was administered in patients undergoing percutaneous coronary revascularization post-MI (59).

EPOR has also been localized in renal tubular and mesangial cells (60). In animal models of kidney injury, such as ischemic-reperfusion injury, erythropoiesis stimulating agents (ESA), including EPO derivatives, have improved disease severity via anti-apoptotic effects (61, 62). However, this beneficial effect has largely not been reflected in clinical trials. A meta-analysis of clinical trials found no clear benefit to ESAs in the development of acute kidney injury primarily following cardiac surgery, in renal transplant outcomes, or in CKD progression after anemia correction (63).

Therefore, tissue-protective effects of EPO have been largely demonstrated in numerous models of organ injury, but their clinical translation has provided inconsistent results, possibly as consequence of suboptimal dosing and timing. Whether selective activation of non-erythropoietic EPOR would improve safety/efficacy profile of EPO is worth investigating.

EPO's Anti-oxidative and Anti-apoptotic Effects

Oxidative stress contributes to tissue damage in the brain, kidney, heart and other organs. The discovery that EPO has direct and indirect anti-oxidative effects supports its use as a tissue-protective molecule. Anti-oxidative properties of EPO are in part independent from its role in countering apoptosis. EPO increases gene expression of Heme-Oxygenase 1 and other anti-oxidative enzymes, like superoxide dismutase, catalase, and glutathione peroxidase, directly on the cells, without the involvement of erythroid cell progenitors (64).

Several studies in different disease models and tissues identified the JAK2-STAT-Bcl2 pathway as one of the main anti-apoptotic mechanisms of EPO, through the induction of anti-apoptotic molecules, Bcl-2 and Bcl-Xl, and the inhibition of pro-apoptotic molecules, Bax and Bak (38). In erythroid cells, EPO-EPOR interactions prevent apoptosis through STAT5 signaling (65). In a murine model of acute encephalopathy due to cerebral malaria, EPO was associated with a dose dependent improvement in survival, together with a significantly reduced number of apoptotic cells (66). In a middle-cerebral artery model of ischemic injury in rats, EPO rescued neurons from apoptosis in a time-dependent manner, through activation of extracellular signal-regulated kinases and PI3K (67). Furthermore, EPO has been noted to exert direct protective effects on pancreatic β islet cells in diabetes mouse models (68), and, in neonatal porcine islet cells, EPO's anti-apoptotic effect occurs through upregulation of Bcl-2 mRNA and downregulation of Bax and caspase-3 mRNA (69).

EPO's Immunomodulatory Effects

Innate Immunity

Erythropoietin's immunomodulatory activity has been demonstrated in both innate and adaptive immune pathways (70) (**Table 1**). In animal models of various autoimmune diseases, EPO reduced disease severity and was associated with decreased levels of proinflammatory cytokines. In a rat model of experimental autoimmune encephalomyelitis, EPO administration resulted in a dose-dependent delay in disease onset and decreased disease severity, as well as decreased inflammatory cells including macrophages, microglia, dendritic cells and monocytes. In this model, EPO also delayed the rise in tumor necrosis factor (TNF) levels and decreased the peak of IL-6 levels in the spinal cord (72). Nairz et al.

TABLE 1 | Role of various cell subsets in SLE pathogenesis and effects of EPO.

Role in SLE	EPO effects
Innate Immune cells	
Dendritic cells	
cDCs are reduced, favoring a proinflammatory environment. pDCs produce high amounts of type I IFN that stimulate B cell proliferation, inflammation and loss of tolerance, promoting SLE development.	- In mice with cerebral malaria, EPO inhibits DCs differentiation and their expression of CD80, CD86, and TLRs (71) - EPO reduces number of DCs in rat EAE model (72)
Macrophages	
Macrophages have impaired function and cell clearance ability. Kidney macrophage infiltrates correlate with disease activity	- EPO inhibits NF-kB and reduces expression of pro-inflammatory genes (Nos2, TNF-α, and IL-6) in mice (73) - EPO downregulates the expression of inflammatory cytokines by macrophages (71) - In pristane-induced lupus-like murine model, EPO increases phagocytosis of apoptotic cells by macrophages and reduces accumulation of dying cells (74)
Adaptive Immunity	
Th1	
SLE patients show altered cytokine profile, including decreased IL-2 plasma levels, which contribute to the imbalance in T cell subsets	- EPO reduces Th1 proliferation, without affecting cell survival (75) - EPO reduces Th1 in MRL/lpr mice (76) - EPO decreases Th1 in rats with EAN (77)
Th2	
IL-10 plasma levels, main drivers of Th2 differentiation, are increased and correlate with SLE disease activity	- EPO promotes Th2 differentiation in rat model of EAN (77) - It increases Th2 cells in MRL/lpr mice (76)
Th17	
Th17 are increased and promote inflammation and tissue damage. These cells are found in kidney and skin infiltrates	- EPO prevents RORC expression and Th17 induction (78) - It promotes Th17 conversion into Treg (78) - EPO reduces Th17 in MRL/lpr mice and in pristane-induced SLE in mice (76, 78)
Treg	
Treg are decreased or defective, contributing to a proinflammatory environment and loss of self-tolerance	- EPO promotes Treg induction through the release of active TGF-β by APCs (79) - EPO increases Treg in lymph nodes and in CNS in mice with EAE (72) - EPO increases Treg in MRL/lpr mice (76, 78) - EPO increases Treg in heart-transplanted mice (79)
B cells	
B cells produce autoantibodies and function as defective APCs that mediate T cells' loss of tolerance	- No direct effects of EPO on B cells have been reported.

EPO, erythropoietin; SLE, systemic lupus erythematosus; cDCs, conventional dendritic cells; pDCs, plasmacytoid dendritic cells; IFN, interferon; TLRs, Toll-like receptors; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nos2, nitric oxide synthase 2; TNF-α, tumor necrosis factor α; EAN, experimental autoimmune neuritis; RORC, RAR-related orphan receptor C; TGF-β, transforming growth factor β; Treg, regulatory T cells; APCs, antigen presenting cells; EAE, experimental autoimmune encephalomyelitis; CNS, central nervous system.

showed that EPO inhibits NF-kB and subsequently reduces expression of proinflammatory genes (Nos2, TNF-α, and IL-6) in murine macrophages. Consistently, EPO administration reduced disease severity in experimental mouse models of autoimmune colitis. The anti-inflammatory effects of EPO, in contrast, impaired clearance of bacterial colonies in *Salmonella typhimurium*-infected mice, reducing animal survival (73). In mice with collagen-induced arthritis, EPO significantly reduced disease severity, oxidative damage, levels of proinflammatory cytokine TNF-α and chemokines MIP-1a and MIP-2, neutrophil infiltration, and the levels of chondrocyte apoptosis (80).

Both murine and human DCs express EPOR, suggesting that DCs can participate in the immunomodulatory properties of EPO. In DCs, EPO/EPOR signaling is more dependent on STAT3 than STAT5 (81). In studies of mice with cerebral malaria, EPO treatment significantly inhibited DCs differentiation and reduced expression of costimulatory markers CD80 and CD86, and TLRs (71).

Erythropoietin has also been demonstrated to play a role in macrophage clearance of apoptotic cells. The "find-me signal" sphingosine 1-phosphate released by dying cells activates EPO signaling in macrophages and, through upregulation of peroxisome proliferator activated receptor-γ (PPARγ), improves clearance of apoptotic cells (74). EPO-derivative ARA290 decreases expression of TNF-α and iNOS in LPS-treated macrophages and increases phagocytosis of apoptotic cells as well (82).

Adaptive Immunity

Both human peripheral blood T and B lymphocytes express EPOR (83), but the effects of EPO/EPOR interaction have been mainly characterized in T cells subsets.

Th1

Our previous experiments showed that EPO reduces Tconv proliferation in a dose-dependent manner, without affecting

cell survival, and reduces Th1 polarization. These effects are mediated by the homodimeric EPO-R expressed on T cells that interferes with signaling downstream of the IL-2R β chain, required for Tconv functions (75). The result is supported by the fact that ARA290 affects proliferation of anti-CD3/anti-CD28 mAb-stimulated CD4+ T cells (75).

Th2

Th2 differentiation of human naïve CD4+ T cells is not affected by EPO *in vitro* (75). Conversely, *in vivo* studies in experimental autoimmune neuritis model in rats show that treatment with EPO or ARA290 promotes Th2 differentiation and, together with Th1 and Th17 reduction and Treg increase, improves the disease (77, 84).

Th17

Th17 cells are strongly linked to autoimmunity and have a main role in SLE pathogenesis.

In vitro treatment with EPO of CD4+ T cells under Th17 polarizing conditions, prevents Th17 master regulator RAR-related orphan receptor C (RORC) and Th17 gene expression and Th17 cell induction, even after exposure to high concentrations of NaCl, a potent Th17 inducer, without affecting cell survival (78). EPO-EPOR interaction also prevents serine-threonine protein kinase-1 (SGK1) phosphorylation, required for RORC activity. SGK1 phosphorylation is dependent upon p38 mitogen-activated protein kinase, which is counteracted by EPO (78). *In vitro* experiments confirmed that EPO prevents Th17 induction and promotes the conversion of Th17 into Treg (78).

Treg

In vitro, EPO promotes the release of active TGF-β from APCs. As TGF-β is the main driver of naïve CD4+ T cell conversion into Treg, EPO thus promotes Treg induction. Importantly, while EPO inhibits Tconv proliferation, it does not affect Treg function once they are formed. Indeed, EPO uncouples signaling downstream of the IL-2R β chain, which is already silenced in Treg by internal phosphatases, leaving IL-2R γ chain signaling, crucial for T cells, unaffected (79).

EPO treatment increases Treg also *in vivo* in experimental models of autoimmune encephalitis (85), SLE (76, 78) and organ transplantation (79). Importantly, the administration of EPO in doses required to correct anemia resulted in increased frequency of peripheral Treg in humans with CKD (79).

EPO IN SLE

Anti-EPO and Anti-EPOR Autoantibodies

Most EPO-related research in SLE has focused on the association between anemia and autoantibodies to EPO and EPOR. Autoantibodies to EPO in patients with SLE were first demonstrated by Tziuofas et al. (86). Since then, several studies have reported associations between the presence of anti-EPO antibodies and hematological (EPO or hemoglobin/hematocrit levels) and SLE-related parameters (SLE disease activity, complement levels or anti-dsDNA antibody levels) (86–88). Overall, these studies found an impaired EPO response in anemic SLE patients, suggesting that autoantibodies may act as EPO antagonists (87, 88). However, other reports indicate that anti-EPO antibodies may just interfere with serum EPO measurement rather than inhibit EPO activity (88).

Luo et al. (89) found that anti-EPOR antibodies in SLE patients were associated with more severe anemia, higher disease activity, augmented anti-dsDNA antibody levels, and lower C3 (increased complement consumption, a sign of disease activity). Notably, Hara et al. specifically looked at 46 patients with biopsy-proven lupus nephritis and detected anti-EPOR antibodies in 18 patients. Those with anti-EPOR antibodies had significantly higher SLE disease activity and more severe anemia, suggesting that anti-EPOR antibodies have inhibitory function. Although these groups shared no differences in anti-dsDNA antibodies, complement levels, or renal function at time of biopsy, those with anti-EPOR antibodies had a higher disease activity index, and the presence of anti-EPOR antibodies was an independent risk factor for CKD progression (90).

Overall, anti-EPO and anti-EPOR antibodies correlate with SLE disease severity and may be associated with poor kidney prognosis, providing associative evidence that, by inhibiting EPOR immune modulatory effects, they may also fuel the autoimmune response.

EPO's Effects in Murine SLE Models

Different murine models have been developed to investigate pathogenic mechanisms of SLE and to identify potential new targets for therapy (91). While spontaneous models of lupus are principally used to study the genetic susceptibility to the disease, induced models help in defining the role of environmental factors in lupus pathogenesis and identifying mechanisms responsible for the onset and progression of disease. MRL/lpr mice, a spontaneous model of SLE, are characterized by a mutation in Fas gene and develop severe lymphoproliferative disease with lymphadenopathy, splenomegaly, proteinuric nephropathy and skin lesions (92). This strain also shows behavioral abnormalities and cerebritis that resemble neuropsychiatric involvement in SLE (93).

In 2018, Zhang et al. showed that MRL/lpr mice that received EPO for 10 weeks had less urinary protein, lower serum anti-dsDNA antibody levels, lower renal histopathologic scores with less IgG/C3 deposition in glomeruli, and decreased cytokine levels in the kidneys compared to controls. They also found that mice treated with EPO had fewer Th1 and Th17 cells and more Th2 and Treg cells (76).

Another study by Huang et al. (82) found that administration of EPO-derived helix-B peptide (ARA290) to MRL/lpr mice significantly decreased serum levels of antinuclear antibodies (ANA), anti-dsDNA antibodies, creatinine, cytokine levels (IL-6, MCP-1, TNF-α), renal deposition of IgG, and quantity of apoptotic cells in the kidney. Similar results were found in pristane-induced SLE mice. Importantly, these results were obtained without significant changes in erythropoiesis (82).

Mice that lacked EPOR selectively on macrophages developed lupus-like symptoms. At 55 weeks of age, the mice had significantly increased anti-dsDNA, antinuclear, and anti-Smith antibodies, pathologic evidence of increased glomerular

deposition of IgG, IgA, and C3, and increased glomerular size, cellularity and infiltration of immune cells compared to controls. They also developed higher proteinuria and serum creatinine and blood urea nitrogen (BUN) concentrations, along with increased IL-6, TNF-α, IFN-α, and IFN-β levels, while TGF-β decreased, suggesting that EPO/EPOR signaling in macrophages is key to maintaining self-tolerance (74).

Furthermore, in pristane-induced lupus-like murine model, EPO therapy increased phagocytosis of apoptotic cells by macrophages and correspondingly decreased accumulation of dying cells. These EPO-treated mice had decreased serum concentrations of anti-dsDNA antibodies, and of IL-6, MCP-1, and TNF-α levels. They also showed decreased glomerular IgG deposition and improved renal function, as indicated by decreased urinary albumin and serum creatinine (74). Mechanistically, these data have been linked to the S1P-EPO-PPARγ pathway in macrophages that is crucial for apoptotic cell phagocytosis (74).

As demonstrated by these studies, EPO treatment reduced disease severity in both pristane-induced and spontaneous MLR/lpr lupus models. More recently, it has been shown that these effects are linked to a direct action of EPO on T cells (78). In these models of lupus nephritis, in which *Epo* gene expression is reduced, EPO treatment prevents Th17 cell induction and increases the Treg/Th17 and Th2/Th1 cell ratio. In pristane-induced lupus nephritis, EPO deficiency selectively on CD4+ T cell resulted in increased susceptibility to the disease (more proteinuria and severe renal involvement) and conferred resistance to the inhibitory effects of EPO on Th17 cell induction (78).

IS EPOR A TARGET FOR FUTURE IMMUNE-MODULATING TREATMENTS FOR SLE?

Erythropoiesis-stimulating agents are already currently used in patients with lupus nephritis for CKD-associated anemia. One cross-sectional study of 12,533 adult patients with ESKD secondary to lupus nephritis found that 4,288 (34%) were receiving ESA therapy at the time of renal replacement therapy (RRT) initiation (94). However, no study has assessed the effect of EPO on renal outcomes in lupus nephritis in humans, including in earlier stages of active disease prior to progression to ESKD.

EPO has immunomodulatory properties that target several pathophysiological mechanisms of SLE. Specifically, EPO has been shown to attenuate proinflammatory cytokine levels, enhance apoptosis and cell clearance, and decrease proliferation of Tconv while promoting Treg induction. Given this background and the EPO-associated positive effects on disease severity in murine models of SLE, EPO may warrant further evaluation in clinical studies including SLE patients.

Notably, EPO administration carries the risk of thrombosis or stroke, especially in patients with a pro-thrombotic disease, like those with SLE. This highlights the potential utility of newer non-hematopoietic EPO-derivatives including carbamylated EPO or ARA290. Although some studies have demonstrated improved lupus nephritis disease activity in mouse models receiving ARA290 (82), others have found inconsistencies between EPO's and ARA290's effects, possibly highlighting the importance of both EPORs in disease pathophysiology (75, 79). Additional studies are needed to clarify the immunomodulating effects of these derivatives and their therapeutic role in SLE.

AUTHOR CONTRIBUTIONS

ME wrote manuscript the initial draft. CC participated in the manuscript writing. PC supervised the manuscript. All authors contributed to the article and approved the submitted version.

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Plasma Sphingolipid Profile Associated With Subclinical Atherosclerosis and Clinical Disease Markers of Systemic Lupus Erythematosus: Potential Predictive Value

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Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects females more than males, with African Americans developing more severe manifestation of the disease. SLE patients are at increased risk for cardiovascular disease (CVD), and SLE women 35-44 years old have 50 fold the incidence rate of CVD. Because SLE patients do not follow the typical age and gender pattern for CVD, but instead an accelerated disease course, the traditional biomarkers of elevated LDL and total cholesterol levels do not accurately assess their CVD risk. Recently, we have reported that African American SLE patients had higher ceramide, hexosylceramide, sphingosine and dihydrosphingosine 1-phosphate levels compared to their healthy controls, and those with atherosclerosis had higher sphingomyelin and sphingoid bases levels than those without (PLoS One. 2019; e0224496). In the current study, we sought to identify sphingolipid species that correlate with and pose the potential to predict atherosclerosis severity in African American SLE patients. Plasma samples from a group of African American predominantly female SLE patients with well-defined carotid atherosclerotic plaque burden were analyzed for sphingolipidomics using targeted mass spectroscopy. The data demonstrated that at baseline, plaque area and C3 values correlated inversely with most lactoceramide species. After one-year follow-up visit, values of the change of plaque area correlated positively with the lactoceramide species. There was no correlation between LDL-C concentrations and lactoceramide species. Taken together, lactocylcermide levels may have a 'predictive' value and sphingolipidomics have an added benefit to currently available tools in early diagnosis and prognosis of African American SLE patients with CVD.

Keywords: sphingolipid, sphingomyelin, ceramide, sphingosine, lactosylceramide, lupus, LDL, atherosclerosis

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with various organ involvement and severity. The majority (90%) of lupus patients are females, and African American women are three times more likely than white women to have lupus and develop severe symptoms (1–4). Despite the dyslipidemia and accelerated cardiovascular disease (CVD) associated with SLE (5), the significance of the conventional plasma lipid panel (e.g., cholesterol and triglycerides) in the diagnosis/prognosis of CVD in SLE patients has been in question. For instance, the efficacy of statins to prevent atherosclerosis in SLE was found to be inconsistent (6). Furthermore, African Americans have increased risk of CVD although normally they have lower triglycerides and higher HDL cholesterol levels than other ethnicities (7).

Sphingolipids are both structural lipids and signaling molecules that are associated with cellular membranes and plasma lipoprotein, and their metabolism is tightly regulated to maintain homeostasis (8, 9). Sphingolipids in the blood are carried on circulating lipoprotein particles (HDL, LDL, and VLDL), and their use as disease biomarkers has been explored (9). Dysregulation of the sphingolipid pathway has been described in several inflammatory and immune-mediated diseases (10, 11), and alterations in the sphingolipid pathway in SLE and some of its related complications have been reported (12–16). A cross sectional study on a European SLE cohort showed that dysregulations in circulating sphingolipids is associated with clinical systemic disease activity and renal disease activity (17). In another cross-sectional study, lupus nephritis patients were stratified by severity of renal impairment, and the results showed that C16:0, C18:0, C20:0, and C24:1 ceramides were significantly elevated in the plasma of lupus nephritis patients when compared to healthy controls, and SLE patients without renal impairment (18). Ceramide C24:1 showed the most potential of being used as a biomarker of lupus nephritis, as it remained strongly elevated in lupus nephritis patients (p =0.0001), even when compared to SLE patients without kidney disease (18). These data show the potential value of assessing changes in circulating sphingolipids to identify a biomarker(s) for early detection of SLE and its comorbidities.

We have recently demonstrated that healthy African Americans have higher sphingomyelin (SM) levels and lower lactosylceramide (Lact-Cer) levels compared to healthy whites, and that SLE patients, irrespective of race, have higher levels of ceramide, and sphingoid bases [sphingosine and dihydrosphingosine (dhSph)] and their phosphates [sphingosine 1-phosphate (S1P) and dihydrosphingosine 1-phosphate (dhSph-1P)] compared to healthy participants (19). We also showed that compared to African American healthy controls, African American SLE patients have higher ceramide, hexosylceramide (Hex-Cer), sphingosine and dhSph-1P levels; and that African American SLE patients with atherosclerosis have higher sphingoid bases and SM levels compared to African American SLE patients without atherosclerosis (19). Notably, plasma levels of sphingosine, C16:0 ceramide/S1P ratio and C24:1 ceramide/S1P ratio significantly correlated with SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) in the African American but not white SLE patients. In the present study, we investigated plasma sphingolipids as potential biomarkers that can predict or indicate atherosclerosis severity and/or established clinical SLE disease markers in African American SLE patients. Using targeted sphingolipidomics, plasma samples from a unique well-characterized African Americans lupus cohort with subclinical atherosclerosis (20, 21) were analyzed.

MATERIALS AND METHODS

Study Participants

Banked plasma samples were utilized from participants who were previously recruited for a cross sectional within-lupus case-control study to evaluate novel and traditional risk factors for accelerated atherosclerosis in a largely African American SLE population (20). Fifty-one participants with SLE but without a history of clinical cardiovascular events were enrolled. At entry, participants met at least four of the 1997 revised American College of Rheumatology (ACR) SLE criteria (22). Traditional risk factors for atherosclerosis assessed by interview and chart review were history of major cardiovascular event or peripheral vascular disease (for exclusion criteria), number of years since SLE diagnosis, history of hypertension, diabetes, obesity, hypercholesterolemia, or smoking (current). Sera and plasma samples were analyzed for lipids and lupus activity markers. Urine protein, creatinine, and cell count; serum C3, C4, and anti-double-stranded DNA (dsDNA) antibody levels; complete blood count; complete metabolic panel; and plasma triglycerides, VLDL, LDL, and HDL cholesterol (VLDL-C, LDL-C, and HDL-C), and total cholesterol were analyzed at the CLIA-certified Medical University of South Carolina (MUSC) Clinical Chemistry, Hematology, and Immunology Laboratories. Medication histories were performed by interview and chart review; the following medications were recorded as either present or absent: immunomodulators (mycophenolate mofetil, mycophenolic acid, azathioprine, methotrexate, or

Abbreviations: ACS, Acute coronary syndrome; ACR, American College of Rheumatology; ACE, Angiotensin converting enzyme; ARBs, Angiotensin receptor blockers; dsDNA, Anti-double-stranded DNA; CVD, Cardiovascular Disease; Cer, Ceramide; CERT, Ceramide risk score; CVA, Cerebrovascular accident; CLIA, Clinical Laboratory Improvement Amendments; C3, Complement protein C3; C4, Complement protein C4; CAD, Coronary artery disease; dhSph, Dihydrosphingosine; dhSph-1P, Dihydrosphingosine 1-phosphate; Hex-Cer, Hexosylceramide; HDL-C, High-density lipoprotein cholesterol; IQR, Interquartile range; Lact-Cer, Lactosylceramide; LDL-C, Low-density lipoprotein cholesterol; MUSC, Medical University of South Carolina; MI, Myocardial infarction; PC, Phosphatidylcholine; SIC, Sphingolipids inclusive CAD; SM, Sphingomyelin; S1P, Sphingosine 1-phosphate; SK, Sphingosine kinase; SLE, Systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; SLICC, Systemic Lupus International Collaborating Clinics Damage Index; TPA, Total plaque area; VLDL, Very low-density lipoprotein.

hydroxychloroquine), angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), prednisone and statins.

This cohort was then followed prospectively, and carotid ultrasound measurements were performed to determine total plaque area (TPA) in both carotids at baseline (visit 1) and one year later (visit 2). TPA was then reported as % TPA or a percent for age and sex-matched controls. Participants were labeled as having accelerated atherosclerosis (cases) if their age and sex-adjusted TPA was greater than the mean of historical controls in a hypertension stroke prevention clinic (23). The rationale for the use of carotid TPA as a marker of clinically relevant atherosclerosis and its measurement with ultrasound was explained previously (21). The historical control population in that study was used to reduce the confounding effect of age on TPA rather than to directly compare TPA in SLE and non-SLE populations (20). Because the goal in the initial study was to determine markers of subclinical atherosclerosis, participants with a history of clinical CVD such as myocardial infarction (MI); stroke (cerebrovascular accident, CVA); or documented peripheral, coronary, or carotid artery functionally significant narrowing were not included in the study. Those with a serum creatinine of > 3.0 were excluded due to the confounding effect of chronic kidney disease on the progression of atherosclerosis. Blood was collected at visits one and two (approximately 12 months apart), immediately processed for platelet-rich plasma, and frozen at -80°C for later, retrospective analysis. A subset (n =39) of the original (n =51) participants was selected for sphingolipid analysis, based upon the availability of their banked plasma samples.

Sphingolipid Extraction and Analysis

Mass spectroscopy was used to measure plasma levels of individual species of five classes of sphingolipids: ceramides, sphingoid bases: sphingosine and dhSph and their phosphates (S1P and dhSph-1P, respectively), SM, and the glycosphingolipids Lact-Cer and Hex-Cer as previously described (24–29). Briefly, 100 µl of de-identified plasma sample (collected in EDTA as anti-coagulant and stored at -80°C) from each participant was spiked with internal standards and the sphingolipid complement in each sample was extracted. The sphingolipids in plasma extracts were separated and their masses quantitated using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) at the MUSC Lipidomics Shared Resource. Lipids eluted during chromatography were detected and quantitated using a Thermo Scientific Quantum Access triple quadruple mass spectrometer equipped with an electrospray ion source (ESI) operating in multiple reaction monitoring (MRM) positive ion mode. Chromatographic separations were obtained under a gradient elution of a Peeke Scientific C8 Column (Redwood City, CA). Quantitative analyses were based on calibration curves generated by injecting known amounts of the target analytes and an equal amount of the internal standards. A listing of the internal standards used, and of the sphingolipids with available calibration standards was previously published (24). The calibration standards were obtained predominately from the MUSC Lipidomics Share Resource facility, and from commercially available sources, Avanti Polar Lipids Inc. and Matreya LLC. Molecular species of sphingolipids, which do not have available standards, were quantified using the calibration curve of the closest eluting counterpart. The final concentrations of analytes in the samples were determined using the appropriate corrections for sample loss based on internal standard recovery calculations. The resulting data was then normalized to the volume of sample analyzed. Final results were reported as pmol/ml plasma.

Statistical Analyses

Descriptive statistics were used to characterize the study participants with respect to demographics and clinical variables. Because of non-normality of many of the study variables, Spearman rank correlations were used to assess associations between sphingolipids and measures of atherosclerosis, and correlations with p-values <0.05 were highlighted. For correlations assessing change in total plaque area, partial Spearman correlations that adjusted for baseline total plaque area were also reported. With a sample size of n =39 participants, our study had 80% power to detect correlations as small as 0.44 with 2-sided testing and alpha=0.05. Wilcoxon rank sum tests were used to compare baseline sphingolipid levels between patients where were (n =4) and were not (n =35) on statins throughout the course of the follow-up time period. *p-values* < 0.05 were considered statistically significant, and as this was largely a hypothesis generating study, no adjustments were made for multiple hypothesis testing. Analyses were conducted using SAS v9.4 (SAS Institute, Cary, NC).

RESULTS

Baseline Clinical Characteristics and Plasma Sphingolipidomics

All of the SLE patients of this study (n =39) were African American, and most were (90%) females. The clinical characteristics at baseline (visit 1) are summarized in **Table 1**. The mean age at baseline was 43 years, with an average of 10-year duration of SLE. Serum concentrations of triglycerides and cholesterol in lipoprotein fractions were within the normal range **(Table 1)** and were similar to those formerly reported for their matched controls (21). Notably, the mean total cholesterol was found to be 10% lower among SLE patients compared with the matched controls (21); hydroxychloroquine, the drug that may have been used to treat the SLE patients before baseline, was previously shown to lower total cholesterol levels by about 15% after starting treatment (30).

The descriptive statistics of the baseline plasma sphingolipids for the large subset of SLE patients (n =35) who were not consistently on statins throughout the study are presented in **Table 2**. The mean ± SD and the median with IQR of the measured medium-, long- and very long-chain SM, ceramide, and glycosphingolipid (Lact-Cer and Hex-Cer) species, and sphingoid bases (sphingosine and dhSph) and their phosphates (S1P and dhSph-1P, respectively) are reported, as are the C16:0 ceramide/S1P, C24:1 ceramide/S1P, C16:0 ceramide/C24:0 ceramide, C18:0 ceramide/C24:0 ceramide, and C24:1

TABLE 1 | Baseline (visit 1) demographics and clinical characteristics.

Variable	Mean	Standard Deviation	Median	IQR	
				From	To
Age (yrs.)	43.1	± 13.0	45.8	28.1	55.3
Years with SLE (yrs.)	10.1	± 8.1	7.0	4.3	14.5
Total plaque area (% of controls)* at baseline	80.4	± 114.3	54.5	0.0	97.0
Total plaque area (cm^2) at visit 1 (baseline)	0.29	± 0.41	0.12	0.00	0.45
Total plaque area (cm^2) at visit 2	0.17	± 0.34	0.00	0.00	0.19
Change in plaque (cm^2)**	-0.12	± 0.44	0.00	-0.15	0.00
Blood pressure: systolic	131.7	± 21.1	130.0	116.0	144.0
Blood pressure: diastolic	78.2	± 13.9	77.0	66.0	89.0
Waist-hip ratio	0.8	± 0.2	0.8	0.8	0.9
SLEDAI score	4.1	± 3.0	4.0	2.0	6.0
SLICC score	1.2	± 1.5	1.0	0.0	2.0
LDL-C (mg/dl)	106.8	± 39.4	102.0	77.0	124.0
HDL-C (mg/dl)	47.1	± 12.7	46.0	38.0	55.0
Triglycerides (mg/dl)	93.7	± 39.8	91.0	60.0	118.0
C3 (mg/dl)	104.2	± 27.7	106.5	89.6	125.2
C4 (mg/dl)	18.0	± 5.7	17.4	13.6	22.8
dsDNA antibodies (IU/ml)	140.9	± 184.3	76.7	4.9	221.3
Urine protein/creatinine	0.3	± 0.4	0.1	0.1	0.2

N = 39 (Females: 35 (89.7%), Males: 4 (10.3%), All African Americans.

**% of age- and sex-matched historical controls.*

***Absolute change in plaque from baseline (visit 1) to visit 2 (approximately 12 months apart).*

IQR, Interquartile range; SLEDAI, Systemic lupus erythematosus disease activity index; SLICC, Systemic Lupus International Collaborating Clinics; LDL-, HDL-C; HDL-, LDL-cholesterol.

ceramide/C24:0 ceramide ratios **(Table 2)**. The baseline plasma sphingolipids of all participating SLE patients (n =39) and those who used statins consistently over the period extending from visit 1 to visit 2 (n =4) are presented in **Table 4**. The mean sphingolipid levels did not differ by hydroxylchloroquine use or by prednisone dose (data not shown). However, for those patients who used ACE inhibitors (N =9), levels of C14:0, C16:0, C18:0, C22:1, C24:1 ceramides and C16:0 ceramide/S1P ratio were significantly higher ($p<0.05$) than in those who did not (**Table S1**). For those who used ARBs (N =3), C16:0 ceramide/C24:0 ceramide, C18:0 ceramide/C24:0 ceramide, and C24:1 ceramide/C24:0 ceramide ratios were significantly higher ($p<0.05$), but levels of C20:0 and C24:0 SM were lower ($p<0.05$) than in those who did not (**Table S2**).

At visit 1, the mean TPA for this study cohort was about 80% of the mean TPA of age- and sex-matched historical controls. One year after visit 1 (i.e., visit 2), TPA (cm^2) was minimally reduced but not with statistical significance [mean ± SD: -0.12 ±0.44; median with interquartile range (IQR): 0.00 (-0.15, 0.00)]; 51.3% of the patients experienced declines in TPA during the course of the study. As noted in **Table 1**, there is great heterogeneity in the lupus population, with some patients have lower levels than controls and some have higher levels. In addition, the control participants were "disease" controls with hypertension in a prevention clinic. As shown also in **Table 5**, the SLEDAI scores in this cohort correlate negatively with Systemic Lupus International Collaborating Clinics Damage Index (SLICC) scores, as well as C3 and C4 levels, whereas SLICC scores correlate positively with C3 and C4 levels. Levels of dsDNA antibodies were found to correlate negatively with SLICC scores, and also with C3 and C4 levels. Characteristically, early SLE markers (e.g., dsDNA) are associated with disease activity, whereas later SLE markers (e.g., low C3 and C4 levels) are associated with organ damage (31). Notably, as shown in **Table 5**, levels of plasma triglycerides, LDL-C, and HDL-C were found not to correlate with any of the traditional SLE makers (SLEDAI, SLICC, C3, C4, dsDNA).

Lactosylceramides Correlate Negatively With Plaque Area and C3, While Sphingomyelins, Ceramides, and Hexosylceramides Correlate Positively With LDL-C

Correlations between concentrations of plasma sphingolipids and clinical variables, including baseline (visit 1) plaque and change in plaque at visit 2 are presented in **Table 3**. For this correlation analysis, participants on statins at both visits (n =4) were excluded, since the statins themselves may impact sphingolipids (32). At baseline, TPA values are shown to correlate negatively with the concentrations of the two most dominant Lact-Cer species (C16:0 and C24:1), Lact-Cer species with long-chain saturated fatty acids (C20:0, C22:0, and C24:0), and total Lact-Cer. However, TPA values are shown to correlate positively with the concentrations of C20:0, C22:0, and total SM, and the very long-chain ceramide species C24:0, C26:0 and C26:1 **(Table 3)**. At visit 2, change in TPA from baseline is shown to correlate positively with the two dominant Lact-Cer species (C16, and C24:1) and total Lact-Cer, but negatively with C14:0 SM concentration. No statistically significant correlations were found between the values of the change of TPA from baseline and concentrations of ceramide and Hex-Cer species; however, negative correlations were found with concentrations of sphingosine, dhSph and dhSph-1P. After adjusting for age

TABLE 2 | Baseline descriptive statistics of plasma sphingolipids among study participants (n =35) not consistently on statins throughout the study.

Sphingolipids [pmol/100 µl plasma]	Mean	Standard Deviation	Median	IQR	
				From	To
Sphingomyelin (SM)					
C14:0 SM	1041.00	± 388.85	916.53	808.4	**1196.4**
C16:0 SM	18500.00	± 3788.00	17611.00	**15786.1**	21503.6
C18:0 SM	1340.00	± 218.17	1314.00	1157.1	1489.1
C18:1 SM	665.61	± 132.24	655.66	554.3	735.5
C20:0 SM	720.25	± 118.82	695.23	642.7	809.7
C20:1 SM	294.20	± 48.57	291.66	257.5	327.4
C22:0 SM	1417.00	± 239.45	1413.00	1260.7	1502.7
C22:1 SM	1082.00	± 147.11	1080.00	956.2	1169.4
C24:0 SM	1217.00	± 237.37	1202.00	1033.3	1355.6
C24:1 SM	2966.00	± 344.44	3037.00	2697.7	3316.0
C26:0 SM	8.57	± 1.96	8.60	7.1	9.9
C26:1 SM	21.12	± 5.25	20.61	16.7	24.7
Total SM	29272.00	± 4667.00	28279.00	25652.7	33846.5
Ceramide (Cer)					
C14:0 Cer	3.24	± 0.96	3.00	2.7	3.6
C16:0 Cer	53.35	± 20.74	52.02	40.2	59.9
C18:0 Cer	16.42	± 7.12	14.64	12.7	20.9
C18:1 Cer	5.13	± 2.67	4.40	3.2	6.3
C20:0 Cer	33.69	± 14.90	31.52	26.4	38.4
C20:1 Cer	6.22	± 2.49	5.94	4.4	7.7
C20:4 Cer	0.04	± 0.03	0.04	0.0	0.1
C22:0 Cer	138.76	± 34.06	140.04	110.5	153.6
C22:1 Cer	50.73	14.23	47.73	41.4	63.7
C24:0 Cer	582.25	± 197.05	555.36	477.3	703.7
C24:1 Cer	228.19	± 64.18	236.11	193.8	276.0
C26:0 Cer	18.36	± 9.94	17.13	10.8	22.9
C26:1 Cer	9.87	± 4.44	9.66	7.2	12.5
Total Cer	1146.00	± 327.38	1147.00	973.2	1393.8
Dihydro-C16:0 Cer	2.12	± 1.05	1.83	1.4	2.7
Lactosylceramide (Lact-Cer)					
C14:0 Lact-Cer	9.64	± 4.29	10.23	6.6	11.4
C16:0 Lact-Cer	265.62	± 91.51	272.41	186.7	319.0
C18:0 Lact-Cer	11.41	± 4.53	10.55	8.2	13.2
C18:1 Lact-Cer	8.07	± 4.45	6.23	4.4	11.2
C20:0 Lact-Cer	2.84	± 1.19	2.72	2.1	3.2
C20:1 Lact-Cer	0.29	± 0.17	0.24	0.2	0.4
C22:0 Lact-Cer	8.78	± 3.55	8.34	6.1	10.5
C22:1 Lact-Cer	0.57	± 0.25	0.50	0.4	0.7
C24:0 Lact-Cer	2.50	± 0.88	2.29	1.8	3.0
C24:1 Lact-Cer	32.01	± 11.91	30.34	24.4	37.6
C26:0 Lact-Cer	0.11	± 0.06	0.11	0.1	0.1
C26:1 Lact-Cer	0.11	± 0.04	0.11	0.1	0.1
Total Lact-Cer	341.95	± 113.27	323.16	247.7	420.7
Hexosylceramide (Hex-Cer)					
C14:0 Hex-Cer	0.57	± 0.27	0.50	0.3	0.7
C16:0 Hex-Cer	76.81	± 28.36	75.70	57.1	95.4
C18:0 Hex-Cer	0.56	± 0.25	0.49	0.4	0.6
C18:1 Hex-Cer	0.19	± 0.08	0.19	0.1	0.2
C20:0 Hex-Cer	0.96	± 0.38	0.87	0.7	1.1
C20:1 Hex-Cer	0.12	± 0.07	0.11	0.1	0.1
C22:0 Hex -Cer	41.51	± 11.16	42.26	34.9	49.0
C22:1 Hex -Cer	1.51	± 0.52	1.46	1.0	1.8
C24:0 Hex -Cer	57.27	± 15.32	56.69	44.9	74.0
C24:1 Hex -Cer	72.97	± 23.39	68.81	55.5	88.7
C26:0 Hex -Cer	0.90	± 0.37	0.88	0.6	1.2
C26:1 Hex -Cer	0.46	± 0.19	0.40	0.3	0.6
Total Hex-Cer	253.83	± 66.38	234.1	205.53	296.64
Dihydrosphingosine (dhSph)	0.58	± 0.26	0.53	0.4	0.7
Sphingosine	1.86	± 0.76	1.58	1.3	2.3
dhSph 1-phosphate (dhSph-1P)	16.18	± 3.95	16.48	13.3	19.5

(Continued)

TABLE 2 | Continued

Sphingolipids [pmol/100 µl plasma]	Mean	Standard Deviation	Median	IQR	
				From	To
Sphingosine 1-phosphate (S1P)	60.17	± 12.55	56.99	51.0	65.5
C16:0 Cer: S1P Ratio	0.92	± 0.39	0.91	0.6	1.1
C24:1 Cer: S1P Ratio	3.91	± 1.28	3.97	3.0	4.5
C16:0 Cer: C24:0 Cer Ratio	0.095	± 0.03	0.09	0.07	0.12
C18:0 Cer: C24:0 Cer Ratio	0.03	± 0.1	0.03	0.02	0.04
C24:1 Cer: C24:0 Cer Ratio	0.41	± 0.07	0.37	0.34	0.46

N = 35, not consistently on statins throughout the study.
IQR, Interquartile range.

(using partial correlation), the correlation between change in plaque area and the sphingolipids became non-significant for C14:0 SM, C20:0 SM, C14:0 Lact-Cer, and C16:0 Hex-Cer.

The data in **Table 3** show that, in this study cohort, LDL-C concentrations strongly and positively correlate with concentrations of the majority of SM, ceramide, and Hex-Cer species, as well as C16:0 dihydroceramide, sphingosine, dhSph, C16:0 Cer/S1P and C24:1 Cer/S1P. In contrast, there is no significant correlations identified between concentrations of LDL-C and those of Lact-Cer species, except for C26:0 Lact-Cer, which exists in the circulation in a barely detectable amount **(Table 1)**. As shown in **Table 3**, there is no significant correlation present between HDL-C concentration and the concentration of any of the plasma sphingolipids.

Table 3 also show that triglyceride concentrations correlate positively with concentrations of SM (C16:0, C18:1, and total), ceramide (C18:0, C18:1, C20:0, C20:1, C22:0, C22:1, and total), dh-Sph and sphingosine. However, triglyceride concentrations were found to have significantly negative correlation with the second dominant Lact-Cer species (C24:1 Lact-Cer), with statistically non-significant negative correlations with the concentrations of the remaining of Lact-Cer species. As shown in **Table 3**, triglyceride concentrations do not correlate with of any of the Hex-Cer species concentrations.

In this study, the older the patient the higher the concentrations of SM species (C20:0, C20:1, C22:0, C22:1, C24:0, and C24:1) and ceramide species (C20:0 and C20:1), and the lower the concentrations of Lact-Cer species (C16:0, C20:0, C22:0, C22:1, C24:0, C24:1, and total Lact-cer) and Hex-Cer species (C16:0, C20:0, and total Hex-Cer) **(Table 3)**. The duration of SLE prior to visit 1, concentrations of dsDNA antibodies, and SLEDAI scores, do not seem to have a significant effect on the concentrations of plasma sphingolipids. However, C3 concentrations were found to correlate negatively with almost all Lact-Cer species, and positively with three long-chain SM species. C4 concentrations, on the other hand, showed associations with concentrations of only few scarce plasma sphingolipid species **(Table 3)**.

Circulating C24:1 Ceramide in African American with SLE Could Be Elevated in Response to Statins

Although this study was not purposely designed to study the effect of statins on the development of atherosclerosis in African American SLE patients, we compared the baseline (visit 1) concentrations of plasma sphingolipids in patients who consistently used statins over the period extending from visit 1 to visit 2 (n =4) with those were not indicated as being prescribed any statins (n =35). The data in **Table 4** show that C24:1 ceramide and C24:1 ceramide/S1P ratio were significantly higher in the former group of patients. The concentration of C26:0 Lact-Cer, one of the least detectable plasma sphingolipid species, was also higher in the SLE patients who consistently used statins compared to those who did not **(Table 4)**.

DISCUSSION

In the present study, we examined the concentrations of sphingolipid species in plasma from a well-characterized cohort of predominantly African American female SLE patients as potential biomarkers that can be associated with atherosclerosis severity and/or with clinical SLE disease markers. Defined carotid atherosclerotic plaque burden measurements at baseline and after one year were analyzed for correlations with the baseline concentrations of plasma sphingolipid species. The data demonstrated that TPA and C3 values correlated inversely with concentrations of the two most dominant Lact-Cer species (C16:0 and C24:1) and of Lact-Cer species with long-chain saturated fatty acids. Because lower C3 levels is a marker for increasing SLE disease activity (31), it is then plausible to assume that higher levels of Lact-Cer would be associated with SLE disease activity. As shown in **Table 5**, the correlations between C3 and each of TPA and the change in plaque from baseline are not statistically significant, which is consistent with the characteristics of our study cohort, who is a largely stable SLE population with little activity.

Lact-Cer is an integral component of the cell outer membrane, serving as a mediator to transduce external stimuli, which may contribute to mortality and morbidity in humans as well as in animal models (recently reviewed in 33). Lact-Cer is synthesized by the action of Lact-Cer synthase, which can be activated by several inflammatory and stress factors. The generated Lact-Cer activates nicotinamide adenine dihydrogen phosphate (NADPH) oxidase producing reactive oxygen species (ROS) and a highly oxidative stress environment, which triggers a cascade of signaling molecules and pathways causing mitochondrial dysfunction and contributing to inflammation,

TABLE 3 | Correlations between plasma sphingolipids and clinical variables, including baseline (visit 1) plaque area and change in plaque at visit 2.

Sphingolipids	Total plaque area %*	Change in Plaque**	Age	Years with SLE	BP Syst.	BP Diast.	Waist-hip ratio	SLEDAI score	SLICC score	LDL-C	HDL-C	TG	C3	C4	dsDNA	Urine protein/ creatinine
Sphingomyelin (SM)																
C14:0 SM	0.30	***-0.35***	0.26	-0.02	0.03	0.03	0.13	0.15	-0.09	***0.77***	0.33	0.32	0.10	-0.11	-0.002	0.30
C16:0 SM	0.28	-0.02	0.08	0.01	0.01	0.19	0.15	-0.06	0.08	***0.83***	0.24	***0.39***	-0.05	0.08	0.01	***0.35***
C18:0 SM	0.03	-0.12	0.02	-0.16	-0.24	-0.07	-0.02	-0.09	-0.003	***0.60***	0.10	0.31	0.05	0.16	-0.02	-0.14
C18:1 SM	0.04	-0.04	0.25	0.02	-0.05	-0.10	-0.05	-0.23	0.30	***0.52***	0.11	***0.44***	0.26	0.26	-0.11	0.04
C20:0 SM	***0.41***	-0.28	***0.47***	***0.35***	-0.06	-0.12	0.13	0.09	-0.02	***0.43***	0.28	0.09	***0.36***	0.05	-0.08	-0.12
C20:1 SM	0.23	-0.16	***0.47***	0.25	0.02	-0.20	-0.19	-0.09	0.31	***0.35***	0.23	0.24	***0.37***	0.12	-0.11	-0.03
C22:0 SM	***0.35***	-0.00	***0.34***	0.30	-0.005	0.11	0.32	0.07	0.01	***0.57***	0.15	0.22	0.26	0.07	-0.09	-0.05
C22:1 SM	0.15	-0.25	***0.51***	0.27	0.10	-0.08	0.21	-0.08	0.24	***0.43***	0.10	0.32	***0.45***	0.10	-0.08	0.10
C24:0 SM	0.26	0.23	***0.35***	0.29	0.19	0.22	0.18	-0.01	0.20	0.30	0.20	0.01	0.23	0.12	-0.19	-0.18
C24:1 SM	0.27	0.24	***0.36***	0.30	0.01	-0.17	0.09	-0.25	0.31	***0.36***	0.15	0.13	0.22	0.23	-0.30	0.04
C26:0 SM	-0.005	0.15	-0.02	0.11	0.14	***0.38***	-0.07	0.11	0.09	0.01	0.17	-0.10	-0.20	-0.10	0.05	-0.19
C26:1 SM	0.24	0.22	0.21	0.17	0.03	0.10	0.05	-0.17	0.18	0.28	0.14	0.04	-0.07	0.15	-0.15	-0.09
Total SM	***0.34***	-0.09	0.20	0.09	0.002	0.13	0.19	-0.08	0.13	***0.88***	0.23	***0.44***	0.10	0.16	-0.07	0.29
Ceramide (Cer)																
C14:0 Cer	0.06	-0.06	0.13	0.02	-0.03	0.25	0.26	-0.13	0.12	***0.69***	-0.05	0.31	0.08	0.02	0.09	0.31
C16:0 Cer	-0.01	0.25	-0.04	-0.11	0.04	0.31	0.27	-0.15	0.22	***0.55***	-0.09	0.27	-0.14	0.08	0.27	***0.47***
C18:0 Cer	0.13	0.05	0.04	0.03	0.01	0.13	0.14	-0.11	0.16	***0.58***	0.04	***0.41***	0.10	0.09	0.18	0.23
C18:1 Cer	0.15	-0.04	0.20	0.03	0.10	0.09	0.10	-0.18	0.30	***0.64***	0.11	***0.40***	0.24	0.21	0.07	0.19
C20:0 Cer	0.15	-0.10	***0.36***	0.30	0.23	***0.35***	0.15	0.05	0.25	***0.51***	0.22	***0.44***	0.33	0.09	0.06	0.32
C20:1 Cer	0.18	0.00	***0.39***	0.20	0.12	0.10	0.07	-0.14	***0.37***	***0.57***	0.22	***0.40***	0.33	0.24	0.05	0.24
C20:4 Cer	0.26	-0.03	-0.005	-0.06	-0.06	-0.02	0.03	-0.12	0.15	0.18	0.25	-0.12	0.23	0.11	-0.14	0.22
C22:0 Cer	0.001	0.07	-0.06	0.02	0.02	0.27	0.30	0.02	0.11	***0.65***	0.14	***0.36***	0.12	0.17	0.02	0.09
C22:1 Cer	0.13	-0.14	0.18	0.08	0.01	0.18	0.28	-0.09	0.15	***0.72***	0.04	***0.51***	0.28	0.17	0.05	0.26
C24:0 Cer	***0.36***	-0.01	0.18	0.11	0.05	0.20	0.32	-0.07	0.04	***0.70***	0.19	0.26	0.19	0.22	-0.03	0.08
C24:1 Cer	0.13	0.02	0.07	0.10	-0.09	0.03	0.28	-0.23	0.16	***0.57***	-0.02	0.33	0.25	0.26	-0.05	0.21
C26:0 Cer	***0.42***	-0.01	0.11	-0.03	0.03	0.20	0.29	-0.14	0.02	***0.57***	0.14	0.18	0.06	0.32	-0.02	0.02
C26:1 Cer	***0.46***	0.13	0.15	0.03	0.04	0.15	0.18	-0.26	0.16	***0.47***	0.08	0.20	0.03	***0.36***	-0.03	0.17
Total Cer	0.29	0.01	0.15	0.11	0.03	0.19	0.33	-0.08	0.10	***0.72***	0.14	***0.35***	0.20	0.22	0.04	0.21
Dihydro-C16:0 Cer	0.16	-0.00	-0.10	0.02	-0.07	0.26	0.15	0.09	0.04	***0.76***	0.25	0.19	-0.13	0.11	0.10	0.25
Lactosylceramide (Lact-Cer)																
C14:0 Lact-Cer	-0.26	0.27	-0.21	-0.16	-0.14	-0.05	-0.17	0.03	-0.02	0.23	0.20	-0.07	***-0.35***	-0.28	0.20	0.02
C16:0 Lact-Cer	***-0.35***	***0.45***	***-0.38***	-0.20	-0.10	0.11	-0.05	-0.02	-0.01	0.18	0.05	-0.19	-0.33	-0.14	0.15	-0.14
C18:0 Lact-Cer	-0.18	0.12	-0.23	-0.25	-0.01	0.11	-0.12	-0.06	0.10	0.15	-0.04	-0.12	***-0.44-0.44***	-0.18	0.12	0.04
C18:1 Lact-Cer	-0.07	0.25	-0.04	0.04	0.13	0.23	-0.02	-0.03	***0.34***	0.32	0.03	0.12	-0.19	0.07	0.01	0.17
C20:0 Lact-Cer	***-0.38***	0.04	***-0.36***	-0.10	-0.24	0.01	0.06	0.22	-0.04	-0.09	-0.24	-0.16	***-0.39***	-0.26	0.30	0.01
C20:1 Lact-Cer	-0.22	0.16	-0.11	-0.17	0.21	0.24	-0.10	0.11	-0.06	-0.15	-0.20	-0.02	***-0.47***	-0.31	0.25	0.17
C22:0 Lact-Cer	***-0.43***	0.23	***-0.36***	-0.21	-0.12	-0.02	0.12	0.19	-0.15	-0.05	-0.16	-0.20	***-0.35***	-0.30	0.25	0.13
C22:1 Lact-Cer	-0.31	0.22	***-0.42***	-0.19	-0.09	0.01	-0.04	0.21	-0.01	0.02	0.05	-0.28	***-0.46***	***-0.34***	0.20	0.13
C24:0 Lact-Cer	***-0.40***	0.15	***-0.39***	-0.27	-0.22	-0.19	0.09	0.24	***-0.34***	-0.18	-0.13	-0.26	***-0.44***	***-0.48***	0.24	0.06
C24:1 Lact-Cer	***-0.36***	***0.41***	***-0.46***	-0.22	-0.25	-0.14	0.09	0.01	-0.06	-0.08	0.01	***-0.40***	***-0.32***	-0.21	0.04	-0.14
C26:0 Lact-Cer	-0.14	0.25	-0.03	-0.16	0.004	0.21	-0.09	0.07	-0.25	***0.40***	0.13	0.01	***-0.34***	-0.32	0.27	0.16
C26:1 Lact-Cer	0.01	0.31	-0.09	-0.06	-0.20	-0.04	-0.01	-0.06	-0.08	0.10	0.04	0.03	-0.05	-0.003	-0.01	0.09
Total Lact-Cer	***-0.35***	***0.45***	***-0.36***	-0.23	-0.14	0.05	-0.04	-0.01	-0.02	0.18	0.05	-0.19	-0.33	-0.19	0.15	-0.11

(Continued)

TABLE 3 | Continued

Sphingolipids	Total plaque area %*	Change in Plaque**	Age	Years with SLE	BP Syst.	BP Diast.	Waist-hip ratio	SLEDAI score	SLICC score	LDL-C	HDL-C	TG	C3	C4	dsDNA	Urine protein/ creatinine
Hexosylceramide (Hex-Cer)																
C14:0 Hex-Cer	0.03	-0.11	-0.26	-0.07	-0.29	-0.05	-0.12	0.06	-0.06	***0.50***	0.28	0.24	***-0.34***	-0.11	0.07	0.15
C16:0 Hex-Cer	-0.28	0.20	***-0.40***	-0.23	-0.09	0.26	0.01	0.07	0.05	***0.49***	0.17	0.06	-0.32	-0.04	0.21	0.15
C18:0 Hex-Cer	0.16	-0.08	-0.14	0.04	***-0.43***	***-0.34***	-0.21	0.03	***-0.36***	0.25	0.11	-0.20	-0.26	***-0.34***	-0.06	***-0.34***
C18:1 Hex-Cer	-0.04	0.02	-0.15	0.11	-0.25	-0.15	-0.14	0.13	-0.05	***0.43***	0.19	0.15	-0.24	-0.26	0.19	-0.04
C20:0 Hex-Cer	0.001	0.03	***-0.40***	-0.05	***-0.40***	-0.17	-0.13	0.14	***-0.38***	0.20	0.27	-0.30	***-0.35***	-0.21	0.09	-0.23
C20:1 Hex-Cer	-0.08	-0.05	-0.31	-0.05	-0.24	-0.15	-0.24	0.08	-0.13	0.25	0.01	0.04	***-0.43***	-0.09	0.003	-0.16
C22:0 Hex -Cer	-0.02	0.13	-0.31	-0.20	-0.19	0.15	0.08	0.33	-0.29	***0.45***	0.21	-0.04	-0.28	-0.21	0.07	-0.06
C22:1 Hex -Cer	0.27	-0.16	-0.14	0.12	-0.21	-0.16	-0.05	0.08	-0.19	***0.46***	0.16	0.16	-0.14	-0.18	-0.12	-0.01
C24:0 Hex -Cer	0.02	0.13	-0.18	-0.14	-0.31	-0.02	0.11	0.18	***-0.33***	***0.45***	0.17	0.05	-0.28	-0.18	0.06	-0.003
C24:1 Hex -Cer	0.002	0.07	-0.31	-0.11	-0.29	-0.16	0.002	0.07	-0.24	***0.39***	0.16	-0.07	-0.20	-0.18	-0.09	-0.07
C26:0 Hex -Cer	0.20	-0.02	0.03	-0.05	-0.19	0.05	0.27	0.10	-0.26	***0.79***	0.22	0.27	-0.11	-0.03	-0.01	0.03
C26:1 Hex -Cer	0.27	-0.08	-0.09	0.13	-0.18	-0.09	0.01	0.01	-0.13	***0.50***	0.11	0.14	-0.10	-0.01	-0.14	0.09
Total Hex-Cer	-0.10	0.10	***-0.35***	-0.21	-0.23	0.04	-0.01	0.15	-0.20	***0.53***	0.25	0.01	-0.33	-0.18	0.05	0.02
Dihydrosphingosine (dhSph)	-0.01	***-0.42***	0.02	0.02	-0.06	0.05	0.03	0.27	-0.12	***0.59***	-0.12	***0.48***	-0.10	-0.21	0.14	0.17
Sphingosine	-0.10	***-0.40***	0.13	0.12	0.06	0.04	0.004	0.09	0.09	***0.39***	-0.24	***0.52***	0.15	-0.08	0.004	-0.001
dhSph 1-phosphate (dhSph-1P)	-0.16	***-0.41***	-0.21	-0.05	-0.31	-0.07	-0.10	0.18	-0.32	0.20	-0.22	0.29	-0.25	-0.30	0.05	-0.15
Sphingosine 1-phosphate (S1P)	-0.04	-0.22	0.03	0.17	-0.07	0.02	0.20	-0.03	0.005	0.10	-0.25	0.31	0.21	0.04	-0.07	-0.24
C16:0 Cer: S1P Ratio	-0.06	0.30	-0.12	-0.20	0.15	***0.36***	0.15	-0.03	0.20	***0.38***	0.04	0.12	-0.29	0.03	0.30	***0.52***
C24:1 Cer: S1P Ratio	0.04	0.18	0.03	0 .005	-0.005	0.01	0.15	-0.19	0.21	***0.40***	0.08	0.11	0.03	0.21	0.04	***0.37***
C16:0 Cer: C24:0 Cer Ratio	***-0.44***	***0.45***	-0.27	-0.24	0.01	0.19	-0.11	0.00	0.19	-0.09	-0.18	0.00	-0.29	-0.21	0.23	0.25
C18:0 Cer: C24:0 Cer Ratio	-0.18	0.16	-0.04	0.03	0.01	0.05	-0.23	-0.12	0.28	0.04	-0.11	0.23	-0.01	-0.08	0.07	0.20
C24:1 Cer: C24:0 Cer Ratio	-0.33	0.18	-0.14	0.01	-0.01	-0.11	-0.17	-0.24	0.22	-0.30	-0.31	0.13	0.06	0.05	-0.16	0.15

*N =35. Data presented are correlations, where **bold italics** is statistically significant at < 0.05 **(black)**, < 0.01 **(red)**, < 0.001 **(blue)**, < 0.00001 **(violet)**, & Underlined; p=0.051 to p=0.059.*

**% of age- and sex-matched historical controls. **Absolute change in plaque from baseline (visit 1) to visit 2. BP, blood pressure; Syst., systolic; Diast., diastolic; SLEDAI, Systemic lupus erythematosus disease activity index; SLICC, Systemic Lupus International Collaborating Clinics; LDL-; HDL-C, HDL-; LDL-cholesterol; TG, triglycerides.*

TABLE 4 | Comparisons in plasma sphingolipids between patients who did versus did not use statins consistently from visit 1 to visit 2.

Sphingolipids [pmol/100 µl plasma]	Statins use not noted or not consistent N = 35	Statins use noted at baseline & visit 2 N = 4*	All participants at baseline N = 39	*P value*
Sphingomyelin (SM)				
C14:0 SM	916.53 (808.35, 1196.43)	1275.78 (1032.84, 1385.57)	931.08 (818.61, 1224.12)	0.16
C16:0 SM	17610.51 (15786.12, 21503.61)	20776.01 (16950.38, 24063.89)	17610.51 (16006.07, 22298.84)	0.29
C18:0 SM	1313.67 (1157.13, 1489.11)	1305.51 (1202.01, 1515.85)	1313.67 (1176.7, 1489.11)	0.85
C18:1 SM	655.66 (554.3, 735.49)	658.74 (551.3, 788.96)	655.66 (554.3, 745.75)	0.82
C20:0 SM	695.23 (642.72, 809.72)	722.95 (663.28, 753.61)	696.55 (642.72, 776.23)	0.96
C20:1 SM	291.66 (257.5, 327.39)	288.9 (256.95, 337)	291.66 (257.5, 327.39)	0.93
C22:0 SM	1413.17 (1260.69, 1502.66)	1305.04 (1053.79, 1565.89)	1413.17 (1250.71, 1503.8)	0.71
C22:1 SM	1079.84 (956.19, 1169.35)	1079.04 (1002.3, 1115.32)	1079.84 (956.19, 1161.2)	0.82
C24:0 SM	1202.42 (1033.28, 1355.55)	1160.99 (970.69, 1503.12)	1202.42 (1028.03, 1355.55)	0.78
C24:1 SM	3037.07 (2697.7, 3316.04)	3053.71 (2847.12, 3389.29)	3037.07 (2729.04, 3316.04)	0.43
C26 SM	8.6 (7.11, 9.93)	7.75 (7.08, 9.1)	8.3 (7.11, 9.93)	0.68
C26:1 SM	20.61 (16.69, 24.72)	21.32 (19.16, 23.84)	20.61 (16.95, 24.72)	0.71
Total SM	28279.45 (25652.71, 33846.53)	31324.86 (27721.74, 35286.62)	28279.45 (25889.17, 34238.7)	0.29
Ceramide (Cer)				
C14:0 Cer	3 (2.69, 3.56)	3.68 (2.97, 4.19)	3.08 (2.69, 3.56)	0.31
C16:0 Cer	52.02 (40.17, 59.92)	68.08 (47.76, 89.24)	52.5 (40.17, 61.65)	0.27
C18:0 Cer	14.64 (12.73, 20.86)	19.37 (11.3, 25.38)	14.72 (12.73, 21.36)	0.52
C18:1 Cer	4.4 (3.22, 6.27)	6.6 (3.59, 8.65)	4.62 (3.22, 6.55)	0.43
C20:0 Cer	31.52 (26.38, 38.41)	40.54 (29.37, 45.03)	31.55 (26.38, 40.21)	0.33
C20:1 Cer	5.94 (4.42, 7.69)	8.2 (4.86, 10.21)	6.11 (4.42, 8.22)	0.31
C20:4 Cer	0.04 (0.02, 0.05)	0.03 (0.01, 0.03)	0.04 (0.02, 0.05)	0.23
C22:0 Cer	140.04 (110.52, 153.59)	147.93 (117.7, 185.07)	140.04 (110.52, 154.65)	0.55
C22:1 Cer	47.73 (41.4, 63.73)	54.95 (49.94, 66.44)	48.85 (42.47, 63.73)	0.25
C24:0 Cer	555.36 (477.31, 703.67)	718.61 (557.31, 843.1)	557.2 (500.52, 722.32)	0.25
C24:1 Cer	236.11 (193.81, 276.02)	289.37 (265.97, 340.6)	241.5 (194.18, 285.11)	***0.033***
C26:0 Cer	17.13 (10.75, 22.87)	23.06 (20.99, 23.12)	17.51 (12.53, 23.11)	0.13
C26:1 Cer	9.66 (7.16, 12.53)	11.67 (10.39, 12.5)	9.69 (7.41, 12.53)	0.25
Total Cer	1147.36 (973.23, 1393.75)	1351.33 (1186.5, 1589.23)	1166.66 (979.87, 1393.84)	0.13
Dihydro-C16:0 Cer	1.83 (1.36, 2.73)	2.41 (2.21, 2.77)	2.03 (1.37, 2.73)	0.19
Lactosylceramide (Lact-Cer)				
C14:0 Lact-Cer	10.23 (6.62, 11.37)	9.86 (7.93, 14.01)	10.23 (7.14, 11.37)	0.49
C16:0 Lact-Cer	272.41 (186.65, 319.04)	263.9 (242.73, 275.74)	269.1 (209.73, 314.68)	0.89
C18:0 Lact-Cer	10.55 (8.23, 13.17)	13.71 (11.3, 16.03)	10.56 (8.62, 13.69)	0.21
C18:1 Lact-Cer	6.23 (4.4, 11.23)	6.14 (5.35, 7.09)	6.23 (4.51, 10.54)	0.96
C20:0 Lact-Cer	2.72 (2.1, 3.19)	3.5 (2.27, 4.32)	2.74 (2.1, 3.52)	0.33
C20:1 Lact-Cer	0.24 (0.16, 0.43)	0.33 (0.26, 0.4)	0.25 (0.17, 0.41)	0.38
C22:0 Lact-Cer	8.34 (6.13, 10.46)	9.76 (8.35, 11)	8.66 (6.49, 10.6)	0.43
C22:1 Lact-Cer	0.5 (0.39, 0.74)	0.46 (0.44, 0.51)	0.5 (0.41, 0.7)	0.55
C24:0 Lact-Cer	2.29 (1.83, 2.99)	2.9 (2.19, 3.92)	2.29 (1.86, 3.16)	0.31
C24:1 Lact-Cer	30.34 (24.44, 37.56)	33.25 (25.09, 35.51)	32.07 (24.44, 36.81)	0.93
C26:0 Lact-Cer	0.11 (0.07, 0.14)	0.21 (0.15, 0.24)	0.12 (0.07, 0.14)	***0.018***
C26:1 Lact-Cer	0.11 (0.08, 0.14)	0.1 (0.09, 0.1)	0.11 (0.08, 0.14)	0.52
Total Lact-cer	323.16 (247.73, 420.67)	345.62 (313.98, 361.05)	340.55 (260.45, 413.83)	0.93
Hexosylceramide (Hex-Cer)				
C14:0 Hex-Cer	0.5 (0.34, 0.74)	0.7 (0.52, 0.76)	0.52 (0.38, 0.74)	0.4
C16:0 Hex-Cer	75.7 (57.13, 95.44)	71.05 (67.14, 83.92)	72.92 (58.12, 94.92)	0.89
C18:0 Hex-Cer	0.49 (0.39, 0.61)	0.62 (0.41, 0.73)	0.51 (0.39, 0.63)	0.75
C18:1 Hex-Cer	0.19 (0.13, 0.24)	0.17 (0.1, 0.22)	0.19 (0.13, 0.24)	0.52
C20:0 Hex-Cer	0.87 (0.68, 1.12)	0.92 (0.7, 0.92)	0.89 (0.68, 1.06)	0.55
C20:1 Hex-Cer	0.11 (0.07, 0.13)	0.08 (0.04, 0.1)	0.1 (0.06, 0.13)	0.13
C22:0 Hex -Cer	42.26 (34.92, 49.04)	40.79 (26.51, 54.43)	42.26 (34.7, 49.25)	1
C22:1 Hex -Cer	1.46 (1.03, 1.84)	1.44 (1.06, 1.86)	1.46 (1.03, 1.84)	0.93
C24:0 Hex -Cer	56.69 (44.91, 74.04)	64.66 (42.6, 85.72)	56.69 (44.91, 74.99)	0.58
C24:1 Hex -Cer	68.81 (55.5, 88.69)	86.64 (61.15, 110.17)	68.81 (55.5, 90.39)	0.38
C26:0 Hex -Cer	0.88 (0.62, 1.15)	0.97 (0.68, 1.37)	0.88 (0.62, 1.16)	0.52
C26:1 Hex -Cer	0.4 (0.32, 0.56)	0.59 (0.46, 0.68)	0.46 (0.32, 0.61)	0.14
Total Hex-Cer	234.1 (205.53, 296.64)	271.68 (218.23, 324.04)	234.1 (214.22, 301.04)	0.71
Dihydrosphingosine (dhSph)	0.53 (0.39, 0.72)	0.4 (0.34, 0.53)	0.52 (0.37, 0.68)	0.23
Sphingosine	1.58 (1.33, 2.29)	1.3 (1.17, 1.58)	1.53 (1.27, 2.26)	0.23

(Continued)

TABLE 4 | Continued

Sphingolipids [pmol/100 µl plasma]	Statins use not noted or not consistent N = 35	Statins use noted at baseline & visit 2 N = 4*	All participants at baseline N = 39	*P value*
dhSph 1-phosphate (dhSph-1P)	16.48 (13.28, 19.52)	12.07 (9.92, 15.46)	16.08 (13.12, 19.03)	0.09
Sphingosine 1-phosphate (S1P)	56.99 (51.03, 65.53)	47.58 (44.08, 56.37)	56.47 (49.64, 65.39)	0.08
C16:0 Cer: S1P Ratio	0.91 (0.64, 1.06)	1.41 (1.02, 1.65)	0.91 (0.65, 1.12)	0.06
C24:1 Cer: S1P Ratio	3.97 (3.01, 4.53)	6.2 (5.58, 6.56)	4.07 (3.15, 5.02)	***0.004***
C16:0 Cer: C24:0 Cer Ratio	0.09 (0.07, 0.12)	0.1 (0.07, 0.12)	0.09 (0.07, 0.12)	0.96
C18:0 Cer: C24:0 Cer Ratio	0.03 (0.02, 0.04)	0.03 (0.02, 0.04)	0.03 (0.02, 0.04)	0.82
C24:1 Cer: C24:0 Cer Ratio	0.37 (0.34, 0.46)	0.47 (0.37, 0.52)	0.38 (0.34, 0.5)	0.46

Females, data presented are median values and interquartile ranges (IQRs),* ***bold italics**: statistically significant at < 0.05.*

atherosclerosis, CVD, diabetes, and skin conditions (33). Thus, the uncovering of the Lact-Cer-mediated oxidative stress pathway could facilitate our understanding of the progression of atherosclerosis and CVD in SLE. In general, relapsing/remitting SLE disease reduces over time, while long quiescent disease increases. Therefore, the inverse correlation between TPA and the Lact-Cer species observed in this stable SLE cohort may be an indication of prior disease activity, when Lact-Cer may have been elevated. From the current retrospective study, it is not possible to conclude whether increases in Lact-Cer levels would be pathologic. Baseline TPA reflects progression prior to the baseline and may be an accumulation from prior disease flares (with low C3) that have resolved (with normalization of C3). This is consistent with the observations in the Johns Hopkins cohort that combined chronic activity associates with cumulative risk (31). The baseline Lact-Cer species might reflect risk for future progression in the following year in this group of patients with low disease activity.

Sphingolipids are both structural lipids and signaling molecules, and their synthesis and degradation are tightly regulated (8, 9). Cellular accumulations of ceramide have been associated with apoptosis and cell death (8, 34), whereas S1P was found to promote endothelial integrity and lymphocyte migration (35). Sphingolipids in blood are carried on circulating lipoprotein particles (HDL, LDL, and VLDL) and their use as disease biomarkers has been explored (9). Emerging clinical data during the past decade have shown that sphingolipids are of not only ample biochemical interest but also have a possible diagnostic value. Sphingolipids were recently evaluated *via* targeted lipidomics to determine if sphingolipid levels in the circulation would be a valuable cholesterol-independent biomarker for coronary artery disease (CAD) (36). Poss et al. developed a sphingolipids inclusive CAD (SIC) risk score, which was found to have better discriminatory power for CVD than the long-established LDL-C levels (C-statistics of 0.79 and 0.69, respectively) (36). The significance of such a score suggests that plasma sphingolipids could be an added clinical characteristic used to assess more accurately not only the risk, but also the diagnosis and prognosis of CVD in SLE. Future longitudinal studies would evaluate progression of atherosclerotic plaques in patients with SLE earlier in the course of their disease and thus with more disease activity.

In our study, LDL-C concentrations were found to strongly and positively correlate with concentrations of the majority of SM, ceramide, and Hex-Cer species, as well as dihydroceramide, sphingosine, dhSph, C16:0 Cer/S1P and C24:1 Cer/S1P, but we found no significant correlations between concentrations of LDL-C and Lact-Cer species, or between concentrations of HDL-C and any of the plasma sphingolipids. Triglyceride concentrations were found to correlate positively with concentrations of a number of SM and ceramide species, also with dh-Sph and sphingosine, but negatively with only C24:1 Lact-Cer, the second dominant Lact-Cer species. The functional significance of these observations and the possible metabolic pathways behind them are yet to be determined.

Jiang et al. reported lower mean plasma SM levels in whites compared with other ethnic groups (37), and showed that plasma SM is associated with subclinical atherosclerotic disease (38). We have recently reported that healthy African Americans have higher SM levels and lower Lact-Cer levels compared to healthy whites; and that SLE patients, irrespective of race, have higher levels of ceramides, and sphingoid bases and their phosphates compared to healthy participants (19). We also showed that African American SLE patients have higher levels of ceramides, Hex-Cer, sphingosine, and dhSph-1P compared to healthy African Americans. Furthermore, within African American SLE patients, those with atherosclerosis were found to have higher levels of SM (most SM species and total SM) and sphingoid bases (sphingosine and dhSph), lower levels of C24:1 Lact-Cer, and no significant differences in ceramide species levels compared to those without (19). These reports are in accordance with our current data demonstrating that TPA in African American SLE patients correlates positively with C20:0, C22:0 and total SM concentrations, while correlates negatively with concentrations of C24:1 Lact-Cer, several other Lact-Cer species and total Lact-Cer. Remarkably, in the current study, the concentrations of C24:0, C26:0 and C26:1 ceramide species, but not total ceramide correlated positively with TPA. This observation highlights the significance of the determination of individual sphingolipid species levels as a more sensitive-to-change parameter than total levels. Importantly, the longitudinal measurement of TPA shows that TPA increased in size with increased levels of C16:0, C24:1 and total Lact-Cer, and with decreased levels of sphingoid bases (sphingosine and dhSph) and dhSph-1P levels.

A role of the glycosphingolipid pathway in atherosclerosis was previously investigated. Measured concentrations of glycosphingolipids in human aortic intima and media from

TABLE 5 | Correlations between normalized carotid plaque area and markers of dyslipidemia and of SLE disease activity and damage.

	Total plaque area %*	Change in plaque**	Age	Years with SLE	BP Syst.	BP Diast.	Waist-hip ratio	SLEDAI score	SLICC score	LDL-C	HDL-C	TG	C3	C4	dsDNA	Urine protein/ creatinine
Total plaque area %*																
Change in plaque**	***-0.61***															
Age	***0.44***	-0.01														
Years with SLE	***0.34***	-0.01	***0.40***													
BP Syst.	-0.07	-0.18	0.29	0.07												
BP Diast.	-0.06	0.03	-0.04	-0.09	***0.68***											
Waist-hip ratio	-0.11	-0.05	-0.09	0.06	0.14	0.14										
SLEDAI score	-0.17	-0.07	-0.27	-0.25	0.21	0.31	-0.08									
SLICC score	0.11	***0.36***	***0.33***	***0.36***	0.23	0.20	-0.06	***-0.42***								
LDL-C	0.24	0.13	0.14	-0.03	-0.26	-0.02	0.13	-0.001	0.03							
HDL-C	0.14	0.21	0.14	-0.03	0.15	0.12	-0.18	0.30	0.12	0.21						
TG	0.06	-0.14	0.21	0.05	0.07	0.07	0.08	-0.08	0.002	***0.42***	-0.32					
C3	0.26	0.04	***0.37***	0.21	0.05	-0.06	0.27	***-0.35***	***0.39***	0.13	-0.0004	0.06				
C4	0.24	0.07	0.02	0.23	-0.02	0.05	***0.33***	***-0.43***	***0.41***	0.09	-0.18	0.20	***0.57***			
dsDNA	-0.22	-0.11	-0.16	-0.14	-0.05	0.02	0.01	0.31	***-0.47***	-0.04	-0.14	0.07	***-0.51***	***-0.44***		
Urine protein/ creatinine	0.21	0.02	0.20	0.17	0.11	0.21	0.04	0.01	0.16	0.17	0.01	0.22	-0.20	-0.05	***0.33***	

N =39. *Data presented are r values where* ***bold italics*** *is statistically significant at < 0.05* ***(black)****, < 0.01* ***(red)****, < 0.001* ***(blue)****, < 0.00001* ***(violet)****, & Underlined: p=0.051 to p=0.059. *% of age- and sex-matched historical controls. **Absolute change in plaque from baseline (visit 1) to visit 2. BP, blood pressure; Syst., systolic; Diast., diastolic; SLEDAI, Systemic lupus erythematosus disease activity index; SLICC, Systemic Lupus International Collaborating Clinics; LDL-; HDL-C, HDL-; LDL-cholesterol; TG, triglycerides.*

patients who died of atherosclerosis showed that the level of LacCer was elevated 5-fold compared to unaffected intima (39). Fatty streaks also were found to accumulate several fold higher levels of glycosphingolipids (glucosylceramides, Lact-Cer and GM3) than normal regions of human aorta (40). Concentrations of Lact-Cer species measured in serum from CAD patients were found to be significantly associated with the fatal outcome of CAD, independently of traditional risk factors (32). Furthermore, it was shown that concentration of C18:0 Lact-Cer in plasma from 581 patients, who underwent coronary angiography for acute coronary syndrome (ACS) or stable CAD was associated with vulnerable plaques, as characterized using intra vascular ultra sound virtual histology (IVUS-VH) and near-infrared spectroscopy (NIRS) imaging, and with 1-year major adverse cardiac events (composite endpoint of death or ACS) (41). Since our previous study showed that healthy African Americans have lower plasma Lact-Cer levels compared to healthy whites (19), and our current data show that plasma Lact-Cer levels in African Americans with SLE correlate negatively with TPA, this raises the question whether African Americans have inherent tendency towards accumulating Lact-Cer in tissues or not effluxing Lact-Cer into the circulation. This hypothesis warrants further investigation.

Serum ceramide concentrations have been shown to have independent predictive value for CVD, including CAD, stroke, heart failure and atrial fibrillation (reviewed in 42), despite the fact that a direct cause-effect relationship between CVD and serum ceramide has not been established yet. A ceramide risk score (CERT1) which was based on C16:0, C18:0, and C24:1 ceramide concentrations and their ratios to C24:0 ceramide was developed for clinical use and was found to identify high-risk coronary heart disease patients beyond LDL-C concentration (43, 44). Based on CERT1, patients are stratified into four risk categories, where a linear CVD risk increase is associated with the increasing score both in patients with a stable coronary heart disease and ACS. The CERT1 ceramide score has been implemented in clinical use both in Finland and at Mayo Clinic in the USA (42). In our study, among the three CERT1 ceramide ratios, only C16:0 ceramide/C24:0 ceramide ratio was negatively correlated with TPA (% of control) but positively correlated with change in TPA ($p< 0.01$) (**Table 3**). It is important to mention here that TPA% of "disease" controls would be different when compared to a control healthy population.

As an upgrade of CERT1, CERT2 has been recently developed by incorporating distinct phosphatidylcholines (PCs) into the score (45); PCs have been shown to have prognostic value for CVD events (46). The CERT2 score have one ceramide/ceramide ratio, two ceramide/PC ratios and a single PC, whereas the original CERT1 score contains three single ceramides and three ceramide/ceramide ratios. The ceramide-PC ratio components of the CERT2 test showed higher hazard ratios for CVD events than any other ceramide-ceramide ratio (45). The CERT2 score was also significantly associated with inflammatory markers (hs-CRP and IL-6) (47), which suggests that CERT2 could assess both plaque burden and inflammatory residual risk

in patients with CVD. Interestingly, patients with renal dysfunction were found to have higher CERT2 scores, while associations of CERT2 with high blood pressure and diabetes were found to be much weaker (47). Furthermore, CERT2 was significantly associated with LDL-C and triglycerides levels; however, CERT2 was prognostic even after adjustment for LDL-C and triglyceride levels (47). In our study, we investigated whether there was any association between circulating creatinine and sphingolipid levels in patients who may have had renal involvement, although creatinine > 3.0 was an exclusion criterion. We found that C22:1, C24:0, C24:1, C26:1 and total Hex-Cers as well as C24:0 Lact-Cer were negatively correlated with serum creatinine ($p<0.05$), but C24:0 SM was positively correlated with serum creatinine ($p<0.05$). Our current data suggest that the use of plasma concentrations of glycosphingolipids (Lact-Cer and Hex-Cer species) with the CERT2 risk estimation tool may improve the stratification of SLE patients for their risk of CVD events.

Fiedorowicz et al. assessed ceramide and S1P serum concentrations in patients with acute ischaemic stroke, transient ischemic attack, and age-matched neurological patients without cerebral ischaemia and recognized the two ratios, S1P/C 24:1 ceramide, and C 24:0 ceramide/C 24:1 ceramide, with a diagnostic potential in ischaemic stroke (48). In our current study, TPA values correlated positively with concentrations of very long-chain ceramide species (C24:0, C26:0 and C26:1). However, C16:0 ceramide/S1P ratio and C24:1 ceramide/S1P ratio correlated positively with LDL-C concentration and the urine protein/creatinine ratio **(Table 3)**. We have previously reported that SLEDAI significantly correlates with plasma C16:0 ceramide/S1P ratio and C24:1 ceramide/S1P ratio in the African American, but not white SLE patients (19). In this and our previous studies the nephritis comorbidity has been excluded; however, it is possible that the plasma S1P fraction bound to albumin (25) is excreted with the urine (albuminuria), which may alter/inflate the ceramide/S1P ratio possibly causing alterations in correlations with TPA and other clinical variables.

Although this study was not purposely designed to study the effect of statins on the development of atherosclerosis in African American SLE patients, the data showed that 'undesirably' C24:1 ceramide and C24:1 ceramide/S1P ratio were significantly higher in the few patients who used statins. The data for statin use in atherosclerosis prevention in SLE, whether in SLE patients or SLE mouse models, has been inconsistent despite reductions in cholesterol levels (reviewed in 6). However, it is reasonable to use statins in SLE patients with traditional CVD risk factors. Therefore, in future studies, statins use could be analyzed as a confounding factor in determining the associations of plasma sphingolipid concentrations with traditional SLE markers. Furthermore, as statins remain the current recommendation to the risk-based approach to CVD treatment, the use of statins in African American SLE patients is not to be discouraged without clinical outcome data; however, the effectiveness of statin treatment to prevent atherosclerosis progression in African American SLE patients warrants further attention. Again, a limitation of our study is that there are only four patients, who were on statins, which makes it difficult to draw any definitive conclusions on the effect of statins on the development of atherosclerosis in African American SLE patients.

In this study, the older the patient the higher the concentrations of long- and very long-chain SM species, the higher the concentrations of C20:0 ceramide species, and the lower the concentrations of long- and very long-chain Lact-Cer species **(Table 3)**. The duration of SLE prior to visit 1, SLEDAI scores, and concentrations of C4 dsDNA antibodies do not seem to influence the concentrations of plasma sphingolipids. However, C3 concentrations, which were found to correlate negatively with almost all Lact-Cer species, and positively with three long-chain SM species, indicate that these sphingolipid measurements could have an added value in assessing the prognosis of CVD in SLE patients. From the current retrospective study, though it is not possible to infer whether increased Lact-Cer levels would be compensatory or pathologic.

Taken together, the data demonstrate that sphingolipidomics have the potential to be used as an early diagnostic tool of atherosclerosis in SLE and may have an added benefit to the currently available tools in the diagnosis, prognosis, and treatment of the disease. Longitudinal studies are warranted to proof the potential sphingolipid markers for early diagnosis of SLE comorbidities, including CVD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical University of South Carolina Institutional Review Board (IRB), (Protocol number: HR1623). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH and JO designed the study. SH administered and supervised the project and wrote the original draft. JO provided the patient plasma samples and the corresponding clinical data. SH and JO validated and interpreted the data. WT and OH contributed to the laboratory work. PN and DW did the statistical analyses. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Causal Effects of Gut Microbiome on Systemic Lupus Erythematosus: A Two-Sample Mendelian Randomization Study

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The observational association between gut microbiome and systemic lupus erythematosus (SLE) has been well documented. However, whether the association is causal remains unclear. The present study used publicly available genome-wide association study (GWAS) summary data to perform two-sample Mendelian randomization (MR), aiming to examine the causal links between gut microbiome and SLE. Two sets of MR analyses were conducted. A group of single nucleotide polymorphisms (SNPs) that less than the genome-wide statistical significance threshold (5×10^{-8}) served as instrumental variables. To obtain a comprehensive conclusion, the other group where SNPs were smaller than the locus-wide significance level (1×10^{-5}) were selected as instrumental variables. Based on the locus-wide significance level, the results indicated that there were causal effects of gut microbiome components on SLE risk. The inverse variance weighted (IVW) method suggested that *Bacilli* and *Lactobacillales* were positively correlated with the risk of SLE and *Bacillales*, *Coprobacter* and *Lachnospira* were negatively correlated with SLE risk. The results of weighted median method supported that *Bacilli*, *Lactobacillales*, and *Eggerthella* were risk factors for SLE and *Bacillales* and *Coprobacter* served as protective factors for SLE. The estimates of MR Egger suggested that genetically predicted *Ruminiclostridium6* was negatively associated with SLE. Based on the genome-wide statistical significance threshold, the results showed that *Actinobacteria* might reduce the SLE risk. However,

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Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) detected significant horizontal pleiotropy between the instrumental variables of *Ruminiclostridium6* and outcome. This study support that there are beneficial or detrimental causal effects of gut microbiome components on SLE risk.

Keywords: autoimmune disease, Mendelian randomization, gut microbiome, systemic lupus erythematosus, causality

1 INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune connective tissue disease involving multiple organs, and it presents with a range of clinical symptoms, including skin rash, pericarditis, nephritis, and neurological and hematological involvement. Loss of tolerance to autoantigens is one of the hallmarks of SLE. Genetic, hormonal, and environmental factors interact in susceptible individuals, resulting in autoantibodies deposition and abnormal production of proinflammatory cytokines (1). In addition, ultraviolet light and infections induce DNA damage and apoptosis which increase exposure to autoantigens are potential triggers for SLE as well (2). The current treatment strategy is mainly the use of non-selective immunosuppressive agents. Long-term use of immunosuppressants weakens the immunity and results in severe infections (3). Therefore, it is imperative to explore the etiology of SLE to facilitate the development of treatment strategies with low damage or even no side effects.

Recently, the causal link between the gut microbiome composition and SLE risk has attracted widespread attention. The intestinal microbiota plays a critical role in the maturation of the host immune response and provide protection against pathogen overgrowth (4). A study demonstrated that the gut microbiome was related to the dynamics of human immune cells, suggesting that the gut microbiome drove the modulation of the immune system (5). The dysbiosis of gut microbiome affected immune responses, which contributed to the occurrence of autoimmune diseases (6). One possible explanation was that the presence of commensal gut microbiome influenced the autoimmune responses to nuclear antigens (7). Several studies indicated that SLE patients had dysbiosis of gut microbiome and decreased species richness (8, 9). Furthermore, the decrease in species diversity was particularly significant in patients with high SLE activity index (10), suggesting that intestinal flora might be involved in the immune pathogenesis of autoimmune diseases. Nevertheless, it remains unclear as to whether there is a causal relationship between gut microbiome and SLE.

Mendelian randomization (MR) is an approach integrating summary data of genome-wide association study (GWAS), and hence, the impact of confounding factors (e.g., environment) is minimized. MR is a common method to infer whether there are causal relationships between exposure and complex outcomes. Genetic variants that are significantly related to exposure are selected as instrumental variables to infer the causality (11). The instrumental variables that affect the exposure will affect the results proportionally if the exposure is causal. In the current study, the two-sample MR was conducted to examine if there is a causal relationship between gut microbiome composition and SLE risk.

2 MATERIALS AND METHODS

2.1 Data Sources and SNP Selection

Single-nucleotide polymorphisms (SNPs) related to human gut microbiome composition were selected as instrumental variables from a GWAS with 18,473 individuals, including 122,110 variant sites (12). It was a multi-ethnic large-scale GWAS that recruited 25 population-based cohorts from the United States, Canada, Israel, the Netherlands, Belgium, Sweden, South Korea, Germany, Denmark, Finland, and the UK to explore the association between autosomal human genetic variants and the gut microbiome. Effect estimates of the SNPs related to SLE risk were extracted from a large SLE GWAS, which involved 7,219 cases and 15,991 controls of European ancestry (13).

To ensure the authenticity and accuracy of the conclusions on the causal link between gut microbiome and SLE risk, the following quality control steps were used to select optimal instrument variables. First, SNPs significantly related to gut microbiome were selected as instrumental variables. Two thresholds were used to select the instrumental variable. A set of SNPs less than the genome-wide statistical significance threshold (5×10^{-8}) served as instrumental variables. In order to obtain more comprehensive results, the other group where SNPs are smaller than the locus-wide significance level (1×10^{-5}) was selected as instrumental variables. Second, the minor allele frequency (MAF) threshold of variants of interest was 0.01. Third, one of the principles of the MR approach is that there is no linkage disequilibrium (LD) among the included instrumental variables, since the presence of strong LD might result in biased results. In the current study, the clumping process ($R^2 < 0.001$ and clumping distance = 10,000kb) were conducted to assess the LD between the included SNPs. Fourth, an important step of MR is to ensure that the effects of the SNPs on the exposure correspond to the same allele as the effects on the outcome. In accordance with the principle, palindromic SNPs would not be included in the instrumental variables. Fifth, when SNPs related to exposure were absent in the outcome GWAS, the proxy SNPs significantly associated with the variants of interest were selected ($r^2 > 0.8$).

2.2 The Assumptions of MR

To minimize the impact of bias on the results, the MR method must conform to three important assumptions. First,

instrumental variables are independent of confounders that influence exposure and outcome. Second, the variants of interest used in the analysis should be significantly associated with exposure. *F* statistic is generally performed to assess the strength of the relevance between instrumental variables and exposure. The formula of *F* statistic is $F = R^2(n\text{-}k\text{-}1)/k(1\text{-}R^2)$. R^2 represents the exposure variance explained by the selected SNPs, *n* is the sample size, and *k* represent the number of included instrumental variables. If *F* is less than 10, there is a weak association between instrumental variables and exposure. Third, instrumental variables affect outcomes only through exposure, which means that there is no horizontal pleiotropy effect between instrumental variables and outcome.

2.3 MR Estimates

In the current study, high-efficiency methods including inverse variance weighted (IVW), MR-Egger, weighted median, and weighted mode were used to infer whether there was causal effect of human gut microbiome composition on SLE risk. IVW is essentially a meta-analysis method, which converts to a weighted regression of the outcome effects of instrumental variables on the exposure effects to obtain an overall estimate of the impact of gut microbiome on the risk of SLE, where the intercept is limited to zero (14). When there is no horizontal pleiotropy, IVW can avoid the impact of confounding factors to obtain unbiased estimates. MR-Egger may be strongly influenced by outlying genetic variables, leading to inaccurate estimates. However, even if all selected instrumental variables are invalid, the MR-Egger method can still provide unbiased estimates. The weighted median can provide consistent estimates of the causal effects, even if as many as 50% of the information in the analysis comes from variations of interest are invalid instrumental variables. The weighted median method has some important advantages over the MR-Egger since it improves the accuracy of the results. When most instrumental variables with similar causal estimates are valid, the weighted mode approach is still valid even if the other instrumental variables do not meet the requirements of MR method for causal inference (15).

The MR-Egger regression was conducted to assess whether the included SNPs had potential horizontal pleiotropic effects. MR-Egger regression is a method, which has the property that both detect and adjust for pleiotropy in the MR analysis, and get a causal effect estimate (16) and examine whether the results are driven by the directional horizontal pleiotropy (17). Given the lower accuracy and statistical power of MR-Egger regression, Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) was performed to detect any outliers reflecting likely pleiotropic biases and correct horizontal pleiotropy. Furthermore, Cochran's *Q* statistic was used to quantify the heterogeneity among the selected SNPs. To determine whether there were potential strong influence SNPs, the leave-one-out sensitivity analysis was performed to verify the reliability and stability of the causal effect estimates. Statistical analyses were performed using R software (version 4.0.2, TwoSampleMR package).

3 RESULTS

3.1 Instrumental Variables Selection

Initially, 14,587 (locus-wide significance level, $P < 1 \times 10^{-5}$) and 456 (genome-wide statistical significance threshold, $P < 5 \times 10^{-8}$) SNPs were identified as instrumental variables from a large-scale GWAS. It contained 211 bacterial traits, including five biological classifications: phylum, class, order, family, and genus. After removing SNPs that had LD effects and independence from SLE, 2,105 ($P < 1 \times 10^{-5}$) and 13 ($P < 5 \times 10^{-8}$) SNPs were selected as instrumental variables. The main information of SNPs including effect allele, other allele, beta, SE, and *P* value were collected systematically for further analysis.

3.2 Two-Sample MR Analysis

3.2.1 Locus-Wide Significance Level

The results of IVW analyses demonstrated that *Bacilli* (odds ratio (OR) = 1.40, 95% confidence interval (CI), 1.02–1.93, $P = 0.037$) and *Lactobacillales* (OR = 1.40, 95% CI, 1.01–1.95, $P = 0.045$) were positively correlated with the risk of SLE and *Bacillales* (OR = 0.85, 95% CI, 0.74–0.98, $P = 0.022$), *Coprobacter* (OR = 0.78, 95% CI, 0.64–0.95, $P = 0.014$), and *Lachnospira* (OR = 0.60, 95% CI, 0.38–0.94, $P = 0.027$) were negatively correlated with SLE risk (**Table 1**). The MR estimates of weighted median indicated that *Bacilli* (OR = 1.59, 95% CI, 1.06–2.39, $P = 0.027$), *Lactobacillales* (OR = 1.73, 95% CI, 1.13–2.64, $P = 0.011$), and *Eggerthella* (OR = 1.41, 95% CI, 1.05–1.90, $P = 0.022$) were risk factors for SLE, and *Bacillales* (OR = 0.81, 95% CI, 0.67–0.96, $P = 0.018$) and *Coprobacter* (OR = 0.77, 95% CI, 0.59–0.99, $P = 0.043$) served as protective factors for SLE (**Table 1**). The estimates of MR Egger suggested that genetically predicted *Ruminiclostridium6* were negatively associated with SLE (OR = 0.35, 95% CI, 0.15–0.83, $P = 0.040$). The detailed statistical results of the 211 intestinal floras were shown in **Supplementary Table S1**.

The horizontal pleiotropy between instrumental variables and outcome was assessed by MR-Egger regression, and the results showed that there was no evidence of horizontal pleiotropy (**Table 1**). No outliers were found in the analysis of *Bacilli* ($P = 0.191$), *Lactobacillales* ($P = 0.213$), *Bacillales* ($P = 0.403$), *Coprobacter* ($P = 0.365$), and *Lachnospira* ($P = 0.301$) by MR-PRESSO. MR-PRESSO suggested that there was significant horizontal pleiotropy between the instrumental variables of *Eggerthella* and outcome ($P = 0.041$), and rs1784446 was identified as outlier. However, the results did not change significantly after removing the SNP (OR = 1.42, 95% CI, 1.06–1.90, $P = 0.020$). In the analysis of *Ruminiclostridium6*, MR-PRESSO found there was significant horizontal pleiotropy ($P = 0.045$) and rs61060922 was identified as a pleiotropic SNP. After removing the outlier, the results changed substantially (OR = 0.48, 95% CI, 0.22–1.04, $P = 0.101$). The detailed information of the instrumental variables was shown in **Table 2**. The *F* statistics of the SNPs were all greater than 10, indicating that there was no weak instrumental variables bias (**Table 1**). Thus, the two-sample MR estimates found that *Bacilli* (**Figure 1**), *Eggerthella* (**Supplementary Figure S1**), and

TABLE 1 | MR results of causal links between gut microbiome and SLE risk ($P < 1 \times 10^{-5}$).

Classification		Nsnp	Methods	Beta	SE	OR (95% CI)	*P* value	Horizontal pleiotropy			Heterogeneity		*F* statistic
								Egger intercept	SE	*P* value	Cochran's *Q*	*P* value	
Class	*Bacilli*	16	MR Egger	0.61	0.44	1.84 (0.77–4.38)	0.189	-0.02	0.03	0.519	19.63	0.142	24.73
			Weighted median	0.46	0.21	1.59 (1.06–2.39)	0.027						
			Inverse variance weighted	0.34	0.16	1.40 (1.02–1.93)	0.037						
			Weighted mode	0.65	0.34	1.91 (0.98–3.75)	0.078						
Order	*Lactobacillales*	14	MR Egger	0.22	0.44	1.24 (0.52–2.94)	0.634	0.01	0.03	0.769	16.71	0.161	25.54
			Weighted median	0.55	0.22	1.73 (1.13–2.64)	0.011						
			Inverse variance weighted	0.34	0.17	1.40 (1.01–1.95)	0.045						
			Weighted mode	0.57	0.33	1.78 (0.93–3.34)	0.107						
	Bacillales	11	MR Egger	0.07	0.30	1.08 (0.60–1.95)	0.813	-0.03	0.04	0.448	7.62	0.666	24.69
			Weighted median	-0.22	0.09	0.81 (0.67–0.96)	0.018						
			Inverse variance weighted	-0.16	0.07	0.85 (0.74–0.98)	0.022						
			Weighted mode	-0.28	0.14	0.76 (0.57–1.01)	0.080						
Genus	*Coprobacter*	12	MR Egger	-0.01	0.38	0.99 (0.47–2.08)	0.975	-0.03	0.04	0.533	12.35	0.262	26.60
			Weighted median	-0.26	0.13	0.77 (0.59–0.99)	0.043						
			Inverse variance weighted	-0.25	0.10	0.78 (0.64–0.95)	0.014						
			Weighted mode	-0.40	0.24	0.67 (0.42–1.07)	0.121						
	Eggerthella	10	MR Egger	0.55	0.70	1.73 (0.44–6.79)	0.453	-0.03	0.08	0.675	19.35	0.022	22.42
			Weighted median	0.35	0.15	1.41 (1.05–1.90)	0.022						
			Inverse variance weighted	0.25	0.15	1.29 (0.95–1.74)	0.097						
			Weighted mode	0.36	0.18	1.43 (0.99–2.06)	0.085						
	Lachnospira	7	MR Egger	-0.31	1.11	0.73 (0.08–6.48)	0.790	-0.01	0.07	0.861	8.21	0.145	10.83
			Weighted median	-0.49	0.26	0.61 (0.37–1.02)	0.059						
			Inverse variance weighted	-0.51	0.23	0.60 (0.38–0.94)	0.027						
			Weighted mode	-0.49	0.37	0.61 (0.30–1.26)	0.231						
	Ruminiclostridium6	11	MR Egger	-1.04	0.43	0.35 (0.15–0.83)	0.040	0.10	0.05	0.065	13.11	0.158	23.83
			Weighted median	-0.37	0.22	0.69 (0.45–1.08)	0.102						
			Inverse variance weighted	-0.20	0.19	0.82 (0.56–1.19)	0.293						
			Weighted mode	-0.47	0.33	0.63 (0.33–1.21)	0.193						

SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; OR, odds ratio.

TABLE 2 | SNPs used as instrumental variables from gut microbiome and SLE GWASs ($P < 1 \times 10^{-5}$).

Bacterial traits	SNP	Effect allele	Other allele	Gut microbiome			SLE			Proxy SNP	Target effect allele	Target other allele
				Beta	SE	*P* value	Beta	SE	*P* value			
Bacilli	rs11110282	A	G	-0.10	0.02	2.08E-06	-0.01	0.07	0.888	–	–	–
	rs12642660	A	G	-0.08	0.02	8.32E-07	-0.11	0.04	0.014	–	–	–
	rs12797734	C	T	0.06	0.01	5.58E-06	0.03	0.03	0.344	–	–	–
	rs2370083	G	T	-0.08	0.02	5.98E-06	-0.09	0.05	0.068	–	–	–
	rs28564647	G	T	-0.06	0.02	5.98E-06	0.04	0.04	0.301	–	–	–
	rs2952251	G	A	-0.06	0.01	6.59E-07	-0.05	0.04	0.160	–	–	–
	rs35344081	A	G	0.06	0.01	9.35E-07	-0.02	0.03	0.560	–	–	–
	rs3911531	C	T	-0.06	0.01	8.53E-06	-0.01	0.04	0.800	–	–	–
	rs4028634	C	T	0.05	0.01	3.08E-06	0.02	0.03	0.568	–	–	–
	rs4459992	C	T	0.05	0.01	9.31E-06	0.05	0.03	0.096	–	–	–
	rs4968759	A	G	-0.05	0.01	9.14E-06	0.04	0.03	0.201	–	–	–
	rs57872228	C	T	-0.07	0.01	7.28E-07	0.01	0.04	0.821	–	–	–
	rs694949	G	A	-0.08	0.02	5.00E-06	-0.08	0.05	0.078	–	–	–
	rs7666190	A	C	-0.10	0.02	7.60E-06	0.04	0.06	0.515	–	–	–
	rs78938557	C	T	0.11	0.02	9.54E-07	-0.04	0.06	0.505	–	–	–
	rs9581006	C	T	0.23	0.05	1.80E-06	0.14	0.07	0.054	–	–	–
Lactobacillales	rs11110282	A	G	-0.11	0.02	1.64E-06	-0.01	0.07	0.888	–	–	–
	rs11627423	A	C	0.05	0.01	8.94E-06	0.03	0.03	0.374	–	–	–
	rs11639594	A	C	0.05	0.01	9.53E-06	0.03	0.03	0.294	–	–	–
	rs12642660	A	G	-0.07	0.02	2.42E-06	-0.11	0.04	0.014	–	–	–
	rs12797734	C	T	0.06	0.01	6.07E-06	0.03	0.03	0.344	–	–	–
	rs2370083	G	T	-0.08	0.02	3.84E-06	-0.09	0.05	0.068	–	–	–
	rs2952251	G	A	-0.06	0.01	2.00E-07	-0.05	0.04	0.160	–	–	–
	rs34989881	A	G	0.11	0.02	7.26E-06	-0.11	0.06	0.081	–	–	–
	rs35344081	A	G	0.06	0.01	3.70E-07	-0.02	0.03	0.560	–	–	–
	rs4028634	C	T	0.05	0.01	1.89E-06	0.02	0.03	0.568	–	–	–
	rs57872228	C	T	-0.07	0.01	2.08E-06	0.01	0.04	0.821	–	–	–
	rs78938557	C	T	0.11	0.02	2.01E-06	-0.04	0.06	0.505	–	–	–
	rs7919839	C	T	0.08	0.02	8.97E-06	-0.01	0.05	0.846	–	–	–
	rs9581006	C	T	0.23	0.05	1.77E-06	0.14	0.07	0.054	–	–	–
Bacillales	rs10410917	C	T	0.11	0.02	5.50E-06	-0.03	0.03	0.259	–	–	–
	rs821056	A	G	0.21	0.04	7.43E-06	-0.01	0.04	0.789	–	–	–
	rs11608727	G	T	0.13	0.03	6.17E-06	0.05	0.03	0.127	–	–	–
	rs12498725	A	G	-0.18	0.04	1.78E-06	0.01	0.04	0.782	–	–	–
	rs182923	T	C	0.14	0.03	4.61E-06	-0.05	0.04	0.214	–	–	–
	rs4617108	A	G	0.25	0.05	1.04E-06	-0.05	0.06	0.377	–	–	–
	rs55793055	C	T	-0.11	0.03	7.79E-06	0.04	0.03	0.126	–	–	–
	rs62141894	A	G	0.14	0.03	6.90E-06	-0.02	0.03	0.556	–	–	–
	rs62640857	A	G	0.15	0.03	3.58E-06	-0.04	0.04	0.346	–	–	–
	rs7611581	G	T	-0.11	0.02	3.00E-06	0.03	0.02	0.231	–	–	–
	rs875142	A	G	0.13	0.03	5.13E-06	-0.04	0.03	0.181	–	–	–
Coprobacter	rs11532348	C	T	-0.10	0.02	4.34E-06	-0.01	0.03	0.753	–	–	–
	rs12684609	C	T	0.10	0.02	5.70E-06	-0.02	0.03	0.487	–	–	–
	rs12996055	A	C	0.09	0.02	5.03E-06	-0.04	0.03	0.229	–	–	–
	rs143180826	C	T	0.17	0.04	6.60E-06	0.05	0.06	0.431	–	–	–
	rs143662916	C	T	0.25	0.05	3.07E-06	-0.11	0.07	0.143	–	–	–
	rs189356	A	G	0.08	0.02	7.59E-06	-0.05	0.03	0.071	–	–	–
	rs213863	C	T	-0.09	0.02	3.78E-06	0.04	0.03	0.217	–	–	–
	rs28402691	C	T	0.11	0.02	7.14E-06	0.02	0.03	0.493	–	–	–
	rs305411	A	G	0.13	0.03	8.82E-07	-0.08	0.05	0.082	–	–	–
	rs3828477	G	T	-0.09	0.02	2.41E-06	0.04	0.03	0.220	–	–	–
	rs72821405	C	T	-0.15	0.03	4.27E-06	0.01	0.04	0.816	–	–	–
	rs74919520	A	G	0.12	0.03	6.39E-06	-0.11	0.05	0.021	–	–	–
Eggerthella	rs112205261	C	T	-0.19	0.04	3.48E-06	0.13	0.06	0.032	–	–	–
	rs116603267	A	G	0.16	0.04	8.39E-06	-0.07	0.06	0.221	–	–	–
	rs1784446	G	A	0.09	0.02	5.05E-06	-0.07	0.03	0.019	–	–	–
	rs2223081	G	A	0.10	0.02	3.04E-06	0.02	0.03	0.563	–	–	–
	rs2240838	A	G	0.09	0.02	1.46E-06	0.05	0.03	0.085	–	–	–
	rs3851328	G	T	-0.11	0.02	1.38E-06	0.04	0.03	0.215	–	–	–
	rs4985746	A	G	0.11	0.02	4.63E-06	-0.08	0.04	0.020	–	–	–
	rs6430926	C	T	0.09	0.02	6.10E-06	0.03	0.03	0.247	–	–	–

(Continued)

TABLE 2 | Continued

Bacterial traits	SNP	Effect allele	Other allele	Gut microbiome			SLE			Proxy SNP	Target effect allele	Target other allele
				Beta	SE	*P* value	Beta	SE	*P* value			
	rs6678488	A	C	0.09	0.02	6.20E-06	-0.02	0.04	0.587	–	–	–
	rs76663501	C	T	0.17	0.04	5.97E-06	-0.08	0.06	0.179	–	–	–
Lachnospira	rs13157098	A	G	-0.08	0.02	8.76E-07	-0.06	0.05	0.196	–	–	–
	rs159484	A	G	0.08	0.02	7.37E-06	-0.03	0.04	0.495	–	–	–
	rs2520509	G	A	0.05	0.01	8.52E-06	-0.06	0.03	0.032	–	–	–
	rs4686798	C	T	0.05	0.01	4.51E-06	-0.03	0.03	0.248	–	–	–
	rs4923324	A	G	-0.06	0.01	1.63E-06	0.07	0.03	0.031	–	–	–
	rs56791201	C	T	0.05	0.01	2.77E-06	-0.01	0.02	0.626	–	–	–
	rs75566846	A	C	0.12	0.03	5.88E-06	-0.11	0.07	0.152	–	–	–
Ruminiclostridium6	rs10829821	C	T	-0.10	0.02	4.89E-06	-0.02	0.06	0.728	rs11017525	A	G
	rs116969552	A	G	-0.17	0.04	8.01E-06	0.05	0.08	0.533	–	–	–
	rs11992182	A	C	0.06	0.01	2.69E-06	0.05	0.03	0.095	–	–	–
	rs1377110	G	A	0.06	0.01	2.20E-06	0.03	0.04	0.416	–	–	–
	rs61060922	G	T	0.16	0.03	8.98E-07	-0.19	0.06	0.002	–	–	–
	rs663262	C	T	-0.13	0.03	3.63E-06	-0.06	0.06	0.312	–	–	–
	rs67479537	C	T	0.12	0.03	5.85E-06	-0.07	0.06	0.236	–	–	–
	rs71414120	G	T	0.20	0.04	8.77E-07	-0.08	0.06	0.179	–	–	–
	rs72991535	G	T	0.14	0.03	3.42E-06	-0.07	0.07	0.306	–	–	–
	rs79968172	A	G	0.11	0.02	2.23E-06	0.07	0.06	0.244	–	–	–
	rs9555756	A	C	-0.08	0.02	3.73E-06	0.05	0.04	0.194	–	–	–

SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism.

Lactobacillales (**Supplementary Figure S2**) were positively related to SLE risk, and *Coprobacter* (**Supplementary Figure S3**), *Bacillales* (**Supplementary Figure S4**), and *Lachnospira* (**Supplementary Figure S5**) played protective roles in the pathogenesis of SLE.

3.2.2 Genome-Wide Statistical Significance Threshold

When MR analysis was performed with gut microbiome as a whole, the results of IVW (OR = 1.20, 95% CI, 0.96–1.52, P = 0.114), MR Egger (OR = 0.74, 95% CI, 0.34–1.58, P = 0.448), weighted median (OR = 1.15, 95% CI, 0.92–1.43, P = 0.221), and weighted mode (OR = 1.26, 95% CI, 0.98–1.63, P = 0.099) showed that gut microbiome was not associated with SLE risk (**Table 3** and **Supplementary Figure S6**). The detailed information of the instrumental variables was shown in **Supplementary Table S2**. MR-Egger regression showed that there was no horizontal pleiotropy between instrumental variables and outcome (P = 0.213). In addition, the results of Cochrane Q statistics showed no significant heterogeneity (P = 0.052) and the F statistics was greater than 10. The results of gut microbiome classification indicated that *Actinobacteria* might reduce the risk of SLE (OR = 0.52, 95% CI, 0.29–0.95, P = 0.033) (**Table 3**). Heterogeneity and horizontal pleiotropy could not be examined due to the limited number of included SNPs.

4 DISCUSSION

This two-sample MR study suggested that the levels of *Bacillales*, *Coprobacter*, *Lachnospira*, and *Actinobacteria* were negatively related to the risk of SLE, and *Bacilli*, *Lactobacillales*, and *Eggerthella* might be the risk factors for SLE onset. However, since there were fewer instrumental variables reaching genome-wide statistical significance threshold, the results and the precision of *Actinobacteria* might have been compromised.

The gastrointestinal mucosal surface of the body is abundantly colonized by trillions of symbiotic gut microbiome which participate in the modulation and maintenance of the host immune system. Therefore, the dysbiosis of gut microbiome interacts with the intestinal mucosal immune system closely (6). Several studies found that autoimmune diseases were often accompanied by gut microbiome dysbiosis or altered microbiome. The distribution of microbes from phylum to genus levels of different taxa was different between healthy subjects and early rheumatoid arthritis (RA) patients, and the difference in microbial diversity and classification indicated that gut microbes might be involved in the pathogenesis of early RA (18). Compared with healthy controls, RA patients had varying degrees of alterations in gut microbiome composition, including *Bacteroides* (19, 20), *Prevotella* (21), *Verrucomicrobiae* (22), and *Salivarius* (23). Jangi et al. (23) found an increase in *Methanobrevibacter* and *Akkermansia* in multiple sclerosis (MS) patients, and *Methanobrevibacter* was involved in the immunomodulatory process due to its ability to recruit inflammatory cells (24). As an autoimmune disease closely related to intestinal microbes, the occurrence of inflammatory bowel disease (IBD) was often accompanied by gut microbiome dysbiosis. A study suggested that the human intestinal microbiome had an important influence on the drug metabolism and efficacy of IBD (25). Currently, there are limited studies on the association between the candidate intestinal bacteria found in this study and complex traits. Some studies indicated that compared with healthy controls, the abundance of *Bacilli* was increased in encephalitis (26) and Graves' disease patients (27). Increased *Lactobacillales* abundance

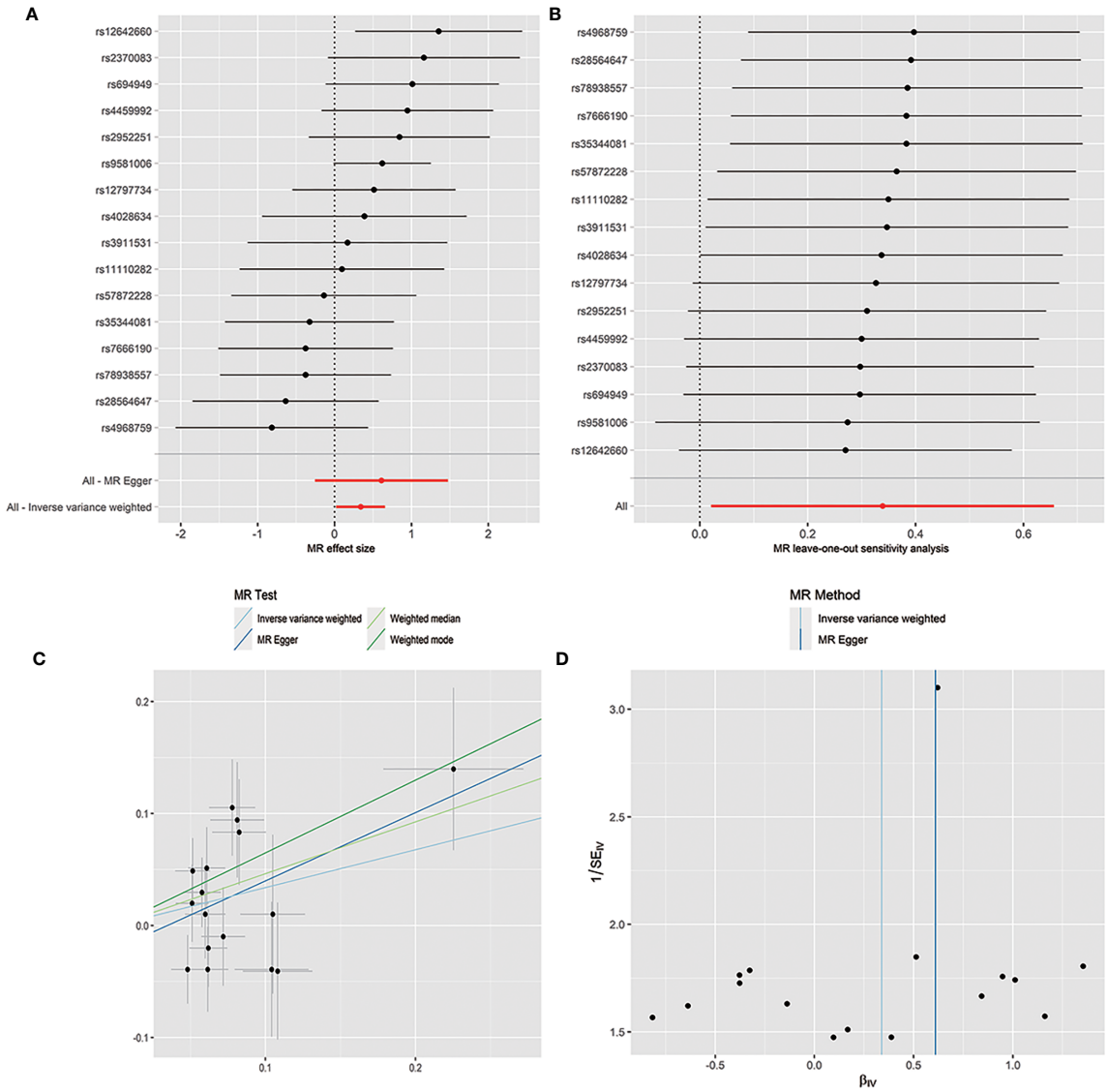

FIGURE 1 | Forest plot **(A)**, sensitivity analysis **(B)**, scatter plot **(C)**, and funnel plot **(D)** of the causal effect of *Bacilli* on SLE risk.

was observed in autoimmune liver disease (28), atopic dermatitis (29), type 1 diabetes (30), and Graves' disease (27) patients. These studies indicated that the *Bacilli* and *Lactobacillales* might have the effects of promoting inflammation. The abundance of *Lachnospira* was decreased in ankylosing spondylitis (31), type 1 diabetes (32), and IgE-associated allergic disease (33) patients, and the *Lachnospira* contributed to the alleviation of inflammation in HIV-infected patients (34), suggesting that *Lachnospira* might have a protective role in inflammatory conditions. These results were consistent with the present study. However, the mechanisms by which these intestinal floras exert beneficial or detrimental effects on the immune-mediated inflammatory disease remain to be further studied.

Recently, numerous human and rodent model studies have been conducted to infer the association between SLE and gut microbiome. A study found that SLE patients, especially those in the active phase, had dysbiosis in the intestinal flora (35). Luo et al. (36) found that the microbiome of active SLE patients changed compared with the non-SLE controls and the use of non-selective immunosuppressive therapies, such as dexamethasone and azathioprine, might have a broad impact on the diversity and abundance of gut microbiome. A study indicated that primary Sjögren's syndrome (pSS) and SLE patients shared similar alterations in the composition of gut microbiome, both showing a lower bacterial abundance and *Firmicutes*/*Bacteroidetes* ratio and a higher *Bacteroides* species richness, which could distinguish patients from individuals in the general population (37). A rodent model indicated that there were significant differences in the composition of gut microbiome between pre-disease and diseased NZB/W F1

TABLE 3 | MR results of causal links between gut microbiome and SLE risk ($P < 5 \times 10^{-8}$).

Classification		Nsnp	Methods	Beta	SE	OR (95% CI)	*P* value	Horizontal pleiotropy			Heterogeneity		*F* statistic
								Egger intercept	SE	*P* value	Cochran's *Q*	*P* value	
Total		13	MR Egger	-0.31	0.39	0.74 (0.34–1.58)	0.448	0.06	0.05	0.213	19.51	0.052	53.03
			Weighted median	0.14	0.11	1.15 (0.92–1.43)	0.221						
			Inverse variance weighted	0.19	0.12	1.20 (0.96–1.52)	0.114						
			Weighted mode	0.23	0.13	1.26 (0.98–1.63)	0.099						
Class	*Actinobacteria*	1	Wald ratio	-0.65	0.30	0.52 (0.29–0.95)	0.033	–	–	–	–	–	–
	Melainabacteria	1	Wald ratio	-0.06	0.21	0.94 (0.62–1.41)	0.766	–	–	–	–	–	–
Family	*Bifidobacteriaceae*	2	Inverse variance weighted	-0.31	0.42	0.73 (0.32–1.68)	0.459	–	–	–	–	–	–
	Streptococcaceae	1	Wald ratio	0.08	0.63	1.08 (0.32–3.68)	0.903	–	–	–	–	–	–
Genus	*Allisonella*	1	Wald ratio	0.34	0.18	1.40 (0.99–2.00)	0.059	–	–	–	–	–	–
	Bifidobacterium	2	Inverse variance weighted	-0.31	0.41	0.73 (0.33–1.65)	0.456	–	–	–	–	–	–
Order	*Bifidobacteriales*	2	Inverse variance weighted	-0.31	0.42	0.73 (0.32–1.68)	0.459	–	–	–	–	–	–

SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; OR, odds ratio.

mice, as well as between untreated group and immunosuppressive drug treatment group (36). With the progression of diseases and drug treatment, the microbiome tended to become more diverse. The fecal microbiome of SLE mice induced the production of anti-dsDNA antibodies and stimulated inflammation, and changed the expression of SLE susceptibility genes in germfree mice (38). Consistently, Choi et al. (39) demonstrated that when transferred to sterile syngeneic C57BL/6 mice, the intestinal microbes of triple congenic lupus-prone mice stimulated autoantibodies production and modulated immune cells. Intriguingly, the horizontal transfer of intestinal flora between co-bred triple congenic lupus-prone mice and syngeneic mice could mitigate the autoimmune pathogenesis.

However, even though most studies showed that SLE patients were usually accompanied by gut microbiome dysbiosis, it might only be a clinical sign of SLE and there was no causal effect on SLE risk and gut microbiome dysbiosis. First, the use of non-selective immunosuppressive agents in SLE patients could lead to alterations in gut microbiome. Second, the intestinal flora of patients with active and inactive SLE might be different, and many studies did not take into account grouping of patients. Third, the composition of gut microbiome might be different due to the inconsistency of gender ratio and ethnicities in different studies. Fourth, although studies found that SLE patients had the phenotype of gut microbiome dysbiosis, the results of changes in specific strains were not consistent. The existence of these uncertain factors obstructed the inference of the causal link between gut microbiome and SLE risk.

The main advantage of this study was that the implementation of MR approach diminished the interference of confounding factors and reverse causality on the results, which might be more convincing than observational studies. To the best of our knowledge, the study is the first MR analysis on this topic. However, some limitations should be mentioned. First, our study was unable to determine whether overlapping participants were involved in the exposure and outcome GWAS used in the two sample MR analyses. Nevertheless, the deviation from participants overlap could be minimized by the *F* statistic (40). Second, since the original research lacked demographic data (e.g., gender and race), further subgroup analysis was impossible. Third, in view of the biological plausibility and the multi-stage statistical process, applying a rigorous multiple testing correction would likely have been overly conservative, which may neglect potential strains that are causally related to SLE. Therefore, we did not account for multiple testing. Fourth, since the majority of participants in the GWAS were of European ancestry, extrapolation of the results of the study to other ethnic groups might be limited.

In summary, this MR study suggests causal effects of gut microbiome on SLE. Several types of intestinal bacteria identified in this study that potentially reduced the occurrence of SLE may have the prospects for the prevention and treatment of SLE.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

H-FP and D-QY conceived the presented idea. KX, PW, and ZX performed the computations and manuscript writing. Y-QH, Y-SH, YC, Y-TF, K-JY, and J-XH were involved in acquisition of data. JW, Z-DW, X-KY, and D-GW were involved in interpretation of data. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.667097/full#supplementary-material

Supplementary Figure 1 | Forest plot **(A)**, sensitivity analysis **(B)**, scatter plot **(C)**, and funnel plot **(D)** of the causal effect of *Eggerthella* on SLE risk.

Supplementary Figure 2 | Forest plot **(A)**, sensitivity analysis **(B)**, scatter plot **(C)**, and funnel plot **(D)** of the causal effect of *Lactobacillales* on SLE risk.

Supplementary Figure 3 | Forest plot **(A)**, sensitivity analysis **(B)**, scatter plot **(C)**, and funnel plot **(D)** of the causal effect of *Coprobacter* on SLE risk.

Supplementary Figure 4 | Forest plot **(A)**, sensitivity analysis **(B)**, scatter plot **(C)**, and funnel plot **(D)** of the causal effect of *Bacillales* on SLE risk.

Supplementary Figure 5 | Forest plot **(A)**, sensitivity analysis **(B)**, scatter plot **(C)**, and funnel plot **(D)** of the causal effect of *Lachnospira* on SLE risk.

Supplementary Figure 6 | Forest plot **(A)**, sensitivity analysis **(B)**, scatter plot **(C)**, and funnel plot **(D)** of the causal effect of the whole gut microbiome on SLE risk ($P < 5 \times 10^{-8}$).

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Accumulation of Senescent Neural Cells in Murine Lupus With Depression-Like Behavior

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Neuropsychiatric manifestations targeting the central, peripheral, and autonomic nervous system are common in systemic lupus erythematosus (SLE); collectively, these symptoms are termed neuropsychiatric SLE (NPSLE). Among a wide variety of neuropsychiatric symptoms, depression is observed in about 24-39% of SLE patients. Several cytokines and chemokines have been identified as biomarkers or therapeutic targets of NPSLE; in particular, the levels of type 1 interferons, TNFs, and IL-6 are elevated in SLE patient's cerebrospinal fluid (CSF), and these factors contribute to the pathology of depression. Here, we show that senescent neural cells accumulate in the hippocampal cornu ammonis 3 (CA3) region in MRL/lpr SLE model mice with depressive behavior. Furthermore, oral administration of fisetin, a senolytic drug, reduced the number of senescent neural cells and reduced depressive behavior in the MRL/lpr mice. In addition, transcription of several senescence and senescence-associated secretory phenotype (SASP) factors in the hippocampal region also decreased after fisetin treatment in the MRL/lpr mice. These results indicate that the accumulation of senescent neural cells in the hippocampus plays a role in NPSLE pathogenesis, and therapies targeting senescent cells may represent a candidate approach to treat NPSLE.

Keywords: systemic lupus erythematosus, senescence, depression, inflammation, SASP (senescence-associated secretory phenotype)

INTRODUCTION

Systemic lupus erythematosus (SLE) is a currently incurable autoimmune disease characterized by hyperactive immune cells, serum autoantibodies, and multiple organ damage involving the kidney, skin, vasculature, and brain (1). Neuropsychiatric manifestations targeting the central, peripheral, and autonomic nervous system are common in SLE; collectively, these symptoms are called

neuropsychiatric SLE (NPSLE). Up to 75% of patients experience central nervous system (CNS) involvement, and 60% of SLE patients experience autonomic symptoms (2–4). Among a wide variety of neuropsychiatric symptoms, depression is observed in about 24-39% of SLE patients (5). Various immune effectors contribute to SLE pathogenesis, including autoantibodies, cytokines, and cell-mediated inflammation (2, 4, 6, 7); however, the detailed mechanism underlying NPSLE remains largely unknown (2, 4, 6, 7).

Cellular senescence is a state of irreversible cell cycle arrest in which an adaptive response is induced by multiple stressors (8, 9). Although senescence serves as a defense mechanism that limits tumorigenesis to maintain tissue homeostasis, accumulation of senescent cells causes age-related disease and chronic inflammation in lung, kidney, heart, and muscle, through the secretion of pro-inflammatory molecules including cytokines, chemokines, and proteases; collectively, these factors are referred to as the senescence-associated secretory phenotype (SASP) (8–13). Prolonged exposure to the SASP leads to pathological changes that contribute to tissue and organ decline (8). Senescent cells contribute to the neurodegeneration and pathogenesis of the brain observed in Alzheimer's disease, Parkinson's disease, and multiple sclerosis (14–18). For example, in Alzheimer's disease model mice, astrocytes, microglia, and oligodendrocyte progenitor cells have features of senescence, and elimination of senescent cells *via* genetic or pharmacological treatment attenuates neuroinflammation and cognitive deficits (17, 18). Chronic neuroinflammation is one of the hallmarks of Parkinson's disease. The expression levels of pro-inflammatory factors and proteases, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), and interferon-gamma (IFN-γ) and metalloproteinase-3 (MMP-3), which are canonical SASP factors, are elevated in the brains of patients with Parkinson's disease (19). Furthermore, the number of senescent astrocytes and dopaminergic neurons is elevated, and these senescent cells have the potential to contribute to pathology (14, 15). Although the mechanism by which cellular senescence is linked to neurodegeneration is not fully understood, the accumulation of senescent cells may trigger a chronic inflammatory response that contributes to synapse damage and cognitive decline (20). NPSLE causes a disruption of the blood–brain barrier, which is directly caused by cytokines and complement proteins (21). Pro-inflammatory cytokines and chemokines related to neuroinflammation in NPSLE were identified in the cerebrospinal fluid (CSF) of SLE patients for use as biomarkers or therapeutic targets; in particular, the levels of type 1 interferons, TNFs, and IL-6 are elevated and contribute to the pathology of depression (4, 6, 8, 22). Overexpression of these pro-inflammatory factors in the CSF of NPSLE patients is hypothesized to cause cellular senescence in CNS; however, to the best of our knowledge, cellular senescence in the CNS has not been evaluated in patients with lupus (22–30).

In this study, we sought to determine the relationship between senescence and depression in SLE by investigating cellular senescence in the hippocampus, which is associated with depression (31–34), in MRL/*lpr* SLE model mice. In addition, we investigated whether senolytics, small molecules that selectively eliminate senescent cells, reduce the observed number of senescent cells and consequently reduce depression symptoms in MRL/*lpr* SLE model mice.

MATERIALS AND METHODS

Mice

The Committee of the Animal Experimentation Center of the Sapporo Medical University School of Medicine approved all animal protocols (#17-080 and #21-051). Mice were maintained in an enclosed, specific pathogen–free facility with a 12 h light and dark cycle. Female MRL/*lpr* mice were used as an SLE mouse model, and haplotype-matched female MRL/*MPJ* mice were used as phenotypic controls (Sankyo Lab Service, Tokyo, Japan). For pathological analysis, four MRL/*MPJ* mice and five MRL/*lpr* mice were used and euthanized at 18 weeks of age. For senolytic treatment, twenty-four MRL/*MPJ* mice and twenty-four MRL/*lpr* mice were used and euthanized at 22 weeks of age, and tissue samples were harvested.

Behavioral Analysis

The tail suspension test was performed to assess depression-like behavior (35–39). Mice were suspended by their tails with tape 60 cm above the floor for 6 min, and the time of immobility was measured. Each mouse was tested only once. The time of immobility was defined as the time when the animal stopped struggling for ≥ 1 s, which was measured using a video tracking system (ANY-maze; Muromachi Kikai, Tokyo, Japan).

Cell Culture and Senescence Induction

Neuro-2a cells (Cell No. IFO50081), which are a mouse brain–derived neuroblast cell line, were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and maintained in Eagle's Minimal Essential Medium with non-essential amino acids and 10% fetal bovine serum. Cells were tested for mycoplasma using the e-Myco Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Seongnam-si, South Korea). Cellular senescence was induced by X-ray irradiation. Neuro-2a cells were exposed to 10 Gy irradiation using an X-Ray Irradiator (MBR-1520-3; HITACHI, Tokyo, Japan), and 3 days later the cells were passaged to avoid confluency. Six days after irradiation, Neuro-2a cells were harvested and subjected to SA-β-Gal staining, PCR analysis, and pharmacological experiments. To detect cellular senescence, we performed senescence-associated β-Galactosidase (SA-β-Gal) staining using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA). Cells were observed using an inverted microscope (Primovert; ZEISS), and the percentage of SA β-Gal–positive cells was calculated by dividing the number of SA β-Gal–positive cells by the total number of cells observed. The cell size was measured using the ImageJ software (National Institutes of Health). Briefly, the cell body was outlined using the drawing/selection tools, and the area was measured using the analyze tool.

In Vitro Senolytic Treatment

For senolytic treatment, fisetin flavonoid, which is found in many fruits and vegetables and was previously identified as a senolytic compound (40), was used. Fisetin (S2298) was purchased from Selleck (Houston, TX, USA) and dissolved in DMSO before use. Irradiated senescent Neuro-2a cells and non-irradiated Neuro-2a cells were seeded on a 96-well black/clear bottom plate at 40,000 cells and 10,000 cells per well, respectively. After senescence induction for 6 days, 5μM, 10μM, or 20μM fisetin was added and the cells were incubated for 48 h. The concentration of fisetin used was based upon a previous study that reported its senolytic effects (40). Cell number and cellular senescence were determined by DAPI staining and SPiDER-β-Gal staining, respectively. Briefly, cells were washed twice with PBS, fixed in 4% paraformaldehyde at room temperature for 5 min, and washed twice with PBS. Sections were incubated in 20 μM SPiDER-β-gal (Dojindo) in solution in McIlvaine buffer (pH 6.0) for 60 min at 37°C. After washing of tissue sections, nuclei were stained with DAPI. Cells were observed using fluorescence microscopy (Axio Observer7; ZEISS).

In Vivo Senolytic Treatment

Eighteen-week–old MRL/*MPJ* mice (n = 24) and MRL/*lpr* mice (n = 24) were randomized for pharmacological treatment analysis. For oral administration, mice were gavaged with 100 mg/kg fisetin (Tokyo Chemical Industry, Tokyo, Japan) (MRL/*MPJ*: n = 12 and MRL/lpr: n = 12) or vehicle (20% PEG 400) (MRL/*MPJ*: n = 12 and MRL/*lpr*: n = 12) for 5 days every week for 4 weeks.

Immunohistochemistry and SPiDER-β-Gal Staining

Brain samples were fixed in 4% paraformaldehyde overnight. The following day, the tissues were transferred to 20% sucrose in phosphate buffer, incubated overnight, frozen in OCT compound in liquid nitrogen, and stored at −80°C until use. Cryosections (8 μm thick) were prepared using a cryostat. The sections were incubated in 0.01 M PBS containing 0.3% Triton-X (PBS-T) and treated with 2% BSA for 60 min at RT. After washing with 0.01 M PBS-T, the sections were incubated with primary antibodies at 4°C overnight, followed by secondary staining. Alexa Fluor 594–conjugated anti-GFAP (1:100; 644708; Biolegend), anti–Iba-1 (1:400; 019-19741; Wako, Osaka, Japan), and anti-NeuN (1:150; 2697501; Proteintech, Rosemont, IL, USA) were used as primary antibodies. Cy3-conjugated IgG (1:400; Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary antibody, and nuclei were stained with DAPI (1:1000; Dojindo). After washing, tissue sections were mounted with Vectashield (Vector Laboratories). For the SPiDER-β-gal stain, tissue sections were incubated in 20 μM SPiDER-β-gal (Dojindo) in solution in McIlvaine buffer (pH 6.0) for 60 min at 37°C. After washing of tissue sections, nuclei were stained with DAPI, and tissue sections were mounted with VECTASHIELD. Sections were observed by fluorescence microscopy [Axio Observer7 (ZEISS) or BZ-X700 (Keyence)].

RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated using the Tri Reagent (Molecular Research Center, Cincinnati, OH, USA), and RNA was reverse transcribed into cDNA using the iScript Advanced cDNA Synthesis Kit (1725038; Bio-Rad, Hercules, CA, USA). Quantitative PCR was performed using SsoAdvanced Universal SYBR Green Supermix (172-5270; Bio-Rad) on a QuantStudio3 Real-Time PCR System (Applied Biosystems). Cycling conditions were as follows: 95°C for 20 s, followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). Transcription levels were normalized against the corresponding levels of housekeeping genes listed in **Supplementary Table 1**. Specific primer sequences used for PCR are listed in **Supplementary Table 1**. The ΔΔCt method was used to compare data.

Statistical Analysis

Quantitative data are shown as means and standard errors in dot plots generated by ggplot2, a plotting system for R based on The Grammar of Graphics (The R Foundation for Statistical Computing, Vienna, Austria). Normality was assessed using the Shapiro–Wilk test. The pairwise t-test was used for comparison between two groups, and a one-way analysis of variance (ANOVA) was conducted to assess differences among three groups or more. Pairwise comparisons were made only when one-way ANOVA indicated statistical significance. P-values for multiple comparisons were adjusted by the Tukey method. Statistical analyses were performed using EZR, a graphical user interface for R (41). Two-sided P-values less than 0.05 were considered statistically significant.

RESULTS

MRL/lpr Mice Exhibit a Depression-Like Phenotype and Neuroinflammation in the Hippocampus

We used 18-week-old MRL/*lpr* mice as a SLE model and MRL/*MPJ* mice as controls. The presence of depression-like behavior was evaluated by tail suspension test. The immobility time observed during the tail suspension test was significantly elevated in MRL/*lpr* mice relative to the MRL/MPJ mice, which indicated that the MRL/*lpr* mice exhibited a depression-like phenotype (**Figures 1A, B**). To determine whether MRL/*lpr* mice exhibited neuroinflammation, we counted GFAP-positive astrocytes and Iba-1–positive microglia in the hippocampus, which are regions that may be important in regulation of emotion in brains of MRL/*lpr* mice (42). MRL/*lpr* mice had more GFAP-positive astrocytes in the cornu ammonis 3 (CA3) region than MRL/*MPJ* mice (**Figures 1C, D**; P=0.009). In the dentate gyrus (DG) region, the number of GFAP-positive astrocytes did not significantly differ between the MRL/*lpr* and MRL/*MPJ* mice (**Figures 1E, F**; P=0.108). Senescent cells contribute to neuroinflammation (14–18, 43); therefore, we

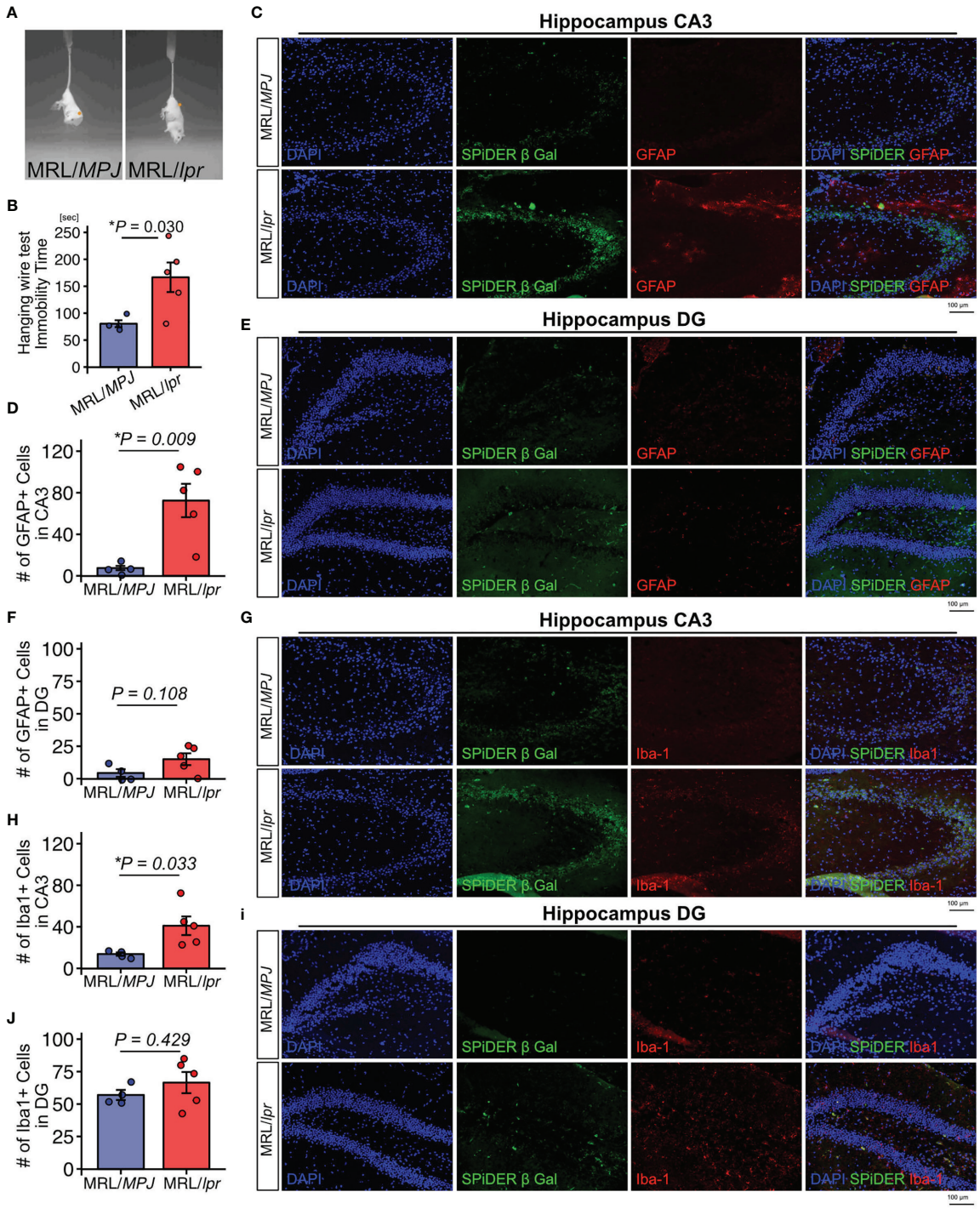

FIGURE 1 | MRL/*lpr* mice exhibit a depression-like phenotype and have higher proportions of GFAP-positive and Iba-1–positive cells in the hippocampus. **(A)** Representative images of tail suspension test of MRL/*lpr* mice (SLE model) and MRL/*MPJ* mice (controls). **(B)** Quantitation of immobility time in MRL/MPJ and MRL/lpr mice during tail suspension test. **(C)** Representative images of GFAP immunostaining of the hippocampus CA3 regions from MRL/*MPJ* and MRL/*lpr* mice and **(D)** the corresponding quantitative data. **(E)** Representative images of GFAP immunostaining of the hippocampus DG regions from MRL/*MPJ* and MRL/*lpr* mice and **(F)** the corresponding quantitative data. **(G)** Representative images of Iba-1 immunostaining of the hippocampus CA3 regions from MRL/*MPJ* and MRL/*lpr* mice and **(H)** the corresponding quantitative data. **(I)** Representative images of Iba-1 immunostaining of the hippocampus DG regions from MRL/*MPJ* and MRL/*lpr* mice and **(J)** the corresponding quantitative data. Quantitative data are shown as means ± SEs in dot plots. P-values were determined by two-tailed Student's t-test. (*P < 0.05)
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counted the number of senescent cells, using the marker SPiDER-β-Gal, in GFAP-positive astrocytes. We could not identify SPiDER-β-Gal–positive astrocytes in either MRL/*lpr* or MRL/*MPJ*. The number of Iba-1 positive microglia was higher in MRL/*lpr* mice than in MRL/*MPJ* mice in the CA3 region (**Figures 1G, H**; P = 0.033), but not in the DG region (**Figures 3C, D**; P = 0.429). Small numbers of SPiDER-β-Gal–positive microglia were present in both of MRL/*lpr* and MRL/*MPJ* mice; however, microglia were not the major population of SPiDER-β-Gal–positive senescent cells (**Figures 1I, J**).

MRL/lpr Mice Have a Higher Proportion of Senescent NeuN+ Cells in CA3 Hippocampus

Next, to determine whether neurons exhibited features of senescence, we performed SPiDER-β-Gal staining and immunofluorescence with a NeuN antibody. SPiDER-β-Gal intensity in NeuN-positive cells was significantly higher in MRL/*lpr* mice than in MRL/MPJ mice in the hippocampus CA3 region (**Figures 2A–C**; P = 0.037). By contrast, in the DG region, SPiDER β-Gal expression was not detectable in either MRL/*lpr* or MRL/*MPJ* mice (**Figure 2B**).

Neuro-2a Cells Induced to Senesce by Irradiation Exhibit a Neuroinflammatory Phenotype

These histological analyses indicated that neural cells in the CA3 region were a major population of senescent cells in lupus model mice with a depression-like phenotype. We next investigated whether senescent neural cells exhibited the features of cells that induce neuroinflammation in NPSLE. To induce senescence, we exposed Neuro-2a cells to 10 Gy irradiation and passaged them 3 days later to avoid the over-confluency that occurs post-irradiation due to Neuro-2a cell enlargement. Six days after irradiation, we harvested the cells and subjected them to SA-β-Gal staining and quantitative PCR analysis. Irradiated Neuro-2a cells exhibited senescent features including SA-β-Gal expression (**Figures 3A, B**), elevated cell size (**Figure 3C**), and upregulation of *Cdkn2a* (Ink4a and Arf), *Cdkn2b*, *Cdkn1a*, and *Trp53* (**Figure 3D**). The irradiated Neuro-2a cells also expressed high levels of genes encoded by SASP factors, including *Tnfa*, *Serpine1*, *Il6*, and *Il1b*, all of which are also upregulated in NPSLE (**Figures 3E, F**) (4).

Fisetin Treatment Selectively Kills SPiDER-β-Gal–Positive Senescence Neural Cells *In Vitro*

Next, we investigated whether the senolytic drug fisetin would selectively kill senescent neural cells. Fisetin, a flavonoid found in many fruits and vegetables, was previously identified as a senolytic compound (40). In addition, fisetin exhibits brain uptake potential and penetrates the blood–brain barrier more effectively than other flavonoids, including quercetin, luteolin, and myricetin (44–46). Hence, we used fisetin as a senolytic

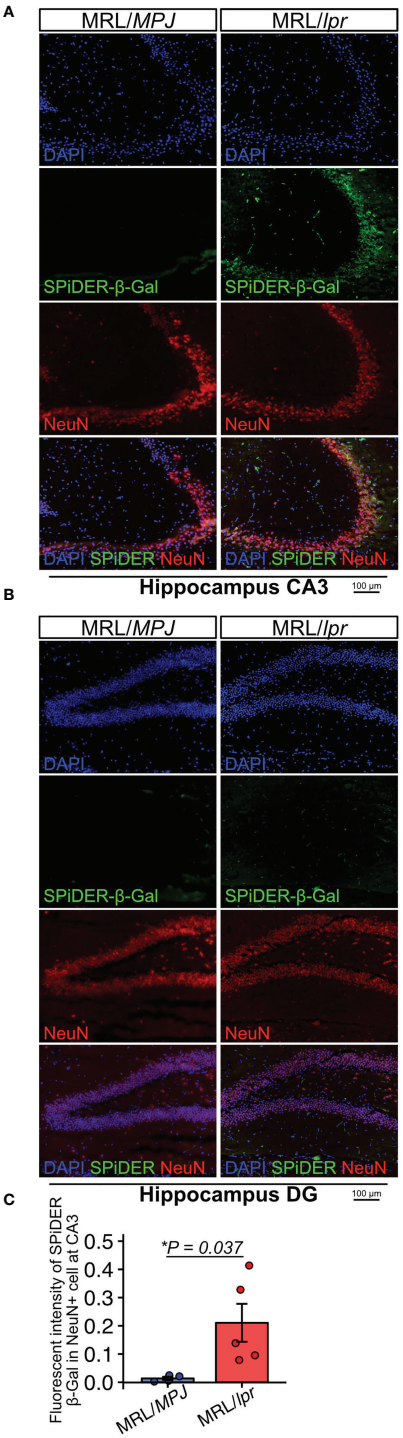

FIGURE 2 | MRL/lpr mice have higher numbers of SPiDER-β-Gal– and NeuN-positive cells in the hippocampus. **(A, B)** Representative images of NeuN immunostaining and SPiDER-β-Gal staining of the hippocampus CA3 and DG regions in MRL/*MPJ* and MRL/*lpr* mice. **(C)** Quantitation of SPiDER-β-Gal intensity in NeuN-positive cells in MRL/*MPJ* and MRL/*lpr* mice. Quantitative data are shown as means ± SEs in dot plots. P-values were determined by two-tailed Student's t-test. (*P < 0.05).

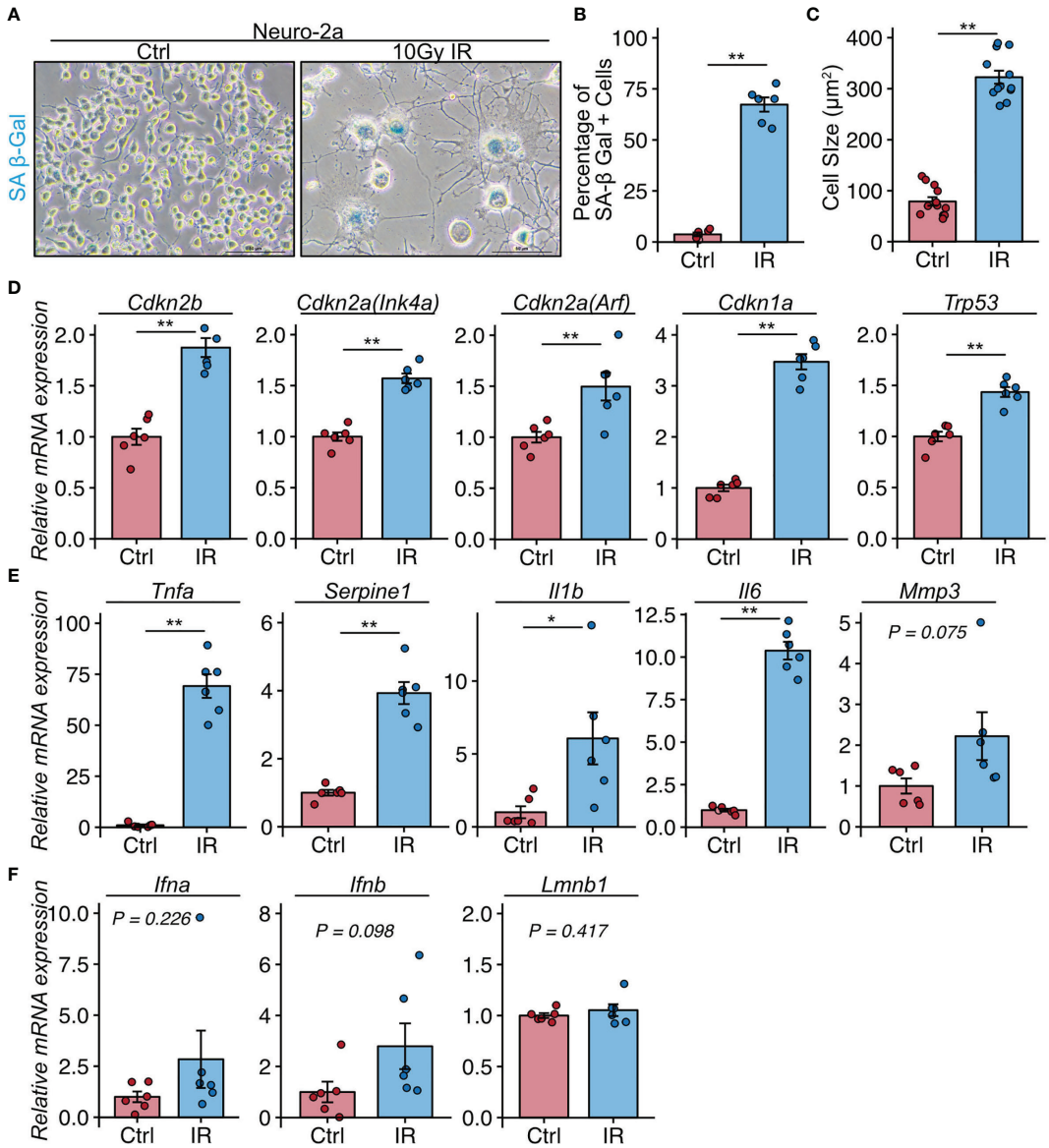

FIGURE 3 | Neuro-2a cells induced to undergo senescence by irradiation exhibit a neuroinflammatory phenotype. **(A)** Representative images of SA-β-Gal expression after 10 Gy ionizing radiation (IR) in Neuro-2a cells in randomly chosen fields of view (n = 6 per group). **(B, C)** Quantitation of SA-β-Gal–positive cells and the cell size. **(D–F)** Relative mRNA expression of senescence and SASP-related genes in Neuro-2a cells with or without 10 Gy IR. Quantitative data are shown as means ± SEs in dot plots. P-values were determined by two-tailed Student's t-test. (*P < 0.05, **P < 0.01).

compound in this study. Unirradiated and irradiated Neuro-2a cells were treated with serial concentrations of fisetin (0–20 μM) for 48 h. The observed number of irradiated Neuro-2a cells significantly decreased in a dose-dependent manner (**Figures 4A, B**), and the number of control Neuro-2a cells significantly decreased after treatment with 20 μM fisetin (**Figures 4A, B**). SPiDER-β-Gal expression in irradiated Neuro-2a cells was significantly reduced at doses of 5, 10, and 20 μM (**Figure 4C**). Doses of 5 and 10 μM fisetin decreased the fraction of SPiDER-β-Gal–positive senescent Neuro-2a cells without affecting non-irradiated proliferating cells.

Fisetin Treatment Reduced the Prevalence of the Depression-Like Phenotype and Number of Senescent Cells *In Vivo*

To examine the senolytic effect of fisetin *in vivo*, we orally administered fisetin (100 mg/kg) or 20% PEG400 (as a control) to MRL/*lpr* (n=12 and 12, respectively) and MRL/MPJ (n=6 and 6, respectively) mice for 5 days every week for 4 weeks (**Figure 5A**). During this 4-week period, two MRL/lpr mice in the vehicle group died. Fifty percent of MRL/lpr mice die from renal failure by 24 weeks of age (47). After this 4-week period, we found that fisetin treatment reduced the prevalence of

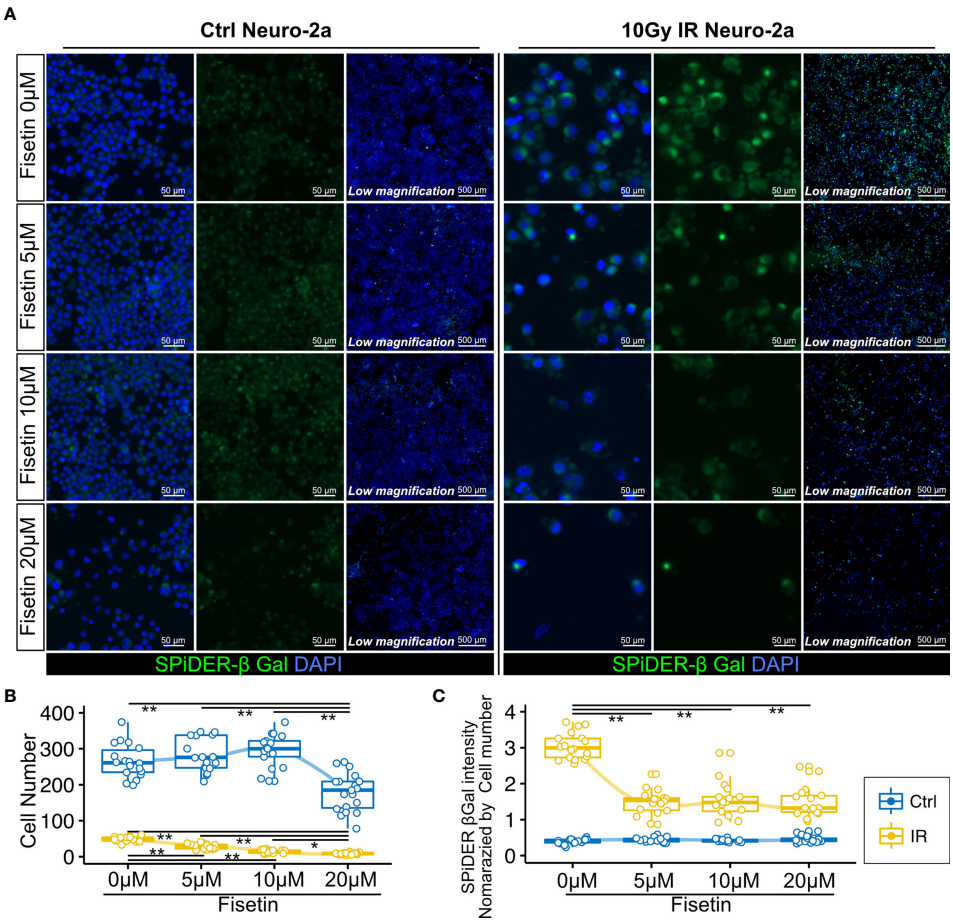

FIGURE 4 | Effect of fisetin treatment on senescent Neuro-2a cells *in vitro*. **(A)** Representative images of SPiDER-β-Gal and F-actin staining in Neuro-2a cells with or without 10 Gy IR. **(B, C)** Quantitation of cell number and SPiDER-β-Gal intensity in Neuro-2a cells treated with the indicated concentrations of fisetin (0–20 μM) for 48 h. Quantitative data are shown as medians with IQRs and 1.5 times the IQR, and are displayed as dot plots and box-and-whisker plots. P-values were determined by one-way ANOVA adjusted by the Tukey method. P-values were determined by two-tailed Student's t-test. (*P < 0.05 and **P < 0.01).

depression-like behavior in the MRL/*lpr* mice (**Figure 5B**). Fisetin treatment also reduced the observed SPiDER-β-Gal expression level in NeuN+ cells in the CA3 region (**Figures 5C, D**). PCR analysis showed that fisetin treatment reduced the mRNA transcription levels of *Cdkn1a* and *Cdkn2a (Arf)*, which are senescence factors, and *Ifna* and *Ifnb*, which are known SASP factors, in the hippocampus of the MRL/*lpr* mice (**Figures 5E, F**). In addition, vehicle-treated MRL/*lpr* mice exhibited significantly increased levels of Trp53, Il6, and Mmp3 mRNA transcription relative to the vehicle-treated MRL/MPJ mice, but no significant difference was observed for the fisetin-treated MRL/*lpr* mice relative to the vehicle-treated MRL/MPJ mice (**Figures 5E, F**).

DISCUSSION

Senescent cells limit their own proliferation but remain metabolically active, secreting a variety of factors including: inflammatory cytokines such as IL-6, IL-8, and TNF-α; chemokines; growth factors such as TGF-β; matrix metalloproteinases (MMPs); and micro-RNAs. Collectively, these secreted factors are referred to as the SASP (48). The SASP is considered a hallmark of cellular senescence when combined with other senescence markers such as the cytoplasmic marker SA-β-gal and the nuclear biomarkers $p16^{INK4a}$, $p21^{WAF1/Cip1}$, Ki67, and γH2AX (48–50). In this study, we show that MRL/lpr lupus-prone mice accumulate senescent NeuN-positive cells in the hippocampus. In addition, neural cells induced to undergo senescence increased mRNA expression of genes encoding SASP-related factors such as *Tnfa*, *Serpine1*, *Il6*, and *Il1b*, all of which are elevated in NPSLE (4). Because neurons are post-mitotic, non-cycling cells (those permanently in the G0 phase of the cell cycle), neuronal senescence, like that observed in other post-mitotic cells, relies on mechanisms other than proliferation arrest. Although it is not a fully specific marker, SA-β-Gal is considered to be a useful marker of cellular senescence, and the number of SA-β-Gal–

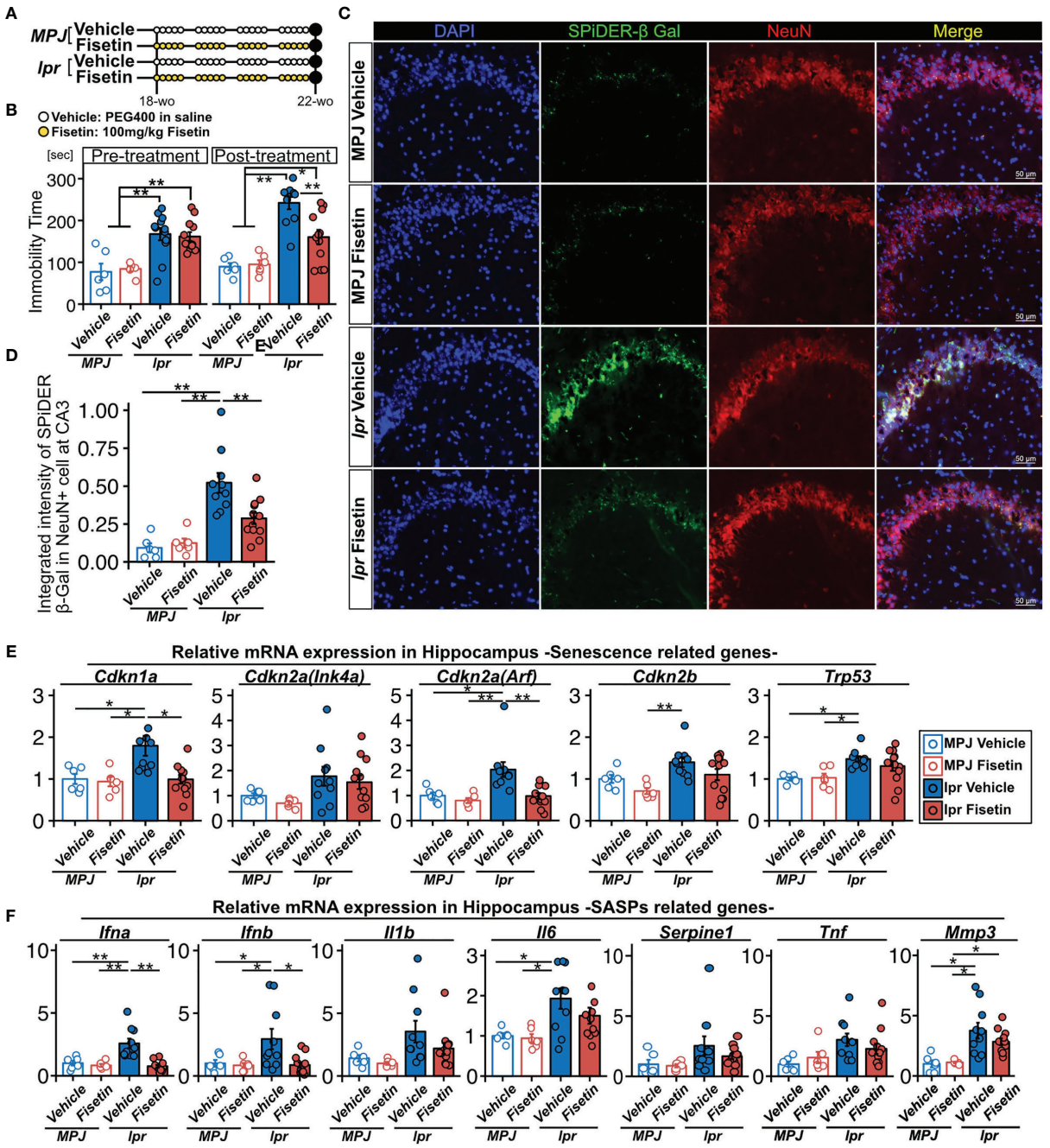

FIGURE 5 | Effect of fisetin treatment on depression-like behavior in MRL/*lpr* mice. **(A)** Schematic diagram of the procedure for oral administration of fisetin to MRL/lpr and MRL/MPJ mice. **(B)** Quantification of the immobility time from the tail suspension test for MRL/*MPJ* and MRL/*lpr* mice with or without fisetin treatment. **(C)** Representative images of NeuN SPiDER-β-Gal immunostaining of the hippocampus CA3 regions and **(D)** the corresponding quantification of SPiDER-β-Gal intensity in NeuN-positive cells in MRL/*MPJ* and MRL/*lpr* mice with or without fisetin treatment. Relative mRNA transcription levels of **(E)** senescence- and **(F)** SASP-related genes in hippocampus isolated from MRL/*MPJ* and MRL/*lpr* mice with or without fisetin treatment. Quantitative data are shown as means ± SEs in dot plots. P-values were determined by two-tailed Student's t-test. (*P < 0.05 and **P < 0.01).

positive neurons increases in aging mice and rats (51, 52). Furthermore, long-term culture–induced senescent neuronal cells exhibit elevated transcription levels of SASP genes (53). Several cytokines and chemokines were identified as biomarkers or therapeutic targets of NPSLE; in particular, type-1 interferons, TNFs, IL-6, and PAI-1, which are major components of the SASP, are present at elevated levels in the CSF of SLE patients (4, 6, 54, 55). Our results showed that the hippocampus isolated

from MRL/*lpr* mice and irradiated senescent Neuro-2a cells exhibit upregulation of the transcription levels of SASP factors, supporting the idea that senescent neural cells contribute to the elevation of cytokines and chemokines in CSF.

In this study, the SA-β-Gal–positive senescent neural cells were observed in the CA3 region of the hippocampus, which is associated with depression (31–34). SLE and MRL/*lpr* mouse brain have an elevated population of damaged neural cells that express Fluoro Jade B (FJB) and also exhibit upregulation of ubiquitin in the CA3 region (56, 57). FJB dye is an anionic fluorescein derivative used for visualization of neuronal degeneration in brain tissue sections (58, 59), and ubiquitin binds to damaged or misfolded proteins (60). Most protein damage is not reversible, and degradation by the ubiquitin–proteasome system (UPS) eliminates damaged proteins (50, 61). Activation of the UPS is a key characteristic of the senescent state (50, 62).

We also demonstrated that fisetin exerts a potent senolytic effect in neural cells *in vivo* and *in vitro*. Several senolytic compounds have been reported, e.g., flavonoids, quercetin, curcumin, and luteolin (12, 13, 40). We used fisetin to target CNS senescence because it has higher brain uptake potential and more effective blood–brain barrier penetration than other flavonoids such as quercetin, luteolin, and myricetin (44–46). *In vivo*, fisetin treatment reduced the observed depression-like behavior in the mice and the number of senescent cells in the CA3 region of the hippocampus. In this study, fisetin treatment also reduced the transcription levels of several senescence- and SASP-related genes in the hippocampus. For example, the transcription level of the senescence gene *Cdkn1a* markedly increased in vehicle-treated MRL/*lpr* mice, and the level decreased after fisetin treatment. The number of p21-expressing NeuN-positive cells increases in older depressed patients relative to non-depressed older patients (63). In our study, type-I interferons, known SASP factors, are upregulated in MRL/lpr mice. Therapeutic administration of type-I interferons to mice with hepatitis C or other malignancies induces SLE-like psychiatric symptoms, including sickness behavior associated with depression, and inhibition of the type-I interferon receptor reduces anxiety-like behavior and cognitive deficits in lupus-prone mice (4, 54). If neural senescent cells produce type-I interferons, thereby exacerbating the development of NPSLE, senolytics targeting the causative cells may be effective treatments for NPSLE. IL-6 is a known pro-inflammatory SASP factor (8–13) that is upregulated in the hippocampus of MRL/lpr mice. The level of IL-6 observed in CFS is higher in NPSLE patients with an acute confusional state (ACS), which includes anxiety disorders, cognitive dysfunction, mood disorders, and psychosis, relative to those with diffuse NPSLE, states other than ACS, or those with focal NPSLE, which suggests that the IL-6 level observed in CFS may indicate the severity of NPSLE (64, 65). In this study, fisetin administration reduced the high transcription level of IL-6 in the hippocampus of MRL/lpr mice. Fisetin treatment causes a reduction of the transcription level of IL-6 in senescent cells in pulmonary fibrosis and aging-related pathology (40, 66). Although the varied and complex pathogenic pathways complicate the development of NPSLE therapies, our data indicate that fisetin treatment targeted specifically to NPSLE senescent neural cells results in inhibition of SASP factors such as type-I interferon and IL-6, suggesting that fisetin is a candidate NPSLE therapeutic. Fisetin not only has potential as a senolytic in neuronal cells, but also acts as a neuroprotective agent *via* its antioxidant, antitumor, anti-inflammatory, and anti-apoptosis effects (45, 67, 68). Hence, fisetin could be a candidate drug for CNS disorders by targeting neural cell populations. Our findings indicate that neural cells are a major population of senescent cells in the lupus-prone mouse model, whereas other studies reported that the major populations of senescent cells in Alzheimer's model mice are astrocytes, microglia, and oligodendrocyte progenitor cells (17, 18). Furthermore, those studies used other senolytic compounds, dasatinib and quercetin (D+Q) and ABT263, to treat Alzheimer's model mice, and administration of both senolytic compounds alleviated cognitive deficits and decreased the abundance of senescent cells in the brain (17, 18). Further study will be needed to identify the most effective senolytic compound for NPSLE.

In conclusion, our study highlights the accumulation of senescent neural cells in hippocampus of lupus-prone model mice. Oral administration of fisetin, a senolytic drug, reduced the number of senescent neural cells observed, the SASP expression level, and depressive behavior in MRL/lpr mice. These results indicate that the accumulation of senescent neural cells in the hippocampus plays a role in NPSLE pathogenesis, and therapies targeting senescent cells may represent candidate therapeutics for the treatment of NPSLE.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by The Committee of the Animal Experimentation Center of the Sapporo Medical University School of Medicine.

AUTHOR CONTRIBUTIONS

YS: Conception and design, Collection and/or assembly of data, Manuscript writing, MM: Collection and/or assembly of data, SY: Data collection, TS: Data collection, NM: Data collection, MF: Manuscript writing, TSC: Conception and design,

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N-Formyl Methionine Peptide-Mediated Neutrophil Activation in Systemic Sclerosis

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Exaggerated neutrophil activation and formation of neutrophil extracellular traps (NETs) are reported in systemic sclerosis (SSc) but its involvement in SSc pathogenesis is not clear. In the present study we assessed markers of neutrophil activation and NET formation in SSc patients in relation to markers of inflammation and disease phenotype. Factors promoting neutrophil activation in SSc remain largely unknown. Among the neutrophil activating factors, mitochondrial-derived N-formyl methionine (fMet) has been reported in several autoinflammatory conditions. The aim of the current study is to assess whether SSc patients have elevated levels of fMet and the role of fMet in neutrophil-mediated inflammation on SSc pathogenesis. Markers of neutrophil activation (calprotectin, NETs) and levels of fMet were analyzed in plasma from two SSc cohorts (n=80 and n=20, respectively) using ELISA. Neutrophil activation assays were performed in presence or absence of formyl peptide receptor 1 (FPR1) inhibitor cyclosporin H. Elevated levels of calprotectin and NETs were observed in SSc patients as compared to healthy controls ($p<0.0001$) associating with SSc clinical disease characteristics. Further, SSc patients had elevated levels of circulating fMet as compared to healthy controls ($p<0.0001$). Consistent with a role for fMet-mediated neutrophil activation, fMet levels correlated with levels of calprotectin and NETs ($r=0.34$, $p=0.002$; $r=0.29$, $p<0.01$ respectively). Additionally, plasma samples from SSc patients with high levels of fMet induced *de novo* neutrophil activation through FPR1-dependent mechanisms. Our data for the first time implicates an important role for the mitochondrial component fMet in promoting neutrophil-mediated inflammation in SSc.

Keywords: systemic sclerosis, autoimmunity, neutrophils (PMNs), neutrophil extracellular traps (NET), mitochondrial peptides, clinical biomarkers

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INTRODUCTION

Systemic sclerosis (SSc) is a rare chronic autoimmune disease characterized by vasculopathy, inflammation, and fibrosis of the skin and internal organs. Depending on the distribution of skin fibrosis, SSc is clinically subdivided into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) (1, 2). The pathogenesis of SSc is complex and the exact etiology of the disease is still

unknown. The role of the adaptive immune system with autoreactive T and B cells producing autoantibodies in SSc pathogenesis has been well established (3, 4). Additionally, several studies have suggested the involvement of the innate immune system in early pathogenic events in SSc but this has not been explored in detail (5).

Among the innate immune cells, neutrophils are prominent contributors of inflammation in several autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), and have also been implicated in SSc pathogenesis (6–9). While neutrophils play a protective role against invading pathogens through reactive oxygen species (ROS) production, phagocytosis and formation of neutrophil extracellular traps (NETs) (10, 11), unrestrained neutrophil activation may lead to inflammation, immune dysregulation and tissue damage. Neutrophils are the most abundant immune cells in the circulation, and in SSc patient's blood neutrophil counts are elevated and correlated with disease severity (12). Neutrophils were also increased in skin biopsies obtained from forearm lesions in SSc patients compared with controls (13). Moreover, neutrophil involvement in induction of endothelial cell apoptosis, an early event leading to fibrosis, has been reported in SSc (14). These studies indicate a critical pathogenic or modulatory role of neutrophils in SSc.

Markers of neutrophil activation, such as elevated levels of circulating calprotectin and NET formation have been previously reported in several autoimmune disorders and evolved as excellent biomarkers for inflammatory processes (6, 8, 15). Calprotectin (S100A8/A9) is a calcium-binding protein abundantly expressed in neutrophils and is essential for initiating immune responses to non-infectious inflammatory processes (16, 17). NET formation is the key feature of neutrophil cell death in which nuclear DNA is extruded together with cytoplasmic and granular contents in web-like structures that can function to entrap and eliminate extracellular pathogens (18). Elevated levels of neutrophil activation markers during an inflammatory response may be detrimental and cause bystander injury that could be perpetual or non-resolving. Among these markers, separate studies have found increased soluble levels of calprotectin, an acute phase protein expressed by neutrophils and NETs in SSc patients and associations with SSc disease manifestations were also reported in these studies (19–22). Additionally, filaments of DNA coming from elastase positive cells, such as neutrophils can be seen in SSc skin (23). Although these studies point to a role of neutrophils in SSc, a comprehensive study of neutrophil activation markers in the circulation of SSc patients and its contribution to inflammation and disease phenotype needs to be carefully addressed.

So far, factors contributing to neutrophil-mediated activation in a sterile pro-inflammatory environment like SSc remains largely unknown. During inflammation, neutrophils are activated by various molecules, including cytokines, immune complexes (ICs) and damage-associated molecular patterns (DAMPs) generated by mitochondrial components (11, 24, 25). We and others have reported mitochondrial extrusion and presence of extracellular mitochondrial components in several autoimmune disorders such as SLE and RA (26–28). Given their prokaryotic origin, mitochondria contain several pro-inflammatory components that can engage neutrophils. Among the mitochondrial protein-derived molecules, N-formyl-methionine (fMet) is a potent neutrophil chemoattractant that can trigger a variety of neutrophil functions leading to tissue damage. fMet acts through formyl peptide receptor-1 (FPR1), which is abundantly expressed on neutrophils and when bound by its cognate ligand induces inflammation (29–32). However, its role remains to be explored in SSc.

In the current study, we first assessed the levels of neutrophil activation markers in two independent SSc cohorts and investigated their association with markers of inflammation and SSc disease phenotype. Secondly, we analyzed levels of fMet in relation to neutrophil activation levels. Finally, we assessed whether fMet could contribute to neutrophil activation through FPR1-mediated signaling in SSc patients. Briefly, neutrophil activation markers were elevated in SSc patients and associated with a disease phenotype. We also made the novel observation that elevated fMet levels were associated with neutrophil activation in SSc patients. Finally, plasma samples from SSc patients induced *de novo* neutrophil activation through an FPR1-dependent mechanism suggesting FPR1 as a potential novel therapeutic target in these patients to reduce neutrophil-mediated inflammation.

MATERIALS AND METHODS

Patient Cohorts and Ethical Statement

Plasma samples from two independent SSc cohorts were analyzed in the current study. The distributions of the sex, age, ethnicity, disease subgroups, prevalence of anti-centromere, anti-topoisomerase, anti-RNA Polymerase III, anti-survival of motor neuron 1 (SMN1) and anti- U11/12 ribonucleoprotein (RNP-C3) autoantibodies and clinical characteristics among the SSc patients are detailed in **Table 1**.

Cohort I comprised 80 SSc patients including 13 who presented for evaluation for autologous hematopoietic cell transplantation (HCT) due to severe SSc and 67 patients were recruited from Rheumatology practices primarily in the Seattle, Washington area, with some patients from Alaska, Montana, Oregon and other states. Patients presenting for HCT had diffuse severe SSc (n=11 autologous HCT, n=2 allogeneic HCT), criteria including modified Rodnan skin score ≥16, SSc 3 years or less from onset of first non-Raynaud symptom, and either FVC or DLCO <70% of predicted, or myocardial disease, or history of proteinuria >500mg/124hr as evaluated by transplant protocols with further details provided for autologous HCT in prior publication (33). Patients recruited from clinical Rheumatology practices were assessed through a combination of medical record review, patient administered questionnaires and, when information was insufficient, direct contact with the rheumatologist consultant. Cohort I also included 40 healthy controls recruited from Seattle WA and the surrounding area.

Cohort II consisted of 20 SSc patients and 24 healthy controls recruited from Skane University Hospital, Lund, Sweden. Age- and gender-matched healthy individuals were recruited to

TABLE 1 | Demographic and clinical information on disease and control groups.

Cohort	SSc 1	SSc 2	HC 1	HC 2
Patients (#)	81	20	40	24
Specimen	Plasma	Plasma	Plasma	Plasma
Age in years (Median, range)	47 (20-80)	67 (19-82)	57 (26-71)	35 (18-67)
Disease duration (Median, range)	4.5 (0-29)	8.5 (0-19)	N/A	N/A
Gender (% female)	100	80	85	88
Ethnicity (White, %)	72	95	100	96
lcSSc (%)	31	60	N/A	N/A
dcSSc (%)	65	40	N/A	N/A
ACA+ (%)	23	25	N/A	N/A
Scl70 (%)	20	25	N/A	N/A
RNAPIII (%)	28	15	N/A	N/A
SMN1 (%)	3	N/D	N/A	N/A
p53 (%)	4	N/D	N/A	N/A
Ro52 (%)	16	N/D	N/A	N/A
PM-Scl-75	9	N/D	N/A	N/A
PM-Scl-100	8	N/D	N/A	N/A
Th/To	13	N/D	N/A	N/A
U11/12 RNPC3	16	N/D	N/A	N/A
Skin score	ND	4 (0-43)	N/A	N/A
Telangiectasia (%)	ND	40	N/A	N/A
Digital pitting scar (%)	ND	45	N/A	N/A
NT-pro-BNP	ND	226 (0-1342)	N/A	N/A

lcSSc, Limited cutaneous scleroderma; dcSSc, Diffuse cutaneous scleroderma. No data on lcSSc or dcSSc manifestation in 4% SSc patients from (Cohort I). ACA: Anti-centromere Abs. Scl70: Anti-topoisomerase Abs.
RNAPIII: Anti-RNA Polymerase III. SMN1, Anti-survival of motor neuron 1; U11/12 (RNPC3), Anti-Ribonucleoprotein.

participate in the research studies. Cohort II was included to validate key findings from Cohort I, as well as assess clinical associations. Patients were classified according to the 2013 ACR classification criteria (34) and were stratified as having lcSSc or dcSSc according to the extent of skin involvement. All patients from Cohort II underwent evaluation by ECG, echocardiography, cineradiography of esophagus, high resolution computed tomography of the lungs and pulmonary function tests (spirometry). The patients also had a measurement of serum NT-pro-BNP. During the clinical assessment, the modified Rodnan skin score was used, and clinicians noted the presence or absence of digital pitting scars, ulcers, and telangiectasia. All clinical characteristics are summarized in **Table 1**.

The Cohort I and II used in our study are quite different clinically. The SSc patient population from Cohort I who were being evaluated for HCT is atypical from what is routinely seen in clinic as it is enriched for patients with severe and rapidly progressive SSc. Thus, Cohort I, unlike Cohort II, is skewed to diffuse SSc with more severe disease. In addition, Cohort I differs from Cohort II for ethnicity % and gender % (100% of Cohort I are female) (**Table 1**).

The study was approved by the Institutional Review Board of Fred Hutchinson Cancer Research Center (part of the University of Washington Consortium) and Lund University #2010/544. Informed written consent was obtained from all study participants according to the Declaration of Helsinki.

ELISA-Based Methods

Levels of circulating calprotectin were analyzed using a commercial ELISA kit according to manufacturer's instructions (R&D Systems, Minneapolis MN, USA). Circulating NETs were quantified using a myeloperoxidase (MPO)-DNA ELISA, as described previously (6, 26). Briefly, 96-well microtiter plate (Corning) was coated with anti-MPO antibody (4 μg/ml; Bio-Rad Laboratories, Hercules, CA, USA) overnight at 4°C, followed by blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2 hours at RT. After blocking, plasma samples (1:1000 dilution in 1% BSA in PBS with 2mM EDTA) were added and incubated overnight at 4°C. Anti-DNA-HRP from Cell Death Detection ELISA kit (clone MCA-33; Roche) was added as secondary antibody for 1.5 hour at RT. The reaction was developed with 3,3′,5,5′ tetramethylbenzidine (TMB; BD Biosciences) and ended by the addition of 2N sulfuric acid. Known concentrations of MPO-DNA complexes (rhMPO, R&D Systems; Calf thymus DNA, Trevigon) were used to construct a standard curve. Plasma levels of human formyl methionine (fMet) were analyzed using a commercial ELISA kit according to manufacturer's instructions (My BioSource Inc., San Diego, CA, USA). Absorbance for all ELISA assays were measured at 450 nm with a Synergy plate reader (BioTek).

Neutrophil Activation Assay

Neutrophils were isolated from healthy subjects by layering heparinized blood on Polymorphprep (Axis-Shield, Dundee, UK) density gradient, according to the manufacturer's instructions, or as described previously (7). Red blood cells were lysed with RBC lysis buffer (BioLegend, San Diego, CA USA). For *in vitro* assays, neutrophils were re-suspended in serum-free RPMI-1640 medium (ThermoFisher). Neutrophils were plated at 2.5 - 3 × 10^5 cells/well and were incubated with or without a selective inhibitor of FPR1, cyclosporin H (CsH, 5 μM) for 30 min prior to the addition of stimuli. Antibodies directed against the human FcγRII (CD32) (5 μg/ml; Caprico Biotechnologies, Norcross, GA USA) was also added for 30 min before addition

of stimuli. As stimuli, R848 (2.5 μg/ml), SSc plasma having either high or low levels of fMet (n=15 each) and healthy control plasma (n=6) (1:50 dilution) from Cohort I were used and incubated with the neutrophils for an additional 2 hours. Non-activated neutrophils were used as negative controls. Approximately 90-95% of neutrophils were viable after neutrophil stimulation with plasma samples. Additionally, quantification of neutrophil DNA release (NETosis) were measured as described previously (26). No/low level of NET formation was evident from neutrophils incubated with HC and SSc plasma during the neutrophil activation assays. Neutrophil activation was assessed by analyzing cell surface expression of CD66b (clone G10F5, BioLegend) and CD11b (clone CBRM1/5, BioLegend) by flow cytometry. Data were analyzed by FlowJo (Tree Star Inc, Ashland, OR) and results were presented as relative mean fluorescent intensity (MFI) % of CD66b and CD11b relative to healthy controls (set as 100%). % Inhibition was calculated as 1-(plasma induced activation marker MFI-100)/(plasma induced activation marker MFI in presence of CsH-100) x 100. This value was further subtracted by 100 as a baseline based on healthy controls (set as 100%).

Statistical Analysis

For sample sets with a non-Gaussian distribution, non-parametric tests, Mann-Whitney U test and Spearman's correlation test were used when applicable. For plasma-mediated neutrophil activation studies, Mann-Whitney U test or Wilcoxon's paired test were performed. GraphPad Prism and SPSS software were used for the analysis. P values less than 0.05 were considered significant.

RESULTS

Levels of Soluble Neutrophil Activation Markers Are Elevated in SSc Patients

To investigate if neutrophil activation occurs in SSc, we analyzed levels of calprotectin (S100A8/A9) as well as NETs (MPO-DNA complexes) in plasma samples from a large cohort of patients with SSc (n=80, Cohort I, **Table 1**) as well as in a smaller validation cohort (n=20, Cohort II, **Table 1**) and compared them with levels found in healthy controls (n=40, Cohort I and n= 24, Cohort II). Levels of calprotectin ($p<0.0001$) and MPO-DNA complexes ($p<0.0001$) were elevated in both SSc cohorts, as compared to healthy controls (**Figure 1**). Additionally, there was correlation of calprotectin and MPO-DNA levels in both SSc cohorts (Cohort I: r=0.30, p=0.006; Cohort II: r=0.48, p=0.03, data not shown). These data clearly indicate neutrophils undergo marked activation and cell death in SSc.

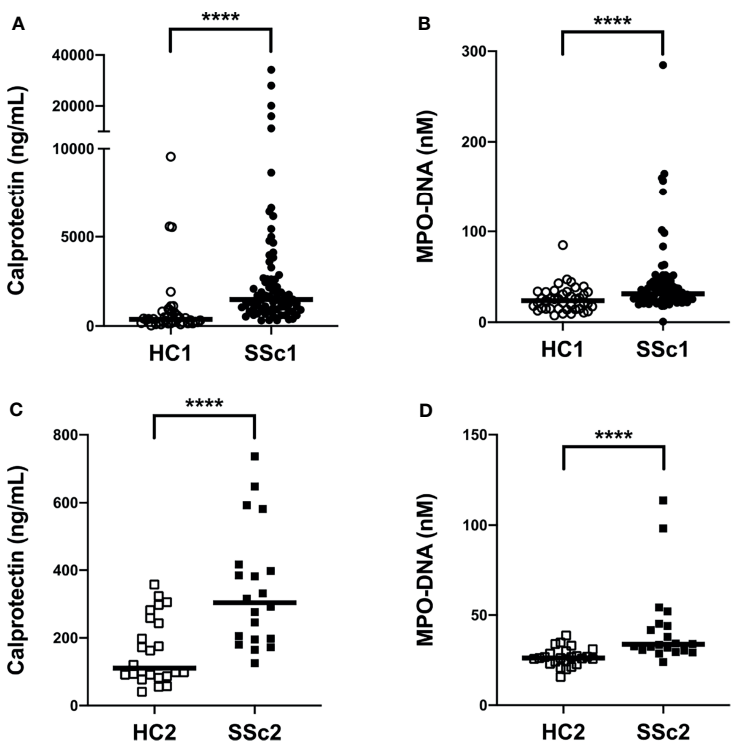

FIGURE 1 | Increased plasma levels of neutrophil activation markers in systemic sclerosis (SSc) patients. **(A, C)** Levels of calprotectin (S100A8/A9) and **(B, D)** neutrophil extracellular traps (NETs, measured as myeloperoxidase-DNA complexes) were analyzed by ELISA in plasma samples of two cohorts of SSc patients and two cohorts of healthy controls (HC). Each symbol represents a single subject. **(A, B)** Cohort I: HC1, White circle (○); SSc1, Black circle (●). **(C, D)** Cohort II: HC2, White square (□); SSc2, Black square (■). Bars represent the median and statistics were determined by Mann-Whitney U test with ****p<0.0001.

Association of Neutrophil Activation Markers With Clinical Variables in SSc Patients

Next, we assessed the clinical correlations of plasma calprotectin and NET levels in the clinically well-characterized SSc Cohort II. Due to limited clinical data on disease activity measures from Cohort I we could not assess their association to neutrophil biomarkers. Elevated levels of calprotectin and NETs in Cohort II SSc patients were correlated with vascular manifestations such as pitting scars ($p<0.05$, **Figures 2A, B**). In addition, SSc patients with telangiectasia had significantly higher levels of circulating NETs ($p<0.05$, **Figure 2C**) but not calprotectin ($p=0.18$, data not shown) as compared to patients without this manifestation. The plasma levels of calprotectin but not NETs positively correlated with their corresponding brain natriuretic peptide (proBNP) levels in the SSc patients in Cohort II ($r= 0.59$, $p=0.01$, **Figure 2D**). A positive correlation between circulating NET levels and skin score was also prominent in the Cohort II SSc patients ($r=0.53$, $p=0.02$, **Figure 2E**). Unexpectedly, no significant correlation was found between plasma calprotectin levels and skin score in the SSc patients ($r=0.34$, $p=0.14$, data not shown). With regards to disease duration, none of the neutrophil biomarkers correlated with disease duration in either of the cohorts. Additionally, no significant differences in neutrophil biomarker levels were present in patients with dcSSc as compared to lcSSc in either of the cohorts. Moreover, presence of common autoantibodies in both Cohort I and Cohort II were assessed, and none of the autoantibodies were associated with presence of neutrophil activation markers at time-point of blood draw (data not shown). Thus, neutrophil activation was associated with several skin- and vascular-related disease phenotypes in SSc patients.

Levels of N-Formyl Methionine Peptides Are Elevated in Patients With SSc

We have recently demonstrated that patients with RA have elevated plasma levels of mitochondrial-derived N-formyl methionine peptides (fMet), a potent neutrophil agonist promoting neutrophil chemotaxis and activation, including NET formation (28). However, whether patients with SSc have elevated levels of fMet, and their potential contribution to neutrophil-mediated inflammation is not known. To address this, we analyzed levels of fMet in plasma samples from both SSc cohorts. As compared to healthy controls, patients with SSc had significantly higher levels of fMet in plasma ($p<0.0001$ and $p=0.03$, respectively, **Figures 3A, B**). Further, increased fMet levels were present in patients with dcSSc as compared to lcSSc in Cohort I ($p=0.04$, **Figure 3C**). With regards to associations with clinical variables, unlike neutrophil activation markers, fMet levels did not associate with clinical characteristics (pitting scar, skin score, proBNP, TA) in Cohort II SSc patients. Additionally, levels of fMet did not correlate with disease duration ($r=-0.08$, $p=0.49$; and $r=-0.32$, $p=0.19$ respectively, data not shown) and common autoantibodies in either of the cohorts.

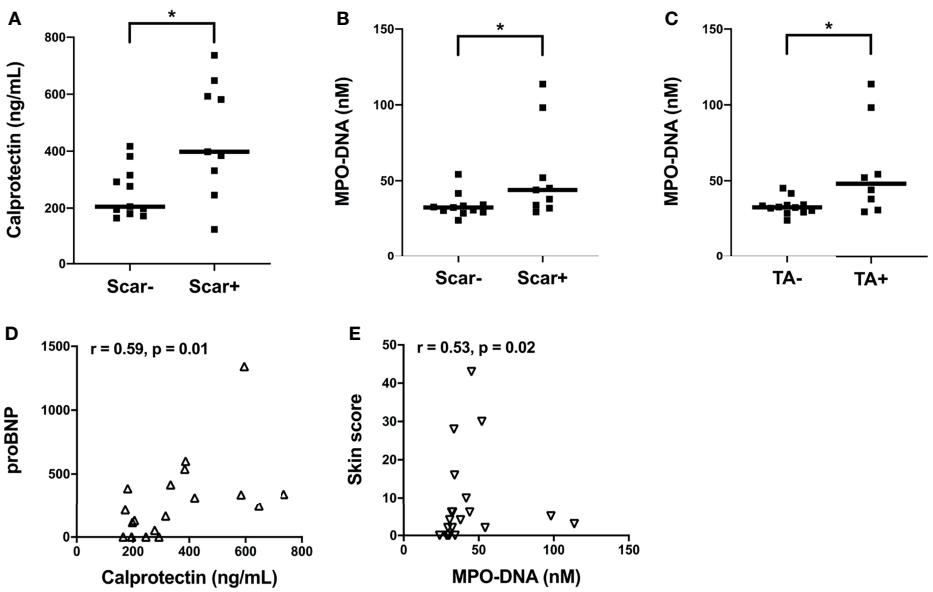

FIGURE 2 | Associations of plasma levels of neutrophil activation markers with clinical parameters in SSc patients from Cohort II. **(A)** Plasma calprotectin levels in the presence and absence of scar tissue manifestation in SSc patients. Plasma NET (myeloperoxidase-DNA complexes) levels in the presence and absence of **(B)** scar tissue manifestation and **(C)** telangiectasia (TA) condition in SSc patients. **(A-C)** Cohort II: SSc2, Black square (■). Shown are the correlation analysis between **(D)** calprotectin levels and brain natriuretic peptide (proBNP) (Δ) and **(E)** NET (myeloperoxidase-DNA complexes) levels and Skin score (∇) in SSc patients. Each symbol represents a single subject. Bars represent the median and statistics were determined by **(A-C)** Mann-Whitney U test with *$p<0.05$ and **(D, E)** Spearman's correlation test.

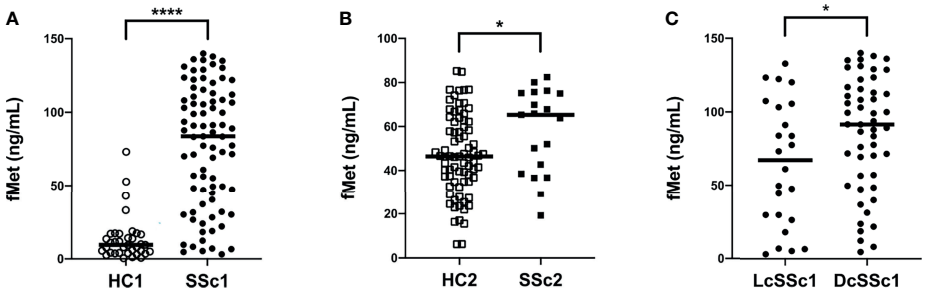

FIGURE 3 | Increased plasma levels of mitochondrial protein fMet in SSc patients. **(A, B)** fMet levels were analyzed by ELISA in two cohorts of SSc patients and healthy controls (HC). **(C)** SSc patients from Cohort I were stratified based on disease phenotype, lcSSc and dcSSc, and assessed for fMet levels. Each symbol represents a single subject. **(A)** Cohort I: HC1, White circle (○); SSc1, Black circle (•). **(B)** Cohort II: HC2, White square (□); SSc2, Black square (■). Bars represent the median and statistics were determined by Mann-Whitney U test with *p<0.05, ****p<0.0001.

Association of Neutrophil Activation Markers With Levels of N-Formyl Methionine Peptides

As fMet is known to induce neutrophil activation through FPR1, we next asked whether plasma levels of fMet were associated with neutrophil activation markers in patients with SSc. In SSc Cohort I, levels of calprotectin (r=0.34, p=0.02, **Figure 4A**) and NETs (r=0.29, p=0.01, **Figure 4B**) correlated significantly with levels of fMet, suggesting fMet-mediated neutrophil activation in these patients. Similar findings were seen in Cohort II, with levels of calprotectin correlating with levels of fMet (r=0.71, p=0.005, **Figure 4C**). However, in contrast, no significant correlation was found between levels of NETs and fMet in Cohort II (r=0.08, p=0.75, **Figure 4D**). In all, neutrophil activation markers are associated with presence of the neutrophil agonist, fMet, in patients with SSc.

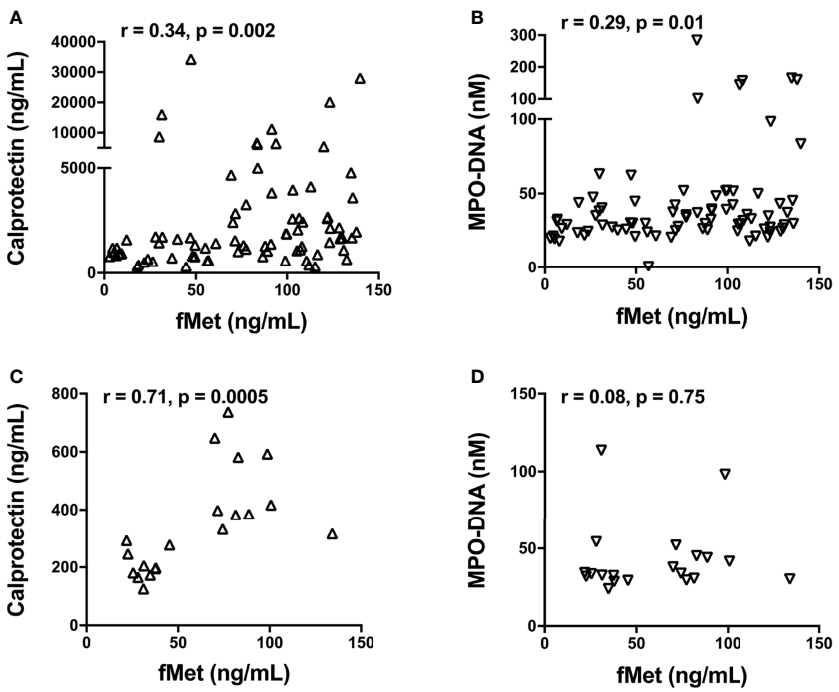

FIGURE 4 | Levels of fMet in SSc patients associate with neutrophil activation markers. Levels of fMet, calprotectin, and NETs (myeloperoxidase-DNA complexes) were analyzed by ELISA. Shown are the correlation analyses between **(A)** calprotectin (Δ) and **(B)** NETs (∇) with fMet levels in SSc patients from Cohort I. **(C)** Calprotectin (Δ) and **(D)** NETs (∇) correlation with fMet levels is shown in SSc patients from Cohort II. Each symbol represents a single subject. Statistics were determined by Spearman's correlation test.

N-Formyl Methionine Peptides Promote Neutrophil Activation Through FPR1 in SSc

Considering the association between elevated levels of fMet and neutrophil activation markers in patients with SSc, we asked whether circulating levels of fMet could promote *de novo* neutrophil activation in an experimental model system. Purified fMet (fMLP: N-Formyl-Met-Leu-Phe) has been previously shown to activate neutrophils in a dose-dependent manner (35). We have also performed dose response curves using fMLP and saw a dose dependent neutrophil activation (CD66b activation marker, **Supplemental Figure 1**). Additionally, we also quantified NET formation induced by purified fMet (fMLP) at various concentrations and did not observe fMLP-mediated NET formation similar to previous studies where fMLP did not induce NET formation (36) (**Supplemental Figure 2**). Thus, purified fMet like molecules such as fMLP does not contribute to NETosis but induces neutrophil activation which were further assessed in this study. Neutrophils, isolated from healthy individuals, were incubated with plasma samples from Cohort I SSc patients and assessed for capacity to induce neutrophil activation by analyzing cell surface expression of CD66b and CD11b by flow cytometry (**Figures 5A, B**). Plasma samples from SSc patients having either high or low levels of fMet were used for this study. SSc plasma with high fMet levels induced marked neutrophil activation, illustrated by upregulation of CD66b ($p=0.02$) and CD11b ($p<0.001$) as compared to healthy controls. Intriguingly, increased neutrophil activation was also observed with SSc plasma with low fMet levels similar to SSc plasma with high fMet levels (CD66b; $p=0.4$ and CD11b; $p=0.5$).

To investigate whether the capacity of plasma to induce neutrophil activation was dependent on fMet, neutrophils were pre-incubated with the specific fMet receptor FPR1 antagonist Cyclosporine H (CsH) prior to addition of plasma samples. We and others have previously shown the specificity of CsH as an FPR1 inhibitor (28, 37). For patients with high levels of fMet, plasma-mediated neutrophil activation was reduced in the presence of the FPR1 inhibitor (CD66b; $p<0.01$ and CD11b; $p<0.0001$). Percentage of inhibition of CD66b and CD11b markers in presence of CsH were 66.7% and 71.6% respectively, suggesting neutrophil activation being partly mediated through the fMet/FPR1 pathway in these patients. Additionally, levels of plasma-mediated neutrophil activation in presence of CsH from patients with high levels of fMet were similar to those observed in healthy controls (CD66b; $p=0.7$ and CD11b; $p=0.5$) emphasizing the contribution of fMet in plasma to neutrophil activation. In contrast to plasma samples with high fMet levels, the FPR1 antagonist CsH could not inhibit neutrophil activation induced by SSc plasma samples with low fMet levels (CD66b; $p=0.5$ and CD11b; $p=0.5$). These results suggest that neutrophil activation is primarily driven by the fMet/FPR1 pathway in patients with high levels of circulating fMet, whereas other mechanisms are operating in low fMet disease states to activate neutrophils.

As fMet levels did not fully explain the neutrophil-activating capacity of plasma, we further assessed if immune complexes (ICs) contributed to the possible activation of neutrophils in SSc patients. We performed neutrophil activation experiments using a FcyRIIA blocking antibody (Clone IV.3) and found a significant decrease in neutrophil activation induced by SSc plasma containing high fMet levels (CD66b; $p<0.0001$ and CD11b; $p=0.02$) as well as low fMet levels (CD66b; $p=0.0002$ and CD11b; $p=0.006$) (**Figures 6A, B**). The percentage of inhibition of CD66b markers from high and low fMet levels SSc plasma by FcyR blocking antibody were 65.2% and 63.3% respectively, and for CD11b markers from high and low fMet levels SSc plasma by FcyR blocking antibody were 18.4% and 33.5% respectively. These observations suggest blocking FcyR significantly abrogates circulating IC-mediated neutrophil activation from the SSc plasma. Thus, both fMet and ICs may contribute to neutrophil activation in SSc.

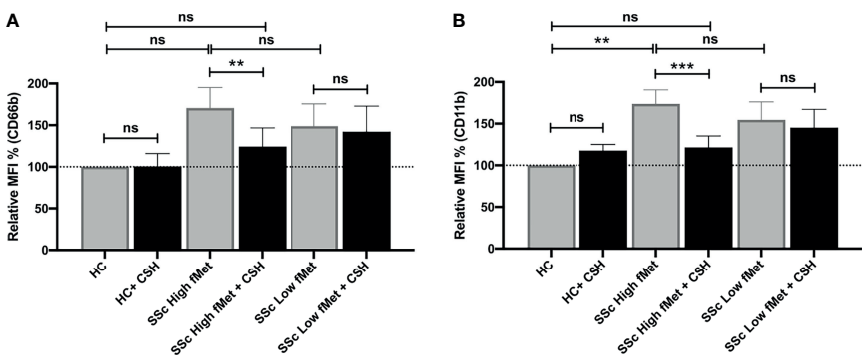

FIGURE 5 | Plasma fMet can induce neutrophil activation in an FPR1-dependent manner. **(A, B)** Plasma from healthy controls, SSc patients containing high fMet levels and low fMet levels were incubated for 2 hours with healthy neutrophils in the presence or absence of Cyclosporine H (CsH) and assessed for the capacity to induce upregulation of neutrophil activation markers **(A)** CD66b and **(B)** CD11b. Bar graphs (means ± SEM) indicate the relative MFI % of CD66b and CD11b which was calculated as CD66b and CD11b MFI induced by stimuli divided by healthy control x 100. All analyses were performed using plasma from patients in Cohort I (HC, n=6; plasma with high fMet, n=15; plasma with low fMet, n=15). Data are combined from two independent experiments. Statistics were performed by Mann-Whitney U test or Wilcoxon's paired test with **p<0.01, ***p<0.001 and ns, non-significant.

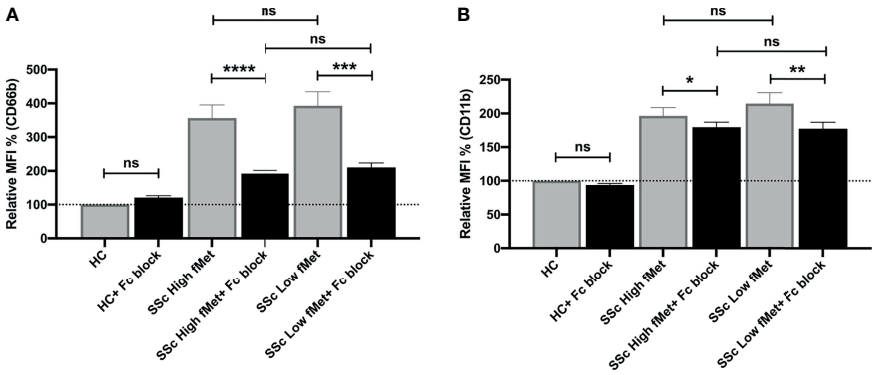

FIGURE 6 | Immune complex mediated neutrophil activation by plasma from SSc patients. **(A, B)** Plasma from healthy controls, SSc patients containing high fMet levels and low fMet levels were incubated for 2 hours with healthy neutrophils in the presence or absence of FcγR blocking antibody (Clone IV.3) and assessed for the capacity to induce upregulation of neutrophil activation markers **(A)** CD66b and **(B)** CD11b. Bar graphs (means ± SEM) indicate the relative MFI % of CD66b and CD11b which was calculated as CD66b and CD11b MFI induced by stimuli divided by healthy control x 100. All analyses were performed using plasma from patients in Cohort I (HC, n=6; plasma with high fMet, n=15; plasma with low fMet, n=15). Data are representative from two independent experiments. Statistics were performed by Mann-Whitney U test or Wilcoxon's paired test with *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001 and ns, non-significant.

DISCUSSION

Owning to its unknown etiology, SSc remains a poorly understood autoimmune disease and this presents one of the greatest challenges to both investigators and physicians. Despite thorough investigations, other than SSc-specific autoantibodies, few biomarkers are validated and/or widely used diagnostically or clinically (38). With respect to early immune cells in SSc pathogenesis, neutrophils are instrumental, infiltrating the lesions and participating in chronic inflammation and fibrosis (5, 39). Although neutrophils are implicated in SSc pathogenesis, neutrophil biomarkers have not been carefully investigated. In the current study we performed a comprehensive analysis on neutrophil activation biomarkers and assessed the role of extracellular mitochondrial components as potential neutrophil activating agents in SSc. Our findings indicate elevated levels of neutrophil activation markers and cell death in SSc. Neutrophil biomarkers associated with SSc disease phenotypes offering potential clinical value to these biomarkers by improving the capacity to monitor disease.

Neutrophils have several effector functions crucial for eliminating invading pathogens. In contrast, uncontrolled activation of neutrophils, including release of inflammatory mediators such as calprotectin can lead to chronic inflammation and immune dysregulation. Calprotectin is primarily produced by neutrophils comprising approximately 45% of its cytoplasmic content (40) and can be released into the extracellular environment upon degranulation. Once extracellular, calprotectin is an efficient damage-associated molecular pattern (DAMP) inducing inflammation by signaling through Toll-like receptor 4 and receptor for advanced glycation end products (RAGE) (41, 42). Thus, in view of its vital role in the physiology of inflammation, calprotectin is a valuable candidate as a neutrophil activation biomarker for inflammation-associated diseases.

Calprotectin (S100A8 and S100A9), either as homodimers, or heterodimer (e.g. calprotectin), have been found at elevated levels in plasma, sera (20, 43), feces (19), saliva (44), BAL fluids (21), and skin (43) of SSc patients. These studies reflect that SSc is not only a skin disease but also affects several visceral organs. Thus, it's possible that neutrophil infiltrates might be present in skin and/or other organs affected by the disease, which needs to be further investigated. With regards to neutrophil activation markers, elevated calprotectin levels in the BAL fluid (21) and serum (20, 43) were associated with extensive lung fibrosis and anti-topoisomerase I (ATA) positivity in the SSc patients. Moreover, fecal calprotectin levels corelated with clinically important features of gastrointestinal (GI) disease and has been explored as a possible biomarker of GI disease in SSc (19). In the same study, plasma calprotectin levels were measured which corelated with systemic inflammation markers like C-reactive protein (CRP) (19). In the current study, we were able to validate elevated levels of calprotectin in SSc patients as well as add to previous findings regarding its potential clinical applications. In particular, our study showed increased calprotectin levels in patients with vasculature-related manifestations like cutaneous pitting scars. Additionally, correlation of calprotectin with proBNP was observed which has been shown to be a useful biomarker for assessing extent of skin fibrosis, degree of restricted pulmonary involvement and a diagnostic marker for pulmonary arterial hypertension (PAH) in SSc (38, 45). These observations further highlight the pathological significance of calprotectin levels in SSc and a promising non-invasive biomarker of SSc.

Effector functions of neutrophils like ROS production has been linked to pathogenesis of SSc. Generation of ROS is crucial for NET formation, a neutrophil cell death process and a well-known biomarker in diseases like RA and SLE (46). Similar to calprotectin, we found elevated levels of NETs (MPO-DNA complexes) in plasma samples of SSc patients, consistent with prior work (22). This study also observed elevated NET levels in

the plasma of active SSc patients (22). Moreover, our study showed correlation of NETs with skin score, a measure of SSc disease progression over time, offering a potential clinical value to NET levels. Association of NETs with vascular manifestations (pitting scars, telangiectasia, nail fold capillary abnormalities) were also observed in our study and the above-mentioned study indicating NETs as a reliable marker to detect the presence of vascular involvement in active SSc patients (22). Elevated level of circulating NETs observed in SSc could be due to increased formation of NETs. Consistent with the hypothesis, prior findings have demonstrated increased capacity of SSc neutrophils to undergo NET formation after stimulation with autologous serum. In addition, neutrophils from SSc patients with severe vascular complications were significantly more prone to releasing NETs compared to other SSc patients (47). Other mechanisms contributing to elevated levels of circulating NETs such as decreased degradation/clearance of NETs in SSc patients are not reported yet and need further investigation. Thus, NET formation might represent a new pathophysiological as well as potential SSc biomarker.

The initial trigger(s) of neutrophil activation in SSc patients remain to be determined. In a recent study, microparticles released from activated platelets expressing the DAMP HMGB1 were abundantly found in blood of SSc patients (22). These microparticles resulted in neutrophil activation and generation of NETs and were further ablated in presence of BoxA, a competitive inhibitor of HMGB1, indicating a platelet-microparticle specific neutrophil activation *via* HMGB1 (22). Although not in the context of SSc, our group has recently demonstrated extracellular mitochondrial N-formyl methionine (fMet) peptides abundantly present in RA patients (28) as well as in systemic vasculitides (Michailidou et al., under revision) suggesting fMet-mediated neutrophil activation possibly being a central process in several autoimmune inflammatory conditions. Whether SSc patients have elevated levels of extracellular mitochondrial fMet and their role in neutrophil-mediated inflammation has so far not been investigated. Consistent with the RA study, elevated level of fMet peptides were found in SSc patients prompting us to investigate its role in neutrophil activation. Additionally, increased levels of fMet were observed in DcSSc as compared to LcSSc patients, suggesting fMet levels might play an important role in the pathogenesis of DcSSc and could be useful serological marker for evaluating type of SSc disease. Mitochondrial fMet peptides can be sensed by N-formyl peptide receptor FPR1, which has high affinity for fMet, and is expressed on various host cell types but most strongly on neutrophils. Activation of neutrophils by fMet *via* FPR1 triggers a wide variety of downstream effector functions including chemotaxis, degranulation, ROS production, and phagocytosis bridging an association between mitochondrial fMet proteins, FPR1 and neutrophils (48).

Association between mitochondrial proteins and neutrophil activation was shown in our recent study of RA patients (28). Similarly, we found levels of fMet associating with neutrophil activation markers like calprotectin and NETs in SSc patients, supporting the hypothesis of fMet-mediated neutrophil activation. Given that correlations do not inform on causality, we performed neutrophil stimulation studies to identify circulating factors such as fMet present in plasma that are able to activate neutrophils *in vitro*. Our data demonstrate that plasma from SSc patients having high fMet levels in circulation had increased ability to induce activation of neutrophils from healthy blood donors. Blockade of FPR1 by cyclosporin H (CsH) suggested that circulating fMet activates FPR1 signaling in SSc patients and contribute significantly to the immune activation of neutrophils. These observations warrant exploring the inhibition of FPR1 signaling as a potential novel pharmaceutical intervention in SSc, a disease which is in dire need of novel therapeutics. Importance of FPR1 signaling has been previously shown in tissue fibrosis, the hallmark of SSc. FPR1 signaling has been shown to be crucial for neutrophil recruitment to the lung and support fibrosis in an *in vivo* mouse model of SSc (49). FPR1 is also expressed on human fibroblasts and signaling *via* FPR1 in SSc patients has been shown to promote transition of fibroblast-to-myofibroblasts and extracellular matrix deposition leading to tissue fibrosis (50). Thus, fMet proteins critical to FPR1 signaling and neutrophil activation might be an important mechanism contributing to SSc pathogenesis and a potential therapeutic target. There are several potential sources of extracellular mitochondria, including platelet activation, neutrophil death, and tissue damage (26, 27). Which, if any, of these mechanisms operate in SSc to promote release of extracellular mitochondria is under current investigation.

Adding to the complexity of SSc, in contrast to our hypothesis we found activation of neutrophils even upon stimulation with a subgroup of SSc patients having low fMet levels in plasma. The activation of neutrophils did not decrease upon blockage of FPR1 indicating the neutrophil activation was not driven by fMet/FPR1 interactions but due to other circulating factors present in the plasma samples. Among the circulating factors, immune complexes (ICs) containing SSc-specific autoantibodies engaging with nucleic acid or DNA/RNA binding proteins are shown to elicit proinflammatory and profibrotic effects on fibroblasts (51). The pathogenicity of the SSc-ICs has been suggested to be mediated by interaction with Toll-like receptors (TLRs) *via* nucleic acid fragments. Additionally, circulating ICs contributing to neutrophil activation has been shown in autoimmune diseases like SLE (52). However, in the current study, presence of SSc autoantibodies towards intracellular antigens did not associate with levels of circulating NETs (data not shown), but neutrophil activation upon stimulation with SSc plasma was abrogated upon blocking of Fcy receptor, the binding sites of ICs. Thus, fMet is an important driver of neutrophil activation and inflammation in a subgroup of SSc patients with high levels of fMet in circulation. However, other mechanisms, potentially driven through ICs, are operating in patients with low levels of fMet. These results further highlight the heterogeneity of the disease and the need for patient stratification or personalized medicine approach, as different patients might have different immunological pathways activated and might not necessarily benefit from the same treatment.

The limitation of our study includes limited clinical data for SSc Cohort I, as well as lack of longitudinal data, which would have allowed us to assess the prognostic utility of the neutrophil biomarkers. Another limitation of our study includes conducting

experiments on neutrophil activation alone *via* fMet/FPR1 signaling. Although FPR1 is expressed strongly on neutrophils, other innate immune cells like monocytes and macrophages also express FPR1 warranting analyzing these cell types which are also implicated in early SSc pathogenesis (5).

In conclusion, our data for the first time demonstrated that levels of fMet are elevated in the circulation of SSc patients and implicate an important role for the mitochondrial component fMet in promoting neutrophil-mediated activation through FPR1 in SSc. Our data also support the clinical value of neutrophil biomarkers and fMet in monitoring SSc disease, although these observations need to be validated in larger patient cohorts as well as in appropriate animal models. We propose fMet-mediated signaling as a potential therapeutic target promoting anti-inflammatory effects in SSc by ameliorating neutrophil based inflammation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Fred Hutchinson Cancer Research Center (part of the University of Washington Consortium) and Lund University #2010/544. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RK and CL conceived the study. RK, BD, and CL designed experiments, analyzed data, and interpreted results. RK, RS, BD, TW, and MF performed experiments. JN, RH, and MF provided materials and clinical cohorts. RK wrote the manuscript and RH, JN, MF, RS, BD, and CL critically reviewed the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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The Anti-DNA Antibodies: Their Specificities for Unique DNA Structures and Their Unresolved Clinical Impact—A System Criticism and a Hypothesis

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Systemic lupus erythematosus (SLE) is diagnosed and classified by criteria, or by experience, intuition and traditions, and not by scientifically well-defined etiology(ies) or pathogenicity(ies). One central criterion and diagnostic factor is founded on theoretical and analytical approaches based on our imperfect definition of the term "The anti-dsDNA antibody". "The anti-dsDNA antibody" holds an archaic position in SLE as a unique classification criterium and pathogenic factor. In a wider sense, antibodies to unique transcriptionally active or silent DNA structures and chromatin components may have individual and profound nephritogenic impact although not considered yet – not in theoretical nor in descriptive or experimental contexts. This hypothesis is contemplated here. In this analysis, our state-of-the-art conception of these antibodies is probed and found too deficient with respect to their origin, structural DNA specificities and clinical/pathogenic impact. Discoveries of DNA structures and functions started with Miescher's Nuclein (1871), *via* Chargaff, Franklin, Watson and Crick, and continues today. The discoveries have left us with a DNA helix that presents distinct structures expressing unique operations of DNA. All structures are proven immunogenic! Unique autoimmune antibodies are described against e.g. ssDNA, elongated B DNA, bent B DNA, Z DNA, cruciform DNA, or individual components of chromatin. In light of the massive scientific interest in anti-DNA antibodies over decades, it is an unexpected observation that the spectrum of DNA structures has been known for decades without being implemented in clinical immunology. This leads consequently to a critical analysis of historical and contemporary evidence-based data and of ignored and one-dimensional contexts and hypotheses: i.e. "one antibody - one disease". In this study radical viewpoints on the impact of DNA and chromatin immunity/autoimmunity are considered and discussed in context of the pathogenesis of lupus nephritis.

Keywords: systemic lupus erythematosus, anti-DNA antibodies, DNA structures, classification criteria, pathogenicity

INTRODUCTION

This theoretical study critically analyses immunology of DNA and chromatin. The discussion is basically immunological and unlinked from SLE, but elements of the syndrome is discussed, as chromatin autoimmunity is relevant to understand SLE in both historical and contemporary contexts[1].

"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" (Ludwik Fleck[2]) (1).

The citation above is highly relevant as backdrop for this study. The central idea is to reconsider historical data on DNA and anti-dsDNA antibodies in light of contemporary prioritized insight.

History and Scientific Impact of Antibodies to DNA

The first reports on antibodies against dsDNA appeared in 1938 and 1939 in context of bacterial infections (2–4). Two decades later they were described in SLE (5–8). Already here we observe a conflict between the current view that the antibodies are unique biomarkers for SLE [see a relevant contextual discussion of "biomarker" (9)] and the historical facts that the antibodies were first described in patients infected with bacteria. Today, the strong links between anti-DNA antibodies and infections and malignancies is not considered important in contemporary rheumatological contexts (**Table 1** presents the major critical elements in this study).

Anti-DNA antibodies are, nevertheless, important and play informative and controversial roles in history of immunology (10–16), in studies of antibody diversity and immunoglobulin variable region structures and genetics (11, 17–21), in molecular biology (22–26) as well as in rheumatology, infections and in malignancies [see e.g. (12, 13, 25, 27–32)]. In contrast to the considerable amount of studies on phenomenological and basic aspects of anti-dsDNA antibodies[3], we still do not definitively know the critical incitements that promote their production *in vivo*.

Furthermore, there is today not consensus on their targets *in vivo* – whether DNA (13) or non-DNA structures (33, 34). Important scientific data describing antibody specificity against functional DNA structures (**Figure 1**) are in current clinical immunology contexts largely neglected - but erroneously discussed in terms of avidity and not specificity (see below). This is not so in basic DNA research where structure and operation of individual forms of DNA are central elements to understand nature of DNA in biology (discussed below).

These short and decisive statements derive from a large series of studies, from preliminary conclusions, and from a categorical lack of international consensus. This is documented in hypotheses and theory studies [see e.g. (12, 35, 36)]. Conversely, the "Anti-dsDNA antibody" has achieved an aristocratic and time wise pompous status as a diagnostic and a pathogenic factor: See e.g. the Wikipedia-statement: "*Anti-dsDNA antibodies are incredibly specific for SLE*[4]". This statement is from historical and contemporary concepts and data difficult to understand, because the data on the antibodies in non-SLE conditions are neglected or overlooked.

The cited Wikipedia statement is worryingly close to a warning Chalmer has formulated: "Biased under-reporting of research should be outlawed" (37). If we consider current views on a clinical impact of anti-dsDNA antibodies, we have to realize that aspects and data that oppose their status as a prototypical biomarker for SLE are clearly under-reported. [**Figure 2A**, discussed in (13)]. This is clear as deviant reports provide us with unmistakable data on anti-dsDNA antibodies in non-SLE conditions [**Figure 2A**, discussed in (13)]. In contrast, a categorically positive correlation of anti-dsDNA antibodies with SLE and lupus nephritis is reported. The SLE classification criterion - "The anti-dsDNA antibody" - in singular represents a group of unique individual antibodies against DNA structures and probably none of them are unique for SLE. This is discussed in detail below.

DNA STRUCTURES AND ANTI-DNA ANTIBODIES

In the modern history of DNA discoveries, different structures of DNA have been described (38, 39). Their unique roles are basically to facilitate and regulate DNA repair, replication and transcription of genes. Insight into these structures have provided us with basic understanding of genetics and DNA biology. Notably, the structures have a striking, yet largely overlooked relevance in an autoimmune context: Each structure has, aside from its basic function, a unique ability to induce highly specific anti-dsDNA antibodies (see below).

A central research focus has over decades been to describe elements of dsDNA and chromatin fragments as stimulators of B cells and T cells in context of SLE [(12, 24, 30, 40), discussed below, and in (13)]. Autoimmune hepatitis, for example, has recently become a focus in this context (41).

Studies of DNA and chromatin structures have indeed promoted fertile scientific achievements (see **Table 2** for a short history). Settled DNA/chromatin structures have provided us with insight into immunological processes that regulate tolerance for chromatin, but also into basic aspects of the immune system itself (see **Table 2**). Some research directions have, however, been hampered by deficient strategic hypotheses (see central problems described in **Table 1**).

In light of this cognition, it is therefore a substandard statement to underline that modern clinical immunology and rheumatology propose that antibodies to dsDNA are a fundamental single unit and a central SLE classification

[1] References listed in this section are meant as valid examples, and do not intend to represent a complete biography of aspects of anti-dsDNA antibodies.

[2] **Ludwik Fleck** (1896 –1961) was a Polish physician, biologist and philosopher. In the 1930s he developed the philosophical concepts of "*Denkstil*" ("thought style") and the "*Denkkollektiv*" ("thought collective"). Fleck´s concept of the "thought collective" is central in the philosophy of science and in logology (the "science of science") and was a leading force in the Polish school of logology. He used his consept to describe non-linear evolution of scientific ideas in a "thought collective", much as in Thomas Kuhn´s "periodic paradigm shift" concept.

[3] Per August 2021 more than 41.700 publications are found in PubMed using the term "anti-DNA antibodies"

[4] https://en.wikipedia.org/wiki/Anti-dsDNA_antibodies

TABLE 1 | Historical and contemporary definitions of DNA and anti-DNA antibodies.

An important reflection by Ludvik Fleck is a correction to our deficient considerations related to impact of DNA/chromatin and corresponding autoantibodies in clinical medicine:

"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" (1).

The analyses presented here reveal that current knowledge remains vague on central aspects. It is easy to document that central data from historical science are not considered in contemporary knowledge and documentation as outlined below:

•Historical data unmistakably demonstrate that anti-dsDNA antibodies were first described in bacterial infections (1938, 1939) – not in SLE (1957). This is not considered in classification or diagnostic criteria, all of which uniformly inform that "The anti-dsDNA antibody" is specific for SLE: "one antibody – one disease".

•Historical data unmistakably demonstrate that multiple functional DNA structures have individual immunogenic potentials and consequently induce production of unique cognate anti-DNA antibodies. These are not considered in classification criteria, nor in discussions of pathogenicity of unique anti-DNA antibodies. Considered is just the misleading term "The anti-dsDNA antibody": Again leading to the paradigm: "One antibody – one disease".

•Historical and recent data unmistakably demonstrate that anti-DNA and anti-chromatin antibodies execute their pathogenic potential by binding chromatin exposed *in situ* on one hand – other data argue that anti-DNA antibodies bind cross-reactive, intrinsic matrix or basement membrane constituents.

•Till now, no collaborative and/or comparative studies have been performed across the different models of lupus nephritis. This should be regarded as a *sine qua none* to develop consistent causal therapies, meaning therapy aiming at preventing true scientifically verified pathogenic processes. We have today to accept that the processes are in conflict with each other with poor perspectives to be solved.

•Also, there is today a strong need to understand the impact of the steadily increasing number of previous and contemporary classification criteria for SLE. They are not linked to each other in an etiological or pathogenetic context, and they define a large number of heterogenous clinical SLE phenotypes. This makes cohort studies on homogenous SLE phenotypes difficult. Likewise, anti-dsDNA antibodies represent a group of antibody specificities. We need to define what we test for, why, and by which assay principles in order to leave the silently accepted term "one antibody – one disease" behind.

•In conclusion: there is a need to create a bases for new definitions of parameters that may define bases for future studies. Those studies must aim to increase our insight into what SLE classification criteria are, if they are linked through common processes, what the etiology encompasses, and what pathogenic pathways are fundamental in SLE.

criterium. This is stated in the "The 1982 revised American College of Rheumatology (ACR) SLE classification criteria", in

"The Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for systemic lupus erythematosus", and in "The 2019 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Systemic Lupus Erythematosus" (54–56) where the anti-dsDNA antibodies are defined irrespective structural DNA specificity. The focus on "The anti-dsDNA antibody" as a separate and specific criterion has most probably derailed a productive and critical, clinically relevant, research focus on anti-dsDNA antibodies. This relates to definition of them as a diagnostic marker as well as a pathogenic factor in SLE. This dilemma puts the critical focus on specificity versus avidity of these antibodies.

ANTIBODIES AGAINST DNA STRUCTURES: DIVERSITY OF SPECIFICITIES OR DIVERSITY OF AVIDITIES—FACTS AND CONTRAFACTS

The next interpretative problem derived from the fact that "The anti-dsDNA antibody" bind differently in assay systems like enzyme-linked immuno-sorbent assay (ELISA), Crithidia luciliae immune-fluorescent test (CLIFT), the Farr and other assays [see e.g. (57, 58) and **Figure 2B**]. Binding in one or the other assay has been misinterpreted as if "The anti-dsDNA antibody" possesses a spectrum of avidities (59) – and not a spectrum of different unique DNA specificities as may appear in individual assay system, like binding of antibodies against bent B DNA as in core nucleosomes or in plasmid DNA (as in CLIFT), while antibodies to elongated linear B DNA, cruciform dsDNA, ssDNA in transcriptionally active chromatin, are all detected by e.g. ELISA assays using DNA designed for each structure, or to Z DNA formed in high salt as in the Farr assay (38, 60–62). It is relevant to stress that ssDNA in clinical immunology is often erroneously described as denatured DNA, and not as a real functional DNA structure (see below for details).

Thus, different antibodies have distinct specificities, and all autoimmune IgG anti-dsDNA antibodies produced *in vivo* are principally antigen-driven by any of the whole spectrum of DNA structures described in chromatin (38, 39). They are consequently affinity maturated and may all be of high avidity [see e.g. (11, 20, 21, 63, 64)]. Thus, specificity of antibodies for DNA structures may have informative impact on classification of SLE and on pathogenicity in SLE and lupus nephritis [discussed in (65)].

Importantly, this rationalization opens for a new understanding of the distinctions between specificity versus avidity and the consequent pathogenic impact of anti-DNA antibodies. Furthermore, this interpretation strongly supports the view that pathogenic impact of anti-dsDNA antibodies may encompass all possible anti-DNA antibody sub-specificities towards structures exposed in extra-cellular chromatin.

This (still mostly) theoretical discussion puts a focus on the nature, origin and function of individual antibodies recognizing dsDNA in all of its structural forms shaped in biologically active chromatin. This puts the focus on origin of these antibodies.

ORIGIN OF SPECIFIC ANTI-DSDNA ANTIBODIES—A CONCISE ANALYSIS

In the aftermath of description of autoimmunity to dsDNA in 1957 (5–8), the complexity of tolerance-regulation of DNA immunity has led to contemporary studies of the immunogenic impact of dsDNA as presented in NETs (31, 66), secondary necrotic cells SNECs (67), and microparticles [(68, 69) discussed in (16, 35) and below]. For central milestones important for our understanding of tolerance and immunity to DNA, see **Table 3**.

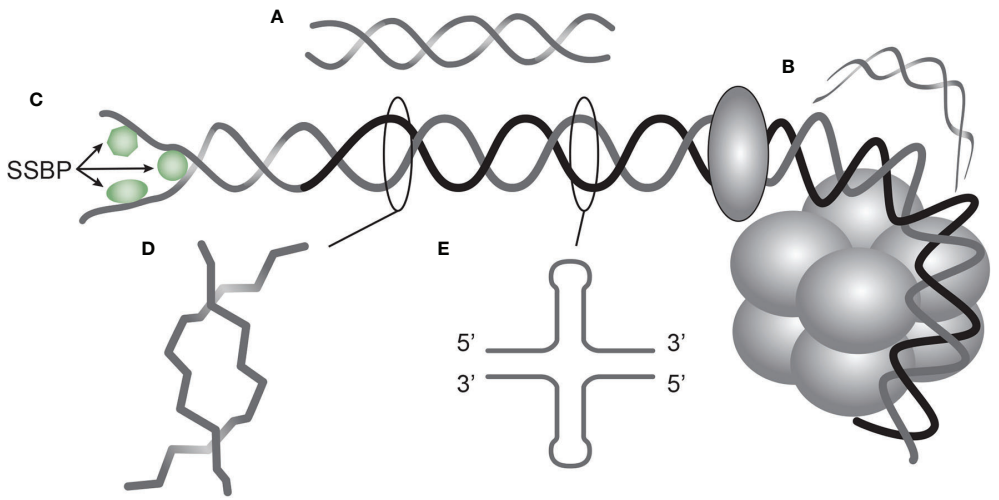

FIGURE 1 | *DNA structures in chromatin express distinct DNA functions, and each structure is a unique antigen.* Elongated (linker) DNA is a relaxed, right-handed. low-energy linear form of B DNA **(A)**, while compacted B DNA as in plasmids (not shown) and in core nucleosomes are defined as bent B DNA **(B)**. In **(C)** the B DNA helix is opened by single-DNA binding proteins (i.e. proteins stabilizing ssDNA and polymerizes involved in replication and repair). In **(D)** Z DNA is demonstrated, which is a left-handed, high energy, supercoiled double helix. Physiologically, Z DNA forms during transcription as a result of torsional strain that depend on interaction of mobile polymerases. Z DNA is predominantly associated with linker DNA and regulate transcription. Cruciform DNA is another structure formed in dsDNA **(E)**, and is different from B and Z DNA. Its generation requires that repeat sequences (palindromes) in one strand is repeated on the other strand in opposite direction. The cruciforms are, like Z DNA, higher energy DNA structures. From an immunogenic point of view, each structure **(A–E)** is unique in terms of inducing highly specific antibodies with potential pathogenic impact if chromatin is exposed in situ. See text for details.

Regrettably, we have to admit that studies on autoimmunity to dsDNA have been less conclusive and thus less successful than the foregoing studies describing structure and function of the DNA helix and chromatin. Still, our insight into clinically relevant DNA-induced autoimmunity is founded on phenomenology, artificial experiments and hypothetical interpretations [discussed in (13)]. We can, however, argue for the view that DNA *structure-specific* antibodies are selectively induced by individual DNA configurations present in chromatin (**Figure 1**), and not merely by the vast number of cross-reactive non-DNA, non-polynucleotide structures [see detailed discussion below, and (13, 70)]. In light of this, "The anti-dsDNA antibody" is a critically inconsistent and erroneous term that does not open for further insight into the clinical impact of anti-DNA antibodies.

Autoimmunity Versus Immunity of DNA—Two Roads Leading to the Same Center?

In order to probe hypotheses linked to experimental and empirical studies aimed to describe origin of anti-dsDNA antibodies, we need to settle a semantic distinction: Anti-dsDNA antibodies may be the result of immune responses to DNA-protein complexes in context of 2 principally different mechanisms for termination of tolerance: *Autoimmunity versus immunity* (see **Figure 3A–D**, for principle models).

This distinction is important to bring to the discussion forum; *autoimmunity* signifies an autoimmune response promoted solely by autologous dsDNA in complex with chromatin-derived autologous proteins, while *immunity* implies immune responses to dsDNA/chromatin in complex with a non-self (like infection-derived) DNA-binding protein component. In general, antibodies to dsDNA generated *in vivo* is most probably a result of both categories of immunity (concise models as described in **Figure 3C, D**, respectively).

There are many reasons to argue for the validity of these models to generate anti-DNA antibodies. These arguments were basically presented as *a theoretical model for the future* by Radic and Weigert in 1994 [presented in **Figure 3A** (11)] and as experimental evidence-based models by Marion et al., Pisetsky et al., and Rekvig et al. (32, 71–73). In absence of responsive T cells a model for tolerance is presented (**Figure 3B**), and imply no help for DNA/chromatin-specific B cells. **Figure 3** presents a basic model in scenarios linked to both immunity and autoimmunity of DNA (**Figure 3C, D**, respectively). The basic model promoted by Radic and Weigert predicts a molecular and cellular prototype model also for linked production of antibodies to DNA, histones and other chromatin associated proteins, in accordance with theoretical reflections provided by Craft and Hardin already in 1987 (74). The immunity model (**Figure 3C**) and the autoimmune model (**Figure 3D**) are validated by descriptive observations and experimental data [see thorough discussions in reference (12, 13)].

Autoimmunity to dsDNA: An Autologous Origin of Key Proteins That Render dsDNA Immunogenic

Still, we have not satisfactorily determined which molecular and cellular processes that are operational to promote production of anti-dsDNA antibodies *in vivo* (12, 13, 32, 75, 76), although DNA seems to be the B cell antigen (14, 16). A key question is why it is so difficult to experimentally induce anti-dsDNA

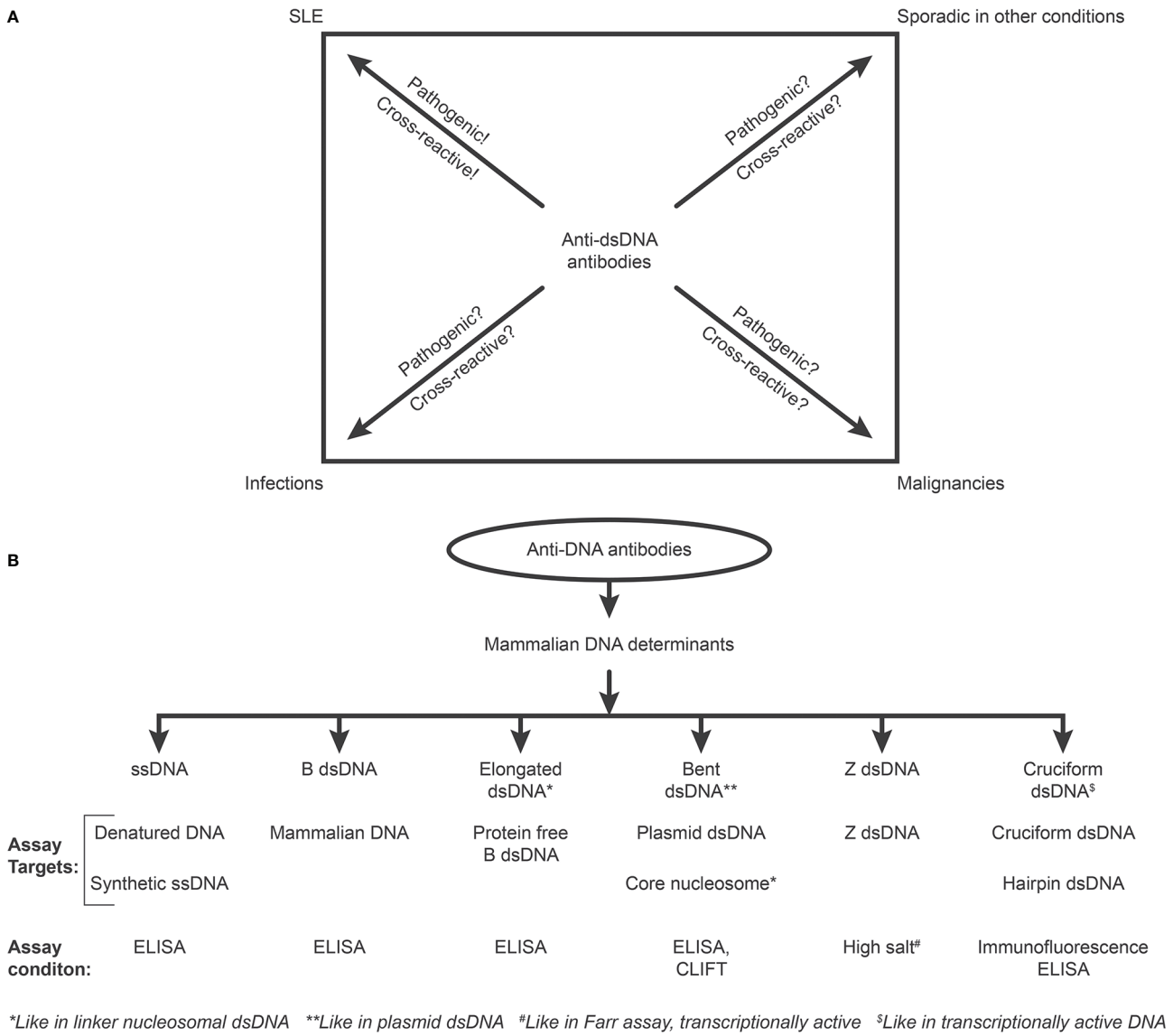

FIGURE 2 | Critical questions related to the SLE classification criterion "The anti-dsDNA antibody" (criterion 11 in ACR) or "Anti-dsDNA" (criterion 6, Immunological criteria, SLICC). Principal problems are linked to the inadequate terminology of the anti-dsDNA antibodies. One problem [demonstrated in **(A)**] illustrates that "The anti-dsDNA antibody" is not unique for SLE, but appears regularly in context of infections, malignancies, and sporadic in other conditions. Little is examined whether anti-dsDNA antibodies are pathogenic and cross-reactive in the latter conditions [question marks in **(A)**], as they truly are in SLE. The second dominant problem considered for the "Anti-dsDNA antibody" is that the antibodies are presented as if "it" is monospecific for dsDNA without further specifications of target DNA structures. This has over decades crystallized the conception that different assay systems detect antibodies possessing different avidities but not different specificities! This conflict is principally [demonstrated in **(B)**]. The "ssDNA/dsDNA" structures are categorized in 6 main sets. Antibodies against all these dsDNA structures have been identified by conventional assay systems, like ELISA in physiological salt (ssDNA, B DNA, elongated B DNA, bent B dsDNA), in high salt (Z dsDNA), and cruciform dsDNA in addition to heterogeneous binding to proteins and phospholipids. The idiom that anti-dsDNA antibodies bind dsDNA in a singular form as in the ACR or SLICC classification systems must be challenged by the multifaceted recognition pattern of anti-dsDNA antibodies as informed about in **(B)** Thus, data in this figure require that assay systems for anti-dsDNA antibodies relates to categorized structural DNA specificities. Lack of implementation of the structural and molecular recognition pattern recognized by individual anti-dsDNA antibodies undermine the potential clinical impact of anti-dsDNA antibody sub-specificities.

antibodies *in vivo* without introducing non-self carrier proteins in complex with DNA/chromatin fragments. Examples are provided in **Figures 4** and **5**, for induction of anti-structural DNA antibodies and anti-chromatin antibodies, respectively by DNA/chromatin-polyomavirus T antigen complexes. This is consistent with high propensity to e.g. viral infections in SLE and cancers (71, 77, 78).

There are indeed studies that have demonstrated an immunogenic potential of purely autologous chromatin. Voll et al. demonstrated that histone-specific T cells (79), or release of

TABLE 2 | Central scientists and the resolution of the DNA structure.

Miesher et al. (42, 43)
•Described in 1871 Nuclein later known as DNA.
Levene et al. (44, 45)
•Discovered ribose in 1909 and deoxyribose in 1929.
•Suggested the structure of nucleic acids as a repeating nucleotide tetramer.
•DNA contained adenine, guanine, thymine, cytosine, deoxyribose, phosphate group.
Chargaff et al. (46–48)
Defined in 1950, 1952 the 2 Chargaff rules
•In double-stranded DNA, guanine units is equal to cytosine units, adenine units is equal to thymine units.
•The composition of DNA varies between species.
Franklin et al. (49–51)
•In 1952 Franklin produced high-resolution photographs of crystallized DNA fibers, interpreted as a helical structure. She and Chargaff were close to defining the structure of DNA.
•Franklin described the A and B forms of DNA.
Watson and Crick (52, 53)
•Used X-ray data from Franklin when they solved the helical structure of DNA in which A pairs with T, and C with G (equal to Chargaff's 1. rule).

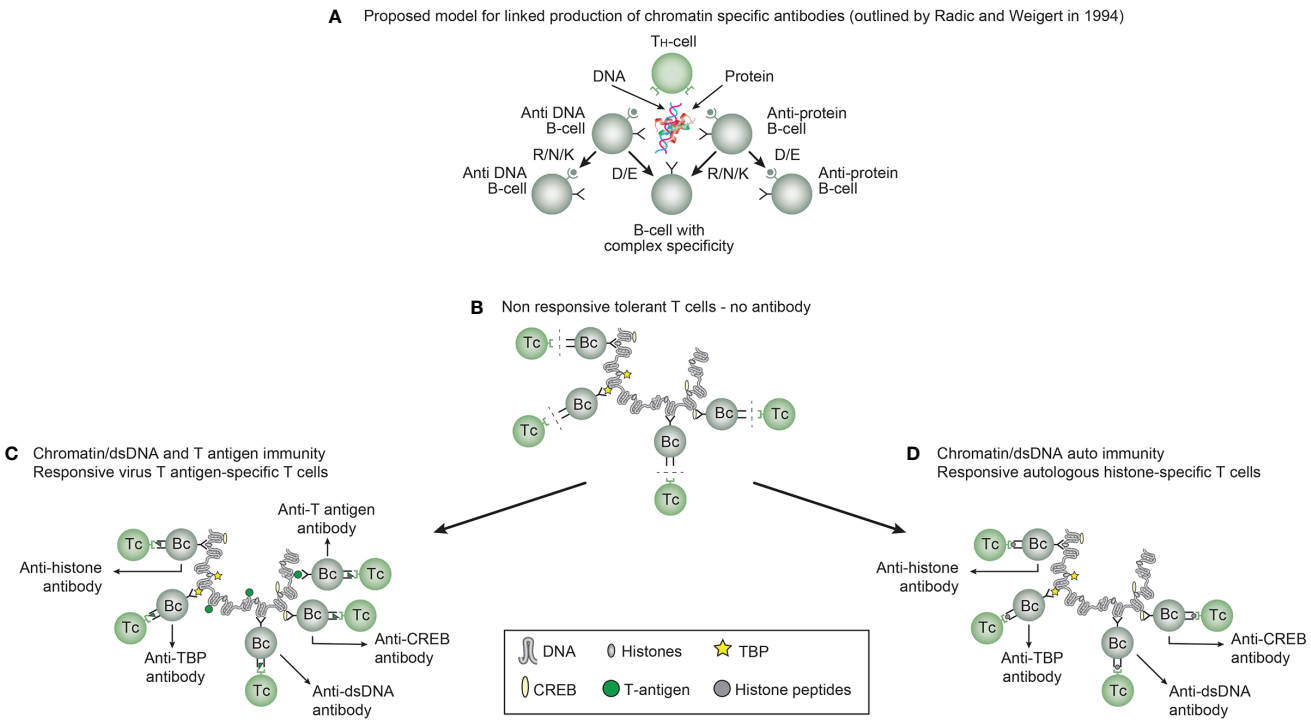

FIGURE 3 | *Models for how production of anti-DNA antibodies may be initiated.* In order to understand results of experimental and empirical studies aimed to describe origin of anti-dsDNA antibodies, we need to settle a semantic distinction: Anti-dsDNA antibodies may be the result of immune responses to DNA-protein complexes in two different contexts: *immunity versus autoimmunity* **(A–D)**. *Autoimmunity* signifies an autoimmune response promoted solely by autologous dsDNA in complex with chromatin-derived autologous proteins. *Immunity* implies immune responses to dsDNA/chromatin in complex with a non-self (like infection-derived) DNA-binding protein component. In general, antibodies to dsDNA generated *in vivo* is most probably a result of both categories of immunity. There are many reasons to argue for the validity of these models to generate anti-DNA antibodies. These arguments were basically presented as *a theoretical model for the future* by Radic and Weigert in 1994 [presented in **(A)**]. In this model, aspects of affinity maturation is demonstrated as the B cell Ig variable regions are undergoing mutations to basic or acidic residues (This figure, Panel a, is re-drawn from a figure in reference (11), and provided by courtesy of Dr. Marko Radic, University of Tennessee Health Science Center). Deriving from this theoretical model, functional evidence-based models by Marion et al., Pisetsky, et al., and Rekvig et al. (32, 71–73) are demonstrated. In absence of responsive T cells a model for tolerance is presented **(B)**, and imply no help for DNA/chromatin-specific B cells. The distinction between immunity and autoimmunity is demonstrated in Panels c an d, respectively. The principle difference relies on the specificity of the T cells. In immunity, the T cells are specific for and engaged by non-self derived DNA-binding proteins **(C)**, while in autoimmunity, the T cells are engaged by autologous, chromatin-derived proteins like histones **(D)**. The basic model promoted by Radic and Weigert predicts a molecular and cellular prototype model also for linked production of antibodies to DNA, histones and other chromatin associated proteins, in accordance with theoretical reflections provided by Craft and Hardin already in 1987 (74). The repertoire of chromatin-specific autoantibodies is from theoretic consideration the same for the models presented in **(C, D)** (see text for details).

chromatin-HMGB1 (High Mobility Group Box 1) complexes (80), promoted production of anti-dsDNA antibodies. HMGB1-containing nucleosomes from apoptotic cells were demonstrated to induce anti-dsDNA and anti-histone antibody responses, whereas nucleosomes taken from living cells did not (80).

Sisirak et al. (81) demonstrated that DNase 1L3 knock-out mice spontaneously produced anti-dsDNA antibodies and developed lupus nephritis. This study represented an experimental correlate to observations that an inherited null mutation of the DNase 1L3 gene is associated with early-onset SLE phenotype and development of lupus nephritis (82). In their study, Sisirak et al. made the logic conclusion that extracellular chromatin is a potential self-antigen normally digested by circulating DNase 1L3. This was further investigated by the Boris Reizis group, where the central effect on DNase 1L3 in prevention of autoimmunity towards DNA was ascertained, and that autoantibody-mediated inhibition of DNase1L3 activity facilitated anti-dsDNA autoreactivity in patients with severe sporadic SLE (81, 83). Restoration of DNase 1L3 activity could therefore represent a causal therapeutic approach to control the manifestations of SLE promoted by exposure of chromatin [discussed in (81, 83)]. Soni and Reizis provide strong arguments for the view that DNA may represent an epicenter in SLE as immunogen and pathogenic factor as extra-cellular target for the anti-dsDNA antibodies [(16), see also (31)].

The role of HMGB1- chromatin complexes to *promote* anti-DNA antibody production, and the role of DNase 1L3 to *prevent* production of anti-dsDNA antibodies represent important conceptual advantages in our search for understanding the molecular and cellular origin of anti-dsDNA antibodies.

Immunity to dsDNA: An Infectious Origin of Key Proteins That Render dsDNA Immunogenic

Valid historical data argue for the view that anti-dsDNA antibodies are not unique for SLE (**Figure 2A**). Infections are commonly encountered in both SLE and in malignant diseases (73, 77, 78, 84, 85), a fact that may causally link anti-dsDNA antibodies to diseases prone to infections.

From studies of infectious-related anti-dsDNA antibody responses, we have insight into basic aspects of the molecular and cellular requirements to fulfill stimulation of the immune system [discussed in (11–13, 36, 70)]. One fairly well documented experimental model proclaims that DNA, as a hapten-like structure, a term introduced by Sercarz et al. (40, 86), must be complexed with certain immunogenic, *in vivo* expressed infection-derived DNA-binding carrier proteins [**Figure 3C** (27, 71, 75, 87)]. This has its experimental counterpart in using artificial carrier proteins like the widely used methylated bovine serum albumin [see e.g. (75, 87–89)].

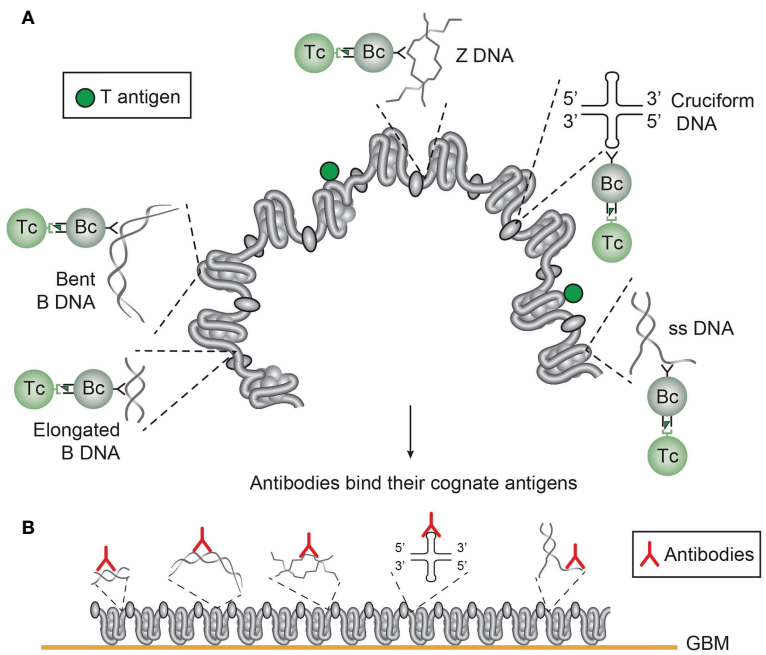

FIGURE 4 | Several unique DNA structures are accessible for B cells that present immunogenic peptides from non-self DNA-binding proteins (here exemplified by polyomavirus T antigen). As indicated, all these exemplified structures are solvent phase and accessible to B cells **(A)**. In this figure, polyomavirus T antigen is associated with chromatin in infected cells, and all DNA-specific B cells that bind DNA/chromatin-T antigen complexes present T antigen peptides to responsive cognate T helper cells. The cognate interaction of DNA structure-specific B cells and T antigen peptide-specific T helper cells promote production of a repertoire of DNA structure-specific antibodies. Since these antigens are accessible to B cell antigen receptors, circulating antibodies may have access to, and bind, the same specter of antigens in chromatin exposed in e.g. glomerulus basement membranes **(B)**. This model emerges from published experimental data on immunogenicity of the selected DNA structures as is discussed in the present text. This model is also valid in a true autoimmune context. Responsive histone-specific T cells may fully substitute T antigen-specific T cells. This will allow the same specter of DNA structure-specific antibodies (see text for details).

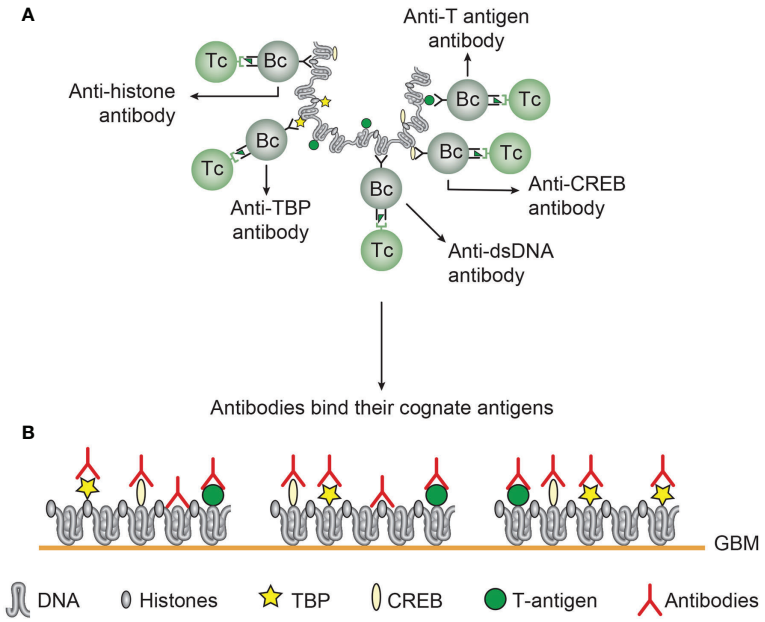

FIGURE 5 | *Chromatin structures and immunogenic chromatin-associated proteins are accessible and may stimulate B and T cells.* In the figure all the selected molecules are accessible to B cells. As in **(A)**, polyomavirus T antigen is associated with chromatin in virus-infected cells, and all chromatin-specific B cells may present T antigen peptides to cognate T helper cells. This results in production of antibodies against unique DNA and chromatin/protein structures. Since these antigens are accessible to B cell antigen receptors, circulating antibodies may have the potential to bind the same specter of accessible antigens in chromatin exposed in e.g. glomerulus basement membranes **(B)**. This demonstrates that chromatin-specific antibodies per se may have pathogenic potentials, and not only anti-dsDNA antibodies. This model emerges from experimental data (see text). [This figure is modified from: **Figure 5** in reference (35)].

In somewhat different, but important and thought-provoking studies, Pisetsky and co-workers investigated the autoimmunogenic properties of bacterial DNA (32, 90, 91). They took logic and important conceptual steps forwards by implementing CpG motifs as an additional (adjuvant-like) stimulation of the innate immune system (91–95).

Autoimmunity to DNA: The Possible Role of Secondary Necrotic Cells (SNECs), Microparticles, or Neutrophil Extracellular Traps (NETs)

Alternatively, reduced clearance of apoptotic cells and consequently accumulation of SNECs (96, 97), microparticles (68), or exposure of NETs (31, 66, 98), have over time been attributed central roles in promotion of autoimmunity to native or to apoptosis-related modified chromatin-associated proteins and dsDNA possibly involved in e.g. lupus nephritis (99–102). However, no formal experimental evidences are presented that anti-dsDNA antibodies are *de facto* induced by SNECs or NETs. It is problematic that the relevant literature is categorized over decades as hypothesis and theories studies [see e.g. (16, 31, 66)], but not funded on solid reproducible experimental data. Such structures may, nevertheless, induce immunity towards proteins that are modified in e.g. NETs (in an altered self context), and may have central pathophysiological roles as targets for relevant autoantibodies (31).

Cross-Stimulation of Anti-dsDNA Antibodies by Phospholipids, Peptides and Proteins

Non-DNA structures may promote production of anti-dsDNA antibodies [see e.g. (13, 65, 103–108)]. Such structures may encompass phospholipids that may share backbone structures similar to dsDNA (109), or peptides/protein structures with no apparent similarities to dsDNA (104, 108, 110, 111). A perfect example of evolution of anti-dsDNA antibodies that may have been promoted by a non-DNA structure is described by Wellman et al. (112). The IgG antibody with the heavy chain variable region in germline configuration did not bind DNA, while somatic mutations introduced during affinity maturation resulted in binding of the antibody to dsDNA.

However, critical questions must be raised in this context. No doubt that proteins or peptides may induce anti-dsDNA antibodies, but is this phenomenon exceptional? Have proteins and polypeptides the potential to induce antibodies against *all* different DNA structures and affinity maturate and converge specificity of the antibodies towards the manifold of DNA structures? Except for the Wellman-study, no published data yet provide answers to these questions.

These observations and discussions have till now not precipitated any conclusive evidence-based conclusions, although strong arguments can be raised that in sum support homologous stimulation of the immune system by native dsDNA

TABLE 3 | Central scientists and milestone studies of anti-dsDNA antibodies.

Autoimmunity towards dsDNA were after 1957 centered around SLE (30, 209). Its relation to infections, as described in 1938-1939 was over time neglected.
Winkenwerder et al. (4), **Sevag et al.** (2, 210), **Menzel et al.** (3)
•They described in 1938-1939 antibodies to DNA in bacterial infections.
•Their data challenge the dogma of anti-dsDNA antibodies as a central biomarker for SLE.
Ceppellini et al. (5), **Robbins et al.** (7), **Miescher et al.** (6), **Seligmann et al.** (8)
•Described in 1957 antibodies to DNA in SLE.
•Their discovery formed later the basis for the dogma of anti-dsDNA antibodies as a central biomarker for SLE.
Sercarz et al. (40, 86)
•Proposed the hapten-carrier model for B and T cell cooperation in autoimmunity. This concept had a considerable impact on experimental studies on immunogenicity of DNA.
Tonegawa et al. (17)
•Described in 1983 somatic mutations in the N-terminal part of the variable region of an antibody as a mechanism for generation of antibody diversity.
Hood et al. (18)
•They proposed a model for variable region gene rearrangement mediated by proteins which recognize the same conserved sequences adjacent to both light and heavy chain immunoglobulin gene segments.
•An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH.
Weigert et al. (211–213)
•He discovered a fundamental mechanism of B cell tolerance which he entitled *receptor editing*.
•Weigert was the first to describe immunoglobulin variable region somatic hypermutation which is an adaptive mechanism to increase avidity, and to converge specificity towards the immunogen.
Stollar et al. (10, 61, 70, 214)
Central pioneer on
•Immunogenic potential of DNA structures
•immunochemical characterization of DNA and
•genetical mapping of anti-DNA antibodies.
Schwartz et al. (215)
•Immunogenicity of DNA and anti-dsDNA antibodies, centralized around SLE
Isenberg et al. (216, 217)
•Clinical impact of anti-DNA antibodies, analyses of large SLE cohorts.
Tsokos et al. (30, 209, 218)
•Performed studies of cellular and molecular pathogenic processes of systemic lupus erythematosus (SLE).
•Central in the field of molecular abnormalities of immunity in SLE.
Pisetsky et al. (12, 219)
Studies on the immunological properties of DNA as related to two main topics:
•The induction of anti-DNA responses in systemic lupus erythematosus
•the stimulation of innate immunity by bacterial DNA.
Reizis et al. (16)
•The Reizis group provide strong arguments for the view that DNA may represent an epicenter in SLE as B cell antigen and pathogenic factor as extra-cellular target for the anti-dsDNA antibodies.
•They propose and provide data that DNase 1L3 prevents autoimmunity towards DNA.
Winkler et al. (112)
•Provided evidence that an affinity-maturated DNA specific autoantibody emerged from an antibody with undetectable affinity for DNA. The somatically mutated heavy chain variable region from the DNA-specific antibody was reverted by site-directed mutagenesis to germline configuration with loss of specificity for DNA. They made the important conclusion that affinity-maturated autoantibodies may develop during a normal immune response from non-autoimmune B cells. In light of high rates of infections their study may have high impact to understand origin of anti-dsDNA antibodies. This adds to data demonstrating that (nucleosomal) dsDNA also directly have immunogenic potential when complexed with an immunogenic carrier protein.
Other central contemporary scientists (14, 152, 187, 220–224)
•They have investigated origin, clinical and pathogenic impact of anti-DNA antibodies. They are all important and they are referred to in this study.

in complex with a T cell-specific immunogenic carrier protein as a central trajectory *in vivo* (13).

THE BIOLOGY OF UNIQUE FORMS OF DNA AND THEIR IMMUNOGENICITY *IN VIVO*

This discussion is based on the contributions provided by historically important scientific pioneers, their observations and consequent interpretations: Characterization of dsDNA and subsequent description of antibodies to dsDNA. A common thread leads from the revolutionary discoveries of DNA and its structures by Miescher et al. in 1871 (42), Levene et al. in 1903 (44), Chargaff et al. in 1950 (46), Franklin et al. in 1953 (49), Watson and Crick in 1953 (52, 53), up to studies of DNA´s structure and function in chromatin by groups of Olins and Olins (113), Kornberg (114, 115), Klug (116), Laskey (117), and others. They contributed to our understanding of structure and biology of the symbiosis of dsDNA and chromatin-associated regulatory proteins (see **Table 2**).

Franklin was the first to describe unique forms of DNA beyond its pure helix structure: The A and B DNA (49). The B DNA was later described as a dynamic bi-structural DNA shape: elongated (118, 119) or bent (120) B DNA, while ssDNA (121, 122), Z DNA (61), cruciform DNA (123), and other structures

were all described in context of specific functions of DNA (see detailed discussion below, **Figure 1**, and (124, 125).

In the history of "The anti-dsDNA antibody" few random attempts have been performed to determine whether all structural DNA-specificities are strongly associated with SLE. Thus, it is not established if unique anti-dsDNA antibody specificities are linked to distinct SLE classification criteria or even to non-SLE related disorders (13). These obvious problems are not considered in the recent expansion of the SLE classification criteria (54–56). In the following sections the roles of DNA structures, functions and their cognate antibodies will be summarized and discussed.

B DNA

Structure and Biology of B DNA

B DNA is the most disseminated DNA structure in the human genome. The fundamental composition (47, 48) and structure of the B form DNA as a right-handed double helix (49, 50, 126) reflect in many ways the basic B DNA in its relaxed low energy conformation. Changes in the B DNA structure reflect dynamic conversion of the basic structure into variants like ssDNA, Z DNA, cruciform DNA, bent DNA and others (see **Figures 1** and **4**). Such activation-related structures have their own, unique ability to induce highly specific immune responses, with relevance to the impact of anti-dsDNA in SLE and lupus nephritis.

The B DNA is reversibly transformed into two different B DNA structures with impact on specific immune responses: The elongated linker B DNA and the bent B DNA formed in the core nucleosome (for other structures, see below).

Elongated B DNA

Linker DNA is a stretched elongated B DNA. Its name defines its context, a link between core nucleosomes, shaping the electron microscopy picture of beads on a string (113). The histone H1 binds to linker DNA where DNA connects the fundamental chromatin units, the core nucleosomes. The role of histone H1 in chromatin is manifold, and H1 contributes to chromatin compaction (127). H1 is a central molecule that basically unmask DNA and contribute to regulation of transcription and other effects involving DNA (128, 129). Thus, H1 is highly mobile in the nucleus, which may indicate its strategic ability to expose B DNA to DNA regulatory proteins.

Bent dsDNA

As H1 slides along linker DNA, the histone octamer (two copies each of the four core histones H2A, H2B, H3 and H4) slides along B DNA and form bent DNA (120, 130–132), thus facilitating further effects of regulatory proteins like high-mobility group proteins to bend DNA into various degrees of flexible conformations (133–135). Studies on kinetoplast DNA [a network of circular DNA (136)] have demonstrated that certain sequences cause DNA to be highly bent, and that other sequences bend in response to binding of proteins (137). Thus, bent B DNA is a widely spread structure in chromatin, which may impact its immunogenic power.

B DNA structures undergo transformation between elongated and bent B DNA necessary to promote transcription and replication (132, 138, 139). The amount of bent DNA is therefore substantial (132). Summarizing this information, functional alterations of the DNA structure generate unique stimulators of the adaptive immune system in substantial amounts.

Spontaneous Production of Anti-B DNA Antibodies

The origin and clinical impact of anti-B DNA antibodies (termed anti-mammalian dsDNA antibodies in clinical and immunological contexts) have been difficult to understand. Therefore, immune responses towards B DNA has been, and is still being regarded as enigmatic. The reason for this derives from two problems: DNA immunogenicity, and affinity maturation of ongoing immune responses against peptides or phospholipids mimicking or apparently not mimicking DNA although stimulating to anti-B DNA antibody production.

In many lupus-prone murine models [for review, see e.g. (140, 141)], antibodies against B DNA appear spontaneously. They distinctively recognize elongated and/or bent dsDNA as in chromatin and kinetoplasts in different assay systems (57). These spontaneously produced antibodies are pathogenic (but not always)! by promoting lupus nephritis (65), dermatitis (142) and some forms of cerebral lupus (143, 144). The capacity of some anti-DNA antibodies to promote inflammation in the kidneys is more rigorously documented than in the skin or brain. The reason for this is the documented devastating effect exerted by the organ-selective silencing of the renal endonuclease DNase 1. This leads to a consequent accumulation of extra-cellular large chromatin fragments in glomerular matrices and membranes where they are targeted by anti-dsDNA antibodies [(65) see below]. 345-352

Experimental Production of Anti-B DNA Antibodies

Over the years, attempts to induce anti-B DNA antibodies have mostly failed [see e.g. (61, 76), reviewed in (13)]. Anti-DNA antibodies have been induced by other DNA structures like chemically modified DNA and synthetic polydeoxyribo-nucleotides that differ from native DNA [discussed in (70)]. After a period where B DNA was regarded as non-immunogenic, clear exceptions from these negative results have appeared.

The current contemporary view is that experimental induction of anti-B DNA operates according to mechanisms described above linked to autoimmunity or immunity against mammalian dsDNA. The early experiments were performed using new hapten-carrier principles: To engage T helper cells, a DNA binding peptide, Fus 1derived from Trypanosoma cruzii, induced in complex with mammalian B DNA strong anti-B DNA antibodies in non-autoimmune mice (14, 145). Immunoglobulin analyses demonstrated that the IgG heavy chain variable regions were structurally similar to those produced spontaneously in autoimmune (NZBxNZW)F1 mice

(63, 145). An analogous approach was independently developed using the DNA-binding polyomavirus BK large T antigen as carrier protein for the hapten-analogous B DNA. The *in vivo* generation of this hapten-carrier complex promoted production of lupus-like autoantibodies to mammalian dsDNA and to chromatin-associated proteins [(73, 84), and references herein]. Experimental induction of antibodies recognizing the kinetoplast DNA of Crithidia luciliae along with elongated B DNA in ELISA were observed in both the Fus 1-DNA and the T antigen-DNA models.

The T antigen model was principally confirmed in another experimental system. Dong et al. induced antibodies to p53 by immunizing non-autoimmune mice with purified p53-T antigen complex (146). These results demonstrate that infections, commonly encountered in SLE (32) and in cancers (77, 78) may be involved in systemic autoimmunity, and explain why anti-dsDNA antibodies principally cannot serve as a unique biomarker for SLE.

Pathogenic Impact of Anti-B DNA Antibodies

There is an international consensus that antibodies to dsDNA and to chromatin antigens have pathogenic potentials. There is, however, no consensus as to how and why these antibodies may be pathogenic (65). Two main directions in international science dominate the discussions: *i.* In context of lupus nephritis, antibodies bind chromatin fragments exposed in the mesangial matrix and in GBM [(147–150), discussed in (65)], or *ii.* Antibodies bind inherent matrix and GBM structures through cross-reactions [discussed in (151, 152)]. Antibodies against chromatin ligands *and* intrinsic glomerular constituents have been eluted from nephritic kidneys (153, 154). The main problem with those studies is that each of them claim to explain the nephritic potential of anti-dsDNA antibodies [discussed in depth in (33, 65)]. These contradictory results have not promoted critical, comparative studies. Before such studies are performed and interpreted, we will not reach consensus on which model(s) is (are) correct and which strategy for causal therapy may be developed (principally discussed below).

SSDNA

Structure and Biology of ssDNA

The ssDNA structure appears in two different contexts: *i.* as intended/not-intended denatured ssDNA in analytical contexts, or *ii.* related to stabilize transcriptionally active DNA (121, 122).

The ssDNA structure is not stable. Single-stranded DNA-binding proteins (SSB) hold the ssDNA intact and exposed during the course of its function: DNA transcription, recombination and repair (155), and to serve as template for opposite strand DNA synthesis [(156), for further reading, see e.g. (157, 158)]. Thus, ssDNA regions may be present in total cellular DNA at considerable amount, which may point at an immunogenic impact of ssDNA and a pathogenic impact of anti-ssDNA antibodies.

Immunity of ssDNA Regions

Anti-ssDNA antibodies may be induced *in vivo* when functional chromatin-associated ssDNA is presented to the immune system (13). Therefore, anti-ssDNA antibodies may bind ssDNA regions in chromatin fragments also when they are exposed in e.g. GBM and thereby promote renal antibody-mediated inflammatory events. This is substantiated, but not seriously considered, by the fact that anti-ssDNA antibodies can be detected in sera and renal eluates from SLE patients with lupus nephritis (153, 159, 160).

Spontaneous and Experimental Production of Anti-ssDNA Antibodies and Their Pathogenic Impact

Autoantibodies against ssDNA has been known for decades (13). They have been detected in SLE and other conditions (161), and they are readily induced experimentally (162–166). In one study from 1989, Vaishnav and Antony injected ssDNA in complex with a carrier protein (mBSA) and observed, as the first ever, appearance of anti-dsDNA antibodies (163). This study was at that time important and challenged the concept of non-immunogenicity of DNA including B DNA, but was not considered important. In later studies and discussions their results were regrettably neglected.

In my training, I was stressed to treat DNA as target in anti-dsDNA antibody assays with S1 nuclease to avoid detection of anti-ssDNA antibodies in clinical contexts (167). Therefore, antibodies against ssDNA regions have been disregarded in clinical contexts, although they have been detected in nephritic kidneys (153). Thus, also anti-ssDNA antibodies may affinity maturate and form high avidity antibodies with potential to promote lupus nephritis and dermatitis when chromatin fragments are exposed in situ.

Z DNA

Structure and Biology of Z-DNA

Z DNA is structurally and functionally integrated in the human genome (168) and is involved in various human diseases (see (169, 170) and references therein). Z-DNA is a left-handed, high energy supercoiled double helix, as opposed to the right-handed B-DNA helix. Physiologically, Z DNA forms *in vivo* and in cell cultures during transcription (171) as a result of torsional strain that depends on interaction of mobile polymerases and other proteins (172, 173). Since the placement of nucleosomes influences the binding of transcription factors, Z-DNA is thought to directly regulate the rate of transcription.

Z-DNA is reported to be formed in elongated B DNA and not associated with the core nucleosome unit, which are normally located after Z-DNA structures (174). Concerns have, however, been expressed by Mulholland et al. (175), who have demonstrated that Z DNA may also be formed in the core nucleosomal complex. This indicates that Z DNA may be more abundant in chromatin with an increased probability for immunogenicity and a pathogenic potential of anti-Z DNA antibodies. A pathogenic potential of anti-Z

DNA antibodies has not been proven by firm descriptive or experimental studies.

Spontaneous Production of Anti-Z DNA Antibodies

Specific anti-Z-DNA antibodies are associated with SLE (176, 177). Significant amounts of anti Z-DNA antibodies were found in SLE patients but not in other rheumatic diseases – analyses in infections or malignancies are, however, not reported. Highest levels of antibodies were associated with the most active stages of SLE.

Experimentally Induced Anti-Z DNA Antibodies

In the period when immunogenicity of DNA was a major problematic focus in clinically related immunology, B DNA was regarded as non-immunogenic (76, 178). In contrast, anti-Z DNA antibodies were readily induced by conventional immunization protocols (87, 179). These contrasting results were insightfully discussed (61), and subsequent experiments revealed that mBSA as carrier protein was functional for Z DNA, but not for B DNA although other carrier proteins had the potential to render B DNA immunogenic [see e.g. (14, 180)].

Anti-Z DNA Antibodies: Potential Pathogenic Impact

IgG antibodies to Z DNA are found in SLE, and they have been experimentally induced in non-autoimmune mice (see above). This confers to affinity maturated antibodies with potentially high avidity. When we consider the fact that Z DNA is involved in transcription and recombination, Z DNA may be abundantly exposed in chromatin, and also in chromatin fragments released and exposed *in situ* in e.g. GBM. This may open for a pathogenic potential for these antibodies (see a theoretical model discussed in **Figure 4**). If anti-Z DNA antibodies indeed are pathogenic has, however, not been investigated.

CRUCIFORM DSDNA

Structure and Biology of Cruciform DNA

Cruciform DNA is structurally different from B and Z DNA. Its formation requires that inverted sequences (palindromes) present in one strand is repeated on the other strand in opposite direction, thus allowing formation of hairpin or cruciform DNA structures. There is a minimum limit of the number of nucleotides in the inverted repeats to form a stable cruciform structure by negative DNA supercoiling. The cruciform structures are, like Z DNA, higher energy DNA structures [for details see (123, 181)].

Cruciform DNA structures are central in a wide range of biological processes, including replication, regulation of gene expression, nucleosome structure and recombination. Several regulatory proteins bind preferentially, but not exclusively to cruciform structures, and regulate homeostasis of the biological functions of DNA (123, 182, 183).

Spontaneously Produced Anti-Cruciform DNA Autoantibodies: Still Not Analyzed

Cruciform DNA-specific antibodies have not been reported in a clinical context, and no attempts to detect these antibodies in context of rheumatology or infectivity have been published.

Experimental Anti-Cruciform DNA Antibodies

Antibodies were induced experimentally in 1987 by Frappier et al. against a cruciform structure presented by a heteroduplex DNA molecule (184). Their well characterized monoclonal antibodies have later been used to study expression and biology of cruciform DNA (182, 185). Notably, also antibodies towards another complex form of DNA, quadruplex DNA, was generated from non-immunized motheaten mice (182, 185). This may indicate that anti-cruciform/anti-quadruplex antibodies may be formed in autoimmune phenotypes., although not investigated yet.

Possible Pathogenic Impact of Anti-Cruciform Autoantibodies—A Hypothesis

Pathogenic impact of anti-cruciform DNA antibodies has not been investigated. This is a consequence of the fact that there are no published reports on true autoimmune anti-cruciform antibodies linked to autoimmune diseases. However, when we consider the central functions of cruciform DNA in biology, and that cruciform DNA structures are abundant in chromatin, these structures are expected to be recognized by the cognate immune system.

UNIQUE DNA STRUCTURES AS STIMULATORS AND TARGETS FOR ANTIBODIES—A CONCLUSION

In this theoretical study, available information linked to immune responses to various structural forms of DNA is contemplated and interpreted: *i.* the role of infection in initiation of anti-DNA production, *ii.* the possible influence of microbiota that turns out to be unbalanced in lupus (186), *iii.* the molecular and structural properties of ssDNA/dsDNA in chromatin and their interaction with B cells (afferent immunogenic stimulus) and anti-DNA antibodies (efferent pathogenic stimulus) (12, 13).

DNA as a native structure is immunogenic and auto-immunogenic *in vivo*. The emerging antibodies do not care what initiates them but their existence is undeniable. Their clinical impact is, however, tremendous. In this picture chromatin exposed *in situ* is a common denominator as target for the whole specter of induced anti-DNA/anti-chromatin antibodies. Cross-reactions with membrane ligands play assumedly an inferior pathogenic role, because it is unlikely that the whole universe of DNA/chromatin-specific autoantibodies cross-react with the small repertoire of protein ligands that make up matrices and membranes.

WHICH ANTI-CHROMATIN ANTIBODIES ARE NEPHRITOGENIC—A HYPOTHESIS

In this section anti-DNA antibodies as principal initiators of lupus nephritis will be discussed. Secondary inflammatory

mediators and processes will not be emphasized here. Anti-dsDNA antibodies (in the contexts discussed above) are among several anti-chromatin antibodies involved in lupus nephritis. We still, however, do not agree on the nature of inducers and glomerular targets of the anti-dsDNA antibodies - whether dsDNA (11, 149, 150, 187), nucleosomes or apoptotic chromatin (147, 148, 188–190) or non-dsDNA cross-reactive structures [see e.g. (13, 65, 103–108)]. This signifies that we today are not able to explain the nephritic process. We can, however, deduce some basic principles and propose some data-based paradigms.

If autoantibodies bind directly to intrinsic ligands in the matrix or GBM, then this mode is equivalent to a Type II antibody-dependent inflammation. If autoantibodies form immune complexes with chromatin fragments *in situ* or in circulation prior to deposition in e.g. GBM, this mode is consistent with a Type III immune complex-mediated inflammation [for review, see (191)].

The chromatin model is complex, and involve a spectrum of chromatin-specific antibodies as indicated in **Figures 4** and **5**. Immunogenic chromatin stimulates production of different anti-chromatin antibodies. The model in **Figure 4** informs about how immunogenic DNA structures may stimulate production of cognate DNA-specific anti-DNA autoantibodies. On the other hand, immunogenic chromatin has the potential to promote production of a spectrum of anti-chromatin antibodies, like DNA, histones, transcription factors ((84, 180), **Figure 5**). Collectively. these antibodies have not been seriously considered as individual promoters of lupus nephritis, with the exception of "The anti-dsDNA antibody". Since all these antibodies, with the exception of anti-cruciform antibodies (not analyzed yet), are induced in SLE (57, 58, 176), the DNA structures must have been accessible to B cells. Then it is likely that the antibodies recognize the same universe of DNA structures and chromatin-associated proteins (**Figures 4** and **5**) when chromatin fragments are exposed in e.g. glomeruli.

Although the spectrum of chromatin autoantibodies may bind chromatin in situ, this does not necessarily imply that each specificity is individually nephritogenic since the density of each target molecule may be too low to initiate e.g. complement activation. However, they may all contribute to the nephritogenic process in concert with other chromatin-specific antibodies. This hypothesis is consistent with the fact that non-anti-DNA IgG antibodies are eluted from lupus nephritic kidneys [(153), discussed in (153, 159, 160)].

This process is also consistent with previous data demonstrating that *in vivo*-bound IgG antibodies co-localize with electron-dense chromatin fragments in the mesangial matrix and in GBM [discussed in (65, 148, 188, 192, 193)]. In addition, antibodies in glomerular eluates demonstrated higher intrinsic affinity for DNA compared to autologous serum antibodies (194).

Data that emerge from these analyses were not consistent with antibody-binding to membrane constituents, as e.g. laminin antibodies added to the sections bound normal GBM and not electron-dense chromatin fragments [see **Figure 3** in reference (65)]. Collectively, these data favor a Type III inflammatory model involved in lupus nephritis, although autoimmune T cells may also be involved (195).

The arguments favoring Type II and Type III nephritis derive from studies over decades, preliminary conclusions and from a lack of international consensus (see e.g. contrasting viewpoints in (13, 33, 34, 65, 149, 196). The two models have their advocates, but still a comparative study is awaited.

Serologically Active, Clinically Quiescent Patients—Why Are Not Anti-DNA Antibodies Always Pathogenic?

This question relates to the statement that describes "serologically active, clinically quiescent" patients (197, 198). The term describes patients that have long-lasting high levels of anti-DNA antibodies without experiencing any inflammatory flare of their disease. This is in fact a core problem aimed to understand the complexity of the pathogenic impact of anti-DNA antibodies: When and how is the anti-dsDNA antibody pathogenic?

Two explanations may allow an understanding of this apparent paradox. Either, the antibodies do not possess an *a priori* nephritogenic potential just because of their presence. This implies that the target(s) for the anti-DNA antibodies is not constitutively expressed and exposed *in vivo*, i.e. they are not an intrinsic part of e.g. GBM. Only in situations where e.g. chromatin accumulate extra-cellularly, the antibodies find their partner and upon binding promote inflammation (188, 199, 200). The loss of DNase 1 endonuclease activity in kidneys but not in other organs (201) may also explain why kidneys are more affected by anti-DNA antibodies as DNase 1 deficiency promotes glomerular exposure of chromatin fragments (202).

The alternative explanation could be that the antibodies account for inflammation if they cross-react with intrinsic membrane constituents like laminin, collagen or entactin. Without cross-reactive potential the anti-DNA antibodies behave as a clinical epiphenomenon [see above, discussed in detail in (13, 65)]. These two models have one perspective in common: They both provide a fair explanation as to why anti-DNA antibodies are not always pathogenic and why patients may be "serologically active, clinically quiescent". A comparative research initiative to solve the real process is an important challenge to us.

Pathogenicity of Anti-DNA Antibodies—Does Immunoglobulin Class Matter?

A potentially important pathogenic aspect of anti-DNA antibodies adheres to the impact of their immunoglobulin class (203). Although IgM antibodies possess low intrinsic affinity their avidity is generally high. This, and the fact that a single IgM molecule is a potent complement activator (204, 205), whereas single IgG molecule hardly activate complement (206), could indicate that IgM anti-DNA antibodies are more pathogenic than IgG. The opposite seems to be true (203). In their study, Wang and Xia conclude that IgG but not IgM correlate with activity of human lupus nephritis (203). Most pathogenic

antibodies are of the IgG class in SLE patients (207). According to e.g. Gronwall et al., IgM antibodies correlated with enhanced removal of apoptotic material and reduced activity of lupus erythematosus (208). These observations implicate that IgG anti-dsDNA antibodies exert a stronger pathogenic impact than IgM antibodies with corresponding DNA specificity.

CONCLUDING REMARKS

In current criteria to classify SLE, "The anti-dsDNA antibody" possesses an archaic position. "The anti-dsDNA antibody"-terminology is neither founded on current knowledge, nor on established insight into unique DNA structures related to distinct DNA-associated operations. *In that sense, any structure-specific anti-dsDNA antibody, detected in any assay using any DNA molecule is valid as a criterium for SLE.* A simple hypothesis - not examined yet - may be quite obvious: The more readily an antibody is induced, the less specific is the antibody for SLE, but may appear in divergent conditions. In other words, antibodies against ssDNA or Z DNA may be less specific for SLE than anti-bent B DNA (extrapolated from data discussed above and in **Figure 2**).

Anti-DNA antibodies are essential in clinical medicine, and particularly in SLE. The autoantibodies are, although as an interim measure, used to diagnose SLE and to classify SLE patients. The antibodies are a central pathogenic factor, and they promote lupus nephritis alone or in combination with other anti-chromatin antibodies. What we need to comprehend from this enormous amount of data and knowledge is to understand what makes the anti-DNA antibody pathogenic - and in which context.

ETHICS STATEMENT

The present manuscript is a review on murine and human SLE and lupus nephritis. 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 has approved it for publication.

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Emerging Molecular Markers Towards Potential Diagnostic Panels for Lupus

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Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease which can affect various tissues and organs, posing significant challenges for clinical diagnosis and treatment. The etiology of SLE is highly complex with contributions from environmental factors, stochastic factors as well as genetic susceptibility. The current criteria for diagnosing SLE is based primarily on a combination of clinical presentations and traditional lab testing. However, these tests have suboptimal sensitivity and specificity. They are unable to indicate disease cause or guide physicians in decision-making for treatment. Therefore, there is an urgent need to develop a more accurate and robust tool for effective clinical management and drug development in lupus patients. It is fortunate that the emerging Omics have empowered scientists in the discovery and identification of potential novel biomarkers of SLE, especially the markers from blood, urine, cerebrospinal fluids (CSF), and other bodily fluids. However, many of these markers have not been carefully validated for clinical use. In addition, it is apparent that individual biomarkers lack sensitivity or specificity. This review summarizes the sensitivity, specificity and diagnostic value of emerging biomarkers from recent studies, and discusses the potential of these markers in the development of biomarker panel based diagnostics or disease monitoring system in SLE.

Keywords: omics, biomarker panel, SLE, disease monitoring, lupus nephritis, neuropsychiatric lupus (NPSLE)

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1 INTRODUCTION

A Biomarker is generally defined as a measurable physical, genetic, biological, or biochemical factor that can reflect normal or abnormal biological process when altered. A biomarker should indicate changes that associate with the pathological features and/or presentations of a disease with diagnostic or prognostic potential. They are a crucial component of personalized medicine (1). An optimal biomarker with good sensitivity and specificity can be readily measured in patient-derived samples that are ideally obtained in a minimally invasive way—such as blood, urine or other body fluids. The tests for biomarkers should be reliable, reproducible, and affordable.

Systemic lupus erythematosus (SLE) is a complex autoimmune disease that can affect multiple organ systems and exhibits various signs and symptoms, hence posing significant challenges in diagnosis and treatment. SLE is defined by the detection of elevated autoantibodies in circulation (2) along with abnormal presentation of B and T lymphocytes (3). SLE etiology is multifactorial,

contributed by environmental, stochastic, and genetic factors (4). Kaul A et al. stated "Genetic interactions along with environmental factors, particularly UV light exposure, Epstein-Barr virus infection, and hormonal factors might initiate the disease, resulting in immune dysregulation at the level of cytokines, T cells, B cells and macrophages" (5). Consequently, this results in a breach in immune tolerance where the T cells identify self-antigens and deliver assistance to the auto-reactive B cells. These B cells generate a diversified repertoire of autoantibodies. The SLE autoantibodies are able to minimize the extent of organ damage by forming immune complexes by binding to host tissue which are then deposited in vascular tissue resulting in the activation of the immune system. SLE affects various organs, however the most common ones are the kidneys, lungs, skin, joints, components of blood, as well as the central nervous system. Disease severity, treatment response, as well as the array of clinical involvement differs from patient to patient posing considerable challenges in the diagnosis and control of SLE (6).

At present, the criteria for diagnosis of lupus is based primarily on the presence of clinical manifestations in the form of joint pain, skin rashes, glomerular nephritis, symptoms of neuropsychiatric illnesses as well as the results of lab tests such as the presence of antinuclear antibodies, ANA and anti-dsDNA antibodies in particular. The EULAR/ACR classification criteria for SLE (2019) necessitates at least one positive ANA for entry (6), followed by additive weighted criteria which is grouped in seven clinical domains (constitutional, hematological, neuropsychiatric, mucocutaneous, serosal, musculoskeletal, and renal) and three immunological domains (antiphospholipid antibodies, complement proteins, SLE-specific antibodies) that are weighted from 2 to 10 points. Patients that accumulate 10 or more points are classified. In the validation cohort, the new criteria had a sensitivity of 96.1% and specificity of 93.4% (6).

However, currently accessible laboratory markers for SLE diagnosis are suboptimal. Such is the case for the ANA test which has high overall sensitivity (94%) but comparatively low specificity (61%) (7, 8). On the other hand, anti-dsDNA and anti-Sm antibody have good specificity for SLE but low sensitivity as a result of its transient presence (8). To achieve better therapeutic outcomes, it is necessary to continuously assess and monitor the disease progression as well as predict the future disease course. There is a need for more accurate and robust biomarkers for SLE to monitor the disease progression, evaluate treatment response, and predict future flares in an organ-specific manner. As outlined in **Figure 1**, the development of biomarkers or a biomarker panel for predicting lupus flare-ups include patient recruitment, sample collection, biobanking, Omics-based biomarker discovery, statistical and bioinformatics analysis of the potential biomarkers, validations studies using a cross-sectional cohort and a longitudinal cohort, ranking of biomarker performance and selection of biomarker panel using mathematical models and machine learning, clinical trials of biomarker panel, and the development of biomarker panel based point-of-care devices for disease monitoring of lupus patients. This review includes a summary of the recent findings of biomarkers in SLE, and a discussion of their advantages and limitations, especially their potential utility in the future of lupus treatment. We reviewed the biomarkers derived from biological fluids including serum or plasma, CSF, and urine based on sensitivity, specificity and Area-under-the-curve (AUC) as demonstrated by Receiver Operating Characteristic (ROC) curve analysis.

In this review, by using the following keywords: "SLE" or "Systemic lupus erythematosus", "detection" or "diagnosis", "biomarker" or "marker" and "AUC" or "ROC" to search the Pubmed, we retrieved "255" relevant research articles. Among these, we filtered out 193 papers which didn't include an analysis of biomarker performance such as sensitivity, specificity and statistical significance (p-value), or they did not satisfy the following criteria: the candidate biomarkers exhibited an overall AUC > 0.8 with p-value <0.05 and sample size > 10 per group. The final 62 papers were selected to perform further comparison analysis on those promising biomarker candidates as presented in **Tables 1**–**4**.

2 BIOMARKERS IN SLE

Manifestations of SLE are linked with various autoantibodies that result in immune complex formation, deposition, and other immune system processes. The clinical presentation as well as pathogenesis is complex making SLE challenging to understand and define (91). According to the 2019 European League Against Rheumatism/American College of Rheumatology, the classification criteria for SLE had a sensitivity of 96.1% and a specificity of 93.4% using positive ANA as an entry criteria, weighted criteria in 7 clinical areas (neuropsychiatric, hematologic, constitutional, mucocutaneous, serosal, musculoskeletal and renal), 3 immunologic domains (antiphospholipid antibodies, low complements, anti-Sm, and anti-dsDNA as SLE-specific antibodies), and a classification threshold score of ≥10 (out of a theoretical maximum of 51) (6).

ANA has a long history serving as a classical clinical marker for the detection and screening of autoantibodies in autoimmune diseases including SLE, however, the sensitivity as well as accuracy of the ANA tests in diagnosis is not satisfactory due to false positives and negatives in previous reports (92–95). Therefore, standardization of ANA-based diagnostic tests in autoimmune diseases are highly recommended (92, 96), including the integration of immunofluorescence ANA (IFA) test with solid phase assays (SPA) such as bead-based high-throughput and/or multiplexing assays (92, 96). Previous studies established that complement components and anti-dsDNA antibody levels have diagnostic, prognostic and predictive values for SLE even before the first clinical signs of disease exacerbation (97–99).

2.1 Autoantibody Markers in SLE

Autoantibodies are a major clinical manifestation and the first serological marker of SLE. In **Tables 1A, B**, we summarized autoantibody biomarkers included in the SLE Classification

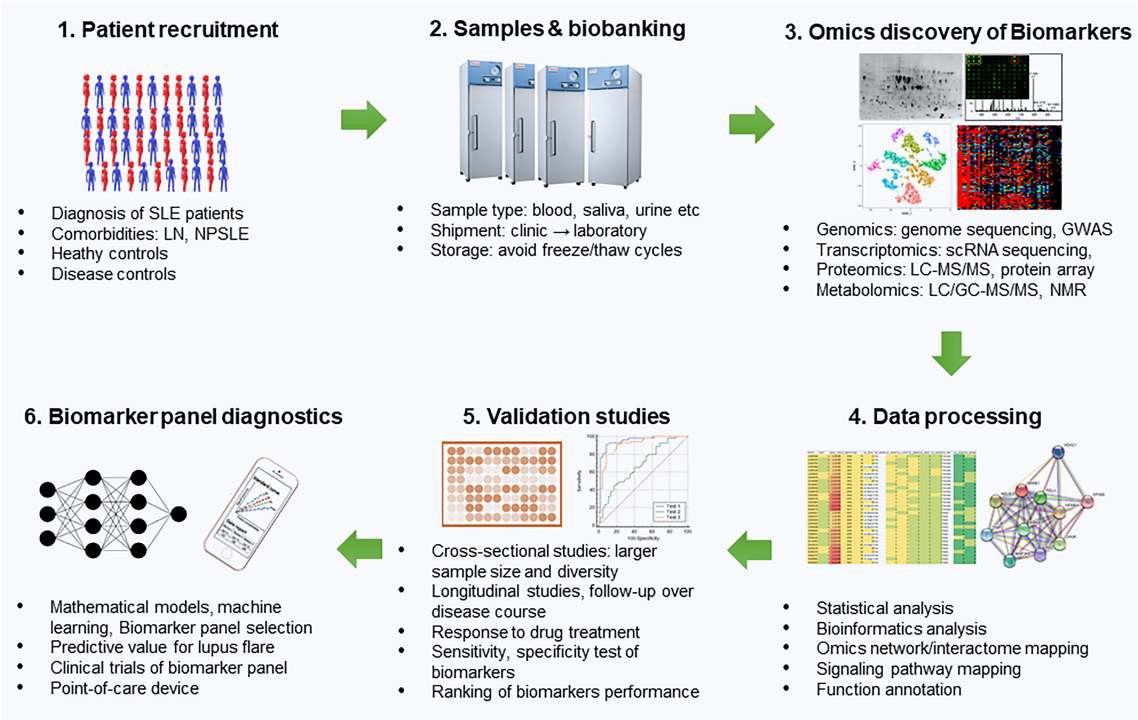

FIGURE 1 | Development of Biomarker panels for predicting lupus flares. This includes patient recruitment, sample collection, biobanking, Omics-based biomarker discovery, statistical and bioinformatics analysis of the potential biomarkers, validation studies using a cross-sectional cohort and a longitudinal cohort, ranking of biomarker performance and selection of biomarker panel using mathematical models and machine learning, clinical trials of biomarker panel and the development of biomarker panel based point-of-care devices for disease monitoring of lupus patients. SLE, systemic lupus erythematosus; LN, lupus nephritis; NPSLE, neuropsychiatric SLE; GWAS, genome-wide-association studies; scRNA, sequencing single cell; RNA, sequencing; LC, liquid chromatography; MS, mass spectrometry; NMR, Nuclear magnetic resonance.

Criteria as well as recently discovered novel autoantibody biomarkers in SLE. A systematic review of Medline, Embase, and the Cochrane database identified 13,080 patients from 64 studies with ANA reported by immunofluorescence on HEp-2 cells. A meta-regression of the ANA operating characteristics found a sensitivity of 97.8% for ANA ≥ 1:80, supporting the use of ANA as an entry criterion (100). Interestingly, about 180 antibodies against various autoantigens were identified in SLE patients which may also be able to indicate comorbidities (2). When using healthy controls and other disease controls, the specificity of anti-dsDNA in diagnosing SLE reached as high as 100% and 97%, respectively (101). The specificity of anti-Sm was 100% in the diagnosis of SLE (14). A high titer of anti-Sm antibody is highly SLE-specific despite the fact that low-titer anti-Sm antibodies in ELISA have been reported in other diseases (102).

In addition to the above autoantibodies which have been included in the criteria, there are more emerging autoantibodies that have demonstrated potential as biomarkers of SLE. An IgG autoantibody panel against six extractable nuclear antigens (ENA): SS-A (Ro 52, Ro 60), SS-B, Sm, RNP/Sm, Scl-70 and Jo-1, namely "ENA-6 Profile" is beneficial for the diagnosis of systemic autoimmune rheumatic diseases (14). The results revealed anti-Sm/RNP as an important marker for the diagnosis of SLE (AUC = 0.942) with 75% sensitivity and 100% specificity, anti-Jo-1 (AUC = 0.915) with 83% sensitivity and 90% specificity, anti-Scl-70 (AUC = 0.899) with 96% sensitivity and 80% specificity (14). A peptide array screening revealed 4 autoantibodies that were bound by acidic ribosomal phosphoprotein (P0)-4, acidic ribosomal phosphoprotein (P0)-11, DNA topoisomerase 1 (full length)-1, and U1-SnRNP 68/70 KDa-1, respectively. The AUC for diagnosing SLE based on these peptides were 0.91, 0.90, 0.93, and 0.91, respectively (10). Serum anti-collectin11 levels was significantly higher in the SLE group and the AUC was 0.806 for the diagnosis of SLE. Additional analysis showed that the positivity rate of anti-collectin11 was very high in SLE patients for whom both anti-dsDNA and anti-Sm antibody were negative. The nervous system and gastrointestinal system involvement are most common in the patients with positive anti-collectin11 (9). A study revealed that serum anti-ribosomal P protein antibody (anti-P) was positive in 38 out of 102 SLE patients (37.3%), and the specificity of anti-P was 96.1% (103). Another study revealed that the specificity and sensitivity of anti- P for SLE diagnosis were 99.4% and 14.2%, respectively in Caucasians, who were generally associated with lower anti-Rib-P antibody levels (104). IgG autoantibodies to histones H4 (HIST1H4A), H2A type 2-A (HIST2H2AA3) and

TABLE 1A | Emerging diagnostic markers of SLE.

Marker	Specimen	Number	Method	Sensitivity	Specificity	AUC	p-Value	Reference
Anti-collectin 11	serum	30/90(SLE/NSLE)	ELISA	n/a/	n/a/	0.806	P<0.001	(9)
Anti -(P0)-4	serum	50/25#	Protein array	n/a/	n/a/	0.91	P < 0.05	(10)
Anti (P0)-11	serum	50/25#	Protein array	n/a/	n/a/	0.90	P < 0.05	(10)
Anti-DNA topoisomerase 1	serum	50/25#	Protein array	n/a/	n/a/	0.93	P < 0.05	(10)
Anti U1-SnRNP 68/70	serum	50/25#	Protein array	n/a/	n/a/	0.91	P < 0.05	(10)
Anti HIST1H4A-IgG	serum	153/81#	ELISA	95%	90%	0.97	p<0.001	(11)
Anti-alpha-1,6-glucan	serum	30/30#	ELISA	93.3%	73.3%	0.863	p = 0.000	(12)
Anti-Tyro3	serum	70/70#	ELISA	n/a/	n/a/	0.871	p < 0.0001	(13)
ENA6 sm	serum	30/30#	ELISA	70%	100%	0.844	P < 0.001	(14)
Sm/RNP	serum	30/30#	ELISA	75%	100%	0.942	P < 0.0005	(14)
Jo-1	serum	30/30#	ELISA	83%	90%	0.915	P < 0.0005	(14)
SCL-70	serum	30/30#	ELISA	96%	80%	0.899	P < 0.0005	(14)
Angiostatin	urine	100/21#	ELISA	n/a/	n/a/	0.93	P<0.0001	(15)
BCDF	serum	36/24#	ELISA	80.6%	70.8%	0.861	p < 0.001	(16)
C3dg	plasma	169/170#	ELISA	84%	94%	0.96	p < 0.001	(17)
C3dg/C3	plasma	169/170#	ELISA	67%	97%	0.89	p < 0.001	(17)
Cyr61	serum	110/100#	ELISA	n/a/	n/a/	0.830	P < 0.001	(18)
FAS	serum	28/9#	Protein array	n/a/	n/a/	0.91	P < 0.01	(19)
IFI27	blood	61/20#	PCR	n/a/	n/a/	91.08	P < 0.01	(20)
IGFBP2	serum	28/9#	Protein array	n/a/	n/a/	0.97	P < 0.01	(19)
IgM	serum	36/24#	ELISA	97.2%	87.5%	0.902	p < 0.001	(16)
MMP-9	serum	36/30#	ELISA	97.2%	n/a/	0.984	P<0.001	(21)
MMP10	serum	28/9#	Protein array	n/a/	n/a/	0.91	P < 0.01	(19)
MLKL	blood	59/30#	PCR	81.36%	93.3%	0.928	P < 0.05	(22)
OPN	serum	28/9#	Protein array	n/a/	n/a/	1.00	P < 0.01	(19)
S100A4	plasma	52/43#	ELISA	95.5%	93.0%	0.989	P < 0.001	(23)
S100A12	plasma	52/43#	ELISA	70.5%	83.7%	0.807	P < 0.001	(23)
Sema3A	serum	170/150#	ELISA	80.6%	77.5%	0.876	P<0.01	(24)
suPAR	plasma	89/29#	ELISA	82.02%	79.31%	0.85	p = 0.0001	(25)
Siglec5	serum	28/9#	Protein array	n/a/	n/a/	0.96	P < 0.01	(19)
sTNFR1	serum	28/9#	Protein array	n/a/	n/a/	0.99	P < 0.01	(19)
sTNFRII	serum	28/9#	Protein array	n/a/	n/a/	1.00	P < 0.01	(19)
GAS5	plasma	163/80#	qRT-PCR	65.03%	93.75%	0.819	P = 0.003	(26)
circPTPN22	PBMCs	49/37#	qRT-PCR	n/a/	n/a/	0.918	P < 0.001	(27)
circRNA407176	PBMCs	122/102 #	qRT-PCR	76.90%	76.90%	0.806	P<0.001	(28)

#SLE vs health controls; SLE/NSLE, SLE vs rheumatoid arthritis (RA); primary Sjogren's Syndrome (SS) and healthy control (HC); n/a, data not available.

TABLE 1B | Emerging activity markers of SLE.

Marker	Specimen	Number	Method	Sensitivity	Specificity	AUC	p-Value	Reference
Ang2	serum	43/30(A/S)	ELISA	81%	89%	0.88	P < 0.001	(29)
CXCL13	serum	36/18(A/S)	ELISA	n/a/	n/a/	0.829	P<0.001	(30)
CXCL13	serum	50/30(A/S)	ELISA	100%	96%	0.989	P < 0.01	(31)
Galectin-9	serum	50/27(A/S)	ELISA	84%	72%	0.84	P < 0.001	(32)
IP-10	serum	27/19(A/S)	ELISA	81.5%	73.7%	0.807	p<0.0001	(33)
IL-17	serum	72/70(A/S)	ELISA	93.3%	92.9%	0.95	P<0.001	(34)
IL-6	serum	72/70(A/S)	ELISA	90.%	90.5%	0.93	P<0.001	(34)
PGLYRP2	serum	30/15(A/S)	ELISA	n/a/	n/a/	0.841	P < 0.01	(35)
PTX3	plasma	64/60(A/S)	ELISA	100%	80%	0.92	P < 0.05	(36)
sTim-3	serum	93/22(A/S)	ELISA	75.3%	81.8%	0.85	p<0.0001	(37)
miR-181a	serum	64/36(A/S)	qRT-PCR	n/a/	n/a/	0.885	P < 0.05	(38)
miR-203	serum	64/36(A/S)	qRT-PCR	n/a/	n/a/	0.843	P < 0.05	(38)
circ_0082689	PBMCs	24/114(A/S+H)	qRT-PCR	87.5%	89.1%	0.913	<0.0001	(39)
circ_0082688	PBMCs	24/114(A/S+H)	qRT-PCR	91.6%	80%	0.924	<0.0001	(39)

A/S, active SLE vs stable SLE; A/S+H, active SLE vs (stable SLE, HC); n/a, data not available.

H2A type 2-C (HIST2H2AC) were analyzed in 153 SLE patients and 81 healthy controls and the results showed that HIST1H4A-IgG was shown to be the marker with the best individual diagnostic performance for SLE vs healthy control (AUC = 0.97, sensitivity of 95% at 90% specificity) (11). Another study demonstrated that SLE patients displayed a higher reactivity with the modified equivalent of histone peptides. Reactivity with H4pac showed both a high sensitivity (89%) and specificity (91%) for SLE, while H2Bpac exhibited a high specificity (96%) but lower sensitivity (69%). Reactivity with H3pme appeared to not be specific for SLE. Anti-

TABLE 2 | Molecular markers of lupus nephritis.

Marker	Specimen	Method	Number	Sensitivity	Specificity	AUC	p-Value	Study
Anti-dsDNA	Serum	IDIM	16/25	56.25%	88%	0.705	p =0.0294	(40)
Anti-dsDNA	Serum	ELISA	227/53	65%	65%	0.75	p< 0.001	(41)
Anti-nucleosome	Serum	IDIM	16/25	87.5%	75%	0.807	p =0.0012	(40)
Anti-C1q	Serum	IDIM	16/25	68.75%	84%	0.843	p =0.003	(40)
AαA	Serum	ELISA	40/40	60%	90%	0.701	P=0.001	(42)
Anti-α-enolase	Serum	ELISA	144/70	82.2%	90.5%	0.809	P=0.004	(43)
AGP	Urine	ELISA	98/30	n/a	n/a	0.87	P<0.02	(44)
Ang-2	Serum	ELISA	60/21	58.1%	90.5%	0.748	p=0.002	(45)
Angiostatin	Urine	ELISA	227/53	82%	80%	0.87	P< 0.001	(41)
Angiostatin	Urine	ELISA	42/12	n/a	n/a	0.97	P<0.001	(46)
APRIL	Urine	ELISA	46/15	n/a	n/a	0·781	P < 0·05	(47)
APRIL	Serum	ELISA	47/27	65%	87.5%	0.713	P<0.05	(48)
BAFF	Urine	ELISA	46/15	n/a	n/a	0·825	P < 0·05	(47)
C24:1Cer	Plasma	LC-MS/MS	46/36	n/a	n/a	0.86	P=0.0001	(49)
C24:1Cer	Serum	LC-MS/MS	46/36	n/a	n/a	0.92	P=0.0001	(49)
C3	Urine	ELISA	227/53	73%	74%	0.82	p< 0.001	(41)
C4d	Serum	ELISA	98/77	79%	58%	0.68	P=0.003	(50)
Ceruloplasmin	Urine	ELISA	98/30	n/a	n/a	0.73	P<0.05	(44)
Ceruloplasmin	Urine	ELISA	76/44	n/a	n/a	0.86	p<0.001	(51)
CXCL4	Urine	ELISA	227/53	61%	63%	0.64	P=0.003	(41)
DKK-1	Serum	ELISA	111/70	77.4%	42.5%	0.783	p = 0.045	(52)
Eotaxin	Serum	Milliplex map	80/40	n/a	n/a	0.777	P < 0.001	(53)
HE4	Serum	ELISA	44/30	81.8%	53.3%	0.714	P < 0.05	(54)
HE4	Serum	ELISA	209/32	76.8%	91.1%	0.878	P < 0.001	(55)
IGFBP-2	Serum	ELISA	87/20	n/a	n/a	0.97	P < 0.0001	(56)
IL-17	Serum	ELISA	80/20	n/a	n/a	0.91	P < 0.001	(57)
IL-17	Urine	ELISA	50/20	66.7%	72%	0.717	P=0.006	(58)
IL-23	Serum	ELISA	80/20	n/a	n/a	0.78	P < 0.01	(57)
IP-10	Serum	ELISA	78/58	n/a	n/a	0.77	p= 0.03	(59)
L-PGDS	Urine	ELISA	98/30	n/a	n/a	0.79	P<0.009	(44)
MCP-1	Urine	ELISA	121/20	n/a	n/a	0.75	p < 0.01	(60)
MCP-1	Serum	ELISA	121/20	n/a	n/a	0.43	P<0.001	(60)
MCP-1	Urine	ELISA	47/53	90%	79%	0.87	<0.001	(61)
MCP-1	Urine	ELISA	78/58	93.3%	53.1%	0.78	p= 0.03	(59)
MCP-1	Urine	ELISA	50/20	76.9%	80%	0.869	P=0.000	(58)
NGAL	Urine	ELISA	54/36	98%	100%	0.997	P < 0.001	(62)
NGAL	Urine	ELISA	34/12	70.8%	87.5%	0.755	P = 0.013	(63)
NGAL	Urine	ELISA	54/36	98%	100%	0.997	p < 0.001	(62)
NGAL	Urine	ELISA	50/20	79.5%	80%	0.875	P=0.000	(58)
OPG	Urine	ELISA	58/63	n/a	n/a	0.72	p < 0.001	(64)
OX40	Blood	FC	40/20	90%	70%C	0.90	P<0.01	(65)
OX40L	Serum	ELISA	40/20	80%	60%	0.71	P<.05	(65)
PGRN	Urine	ELISA	154/71	100%	100%	1.000	P < 0.001	(55)
PGRN	Serum	ELISA	154/71	60.5%	100%	0.877	P < 0.001	(55)
Plasmin	Urine	ELISA	113/41	100%	69.9%	0.86	p< 0.001	(66)
sICAM-1	Urine	ELISA	92/20	94.5%	78.9%	0.874	P<0.001	(67)
TGF-1	Urine	ELISA	50/20	64%	68%	0.665	P=0.038	(58)
TRAF6	Serum	qPCR	128/30	n/a	n/a	0.897	P<0.001	(68)
Transferrin	Urine	ELISA	98/30	n/a	n/a	0.84	P<0.05	(44)
Transferrin	Urine	ELISA	76/44	n/a	n/a	0.84	p<0.001	(51)
TWEAK	Urine	ELISA	70/20	62.22%	93.33%	0.815	p < 0.0001	(69)
VCAM1	Urine	ELISA	227/53	66%	69%	0.73	p< 0.001	(41)
VCAM-1	Urine	ELISA	42/12	n/a	n/a	0.98	P<0.001	(46)
VCAM-1	Urine	ELISA	92/20	98.2%	66.7%	0.882	P<0.001	(67)
β2-MG	Urine	Immunoturbidimetry	144/70	81.8%	90.0%	0.845	P=0.001	(43)
miR-125a	plasma	qRT-PCR	26/26	92%	34%	0.67	P=0.048	(70)
miR-142-3p	plasma	qRT-PCR	26/26	80%	55%	0.62	P=0.185	(70)
miR-146	plasma	qRT-PCR	26/26	56%	96%	0.75	P=0.005	(70)
miR-155	plasma	qRT-PCR	26/26	88%	67%	0.82	p< 0.001	(70)
MiR-29c	Urine	RT-PCR	32/20	94%	82%	0.946	P < 0.001	(71)
miR-21	Plasma	qPCR	26/26	n/a	n/a	0.912	P < 0.001	(72)
miR-146a	PBMCs	qRT-PCR	128/30	n/a	n/a	0.821	P<0.001	(68)
miR-200b-5p	plasma	qRT-PCR	101/100	n/a	n/a	0.748	p < 0.001	(73)

(Continued)

TABLE 2 | Continued

Marker	Specimen	Method	Number	Sensitivity	Specificity	AUC	p-Value	Study
miR-141-5p	plasma	qRT-PCR	101/100	n/a	n/a	0.748	p<0.001	(73)
miR-200c-5p	plasma	qRT-PCR	101/100	n/a	n/a	0.723	p<0.001	(73)
circRNA002453	plasma	qRT-PCR	59/27	90%	84.1%	0.906	p < 0.001	(74)

FC, flow cytometry; LC–MS/MS, Liquid Chromatography and Mass spectrometry; "n/a", data not available.

TABLE 3 | Molecular markers of neuropsychiatric SLE (NPSLE) or other SLE comorbidities.

Marker	Specimen	Number	Method	Sensitivity	Specificity	AUC	p-Value	Study
α-Klotho	CSF	34/84	ELISA	82.4%	94.0%	0.94	p= 0.0004	(75)
ANRIL	plasma	65/35	ELISA	54%	73%	0.66	P=0.02	(76)
APOA1-AS	plasma	65/35	ELISA	65%	66%	0.72	P=0.003	(76)
CCL21	plasma	9/9	ELISA	88.9%	75%	0.85	P < 0.01	(77)
IL-6	CSF	32/13	ELISA	87.5%	92.3	0.956	p < 0.0001	(78)
IP-10	plasma	9/9	ELISA	66.7%	100%	0.82	P < 0.01	(77)
NOS3-AS	plasma	65/35	ELISA	80%	66%	0.71	P=0.004	(76)
OPN	CSF	18/25	ELISA	70%	100%	0.88	p < 0.05	(79)
S100B	serum	47/20	ELISA	84%	61.5%	0.742	p = 0.021	(80)
S100B	serum	87/25	LIA	73.9%	79.8%	0.77	p = 0.009	(81)

LIA, luminescence immunoassay.

TABLE 4 | Examples of Biomarker panels for lupus.

Marker	Number	Method	Sensitivity	Specificity	AUC	p-Value	Study
13S1212Cit3,13S1210	60/50	Microarray	n/a	n/a	0.83	P<0.001	(82)
Peaks m/z: 8595, 7170, 7661, 7740, 5806	27/27	MALDI-TOF-MS	92.6%	92.6%	n/a	n/a	(83)
65 specific peptides	34/58	CE-MS	n/a	n/a	0.99	P<0.001	(84)
AGP, CP	31/60	ELISA	n/a	n/a	0.88	P<0.001	(85)
AGP, CP, LPGDS	31/60	ELISA	n/a	n/a	0.90	P<0.001	(85)
AGP, CP,LPGDS,TF	31/60	ELISA	n/a	n/a	0.92	P<0.001	(85)
AGP, CP, LPGDS, TF, VCAM-1	31/60	ELISA	n/a	n/a	0.92	P<0.001	(85)
AGP, CP , LPGDS , TF , VCAM-1 , MCP-1	31/60	ELISA	n/a	n/a	0.92	P<0.001	(85)
Anti-heparan, anti-histone H2B, anti-vimentin	69/203	Antigen array	n/a	n/a	0.845	P < 0.0001	(86)
Anti-α-enolase,β2-MG	144/70	ELISA	91.9%	93.3%	0.927	P=0.004	(43)
MCP-1, TWEAK	70/20	ELISA	86.67%	80.00%	0.887	p < 0.0001	(69)
OPN, adiponectin	14/75	ELISA	81%	67%	0.75	P=0.003	(87)
Plasmin, TFPI	113/41	ELISA	83.8%	86.4%	0.86	p < 0.001	(66)
PGRN(S+U)	154/71	ELISA	100%	100%	1.00	p<0.001	(88)
PG 27:2, proline	32/26	UltraLC	87.5%	76.9%	0.846	P<0.001	(89)
uTGF-1、uNGAL	50/20	ELISA	64.1%	88%	n/a	p<0.001	(58)
miR-21, miR-423, miR-150	26/26	qPCR	79%	83%	0.93	P <.001	(72)
miR-125a, miR-142-3p, miR-146, miR-155	26/26	qRT-PCR	88%	78%	0.89	p< 0.001	(70)
miR-200b-5p, miR-141-5p, miR-200c-5p	101/100	qRT-PCR	80%	93%	0.936	p <0.001	(73)
lnc0597, GAS5	163/80	qRT-PCR	83.44%	93.75%	0.942	P<0.001	(26)
lnc0597,0640,5150,7074,GAS5	240/120	qRT-PCR	95%	85%	0.966	P<0.001	(90)

MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CE, capillary Electrophoresis; LC, liquid chromatography; "n/a", data not available.

H4pac and anti-H2Bpac reactivity demonstrated a strong correlation with disease activity (105). Anti-DNase I antibodies were positive in 35 SLE and 8 control patients, without significant difference between the mean antibody concentrations of the 2 groups. Sensitivity of this test was 64.81%, and specificity 84.62% (106). Anti-alpha-1,6-glucan-IgG levels were significantly elevated in patients with SLE and the sensitivity for detecting SLE was 93.3%, whereas the specificity was 73.3% and the area under the ROC curve was 0.863 (12). Antibodies to cell membrane associated DNA (mDNA) were identified by an indirect immunofluorescence assay using a B cell line fixed but not permeabilized with sensitivity of 65% and specificity of 98% (107). Serum anti-lipocalin IgG levels in patients that have SLE were significantly increased in comparison to patients with RA, pSS, SSc, or healthy controls, efficiently distinguishing SLE from other conditions with 49.5% sensitivity and 90.7% specificity (108).

It is apparent that classical autoantibodies are advantageous for initial testing of lupus in clinical settings; however, individual autoantibodies may not be able to achieve satisfactory sensitivity and specificity at the same time. Therefore, an autoantibody panel or autoantibody array technology may aid in improving lupus diagnostics in the future.

2.2 Serum Protein Markers in SLE

Besides autoantibodies, some serum proteins such as cytokines, chemokines, mediators, adhesion molecules, and complement fragments have also been implicated in SLE as potential markers.

2.2.1 Cytokines

Cytokines are known to play a vital part in the pathophysiology and immunology of SLE. Thus, a number of promising cytokines have been investigated as an SLE diagnostic or prognostic biomarker. Pacheco Y et al. reported 8 cytokines: IL-8, G-CSF, IL-12/23p40, IFNα, TNFα, IL-17A, IL-6, and IL-10 that were elevated in SLE compared to healthy controls (HCs) (109). In another study, IL-17 and IL-6 were found to be in significantly higher levels in SLE patients compared to normal subjects and were associated with active lupus nephritis, anemia, and positively correlated with SLEDAI-2k scores. ROC curve analysis for IL-6 and IL-17 indicated the optimal cutoff level was 12.3 pg/ml and 19.7 pg/ml, respectively, with AUC of 0.93 for IL-6 and AUC of 0.95 for IL-17 (34). Pentraxin 3 (PTX3) is a protein that is known to employ anti-inflammatory as well as protective effects in peripheral inflammatory conditions such as infections, acute myocardial infarctions, and inflammation of the lungs (110). The plasma PTX3 concentration was significantly higher in SLE patients than healthy controls and the cut-off value was 2.8 ng/mL in discriminating SLE from healthy controls with high sensitivity (100%) and high specificity (80%) (36). In another study, PTX3 had a cut-off point of 1.96 ng/mL and displayed a sensitivity of 34% and a specificity of 96% (111). Soluble urokinase plasminogen activator receptor (suPAR) is a biomarker of systemic inflammation. The ROC analysis of suPAR resulted in an AUC of 0.85 and a cut-off value of 3.54 ng/mL with a sensitivity of 82.02% and specificity of 79.31% in discriminating SLE patients from healthy individuals (25). B cell differentiating factor (BCDF) plays a vital role in the differentiation of B cells and increased levels of BCDF was observed in SLE patients in comparison to healthy controls. ROC analysis revealed an AUC of 0.861 for BCDF in discriminating SLE from healthy controls with a sensitivity of 80.6% and a specificity of 70.8% (16). In a study, hepatocyte growth factor (HGF) had significantly increased serum levels in SLE patients compared to healthy controls, but the matrix metalloproteinase-9 (MMP-9) had decreased serum levels in SLE patients. Serum level of HGF was significantly decreased after treatment in SLE patients, but serum level of MMP-9 increased (21). The serum level of Cysteine rich 61 (Cyr61) was higher in SLE patients compared to healthy controls; ROC analysis indicated Cyr61 may have predictive value in the diagnosis of SLE with an AUC of 0.830 (18). Serum growth arrest-specific protein (Gas6) levels in SLE patients were higher than in normal controls, and the sensitivity and specificity were 72.7% and 84%, respectively, with a cut-off value of 25.3 ng/mL when discriminating SLE from normal controls (112).

2.2.2 Chemokines

Chemokines are a family of small (8–10 kDa) chemotactic cytokines that regulate the migration patterns and positions of immune cells (113). Chemokines as well as their attached receptors have a significant role in the pathogenesis of SLE in human and mouse models (114). Some chemokines have been shown to perform very well as biomarkers in the diagnosis and prognosis of SLE. Serum IP-10 could differentiate SLE patients from healthy controls with a sensitivity of 76% and specificity of 70%.

2.2.3 Complement Components

The complement system plays a major role in SLE. Considering that most of the complement system is present within plasma and available, it could be suitable as a biomarker for diagnosis or monitoring of disease activity (115). Hypocomplementemia was included in the classification criteria of SLE, but the measurement of C3 or C4 often reflects disease activity poorly as the sensitivity and specificity of C3 for SLE are 80% and 14%, respectively (17). C3dg, an activation fragment of C3 which is generated following complement activation, was found in higher levels in SLE patient's plasma than in the controls. The ROC analysis indicated that C3dg had an AUC of 0.96, which was superior to C3 in differentiating patients from controls. This suggests that C3dg could be considered as a complement activation measurement for SLE classification criteria (17). Significantly elevated levels of C4d and C3d were detected specifically on T and B-lymphocytes of SLE patients. T-C4d had a sensitivity of 56% and specificity of 80%, and B-C4d had a sensitivity of 60% and specificity of 82% in differentiating SLE from other diseases (116). Complement C4d levels on erythrocytes (EC4d) and B cells (BC4d) were several times higher in SLE patients in comparison to patients with other rheumatic diseases as well as healthy subjects (117).

2.2.4 Other Disease Markers in SLE

Galectin-9 was found to be elevated in patients with SLE, and it correlated with disease activity and tissue factor expression. It correlated well with the IFN score with an AUC of 0.86 (32). The proinflammatory calcium-binding S100 family of proteins plays a pivotal role in the pathogenesis of rheumatic diseases (118). The levels of plasma S100 proteins effectively discriminated between SLE patients and healthy controls, with an AUC of 0.989, 0.678 and 0.807 for plasma levels of S100A4, S100A8/9 and S100A12, respectively, indicating that S100A4 may be a potential diagnostic biomarker for SLE (23). Serum S100B's protein level was increased in NPSLE, reflecting continuing neurological damage (81).

In a recent protein array based study, 48 proteins were upregulated in the serum of SLE patients. Among these, serum levels of AXL, ferritin, and sTNFRII were significantly elevated in patients with active lupus nephritis (LN) in comparison to dormant SLE patients. Interestingly, OPN, sTNFRI, sTNFRII, IGFBP2, SIGLEC5, FAS, and MMP10 displayed capacity to discriminate SLE from healthy controls with an ROC AUC exceeding 90% ($p < 0.001$) (19).

Despite the fact that there is not a satisfactory protein biomarker that can be used in clinic for SLE patients, emerging proteomics may bear great promises in screening for potential candidate biomarkers that could eventually be used to develop a biomarker panel with improved sensitivity and specificity in the

diagnosis or disease monitoring of lupus. Besides the efforts towards the discovery of novel biomarkers, tremendous work in validation studies will be urgently needed to test if the aforementioned markers could truly reflect disease status, especially in multi-cohorts or multicenter settings with an increased sample size and over a disease course.

2.3 MicroRNAs and Long Non-Coding RNAs (LncRNA) as Disease Markers in SLE

Recent studies discovered some microRNAs (miRNAs), negative regulators of protein expression at the post-transcriptional level through mRNA stability reduction and translation inhibition, were closely connected with SLE pathogenesis. Therefore, miRNAs have great potential as diagnostic markers or therapeutic targets of lupus. Circulating miRNAs can easily be identified through non-invasive methods and numerous have been identified as biomarkers of lupus, as summarized in **Tables 1A, B**. In whole peripheral blood, miR-146a and miR-155 were elevated in SLE patients compared to healthy controls (HCs) (119). In a separate study, compared to healthy controls, miR-21, miR-181a and miR-196a were found to be upregulated in SLE patients, with an AUC of 0.73, 0.72 and 0.76, respectively. It was found that miR-196a was a better marker in differentiating SLE patients from healthy controls, whereas miR-21 was a better marker in discriminating mild SLE from severe SLE in patients (120). In another study, plasma miR-21 levels in SLE patients were higher than that of healthy controls, with an AUC of 0.64 when differentiating SLE from healthy controls (121).

Besides microRNA markers, others found dysregulated expression of circRNAs or lncRNAs involved in the pathogenesis of autoimmune diseases (**Table 1**). The levels of hsa_circRNA_407176 and hsa_circRNA_001308 were decreased in both plasma and peripheral blood mononuclear cells (PBMCs) in SLE when compared with healthy controls. In plasma, the AUC of hsa_circRNA_407176 and hsa_circRNA_001308 were 0.599 and 0.662, respectively (28). However, in PBMCs, the AUC of hsa_circRNA_407176, hsa_circRNA_406567, and hsa_circRNA_001308 were 0.806, 0.744, and 0.722, respectively. The study demonstrated that hsa_circRNA_407176 and hsa_circRNA_001308 in plasma and PBMCs could be potential biomarkers for SLE (28). Plasma levels of GAS5 and lnc-DC were significantly decreased in SLE patients compared to healthy controls, while linc0597 was overexpressed in SLE patients; the combination of GAS5 and linc0597 provided better diagnostic accuracy with an AUC of 0.942 (26). Plasma levels of linc0597, lnc0640, and lnc5150 were found elevated, but GAS5 and lnc7074 levels were decreased in SLE patients compared to HCs. The combination of five lncRNAs achieved an AUC ranging from 0.604 to 0.833 when compared to healthy controls in an independent validation phase. This panel of five lncRNAs had high diagnostic accuracy for SLE (AUC = 0.966) and distinguished SLE from RA and pSS (AUC = 0.683 and 0.910, respectively) (90). Miao et al. found patients with higher SLEDAI scores had lower expression levels of circPTPN22, and long-term hormone treatment had significantly increased circPTPN22 levels. ROC curve analysis indicated that circPTPN22 had good diagnostic value for SLE (27). These findings suggest that circulating miRNA, lncRNA and other RNA or DNA fragments in the blood stream may hold great promise as biomarkers for lupus. They are relatively easy to detect using standard polymerase chain reaction (PCR), which is cheaper compared to protein biomarkers assays. However, the unstable nature of these molecules may compromise the accuracy of detection in some cases.

2.4 Disease Activity Markers in SLE

The diagnostic markers of SLE are mainly discussed above, and the markers in this section are disease activity makers, which are mainly used to distinguish active SLE or flare from inactive SLE or to determine disease activity of SLE. Based on the definition by Lupus Foundation of America, "Flare is a measurable increase in disease activity in one or more organ systems involving new or worse clinical signs and symptoms and/or laboratory measurements. It must be considered clinically significant by the assessor and usually there would be at least consideration of a change or an increase in treatment" (122). Circulating angiopoietin2 (Ang2) levels were increased in patients with active SLE compared to healthy controls. A calculated Ang2 cut-off value of >2.0 ng/ml was obtained with a specificity of 89% and sensitivity of 81% in discriminating active from inactive SLE (29). A proliferation-inducing ligand (APRIL) in the serum as well as its intrarenal mRNA levels were associated with resistance to treatment. The serum levels of APRIL at 4 ng/ml could accurately predict the response to treatment with a sensitivity of 65% and a specificity of 87.5% (48). High circulating Osteopontin (OPN) levels preceded increased cumulative disease activity and organ damage in SLE patients, especially in pSLE (123). Serum protein, CXC ligand 13 (CXCL13), plays a key role in chemotaxis of B cells; its levels in SLE patients were significantly increased. The ROC analysis demonstrated that serum CXCL13 level could be useful in identifying active disease from overall SLE patients with considerable accuracy (AUC = 0.829) (30). At a cutoff level of 80 pg/mL, CXCL13 could discriminate active SLE from inactive (AUC = 0.989, sensitivity = 100%, specificity = 96%) (31). Serum and urinary IP-10 levels were found to be significantly elevated in active SLE patients compared to inactive SLE patients with a sensitivity of 81% and specificity of 71% (124). At the optimal cutoff point of 14.41 pg/ml of IP-10, the AUC for IP-10 serum levels that differentiated active pediatric systemic lupus erythematosus (pSLE) from inactive pSLE was 0.807 with a sensitivity of 0.815 and specificity of 0.737 (33). Serum leucine-rich α2-glycoprotein (LRG) was found to be higher in patients with active SLE compared to inactive SLE and healthy controls. Serum LRG significantly correlated with SLEDAI-2K and clinical laboratory variables. ROC analysis revealed that optimal serum LRG cutoff value for active SLE was >45.7 ng/ml, and the AUC of LRG for predicting active SLE was 0.666 (125). Hyperprolactinemia is prevalent in SLE patients and correlated with clinical disease activity and the urine protein-creatinine index (UPCI). An ROC curve analysis of serum prolactin could predict SLE disease activity with a sensitivity of 91.7%, specificity of 58.1%, and AUC of 0.74 (4). In another study, SLE patients exhibited

significantly higher serum levels of miR-181a and lower serum levels of miR-203, which were correlated with SLE disease activity (126). The results suggested both miR-181a and miR-203 have diagnostic values for active SLE, with an AUC of 0.885 and 0.843, respectively (126). These disease activity biomarkers, if validated, may have great potential in monitoring disease activity or predicting lupus flare.

3 DISEASE MARKERS IN LUPUS NEPHRITIS

Lupus nephritis (LN), one of the most common and serious clinical manifestations of SLE, is a leading cause of mortality and morbidity. Various novel immunosuppressive drugs and biological therapies have improved SLE/LN survival rates, however early diagnosis and consistent monitoring of disease flares are still urgently needed for a better therapeutic outcome. The gold standard for diagnosis and prognosis of LN in modern medicine is renal biopsy; however, it should not be used for routine or repeated monitoring of disease activity and treatment response due to its invasive nature (66). During these past years, emerging studies have focused on screening and searching for non-invasive biomarkers which could reflect renal pathology or disease activity in LN, as summarized in **Table 2**.

3.1 Autoantibodies in LN

Classical autoantibodies, including anti-dsDNA, anti-cardiolipin, anti-ribosomal P, anti-SSA/Ro, anti-Sm, anti-endothelial cells, anti-epithelial cells, anti-glomerular matrix, and anti-glomerular basement membrane (GBM) antibodies have been found to be associated with LN (3). Pesickova et al. found that anti-CRP antibodies were detected solely in patients that had active renal disease and levels of antibody present correlated with SLEDAI (127). Anti-C1q antibody was found to have a strong association with LN (40). When anti-C1q was greater than 134 U/ml, there was a 15-fold increased risk of LN, with a specificity of 92% and sensitivity of 56%. Serum alpha-actinin antibody (AαA) was significantly lower in LN in comparison to SLE patients without nephritis. Serum AαA at cut-off levels ≤ 59.5 pg/ml could be used to discriminate between the two groups with sensitivity of 60%, specificity of 90%, and positive predictive value of 85.7% (42).

3.2 Potential Protein Markers in LN

Besides autoantibodies, some serum protein markers have been indicated to be involved in LN (**Table 2**). Serum human epididymis protein 4 (HE4) levels were significantly higher in LN patients that were positive for anti-dsDNA antibody with low C3. HE4 had a predictive value for LN with an optimal cutoff of 64.8 pM, AUC of 0.714, sensitivity of 81.8%, and specificity of 53.3% according to the ROC curve (54). When the cutoff value was 150.1 pM, the sensitivity and specificity reached 76.8% and 91.1%, respectively in the diagnosis of LN (55). Urinary clusterin was significantly elevated in LN patients with tubulointerstitial renal lesions. ROC curve analysis was used to diagnose the cases who progressed to ESRD, and they found that at the optimal cutoff point of urinary clusterin, the AUC was 0.804 with sensitivity of 72% and specificity of 82% (128). Neutrophil gelatinase-associated lipocalin (NGAL) was identified as an early marker in the kidney after ischemic or nephrotoxic injury. NGAL was easily detected in the urine and blood soon after acute kidney injury, and uNGAL could discriminate patients with nephritis from those without nephritis, with the best cut-off value of 13.66 ng/ml, AUC of 0.959 with sensitivity of 92% and specificity of 75% (129). At the cutoff value of 80 ng/mL, uNGAL levels serve as a predictor for the presence of LN with a high AUC of 0.997 with good sensitivity (98%) and specificity (100%) (62). In an independent study, at a cutoff value of 91.25 ng/mg creatinine, uNGAL had a sensitivity of 0.89 and a specificity of 0.67 (130). C4d levels were significantly increased in patients with SLE. According to ROC curve analysis, C4d levels could discriminate between high and low disease activity exhibiting a positive predictive value of 68% (50). At high disease activity, C4d levels were correlated predominantly with lupus nephritis and exhibited a sensitivity of 79% (50). Complement factor H-related proteins (CFHRs), consisting of proteins CFHR1 through CFHR5, are a part of the broader factor H/CFHR family. The levels of CFHR3 and CFHR5 found in plasma were higher in patients with lupus nephritis than in healthy individuals, and patients with both high CFHR3 and high CFHR5 exhibited the shortest progression-free survival (131).

The levels of IL-17 and IL-23 were found to be higher in patients with active LN compared to patients with inactive LN or healthy controls (57). The AUC of IL-17 to predict the activity of LN (SLEDAI > 9) was 0.91, whereas the AUC of IL-23 to predict the activity of LN (SLEDAI > 9) was 0.78 (57). In LN patients, plasma eotaxin, TNF-α, interleukin-17-α, interleukin-10, and interleukin-15 were significantly increased compared to the SLE non-nephritis group (53). Urine angiostatin displayed higher specificity and sensitivity in discriminating active renal SLE from active non-renal SLE with an AUC of 0.87 and correlated significantly with proteinuria (41). Higher levels of serum and urine Dickkopf-1 (DKK-1) proteins were detected in SLE patients compared to healthy subjects. DKK-1 levels especially were higher in patients with LN in comparison to non-nephritis SLE patients (52). Urine APRIL (uAPRIL) and BAFF (uBAFF) levels were significantly increased in LN, and ROC curve examination of uBAFF and uAPRIL showed an AUC of 0.825 and 0.781, respectively, in distinguishing between nephritic and non-nephritic SLE patients (47). Urinary monocyte chemoattractant protein 1(uMCP-1) level was significantly higher in LN and correlated well with LN disease activity. The cut-off value of uMCP-1 was 82 pg/ml, where AUC was 0.727 with a sensitivity of 88.5% and specificity of 46.3% in identifying LN (132). These levels fell with treatment and could have potential to predict a poor response and subsequent relapse of LN (60). Serum and urine progranulin (PGRN) levels were significantly higher in LN and closely associated with the

disease activity of LN (55). Urine CD163 levels were significantly higher in patients with active LN than healthy controls and ROC curves showed an AUC of 0.998 in the predefined groups of active and inactive LN (133).

Urinary osteoprotegerin (uOPG) was significantly higher in active LN. It showed modest correlation with disease activity with a potential to predict poor response to treatment and relapse of LN (64). Urinary and serum IP-10 could be potentially useful markers of lupus activity in differentiating active from inactive lupus, and their AUC was 0.68 and 0.77, respectively (59). Urine plasmin could discriminate active LN from inactive disease with an AUC of 0.84 (66). Some ceramides (Cer) such as C16cer, C18Cer, C20Cer, and C24:1Cer were elevated in serum and plasma samples of patients with LN with impaired renal function compared to healthy controls, as well as non-nephritic SLE patients (49). In this study, C24:1dhCer was implicated as a potent biomarker for renal impairment in patients suffering from SLE (49). Urinary levels of transferrin (TF) and ceruloplasmin (CP) were significantly higher in patients with LN compared to those without LN, with an AUC of 0.84 and 0.86, respectively in discriminating LN from non-LN controls (51). Urinary angiostatin and vascular cell adhesion molecule-1 (VCAM-1) exhibited outstanding potential with an AUC of 0.97 and 0.98, respectively to predict renal biopsy activity index score ≥ 7, which can be associated with poor long-term prognosis (46). However, the urinary angiostatin was not able to discriminate LN patients from other CKD patients with an AUC 0.56 (15). Urinary soluble cellular adhesion molecules (sVCAM-1) and VCAM-1 levels were significantly elevated in LN patients compared to the controls, and the ROC curve of urine sICAM-1 showed an AUC of 0.874 with high sensitivity (0.945) and specificity (0.789), whereas the AUC of VCAM-1 was 0.882 with a sensitivity of 0.982 and specificity of 0.667 (67). The level of urinary transforming growth factor beta 1 (uTGF-β1) and urinary interleukin 17 (uIL-17) were significantly higher in severe LN than control groups. The AUC values of uTGF-β1 and uIL-17 were 0.665 and 0.717, with a cut-off value of 27.13 pg/ml and 36.62 pg/ml, respectively (58). Angiopoietin-2 (Ang2) level was increased in SLE patients in comparison to the control, and it was significantly higher in the LN patients than in SLE patients that did not have nephritis. Ang2 positively correlated with SLEDAI, 24 hours proteinuria, as well as histological activity index (45). Ang2 could indicate the degree of endothelial activation and may potentially be used as a biomarker for both disease activity and renal involvement in SLE patients. However, Ang2 level could not distinguish between proliferative and non-proliferative lesions in LN (45). TNF receptor associated factor 6 (TRAF6) was upregulated in LN patients and was related to LN activity. It positively correlated with serum IL-1β, IL-6, IL-8, as well as TNF-α activity. The AUC of TRAF6 for the diagnosis of LN was 0.897 (68). Stanley et al. found that urinary IL- 7, IL- 12p40, IL- 15, IP- 10 and TARC levels were significantly higher in patients with active LN in comparison to those with inactive SLE as well as healthy controls. It also correlated with renal SLEDAI and physicians global assessment of disease activity (134).

3.3 MicroRNA as Disease Markers in LN

A growing body of evidence indicates that microRNAs participate in LN development and kidney fibrosis (135, 136). Since miRNAs are present in body fluids with high stability and can be sampled non-invasively, some of them have been reported as potentially advantageous as diagnostic and prognostic biomarkers for a variety of human diseases (137). Recent findings of miRNAs as potential LN biomarkers were summarized in **Table 2**. For example, miR-146a expression was significantly reduced in LN and was found to be associated with LN activity. The AUC of miR-146a for the diagnosis of LN was 0.821, and the AUC of miR-146a for differentiating LN activity was 0.921 (68). The levels of circulating miR-21 was significantly increased in LN patients compared to healthy controls, and ROC analysis indicated that miR-21 was better at discriminating LN patients from controls with an AUC of 0.912 (72). The multivariate ROC curve analysis showed that the plasma circulating miR-125a, miR-142-3p, miR-146, and miR-155 together could distinguish most of the patients with LN from controls with an AUC of 0.89, sensitivity of 88%, and specificity of 78% (70). Levels of MiR-29c in urinary exosomes displayed a negative correlation with the histological chronicity index as well as glomerular sclerosis. MiR-29c expression levels could predict the degree of chronicity in LN patients with a remarkable AUC of 0.946, sensitivity of 94% and specificity of 82%, respectively (71). Kidney biopsies from LN patients revealed elevated lncRNA RP11-2B6.2 levels and was positively correlated with IFN scores and disease activity (138). There was a statistically significantly decrease in Lnc3643 levels of SLE patients with proteinuria compared with those without (139). LN may be discriminated from SLE without nephritis through lnc-DC (26).

4 DISEASE MARKERS FOR NEUROPSYCHIATRIC SLE (NPSLE) AND SLE WITH OTHER COMORBIDITIES

4.1 Markers for Neuropsychiatric Systemic Lupus Erythematosus (NPSLE)

NPSLE, one of the most serious complications associated lupus, affects both the central and peripheral nervous systems. NPSLE manifestations are associated with varying degrees of morbidity that differ in presentation and severity between patients. They are often times difficult to differentiate from other neuropsychiatric conditions with a different etiology (140). There is no gold standard diagnostic approach for NPSLE that exists. There are however various clinical, laboratory, and radiographic findings reported for differential diagnosis of neuropsychiatric conditions that are associated with SLE. The NPSLE diagnosis remains a challenge — no diagnostic test is available, and differential diagnosis is often obtained through a process of elimination. Many factors contribute to the lack of consistency such as variation in study design, study methodology, patient selection criteria and the rarity of some neuropsychiatric syndromes (140). Increasing interest has been focused on the identifying biomarkers vital to the origin of the disease and as a result, correlated closely

with disease activity and outcome. Some potential biomarkers that have been reported in NPSLE patients are summarized below in **Table 3**. The capability for S100B protein levels to differentiate between patients with and without NPSLE was analyzed and it showed good discriminatory capacity for NPSLE (AUC = 0.77) and a better capacity for acute NPSLE (AUC = 0.82). At the cut-off point of 0.125ng/ml, S100B levels would provide a sensitivity of 73.9% and a specificity of 79.8% in differentiating NPSLE. In the case of acute NPSLE, this cut-off value would deliver a sensitivity of 77.8% and a specificity of 79.8% (81). An independent study revealed S100B had a discriminating value for NPSLE patients with peripheral polyneuropathy with an AUC of 0.706 (80). The CSF α-Klotho levels showed decent discriminatory capability for NPSLE (AUC = 0.94). The cut-off point ≤ 230.2 pg/ml would deliver a sensitivity of 82.4% and a specificity of 94.0% for NPSLE (75). By using a Surface-enhanced laser desorption/ionization (SELDI) technique, a panel of m/z peaks at 8595, 7170, 7661, 7740, and 5806 were identified to build a diagnostic decision tree model which could recognize NPSLE with a sensitivity of 92.6% based on training group samples (83). ROC curve analysis showed that the sensitivity and specificity of CSF IL-6 for the diagnosis of lupus psychosis (LP) were 87.5% and 92.3%, respectively, and the AUC was 0.9567 with a cut-off value of 4.3 pg/ml (78). The CSF concentration of OPN was significantly higher in NPSLE than in non-NPSLE. When the cut-off value of OPN in CSF was at 963.4 ng/ml, the sensitivity and specificity for the diagnosis of NPSLE were 70% and 100%, respectively (79). Lipocalin-2 (LCN2) was upregulated in the CSF of NPSLE patients across two different ethnicities, demonstrating that CSF LCN2 may be a novel biomarker for NPSLE (141).

Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) autoantibodies were found to be significantly elevated in SLE patients, particularly in patients with NPSLE (142). Anti-DNA/NR2 antibodies in NPSLE were higher than those in healthy controls, indicating that anti-DNA/NR2 antibodies may be a predictive factor in post-steroid neuropsychiatric manifestation (PSNP) -SLE (143). Anti-Suprabasin (SBSN) antibodies were significantly higher in the CSF of the NPSLE group compared to the non-NPSLE group, indicating that anti-SBSN could potentially be a novel marker for the evaluation of suspicious NPSLE (144). The levels of anti-UCHL-1 autoantibodies in the NPSLE group were significantly higher than in the control group, and the positive rate of anti-ubiquitin C-terminal hydrolase L1 (anti-UCHL-1) autoantibodies in the NPSLE group was 23.7% (145). Significantly higher anti-microtubule-associated protein-2 (anti-MAP-2) antibody titers were discovered in the CSF of patients with NPSLE compared to the CSF of non-NPSLE controls. Anti-MAP-2 antibody prevalence was 33.3% in NPSLE (146).

4.2 Disease Markers in SLE With Cardiovascular Diseases (CVD)

Cardiovascular diseases, one of the most serious complications associated SLE, have emerged as a leading cause of illness and mortality. There are several novel biomarkers that have been reported in recent studies in addition to anti-phospholipid antibodies, as summarized in **Table 3**. Some lncRNAs were found to be relevant to atherosclerosis such as antisense lncRNA of INK4b/ARF/INK4a locus (ANRIL), antisense lncRNA of NOS3 (NOS3-AS), and antisense transcript of APOA1 (APOA1-AS) which were increased in atherosclerotic SLE patients than non-atherosclerotic SLE patients. Multivariate analysis identified these lncRNAs as independent predictors for atherosclerosis in SLE (76). SLE patients with a CVD history had higher serum levels of both S100A8/A9 and S100A12 compared to patients without CVD or venous thromboembolism (147). Meta-analysis revealed an increased risk of recurrent major adverse cardiac events in patients with high IgG anti-cardiolipin antibodies both at 12 and 24 months (148). Anti-HDL antibodies were associated with higher risk of CVD in SLE patients, and anti-PON1 antibodies were associated with intima-media thickness in SLE (149). Serum E-selectin was increased in SLE patients and particularly associated with atherosclerosis in patients with SLE (150). Serum annexin A5 was found as an independent predictive variable for endothelial dysfunction in SLE patients (151).

4.3 Disease Markers of SLE With Lung Complications

Respiratory tract complications are highly frequent in SLE patients, yet there are only a limited number of studies assessing risk factors or biomarkers that might be able to predict pulmonary manifestations in SLE (SLEpulm), as summarized in **Table 3**. Chemokine (C-C motif) ligand 21 (CCL21) and CXCL10 (IP-10) levels were significantly higher in SLEpulm than SLE without pulmonary manifestations. ROC analysis demonstrated that CCL21 could discriminate SLEpulm from non-pulmonary SLE with an AUC of 0.85, sensitivity of 88.90% and specificity of 75%; likewise, CXCL10 had a good discriminatory value for SLEpulm (AUC = 0.82; sensitivity = 66.67%, specificity% = 100%) (77). Plasma Cyr61 concentration in SLE-associated pulmonary arterial hypertension (SLE-PAH) patients was significantly higher than matched SLE-non-PAH patients and healthy controls. Cyr61 level ≥140.7 pg/ml was indicated to be an independent risk factor for developing PAH in SLE patients (152).

4.4 Disease Markers of Cutaneous Lupus Erythematosus (CLE)

Cutaneous lupus erythematosus (CLE) is a frequent manifestation in SLE patients and can also exist as a single entity without associated systemic autoimmunity (153). It can persist for many years and impair quality of life, including vocational disability. Recently, some potential biomarkers have been identified to distinguish CLE from other types of SLE as summarized in **Table 3**. For example, CD40 was intensely expressed in all subacute cutaneous lupus erythematosus (SCLE), discoid LE (DLE), and dermatomyositis (DM) lesions (154). The CCR4 ligand TARC/CCL17 was found to be strongly expressed in skin lesions and its levels were elevated in CLE patient's serum (155). Soluble E-selectin was significantly elevated in DLE patients with wide-spread lesions and correlated significantly with active cutaneous skin lesions

(156). Serum IL-17A and IL-17F concentrations were increased in DLE and SLE patients (157). In addition, anti-C1q antibody levels were correlated with cutaneous Caspase 3 expression in SCLE patients (158). The gene expression of Tyrosine kinase 2 (TYK2), interferon regulatory factor 5 (IRF5), and CTLA4 are associated with SLE and conferred risk for DLE and SCLE (159). SCLE patients had significantly higher levels of anti-C1q antibodies and serum C1q circulating immune complexes (C1q-CIC) levels in comparison to healthy controls. Anti-Laminin-1 antibodies were found in the sera of cutaneous lupus erythematosus patients (160).

5 BIOMARKER PANELS FOR SLE: THE DESTINATION

SLE is a complex autoimmune condition affecting multiple organ systems and displays a variety of clinical signs and symptoms. Therefore, it is difficult to accurately diagnose or evaluate the prognosis of SLE with a single biomarker. Combinations of different biomarkers have been explored in the diagnostic or prognostic assessment of SLE to improve its sensitivity and specificity as summarized in **Table 4**. A combination of 13S1212Cit3-IgM with 13S1210-IgG (termed "COPSLE" for the combination of peptides for SLE) was more effective for SLE diagnosis, with an AUC of 0.830 and a positive rate of 73.33%. This combination could be utilized for the identification of 80.0% of SLE patients found negative for anti-Smith, anti-dsDNA, and anti-cardiolipin (ACA) antibodies (82). The combined model of fecal phosphatidylglycerol and proline resulted in an AUC of 0.846 with a good diagnostic value (89). A biomarker panel with 65 peptides were applied to the discovery cohort and resulted in an AUC of 0.99 in discriminating SLE from healthy controls (84). The combination of urine plasmin and tissue factor pathway inhibitor (TFPI) discriminated active LN from inactive LN with an AUC of 0.86, exceeding the specificity as well as positive predictive value of traditional individual markers such as anti-dsDNA and complement C3 (66). The combination of adiponectin and OPN predicted chronic LN damage with an AUC of 0.75, sensitivity of 81% and specificity of 67% (87). The combination of uTGF-β1 and uNGAL exhibited a sensitivity of 64.1% and specificity of 88% for LN (58). The combination of miR-21, miR-423, and miR-146 could differentiate LN from controls with an excellent AUC of 0.93, sensitivity of 79%, and specificity of 83% (72). The combination of plasma circulating miR-125a, miR-142-3p, miR-146, and miR-155 together could distinguish most of the patients with LN from controls with an AUC of 0.89, sensitivity of 88%, and specificity of 78% (70). A combined model of uMCP-1 and uTWEAK showed an AUC of 0.887, sensitivity of 86.67% and specificity of 80.00% to discriminate active LN, and an AUC of 0.778, sensitivity of 75% and specificity of 81.82% to discriminate LN with poor outcome (69). The combination of miR-200b-5p, miR-141-5p, and miR-200c-5p disclosed a greater diagnostic value for LN with an AUC of 0.936, sensitivity of 80%, and specificity of 93% (73). Urinary alpha-1-acid glycoprotein (AGP), ceruloplasmin (CP), VCAM-1, MCP-1, and Lipocalin-like prostaglandin D synthase (LPGDS) levels were significantly higher in those patients with active LN than non-LN patients. The model including both AGP and CP resulted in an AUC of 0.88. With the addition of LPGDS to this model, the AUC increased to 0.90, and further increased to 0.92 upon the addition of TF. The addition of VCAM-1 and MCP-1 into this model however did not increase the AUC (85). The combination of IgG autoantibodies against heparan sulphate, histone H2B, and vimentin could differentiate NPSLE from non-NPSLE with an AUC of 0.845 (86). The combination of urinary VCAM-1, CystatinC, and KIM-1 discriminated proliferative LN from membranous LN with an AUC of 0.80 (95%CI: 0.69–0.90) (161). A combination of five urinary proteins, namely L-PGDS, transferrin, ceruloplasmin, MCP-1, and sVCAM-1 was a good predictor of active LN (AUC= 0.898).A combined model of L-PGDS, transferrin, alpha-1-acid glycoprotein (AGP-1), ceruloplasmin, MCP-1 and sVCAM-1 predicted response to rituximab treatment at 12 months (AUC = 0.818) (162). Proinflammatory high-density lipoprotein (HDL) (piHDL), leptin, plasma soluble TNF-like weak inducer of apoptosis (sTWEAK), and homocysteine when combined with clinical variables such as age and diabetes, could create a risk profile as "predictors of risk for elevated flares, damage progression, and increased cardiovascular disease in patients with SLE (PREDICTS)". The PREDICTS profile could accurately identify patients with SLE at risk for future subclinical atherosclerosis progression (163).

With the rapid development of single-cell RNA sequencing (scRNA seq) and its application in the profiling of genes associated with SLE or LN (164–166), more novel biomarkers or biomarker panels may be emerging. However, these gene expression data must be validated at the protein level before moving to biomarker detection in clinical settings. It is advantageous to combine multiple LN biomarkers to constitute a biomarker panel to improve the sensitivity or specificity for disease diagnosis, especially in discriminating LN from controls, or discriminating active LN from inactive LN. The reason for this is that different biomarkers represent different biological activity behind LN and collectively they reflect various aspects of this multifactorial disease, hence improving the diagnostic value for LN. However, current biomarker panels are largely limited to a combination of biomarkers from the same categories such as microRNA panel, autoantibody panel, cytokine panel, peptide panel, metabolite panel etc. This is clearly a huge restraint in developing a robust biomarker panel for LN. Moving forward, we should combine the most promising biomarkers across different categories as mentioned above, incorporate pathological markers, and some robust descriptive clinical scores to develop more accurate and clinically useable biomarker panels for LN.

Besides the biomarker panels described in previous studies (**Table 4**), novel biomarker panels may be identified based on future validation results from the promising individual biomarkers as listed in **Tables 1A, B**, for diagnostic a

biomarker panel and a disease activity biomarker panel, respectively. For a diagnostic biomarker panel of SLE, based on the preliminary data of the performance of individual markers, anti-HIST1H4A, S100A4, C3dg, TNFRII and IGFBP2 seem to be good candidates if they are validated by other research groups. Likewise, CXCL13, PTX3, IL-6 and IL-17 seem to be promising candidates to constitute a disease activity biomarker panel for SLE if they are validated. In addition, urinary CD163, PGRN, VCAM1, NGAL and Angiostatin seem to have good discriminative capability in the diagnosis and prognosis of active LN. That being said, beyond the biological validity of these biomarker candidates, there are three additional factors impacting the selection of biomarkers for a successful biomarker panel: (1) Technical compatibility of each biomarker within the panel during detection: the ability to detect all biomarkers within the panel in one assay is needed to make the panel viable for clinical use; (2) Availability of high-quality antibodies for each biomarker within the panel: a careful selection of high-quality antibodies with good affinity and specificity is key to build a biomarker panel based assay. (3) A statistically meaningful biomarker panel: it is critical to use multivariable model to generate a statistically sound biomarker panel so that the later stage evaluation of diagnostic or prognostic ability of the panel may have a satisfactory outcome.

6 CONCLUSION

Accurate diagnosis and early treatment can significantly improve therapeutic outcome and prognosis for SLE. Therefore, a good molecular diagnosis is desirable for SLE to reflect disease activity, monitor drug response, and predict flares. Various Omics technologies are promising in identifying novel and robust biomarkers for SLE.

As SLE is a multi-factorial disease with multiple molecular and pathological alterations, individual biomarkers are insufficient in satisfying the clinical need in diagnosis and disease monitoring with desirable sensitivity and specificity. Fortunately, the combination of molecularly and pathologically relevant biomarkers of SLE may significantly improve the accuracy and robustness for disease detection and prediction. Ultimately, the incorporation of these biochemical markers in mainstream clinical care will require validated, standardized laboratory tests that are available worldwide. Such tests need to be robust, reliable, easy-to-perform, and affordable. In addition, standardization of relevant biomarkers must be established, because the relevant biomarker values are usually not very consistent across different studies. For this, the following challenges in LN biomarker studies need to be tackled: (1) The sample size for most studies were relatively small, due to the limited collaborative lupus biomarker research consortium and access to a centralized clinical sample bank. On the other hand, the heterogenous nature of lupus requires a larger sample size in order to identify a statistically meaningful biomarker for SLE and LN. (2) Many of these studies only included healthy donors as controls and fewer studies had disease controls such as other autoimmune diseases or relevant chronic diseases. (3) Commercial ELISA kits have often been directly used as a quantitative method. However, a more careful test, especially the validation of these kits in various sample types by different research groups are still lacking, particularly prior to large-cohort validation studies. Mass spectrometry may be used to further confirm the target biomarker, which is thought to be selectively bound by the capture antibody in the ELISA kit. (4) Autoantibodies and immune complexes are abundant in the blood samples of lupus patients, which may inevitably interfere with the assay *via* competition with capture antibody or detection antibody in the ELISA kit and generate misleading results. All these challenges have to be tackled before a clinically useful biomarker or biomarker panel is identified.

With the development of artificial intelligence and machine learning technology, SLE and LN biomarkers discovered from SLE Omics studies may be categorized according to age, sex, ethnicity, geography, genetic etiology, molecular and cellular mechanism, pathological changes in patients to inform disease cause and drug response, and to guide personalized medication for SLE.

AUTHOR CONTRIBUTIONS

GT and TW conceived this work. GT prepared the first draft of the manuscript. BB, YZ, and TW edited the manuscript. All authors contributed to the article and approved the submitted version.

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GLOSSARY

AGP	alpha-1-acid glycoprotein
Ang2	Angiopoietin-2
Ang2	angiopoietin2
anti-MAP-2	anti-microtubule-associated protein-2
anti-P	anti-ribosomal P protein antibody
anti-UCHL-1	anti-ubiquitin C-terminal hydrolase L1
APRIL	A proliferation-inducing ligand
AUC	area-under-the-curve
AαA	alpha-actinin antibody
BAFF	tumor necrosis factor family
BCDF	B cell differentiating factor
CCL21	Chemokine (C-C motif) ligand 21
CFHRs	Complement factor H-related proteins
CLE	Cutaneous lupus erythematosus
CNS	central nervous system
COPSLE	combination of peptides for SLE
CP	ceruloplasmin
CSF	cerebral spinal fluids
CVD	cardiovascular diseases
CXCL13	CXC ligand 13 protein
Cyr61	Cysteine rich 61
DKK-1	Dickkopf-1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gas6	growth arrest-specific protein
HCs	health controls
HE4	human epididymis protein 4
HGF	hepatocyte growth factor
HIST1H4A	histones H4A
IFI27	Interferon Alpha Inducible Protein 27
IP-10 IFN-&gamma	inducible protein 10
IRF5	interferon regulatory factor 5
LCN2	lipocalin-2
LN	Lupus nephritis
LP	lupus psychosis
LPGDS	lipocalin-like prostaglandin D synthase
LRG	leucine-rich α2-glycoprotein
MCP-1	monocyte chemoattractant protein 1
mDNA	membrane associated DNA
miRNAs	microRNAs
MLKL	Mixed lineage kinase domain-like pseudokinase
MMP-9	matrix metalloproteinase-9
NGAL	Neutrophil gelatinase-associated lipocalin
NPSLE	neuropsychiatric SLE
OPG	Osteoprotegerin
OX40L	ligand for OX40
PG	phosphatidylglycerol
PGRN	progranulin
pSLE	pediatric systemic lupus erythematosus
PTX3	Pentraxin 3
ROC	Receiver Operating Characteristic
SLE	systemic lupus erythematosus
SLE-PAH	systemic lupus erythematosus-associated pulmonary arterial hypertension
suPAR	Soluble urokinase plasminogen activator receptor
TF	transferrin
TFPI	tissue factor pathway inhibitor
TNFSF4/OX40	TNF superfamily member 4
TRAF6	TNF receptor associated factor 6
TYK2	Tyrosine kinase 2
UPCI	urine protein-creatinine index
uTGF-β1	urinary transforming growth factor beta 1
VCAM-1	vascular cell adhesion molecule-1

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ORIGINAL RESEARCH
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Discovery of Novel Circulating Immune Complexes in Lupus Nephritis Using Immunoproteomics

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Objective: The goal is to discover novel circulating immune complexes (ICx) in the serum of lupus nephritis (LN) as potential biomarkers.

Methods: Protein A/G magnetic beads or C1q-coated plates were used to capture ICx in the serum of LN, followed by the identification of immunoglobulin-binding proteins using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Bioinformatic approaches and single-cell RNA sequencing (scRNA Seq) databases were used to select potential candidate ICx markers in LN. The selected ICx markers were further validated using ELISA.

Results: A total of 300 immunoglobulin-binding proteins were discovered in the screening, among which 77 proteins were detectable only in LN samples. Bioinformatics-assisted selection allowed us to further identify 10 potential immunoglobulin-binding proteins, which form ICx as potential biomarkers in LN. In a validation cohort of 62 LN patients and 21 healthy controls (HC), we found that prolyl 3-hydroxylase 1 (P3H1), phosphatase and actin regulator 4 (PHACTR4), and regulator of G-protein signaling 12 (RGS12) ICx exhibited discriminative capability in distinguishing LN from HC, with an area under the curve (AUC) values of 0.82, 0.99, and 0.90, respectively. Furthermore, a biomarker panel comprising CD14, CD34, cystatin A, myocyte enhancer factor 2C (MEF2C), RGS12, and ubiquitin C (UBC) ICx could distinguish active LN from inactive LN with an AUC value of 0.85, which is comparable to or better than pathological parameters such as renal activity index (AI) and renal chronicity index (CI).

Conclusion: Immunoproteomics-based discovery studies have enabled us to identify circulating immune complexes as potential biomarkers of LN.

Keywords: immunoproteomics, immune complex (ICx), biomarkers, biomarker panel, systemic lupus erythematosus, lupus nephritis

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifactorial and heterogeneous autoimmune disease, manifested by autoantibody production and altered type I interferon expression and regulation (1–3). About 60% of SLE patients are eventually advanced into potentially fatal lupus nephritis (LN) (4, 5). LN is a leading cause of mortality in SLE patients, and the treatment of LN has become a significant social and economic burden in the United States (6). Unfortunately, the diagnosis or disease monitoring of SLE or LN is suboptimal. The current gold standard for clinical diagnosis of LN is renal biopsy, which is invasive and may cause kidney damage (7, 8). Using serum for a liquid biopsy is minimally invasive; therefore, serum biomarkers may have great potential in the diagnosis and disease monitoring of LN patients in clinical settings. Given the heterogenetic nature and unmet needs in precision diagnosis and classification of SLE/LN patients for personalized medication, identification of novel biomarkers, particularly in the form of a biomarker panel, is of paramount importance (9). Omics studies are promising in the discovery of novel serum biomarkers which may aid in accurate diagnosis and disease monitoring of LN clinically (10, 11). Robust serum biomarkers may also be useful in developing point-of-care systems that can be used for home testing of LN.

IgG antinuclear autoantibodies (ANA) against components such as DNA and nucleoprotein are commonly found in the glomeruli and serum of individuals with LN (12). The etiology of LN involves antibody binding to multiple autoantigens (AAgs) (13). Through the Fc–FcR interaction, the intracellular or extracellular AAgs can bind to specific autoantibodies to form immune complexes (ICx), which as a double-edged sword may exert pathological effects or beneficial regulatory effects, depending on the antigen–antibody ratio, antibody subclass, and antigen subcellular location (14, 15). Therefore, disease-associated ICx, particularly AAgs, may not only contribute to the pathogenesis of the disease but also serve as disease biomarkers in autoimmune diseases such as SLE and LN.

Omics technologies, such as genomics, transcriptomics, proteomics, and metabolomics, are rapidly evolving which enable the discovery of putative biomarkers in SLE (16). In particular, single-cell RNA sequencing (scRNA Seq) allows us to investigate transcriptomic profiles at a single-cell resolution, in which the function of rare cell populations and the information on communication among different cell types can extend our understanding of the pathogenesis and heterogeneity of SLE (17). A single-cell analysis of intrinsic renal cells and infiltrating cells from patients with LN may be helpful in defining the pathways of renal injury at a cellular level (18). However, identifying a clinically useable biomarker of SLE/LN is still challenging due to two major issues: (1) heterogeneity of SLE where multifactorial pathogenesis may involve various molecular or signaling pathways in different patients; (2) difficulty in standardization of omics technological platforms and experimental systems which may result in variable preliminary data. To tackle these challenges, we aimed to combine the immunocapture-based proteomics approach with bioinformatics and existing scRNA Seq databases to pinpoint clinically useful biomarkers in SLE or LN.

Abbreviations: ICx, immune complexes; LN, lupus nephritis; HC, healthy control; GN, glomerulonephritis; SLE, systemic lupus erythematosus; ROC, receiver operating curve; AUC, area under the curve; AI, renal Activity Index; CI, renal Chronicity Index; GO, Gene Ontology; BCRs, B cell receptors; scRNA Seq, single-cell RNA sequencing; DEGs, differentially expressed genes; PCA, principal component analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization and Integrated Discovery; LC-MS/MS, liquid chromatography and tandem mass spectrometry.

MATERIALS AND METHODS

Reagents

Protein A- or G-coated magnetic beads were purchased from Millipore Sigma (Saint Louis, MO). The native human C1Q was purchased from Abcam (Boston, MA, USA). Papain solution was purchased from Millipore Sigma, MO. The CD14, CD34, CSTA, UBC, and BST1 antibodies were purchased from R&D Systems (Minneapolis, MN, USA), the P3H1, RGS12, and GUK1 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and the MEF2C and PHACTR4 monoclonal antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA) and Abcam, respectively. The anti-human IgG antibody was purchased from Jackson ImmunoResearch (West Grove, PA).

Patients and Clinical Samples

Serum samples from lupus nephritis and healthy controls were collected at the University of Texas, Southwestern Medical Center at Dallas. The samples were aliquoted and stored at -80°C. All human subject-related procedures were performed following the institutionally approved IRB protocols, and all consents were obtained before sample collection. The detailed demographics and clinical information are summarized in **Table 1**. Active lupus nephritis (LN-active) is defined as Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) greater than 4 and the renal domain of SLEDAI (rSLEDAI) greater than 0. Inactive lupus nephritis (LN-inactive) is defined as SLEDAI less than 4 and rSLEDAI equal to 0. In the immunoproteomic screening study, serum samples from 3 LN patients (SLEDAI = 4, 19, and 20, respectively) or 3 healthy controls were pooled, respectively, for further experiments.

Protein A/G-Based Immunoprecipitation

40 µl of Protein A- or G-coated magnetic beads was gently mixed and washed with 0.1% PBST. 100 µl of 1:100 PBS-diluted pooled serum samples from LN or healthy controls was then mixed and incubated with the Protein A/G magnetic beads at room temperature for 30 min with gentle shaking. The immune complex bound beads were then washed three times with 50 µl PBS. A magnetic stand was used to hold the beads, while the supernatant was removed after washing. The washed beads were suspended in 50 µl of 0.1 mg/ml papain solution (0.04 M EDTA, 0.04 M L-cysteine) and incubated at 37°C for 1 h. The magnetic stand was used to remove the beads, and the resultant supernatant was transferred to a new tube, and 50 µl of 0.06 M

TABLE 1 | Demographics and clinical characteristics of subjects.

LN patients	LN-active	LN-inactive
Total no. of subjects	49	13
Female, no. (%)	80.79%	100%
Age, mean ± SE., years	30.15 ± 8.47	37.84 ± 13.19
Ethnicity, Asian/Black/Hispanic/Hawaii/White, no.	3/23/1/1/21	1/2/0/0/10
During time from LN onset, median (interquartile), years	7 (3–10)	6 (2–12)
SLEDAI, median (interquartile)	8 (8–12)	2 (0–3)
Renal SLEDAI, median (interquartile)	4 (4–8)	4 (4–8)
No. of patients with renal SLEDAI = 0 (%)	2.04%	100%
Protein: creatinine ratio, mg/mg, median (interquartile)	2.79 (1.32–5.31)	0.12 (0.18–0.22)
Serum Cr, mg/dL, median (interquartile)	0.95 (0.71–1.85)	0.75 (0.64–0.81)
No. of patients with DNA positive (%)	33 (67.35%)	5 (38.46%)
Treatment at time of biopsy		
Pred (mg/d)	36 (73.47%)	6 (46.15%)
MMF (g/d)	24 (48.98%)	2 (14.29%)
HCQ (mg/d)	29 (59.18)	6 (46.15%)
Health controls		
Total no. of subjects	21	
Female, no. (%)	71.43%	
Age, mean ± SE., years	30.60 ± 10.04	
Ethnicity, Asian/Black/Hispanic/Hawaii/White, no	3/8/0/0/10	

iodoacetamide was added to terminate the reaction of papain digestion, followed by gel purification and mass-spectrometry analysis.

C1q-Based Capture/Enrichment of Immune Complex

100 µl of 200 µg/ml native human C1q was diluted into PBS, coated onto a 96-well microplate (Thermo Fisher, Waltham, MA), and incubated overnight at room temperature. The microplate was washed three times with 150 µl PBST and blocked, and then 100 µl of 1:100 PBS-diluted pooled serum samples was added into the wells and incubated for 2 h at room temperature. The aqueous portion was removed, and the microplate was washed three times with 150 µl PBST. A 50-µl 0.1-mg/ml papain solution was added to each well and incubated at 37°C for 1 h. Then, 50 µl of 0.06 M iodoacetamide was added into the well to terminate the papain digestion, followed by gel purification and mass spectrometry analysis.

Identification Autoantigens With Mass Spectrometry

Supernatants from the Protein A/G- or C1q-captured immune complex samples were purified by a brief running (≤10 min) of SDS-PAGE. The gel band containing samples was subjected to in-gel digestion (19). An aliquot of the tryptic digest (in 2% acetonitrile/0.1% formic acid in water) was fractionated through liquid chromatography and then analyzed by an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific™) interfaced with a Dionex UltiMate 3000 Binary RSLCnano System. Peptides were separated onto an Acclaim™ PepMap™ C18 column at a flow rate of 300 nl/min. Gradient conditions were 3%–22% B for 40 min; 22%–35% B for 10 min; 35%–90% B for 10 min; and 90% B held for 10 min (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile). The peptides were analyzed using a data-dependent acquisition method; Orbitrap Fusion was operated with the measurement of FTMS1 at resolutions of 120,000 FWHM, scan range of 350–1,500 m/z, AGC target 2E5, and maximum injection time of 50 ms. During a maximum 3-s cycle time, the ITMS2 spectra were collected at a rapid scan rate mode, with HCD NCE 35, 1.6-m/z isolation window, AGC target 1E4, maximum injection time of 35 ms, and dynamic exclusion which was employed for 35 s. The raw data files were processed using Thermo Scientific™ Proteome Discoverer™ software version 1.4, and spectra were searched against the UniProt Homo sapiens database using the Sequest search engine v2.3.02 (Matrix Sciences, Chicago, IL, USA) run on an in-house server. Search results were trimmed to a 1% FDR for stringency and 5% for relaxed conditions using Percolator. For the trypsin digestion, up to two missed cleavages were allowed. MS tolerance was set at 10 ppm; MS/MS tolerance at 0.6 Da. Carbamidomethylation on cysteine residues was used as fixed modification; oxidation of methionine and phosphorylation of serine, threonine, and tyrosine were set as variable modifications. The sum of scores of individual peptides for identified proteins was used to identify proteins from the immune complex.

Annotation Enrichment Analysis

A total of 77 candidate proteins with protein accession numbers were converted into gene names with DAVID (https://david.ncifcrf.gov) and UniProt (https://www.uniprot.org) within Homo sapiens species. The functional enrichment gene-set analysis for three GO (Gene Ontology) sub-ontologies was performed with ClusteProfiler 4.0 (20) with a p-value cutoff of 0.05 and the "Benjamini–Hochberg" p-Adjust-value method. The "Disease class," "Up tissue," and "Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway" annotations were retrieved from DAVID (21).

Gene Expression Analysis of the Autoantigens in 6 Genomic-Level Databases

Six publicly available SLE or LN databases (**Supplementary Table 3**) were downloaded to cross-validate the autoantigens discovered using immunoproteomics in the present study. In the 6 databases, 3 major genomic technologies were used, including single-cell RNA Sequencing (scRNA Seq), total RNA sequencing (RNA-Seq), and gene expression microarray (RNA-Array), to eliminate bias generated by single technology or sample type. The differentially expressed genes (DEGs) were defined as adjusted p-values (p-adjust) smaller than 0.05 between SLE/LN and healthy controls. In the two scRNA databases, Seurat V4 was used to generate an expression matrix, PCA dimension reduction, and cell cluster as described previously (22). Each cluster cell type was identified with reference-based package SingleR (23) and verified with canonical markers. Then, the DEGs were discovered by DEsingle (24). For two RNA databases, processed count files were directly obtained from GEO, and the DEGs were discovered by DESeq2 (25). The databases from the two RNA-Array were downloaded and standardized, and the DEGs were screened using the GEOquery and Limma (26, 27).

Validation of Immune Complex in LN Using ELISA

ICx in the serum of LN and controls was measured using an in-house-developed ELISA kit. Briefly, to capture antigen-specific ICx, commercial monoclonal antibodies (tested by Western blot) against each autoantigen were coated onto the Immulon 2 HB flat-bottom microtiter plates (Thermo Fisher, MA) overnight at 4°C and then blocked. Serum samples were diluted into 1:100 in reagent diluent, added into the wells, and incubated for 2 h at room temperature. Anti-human IgG antibody (Jackson ImmunoResearch, PA) was diluted into 1:20,000 and added into the wells and incubated for 1.5 h at room temperature followed by color development. The ELISA signal was read by an Epoch plate reader (BioTek, Winooski, VT) at 450 nm, and the blank was subtracted from the OD450 readings of the samples.

Statistical Analysis

All data were analyzed, plotted, or visualized with R 4.1.0 language or ggplot2 (28) package unless stated otherwise. Biomarker-level group-wise comparisons of statistical significance (p-values) were analyzed using the Wilcoxon test. Principal component analysis (PCA) was conducted to transform the serum levels of ICx into uncorrelated principal components, and only the first two components were used for the dot plot. Sensitivity (true positive ratio), 1-specificity (false positive ratio), and area under the curve (AUC) values were determined by receiver-operating characteristic (ROC) analysis using a pROC package (29). The least absolute shrinkage and selection operator (LASSO) (30) was used to evaluate and select candidate serum ICx as the best panel of biomarkers to distinguish LN from HC or LN-active from LN-inactive with the largest value of lambda under 3-fold cross-validation. The correlation between serum ICx levels and clinical parameters was determined by Spearman's correlation coefficient.

RESULTS

Immunoproteomics-Based Discovery of Novel ICx in the Serum of LN

To discover novel ICx that are differentially expressed in SLE or LN, we designed two strategies to capture ICx, as illustrated in **Figure 1**, showing Protein A/G magnetic beads or C1q-based capture of immunoglobulins or the antigen–antibody complex. Pooled serum samples from 3 LN patients or 3 health controls were used to capture and enrich ICx. As shown in **Supplementary Figure 1**, SDS-PAGE was used to visualize the immunocaptured products. After gel purification and in-gel digestion, the peptides were analyzed using liquid chromatography and tandem mass spectrometry (LC-MS/MS). In total, 239 and 61 immunoglobulin or immunoglobulin-binding proteins were discovered *via* Protein A/G and C1q, respectively. Based on the cumulative protein score of each protein, we ended up with identifying 52 (Protein A/G method) and 27 (C1Q method) proteins which were only detectable in the serum of LN but not healthy controls by mass spectrometry, as shown in **Supplementary Table 1** and **Supplementary Table 2**. Among the 77 unique proteins identified, TTR and KRT14 were found in both Protein A/G and C1q procedures. Gene Ontology (GO) enrichment analyses were performed to investigate the enrichment function of these proteins that are expressed in the LN patients, as shown in **Figure 2**. In the biological process (**Figure 2A**), the protein functions of complement activation and B cell activation were significantly enriched which indicated that the 77 proteins may be involved in forming an immune complex during disease development and contribute to the pathogenesis of SLE or LN. The most significant cellular component (CC), immunoglobulin complex, represents 16 immunoglobulins which consist of 11 IgH, 3 IgK, and 2 IgL. Several IgH were found associated with B cell receptor (BCR) analysis of immune-mediated diseases, such as IGHV4-34 and IGHV4-59, which are highly expressed in SLE (31).

Functional Relevance of Immuno-Captured Proteins to SLE or LN

Given that the 77 proteins captured through immunoprecipitation were only detectable in LN but not healthy controls using the immunoproteomic approach, we assumed they may be functionally relevant to SLE or LN. To confirm this, we performed a bioinformatics analysis to determine which proteins may be involved in the disease course of SLE or LN and may potentially serve as a disease biomarker. As shown in **Supplementary Table 1** and **Supplementary Table 2**, among the 77 proteins, 32 of them are highly abundant proteins including keratins, histones, complement system proteins, and albumin and immunoglobulin family proteins which have already been discussed extensively in previous studies (32–36). Therefore, they were eliminated from further analysis in the current study. For the remaining 45 proteins, we performed the following bioinformatics analysis: Database for Annotation, Visualization and Integrated Discovery (DAVID), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology Biological Processes (GO-BP), to determine the relevance of the candidate autoantigens (AAgs) to SLE or LN.

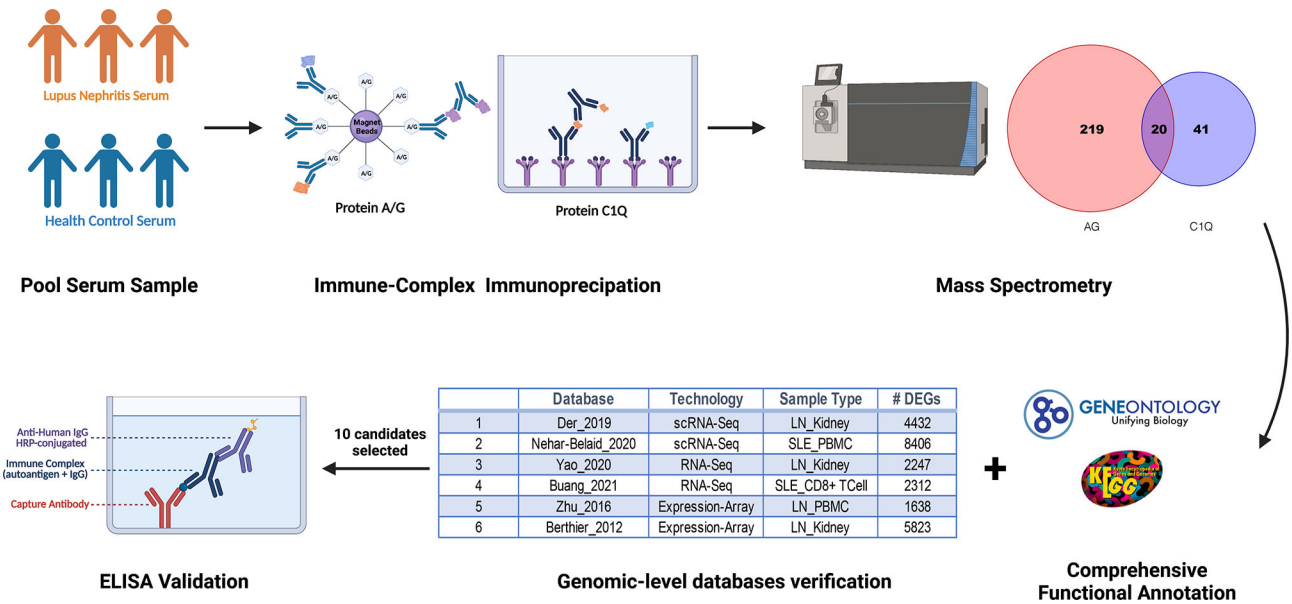

FIGURE 1 | Schematic diagram of discovery and validation of immune complex (ICx) in lupus nephritis (LN) using the immunoproteomic approach. Serum samples from patients with LN or healthy control (n =3 per group) were collected and pooled for Protein A/G or C1q-based ICx capture, followed by the identification of the proteins using mass spectrometry. Candidate ICx were further validated in a larger cohort of LN patients and healthy controls using ELISA. This figure was created using a graphing program from BioRender.com.

In DAVID analysis, we collected 5 features of DAVID functional annotation as described previously (21). As SLE is aberrant in immune function and causes end-organ damage, particularly renal damage (37), the "renal" or "immune" disease-related proteins or the proteins expressed in SLE-affected tissues (such as skin, muscle, bone, joints, kidney, spleen, and brain) were considered "positive" and are indicated in red in **Figure 3**. In KEGG analysis, the proteins involved in "Systemic lupus erythematosus - Homo sapiens (human)" KEGG pathway (https://www.genome.jp/entry/pathway+hsa05322) and its eight related pathways (hsa04060, hsa04514, hsa04610, hsa04612, hsa04630, hsa04660, hsa04662, and hsa04670) were considered "positive" and are indicated in red in **Figure 3**. In GO-BP analysis, the "immune response" and "Inflammatory" annotated proteins were considered "positive" and are indicated in red in **Figure 3**. The "Cumulative Protein Score" is the sum of the peptide scores for proteins in LN patients, which was normalized to be in the range of 0~5.

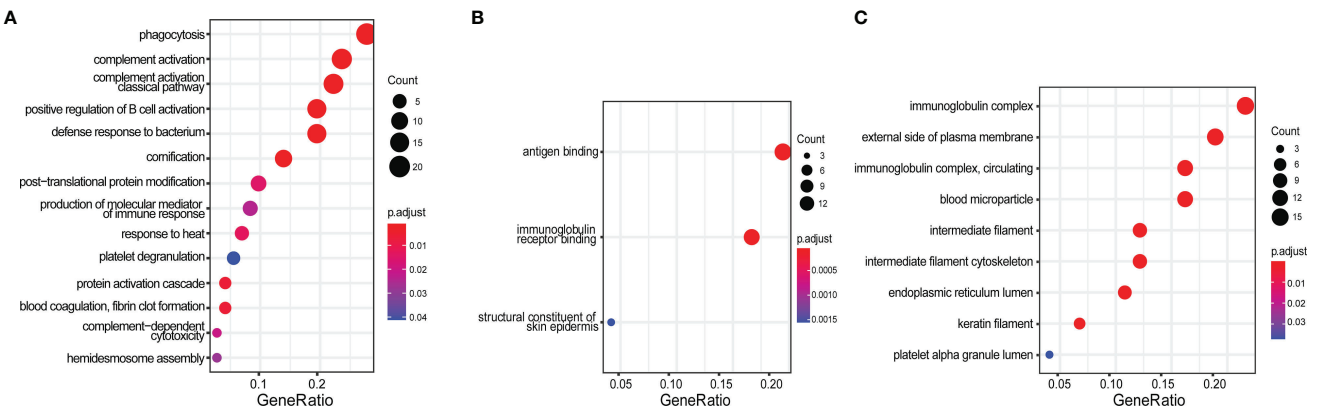

FIGURE 2 | Gene Ontology (GO) enrichment analysis of 77 protein candidates discovered by immunoproteomics. **(A)** Biological process, **(B)** molecular function, and **(C)** cellular component. The gene ratio was calculated by k/n, where k = the size of the unique genes annotated in the specific gene set (e.g., immune complex); n = size of the input genes of interest (e.g., all 77 protein-encoding genes identified from this study).

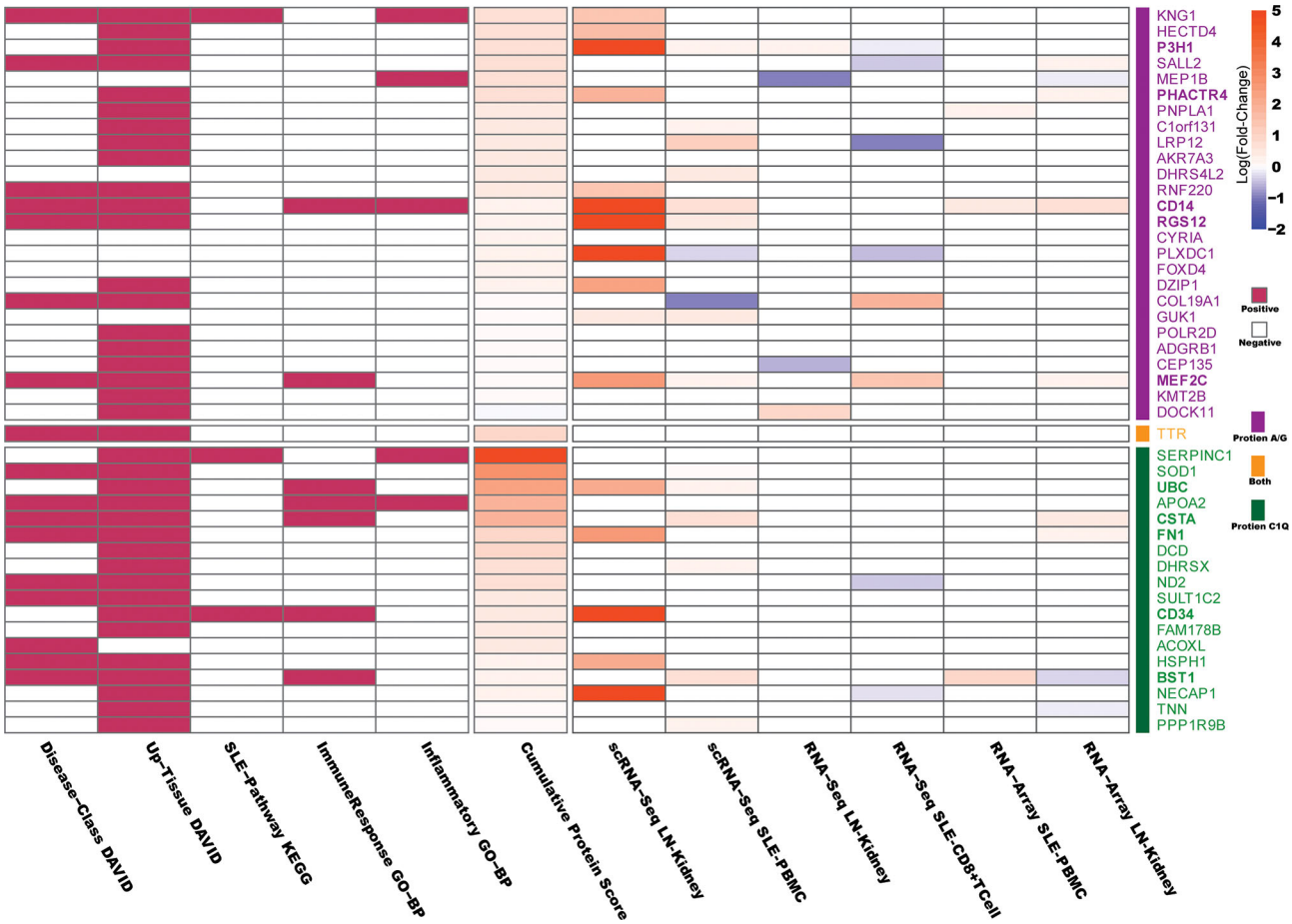

FIGURE 3 | Bioinformatics analysis of the protein candidates. The Database for Annotation, Visualization and Integrated Discovery (DAVID), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology Biological Processes (GO-BP) were used to determine the relevance of the differentially expressed protein candidates to SLE or LN. In DAVID analysis, "renal" or "immune" disease-related proteins or the proteins expressed in SLE-affected tissues (such as skin, muscle, bone, joints, kidney, spleen and brain) were considered "positive." In KEGG analysis, proteins involved in the "Systemic lupus erythematosus - Homo sapiens (human)" KEGG pathway and its eight related pathways were considered "positive." In GO-BP analysis, "immune response" and "Inflammatory" annotated proteins were considered "positive." The "Cumulative Protein Score" is the sum of the peptide scores for each protein identified in LN, which were then normalized to be in the range of 0~5. The Differentially Expressed Genes (DEGs) corresponding to the 45 candidate autoantigens that appeared in six published SLE/LN databases are indicated in the right-hand column of the heatmap, where the filled color represents the log (fold-change) values (p.adjust < 0.05) as indicated in the scale bar. The details of the six databases are presented in **Supplementary Table 3**.

Next, we used 6 published gene expression databases of SLE or LN as detailed in **Supplementary Table 3**, to determine if the genes that encode the autoantigens were differentially expressed in SLE/LN compared to healthy controls. The 6 databases were derived based on data from single-cell RNA sequencing, total RNA sequencing, or gene expression microarray on human SLE/LN genomes from various cell or tissue types (38–43). Differentially expressed genes (DEGs) were defined as adjusted p-values (p-adjust) less than 0.05 between SLE/LN and healthy controls. As shown in **Figure 3**, the filled color represents the log (fold-change) values (p.adjust < 0.05) as indicated in the scale bar.

Two general criteria were used to determine which candidate proteins (antigens) would be selected to move forward for the next phase of validation studies: 1) the protein received at least one "positive" in the comprehensive functional annotations (DAVID/KEGG/GO, **Figure 3**) and 2) the protein-coding genes are "upregulated" in two genomic databases (**Supplementary Table 3**) or highly upregulated in one genomic database. As a result, 10 candidate proteins satisfied both criteria 1 and criteria 2 and were selected for validation.

Validation of Selected Autoantigens/Immune Complexes

A total of 10 candidate AAgs including BST1, CD14, CD34, CSTA, FN1, MEF2C, P3H1, PHACTR4, RGS12, and UBC were selected for validation. Given the fact that the immune-capture process was designed to capture either immunoglobulins or the antigen–antibody complex with Protein A/G- or C1Q-based methods, the proteins/antigens identified by mass spectrometry were presumably

the "bound-form" and originated from the antigen–antibody complex. Therefore, validating serum ICx or bound-form antigens is more reasonable than validating the free-form antigens. We then used monoclonal antibodies against each specific AAg to coat the ELISA plate, and the corresponding ICx were captured and detected on the plate using a secondary antibody, anti-human IgG conjugated with horseradish peroxidase (HRP). In the validation study, we used an independent cohort of 62 LN patients and 21 healthy controls. Detailed patient demographics and clinical characteristics information are presented in **Table 1**. The LN patients were divided into LN-active and LN-inactive based on SLEDAI and rSLEDAI index, as detailed in *Methods*.

As shown in **Figure 4A**, the BST1, PHACTR4, RGS12, and UBC-specific ICx were significantly upregulated in LN-inactive patients, compared to the healthy controls. Furthermore, BST1, CD34, RGS12, and UBC-specific ICx were significantly elevated in LN-inactive compared to LN-active patients (**Figure 4A**). Surprisingly, P3H1 immune complex levels were significantly downregulated in LN-inactive patients, compared to the healthy controls. Also, FN1- and MEF2C-specific ICx were decreased in LN-active compared to healthy controls. This inconsistency with the screening data may be due to the heterogeneity of SLE and the relatively smaller sample size in the screening cohort. Also, some of the commercial monoclonal antibodies may not be optimal (e.g., affinity or epitopes) in maximally capturing serum ICx by ELISA. In addition, it is conceivable that serum CD34 and MEF2C ICx levels may respond to drug treatment, because they were significantly lower in the LN-active group compared to the LN-inactive group following drug treatment (**Supplementary Figure 2**). However, no significant responses to drug treatment were observed in the other ICx as well as in anti-dsDNA autoantibodies (**Supplementary Figure 2**).

To uncover the distribution patterns of the 10 ICx in an unbiased manner, dimension reduction principal component analysis (PCA) was performed using the ELISA validation data. The percentage of variance for the first and second principal components (PCs) were 48.32% and 23.16%, respectively, which were used to map both LN and HC samples (**Figure 4B**). The HC samples were clearly separated from LN patients implying the distinction of their ICx expression patterns. The discriminative capabilities of 10 ICx for LN vs. HC were evaluated with ROC analysis (**Figure 4C**). P3H1, PHACTR4, and RGS12 ICx outperform other markers with AUC values greater than 0.8. Furthermore, when individual ICx were combined into a biomarker panel with the LASSO model, the discriminative ability was significantly improved (**Figure 4D**). In the LN vs. HC group, the AUC value was 0.99, where the greatest positive and negative contributing variables were PHACTR4 and FN1, respectively. In the LN-active vs. LN-inactive group, 4 positive (CD34, MEF2C, RGS12, and UBC) ICx and 2 negative (CD14 and CSTA) ICx contributing variables were identified using the LASSO model. When combined into a panel, these biomarkers can discriminate LN-active from LN-inactive with an AUC value of 0.85. In comparison, when using clinical parameters to discriminate LN-active from LN-inactive, the AUC values were 0.81 for renal chronicity index (CI), 0.62 for renal activity index (AI), 0.58 for white blood cell counts (WBC), and 0.91 for proteinuria. Hence, the LASSO-derived serum biomarker panel (CD14, CD34, CSTA, MEF2C, RGS12, and UBC ICx) outperformed the renal pathology indices such as AI and CI.

Serum ICx May Reflect Pathological Disease Activity

Next, we determined if serum ICx were associated with clinical and pathological parameters and if they had a diagnostic value in reflecting renal pathological change without the need of renal biopsy. As shown in the correlation heatmap in **Figure 5**, most serum ICx exhibited a significant negative correlation with SLEDAI, rSLEDAI, WBC, and proteinuria. Interestingly, serum ICx levels exhibited a negative correlation with AI but a positive correlation with CI, as well as the individual components of AI and CI. This is consistent with the fact that serum ICx was lower in LN-active compared to LN-inactive patients. Besides, the longitudinal studies indicated that BST1, CSTA, and UBC serum ICx levels changed over two time points, which could track with changes in SLEDAI and/or rSLEDAI (**Supplementary Figure 3**).

Gene Expression of Autoantigens in Various Cell Types in SLE

To determine if these AAg-encoding genes are differentially expressed in various cell types in LN, we examined the gene expression of the AAgs at a single-cell resolution using a database of scRNA sequencing of PBMC from 8 SLE adult patients and 5 healthy controls (42). As shown in **Figure 6A**, 8 different cell types were identified from 82,748 cells and each cell population in the SLE and HC groups is displayed in **Figures 6A, B**. CD14, BST1, CSTA, and MEF2C were found to have an enriched gene expression in CD14+ or CD16+ monocytes, and MEF2C was also highly expressed in SLE B cells compared to healthy controls. Furthermore, the significance test using Wilcoxon comparison between SLE and HC in different cell types is presented in **Figure 6C**. P3H1, PHACTR4, and UBC were ubiquitously expressed in all cell types (**Figure 6D**). In the CD4+ T cell cluster, P3H1 and UBC were found upregulated, whereas MEF2C was downregulated in the SLE group, compared to healthy controls. In monocytes, CD14 were highly expressed in CD16+ cells, and MEF2C and FN1 were upregulated in CD14+ cells in SLE compared to healthy controls. UBC exhibited overall the strongest expression across all cell types, and it was significantly upregulated in CD4+ T cells, CD8+ T cells, and natural killer (NK) cells in SLE compared to HC. However, it was significantly downregulated in dendritic cells (DCs) in SLE, compared to HC. The gene expression profiles of these AAgs were also examined in 1,496 cells from LN renal tissues using the scRNA Seq database (details are shown in **Supplementary Figure 4**). The results demonstrate that MEF2C were highly expressed in leukocytes, endothelial cells, and mesangial cells; FN1 exhibited an overall higher gene expression in renal cells compared to PBMCs; UBC was also found highly expressed across all cell types in the kidney, which is consistent with the PBMC data.

In summary, we found that PHACTR4, RGS12, UBC, CSTA, and BST1 are concordantly elevated in both our ICx assays and the public gene expression database, as detailed in **Figure 4** and **Supplementary Table 3**.

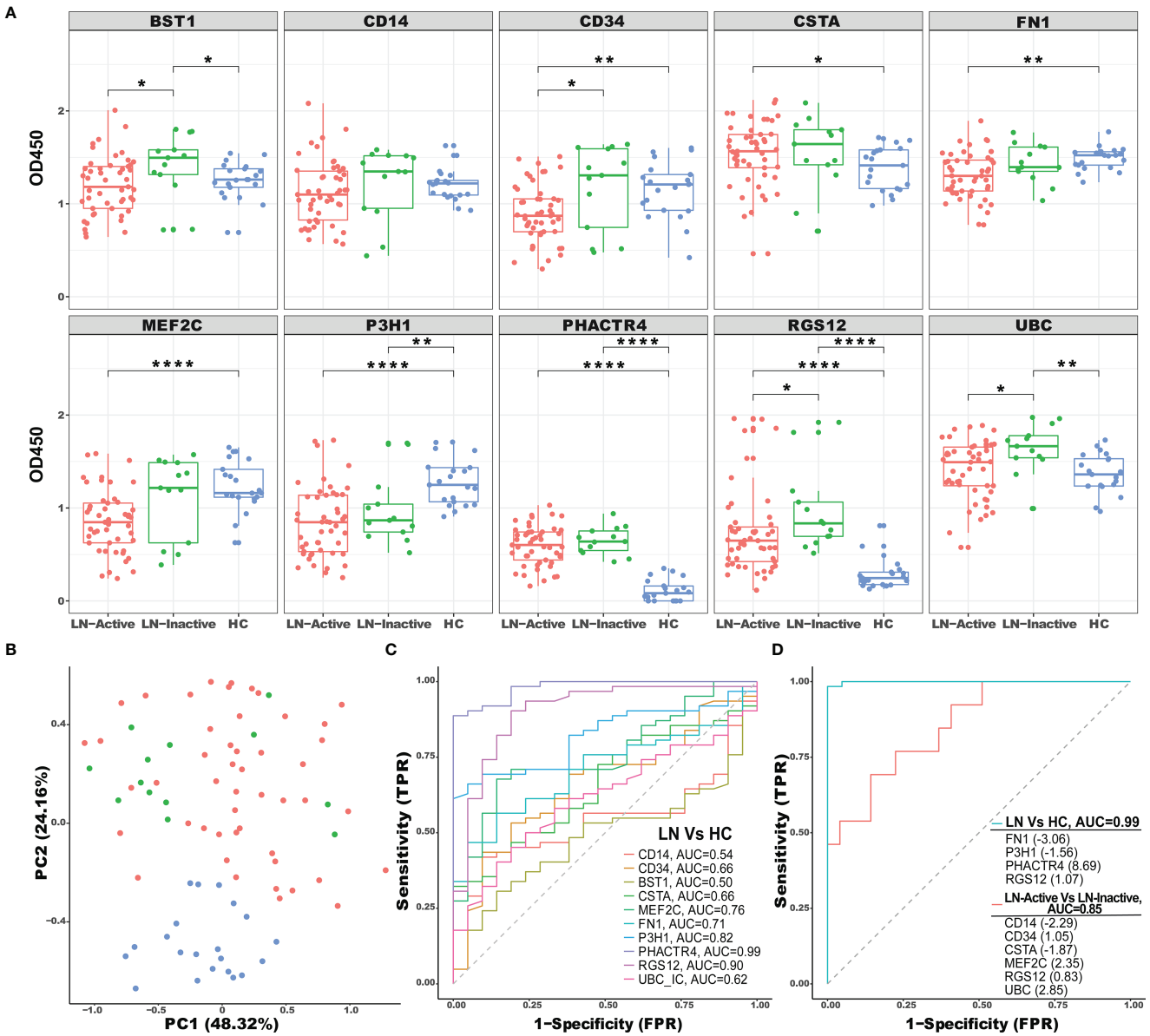

FIGURE 4 | ELISA validation of selected ICx in an independent cohort of LN patients. **(A)** A total of 83 serum samples from healthy controls (N = 21, blue), inactive LN (N = 13, green), and active LN (N = 49, red) were tested by sandwich ELISA for immune complex levels of BST1, CD14, CD34, CSTA, FN1, MEF2C, P3H1, PHACTR4, RGS12, and UBC. Asterisks designate the level of statistical significance: *p < 0.05, **p < 0.01, ****p < 0.0001, using the Wilcoxon test. **(B)** Principal component analysis (PCA) of 10 ICx among HC, inactive LN, and active LN individuals. The two first principal components (PC1, PC2) were plotted. **(C)** The discriminatory abilities of the 10 ICx in distinguishing LN patients from healthy control were examined using ROC analysis. **(D)** Using LASSO regression analysis, the ICx were combined into panels to distinguish LN patients from healthy control or active LN from inactive LN.

DISCUSSION

Experimental disease models of glomerulonephritis (GN) and vasculitis have verified the potential of circulating ICx (antigen–antibody complexes) in causing disease (44). ICx are responsible for the GN of lupus and also contribute to the pathogenesis of other manifestations in SLE (45, 46). ICx play a complicated role in LN, either by depositing on vessels/tissues to cause pathological effects or by interacting with receptors on immune cells to initiate immunological regulations (15). Several assays that indirectly measure circulating ICx have been developed to evaluate ICx-mediated inflammation in patients with SLE; however, currently available assays are insufficient to reliably and reproducibly detect ICx (47). In this study, we employed both Protein A/G magnetic beads and microplate-based C1q to capture, enrich, and purify ICx from lupus serum. In this particular study, it seems that the Protein

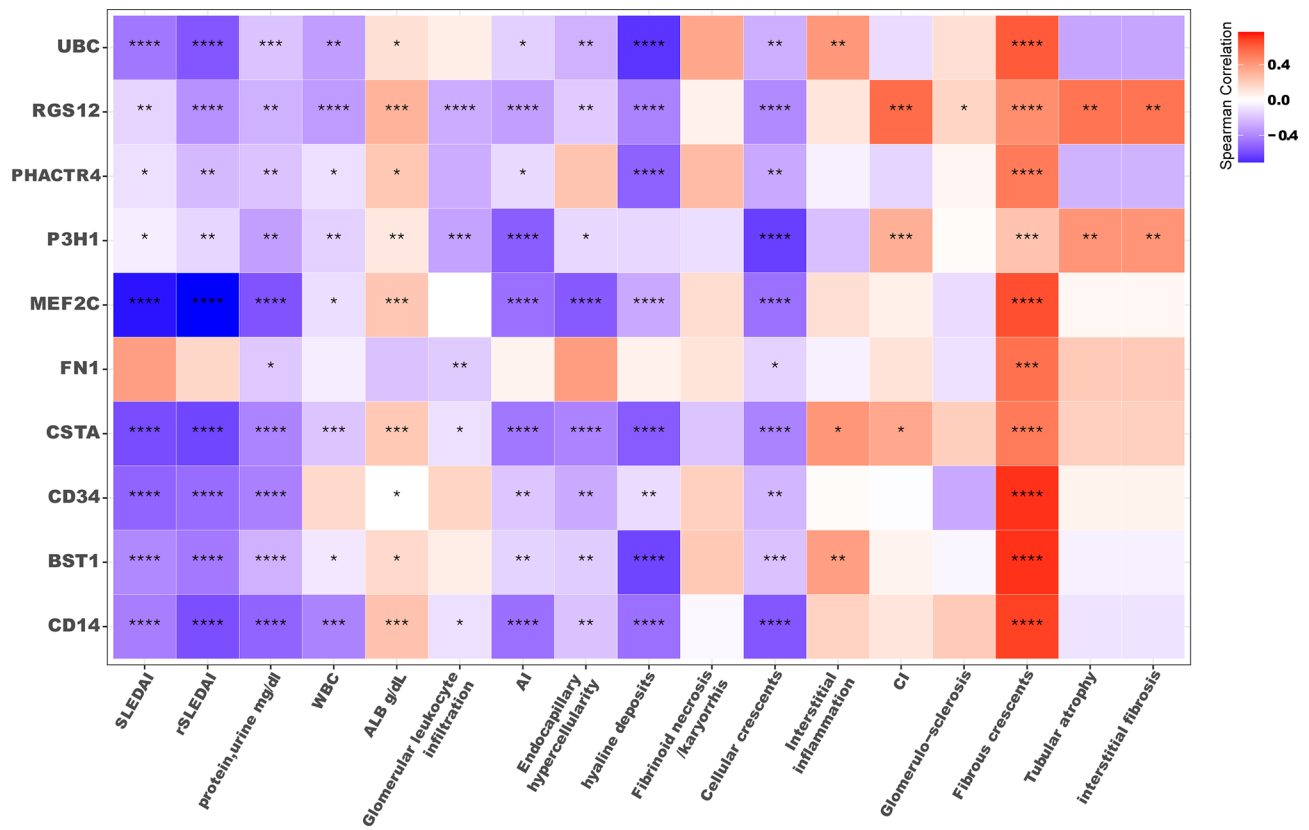

FIGURE 5 | Correlation plot of serum ICx levels with clinical parameters. The color filled in each square represents Spearman's correlation coefficient value, and the corresponding significance level was indicated with asterisks: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. SLEDAI, SLE disease activity index; rSLEDAI, renal SLE disease activity index; WBC, white blood cell count; ALB, the amount of albumin in blood; AI, renal pathology activity index, CI, renal pathology chronicity index.

A/G-based approach is more efficient (239 proteins) in capturing ICx in SLE, compared to the C1q method (61 proteins). This may be due to the affinity differences between Protein A/G and C1q in binding ICx.

The DEGs from six transcriptomics SLE/LN databases were informative to understand the functional aspects of the autoantigens and to select the most relevant proteins/ICx for further validation. However, given the fact that the 6 databases were developed using different technologies, this inevitably poses a challenge to integrate all data which requires statistical framework and tremendous computational resources. To address this challenge, the DEGs were separately calculated using mainstream methods for each technology (24–26) and then aggregated and visualized with a heatmap. The discrepancy in gene expression profiles of the same genes across six databases may be due to the heterogeneity of LN or platform differences. For example, LRP12 and COL19A1 gene expression changes were found to be opposite in SLE between a scRNA database and an RNA database. In another scenario, PLXDC1 expression levels were different in SLE between PBMCs and kidney cells at a single-cell resolution, which suggested that the context of cells is critical.

It is worth noting that PHACTR4 ICx were significantly elevated in LN patients compared to HC (AUC = 0.99), and consistent transcription profiles of this molecule were also found across two kidney databases, based on scRNA Seq and gene expression microarray. Interestingly, previous studies have identified PHACTR4 as a tumor suppressor in several cancers, with functions in PP1 localization and Rb dephosphorylation (48). The aberrant gene expression in the kidney and formation of ICx may contribute to LN pathogenesis. The regulator of G-protein signaling 12 (RGS12) ICx was found upregulated in LN, compared to HC. Interestingly, higher levels of RGS12 ICx were detected in the LN-inactive compared to LN-active group. Furthermore, serum RGS12 ICx exhibited a positive correlation with chronicity index (CI), as well as glomerulosclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis. In a study of rheumatoid arthritis, a significant association was found between RGS12 genetic variation with the response to an immunosuppressive drug (49). These data suggest that RGS12 may be involved in the pathogenesis of SLE and other rheumatic diseases. Notably, P3H1 ICx was the only one found significantly downregulated in LN (AUC = 0.82) compared to HC. P3H1 mRNA was found upregulated in 3 gene expression databases and downregulated in one database. In the PBMC scRNA Seq database, transcriptomic P3H1 was significantly upregulated in CD4+T cells. P3H1 is responsible for the 3-hydroxyproline posttranslational modification of type I collagen; if defective, it may

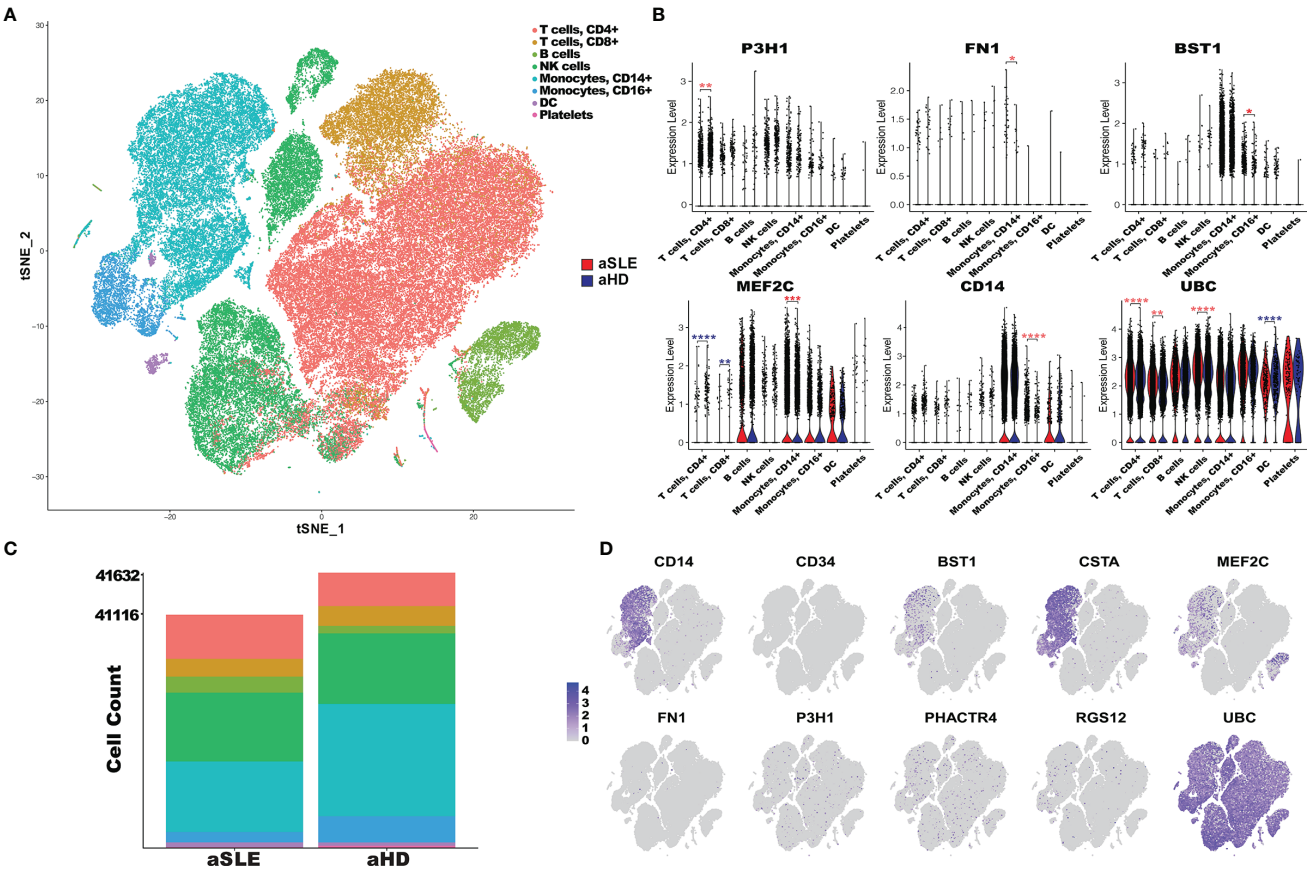

FIGURE 6 | PBMC scRNA expression of autoantigen candidates in SLE patients and healthy controls. **(A)** tSNE plot of 82,748 cells across 23,257 gens from the PBMC sample of 8 adult SLE patients (aSLE) and 5 adult healthy controls (aHD). Clusters were derived from PCA reduction with the first 18 dimensions, and the clusters' cell type was identified with SingleR reference databases and canonical markers. **(B)** The bar plot represents the cell abundance of 8 clusters across aSLE (41,116 cells) and aHD (41,632 cells) groups. **(C)** Violin plots of PBMC scRNA expression in 8 cell type clusters in aSLE (red) and aHD (cyan). The autoantigens' differential expression levels in each cluster were determined using the Wilcoxon test. The asterisks designate the level of significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0. 0001, and increased or decreased expression in aSLE was colored in red and cyan, respectively. **(D)** Feature plots of 10 autoantigens; each dot represents one cell, and the color intensity indicates the gene expression level of autoantigen in the cells, from gray to purple.

cause renal pathology (50). Therefore, P3H1 may be involved in renal pathology and contribute to the pathogenesis of LN. In addition, the fact that serum CD34 and MEF2C ICx but not anti-dsDNA autoantibody levels responded to drug treatment (**Supplementary Figure 2**) may indicate that novel serum ICx may be a better biomarker in assessing drug responses in LN. It is important to highlight that PHACTR4, RGS12, UBC, CSTA, and BST1 are concordantly elevated in both our ICx assays and the public gene expression database, as detailed in **Figure 4** and **Supplementary Table 3**. The reason that the two ICx enrichment methods resulted in different protein profiles may be due to structurally different binding sites in the antibodies and different binding kinetics/affinities for ICx. As indicated in the literature, C1q binds to CH2 domains of the Fc (51), whereas protein A and protein G mainly bind to the CH2-Ch3 domains (52, 53). Thus, it is likely that protein A/G and C1q may select and capture different antibodies in the ICx, which were characterized by mass spectrometry-based sequencing of the protein/antigen.

The caveats of this study include (1) the relatively small sample size especially in the LN-inactive group and (2) the lack of various disease controls such as other autoimmune diseases and other kidney diseases due to the limited access to these samples. In future studies, the increase of the sample size in the LN-inactive group may aid to confirm if and why serum ICx were lower in LN-active compared to LN-inactive. The inclusion of disease controls may be helpful in better establishing the specificity of these ICx biomarkers in LN.

CONCLUSION

Immunoproteomics-based discovery studies have entabled us to identify promising immune complexes in LN, which are associated with clinical parameters including renal pathology indices. These ICx may be useful in diagnostics, disease monitoring, and/or assessing drug responses in LN contingent upon further validation.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (1) partner repository with the dataset identifier PXD031069.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Houston IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TW conceived this study. CT, MF, GT, and TZ performed the experiments and/or collected the data. CT, MF, GT, SZ, BY, YL, TZ, RS, CM, and TW were involved in the data analysis or data interpretation. CT and TW wrote the manuscript. SZ, BY, and CM edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.850015/full#supplementary-material

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S100A8 in Serum, Urine, and Saliva as a Potential Biomarker for Systemic Lupus Erythematosus

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Objectives: This study aimed to elucidate the potential of serum, urine, and saliva S100 calcium-binding protein A8 protein (S100A8) levels as biomarkers for systemic lupus erythematosus (SLE).

Methods: Serum, urine, and saliva samples were obtained from 249 patients with SLE from the Ajou lupus cohort and 52 age- and sex-matched healthy controls (HCs). The concentrations of S100A8 were quantified using an ELISA, and a receiver operating characteristic curve was used to analyze whether they may be used as biomarkers for diagnosing SLE.

Results: Among 249 SLE patients included in our study, the mean SLE disease activity index (SLEDAI)-2K was 7.16 ± 5.61, and the number of patients with lupus flare was 11. Patients with SLE showed a 2.7-fold increase in serum S100A8 levels compared with that in HCs (1,890.6 vs. 709 pg/ml, $p < 0.001$). In urine and saliva, the average S100A8 levels were significantly higher in patients with SLE compared with those in HCs (urine, 2,029.4 vs. 1,096.7 pg/ml, $p = 0.001$; saliva, 290,496.3 vs. 47,742 pg/ml, $p < 0.001$). For SLE diagnosis, the area under the receiver operating characteristic curve was 0.831 for serum S100A8 (95% CI, 0.765–0.897), 0.751 for urine S100A8 (95% CI, 0.648–0.854), and 0.729 for salivary S100A8 (95% CI, 0.646–0.812). Pearson's correlation analysis showed that S100A8 in serum, urine, and saliva was significantly associated with the SLEDAI ($r = 0.267$, $p < 0.001$; $r = 0.274$, $p < 0.001$; and $r = 0.629$, $p < 0.001$, respectively). Among the clinical manifestations, nephritis was the most influential factor related to SLE in the concentration of S100A8 in serum, urine, and saliva.

Conclusion: This is the first study to show that the expression of S100A8 in serum, urine, and saliva is significantly higher in patients with SLE than in HCs and is associated with disease activity markers. Therefore, we suggest that S100A8 protein could be a potential biomarker for SLE.

Keywords: S100A8, systemic lupus erythematosus, biomarkers, biofluids, disease activity

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of autoantibodies owing to the loss of immunological tolerance to autoantigens caused by genetic, hormonal, and environmental factors (1). Dysregulation of the immune system attacks healthy cells and tissues, causing inflammation in multiple organs, including the skin, joints, and kidneys, and results in a wide range of clinical manifestations (2). The classification and diagnosis of SLE are complex and difficult because of the nature of this multisystemic disease and an incomplete understanding of its pathophysiology (3). Although classification criteria that combine diverse clinical symptoms and supportive serologic abnormalities are used to diagnose SLE, the diagnosis does not rely solely on the fulfillment of the classification criteria, and the final diagnosis is left to the clinicians' judgment (4). However, it is challenging to make a prompt diagnosis even for an experienced rheumatologist because of the heterogeneity in both the expression of various clinical symptoms and profiles of autoantibodies. Therefore, serological biomarkers that can meet the currently unmet diagnostic needs are required.

Various biomarkers have been proposed in SLE to overcome the difficulty of diagnosis due to the heterogeneity of the manifestations, many of which have been well validated, and some of which are being used in clinical practice (5). One biomarker with established clinical significance in SLE is S100 calcium-binding protein A8 protein (S100A8) (6). S100A8 is a Ca^{2+}-binding protein belonging to the S100 family that is released from neutrophils as part of neutrophil extracellular traps during an inflammatory response (7). Although mainly known in the heterodimer of S100A8/A9, S100A8 also acts as a damage-associated molecular pattern molecule after release and accumulates in the blood and body compartments, as it is an important regulator of inflammation that promotes the function of innate immune cells through interaction with toll-like receptor 4 and the receptor of advanced glycation end products, which are members of the immunoglobulin superfamily of cell surface molecules (8–10). There is growing experimental and clinical evidence that serum S100A8 levels are higher in patients with SLE than in healthy controls (HCs) and are associated with disease activity, glomerulonephritis, and anti-double-stranded DNA (dsDNA) antibodies (Ab) (11–13). However, the increased serum S100A8 level may be insufficient in its role as an SLE-specific biomarker, given that it is also observed in many inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (14, 15).

There is a growing interest in combining biomarkers to improve the predictive value to obtain an accurate and early diagnosis of the disease. Considering the difficulty in finding candidate biomarkers and the high cost of obtaining them, it is preferable to collect and combine specific biomarkers from several biofluids rather than to use multiple serum biomarkers (16). Among biofluids, researchers mainly use serum, saliva, urine, and tears because of factors such as ease of access, minimization of invasive sampling, and availability of multiple sampling (17). S100A8 protein has also been analyzed as a biomarker in several biofluids; however, there is no comparative evidence for S100A8 homodimer in the saliva and urine of patients with SLE. Hence, in this study, we hypothesized that S100A8 homodimer could be useful as a biomarker for identifying the onset of SLE, and to prove this, we compared the screening ability of S100A8 homodimer in serum, urine, and saliva from patients with SLE and HCs.

Abbreviations: Ab, antibodies; aPL, antiphospholipid; AUC, area under the curve; dsDNA, double-stranded DNA; ESR, erythrocyte sedimentation rate; HCs, healthy controls; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; S100A8, S100 calcium-binding protein A8 protein; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; UPCR, urine protein/creatinine ratio.

MATERIALS AND METHODS

Study Population and Clinical Assessments

This study enrolled 249 patients with SLE from the Ajou lupus cohort at the Department of Rheumatology, Ajou University Hospital, Republic of Korea. SLE diagnosis was based on the 1997 American College of Rheumatology (ACR) criteria or the 2012 Systemic Lupus International Collaborating Clinics (SLICC) classification criteria (18, 19). Patients with other autoimmune diseases, such as Sjogren's syndrome, rheumatoid arthritis, and systemic sclerosis, were excluded. Informed consent was obtained from each patient prior to sample collection, and 249 serum and urine samples and 100 saliva samples were collected, excluding patients who did not want to participate. Demographic, clinical, therapeutic, and laboratory data related to SLE were gathered from the patients' medical records.

In our center, anti-double-stranded DNA (anti-dsDNA) antibodies were assayed using Anti-dsDNA kit (Trinity Biotech, Bray, Ireland), and anti-dsDNA values >7 IU/ml were defined as abnormal. Complement 3 (C3) and complement 4 (C4) levels were measured on Cobas (Roche Diagnostics, Basel, Switzerland), with a normal range of C3 of 90–180 and C4 of 10–40 mg/dl.

Disease activity was assessed using the SLE disease activity index (SLEDAI)-2K at the time of sample collection (20). Lupus flares were defined as a ≥3 point increase in SELENA-SLEDAI according to SELENA-SLEDAI Flare Index (21). Damage was determined by the SLICC/ACR damage index (SDI) score calculated based on 12 different organ damages that occurred after diagnosis of SLE, and significant organ damage was defined as SDI ≥ 1 (22). Fifty-two age- and sex-matched HCs were recruited from the same region. The study protocol was reviewed and approved by the Institutional Review Board of Ajou University Hospital (AJIRB-BMR-SMP-19-403).

ELISA for S100A8 Proteins

Venous blood, unstimulated saliva, and urine were collected from patients with SLE and HCs, and the serum was immediately centrifuged at 15,928 relative centrifugal force (RCF) and saliva at 1,763 RCF for 10 min. The supernatant was collected and stored at −80°C until further analysis. Before ELISA was

conducted, frozen serum, urine, and saliva samples were thawed and then diluted 1:100 in phosphate-buffered saline. S100A8 homodimer concentrations were measured using a commercially available ELISA kit (MBS2022637; MyBioSource, San Diego, CA, USA) for serum and urine. S100A8 homodimer concentrations in saliva were evaluated using the Human S100A8 ELISA kit (R&D Systems, Minneapolis, MN, USA) (cat. No. DY4570-05). All materials were supplied with the kit, and the test was performed according to the manufacturer's instructions.

Statistical Analysis

To determine the baseline differences in populations, Student's *t*-test or Mann–Whitney U test was performed for continuous variables, and the chi-square test or Fisher's exact test was performed for categorical variables. The results were expressed as mean ± SD, and all statistical significance was set at a p-value <0.05. By analyzing the area under the curve (AUC) of the receiver operating characteristic (ROC) curve, we established the utility of S100A8 levels in serum, urine, and saliva as a diagnostic marker to distinguish patients with SLE from HCs. Youden's index (calculated as sensitivity + specificity − 1) was used to determine the cutoff values for S100A8 levels. We also calculated sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Pearson's correlation analysis was conducted to evaluate the relationship between S100A8 levels and the disease activity index. All statistical analyses were performed using SPSS software (version 25.0; IBM Corporation, Armonk, NY, USA).

RESULTS

Baseline Clinical Characteristics of Patients With Systemic Lupus Erythematosus and Healthy Controls

The baseline patient characteristics are presented in **Table 1**. The mean age of patients with SLE was 42.1 ± 11.2 years, and 95.2% were female (no significant difference from the HCs, data not shown). The mean disease duration of SLE was 98.8 ± 73.8 months, the mean SLEDAI-2K was 7.16 ± 5.61, and the number of patients with lupus flare was 11. In patients with SLE, the most common clinical symptom was arthritis (53.8%); a total of 73 patients (29.3%) had lupus nephritis, of which 45 (18.1%) patients had >500 mg/day of urine protein/creatinine ratio (UPCR). Laboratory findings were positive for antinuclear antibody (ANA) in all cases except for seven, and anti-dsDNA Ab was positive in 94 (38.8%), anti-Sm Ab was positive in 28 (11.2%), and antiphospholipid Ab (aPL) was positive in 75 (30.1%) patients. Nearly half of the patients (49.4%) had at least one abnormally low C3 (<90 mg/dl) or C4 (<10 mg/dl) level. The majority of the patients (97.2%) were receiving hydroxychloroquine, and 176 (70.7%) were receiving corticosteroids, with a mean dose of 4.43 ± 6.34 mg. Among the patients with SLE, 93 were taking immunosuppressants, with calcineurin inhibitors being the most common, followed by mycophenolate mofetil, azathioprine, and cyclophosphamide.

TABLE 1 | Demographic and clinical characteristics of patients with systemic lupus erythematosus (SLE) and healthy controls.

Variable	SLE (N = 249)
Age, years	42.1 ± 11.2
Female, no. (%)	237 (95.2)
Disease duration, months	98.8 ± 73.8
Alcohol, no. (%)	72 (41.4)
Smoking, no. (%)	21 (8.4)
Clinical manifestations	
Mucocutaneous, no. (%)	111 (44.6)
Arthritis, no. (%)	134 (53.8)
Nephritis, no. (%)	73 (29.3)
Serositis, no. (%)	10 (4)
Hematologic, no. (%)	73 (29.3)
Central nervous system, no. (%)	5 (2)
Laboratory finding	
Leukocyte,/μl (normal range 3,400–10,600)	4,929.4 ± 2,252.4
Lymphocyte,/μl (normal range 1,600–4,900)	1,419.2 ± 606.5
Platelets, $\times 10^3$/μl (normal range 134–387)	215.1 ± 69.1
ESR, mm/h (normal range 0–25)	15.5 ± 13.6
Complement 3, mg/dl (normal range 90–180)	88.6 ± 25.3
Complement 4, mg/dl (normal range 10–40)	18.2 ± 9.1
Anti-ds DNA (IU/ml) (normal range 0–7)	42.4 ± 111.2
Immunologic finding	
ANA positivity, no. (%)	242 (97.2)
Anti-ds DNA Ab positivity, no. (%)	94 (38.8)
Anti-Sm Ab positivity, no. (%)	28 (11.2)
aPL positivity, no. (%)	75 (30.1)
Low complements (C3 < 90 mg/dl or C4 < 10 mg/dl), no. (%)	123 (49.4)
Urinalysis	
Proteinuria (mg/day)	0.38 ± 0.85
Proteinuria > 0.5 g/day, no. (%)	45 (18.1)
Renal histology (ISN/RPS classification), no. (%)	73 (29.3)
Class II	3 (4.1)
Class III	9 (12.3)
Class IV	29 (39.7)
Class V	15 (20.5)
Class III+V	7 (9.6)
Class IV+V	10 (13.7)
SLEDAI-2K	7.16 ± 5.61
Recent SLE flare, no. (%)	11 (4.4)
SDI score ≥1, no. (%)	27 (10.8)
Treatment	
Hydroxychloroquine, no. (%)	242 (97.2)
NSAIDs, no. (%)	82 (32.9)
GCs, no. (%)	176 (70.7)
Mean GC dose, mg/day (prednisolone-equivalent)	4.43 ± 6.34
Cumulative GC dose, g (prednisolone-equivalent)	11.1 ± 13.3
Immunosuppressants no. (%)	
Azathioprine, no. (%)	27 (10.8)
Mycophenolate mofetil, no. (%)	52 (20.9)
Cyclophosphamide, no. (%)	18 (7.2)
Calcineurin inhibitor, no. (%)	53 (21.3)
ACE inhibitor or ARB, no. (%)	52 (20.9)
Vitamin D, no. (%)	190 (76.3)

Antiphospholipid antibody positivity included persistently positive (>12 weeks positivity) of at least one lupus anticoagulant, anticardiolipin, or anti-beta-2 glycoprotein I IgG or IgM. SLE, systemic lupus erythematosus; ESR, erythrocyte sedimentation rate; ANA, antinuclear antibody; dsDNA, double-strand deoxyribonucleic acid; Ab, antibody; Sm, Smith; aPL, antiphospholipid antibodies; C3, complement 3; C4, complement 4; ISN/RPS, International Society of Nephrology and the Renal Pathology Society; SLEDAI-2K, SLE disease activity index 2000; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index; NSAIDs, non-steroidal anti-inflammatory drugs; GC, glucocorticoid; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; NA, not applicable.

Concentration of S100A8 in Biofluids in Patients With Systemic Lupus Erythematosus and Healthy Controls

As shown in **Figure 1A**, the serum S100A8 levels were significantly higher in patients with SLE than in HCs (1,890.6 ± 1,254.7 vs. 709 ± 413 pg/ml, $p < 0.001$). **Figures 1B, C** show elevated urine and saliva concentrations of S100A8 in patients with SLE compared with those in HCs (2,029.4 ± 2,251.4 vs. 1,096.7 ± 1,422.8 pg/ml, $p = 0.001$; and 290,496.3 ± 513,156.5 vs. 47,742.1 ± 60,875.7 pg/ml; $p < 0.001$, respectively).

Receiver Operating Characteristic Curves for the Diagnosis of Systemic Lupus Erythematosus of S100A8 Levels in Saliva, Urine, and Serum

The ROC curves for serum, urine, and salivary S100A8 levels for discriminating SLE are shown in **Figure 2**. The AUC values for serum, urine, and salivary S100A8 levels were 0.831 for serum S100A8 (95% CI, 0.765–0.897), 0.751 for urine S100A8 (95% CI, 0.648–0.854), and 0.729 for salivary S100A8 (95% CI, 0.646–0.812), with optimal cutoff values of 1,055, 512.5, and 80,269.5 pg/ml, respectively. The diagnostic ability characteristics of the biomarkers, including sensitivity, specificity, PPV, and NPV, are presented in **Table 2**. Of the three biofluid biomarkers, urine S100A8 showed the highest specificity (0.99) with the lowest sensitivity (0.556), and serum and salivary S100A8 showed both higher specificity (0.911) and lower sensitivity (0. 61 and 0.52, respectively) than urine S100A8. At the cutoff value of each biomarker, the highest PPV was 95.7% in serum, and the highest NPV was 80.6% in urine.

Correlations of S100A8 Levels With Disease Activity Index and Clinical Manifestations of Systemic Lupus Erythematosus

Using Pearson's correlation, we confirmed that the S100A8 levels in serum, urine, and saliva were correlated with clinical indices explaining disease activity in SLE (**Table 3**). Serum S100A8 concentrations were positively correlated with erythrocyte sedimentation rate (ESR) ($r = 0.125$, $p = 0.006$), anti-dsDNA

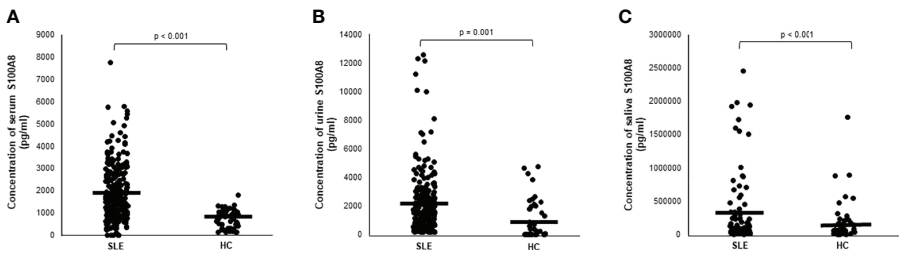

FIGURE 1 | **(A)** Serum level of S100A8 in patients with systemic lupus erythematosus (SLE). **(B)** Urine level of S100A8 in patients with SLE. **(C)** Salivary level of S100A8 in patients with SLE. Central horizontal bar indicates mean value. Statistical analyses were conducted using Mann–Whitney U test.

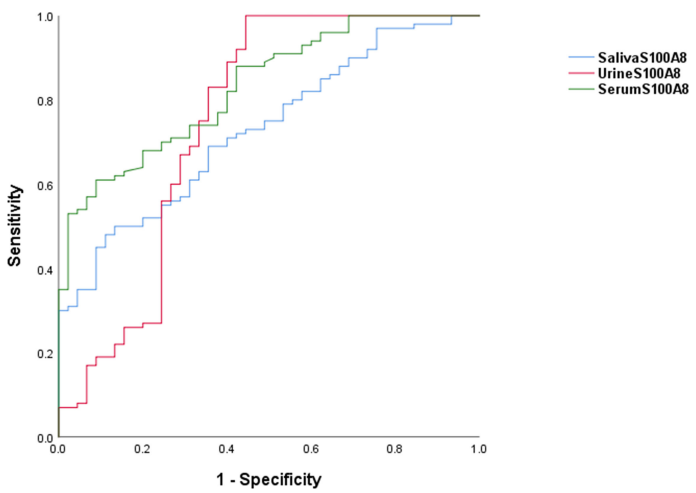

FIGURE 2 | Receiver operating characteristic curves associated with the diagnostic utility of S100A8 in serum, urine, and saliva. For SLE diagnosis, the AUC was 0.831 for the serum S100A8 (95% CI, 0.765–0.897), 0.751 for the urine S100A8 (95% CI, 0.648–0.854), and 0.729 for the salivary S100A8 (95% CI, 0.646–0.812). AUC, area under the receiver operating characteristic curve; SLE, systemic lupus erythematosus.

TABLE 2 | Utility of S100A8 levels in serum, urine, and saliva for diagnosing SLE.

Variable	AUC	p-Value	Cut-off	Sensitivity	Specificity	PPV	NPV
Serum S100A8 (pg/ml)	0.831	<0.001	1055	61%	91.1%	95.7%	37.9%
Urine S100A8 (pg/ml)	0.751	<0.001	512.5	99%	55.6%	63.9%	80.6%
Salivary S100A8 (pg/ml)	0.729	<0.001	80,269.5	52%	91.1%	87.3%	46.4%

SLE, systemic lupus erythematosus; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

TABLE 3 | Correlation between S100A8 level and disease activity markers in patients with SLE.

Disease activity markers	Correlation coefficient, r (p-value)		
	Serum S100A8	Urine S100A8	Salivary S100A8
Leukocyte	0.072 (0.095)	−0.008 (0.91)	0.071 (0.299)
Lymphocyte	0.002 (0.959)	−0.081(0.229)	−0.051 (0.459)
Hemoglobin	−0.017 (0.695)	−0.279 (**<0.001**)	−0.09 (0.192)
Platelet	0.000 (0.248)	−0.135 (0.179)	−0.111 (0.105)
ESR	0.125 (**0.006**)	0.183 (0.006)	0.139 (**0.043**)
Complement 3	−0.205 (**0.001**)	−0.104 (0.119)	−0.107 (0.119)
Complement 4	−0.107 (0.094)	−0.139 (**0.037**)	−0.118 (0.084)
Anti-ds DNA Ab	0.204 (**0.001**)	0.167 (**0.012**)	0.179 (**0.009**)
UPCR	0.127 (**0.014**)	0.177 (**0.018**)	0.012 (0.864)
SLEDAI	0.267 (**<0.001**)	0.274 (**<0.001**)	0.629 (**<0.001**)

Bold values indicate significant p-values.
SLE, systemic lupus erythematosus; ESR, erythrocyte sedimentation rate; dsDNA, double-strand deoxyribonucleic acid; Ab, antibody; UPCR, urine protein/creatinine ratio; SLEDAI, SLE disease activity index.

Ab (r = 0.204, p = 0.001), and UPCR (r = 0.127, p = 0.014) and negatively correlated with complement 3 (r = −0.205, p = 0.001). Similarly, urine S100A8 concentrations were positively correlated with anti-dsDNA Ab (r = 0.167, p = 0.012) and UPCR (r = 0.177, p = 0.018) and negatively correlated with hemoglobin (r = −0.279, p < 0.001) and complement 4 (r = −0.139, p = 0.037). ESR (r = 0.139, p = 0.043) and anti-dsDNA Ab (r = 0.179, p = 0.009) were also positively correlated with salivary S100A8 levels. No significant correlation was found between S100A8 levels and the other indices, including leukocytes, lymphocytes, and platelets. There was a significant positive correlation between the SLEDAI-2K and all biofluid biomarkers (serum, r = 0.267, p < 0.001; urine, r = 0.274, p < 0.001; saliva, r = 0.629, p < 0.001; **Figures 3A–C**).

We further compared the concentrations of S100A8 in serum, urine, and saliva according to clinical manifestations, but the association between clinical manifestations and S100A8 levels was different for each biofluid (**Table 4**). The only significant difference in S100A8 levels in the serum, urine, and saliva was nephritis (serum, p = 0.012; urine, p = 0.015; and saliva, p = 0.003). There was no significant difference in the results of further analysis on the levels of S100A8 according to the lupus nephritis classification. Serum S100A8 levels differed according to fever and central nervous system (CNS) involvement (p < 0.001 and p = 0.049, respectively), and urine and salivary S100A8 showed significant differences in arthritis and malar rash, respectively (p = 0.038 and p = 0.018, respectively). Symptoms of CNS lupus included seizures, headache, and vasculitis, among which three had seizures, and one patient each had headache and vasculitis. We stratified patients with SLE into low disease activity (SLEDAI < 6) and high disease activity (SLEDAI ≥ 6) groups, and S100A8 levels in all biofluids were significantly higher in the high disease activity group (serum, 2,052.6 ± 1,326.8 vs. 1,659 ± 1,107.9 pg/ml, p = 0.011; urine, 2,231.5 ± 2,396.6 vs. 1,638 ± 1,483.8 pg/ml, p = 0.02; and saliva, 487,263 ± 640,283.6 vs. 68,610.5 ± 91,553.7 pg/ml, p < 0.001). Furthermore, in patients with an SDI score ≥1 indicative of chronic damage, S100A8 in serum and saliva was higher than that of no damage.

DISCUSSION

The ability to identify SLE early through a specific sample is particularly critical, as a definitive diagnosis cannot be provided with a single laboratory indication and is difficult to distinguish from other diseases, such as infection and malignancies, due to complex clinical symptoms. Early detection of SLE usually has a significant impact on prognosis through prompt and appropriate treatment (23). Many research groups have proposed various diagnostic markers for SLE; however, early diagnosis of SLE remains challenging (24–26).

Increased serum levels of myeloid calcium-binding proteins in connective tissue diseases, including SLE, were first described in 1990. Since then, S100 proteins have been reported to be upregulated in various inflammatory diseases and malignancies by being involved in regulating cell proliferation and transcriptional factor activity (7, 12, 27, 28). S100A8 (also known as calgranulin A or myeloid-related protein-8), which belongs to the S100 family, forms the calgranulin subfamily, a group of proteins that play a crucial role in the regulation of inflammatory processes, together with S100A9 (also known as calgranulin B or myeloid-related protein-14) and S100A12 (also

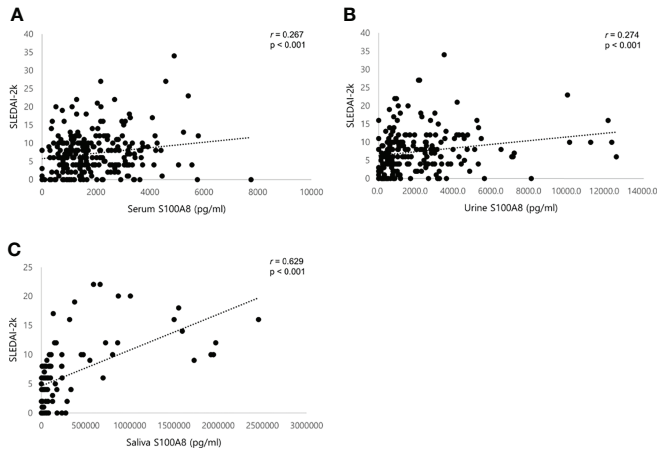

FIGURE 3 | Scatter plots showing positive correlations between S100A8 and SLEDAI-2K in patients with SLE. **(A)** Serum S100A8. **(B)** Urine S100A8. **(C)** Salivary S100A8. SLEDAI, systemic lupus erythematosus (SLE) disease activity index. Statistical analyses were conducted using Pearson's correlation analyses.

TABLE 4 | Comparison of serum, urine, and salivary S100A8 levels according to clinical manifestations in patients with SLE.

Manifestations[a]	Serum S100A8	p-Value	Urine S100A8	p-Value	Salivary S100A8	p-Value
Fever						
(+) = 11 (9)	3,198.4 ± 1,878.4	**<0.001**	1,586.4 ± 1,660.2	0.649	454,849.9 ± 528,660.4	0.316
(–) = 238 (91)	1,830.1 ± 1,189.7		1,954.7 ± 2,259.3		274,241.8 ± 511,714.2	
Oral ulcer						
(+) = 43 (19)	1,815.8 ± 1,223.4	0.668	1,892 ± 2,736.2	0.881	222,365 ± 471,954.7	0.523
(–) = 206 (81)	1,906.2 ± 1,263.5		1,708.9 ± 1289.6		306,477.8 ± 523,824.9	
Malar rash						
(+) = 36 (28)	2,004.2 ± 1,397.1	0.558	2494.3 ± 2860.3	0.155	521,816.2 ± 631,566.5	**0.018**
(–) = 213 (72)	1,871.4 ± 1,231.5		1860.3 ± 2129.4		200,538.6 ± 431,490.6	
Photosensitivity						
(+) = 13 (13)	2,368.7 ± 1,072.5	0.159	1,160.6 ± 1,414.2	0.236	491,065.2 ± 830,429.8	0.42
(–) = 236 (87)	1,864.2 ± 1,260.6		1,981.6 ± 2,268.2		265,009 ± 449,282.8	
Arthritis						
(+) = 134 (61)	1,972.4 ± 1,281.1	0.268	2,226.6 ± 2,503.5	**0.038**	253,206.3 ± 471,825.1	0.366
(–) = 115 (39)	1,795.2 ± 1,221.8		1,619.1 ± 1,855.2		348,821.8 ± 573,424.6	
Alopecia						
(+) = 44 (29)	1,881.5 ± 1,139.4	0.947	2,026.1 ± 2,423.3	0.737	447,309.9 ± 673,524.9	0.109
(–) = 205 (71)	1,893.7 ± 1,295.1		1,911.8 ± 2,177.1		226,445.7 ± 420,262.3	
Nephritis						
(+) = 73 (29)	2,240.7 ± 1,483.4	**0.012**	2,489.6 ± 2,196.6	**0.015**	606,700.8 ± 727,196.3	**0.003**
(–) = 176 (71)	1,745.3 ± 1,119.5		1,782.1 ± 2,018.2		161,313.8 ± 320,203.3	
Serositis						
(+) = 10 (6)	2,313.1 ± 1,609.9	0.278	2,555.4 ± 3,186.4	0.403	460,210 ± 727,252.3	0.406
(–) = 239 (94)	1,872.9 ± 1,238.8		1,916.2 ± 2,197.7		279,663.6 ± 499,906.5	
CNS involvement						
(+) = 5 (1)	2,984.5 ± 1,730.3	**0.049**	1,285.3 ± 1,484	0.509	868,505.5 ± 1,214,124.3	0.617
(–) = 244 (114)	1,868.1 ± 1,237.8		1,956.5 ± 2,253.3		278,700.2 ± 496,521	
High disease activity (SLEDAI >6)						
(+) = 147 (53)	2,052.6 ± 1,326.8	**0.011**	2,231.5 ± 2,396.6	**0.02**	487,263 ± 640,283.6	**<0.001**
(–) = 102 (47)	1,659 ± 1,107.9		1,638 ± 1,483.8		68,610.5 ± 91,533.7	
SDI score ≥1						
(+) =27 (14)	2,413.2 ± 1,413.6	**0.022**	1,914.1 ± 1,389	0.845	773,569.4 ± 742,893.9	**0.015**
(–) = 222 (86)	1,827 ± 1,222.3		1,999.3 ± 2,162.5		211,856.5 ± 421,428.5	

Bold values indicate significant p-values.

SLE, systemic lupus erythematosus; CNS, central nervous system; SLEDAI, SLE disease activity index; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index.

[a]*The number of patients in S100A8 is in parentheses.*

known as calgranulin C), and acts as a homodimer, heterodimer, or heterotetramer with S100A9 to exert biological functions (29). The S100A8/A9 heterodimer, the dominant form in serum, has been observed in patients with SLE as well as in those with cardiovascular disease in SLE and active lupus nephritis and can predict responses to treatment of SLE (6, 11, 13, 30, 31). Although it is not clear whether the small amounts of S100A8 homodimers are comparable to S100A8/A9 heterodimers *in vivo*, the role of S100A8 may also be evident, given that mortality occurred in the early stages of development only in S100A8 target-destroyed mice, whereas S100A9-deficient mice were viable and fertile (32–34). Therefore, the diagnostic values of S100A8 levels in the serum, urine, and saliva were evaluated and compared in the present study.

This is the first report comparing the diagnostic efficacy of S100A8 for SLE diagnosis, and it is novel that S100A8 in urine and saliva, as well as serum, were used. In this study, the mean S100A8 levels in the serum, urine, and saliva were significantly higher in patients with SLE than in HCs. There have been no previous studies comparing patients with SLE with HCs using the homodimer of S100A8; however, a study of 37 patients with SLE showed a significant decrease in serum S100A8 levels after treatment (35). According to the AUC results, the ability of serum S100A8 to diagnose SLE was good (AUC = 0.831), and that of urine and salivary S100A8 was fair (AUC = 0.751 and 0.729, respectively). Our data demonstrated that S100A8 was superior to previous studies evaluating the diagnostic biomarker ability of S100 proteins (S100A4, S100A8/A9, and S100A12) in serum and urine for SLE (6, 13). Furthermore, combining serum S100A8 with high specificity (91.1%) and PPV (95.7%), and urine S100A8 with high sensitivity (99%) and NPV (80.6%) further increased diagnostic accuracy (data not shown).

Salivary S100A8 has been identified as a potential diagnostic biomarker for oral cavity infection or oral cancer; however, to date, no data are available regarding S100 protein expression in the saliva of patients with SLE (36, 37). In Sjogren's syndrome affecting exocrine glands, especially salivary and lacrimal glands, S100A8/A9 has been identified as a biomarker (38). S100A8/A9 levels in saliva have also been found to be higher in patients with systemic sclerosis and inflammatory bowel disease than in HCs (39, 40). In our study, salivary S100A8 concentrations in patients with SLE were significantly higher than those in HCs, and there was also a correlation with clinical indices reflecting disease activity, such as ESR and anti-dsDNA Ab. Salivary S100A8 has a high specificity of 0.911 despite its low diagnostic accuracy (AUC = 0.729; sensitivity, 0.52) compared with serum and urine, is non-invasive, and has the advantages of simple access and storage, making it an inexpensive screening tool.

Another important aspect of this study is that we also analyzed the relationship between disease activity and S100A8 levels in serum, urine, and saliva. The results showed that high S100A8 expression was correlated with low hemoglobin, high ESR, low complement, high anti-dsDNA Ab, and high proteinuria, similar to previous studies (12, 13). In addition, we found a statistically significant positive correlation between the expression of candidate biomarkers and the SLEDAI, one of the most popular and widely used indices for evaluating disease activity in SLE. All biomarkers have been proven to have an obvious association with SLEDAI; in particular, salivary S100A8 had a moderate positive correlation. Most of the recently published studies using serum and urine S100A8 reported that S100A8 concentrations increased as the disease activity of SLE increased (13, 41, 42), and only in a few studies were they not relevant (6). Considering its association with disease activity, S100A8 in serum, urine, and saliva may be efficient in detecting and monitoring disease progression in addition to diagnostic purposes.

We further investigated the correlation between clinical manifestations and S100A8 levels in serum, urine, and saliva. As expected, our data indicate a significant increase in urine S100A8 levels in lupus nephritis compared with extrarenal SLE, and intriguingly, S100A8 in serum and saliva also showed a significant increase in patients with lupus nephritis. Similar to the increase in urine S1008 in lupus nephritis in this study, another study with neuropsychiatric SLE showed an increase in S100 protein in cerebrospinal (CSF) fluid, whereas salivary S100A8 was not found to have a clear correlation with oral manifestations (43). Another noteworthy point is that the level of S100A8 was high in the serum of patients with CNS lupus, but since the sample size was small, additional studies will be needed to assert the diagnostic utility of S100A8 in CNS lupus. In addition, the S100A8 concentrations in serum and saliva showed significant differences according to the organ damage and were similar to the results of comparing the differences in S100 proteins depending on the presence of SDI scores in SLE patients with cardiovascular disease (31). The relationship between other clinical features and S100A8 levels in the serum, urine, and saliva was not consistent.

To the best of our knowledge, this is the first study to investigate the role of S100A8 protein as a diagnostic biomarker and its association with disease activity in patients with SLE using serum, urine, and saliva. It is important to analyze the S100 protein in the saliva of patients with SLE, which is a biofluid that is gradually receiving special attention, as it has been acknowledged that it contains many informative proteins about the disease process (44). Although the diagnostic ability of S100A8 that we have demonstrated is not superior to the diagnostic sensitivity and specificity of previously proposed classification criteria (45), it is worth emphasizing that we have discovered a powerful diagnostic biomarker in various biofluids. Due to the heterogeneous nature of SLE, a single diagnostic marker is not realistic; therefore, biofluid-based biomarkers will be indispensable in the future in terms of reliability, price, easy sampling, safety, and reproducibility. Another strength of our study is that the samples were collected in a consistent process by the same researcher using a cohort, and a prospective follow-up study under the same conditions for monitoring SLE disease activity is possible. Our cohort collects clinical information and biofluid samples of SLE patients annually; therefore, we will demonstrate the ability of S100A8 as a biomarker to predict the flare of SLE in future studies.

However, this study had some limitations. First, some of the samples included in the study may not have belonged to newly

diagnosed patients; thus, concomitant medications such as immunosuppressants may have affected the concentration of S100A8. Second, a cross-sectional study using a cohort sample showed a difference in the number of samples obtained, depending on the type of biofluid, and the number of saliva samples was remarkably small. Finally, saliva biology and circadian variation may have affected the salivary samples, and only unstimulated saliva was collected. Future studies using unstimulated and stimulated saliva collected at the same time will be essential to verify our findings.

In summary, our study provides insights into the potential diagnostic role of S100A8 levels in the serum, urine, and saliva of patients with SLE. Serum, urine, and salivary S100A8 levels have good diagnostic ability, and a combination of various biofluids instead of a single biomarker will demonstrate their usefulness as a robust screening tool. Moreover, these biofluid-based biomarkers will be helpful indicators for monitoring SLE disease activity and predicting treatment response.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Ajou University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-WK acquired, analyzed, and interpreted the data and drafted the work. J-YJ, S-WL, W-YB, and H-AK analyzed and interpreted the data. C-HS conceptualized and designed the work, interpreted the data, and revised the manuscript. All authors approved the submitted version.

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Glomerular Expression of S100A8 in Lupus Nephritis: An Integrated Bioinformatics Analysis

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Introduction: Lupus nephritis (LN) is a major risk factor of morbidity and mortality. Glomerular injury is associated with different pathogeneses and clinical presentations in LN patients. However, the molecular mechanisms involved are not well understood. This study aimed to explore the molecular characteristics and mechanisms of this disease using bioinformatics analysis.

Methods: To characterize glomeruli in LN, microarray datasets GSE113342 and GSE32591 were downloaded from the Gene Expression Omnibus database and analyzed to determine the differentially expressed genes (DEGs) between LN glomeruli and normal glomeruli. Functional enrichment analyses and protein–protein interaction network analyses were then performed. Module analysis was performed using the Search Tool for the Retrieval of Interacting Genes/Proteins and Cytoscape software. Immunofluorescence staining was performed to identify the glomerular expression of S100A8 in various International Society of Nephrology/Renal Pathology Society (ISN/RPS) class LN patients. The image of each glomerulus was acquired using a digital imaging system, and the green fluorescence intensity was quantified using Image-Pro Plus software.

Results: A total of 13 DEGs, consisting of 12 downregulated genes and one upregulated gene (S100A8), were identified in the microarray datasets. The functions and pathways associated with the DEGs mainly include inflammatory response, innate immune response, neutrophil chemotaxis, leukocyte migration, cell adhesion, cell–cell signaling, and infection. We also found that monocytes and activated natural killer cells were upregulated in both GSE113342 and GSE32591. Glomerular S100A8 staining was significantly enhanced compared to that in the controls, especially in class IV.

Conclusions: The DEGs identified in the present study help us understand the underlying molecular mechanisms of LN. Our results show that glomerular S100A8 expression varies in different pathological types; however, further research is required to confirm the role of S100A8 in LN.

Keywords: lupus nephritis, differentially expressed genes, microarray, immunofluorescence, S100A8

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BIOGRAPHICAL NOTE

We use a bioinformatics method to obtain the DEGs between LN glomerulus and normal glomerulus and performed immunofluorescence staining to identify the expression of S100A8 in various ISN/RPS class LN patients.

SIGNIFICANCE AND INNOVATIONS

➢Monocytes and activated NK cells were upregulated in LN glomerulus.

➢Glomerular S100A8 is different in different pathological types.

➢The glomerular S100A8 staining was obviously enhanced compared with the controls, especially in class IV.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by immune inflammation, which can affect multiple organs. It is known to affect the kidneys in approximately 50% of patients (1). Lupus nephritis (LN) is a major risk factor of morbidity and mortality. It was found that glomerular injury, including that of mesangial cells, endothelial cells, and podocytes, is associated with different pathogeneses and clinical presentations in LN patients (2). Intense efforts have been made to elucidate the pathogenesis and molecular mechanisms of LN; however, they are still not well understood (3). Therefore, it is necessary to further explore the molecular characteristics and mechanisms of the disease. To characterize glomeruli in LN using bioinformatics analysis, microarray datasets GSE113342 and GSE32591 were downloaded from the Gene Expression Omnibus (GEO) database and analyzed to determine the differentially expressed genes (DEGs) between the LN glomerulus and normal glomerulus. Functional enrichment analyses and protein–protein interaction (PPI) network analyses were then performed. Module analysis was performed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and Cytoscape software.

S100A8 was identified as one of the 13 DEGs. However, its mRNA expression was different in the abovementioned two microarray datasets. S100A8 (also known as MRP8) is a calcium-binding protein belonging to the S100 family (4). It is mainly expressed in granulocytes and mononuclear blood cells, such as neutrophils and macrophages (5). S100A8 is expressed in various autoimmune diseases, such as systemic sclerosis, psoriasis, dermatomyositis, and Sjogren's syndrome (6). Some studies have also found that serum and urine S100A8 levels are elevated in patients with LN (7, 8). To our knowledge, the glomerular expression of S100A8 in various ISN/RPS class LN patients is unknown. In this study, immunofluorescence staining and semiquantitative analysis were performed, and the relationship between glomerular expression of S100A8 and clinical data, such as disease activity or urinary protein measurements, was explored.

METHODS

Microarray Data

NCBI GEO (http://www.ncbi.nlm.nih.gov/geo) (9) is a public functional genomics data repository with high-throughput gene expression data, chips, and microarrays. We used "lupus nephritis" (keywords) to search, and there were 335 results in the GEO Database. Then, we selected *Homo sapiens*, and 301 results were left. We browsed 301 links in detail. In this study, we want to explore the DEGs between LN glomerulus and normal glomerulus. In the first step, we selected LN kidney samples. And only 5 datasets were left. They were GSE112943, GSE127797, GSE113342, GSE32591, and GSE69438. Then, only glomeruli and tubulointerstitium separated samples were what we needed, and GSE113342 and GSE32591 were selected. The two gene expression datasets GSE113342 (10) and GSE32591 (11) were downloaded. The GSE113342 dataset contained 14 LN glomerulus biopsy samples and six normal glomerulus biopsy samples. GSE32591 contained 32 LN glomerulus biopsy samples and 14 normal glomerulus biopsy samples.

Identification of Differentially Expressed Genes

The GEO2R online tool was used to identify DEGs between LN and normal glomerular biopsy samples. Log fold change (FC) >1 and P-value <0.01 were considered statistically significant. The raw data were checked online using Venn software to detect common DEGs. DEGs with log FC <0 were considered downregulated, whereas DEGs with log FC >0 were considered upregulated.

Functional Enrichment and Protein–Protein Interaction Analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.ncifcrf.gov) (12) is an online biological information database. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and biological systems from large-scale molecular datasets generated using high-throughput experimental technologies (13). Gene Ontology (GO) is a major bioinformatics tool for annotating genes and analyzing their biological processes (14). DAVID 6.8 Bioinformatics Resources was used for pathway annotations. Statistical significance was set at $P < 0.05$. A PPI network was established using the STRING (version 10.0) (15) online search tool, and an interaction with a combined score >0.4 was considered statistically significant. The PPI network was visualized using Cytoscape (version 3.7.2).

Profiling Infiltrating Immune Cells With CIBERSORT in the Glomeruli

To assess the expression changes in immune cells and obtain the proportion of various types of immune cells from the glomerulus, we used the online CIBERSORT algorithm (https://cibersort.stanford.edu/). The GSE113342 and GSE32591 series matrix txt format files were downloaded from the NCBI GEO website, and the glomerular expression data were

selected (GSM3103966, GSM3103968, GSM3103970, GSM3103972, GSM3103974, GSM3103976, GSM3103978, GSM3103980, GSM3103982, GSM3103984, GSM3103986, GSM3103988, GSM3103990, GSM3103992, GSM3103994-99, GSM807889-7934). Differences in 22 immune cells between normal and lupus glomeruli were analyzed.

Patients

All protocols were approved by the ethics committee of the Fujian Provincial Hospital. Overall, 30 LN patients with a mean age of 32.70 ± 12.17 years were included. Six types of pathological classifications (Classes II, III, IV, V, III+V, and IV+V) were used, with five patients in each pathological classification. Normal renal tissues from 5 patients with a mean age of 55.47 ± 8.82 years who underwent nephrectomy due to renal tumors were used as normal controls.

Immunofluorescence Staining of Glomerular S100A8

Renal biopsy specimens were embedded in an OCT mixture (Sakura, Hayward, CA, USA) and sliced into 5-μm frozen sections (16). The mouse anti-S100A8 antibody (Proteintech Group, Inc., Chicago, IL, USA) was used. Rabbit anti-synaptopodin antibody (Proteintech Group, Inc., Chicago, IL, USA) was used as a podocyte marker for double immunofluorescence staining. Goat anti-rabbit IgG/Alexa Fluor 594 antibody and goat anti-mouse IgG/Alexa Fluor 488 antibody (BIOGOT, Nanjing, China) were used to visualize the different proteins. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Images were captured using a fluorescence microscope (Nikon Eclipse C1, Japan). Integral optical density (IOD) and the area ratio (AR) of the positively stained area to the glomerular area were used as semiquantitative values of the expression of S100A8. We used Image-Pro Plus software (17).

Correlation of Glomerular Expression of S100A8 With Clinical and Laboratory Data

The clinical data are from the cohort used for fluorescence staining of S100A8 in our study. Thirty LN patients were used for this correlation analysis. Six types of pathological classifications (Classes II, III, IV, V, III+V, and IV+V) were used, with five patients in each pathological classification.

Statistical Analysis

SPSS (version 21.0; SPSS Inc., Chicago, IL, USA) was used to compare the differences between the two groups using the *t*-test or Mann–Whitney U test. The ratio was calculated using the χ^2 test. Spearman correlation analysis was performed between clinical data and glomerular S100A8 expression levels. Statistical significance was set at $P < 0.05$.

RESULTS

Differentially Expressed Gene Identification

From the GSE113342 dataset, 93 DEGs were successfully identified, including 53 upregulated and 40 downregulated genes. From the GSE32591 dataset, 345 DEGs, involving 97 upregulated and 248 downregulated genes, were observed. Out of all the DEGs, 13 were common between the two datasets, as shown in the Venn diagram (**Figure 1A**). These 13 DEGs discovered between the LN glomerulus and the normal glomerulus based on the two microarray datasets

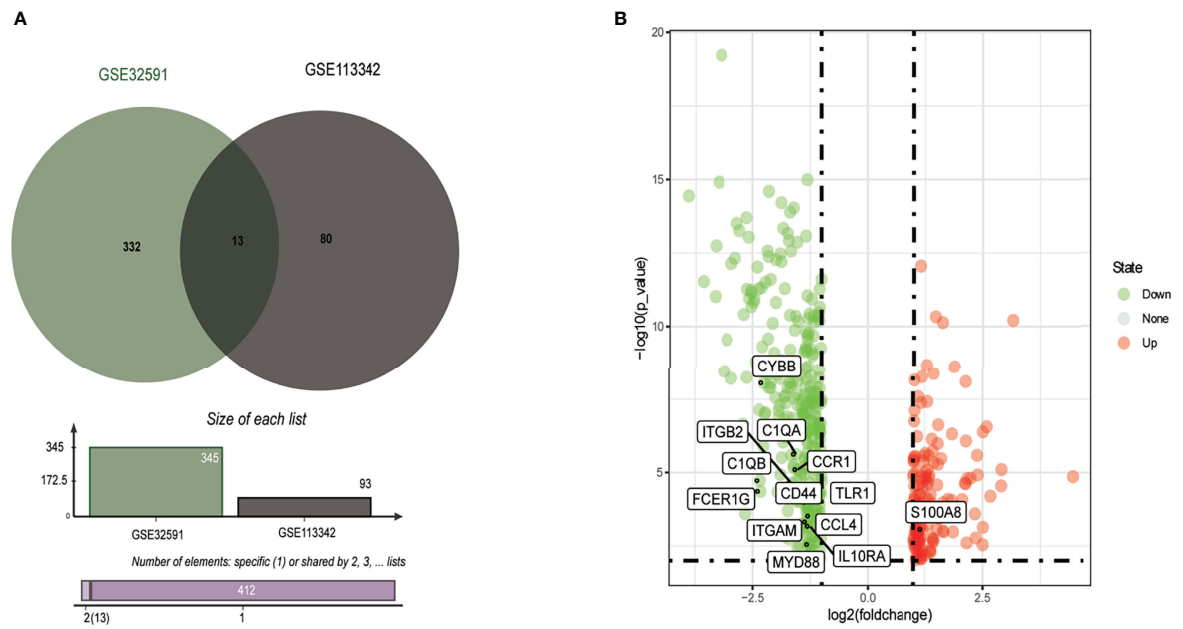

FIGURE 1 | Identification of common DEGs from GSE32591 and GSE113342 datasets. **(A)** Venn diagram of DEGs based on the two GEO datasets. **(B)** Volcano plot of the 13 DEGs. Red, upregulation; green, downregulation. DEGs, differentially expressed genes; GEO, Gene Expression Omnibus.

consisted of 12 downregulated genes and one upregulated gene (S100A8). The 13 DEGs are plotted in **Figure 1B**, where the red and green dots represent upregulated and downregulated genes, respectively.

Gene Ontology Annotation and Kyoto Encyclopedia of Genes and Genomes Pathway Enrichment Analyses

To gain deeper insight into the biological roles of these 13 DEGs, functional and pathway enrichment analyses were performed using DAVID. The enriched GO terms and KEGG pathways are shown in **Table 1** and **Figure 2**. KEGG pathway analysis revealed that the DEGs were mainly associated with infections and the Toll-like receptor (TLR) signaling pathway. GO biological process analysis indicated that the 13 DEGs were significantly associated with inflammatory response, innate immune response, neutrophil chemotaxis, leukocyte migration, cell adhesion, and cell–cell signaling. The top 3 significantly enriched terms regarding changes in cell component of DEGs were plasma membrane, extracellular exosome, and integral component of the plasma membrane. Changes in molecular function are primarily associated with protein binding.

Protein–Protein Interaction and Modular Analysis

A total of 13 DEGs were imported into the PPI network complex, comprising 13 nodes and 57 edges, including 12 downregulated genes and the upregulated gene S100A8 (**Figure 3**). We then applied Cytotype MCODE for further analysis, and the results are shown in **Figure 3B**.

TABLE 1 | GO and KEGG pathway enrichment analysis of DEGs in LN glomerulus.

	Term	Description	Count in gene set	P-value
KEGG_PATHWAY	hsa05133	Pertussis	5	4.06E-06
	hsa05152	Tuberculosis	6	4.34E-06
	hsa05150	*Staphylococcus aureus* infection	4	7.22E-05
	hsa05134	Legionellosis	3	0.003179
	hsa05140	Leishmaniasis	3	0.00544
	hsa05142	Chagas disease (American trypanosomiasis)	3	0.011392
	hsa04620	Toll-like receptor signaling pathway	3	0.011815
Biological processes (BP)	GO:0006954	Inflammatory response	7	1.05E-07
	GO:0045087	Innate immune response	7	2.21E-07
	GO:0030593	Neutrophil chemotaxis	4	1.24E-05
	GO:0050900	Leukocyte migration	4	7.85E-05
	GO:0007155	Cell adhesion	5	2.29E-04
	GO:0007267	Cell–cell signaling	4	6.80E-04
	GO:0007229	Integrin-mediated signaling pathway	3	0.002185
	GO:0019221	Cytokine-mediated signaling pathway	3	0.003787
	GO:0051092	Positive regulation of NF-kappaB transcription factor activity	3	0.003901
	GO:0042742	Defense response to bacterium	3	0.004618
	GO:0071404	Cellular response to low-density lipoprotein particle stimulus	2	0.006415
	GO:0070374	Positive regulation of ERK1 and ERK2 cascade	3	0.006655
	GO:0002523	Leukocyte migration involved in the inflammatory response	2	0.007835
	GO:0016064	Immunoglobulin-mediated immune response	2	0.007835
	GO:0042535	Positive regulation of tumor necrosis factor biosynthetic process	2	0.007835
	GO:0030198	Extracellular matrix organization	3	0.008283
	GO:0034142	Toll-like receptor 4 signaling pathway	2	0.012792
	GO:0051928	Positive regulation of calcium ion transport	2	0.018429
	GO:0002224	Toll-like receptor signaling pathway	2	0.019131
	GO:0002755	MyD88-dependent Toll-like receptor signaling pathway	2	0.023337
	GO:0031623	Receptor internalization	2	0.03031
	GO:0032755	Positive regulation of interleukin-6 production	2	0.031699
	GO:0032760	Positive regulation of tumor necrosis factor production	2	0.033086
	GO:0006955	Immune response	3	0.035053
	GO:0070098	Chemokine-mediated signaling pathway	2	0.049591
Cell component (CC)	GO:0005886	Plasma membrane	10	1.71E-04
	GO:0005602	Complement component C1 complex	2	0.001317
	GO:0009986	Cell surface	4	0.00471
	GO:0005887	Integral component of plasma membrane	5	0.010799
	GO:0008305	Integrin complex	2	0.01764
	GO:0070062	Extracellular exosome	6	0.026716
	GO:0030670	Phagocytic vesicle membrane	2	0.038177
Molecular function (MF)	GO:0005515	Protein binding	13	3.93E-04
	GO:0046982	Protein heterodimerization activity	4	0.003795
	GO:0004872	Receptor activity	3	0.009973
	GO:0001948	Glycoprotein binding	2	0.045254

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; LN, lupus nephritis; ERK, extracellular regulated protein kinases.

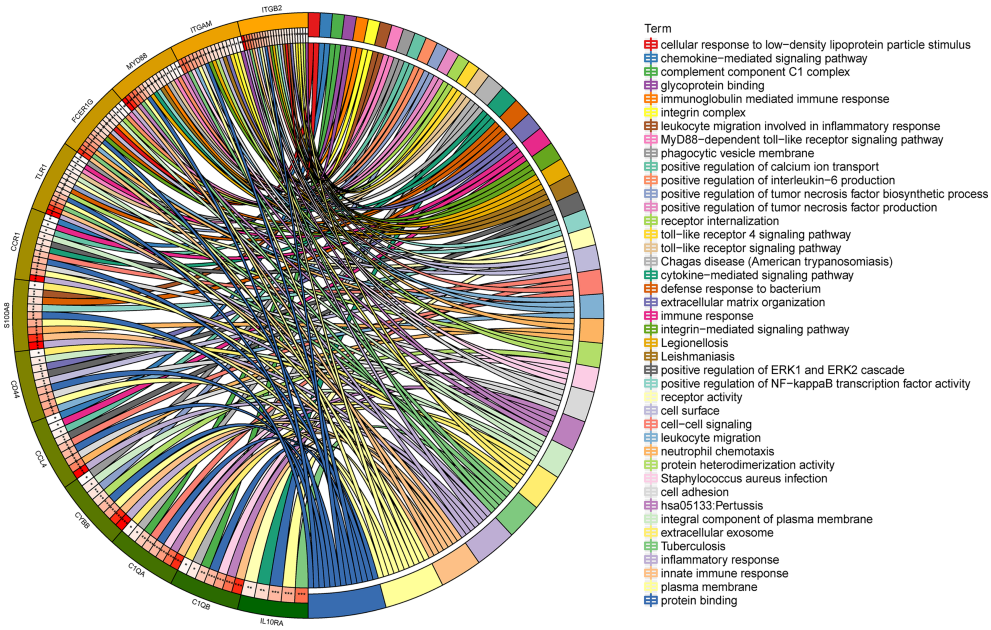

FIGURE 2 | Distribution of integrated DEGs in LN glomerulus for different enriched functions. The DEG enrichment of BP, MF, CC, and KEGG pathways ($P < 0.05$).

Infiltrating Immune Cells in the Glomeruli

We can conclude that monocytes (GSE32591) and eosinophils (GSE113342) accounted for the majority of all infiltrating cells in the glomerulus, especially in patients with LN. The differential expression proportion of immune-infiltrating cells in the LN and normal groups is shown in **Figures 4**, **5**. Based on analyses of the GSE32591 database, we found that memory B cells, follicular helper T cells, regulatory T cells (Tregs), and resting natural killer (NK) cells were downregulated in LN glomeruli. In addition, monocytes and activated NK cells were upregulated. Analyses of the GSE113342 database revealed that naive B cells, plasma cells, and resting mast cells were downregulated, and monocytes and activated NK cells were upregulated, similar to the observations from GSE32591. In addition, activated mast cells and eosinophils were also upregulated.

Clinical and Laboratory Information of the Lupus Nephritis Patients

No significant age- or sex-dependent differences were found among the renal biopsies of different LN groups. There were some differences in Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), Creatinine (Cr), Blood Urea Nitrogen (BUN), C3, C4, and Albumin (Alb) among various ISN/RPS class LN patients. In patients with LN,

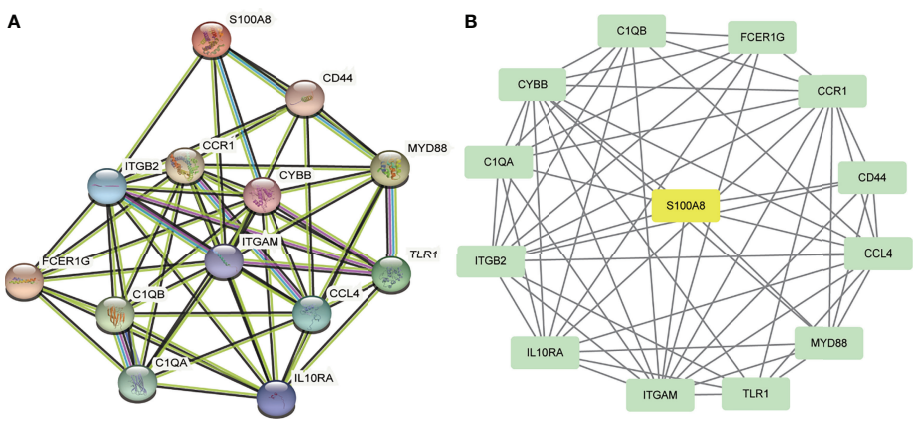

FIGURE 3 | PPI network and the significant module of DEGs. **(A)** The PPI network of DEGs. **(B)** The significant module was obtained from PPI network constructed using Cytoscape with 13 nodes and 57 edges. S100A8 is marked in yellow, and downregulated genes are marked in green.

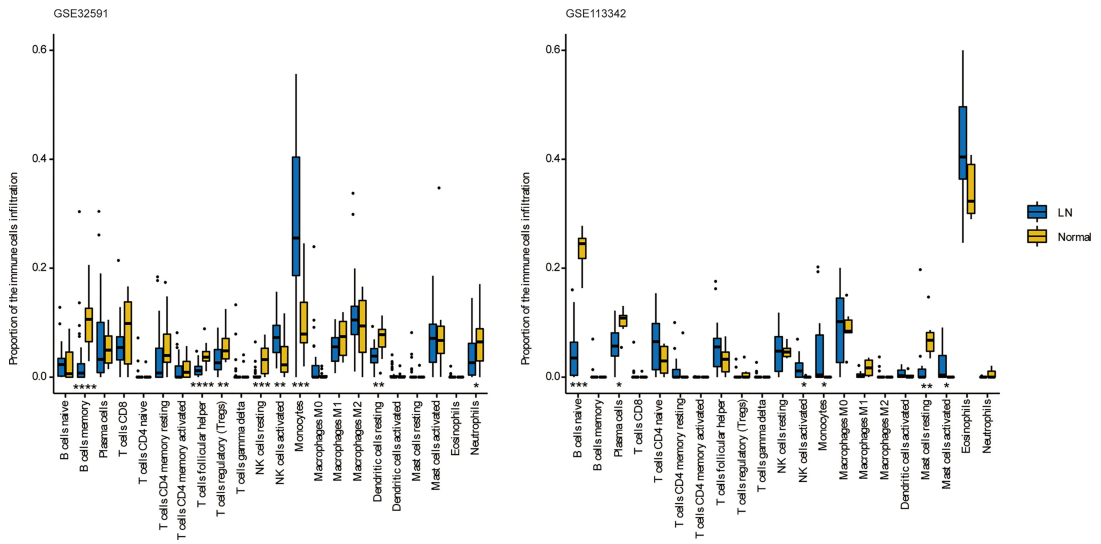

FIGURE 4 | The differences of 22 immune cells between normal and lupus glomeruli. Monocytes and activated NK cells were upregulated in GSE32591 and GSE113342. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

no significant difference in 24-h urinary protein measurements was found. The details are presented in **Table 2**.

Glomerular Expression of S100A8 in Various ISN/RPS Class Lupus Nephritis Patients

Using immunofluorescence microscopy, the glomerular staining of S100A8 in the controls was found to be weak, and the staining in patients with classes II and V was similar to that of the controls. Glomerular staining was markedly enhanced in Class IV. We found the S100A8 proteins to be distributed throughout the glomerulus, and S100A8 did not colocalize with the podocyte marker synaptopodin (**Figure 6**).

We conducted a semiquantitative analysis and found a significant increase in IOD and AR in LN compared with that of the controls. However, no significant differences were found in the glomerular expression of S100A8 between the control and class II groups or control and class V groups (**Tables 2**, **3**).

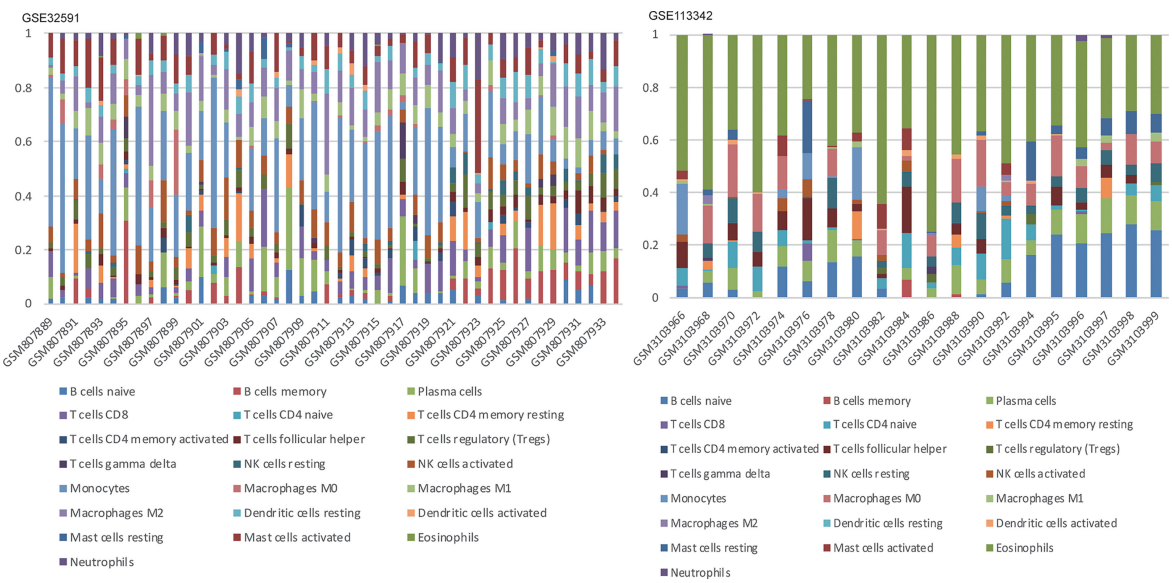

FIGURE 5 | Stacked bar charts of 22 immune cell proportions in the glomeruli. In GSE32591, monocytes accounted for the majority of all infiltrating cells in the glomeruli. While in GSE113342, eosinophils are the majority.

TABLE 2 | Clinical and laboratory information of the LN patients.

Group	Control	LN patients	Class II	Class III	Class IV	Class V	Class III+V	Class IV+V	P-value (Control vs. LN)	P-value (Among LN)
Age (years)	55.47 ± 8.82	32.70 ± 12.17	31.60 ± 11.89	36.9 ± 17.61	31.00 ± 12.75	35.40 ± 11.15	33.40 ± 12.08	30.80 ± 11.97	<0.01	0.05
Sex (F/M)	10/5	23/7	3/2	3/2	4/1	5/0	3/2	5/0	0.49	0.44
SLEDAI	NA	18.57 ± 4.44	14.40 ± 2.97	15.8 ± 2.68	20.4 ± 2.97	17.2 ± 4.15	22.4 ± 3.36	21.2 ± 5.02	NA	0.009
Cr (μmol/L)	75.27 ± 21.30	75.50 ± 72.43	52.00 ± 10.89	64.00 ± 10.08	69.80 ± 10.46	134.20 ± 175.51	56.00 ± 14.25	77.00 ± 31.96	0.99	0.04
BUN (mmol/L)	4.89 ± 0.86	7.00 ± 4.83	5.38 ± 1.53	7.20 ± 1.60	4.92 ± 0.64	8.70 ± 8.47	3.88 ± 0.90	11.90 ± 6.04	0.03	<0.01
24-h UTP or Urine protein (g/24 h)	All negative	1.66 ± 1.71	0.95 ± 1.10	0.70 ± 0.47	1.53 ± 0.65	2.18 ± 1.57	2.16 ± 1.75	2.40 ± 3.25	<0.01	0.54
C3 (g/L)	NA	0.52 ± 0.09	0.53 ± 0.24	0.68 ± 0.37	0.29 ± 0.14	0.89 ± 0.35	0.36 ± 0.16	0.37 ± 0.09	NA	0.007
C4 (g/L)	NA	0.09 ± 0.07	0.08 ± 0.04	0.11 ± 0.08	0.07 ± 0.05	0.18 ± 0.13	0.05 ± 0.03	0.06 ± 0.03	NA	0.06
Alb (g/L)	NA	32.33 ± 6.58	38.20 ± 4.15	33.20 ± 7.40	26.60 ± 7.40	35.80 ± 3.70	31.60 ± 2.61	28.60 ± 6.95	NA	0.04
Glomerular S100A8-AR	0.002 (0.001, 0.005)	0.010 (0.002, 0.028)	0.002 (0.001, 0.005)	0.026 (0.020, 0.075)	0.059 (0.035, 0.107)	0.003 (0.000, 0.003)	0.013 (0.006, 0.033)	0.018 (0.008, 0.034)	0.002	<0.01
Glomerular S100A8-IOD	5.967 (2.149, 16.933)	24.805 (5.647, 87.4068)	6.982 (2.161, 13.752)	82.603 (20.480, 147.444)	227.417 (133.910, 407.012)	1.027 (0.000, 10.880)	39.839 (14.546, 90.071)	62.562 (20.069, 100.833)	0.006	<0.01

Correlation of Glomerular Expression of S100A8 With Clinical and Laboratory Data

The IOD of S100A8 positively correlated with the AR of S100A8. However, the IOD and AR of S100A8 did not correlate with clinical and laboratory data. Correlations were observed among anti-ds DNA, SLEDAI, Cr, BUN, C3, C4, Alb, and 24-h urinary total protein (UTP) in various ISN/RPS class LN patients. The details are presented in **Figure 7**.

DISCUSSION

In this study, we used bioinformatics analysis to identify 13 DEGs (CYBB, C1QA, C1QB, ITGB2, ITGAM, IL10RA, TLR1, MYD88, CCL4, CD44, CCR1, FCER1G, and S100A8) that were common between LN and normal glomeruli based on gene expression profiles obtained from the GSE113342 and GSE32591 datasets.

Proteins expressed by these genes are distributed in a variety of inflammatory cells. They are also chemokine receptors for inflammatory cells (18–28). Through enrichment analysis, we found that these genes were mainly related to the inflammatory response, innate immune response, neutrophil chemotaxis, leukocyte migration, cell adhesion, and cell–cell signaling. These factors are closely related to the pathogenesis of LN.

We also observed differential expression of immune cells, including T cells, B cells, NK cells, and macrophages, in the LN and normal groups. Studies have reported that humoral and cellular immunity is involved in the pathogenesis of LN (29). A variety of autoantibodies that form immune complexes are deposited in the glomerulus, causing kidney tissue damage (30). Various immune cells can infiltrate kidney tissues. B-cell infiltration can produce many antibodies, causing kidney tissue damage and aggravating local inflammation. Activated T cells infiltrate kidney tissue and secrete cytokines, causing kidney damage. Macrophages activate a variety of signaling pathways and promote inflammation (31). They can cause glomerular mesangial matrix proliferation, innate cells, and damage to the structure or function of the kidney tissue (32). Macrophages can also release a large number of chemical and inflammatory mediators that aggravate kidney damage (33).

In this study, we found that S100A8 was differentially regulated in the above two microarray datasets between LN and normal glomeruli. Using immunofluorescence staining, we found that S100A8 levels in the controls were weak. Glomerular staining was markedly enhanced compared to that in the controls, especially in class IV. Protein S100A8 belongs to the calcium-binding S100 protein family and has gained considerable interest as a critical modulator of inflammatory response after its cellular release (34). Basic and clinical studies have suggested a potential link between S100A8 and LN (7, 8). Consistent with our research, Frosch et al. (35) reported that the expression pattern of S100A8 markedly differed between the glomeruli and interstitium in LN. S100A8 expression was significantly increased in the interstitium, paralleling the findings in glomeruli. Intrarenal S100A8 expression is increased in refractory patients with ISN/RPS class III/IV LN (36). Davies et al. (37) found that serum and urine S100A8 levels were elevated in

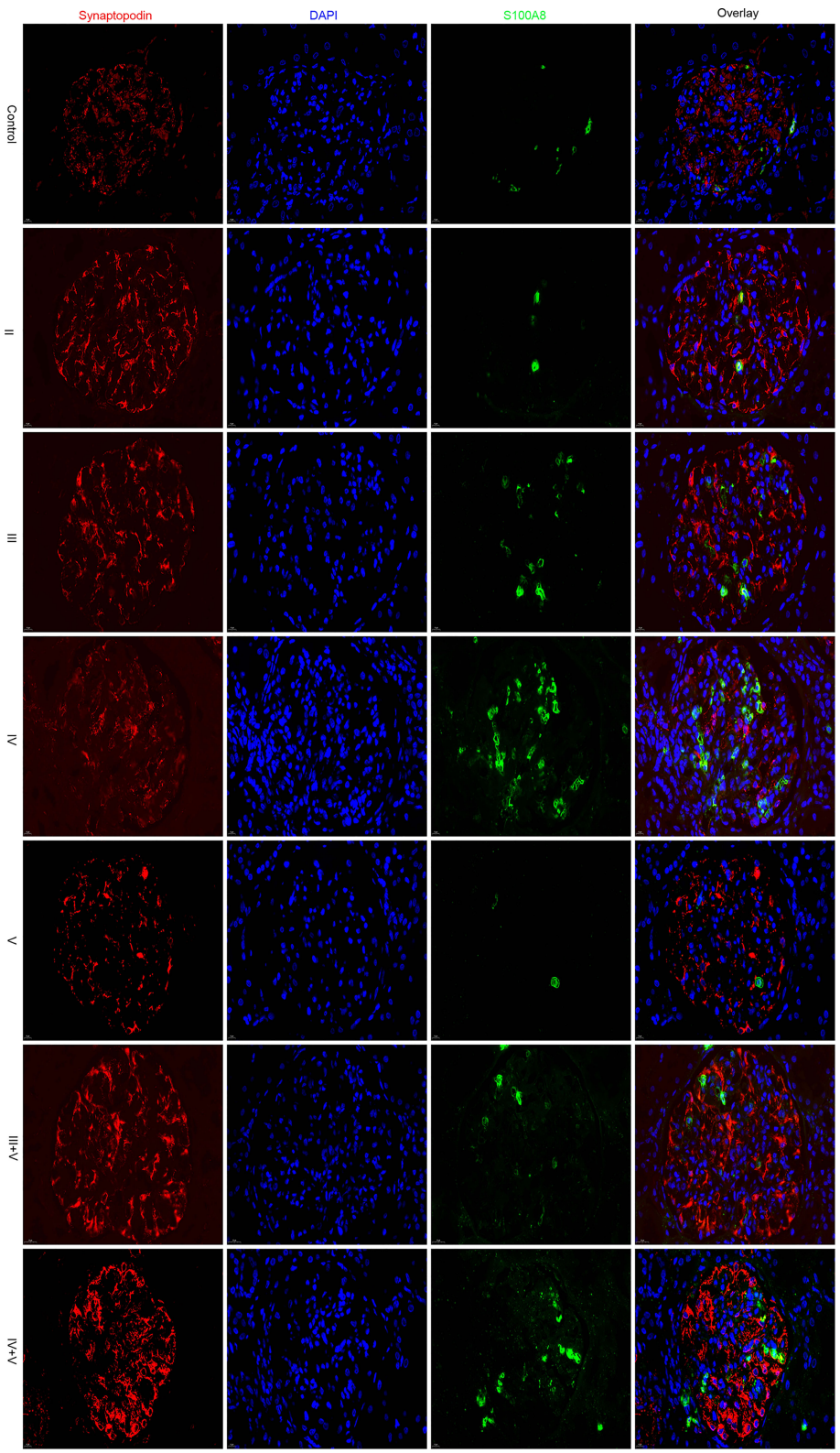

FIGURE 6 | Glomerular expression of S100A8 in various ISN/RPS class LN patients.

TABLE 3 | Glomerular expression of S100A8 in various ISN/RPS class LN patients.

Glomerular S100A8-AR/IOD P value	Control	LN patients	Class II	Class III	Class IV	Class V	Class III+V	Class IV+V
Control	0.002 (0.001, 0.005)/ 5.967 (2.149, 16.933)	0.002/0.006	0.955/0.966	<0.001/0.001	<0.001/<0.001	0.856/0.916	0.002/0.013	<0.001/<0.001
LN patients	0.002/0.006	0.010 (0.002, 0.028)/ 24.805 (5.647, 87.4068)	<0.001/<0.001	0.003/0.387	<0.001/<0.001	0.001/0.005	0.988/0.693	0.601/0.312
Class II	0.955/0.966	<0.001/<0.001	0.002 (0.001, 0.005)/ 6.982 (2.161, 13.752)	<0.001/0.001	<0.001/<0.001	0.814/0.884	<0.001/0.015	<0.001/<0.001
Class III	<0.001/0.001	0.003/0.387	<0.001/0.001	0.026 (0.020, 0.075)/ 82.603 (20.480, 147.444)	<0.001/<0.001	<0.001/0.001	<0.001/0.218	<0.001/0.887
Class IV	<0.001/<0.001	<0.001/<0.001	<0.001/<0.001	<0.001/<0.001	0.059 (0.035, 0.107)/ 227.417 (133.910, 407.012)	<0.001/<0.001	<0.001/<0.001	<0.001/<0.001
Class V	0.856/0.916	0.001/0.005	0.814/0.884	<0.001/0.001	<0.001/<0.001	0.003 (0.000, 0.003)/ 1.027 (0.000, 10.880)	<0.001/0.016	<0.001/<0.001
Class III+V	0.002/0.013	0.988/0.693	<0.001/0.015	<0.001/0.218	<0.001/<0.001	<0.001/0.016	0.013 (0.006, 0.033)/ 39.839 (14.546, 90.071)	0.632/0.208
Class IV+V	<0.001/<0.001	0.601/0.312	<0.001/<0.001	<0.001/0.887	<0.001/<0.001	<0.001/<0.001	0.632/0.208	0.018 (0.008, 0.034)/ 62.562 (20.069, 100.833)

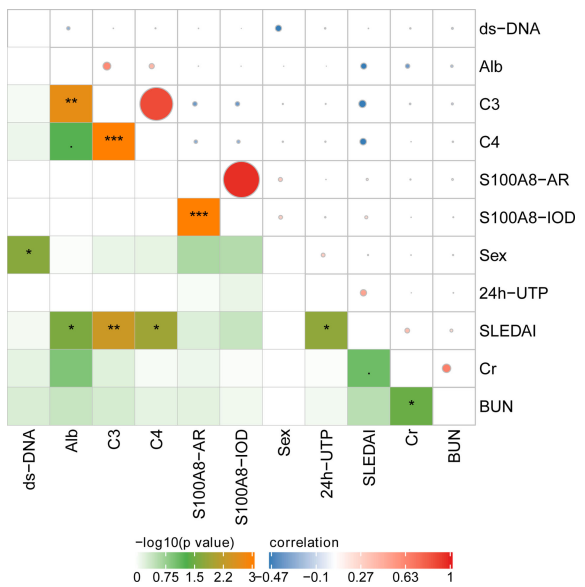

FIGURE 7 | The correlation of glomerular expression of S100A8 with clinical and laboratory data. *P < 0.05; **P < 0.01;***P < 0.001.

patients with SLE, and the urine/serum ratios were elevated in patients with active LN. Tantivitayakul et al. (38) detected S100A8 in infiltrating cells of glomeruli and peritubular capillaries.

Macrophage infiltration is associated with the severity of the inflammatory response, and macrophages express a large amount of S100A8, which participates in the pathogenesis of LN. Staining of S100A8 in patients with classes II and V was similar to that of the controls and was enhanced in classes III, IV, III+V, and IV+V. Therefore, we speculate that the pathogenesis of S100A8 varies in different pathological types. Unfortunately, we did not find a relationship between S100A8 levels and clinical or laboratory data. However, the exact mechanisms of pathogenesis remain unclear. Further research is required to confirm the role of S100A8 in LN. One study reported that S100A8 could be a promising therapeutic target for myocardial ischemia–reperfusion injury (39). This is an important question that needs to be explored in future research.

CONCLUSIONS

We used bioinformatics to determine the DEGs between the LN glomerulus and normal glomerulus. Immunofluorescence staining was used to identify the expression level of S100A8 in various ISN/RPS classes of LN. We found that the number of monocytes and activated NK cells were upregulated in the LN glomeruli, and the glomerular S100A8 level differed in different pathological types. Glomerular S100A8 staining was markedly increased in LN glomeruli compared to that in the controls, especially in class IV. Our results indicate that S100A8 participates in the pathogenesis of LN, and the precise mechanisms of this process need to be explored in our follow-up research.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Fujian Provincial Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WQ collected and analyzed clinical data and drafted the article. PM did immunofluorescence staining. YQ and GF helped WQ to collect and interpret data for the work. CZ and WZ designed this topic and approved the final version of manuscript. LH revised the manuscript carefully.

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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.2022.843576/full#supplementary-material

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

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CD11c^{+} T-bet^{+} CD21^{hi} B Cells Are Negatively Associated With Renal Impairment in Systemic Lupus Erythematosus and Act as a Marker for Nephritis Remission

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Lupus nephritis (LN) is one of the most common manifestations of systemic lupus erythematosus (SLE), characterized by abnormal B cell activation and differentiation to memory or plasma effector cells. However, the role of these cells in the pathogenesis of LN is not fully understood, as well as the effect of induction therapy on B cell subsets, possibly associated with this manifestation, like aged-associated B cells (ABCs). Consequently, we analyzed the molecules defining the ABCs subpopulation (CD11c, T-bet, and CD21) through flow cytometry of blood samples from patients with lupus presenting or not LN, following up a small sub-cohort after six months of induction therapy. The frequency of ABCs resulted higher in LN patients compared to healthy subjects. Unexpectedly, we identified a robust reduction of a CD21^{hi} subset that was almost specific to LN patients. Moreover, several clinical and laboratory lupus features showed strong and significant correlations with this undefined B cell subpopulation. Finally, it was observed that the induction therapy affected not only the frequencies of ABCs and CD21^{hi} subsets but also the phenotype of the CD21^{hi} subset that expressed a higher density of CXCR5. Collectively, our results suggest that ABCs, and more importantly the CD21^{hi} subset, may work to assess therapeutic response since the reduced frequency of CD21^{hi} cells could be associated with the onset of LN.

Keywords: B cells, aged-associated B cells, systemic lupus erythematosus, lupus nephritis, induction theraphy

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by a deleterious immune response that affects several tissues and organs, including kidneys. Lupus nephritis (LN) is one of the most common manifestations of this chronic disease, constituting a poor prognosis feature that increases morbidity and mortality (1–3). Over the years, different factors that contribute to LN pathogenesis have been identified being one of the most relevant, the deleterious function of B cells, as it generates hyper-reactive plasma cells that produce autoantibodies that can cause permanent damage within the glomerular, vascular, and tubulo-interstitial compartments of the kidneys, leading to acute or chronic renal failure (4). Nevertheless, the role of B cells in SLE beyond their differentiation to plasma cells and subsequent autoantibodies secretion is still unclear (5).

Analyzing specific circulating B cell subsets to identify alterations in their frequency or phenotype is one of the main approaches that would allow identifying possible roles of this lymphocyte lineage and their specific subpopulations in autoimmune diseases (6). Accordingly, the discovery and characterization of the Aged-associated B Cells (ABCs) subset has been relevant by its association with pro-inflammatory contexts. ABCs have been found numerically altered in conditions of chronic inflammation that encompass autoimmune diseases (SLE, rheumatoid arthritis, systemic sclerosis, etc.) or chronic infection diseases (HIV infection, malaria, hepatitis C virus infection, etc.) (7–10). This rare B cell subset has been defined by the expression of the α-integrin subunit CD11c and the transcription factor T-bet, as well as a low-level expression of the complement receptor type 2 (CR2)/CD21 (11). Regarding their functional properties, ABCs are highly responsive to innate stimuli such as TLR-7 ligands; they may produce inflammatory cytokines (including TNF-α and IFNγ) and block the generation of conventional B cells progenitors by inducing apoptosis through the expression of high levels of surrogate light chain (11–13). Furthermore, upon activation, these cells can rapidly expand, differentiate and produce antibodies (mainly IgG); in fact, higher frequencies of circulating ABCs have been correlated with elevated titers of autoantibodies in patients with SLE (14).

Although ABCs features are particularly well described in mouse models, there are many unsolved questions about their implication in human autoimmune diseases, including SLE, particularly how they are linked to the evolution of the disease, their impact on patients' treatments, or if alterations in its phenotype could be used as prognostic markers. Consequently, we analyzed the ABCs subset and similar B cell phenotypes in cohorts of SLE patients exhibiting or not LN. We found that patients with LN present low or null frequencies of the atypical "ABC-like" $CD11c^{+}T\text{-}bet^{+}CD21^{hi}$ B cell subset, in contrast with healthy individuals and SLE patients without LN. This subpopulation rendered significant correlations with different features of SLE. Interestingly, both ABCs and $CD21^{hi}$ subsets presented alterations in kidney disease patients after induction therapy, making them attractive candidates to constitute markers that could define patients' status or prognosis in LN onset.

Abbreviations: ABC's, Aged-associated B Cells; CR2 (CD21), Complement Receptor 2; DN, Double Negative B cells; FMO, Fluorescence Minus One; Lk, Leukocyte; Lt, Lymphocyte; Nt, Neutrophil; PBMCs, Peripheral blood mononuclear cells; LN, Lupus Nephritis; SLE, Systemic lupus erythematosus; TLR's, Toll-like receptors; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; eGFR, estimated Glomerular Filtration Rate; α-dsDNA, auto-antibody anti-double strand DNA; MMF, Mycophenolate Mofetil; CYC, cyclophosphamide; ISN/RPS, International Society of Nephrology/Renal Pathology Society; ACR, American College of Rheumatology; gMFI, geometric Mean Fluorescence Intensity; CVID, Common Variable Immunodeficiency; RA, Rheumatoid Arthritis; HCV, Hepatitis C Virus.

METHODS

Patients and Healthy Individuals

We analyzed blood samples from a cohort of 10 patients with SLE without LN (non-LN) in the 5 previous years to recruitment and 17 patients with active LN. They were followed-up in a tertiary care center (Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán in Mexico City, Mexico). All patients without lupus nephritis were confirmed by laboratory and clinical features. All SLE patients fulfilled ACR and SLICC classification criteria for SLE (15, 16). LN was confirmed by a renal biopsy and classified by glomerular disease type using the criteria of the International Society of Nephrology/Renal Pathology Society (ISN/RPS) (17). Notably, the first blood sample of these patients was obtained in a period no longer than three weeks after renal biopsy. Moreover, we included a cohort of 10 age-matched healthy individuals as controls. Exclusion criteria were applied to cohorts with any acute or chronic infection, pregnancy, puerperium, or neoplasia. None of the study participants received any B cell-depleting or other biological therapies. The main demographic and clinical characteristics of all these individuals are depicted in **Table 1**. **Supplementary Table 1** shows that LN patients' groups, either followed or not followed up, do not display significant baseline differences in their clinical characteristics. Additionally, **Supplementary Table 2** displays the immunosuppressants and dosage administered for induction therapy of LN patients.

All recruited patients and healthy individuals signed informed consent before their inclusion. The institutional ethics and research committees of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán approved the study (Ref. 2555) in compliance with the Helsinki declaration.

Multiparametric Flow Cytometry Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradients with Ficoll-Paque (GE Healthcare Life Sciences). Recovered cells were resuspended in RPMI-1640 with phenol red (Gibco), counted, then washed in Cell Staining Buffer (BioLegend) and treated with a human FcX blocker antibody mix (BioLegend®) for 10 minutes and performed the viability staining using Zombie UV (BioLegend) according to manufacturer's instructions. Cells were then immediately stained with the following conjugated monoclonal antibodies: anti-human CD19 Pacific Blue (BioLegend), CD11c PE

TABLE 1 | Demographics, clinical and laboratory features of SLE/LN patients.

Features	SLE non-LN	LN month 0	LN month 6
Gender - #			
Male	2	7	4
Female	8	10	5
Age in years – median	30 (28-32)	26 (20-33)	22 (20-39)
Disease Activity - median			
SLEDAI score (min.-max.)	2 (0-6)	22 (16-35)	8 (2-12)
Laboratory Values - median			
White blood cell count/mL [10^6]	5.4 (4.1-5.82)	6.5 (5.45-9.75)	8.0 (4.7-8.8)
Absolute lymphocyte count/mL [10^6]	1.32 (1.00-1.62)	0.75 (0.48-1.07)	1.12 (0.70-1.74)
Monocytes, %	7.3 (6.8-8.4)	6.3 (4.6-8.8)	8.7 (7.6-9.5)
Neutrophils, %	62.5 (50.7-71.8)	81.1 (74.8-89.1)	72 (64.9-78.9)
Platelet count, K/μL	205 (180-269)	167 (91.5-228)	271 (214-326)
B cells, %	14.7 (10.91-23.78)	22.3 (17.75-24.95)	5.4 (5.05-8.69)
Creatinine, mg/dL	0.6 (0.5-0.7)	2.2 (1.4-3.9)	0.9 (0.6-1.1)
eGFR, mL/min	119 (112-159)	35.4 (27.9-54.8)	107 (74-127)
C3, mg/dL	110 (88.0-118.8)	53 (40.5-60.5)	99 (90.5-120.5)
C4, mg/dL	23.5 (16.7-26.7)	8 (8-12.5)	30 (14.5-38.5)
Anti-dsDNA (UI/mL)	30 (8.3-62.0)	481 (128.1-688.1)	7.9 (4.4-56.9)
Treatments – #			
Mycophenolate Mofetil	1	8	5
Cyclophosphamide	–	9	4
Prednisone	2	17	9
Hydroxychloroquine	6	10	6
Chloroquine	1	4	2
Azathioprine	2	1	–
Classification of Lupus Nephritis by ISN/RPS			
Class IV	–	2	1
Class III+V	–	2	1
Class IV+V	–	13	7
Outcomes - #			
No Remission			2
Partial Remission	–	–	4
Complete Remission	–	–	3
Healthy individuals			
Male		4	
Female		5	
Age in years – median		28 (25-35)	
White blood cell count/mL [10^6]		4.2 (4.1-5.1)	
Absolute lymphocyte count/mL [10^6]		0.81 (0.64-0.92)	
B cells, %		7.6 (5.04-11.25)	

Data presented are the median (IQR), except for SLEDAI score presented as median (min.-max. values). # represents the number of individuals.

(BioLegend), CD21 APC-Fire (BioLegend), CD183/CXCR3 BV605 (BioLegend), CD185/CXCR5 APC (BioLegend), CD197/CCR7 PE-Dazzle 594 (BioLegend) for cell surface detection and anti-T-bet BV711 (BioLegend) for intracellular detection. For surface staining, cells were incubated for 30 minutes at 4°C with the antibody cocktail. After washing the cells, we followed the protocol of True-Nuclear™ Transcription Buffer Set (BioLegend). We then fixed samples with True-Nuclear™ 1X fix buffer and incubated them at room temperature in the dark for 60 minutes. Later, we washed cells two times with True-Nuclear™ 1X perm buffer at 1,500 rpm for 5 minutes, then resuspended the cell pellet in True-Nuclear™ 1X Perm buffer and added the conjugated monoclonal antibody anti-T-bet (BioLegend) and incubated at room temperature in the dark for 30 minutes. Lastly, cells were washed once with cell staining buffer (BioLegend) and then resuspended in 300 μL of the same buffer for immediate flow cytometric analysis on a BD LSRFortessa using FACSDiva software (BD Biosciences).

Up to $1x10^6$ events (cells) were analyzed using FlowJo v10 software (BD Biosciences) with the strategy shown in **Figure 1A**, developed by using Fluorescence Minus One (FMO) controls to define gates plus CompBeads (BD Biosciences) and single stained fluorescent samples to achieve compensation.

Statistical Analysis

Statistical differences in B cell subsets frequencies between cohorts were assessed by Kruskal-Wallis, followed by Dunn's *post-hoc* and the Wilcoxon tests. Statistical differences in baseline characteristics between subgroups of LN patients were evaluated by a U-Mann-Whitney test. Comparative analyses of ABCs and $CD21^{hi}$ cell counts regarding employed therapies in LN patients were also performed by a U-Mann-Whitney test. Correlation between B cell subsets frequencies and clinical or laboratory features were evaluated with Spearman's test. Finally, correlations between ABCs or $CD21^{hi}$ cell absolute numbers with the dosage of induction therapy in LN patients were

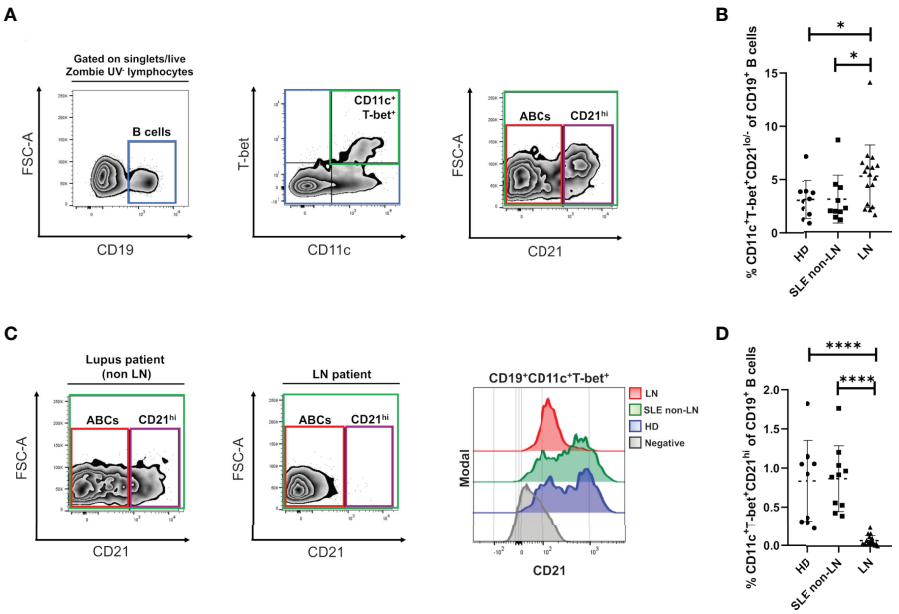

FIGURE 1 | Alterations of ABCs and CD21^{hi} B cell subsets in lupus nephritis. **(A)** Gating strategy for the identification of the indicated B cell subsets in PBMCs, selected from singlets (FSC-A vs. FSC-H), lymphocytes (SSC-A vs. FSC-A) and live Zombie UV^{-} gates. We then selected the CD19 positive cells, to gate over the double positive cells for T-bet and CD11c. Lastly, we segregate these cells by CD21 expression into CD21$^{lo/-}$ (ABCs) and CD21^{hi}. To depict this strategy, we present the data obtained from a representative healthy control. **(B)** Comparative analysis of ABCs frequencies (relative to CD19^{+} B cells) between cohorts of healthy donors (HD), non-LN lupus patients and LN. **(C)** Left: representative zebra plots from a non-LN vs. a LN patient to show lack of the CD21^{hi} subset. Right: representative histograms to evaluate density expression of CD21 over the CD19^{+} T-bet^{+} CD11c^{+} cells. **(D)** Comparative analysis of CD21^{hi} frequencies (relative to CD19^{+} B cells) between the same cohorts. All comparative analysis were assessed by a Kruskal-Wallis test followed by a Dunn's *post-hoc* test. *p ≤ 0.05, ****p ≤ 0.0001.

assessed with Spearman's test. Prism 9 (GraphPad) and R, v. 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria; URL http://www.R-project.org/) were used to analyze and graph all data sets.

RESULTS

The Frequencies of ABC Subset Are Increased in Patients With LN

To understand the alterations of ABCs and their phenotype in the context of LN and patients' response to induction therapy, we determined these cells' frequency in peripheral blood. As mentioned before, we discriminated two CD19^{+} cell subpopulations of interest, both expressing CD11c^{+}, and T-bet^{+} but segregated by their differential expression of CR2 (CD21), thus defining a CD21$^{-/lo}$ subset (or "classical" ABCs) and a CD21^{hi} subset (**Figure 1A**) that were analyzed in three different groups: healthy individuals, patients with SLE (non-LN) and LN patients. As expected, we observed a notably increase of ABCs frequencies in patients with LN compared to healthy individuals and non-LN patients. However, healthy individuals and patients without LN, do not exhibited a significant difference between them (**Figure 1B**). These observations are supported when absolute cell numbers are assessed since ABCs show the same increase trend in LN patients (**Supplementary Figure 1A**).

High Expression of CD21 Defines a Different CD11c^{+} T-bet^{+} B Cell Subset That Is Almost Absent in LN Patients

Interestingly, using our flow cytometry approach, we observed a B cell subset that displays a CD11c^{+} and T-bet^{+} phenotype like ABCs but highly expresses the CD21 marker. We detected this CD21^{hi} subset in healthy individuals (**Figure 1A**) and lupus patients without nephropathy (**Figure 1C**, left panel) but was almost absent in LN (**Figure 1C**, middle panel) as shown in the corresponding representative plots. This observation is better shown through a representative histogram (**Figure 1C**, right panel), revealing that healthy individuals and non-LN lupus patients expressed a higher density of CD21 among CD11c^{+} T-bet^{+} B cells compared to nephropathy patients. The healthy subjects and lupus patients without nephropathy groups showed almost the same frequency mean values when all subjects were compared. In contrast, the group of LN patients displays very significant lower CD21^{hi} cell frequencies (**Figure 1D**). Again, if absolute counts are measured, CD21^{hi} cells numbers are significantly reduced in LN patients compared to the other study groups (**Supplementary Figure 1B**).

The CD21^{hi} B Cell Subset Correlates With Clinical and Laboratory Features of Disease Activity

Trying to understand the relevance of the CD21^{hi} subset, we performed correlation analysis between these cell frequencies of

non-LN/LN patients and clinical/laboratory features typically assessed in lupus patients. Interestingly, these cells showed a positive correlation with levels of complement (C3/C4) and estimated glomerular filtration rate (eGFR). In contrast, the SLE disease activity index (SLEDAI), titer of antibodies anti-dsDNA and serum creatinine concentration showed a negative correlation (**Figure 2A** and **Table 2**). In addition, we developed a correlation matrix with both subsets, in order to compare their clinical profile. We observed an opposed correlation pattern for these B cell subsets and remarkably, we noticed that the $CD21^{hi}$ subset displayed robust and highly significant correlations, better than those shown by ABCs (**Figure 2B** and **Table 2**).

ABCs Subset Decreases While $CD21^{hi}$ Subset Expands After Induction Therapy

To assess the effects of induction therapy on the ABCs and $CD21^{hi}$ compartments, their frequencies were analyzed again after 6 months of treatment. Since this study was performed during COVID19 pandemic, we were only able to follow up nine LN patients from the total cohort: three of them showed complete remission, four patients exhibited partial remission and two did not show response to treatment, according to guidelines of the European League Against Rheumatism (EULAR) and European Renal Association–European Dialysis and Transplant Association (ERA-EDTA) (2). Although induction therapy does not specifically target B cells, the frequency of ABCs in both complete and partial remission patients exhibit a decrease in this subset, however, one of the patients who showed no response indeed displayed an increase while the other a slight decrease in contrast to the patients who responded to treatment (**Figure 3A**). On the other hand, patients who exhibited partial or complete remission shows an increase in the $CD21^{hi}$ subset (**Figure 3B**). Both ABCs (**Supplementary Figure 2A**) and $CD21^{hi}$ (**Supplementary Figure 2B**) absolute cell numbers were also addressed to confirm our observations, showing the same pattern.

The $CD21^{hi}$ Subset Is Characterized by a Higher CXCR5 Expression Than the ABCs Subset

In order to further characterize the $CD21^{hi}$ subset, we measured the expression of three chemokine receptors in patients who responded to treatment (n= 7 patients). We analyzed CXCR3, CCR7, and CXCR5, which play an essential role in B cell migration in inflammatory and homeostatic conditions (18, 19). CXCR3 expression was present in both subsets and exhibited similar levels (**Figure 4A**). In contrast, CCR7 expression was detected neither in ABCs nor $CD21^{hi}$ subset (**Figure 4B**). Interestingly, the expression of CXCR5 was significantly higher in the $CD21^{hi}$ subset than the ABCs subset in all patients (**Figure 4C**). Besides this, it was evident that samples from untreated (before induction) LN patients presented very low frequencies of $CXCR5^{+}$ $CD21^{hi}$, in contrast with those taken from the same patients after they reach complete or partial remission (**Figure 4D**).

DISCUSSION

One of the main factors contributing to SLE development and its clinical manifestations are B cells. Several reports concerning these cells have shown their functional, phenotypic, or activation-related alterations. Therefore, the analyses of different B cell subsets have been useful for understanding the pathogenesis of SLE; among them, the T-bet-expressing ABCs represent one of the most intriguing examples. Previous studies in infectious or immune-mediated diseases have evaluated the T-bet transcription factor, integrin CD11c and complement receptor CD21 in these B cells, as molecules associated with aberrant cell activity. However, the relevance of these cells in the pathological, clinical, and therapeutic contexts during autoimmune disorders such as SLE is still not fully clarified.

According to our analysis of ABCs subset, the presence of these cells was detected in our three study groups. In this regard, it has been previously reported that ABCs are increased in lupus patients compared to healthy controls, with a significant difference even more marked when they develop LN (14, 20, 21). As mentioned, our cohorts do not to display that trend, as we could not detect any difference among healthy subjects and lupus patients without active lupus nephritis. Additionally, the LN group of patients only showed a moderate significant increase compared to healthy donors but no difference with non-LN lupus patients. Of course, this trend could be the result of the limited number of recruited patients in our study but also, it could suggest that ABCs are not necessarily as robust for segregating SLE and LN outcomes. Another possible explanation could be that non active disease is associated with absence of a proinflammatory state that could be the main trigger for the increase in ABCs subpopulation.

Prior reports aimed to understand the abnormal activity of B lymphocytes in lupus have detected alterations of ABCs in peripheral blood. The first report to describe a disruption in a similar B cell compartment described an incremented frequency of a $CD19^{+}$ $CD21^{lo/-}$ B cell subset in patients with common variable immunodeficiency (CVID) and SLE (22, 23). Upon this observation, several reports emerged describing and expanding this cell phenotype (including its T-bet expression), besides its association with different chronic infectious diseases and autoimmune disorders (7–10). Despite that, conflicting data about B cells' CD21 expression in autoimmunity contexts have gone unnoticed. One of the few related approaches was reported by Dash R. et al., which detected an increase in *CR2* (CD21) transcript levels in PBMCs of rheumatoid arthritis patients that negatively correlates with disease activity (24). Regarding SLE, a decrease in the frequency of total $CD19^{+}$ $CD21^{+}$ B cells was previously reported (25). In our present report, the absence of the $CD21^{hi}$ subset in LN patients contrasts with healthy individuals and non-LN patients. Accordingly, the importance of the analysis of CD21 expression can be highlighted. Considering that one of the most important features to development of SLE/LN is the abnormal function of B cells, the integration of these atypical $CD21^{hi}$ B cells to the whole disease landscape, could be relevant to understand phenotypic and functional changes of this cell lineage, thus maybe associated with LN progression.

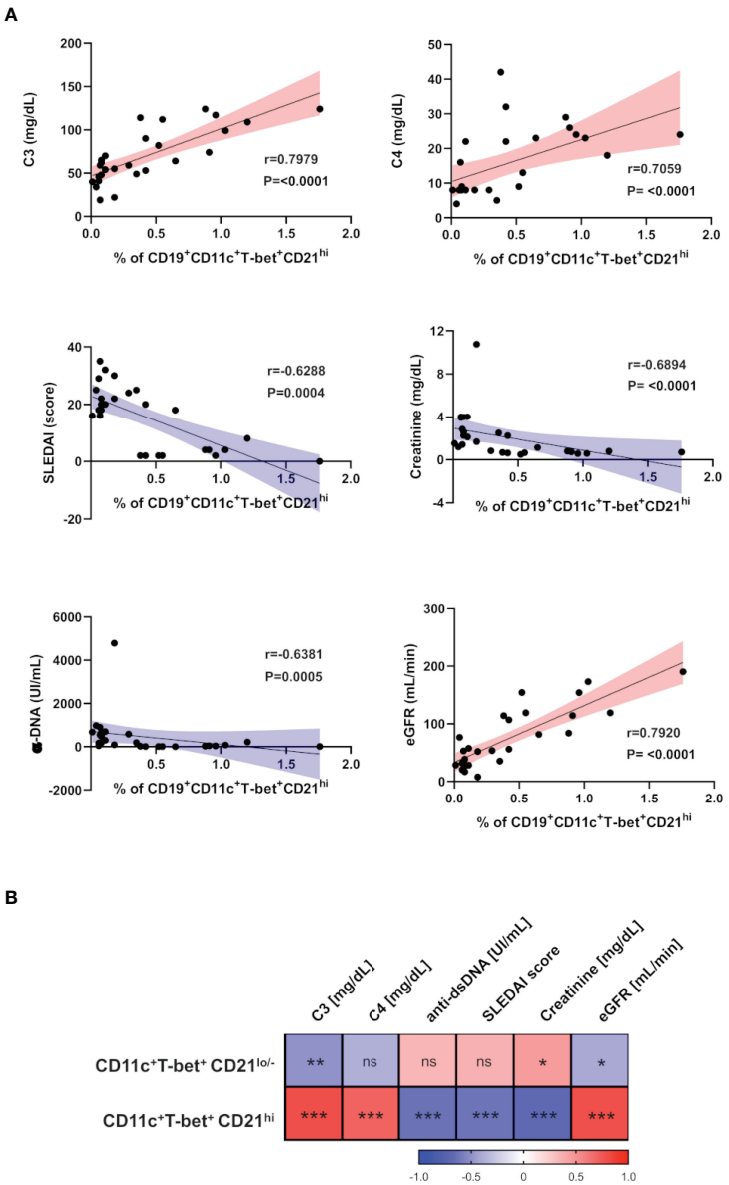

FIGURE 2 | ABCs and CD21^{hi} B cell subsets correlate with different clinical and laboratory parameters in SLE. **(A)** Correlation analysis between peripheral CD21^{hi} subset and levels of C3/C4, SLEDAI score, α-dsDNA, serum creatinine and estimated-GFR. The blue slopes (gradient) present negative correlations and red ones represent positive correlations. All graphs show calculated Spearman's coefficient (r) and p values (all significant). **(B)** Correlation matrix showing a graphical representation of calculated Spearman's coefficient calculations between the B cell subset frequencies and clinical and laboratory variables of non-LN patients (n=10) and LN patients before the induction therapy (n=17). The underlying color scale indicates Spearman's coefficient values. ns, not statistically significant. *p ≤ 0.05 **p ≤ 0.01 ***p ≤ 0.001.

ABCs have also been proposed as a good marker when following SLE patients since their numbers correlate with some clinical and laboratory features, but mainly with auto-antibody titers (14). These findings also showed that the higher frequency of ABCs implies, in most cases, an increase in the activity of the disease (14, 21). Nevertheless, these correlations are weak or not as significant as those detected in the present study for the CD21^{hi} subpopulation that seems to be more associated to an immunological state of homeostasis.

Beyond the mentioned, there are few descriptions about the effects of induction therapy in LN over B cells; some findings have shown decreased frequencies of plasmablasts/plasma cells associated with mycophenolate mofetil (MMF) administration or the selective depletion of naïve B cells in patients treated with cyclophosphamide (CYC), with little or no effect on class-switched memory B cells in both cases (5). More recently, the effects of the immunosuppressive treatment over a IgD^{-} CD27^{-} B cell subpopulation (double negative; DN) were documented in

TABLE 2 | Correlations between ABCs or CD21^{hi} cell frequencies with clinical/laboratory features of SLE/LN patients.

Features	ABCs subset		CD21^{hi} subset	
Correlation Values	**r**	**p**	**r**	**p**
SLEDAI score	0.3576	0.0671	-0.6288	0.0004
Creatinine, mg/dL	0.4624	0.0152	-0.6894	<0.0001
eGFR, ml/min	-0.4045	0.0364	0.7920	<0.0001
C3, mg/dL	-0.5002	0.0079	0.7979	<0.0001
C4, mg/dL	-0.3756	0.0535	0.7059	<0.0001
Anti-dsDNA (UI/mL)	0.3436	0.0856	-0.6381	0.0005

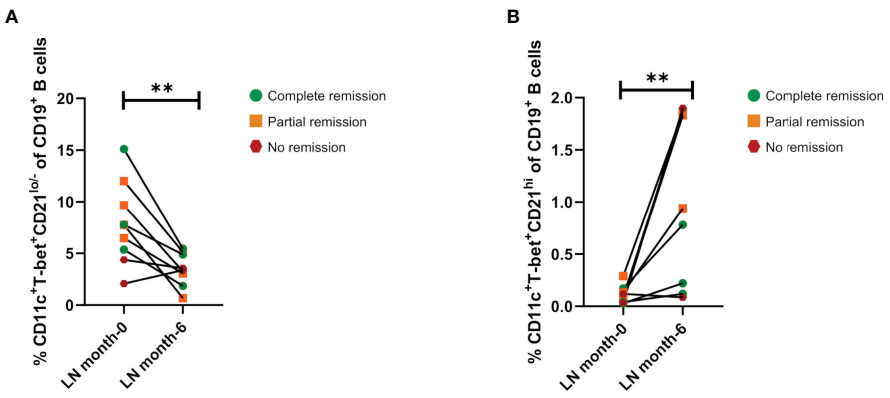

FIGURE 3 | Effect of induction therapy over frequencies of ABCs and CD21^{hi} subsets. **(A)** Comparative analysis of ABCs frequencies (relative to CD19^{+} B cells) between patients at the beginning of induction therapy (month 0), and the same patients after 6 months of treatment (n=9). **(B)** Comparative analysis of CD21^{hi} cell frequencies between patients at the beginning of induction therapy and the same patients after 6 months of treatment (n=9). Both comparative analyses were assessed by a Wilcoxon test. **p ≤ 0.01.

SLE patients with kidney damage, demonstrating a decreased frequency of these cells in patients who responded to therapy (26). To our knowledge, this is the first study about the effect of induction therapy on ABCs or similar B cell phenotypes. Trying to explain the mechanisms involved in ABCs decrease after the immunosuppressive treatment, we could mention the possible role of IL-21: a cytokine that supports the proliferation of this subset (14, 17). Since MMF therapy has been associated with the inhibition of STAT3 phosphorylation (27) and this transcription factor is linked to the stimulation pathway mediated by IL-21, it would not be surprising that MMF could be directly affecting the functionality of ABCs. However, when we analyzed the potential effects of the different immunosuppressants (MMF or CYC) or dosages employed for induction therapy of followed up LN patients in ABCs or CD21^{hi} subsets, we could not detect any significant shift in their absolute counts regarding the administered drugs (**Supplementary Table 3**) nor any significant correlation between these cell numbers and dosage of the same treatments (**Supplementary Table 4**). Therefore, we hypothesize that additional yet unknown factors, independent of the influence of therapeutics, could contribute to numeric changes in these specific B cell subsets in circulation.

On the other hand, the recovery of CD21^{hi} subset, regardless of a direct effect of induction therapy, could be due to a decrease of different CD21 ligands in circulation after treatment, that includes molecules such as IFN-α, DNA, and C3d, which are classically associated with the physiopathology of SLE (28–30). At this point, we cannot discard that CD21^{hi} cells could not represent an independent B cell subset, thus maybe constituting a transitory stage derived from ABCs. However, as we still do not understand the biological function of this subset, many questions about their identity or origins remain.

To gain insight about functional roles of these cells and considering that the expression of chemoreceptors plays an important role in the activation and maturation of B cells, we evaluated the expression of CXCR3, CCR7 and CXCR5 associated with extra-follicular/follicular pathway, to characterize the putative activation/effector sites of the CD21^{hi} population. An increase in B cells' CXCR5 expression has been demonstrated when high levels of serum CXCL13 are detected in patients with SLE and nephropathy (31–33). Conflictingly, it has been stated that higher frequencies of CXCR5^{-} CXCR3^{+} B cells correlate with active SLE, besides the presence of CXCR3^{+} B cells in human kidney tissue (18, 34, 35). Since all these reports do not perform extensive phenotyping of these cells, it is possible that our subsets of interest can be overlapped. As ABCs and the recently defined DN subset DN2 (ABCs-like phenotype) have been characterized by a high expression of CXCR3 in contrast to CXCR5 (10, 14, 21), our results regarding these cells correspond to those defining a CXCR3^{+} CXCR5$^{-/lo}$ CCR7^{-} phenotype for ABCs. On the other

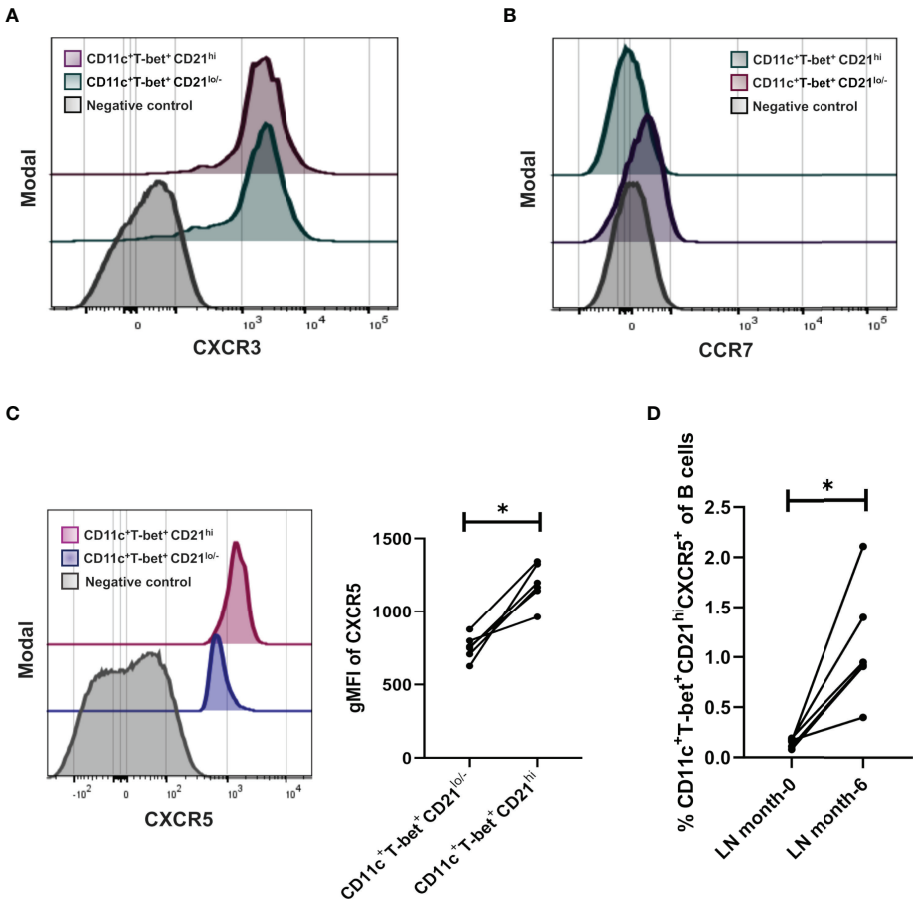

FIGURE 4 | Density expression of chemokine receptors CXCR3, CCR7 and frequencies of CXCR5 in the CD21^{hi} subset. **(A)** Representative histogram of treated LN patients (n=7 responders) to evaluate density expression of CXCR3 between ABCs and CD21^{hi} subsets. **(B)** Representative histogram of treated LN patients to evaluate density expression of CCR7 between ABCs and CD21^{hi} subsets. **(C)** Representative histogram of a treated LN patient to evaluate density expression of CXCR5 between ABCs and CD21^{hi} subsets; further comparative analysis of CD21 gMFI of the mentioned subsets. **(D)** Comparative analysis of CXCR5 frequencies over the CD21^{hi} subset between patients at the beginning of induction therapy and the same patients after 6 months treated. All comparative analysis were assessed by a Wilcoxon test. *p ≤ 0.05.

hand, the neglected CD21^{hi} subset that possesses a higher density of CXCR5 and increases in frequency in post-treatment LN patients, would represent those cells mentioned in the CXCL13/CXCR5 axis reports (31–33). Perhaps by responding to CXCL13, these lymphocytes could migrate towards tertiary lymphoid aggregates to exert putative tissue-associated functions; or maybe, this phenotype could be associated with a follicular pathway that these cells need to follow to be adequately immunologically trained as memory precursors. Accordingly, we recognize that our study is currently restricted by the limited phenotyping of these B cell subsets, that will be further deeply characterized by our group to identify a possible effector role that would promote or cease inflammatory responses.

In summary, we confirmed that ABCs increment their frequency in the circulation of LN patients, and although they cannot be discarded as a factor promoting the pathogenesis of this SLE feature, their numerical alterations could not be as robustly related to LN as those from the non-previously described CD11c^{+} T-bet^{+} CD21^{hi} cells that are almost absent when renal manifestations arise. Importantly, this CD21^{hi} subset could be used as a prognostic factor considering that their numbers strongly correlate with less activity of SLE in our cohort. Furthermore, we found that either ABCs or CD21^{hi} B cell subsets could be considered to assess response to induction therapy in LN. Finally, the identity and functional roles that CD21^{hi} cells perform during the LN onset/course must be studied considering that they may represent a divergent subset different from the already studied ABCs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics and Research Committees of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (Ref. 2555). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VS-H contributed to the design and performance of experiments, analysis, and interpretation of data. SR-R and RC-D performed experiments and analyzed data. DC-V, IN-H, LW-G, JT-R, RR-H, and DM-S assisted in obtaining, processing and preservation of patient samples. LW-G, AA-A, and AV-C recruited and collected patient data, generated, and organized our clinical database. GJ-V supports on flow cytometry analyses. VS-H and NM-D performed bioinformatics and statistical analyses. VS-H, VO-N, DG-M, and JM-M designed experiments, supervised general work, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.892241/full#supplementary-material

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Urine ALCAM, PF4 and VCAM-1 Surpass Conventional Metrics in Identifying Nephritis Disease Activity in Childhood-Onset Systemic Lupus Erythematosus

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Objectives: Serial kidney biopsy for repeat evaluation and monitoring of lupus nephritis (LN) in childhood-onset Systemic Lupus Erythematosus (cSLE) remains challenging, thus non-invasive biomarkers are needed. Here, we evaluate the performance of ten urine protein markers of diverse nature including cytokines, chemokines, and adhesion molecules in distinguishing disease activity in cSLE.

Methods: Eighty-four pediatric patients meeting ≥4 ACR criteria for SLE were prospectively enrolled for urine assay of 10 protein markers normalized to urine creatinine, namely ALCAM, cystatin-C, hemopexin, KIM-1, MCP-1, NGAL, PF-4, Timp-1, TWEAK, and VCAM-1 by ELISA. Samples from active renal (LN) and active non-renal SLE patients were obtained prior to onset/escalation of immunosuppression. SLE disease activity was evaluated using SLEDAI-2000. 59 patients had clinically-active SLE (SLEDAI score ≥4 or having a flare), of whom 29 patients (34.5%) were classified as active renal, and 30 patients (35.7%) were active non-renal. Twenty-five healthy subjects were recruited as controls.

Results: Urine concentrations of ALCAM, KIM-1, PF4 and VCAM-1 were significantly increased in active LN patients versus active non-renal SLE, inactive SLE and healthy controls. Five urine proteins differed significantly between 2 (hemopexin, NGAL, MCP1) or 3 (Cystatin-C, TWEAK) groups only, with the highest levels detected in active LN patients. Urine ALCAM, VCAM-1, PF4 and hemopexin correlated best with total SLEDAI as well as renal-SLEDAI scores ($p < 0.05$). Urine ALCAM, VCAM-1 and hemopexin outperformed conventional laboratory measures (anti-dsDNA, complement C3 and C4) in identifying concurrent SLE disease activity among patients (AUCs 0.75, 0.81, 0.81 respectively), while urine ALCAM, VCAM-1 and PF4 were the best discriminators of renal disease activity in

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cSLE (AUCs 0.83, 0.88, 0.78 respectively), surpassing conventional biomarkers, including proteinuria. Unsupervised Bayesian network analysis based on conditional probabilities re-affirmed urine ALCAM as being most predictive of active LN in cSLE patients.

Conclusion: Urinary ALCAM, PF4, and VCAM-1 are potential biomarkers for predicting kidney disease activity in cSLE and hold potential as surrogate markers of nephritis flares in these patients.

Keywords: ALCAM, VCAM 1, PF4, urine, biomarker, childhood-onset lupus

INTRODUCTION

Childhood-onset systemic lupus erythematosus (cSLE) is a complex, chronic, multisystem autoimmune disease with a significant impact on the affected child or adolescent under 18 years of age. Despite sharing similar pathogenesis with adult-onset SLE (aSLE), the clinical presentation of cSLE is generally more severe, with higher disease activity and damage, requiring more aggressive treatment (1–4). cSLE comprises approximately 15-20% of SLE cases, with a prevalence of 1.89-25.7 per 100,000 children and an annual incidence of less than 1 per 100,000 children, rendering it a rare disease in childhood, with considerably lower rates than adults. Accordingly, clinical research is more challenging, and evidence-based guidelines are lacking (5–8).

Compared to aSLE, cSLE exhibits higher frequencies of kidney, neuropsychiatric, and hematologic involvement (1, 4). Lupus nephritis (LN) continues to be a prominent source of morbidity and mortality in SLE, reported in 30-40% of cSLE patients (3–5, 8–11). LN is more frequent in Hispanics, and African descendants, showing higher levels of disease activity and risk of developing kidney failure compared to non-Hispanic whites (6). Proliferative lesions (focal or diffuse) are the most common biopsy finding in childhood LN, with approximately 10% of affected children progressing to kidney failure within 5 years (3).

Since LN treatment is commonly associated with significant side effects, clinical care must balance optimal control of inflammation and tissue injury with minimization of immunosuppressive therapy side effects. The absence of serologic and biochemical diagnostics that adequately indicate the type and extent of kidney inflammation is one of the obstacles to this approach (12). Currently utilized markers of SLE and LN disease activity, for example anti-double stranded DNA (ds-DNA), serum complement levels, creatinine, and urinary protein excretion have significant limitations. They are inconsistent at predicting approaching disease flares, which may start without any significant alteration in their levels (12, 13). Likewise, proteinuria and measures of kidney function such as serum creatinine lack specificity to lupus-related kidney inflammation and injury. Consequently, reliance on changes in proteinuria or serum creatinine as a marker of LN delays starting adequate therapy, and kidney biopsy remains the gold standard to distinguish between activity and chronicity of LN histopathology. As a result, there is a dire need for identifying biomarkers which reliably signify the degree, nature, and course of kidney inflammation in LN (14).

Urine is a promising body fluid for identifying LN-specific biomarkers. Several studies comparing levels of various biomarkers in serum and urine of active LN reported superiority of urine in predicting LN activity (12, 15–18). In the current study, we investigate the efficacy of a panel of 10 urinary proteins representing groups of molecules hypothesized to be implicated in the pathogenesis of lupus *via* diverse pathways, including cytokines or chemokines and their receptors [e.g., monocyte chemoattractant protein-1 (MCP-1), platelet factor-4 (PF4), and tumor necrosis factor-like weak inducer of apoptosis (TWEAK)], metalloproteinases inhibitors [e.g., tissue inhibitor of metalloproteinase-1 (TIMP-1)], cell adhesion molecules (CAMs) [e.g., activated leukocyte CAM (ALCAM) and vascular CAM-1 (VCAM-1)], acute phase reactant glycoproteins (e.g., hemopexin), and markers of kidney damage [e.g., cystatin-C, kidney injury molecule-1 (KIM-1) and lipocalin2/neutrophil gelatinase-associated lipocalin (NGAL)], as markers of disease flare in a well-phenotyped cSLE cohort. Moreover, these proteins have previously been implicated as biomarkers in adult patients with LN, as discussed below.

PATIENTS AND METHODS

Patients

Eighty-four pediatric patients (≤18 years of age) fulfilling the revised 1997 classification criteria of the American College of Rheumatology (ACR) for SLE (19) were recruited into this study from the Pediatric Nephrology Research Consortium (PNRC) LN-Autoantibodies study cohort, with patients enrolled from pediatric clinics at Connecticut Children's Medical Center, Texas Children's Hospital (TCH), and Children's Healthcare of Atlanta. Institutional review boards (IRBs) at Baylor College of Medicine (H-35050), the University of Connecticut, Emory University, and the University of Houston all gave their approval to the study. Based on good clinical practice and the Declaration of Helsinki, all recruited patients completed an IRB-approved informed consent form.

Prospectively, demographics, clinical characteristics and conservative metrics of disease activity, such as anti-dsDNA, C3 and C4 levels, serum creatinine levels, spot urine protein-to-creatinine ratio (uPCR), and eGFR (assessed by Bedside Schwartz equation) were collected. **Table 1** highlights

demographics and clinical characteristics of all patients. Twenty-five healthy subjects of same sex and age were recruited as controls from TCH's Gynecology and Adolescent Medicine Clinic.

Assessment of SLE Disease Activity and Flares

In the LN-autoantibodies study, enrolled patients were either incident patients who had their samples taken before starting immunosuppression, prevalent patients who had a recent lupus flare (before escalating immunosuppression), or prevalent patients who were in remission (on or off immunosuppression). SLEDAI-2000, an established index in research and clinical practice was used to evaluate SLE disease activity (20). Clinical LN activity was weighed using the renal domain scores of SLEDAI (range 0–16; 0 = inactive LN). Patients were divided into three groups at the time of enrollment: active renal SLE (LN, patients with a renal SLEDAI score of 4 or higher), active non-renal SLE (patients with active symptoms or organ involvement but a renal SLEDAI of 0), and inactive SLE (patients with a total clinical SLEDAI of 0, asymptomatic with no findings of organ activity, subclinical hypocomplementemia, and/or elevated autoantibodies allowed). The Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (SDI) (range 0–47; 0=no SLE damage) was used to assess disease damage (21).

Urine Biomarkers Assays

Prior to batch processing, urine samples were prepared, aliquoted, and frozen at -80°C before 2 hours of collection. Only one aliquot was recovered for each experiment to avoid repeated freeze/thaw cycles. Urine levels of ALCAM, cystatin-C, hemopexin, KIM-1, MCP-1, NGAL, PF-4, Timp-1, TWEAK, and VCAM-1 were measured by means of a human enzyme-linked immunosorbent assay (ELISA) kit. All biomarkers were tested using ELISA kits from R&D Systems (Minneapolis, Minnesota, USA), except for hemopexin (pre-coated ELISA kit from Immunology Consultants, Portland, OR, USA) according to the manufacturer's manual. A microplate reader ELX808 (BioTek Instruments, Winooski, VT) was used to detect optical densities at 450 nm, and sample concentrations were estimated using a standard curve. All measurements were double-checked. Urine samples were diluted 1:2 for ALCAM and KIM-1, 1:5 (MCP-1, NGAL, PF-4, TIMP-1 and TWEAK), 1:50 (hemopexin and cystatin-C) and 1:100 for VCAM-1. All tested dilutions in the initial screening cohort prior to testing in the validation cohort are presented in **Supplementary Table 2**. Urine creatinine was used to standardize the results of urinary protein markers. Biomarker assay performers and readers were blinded to patient groups and clinical information.

Renal Histology

Patients were registered in this experiment if a random spot urine sample was obtainable within 1 week of the kidney biopsy. The renal histopathologic features of the active renal group were evaluated by doing a kidney biopsy assessed by one pediatric nephropathologist, blinded to the patients' biomarker expression data. The International Society of Nephrology/Renal Pathology Society (ISN/RPS) criteria were used to determine LN classification, histologic features of active inflammation, and features of chronicity or degenerative damage associated with LN (22). Biopsy activity and chronicity indices (AI, CI,

TABLE 1 | Patient demographics and clinical characteristics of the cSLE cohort.

Features	All SLE	Active Renal	Active Non-renal	Inactive SLE	Healthy Controls
Number	84	29	30	25	25
Age, mean ± SD	15.22 ± 2.7	15.1 ± 2.7	15.2 ± 2.6	15.4 ± 2.9	15.3 ± 1.85
Females, N(%)	73(86.9)	27 (93.1)	26 (86.7)	20(80)	25 (100)
Race					
Hispanic, N(%)	46 (54.8)	16	16	14	14
African American, N(%)	23 (27.4)	8	10	5	6
Caucasian, N(%)	8 (9.5)	2	2	4	2
Asian, N(%)	5 (5.9)	1	2	2	3
Mixed, N(%)	2 (2.4)	2	0	0	0
SLE disease duration, median (IQR), mos	6.8 (0.2-30)	0.3 (0.1-2.6)	0.7 (0.2-17)	28 (16-55)	–
SLEDAI, median (IQR)	4 (0-11)	12 (8-22)	4 (4-6)	0 (0-2)	–
Historic SLE Manifestations (%)					
Neuropsychiatric	12%	18%	10%	9%	–
Musculoskeletal	60%	64%	57%	56%	–
Kidney disorder	61%	100%	17%	58%	–
Mucocutanous	52%	57%	58%	38%	–
Serositis	22%	32%	14%	21%	–
Hematological	83%	86%	72%	85%	–
Features of renal disease*					
Serum creatinine, mean (SD), mg/dL		0.78 ± 0.11	0.55 ± 0.03	0.62 ± 0.02	–
eGFR, mean (SD), ml/min/1.73m^2		119.1 ± 18.2	115.2 ± 3.9	102 ± 3.7	–
urine PCR, mean (SD), mg/mg		3.29 ± 0.95	0.12 ± 0.08	0.11 ± 0.09	–
Renal SLEDAI score, median (IQR) †		8 (4-12)	0	0	–+

*eGFR, estimated glomerular filtration rate; IQR, Interquartile range; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; IQR, Interquartile range; PCR, Protein Creatinine ratio. †: Range 0-16; 0 = inactive LN, *Healthy controls did not have proteinuria, as determined using a negative urine dipstick.*

respectively) were employed to assess biopsy activity and chronicity in accordance with the National Institute of Health's LN guidelines (23). Activity and chronicity features are given numeric values, which are further used to estimate the AI score (range 0-24; 0= no LN activity) and CI score (range 0-12; 0= no LN chronicity) (23), with AI and CI scores of ≥7 and ≥4, respectively considered as poor prognostic risk factors for LN outcomes upon long-term follow-up (24).

Data Analysis

For interval and ordinal data, means± standard deviations (SD), and ranges were calculated; for categorical variables, frequencies and percentages were calculated. The mean-standard error of the mean was used to express continuous variables (SEM). To check for data normality, the Kolmogorov–Smirnov and Shapiro–Wilk tests were utilized. If the data was not normally distributed, the results were reported as medians and interquartile ranges (IQR). The non-parametric Kruskal-Wallis H (continuous variables) or chi-square (categorical variables) tests were used to compare values between groups. For correlation analysis of continuous and regularly distributed data, Pearson's correlation coefficient was utilized. The nonparametric Spearman's correlation coefficient was used otherwise. Rho values of 0.2–0.4 were rated mild; 0.4–0.6 were considered modest; and >0.6 were considered high. Statistical significance was defined as a two-tailed P-value of less than 0.05.

Receiver operating characteristic curve (ROC) analysis was used to examine the diagnostic accuracy of each biomarker as well as traditional SLE indicators, and the associated area under the curve (AUC; range 0–1) was obtained. The sensitivity, specificity, positive and negative predictive values, and ideal cut-off values were all determined using ROC analysis. GraphPad Prism v.6.0 was used for all statistical analyses (GraphPad, San Diego, CA, USA).

To identify the multi-marker panel of proteins that best discriminate groups of subjects, the predictive projection feature selection technique (25, 26) was implemented using the projpred package in R (version 4.0.3) (27). Model selection was conducted based on a model with the best predictive power (reference model) to locate a simpler model with a smaller number of proteins that maintains comparable prediction performance compared to the reference model (predictive projection). This selection process consisted of two main steps. First, we fitted a Bayesian regularized logistic regression model with horseshoe prior (28, 29), including all 8 proteins as a reference model. Second, we searched for a projected submodel with (at most) 5 proteins that minimized the Kullback-Leibler divergence from the posterior distribution of the reference model to that of the projected model. The selected submodel exhibited a similar predictive performance determined by the mean log predictive density and the mean squared error. Both performance metrics, along with area under the curve (AUC) and prediction accuracy, were evaluated through leave-one-out cross validation (LOOCV) to bypass potential problems of overfitting. The selected proteins of one model and its model performance metrics were compared to those of the counterpart model with adjustment for age, gender (male and female) and race (Caucasian and other) to account for potential confounder effects from these variables.

RESULTS

Study Population Characteristics and Histopathologic Features of Active Lupus Nephritis Subjects

A total of 84 patients with SLE (86.9% female) were enrolled in this study. Their mean age was 15.2 ± 2.7 years. The patients' median SLEDAI score was 4, with scores ranging from 0 to 33. According to their SLEDAI results, 29 patients (34.5%) had active renal disease, 30 patients (35.7%) had active non-renal disease, and 25 patients (29.8%) had clinically inactive SLE. All patients had their SLE disease damage measured using the SLICC damage index, which was classified as 0 or 1 at the time of enrollment. As controls, 25 healthy subjects (all females, mean age 15.31.8) were included.

Whether renal or non-renal, all active SLE patients were sampled before initiating immunosuppression apart from oral prednisolone or intravenous (IV) methylprednisolone. Low dose immunosuppression, in the form of prednisone (59%, median dose 2.5mg/day), hydroxychloroquine (84%), azathioprine (25%), mycophenolate mofetil (50%), or methotrexate (9%) were used as maintenance therapy for inactive SLE patients. For inactive patients who had rituximab (63%), samples were collected at a median of 437 days following the last dosage (IQR 215-716 days). Also, those who had IV methylprednisolone were sampled on average 455 days following their previous dosage (78%) (IQR 387-716 days).

Comparing patients with active renal disease to those with active non-renal and inactive SLE disease, their total SLEDAI scores were significantly higher (median 12; range 4-33) (P<0.0001) (**Table 1**). The median renal SLEDAI score was 8 among the 29 individuals with active LN (range 4-16). The uPCR concentrations ranged from 0.08 to 21.5 mg/mg, with significant increase in active renal patients compared to active non-renal and inactive SLE (P<0.0001). However, both serum creatinine and eGFR did not exhibit any significant differences among the three patient groups (P= 0.1235 and 0.102, respectively). In 15 (51.7%), 20 (69%), and 12 (41.4%) individuals, respectively, pyuria, hematuria, and active urinary casts were found. In twenty-three (79.3%) of the patients, a kidney biopsy was conducted. None of them revealed ISN/RPS class IV LN isolated. ISN/RPS classes VI and V were identified in 6 (26%) patients each, ISN/RPS class III in 3 (13.6%) patients, and mixed class LN (III+V or IV+V) in 5 (21.7%) patients. The proliferative LN subgroup (N = 14) included patients with ISN/RPS class III/IV± V, while the non-proliferative LN subgroup (N = 9) included those with other histological classes of nephritis (ISN/RPS I/II/pure V). In the same setting, histopathologic aspects of LN activity and chronicity were assessed simultaneously (**Table 2**), with a median biopsy activity score of 4 (range 0-17) and chronicity index of 0 (range 0-3).

Urine Levels of Assayed Protein Markers

As a group, the 10 urine biomarker proteins showed ability to discriminate active LN patients from active non-renal SLE patients, based on principal component analysis, as illustrated in **Figure 1A**. Among the 10 assayed biomarkers, particularly noteworthy was the high correlation of urine ALCAM with VCAM1 and PF4. Furthermore, we examined the performance of each individual marker (**Figure 2**). Urine levels of ALCAM, KIM-1, PF4 and VCAM-1 were significantly increased in active LN patients versus all other groups of patients: active non-renal, inactive SLE and controls. However, no significant difference in urine Timp1 concentrations among the 4 groups was detected. Urine concentrations of cystatin-C and TWEAK were significantly higher in patients with active renal disease than in healthy controls (P= 0.0014, 0.0005, respectively). In addition, urine cystatin-C levels were significantly increased in active LN than inactive SLE patients (P=0.0022), while urine TWEAK levels exhibited significant increase in active LN than active non-renal patients (P=0.007). Urine levels of hemopexin and lipocalin2/NGAL were only significantly different between active renal and inactive SLE patients (P=0.009, 0.0018, respectively), whereas urine MCP-1 levels only showed significant difference between active renal and healthy controls (P=0.0027).

Among the active renal lupus patients with a concurrent kidney biopsy, urine hemopexin and KIM-1 levels showed an increase of approximately 4- and 2-folds in the urine of patients with proliferative LN classes in contrast to non-proliferative LN classes. These differences, however, were not statistically significant. Non-significant increases in urine ALCAM, cystatin-C, MCP-1, NGAL, PF4 and VCAM-1 (47%, 48%, 19%, 86%, 15% and 36%) in patients with proliferative LN classes were detected as well. Urine levels of Timp-1 and TWEAK were comparable in proliferative and non-proliferative LN patients. The proliferative LN subgroup had significantly lower serum complement C3 levels (37.1± 4.3 vs. 77.5± 15.1 mg/dl, P = 0.02). Patients with proliferative LN classes exhibited significantly greater levels of pyuria (11 vs. 1, P = 0.003) and hematuria (14 vs. 4, P = 0.003) than those with non-proliferative classes. Consequently, SLEDAI as well as the renal domain of SLEDAI scores were significantly higher in proliferative LN patients [median (IQR) 21(8-33) vs. 12(4-27), P=0.018] and [median (IQR) 12(4-16) vs. 4(4-8), P= 0.012], respectively. The renal biopsy activity index was significantly higher in proliferative LN [median (IQR) 10 (4-12) vs. 0 (0-1), P <0.0001], while the biopsy chronicity index showed no significant difference.

TABLE 2 | Histologic features of the active lupus nephritis patients.

ISN/RPS classification (n=23)	
Class I (Minimal mesangial LN), N (%)	2 (8.7)
Class II (Mesangial proliferative LN), N (%)	1 (4.3)
Class III (Focal LN), N (%)	3 (13.6)
Class IV (Diffuse LN), N (%)	6 (26.1)
Class V (Membranous LN) (pure), N (%)	6 (26.1)
Mixed class III/IV and V, N (%)	5 (21.7)
Histologic features (n = 23)	
Activity Index, median (IQR)§	*4 (0-17)*
Endocapillary proliferation score >0, N (%)	12 (54.5)
Glomerular WBC infiltration score >0, N (%)	9 (40.9)
Hyaline deposits score >0, N (%)	7 (31.8)
Karyorrhexis score >0, N (%)	5 (22.7)
Cellular crescents score >0, N (%)	5 (22.7)
Interstitial inflammation score >0, N (%)	9 (40.9)
No active lesions noted, N (%)	5 (22.7)
Chronicity Index, median (IQR) ¶	*0 (0-3)*
Glomerulosclerosis score >0, N (%)	6 (27.3)
Fibrous crescents score >0, N (%)	0
Tubular atrophy and interstitial fibrosis scores >0, N (%)	7 (31.8)
No chronicity noted, N (%)	15 (65.2)

SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; ISN/RPS, International Society of Nephrology/Renal Pathology Society.
§: Range 0-24; 0 = no LN activity features, ¶: Range 0-12; 0 = no LN chronic change.

Biomarkers' Performance in Distinguishing Global and Renal Disease Activity in SLE Patients

ROC analysis was conducted to evaluate the effectiveness of the ten urine biomarkers in distinguishing active renal from active non-renal and active SLE from inactive SLE participants, in comparison to serum anti-dsDNA and low C3 levels (**Table 3**). Urine VCAM-1 and hemopexin were the most discriminatory proteins (AUC 0.81 both, P =0.009 and <0.0001, respectively) for distinguishing disease activity in cSLE independent of the end organ affected, while urine ALCAM showed good performance (AUC 0.75, P =0.0001). Importantly, urine VCAM-1 and ALCAM showed excellent ability in discriminating renal disease activity among active SLE patients (with sensitivity and specificity vales ranging from 78-92%), whereas urine PF4 displayed good performance in this respect (AUC 0.778, P =0.001), as indicated in **Table 3** and **Figure 3**. All of the biomarkers listed above outperformed anti-dsDNA in discriminating active (renal) cSLE from other cSLE patients.

We also constructed multi-marker panels after adjusting for demographic variables, using predictive projection feature selection. As shown in **Supplementary Table 1**, the multi-marker panel that best distinguished active LN from active non-renal SLE was a panel composed of ALCAM and PF4 (ROC AUC = 0.71, Accuracy = 0.76, after adjusting for demographic variables), while urine ALCAM alone outperformed all multi-marker panels in distinguishing active SLE from inactive SLE.

Correlation of Urine Biomarkers With SLE Disease Activity and Renal Parameters

In SLE patients (N = 84), urine ALCAM and VCAM-1 showed a strong significant correlation with total SLEDAI scores (**Figure 4A**), while urine PF4, hemopexin and cystatin-C revealed good correlations with SLEDAI (r 0.47, P<0.0001; r 0.43, P<0.0001; r 0.42, P<0.0001), respectively. Among active lupus nephritis patients (**Figure 4B**), urine VCAM-1 (r 0.57, P<0.0001), ALCAM (r 0.53, P<0.0001) and PF4 (r 0.50, P<0.0001) exhibited the best correlations with renal SLEDAI.

We then subjected the 10 assayed urine proteins, ethnicity, and various clinical metrics to an unsupervised Bayesian network

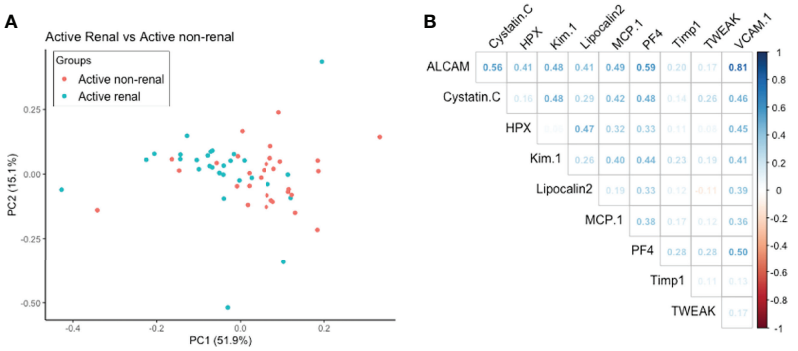

FIGURE 1 | **(A)** Principal Component Analysis aiming to discriminate active renal SLE from active non-renal SLE using 10 urine proteins assayed in the cSLE cohort. Together the first 2 components accounted for 67% of the variance between these two disease groups. **(B)** Correlation of levels of assayed urine protein markers with each other. Correlation coefficient between the 10 markers is represented by density of blue (for positive correlation) or red color (for negative correlation).

analysis (**Figure 5**), to investigate the interdependencies of all changing variables in a model and how they relate to one another, using probability distributions. As predicted, rSLEDAI was strongly linked to proteinuria and the disease group, offering independent validation of this unsupervised approach. Likewise, the close association/correlation of urine Cystatin C with eGFR also supports the validity of this methodology. This independent analysis re-affirmed urine ALCAM as the biomarker having the greatest impact on this complex, based on its "node force", being proportional to the size of each node, correlating strongly with urine VCAM-1, Kim1/Cystatin-C and Lipocalin-2, all of which had weaker impacts on this network.

Correlation of Urine Biomarkers With Biopsy Activity and Chronicity Histopathologic Features

We further investigated the correlation of the assayed biomarkers with histopathologic features of renal biopsy activity and chronicity indices among active LN patients who had concurrent kidney biopsies (N=23). As shown in **Figure 6**, urine KIM-1 was the only protein to correlate significantly with AI score, as well as two activity features, while urine Lipocalin2/NGAL and HPX significantly correlated with the CI scores. The former two proteins also correlated with different chronicity features, in addition to cystatin-C, which correlated with tubular atrophy and interstitial fibrosis.

DISCUSSION

In the present study, we have investigated the performance of ten urine proteins as potential biomarkers for lupus nephritis in a cSLE cohort. We demonstrated that all tested urinary proteins, except for Timp-1, were elevated in active LN patients. Urine ALCAM, KIM-1, PF4 and VCAM-1 were significantly increased in active LN patients in comparison to active non-renal, inactive

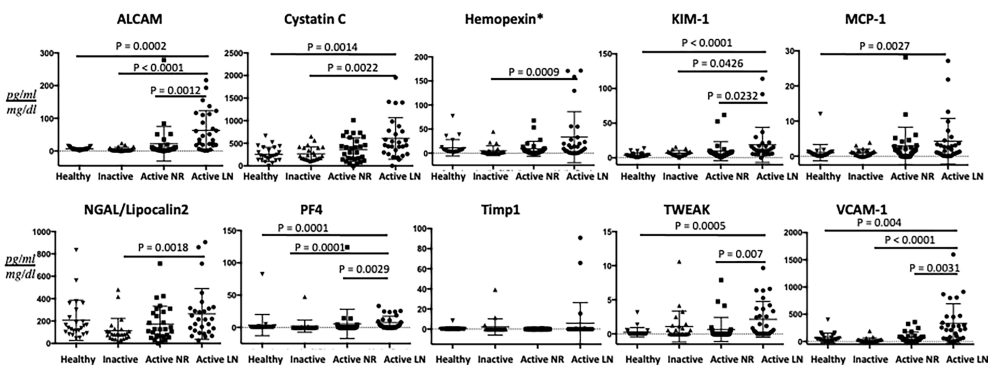

FIGURE 2 | Urine concentrations of the ten assayed proteins in the cSLE cohort. The concentrations of the 10 proteins investigated are shown on the Y-axes. The four groupings are represented by the X-axes (29 active renal; 30 active non-renal; 25 inactive cSLE and 25 healthy controls). Means and SE (error bars) are shown. Only comparisons achieving statistical significance are shown with *P*-values. All biomarkers' values are in pg/ml, normalized to urine Cr, except urine Hemopexin (*) which is expressed as ng/ml normalized to urine Cr.

TABLE 3 | Performance of the protein markers in differentiating SLE and LN disease activity.

Markers	Cut-off	AUC (95%CI)	Specificity	Sensitivity	P
		Active SLE (active renal & active non-renal) Vs. Inactive SLE			
VCAM-1	> 38.5 pg/ng	0.81(0.714 – 0.904)	60.87	74.29	0.009*
Hemopexin	>2.68 ng/ng	0.81 (0.713-0.905)	82.6	75	<0.0001*
ALCAM	>14.7 pg/ng	0.747 (0.64-0.85)	58.7	85.7	0.0001*
MCP-1	>1.402 pg/ng	0.669 (0.549-0.788)	60.87	74.29	0.009*
NGAL	>127.8 pg/ng	0.662 (0.54-0.783)	63	77.14	0.0125*
PF4	>0.182 pg/ng	0.654 (0.535-0.773)	58.7	73.5	0.0188*
Cystatin-C	>423 pg/ng	0.603 (0.478-0.726)	54.38	77.14	0.1158
KIM-1	>8.93 pg/ng	0.59 (0.465-0.714)	50	80	0.1669
Tweak	>2.517 pg/ng	0.588 (0.464-0.711)	28.3	88.9	0.172
Timp-1	>0.125 pg/ng	0.536 (0.408-0.663)	93.5	13.9	0.578
Anti-dsDNA	>30 IU/ml	0.67 (0.55-0.79)	59	77	0.01*
		Active renal Vs. active non-renal SLE			
VCAM-1	>125.7 pg/ng	0.883 (0.787-0.979)	81.8	82.6	<0.0001*
ALCAM	>16.3 pg/ng	0.828 (0.698-0.958)	91.3	78.3	0.0001*
PF4	>0.197 pg/ng	0.778 (0.641-0.916)	82.6	69.57	0.001*
Tweak	>0.1625 pg/ng	0.746 (0.599-0.894)	78.3	73.9	0.004*
KIM-1	>9.066 pg/ng	0.735 (0.586-0.885)	73.9	78.3	0.006*
Cystatin-C	>427.1 pg/ng	0.728 (0.582-0.876)	69.57	69.57	0.008*
Hemopexin	>8.78 ng/ng	0.720 (0.568-0.872)	73.9	73.9	0.0105*
MCP-1	>2.448 pg/ng	0.709 (0.56-0.859)	60.8	78.3	0.015*
NGAL	>59.5 pg/ng	0.646 (0.487-0.805)	95.6	30.4	0.088
Timp-1	>0.242 pg/ng	0.565 (0.398-0.733)	13.04	100	0.448
Anti-dsDNA	>120 IU/ml	0.55 (0.39-0.71)	48	69	0.485

AUC, area under the curve; SLE, systemic lupus erythematosus; CI, confidence interval. Biomarkers are listed in rank order by AUC values from highest to lowest.
**means statistically significant.*

SLE and controls. Urine ALCAM, VCAM-1, PF4 and hemopexin exhibited the best correlations with total SLEDAI as well as renal-SLEDAI scores. Urine ALCAM, VCAM-1 and hemopexin surpassed conventional laboratory metrics (anti-dsDNA, C3 and C4) in detecting clinical disease activity among cSLE patients, whereas ALCAM, VCAM-1 and PF4 levels were the best discriminators of renal disease activity in cSLE patients, outperforming conventional biomarkers including proteinuria. Based on the correlation analysis (**Figure 1B**) and the unsupervised Bayesian network analysis (**Figure 5**), urine ALCAM is likely driving the biomarker potential of VCAM1 and PF4. Indeed, Bayesian analysis also identified urine ALCAM as a driving factor in dictating the expression profiles of other urine markers in aSLE (30).

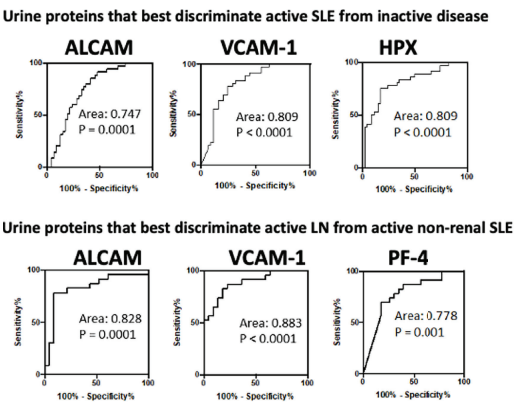

FIGURE 3 | Receiver operating characteristic (ROC) curves for the best 3 urine biomarkers in differentiating **(A)** active cSLE (renal and non-renal) from inactive cSLE patients and **(B)** active renal from active non-renal cSLE patients.

ALCAM, also known as cluster of differentiation-166 (CD-166) is a cell adhesion glycoprotein that is highly expressed on antigen-presenting cells and shows a fundamental role in mediating immune cell adhesion and migration, co-stimulation of T-cells and sustaining T cell activation. The role of ALCAM as a biomarker for inflammation, angiogenesis, diagnosis, prognosis, and treatment response in various cancers has been established (31). In diabetic nephropathy, serum concentrations of ALCAM as well as its expression in kidney tissue were significantly elevated and upregulated in glomeruli and tubules (32). ALCAM expression was also up-regulated in the glomeruli and tubules of MRL/*lpr* lupus-like murine model (33). In recent high-throughput proteomic approaches, urine ALCAM showed promise in predicting LN activity in SLE patients (30). Further validation in two aSLE cohorts confirmed the higher urine ALCAM levels with significant correlations with total and renal SLEDAI scores (34, 35).

The current study is the first to evaluate the performance of urine ALCAM in a cSLE cohort. In addition to being significantly increased in active LN patients, urine ALCAM correlated significantly with total and renal SLEDAI score and exhibited excellent ability to distinguish cSLE patients with active renal disease. Likewise, all multi-marker panels that exhibited outstanding diagnostic performance in distinguishing active (renal) cSLE included urine ALCAM. However, in contrast to findings in aSLE, urine ALCAM levels were not associated with proliferative LN or with renal pathology AI or CI, possibly due to the limited sample

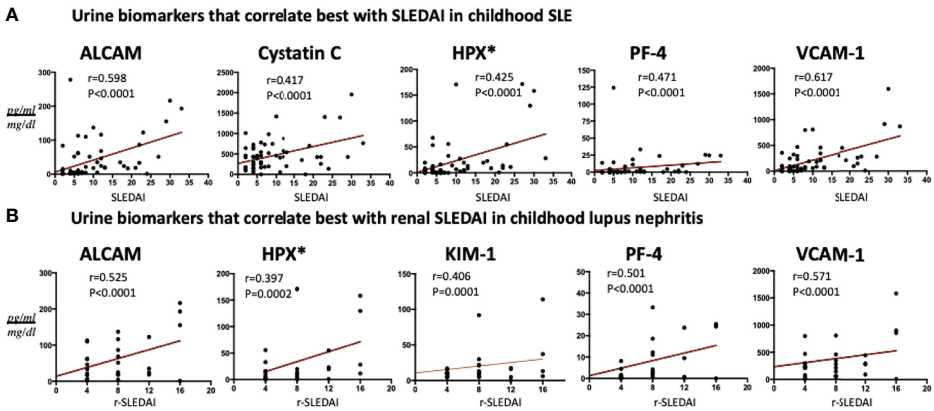

FIGURE 4 | Association of the best 5 urine protein markers with **(A)** SLEDAI in all SLE patients and **(B)** renal-SLEDAI in active renal SLE patients. All biomarkers' values are in pg/ml, normalized to urine Cr, except urine Hemopexin (*) which is expressed as ng/ml normalized to urine Cr.

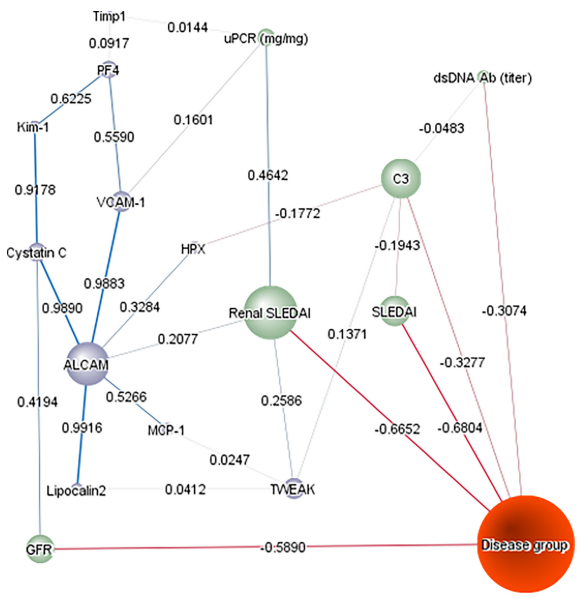

FIGURE 5 | Bayesian Network Analysis. The levels of the 10 urine proteins in the cSLE cohort and their relevant clinical characteristics were analyzed using Bayesian network analysis using BayesiaLab. The presented network was assembled in an unsupervised manner, using the EQ algorithm and a structural coefficient of 0.4. The circular nodes making up the Bayesian Network denote the variables of interest, including urine protein markers (purple-colored), clinical indices (green-colored), and disease group (inactive/active non-renal, active renal; colored orange). The "node force" is denoted by the size of each node, reflecting its effect on other nodes in the network, according to conditional probabilities. The informational or causal dependencies among the variables are represented by the links (arcs) that connect the nodes, including the correlation coefficients between adjacent nodes (as stated), with the thickness of the link being proportional to the correlation coefficient.

size with concurrent renal biopsies. Alternatively, this might be an indication that the molecular determinants of clinical disease activity and renal disease activity in cSLE may be distinct.

VCAM-1 or CD106 is a widely expressed cell adhesion molecule in peripheral circulation being expressed mainly in endothelial cells and glomerular parietal epithelial cells (36). Several studies have shown elevated and strongly correlated serum and urinary VCAM-1 levels with LN activity and severity (14, 18, 34, 37–40). Moreover, some studies reported its association with proliferative LN classes (39, 40), as well as its reduction following treatment (38). In resonance with the studies in aSLE, urine VCAM-1 was significantly elevated in active LN patients versus active non-renal, inactive SLE and healthy controls in our cohort. Urine VCAM-1 was highly predictive of SLE disease activity (when compared with inactive SLE, AUC 0.81) and more specifically with LN disease activity (when compared with active non-renal SLE patients, AUC 0.88). Furthermore, among the ten tested biomarkers, urine ALCAM and VCAM-1 revealed the best correlations with total and renal SLEDAI scores, although they did not reflect concurrent renal pathology activity, as discussed above. Two previous studies explored serum (41) and urine (42) VCAM-1 levels in a pediatric SLE, corroborating the present findings.

Another promising biomarker of SLE and LN is PF4, an anti-angiogenic chemokine functioning *via* an integrin-dependent mechanism to regulate angiogenesis. Anti-fibrotic cytokines (e.g., interferon- γ) are inhibited by PF4, while pro-fibrotic cytokines are promoted (e.g., IL-4 and IL-13). It also boosts the growth of regulatory T cells (43). Additionally, the roles of PF4 in cancer, atherosclerosis, and heparin-induced thrombocytopenia are well-established (44–46). Serum PF4 levels were found to be elevated in systemic sclerosis (47), as well as in the plasma of antiphospholipid syndrome (APS) patients (48), suggesting its role in the pathogenesis of these disorders.

Recent reports (12, 39, 43) have verified urinary PF4 as a promising biomarker distinguishing active LN adult patients and correlating with biopsy activity changes. Consistent with these findings, the current study found urinary PF4 levels to

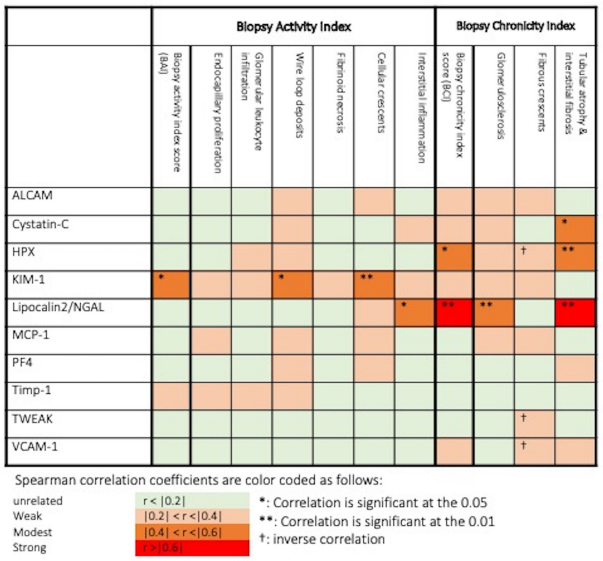

FIGURE 6 | Association of urine biomarkers levels and histopathologic features in biopsied active lupus nephritis patients. *: Correlation is significant at p = 0.05, **: Correlation is significant at p = 0.01, †: inverse correlation. Spearman correlation coefficients are color coded as follows: r <0.2: unrelated, 0.2 ≤ r <0.4: weak, 0.4 ≤ r < 0.6: modest, r ≥ 0.6: strong.

exhibit highest values in active LN patients compared to other SLE groups as well as controls. Additionally, the ability of the biomarker in distinguishing active LN from active non-renal SLE was "very good". Furthermore, PF4 levels were among best biomarkers that correlated with renal SLEDAI as well as total SLEDAI scores. It showed good correlations with urine ALCAM and VCAM-1 concentrations, supporting the established link between VCAM-1 and PF4/CXCL4 and their receptors *via* the crosstalk between neutrophils and bone marrow endothelial cells (49). Similar interactions between these molecules may in part explain the coordinated elevations in these proteins in cSLE. Additional studies in aSLE cohorts provide further support for these proteins, endorsing their role as biomarkers for early detection of LN flare-ups and as potential therapeutic targets in LN. These include studies focusing on these biomarkers highlighting their potential utility in serial biomarker tracking, predicting clinical and pathological activity in LN, and biomarker-directed therapeutic targeting (50–52).

A strength of the present study was the identification of novel urine biomarkers for cSLE and childhood LN activity that had better diagnostic capacity than traditional kidney injury markers (e.g., Cystatin-C and KIM-1). Serum cystatin-C has been recommended as a promising marker of GFR, useful for estimating kidney function in both acute and chronic kidney dysfunction (53, 54). Indeed, its association with eGFR was re-confirmed in the unsupervised Bayesian network analysis. KIM-1 is an immunoglobulin domain and mucin domain-1 bearing protein induced in damaged tubular epithelial cells and is related to interstitial fibrosis and kidney inflammation (55). In our study, urinary cystatin-C and KIM-1 showed significant increases in active LN, and urinary KIM-1 was the only biomarker to correlate significantly with the renal pathology AI score, due to its correlation with cellular crescents and wire loops, an indicator of immune deposition.

The study's limitations included the relatively low number of patients, and its cross-sectional design which prevented authors from interrogating biomarkers for prognosis or recording changes in disease activity over time, without the confounding effects of subject to subject variation. Pure class V LN, which behaves quite differently from proliferative (Class III or IV) lesions, were seen in a high proportion of the active LN subjects examined in this study. As a result, the Activity Index on renal biopsy in this group is rather low, at 4 points. Accordingly, proteinuria in the nephrotic range was the primary presenting feature in these patients, without significant inflammatory infiltrates. Future biomarker studies will have to include larger numbers of LN patients with proliferative renal disease.

To the best of our knowledge, this is the first study to assess the role of urinary ALCAM and PF4 in a cSLE cohort. Urine ALCAM and PF4 appear to have the greatest promise as SLE and lupus nephritis activity indicators, outperforming conventional markers in distinguishing active SLE and LN patients. Urine VCAM-1 and HPX levels have already been studied in many aSLE and cSLE cohorts, confirming earlier findings. Further longitudinal research is needed to confirm the performance of these urine proteins as disease flare predictors compared to traditional markers, as well as to see if combining these urine protein markers with anti-dsDNA and complement levels might offer improved sensitivity and specificity profiles in predicting early SLE relapse.

DATA AVAILABILITY STATEMENT

The LN-autoantibodies clinical dataset and paired urine samples are available upon request, in accordance with thePediatric Nephrology Research Consortium policies (https://pnrconsortium.org/). Anybiomarker data generated and/or analyzed during the current study that were not includedin this published article are available from the corresponding authors upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The institutional review boards (IRBs) at Baylor College of Medicine (H-35050), the University of Connecticut, Emory University, and the University of Houston. Based on good clinical practice and the Declaration of Helsinki, written

informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SS, AH, KV, and TZ performed the experiments. KL, CP, and FI performed the data analyses. MJH performed all pathology analyses. LG, SM, and SW provided patient samples. SW and CM designed the studies and reviewed all data. SS, SW, and CM wrote the manuscript. All authors reviewed the manuscript and concurred with the findings. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Identification and Validation of a Urinary Biomarker Panel to Accurately Diagnose and Predict Response to Therapy in Lupus Nephritis

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Background: We have previously shown that 15 urinary biomarkers (of 129 tested by Luminex), discriminate between active Lupus Nephritis (ALN) and non-LN patients. The aim of this study was to evaluate the ability of these 15 previously-identified urinary biomarkers to predict treatment responses to conventional therapy, and for the most predictive of these biomarkers to validate their utility to identify ALN patients in an independent prospectively-acquired lupus cohort.

Methods: Our study had a 3-stage approach. In stage 1, we used Luminex to examine whether our previously identified urinary biomarkers at the time of the renal flare (± 3 months) or 12 ± 3 months after treatment of biopsy-proven ALN could predict treatment responses. In stage 2, a larger prospectively-acquired cross-sectional cohort was used to further validate the utility of the most predictive urinary biomarkers (identified in stage 1) to detect ALN patients. In this 2nd stage, cut-offs with the best operating characteristics to detect ALN patients were produced for each biomarker and different combinations and/or numbers of elevated biomarkers needed to accurately identify ALN patients were analyzed. In stage 3, we aimed to further corroborate the sensitivity of the cut-offs created in stage 2 to detect ALN patients in a biopsy-proven ALN cohort who had a urine sample collection within 3 months of their biopsy.

Results: Twenty-one patients were included in stage 1. Twelve (57.1%), 4 (19.1%), and 5 (23.8%) patients had a complete (CR), partial (PR) and no (NR) remission at 24 ± 3 months, respectively. The percentage decrease following 12 ± 3 months of treatment for Adiponectin, MCP-1, sVCAM-1, PF4, IL-15 and vWF was significantly higher in patients with CR in comparison to those with PR/NR. In stage 2, a total of 247 SLE patients were included, of

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which 24 (9.7%) had ALN, 79 (31.9%) had LN in remission (RLN) and 144 (58.3%) were non-LN (NLN) patients. Based on the combinations of biomarkers with the best operating characteristics we propose "rule out" and "rule in" ALN criteria. In stage 3, 53 biopsy-proven ALN patients were included, 35 with proliferative LN and 18 with non-proliferative ALN, demonstrating that our "rule in ALN" criteria operate better in detecting active proliferative than non-proliferative classes.

Conclusions: Our results provide further evidence to support the role of Adiponectin, MCP-1, sVCAM-1 and PF4 in the detection of proliferative ALN cases. We further show the clinical utility of measuring multiple rather than a single biomarker and we propose novel "rule in" and "rule out" criteria for the detection of proliferative ALN with excellent operating characteristics.

Keywords: predictors of response, urinary biomarker, biomarkers, lupus nephritis, systemic lupus erythematosus

INTRODUCTION

Lupus nephritis (LN) occurs in up to 65% of patients with Systemic Lupus Erythematosus (SLE), and is most prevalent in younger patients, many of whom are of African, Asian, and Hispanic ancestry (1–3). LN is one of the most common causes of death as well as an important predictor of subsequent mortality in SLE (3–8). It is also associated with a significant morbidity, since up to 20% of patients will progress to end stage renal disease (3, 9), which has a particularly high socioeconomic impact (10, 11).

The gold standard for determining the presence and type of kidney involvement is the kidney biopsy (KB) (12). However, serial biopsies to assess renal activity following treatment are impractical due to their invasive nature and risk of complications. There is also a subset of patients with contraindications that preclude a KB at the time of LN flare. Consequently, the diagnosis of LN and the monitoring of response to treatment has been based on urinary findings of proteinuria, hematuria, pyuria, or casts, and alterations of renal function, such as increased serum creatinine.

The utility of proteinuria as a biomarker has drawbacks. LN-associated proteinuria frequently persists for years after renal injury, especially in patients with nephrotic range proteinuria, normalizing in less than 50% of patients within two years (13). In addition, proteinuria may reflect chronic histologic lesions rather than active inflammation within the kidney, as demonstrated by Malvar et al. who showed that 62% of LN patients who had complete histologic remission on a repeat KB following initiation of therapy were still 'clinically active', as defined by persistent proteinuria (14). Being able to correctly differentiate between residual activity and damage in LN is crucial when treating patients, highlighting the need for new biomarkers in the clinical setting.

Various urinary cytokines, chemokines, pro-inflammatory factors, growth factors and adhesion molecules, have been assessed as potential urinary biomarkers for LN (15–24). Unfortunately, none of them have been able to successfully transition into clinical practice, with the lack of clear cut-offs and algorithms that accurately detect active LN (ALN) being part of the challenge. We have previously shown that 42 urine biomarkers (of 129 tested by Luminex) discriminate between ALN and non-LN patients (NLN). Of these, Clusterin, Cystatin C, NGAL, PF4, vWF, sVCAM-1, GM-CSF, GRO, IL-15, IL-6, MCP-1, Adiponectin, PAI-1, MMP-7 and TIMP-1 were the 15 biomarkers with the most promising results, based on their ability to discriminate between ALN and non-active LN (remission LN, RLN) and/or their correlations with histologic features in the KB (16).

The aim of this study was to evaluate the ability of these 15 previously identified urinary biomarkers to predict treatment responses following initiation of conventional therapy, and for the most predictive of these biomarkers to validate their utility to accurately detect ALN in an independent prospectively acquired lupus cohort.

MATERIAL AND METHODS

Study Design and Patients

Patients with Systemic Lupus Erythematosus (SLE) from the University of Toronto Lupus cohort and the LuNNET cohort (16, 25) were included in the study. All patients met the revised 1997 ACR classification criteria for SLE (26) or had three criteria and a supportive biopsy (skin or kidney).

The study had 3 stages. In stage 1, we used Luminex to examine whether the 15 urinary biomarkers identified in our previous study could predict treatment response. For this stage, the cohort was composed of SLE patients from the LuNNET cohort, recruited from April 2006 to December 2011, all of whom had biopsy proven ALN. The KB was performed ± 3 months from the baseline urine sample collection, with all patients being followed longitudinally for a minimum of 2 years at the University of Toronto Lupus Clinic. Follow-up urine samples were collected every 3-6 months up to 24 ± 3 months.

The response to treatment was established after 24 months of follow-up, using the following criteria: 1) Complete response (CR): reduction in a 24 hour protein excretion to <500 mg/day with normal serum creatinine or serum creatinine within 15% of previous baseline; 2) Partial response (PR): > 50% reduction in proteinuria and to non-nephrotic levels, with serum creatinine

within 25% of previous baseline; and 3) No response (NR): patients who did not achieve CR or PR (2, 27). Samples from 24 healthy controls were also assayed to enable determination of normal biomarker values.

In stage 2 of the study, the most predictive urinary biomarkers for response to treatment that were identified in stage 1 (Adiponectin, MCP-1, sVCAM, PF4, IL-15 and vWF) were assayed using ELISA, to further validate their ability to accurately detect ALN patients. For this second part of the study, a larger cross-sectional cohort was acquired. SLE patients from the University of Toronto Lupus cohort (enrolled within the last 5 years, to assure no overlap with the LuNNET cohort) were consecutively recruited from July 2016 to March 2019, when attending their scheduled clinic appointment. For this cohort, ALN was defined clinically as a LN flare that occurred within the last 12 months from the urine collection, with a 24 hour urinary protein excretion of ≥500mg/day, which was interpreted by the physician in charge as being secondary to active renal inflammation prompting a change in immunosuppressive therapy. Non-ALN patients were divided into two groups: 1) Patients with RLN, defined as the presence of a history of LN but no clinical signs of renal activity at the time of sample collection, with a 24 hour urinary protein excretion of <500mg/day or the presence of chronic proteinuria which was interpreted by the physician in charge as being secondary to damage and not requiring a change in immunosuppressive therapy. Chronic proteinuria was defined as stable proteinuria present for at least 1 year, in the presence of chronic kidney disease (CKD, defined as a eGFR<60ml/min/m^2) and/or other comorbidities known to cause of proteinuria, such as diabetes mellitus and hypertension; and 2) NLN patients, with no history of LN and no clinical signs of ALN (urinary protein excretion <500mg/day) at the time of the urine sampling, but who could have extra-renal SLE activity.

In Stage 3, we aimed to validate the sensitivity of our urinary biomarker cut-offs, established in the cross-sectional cohort, to identify ALN patients, and determine if they operated similarly for proliferative and non-proliferative ALN classes. For this stage, urinary Adiponectin, MCP-1, sVCAM and PF4 were measured using ELISA. All patients had biopsy proven ALN ± 3 months from the urine sample collection.

Urinary Biomarker Assays

All urine samples were spun to remove cellular debris and frozen at –80° C. To avoid repeated freeze/thaws, samples were thawed once on ice, sub-aliquoted, re-frozen at -80°C, and then individual aliquots thawed immediately prior to use. For the first stage of the study, the urinary concentrations of 15 analytes (Clusterin, Cystatin C, NGAL, PF4, vWF, sVCAM-1, GM-CSF, GRO, IL-15, IL-6, MCP-1, Adiponectin, PAI-1, MMP-7 and TIMP-1) were measured by coupled bead assay (Luminex using MILLIPLEX® Map Kits (EMC Millipore Corporation) through Eve Technologies Inc.). Further information regarding the sensitivity and dynamic range of the assays can be found on the company website. For the majority of assays, the urine samples were run undiluted except for Clusterin and Cystatin C, which were diluted 1/50 and TIMP-1, which was diluted 1/5. All analytes were measured in duplicate, with a single sample on each of two separate plates and averaged. Urinary biomarker levels were considered abnormal if they were > 2 SD above the mean of the 24 healthy controls.

For the second and third stage of the study, sVCAM-1 (Cat# DY809), MCP-1 (Cat# DY279), Adiponectin (Cat# DY1065), PF-4 (Cat# DY795), vWF (Cat# DY2764-05) and IL-15 (Cat# DY247) were measured by ELISA, using Duoset and Ancillary Reagent Kits (Cat# DY008) obtained from R&D Systems, and processed following the manufacturer's protocols. Optimal dilutions for each cytokine ELISA were determined in preliminary experiments and were 1/16 for Adiponectin, 1/128 for sVCAM-1, 1/8 for MCP-1, and 1/4 for PF-4, vWF, and IL-15. For the majority of samples IL-15 and vWF concentrations were below the limit of detection and therefore were not pursued further. All samples were run in duplicate, averaged, and their cytokine concentration computed from a ln–ln plot of the cytokine standard curve, with adjustment for the dilution factor. Any samples with raw absorbance values that were under the lower limit of the standard curve using the optimal dilution, were re-run at lower dilutions and those that were below the standard curve at a 1/4 dilution were given the lowest standard curve value for ensuing calculations.

Statistical Analysis

Descriptive statistics were generated for patients' baseline characteristics for the two cohorts, with baseline categorical variables being presented as counts and percentages. Continuous biomarker variables are presented as median and IQR or mean and standard deviation, as appropriate.

In the first stage of the study, logistic regression models were used to determine if the baseline urinary biomarker levels, or the absolute or percentage decrease, after 12 months of therapy predicted CR to treatment at 24 months. For this analysis non-CR (PR and NR) were pooled together. A scatter plot of each of the patient measurements at different time points, as well as a smooth line were plotted to visualize the trend of the curve depending on the response (CR *vs* PR/NR).

In the second stage of the study, the Kruskal-Wallis test was used to assess the differences in biomarker measures between groups. Logistic regression models were used to assess the impact of each of the potential continuous predictors to discriminate between ALN and non-ALN. A binary partitioning method was used to obtain the optimum cut-off for each biomarker that discriminated between ALN and non-ALN (RLN and NLN). Receiver Operating Characteristic curves were generated for each individual biomarker.

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR) and negative likelihood ratios (-LR) with 95% confidence intervals were calculated to determine the accuracy of detecting active LN when: 1 or more, 2 or more or 3 or more biomarkers were elevated. Sensitivity, specificity, PPV and NPV above 80% were considered good and above 90% excellent. Likelihood ratios above 10 for +LR and below 0.1 for –LR were considered to provide strong evidence to rule in or rule out diagnoses (28).

In the third stage of the study, the sensitivity with 95% confidence intervals was calculated for the presence of 2 or more, or 3 or more elevated biomarkers.

All p-values were 2-sided and for the statistical analyses, a $p < 0.05$ was considered to indicate a statistically significant result. Statistical analysis was performed using version 9.4 of the SAS system for Windows, Copyright © 2002-2012 SAS Institute, Inc., Cary, NC.

RESULTS

Only a Subset of Urinary Biomarkers Demonstrate Change Over Time That Associates With Treatment Response

In the first stage of the study, 21 biopsy-proven ALN patients were included, 19 (90.5%) of whom had proliferative LN (see **Table 1**). The mean age at baseline was 32.15 years and 85.7% of patients were female. The predominant ethnicity was Caucasian (47.6%), followed by Asian (23.8%) and Afro-Caribbean (14.3%). The mean SLE disease duration was 3.69 years and the average time since the start of the LN flare to the urine sample collection was 1.19 ± 1.12 months. Twelve (57.1%), 4 (19.1%), and 5 (23.8%) patients had a complete (CR), partial (PR) and no remission (NR), respectively after 24 months of conventional therapy.

Patients who achieved CR, PR and NR were treated similarly. The dose of prednisone used at baseline was similar for the 3 groups (45, 52 and 39 mg for CR, PR and NR, respectively, p=0.241). In the CR group 2 (16%) were treated with Azathioprine, 2 (16%) with Cyclophosphamide and 8 (66.6%) with Mycophenolate. All patients with PR and 4 (80%) of the patients with NR were treated with Mycophenolate. The remaining NR patient was treated with Azathioprine.

The baseline levels of urinary biomarkers did not predict response to therapy at 24 months. However, the percentage decrease in Adiponectin, MCP-1, sVCAM-1, PF4, IL-15 and vWF at 12 ± 3 months predicted response to therapy at 24 months. (**Figure 1** and **Table 2**).

Validation of the Most Predictive Biomarkers in a Cross-Sectional SLE Cohort

A total of 247 SLE patients from the University of Toronto Lupus Clinic were included in stage 2, of whom 24 (9.7%) had ALN, 79 (31.9%) had RLN and 144 (58.3%) had NLN patients. All ALN patients were within 12 months of detection of the LN flare, with a mean time of 6 months between the initiation of the flare and the urine collection. Since our criteria for including ALN were clinical, only 11 (45.8%) had a KB at the time of their LN flare, of whom 10 (41.7%) had a proliferative class, either III or IV with or without class V, and 1 (0.04%) had pure membranous class V. The remaining 13 patients did not have a KB performed at the time of the flare. However, 10 had a prior KB (9 class III or IV with or without class V, and 1 pure class V). **Table 1** shows the baseline demographic and clinical characteristics of the cohort.

Based on the findings from stage 1, 6 urinary biomarkers were measured by ELISA in the cross-sectional cohort, including Adiponectin, MCP-1, sVCAM-1, PF4, vWF and IL-15. vWF and IL-15 were not consistently detectable by ELISA and were not furthered studied. Patients with ALN had higher levels of all 4 remaining analytes, including Adiponectin, MCP-1, sVCAM-1 and PF4, in comparison to patients with RLN and NLN, as shown in **Figure 2** and **Supplementary Table 1**. Cut-offs with the best operating characteristics to detect ALN patients were produced for each biomarker (Adiponectin 18000 pg/ml, MCP-1 1341 pg/ml, sVCAM-1 46000 pg/ml and PF4 134 pg/ml), see **Supplementary Figure 1** for Receiver Operating Characteristic curves. Adiponectin was the most sensitive, with a high NPV (99%) and good -LR (0.09). However, 2 patients that were classified as ALN did not meet the Adiponectin cut-off. These patients were in the 4th and 10th month of the onset of their LN flare. In addition, Adiponectin alone had a low PPV (52%). In contrast, MCP-1 and PF4 had high specificities, but low PPV´s. Thus, no single biomarker appeared to be sufficient to accurately detect ALN patients (**Table 3**).

Identifying the Optimal Biomarker Combination to Accurately Detect ALN

Given that none of the 4 urinary biomarkers by themselves had excellent operating characteristics, we analyzed whether different combinations and/or numbers of elevated biomarkers could more accurately identify ALN patients (**Table 4**). The operating characteristics for any combination of 2 elevated biomarkers were good, with a sensitivity and specificity above 90%, high NPV (99%) and excellent -LR (0.09). These results were similar to those for Adiponectin alone. Even though this combination failed to detect 2 ALN patients, the onset of the LN flare was 12 months prior to the sample collection for both patients and both had a CR at 24 months. This finding suggests that these criteria could rule out ALN cases in a more reliable manner than Adiponectin alone. Nevertheless, the PPV of this combination remained low (50%), with 22 false positives.

When analyzing the different combinations of 2 elevated biomarkers. The MCP-1-Adiponectin and MCP-1-PF4 combinations had specificities and PPVs of 100%, the combination of Adiponectin-PF4 and MCP-1-sVCAM-1 had a lower PPVs, but still excellent +LRs. sVCAM-1-Adiponectin and sVCAM-1-PF4 combinations had good +LRs but had the lowest PPVs.

Overall, the sensitivities for the individual combinations of 2 biomarkers were not excellent, all below 80% and some as low as 30-40%, suggesting that all 4 biomarkers should be tested in other to improve sensitivity (**Table 4**).

Given that the combinations of 2 elevated biomarkers including sVCAM-1 had lower PPV, we assessed whether increasing the cut-off for sVCAM-1 from 46000 to 103700 improved the operating characteristics. By doing this, the PPV and +LR of the combinations of sVCAM-1-Adiponectin and sVCAM-1-PF4 substantially improved as seen in **Table 4**. The number of false positives for the presence of any combination of 2 elevated biomarkers decreased from 22 (sVCAM-1 cut-off of 46000) to 12 (sVCAM-1 cut-off of 103700). Of the remaining 12 false positives, 9 had RLN and 3 were NLN patients. From the RLN group, 4 had their last LN flare ≤ 2 years before the study (1

TABLE 1 | Baseline demographic and clinical characteristics of the patient cohorts[†].

	Stage 1	Stage 2 cross-sectional cohort N = 247		
	ALN (N = 21)	**ALN (N = 24)**	**RLN (N = 79)**	**NLN (N = 144)**
Ethnicity, n (%)				
Caucasian	10 (47.6)	8 (33.3)	41 (51.8)	82 (56.9)
Afro-Caribbean	3 (14.3)	9 (37.5)	14 (17.7)	33 (23.1)
Asian	5 (23.8)	4 (16.6)	11 (13.9)	12 (8.4)
Other	3 (14.3)	3 (12.5)	13 (16.5)	17 (11.9)
Female, n (%)	18 (85.7)	20 (83.3)	64 (81.0)	132 (91.6)
Age (years), Median (IQR)	28.9 (23.5-44.0)	28.6 (24.6-36.1)	41 (28.9-52.4)	38 (29.6-52.7)
Duration SLE (years), Median (IQR)	2.9 (0.1-7.5)	7.7 (3.5-10.2)	9.27 (4.2-17.5)	7.1 (2.8-13.5)
Time from LN flare (months)*, Median (IQR)	1.0 (0-2.0)	5.5 (2.7-10)	48 (24-108)	NA
Antiphospholipid syndrome, n (%)	1 (4.8)	1 (4.2)	9 (11.4)	9 (6.3)
Hypertension, n (%)	5 (23.8)	6 (25.0)	22 (27.8)	19 (13.2)
Diabetes Mellitus, n (%)	0 (0)	0 (0)	6 (7.6)	3 (2.1)
Clinical features, n (%)				
Mucocutaneous	7 (33.3)	4 (16.7)	8 (10.1)	13 (9.0)
Musculoskeletal	8 (38.1)	0 (0)	2 (2.5)	7 (4.9)
Serositis	2 (9.5)	0 (0)	0 (0)	1 (0.7)
Hematologic	3 (14.3)	1 (4.2)	6 (7.6)	13 (9.0)
Central Nervous System	0 (0)	0 (0)	1 (1.3)	1 (0.7)
Vasculitis	1 (4.8)	0 (0)	1 (1.26)	0 (0)
Renal	21 (100)	24 (100)	0 (0)	0 (0)
Fever	2 (9.5)	0 (0)	1 (1.3)	0 (0)
SLEDAI, total score, Median (IQR)	18 (14-24)	10 (6-13)	3 (0-4)	2 (0-4)
SLEDAI, renal, Median (IQR)	12 (8-12)	4 (4-8)	–	–
Anti-dsDNA Ab (IU/ml), Median (IQR)	100 (19-101)	55 (24-254)	15 (1-55)	3 (1-15)
Positive Antiphospholipid Abs, n (%)	4 (19.0)	4 (16.7)	18 (22.8)	33 (22.9)
C3, g/L, Median (IQR)	0.62 (0.35-0.77)	0.86 (0.67-0.99)	0.99 (0.82-1.12)	1.04 (0.85-1.22)
C4, g/L, Median (IQR)	0.07 (0.05-0.14)	0.14 (0.13-0.20)	0.17 (0.13-0.23)	0.19 (0.14-0.25)
Serum Albumin (g/L), Median (IQR)	29 (22-32)	34 (31.5-37.5)	41 (38-43)	42 (39-44)
Serum Creatinine (umol/L), Median (IQR)	89 (71-134)	77.5 (66.5-100)	77 (57-81)	64.5 (56.5-74.5)
eGFR<60 ml/min/m^2, n (%)	6 (28.6)	6 (25)	11 (13.9)	0 (0)
eGFR<30 ml/min/m^2, n (%)	3 (14.3)	2 (8.3)	3 (3.8)	0 (0)
eGFR<15 ml/min/m^2 or RRT, n (%)	0 (0)	0 (0)	0	0 (0)
24-hour Protein excretion (g), Median (IQR)	2.1 (1.5-3.8)	1.1 (0.7-2.7)	0 (0-0.4)	0 (0-0.4)
Kidney biopsy Class, n (%)	21 (100)	11 (45.8)[#]		
I	0 (0)	0 (0)		
II	0 (0)	0 (0)		
III	1 (4.8)	2 (0.08)		
IV	10 (47.6)	5 (0.2)		
V	2 (9.5)	1 (0.04)		
III+V	4 (19)	1(0.04)		
IV + V	4 (19)	2 (0.08)		
VI	0 (0)	0 (0)		
Activity Index, Median (IQR)	11 (6-13)	7 (2.3-9.8)		
Chronicity Index, Median (IQR)	3 (2-4)	3 (2.3-4.8)		
Prednisone, n (%)	21 (100)	22 (91.7)	47 (59.5)	71 (49.3)
Prednisone dose (mg), Median (IQR)	45 (40-50)	15 (10-20)	5 (5-10)	4 (5-7.5)
Antimalarial, n (%)	20 (95.2)	20 (83.3)	67 (84.8)	124 (86.1)
Immunosuppressive, n (%)	21 (100)	23 (95.8)	56 (70.8)	83 (57.6)
Azathioprine, n (%)	3 (14.3)	1 (4.2)	11 (13.9)	31 (21.5)
Azathioprine dose (mg), Median (IQR)	125 (100/150)	100 (100/100)	100 (100/150)	100(75/150)
Mychophenolate, n (%)	16 (76.2)	20 (83.3)	42 (53.2)	37 (25.7)
Mychophenolate dose (g), Median (IQR)	2 (2-3)	2 (2-3)	2 (1.5-2.5)	2 (2-3)
Cyclophosphamide, n (%)	2 (19.5)	0 (0)	0 (0)	0 (0)
Methotrexate, n (%)	–	1(4.2)	3 (3.8)	15 (10.4)
Methotrexate dose (mg), Median (IQR)	–	22.5 (20-25)	12.5 (10-15)	17.5 (15-20)

*[†]Baseline clinical characteristics are at the time of the urine sample collection, *Time from LN flare to urine sample collection (months), [#]Remaining 13 patients did not have a KB at the time of the urine sample collection, of whom 3 did not have a prior KB and 10 had a prior KB (9 class III or IV with or without class V and 1 pure class V).*
eGFR, estimated Glomerular Filtration Rate, RRT, Renal Replacement Therapy.

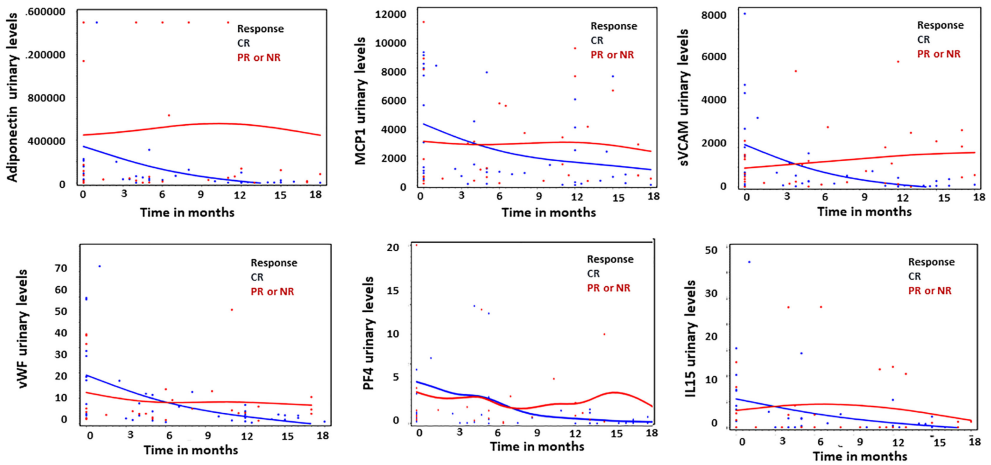

FIGURE 1 | Thumbnail plots illustrating the difference in the amount of urinary biomarkers over time between complete responders (CR; n = 12, blue) and partial or non responders (PR or NR; n = 9, red). Units for all graphs are in pg/μmol, except PF4 expressed in ng/μmol.

of which developed a subsequent flare 2 years later), 3 had chronic proteinuria (all with CKD, 1 of which also had type 2 diabetes mellitus), and 1 had an active urinary tract infection which required antibiotic therapy.

The presence of 3 biomarkers above the established cut-off, irrespective of the combination and the cut-off of sVCAM-1, had excellent specificity, PPV and +LR (**Table 4**). There were only 3 false positives, all with RLN, 2 of whom had their last LN within 2 years of their urine sampling, 1 of whom developed a subsequent flare in the following 2 years. The remaining false positive had an active urinary tract infection. However, this combination had a low NPV (81%) and a -LR above the optimal set point of 0.1 (0.3). Indeed, 6 of 24 ALN patients did not meet this criterion.

A Two-Step Approach Provides the Best Accuracy for Detecting ALN Patients

Based on our results we propose a two-step approach to improve the accuracy of ALN identification (**Figure 3**). In the first step, we propose the following "rule out ALN" criteria. If there are < 2 elevated biomarkers using the lower cut-off for sVCAM-1 (46,000), given the low -LR (0.09) and high NPV (99%), the probability of ALN reduces substantially. For the "rule in ALN" criteria we suggest the following approach. If 2 biomarkers are elevated including the following combinations Adiponectin-MCP-1, Adiponectin-PF4, MCP-1-PF4 and sVCAM-1-MCP-1, given the PPV and +LR, the diagnosis of LN is very probable. On the other hand, if the combination of 2 elevated biomarkers includes sVCAM-1-Adiponectin and sVCAM-1-PF4, then in

TABLE 2 | Logistic regression analysis assessing baseline levels and percentage decrease at 12 ± 3 months as predictors of complete response at month 24 for the urinary biomarkers. N = 21.

	Baseline		% Decrease at month 12	
Biomarkers	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
Adiponectin	1.00 (1.00-1.00)	0.58	NA[1]	NA[1]
MCP-1	1.00 (1.00-1.00)	0.79	NA[2]	NA[2]
sVCAM-1	1.00 (1.00-1.00)	0.53	0.05 (0.006-0.44)	0.007
PF4	0.97 (0.84-1.13)	0.71	0.042 (0.004-0.49)	0.011
vWF	1.02 (0.96-1.08)	0.56	0.045 (0.004-0.54)	0.014
IL-15	1.09 (0.89-1.34)	0.41	0.143 (0.02-0.93)	0.042
Cystatin-C	0.99 (0.97-1.00)	0.21	1.00 (1.00-1.00)	0.94
PAI-1	0.99 (0.99-1.00)	0.23	0.98 (0.96-1.00)	0.13
GM-CSF	1.29 (0.83-1.98)	0.25	1.00 (1.00-1.00)	0.52
Lipocalin	0.99 (0.99-1.00)	0.52	1.00 (1.00-1.00)	0.87
GRO	0.99 (0.99-1.00)	0.69	0.99 (0.99-1.00)	0.36
MMP-7	1.00 (1.00-1.00)	0.72	0.99 (0.99-1.00)	0.86
IL-6	0.99 (0.97-1.02)	0.86	0.99 (0.99-1.00)	0.73
Clusterin	1.00 (1.00-1.00)	0.90	1.00 (0.99-1.00)	0.94
TIMP-1	0.99 (0.98-1.01)	0.92	0.99 (0.99-1.00)	0.14

NA[1] – Not Applicable due to perfect specificity.
NA[2] – Not Applicable due to perfect sensitivity.

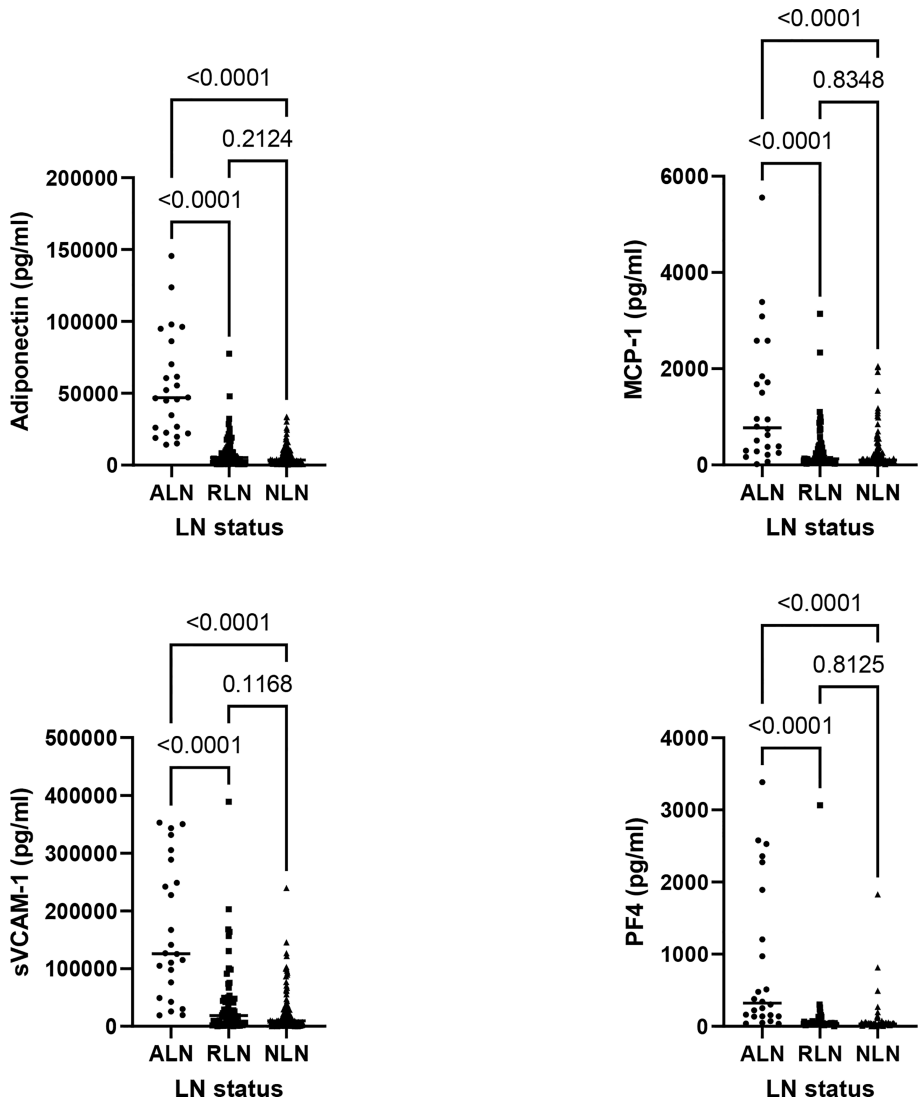

FIGURE 2 | Comparison of biomarker levels between active LN (ALN; n = 24, circles), remission LN (RLN; n = 79, squares) and non LN (NLN; n=144, triangles). For all graphs each symbol represents the determination from a single individual, with the median value for each group indicated by a horizontal line. The Kruskal-Wallis test was used to assess the differences in biomarker levels between ALN, RLN and NLN patients.

order to improve accuracy we suggest increasing the cut-off of sVCAM-1 from 46000 to 103700. If there are 3 or more elevated biomarkers, irrespective of the combination and sVCAM-1 cut-off, taking into consideration the high PPV (96.9%) and +LR (37.3), LN is very likely. Urinary tract infections should be ruled out, as they may cause false positive results.

Our Proposed "Rule Out ALN Criteria" at 12 Months Following ALN Flare Predicts Response to Treatment at 24 Months

In the first stage of the study, we determined that the percentage decrease of Adiponectin, MCP-1, sVCAM-1, PF4, vWF and IL-15 after 12 ± 3 months of treatment predicted response to therapy at 24 months. In order to evaluate if our rule out criteria (presence of < 2 elevated urinary biomarkers, with sVCAM-1 cut-off of 46,000) could also serve as a predictor of response to treatment we analyzed a subpopulation of the cross-sectional cohort who´s urine sample was collected at 12 ± 3 months after their LN flare. Of the twenty-two patients in the, analysis, 12 had < 2 elevated biomarkers, with 11 achieving a CR at 24 months. In contrast only 4 out of 10 patients with ≥ 2 elevated biomarkers achieved a CR (p= 0.02, Fisher´s exact test). The operating characteristics for this subpopulation analysis were as follows: sensitivity (73.3 [95%CI 44.9-92.2]), specificity (85.7 [95%CI 42.1-99.6]), PPV (91.7 [95%CI 61.5-99.8]) and NPV (60.0 [95%CI 26.2-87.8]).

TABLE 3 | Operating characteristics for individual biomarkers.

Biomarkers	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	+LR (95% CI)	-LR (95% CI)
Adiponectin (18000 pg/ml)	91.7 (73.0-99.0)	90.9 (86.3-94.3)	52.4 (36.4-68.0)	99.0 (96.5-99.9	10 (6.50-16)	0.09 (0.02-0.35)
MCP-1 (1341 pg/ml)	37.5 (18.8-59.4)	97.3 (94.2-99.0)	60.0 (32.3-83.7)	93.5 (89.5-96.3)	14 (5.40-36)	0.64 (0.47-0.88)
PF4 (134 pg/ml)	83.3 (62.6-95.3)	93.7 (89.7-96.5)	58.8 (40.7-75.4)	98.1 (95.2-99.5)	13 (7.72-23)	0.18 (0.07-0.44)
sVCAM-1 (46000 pg/ml)	79.2 (57.9-92.9)	81.1 (75.3-86.0)	31.2 (19.9-44.3)	97.3 (93.8-99.1)	4.2 (2.9-5.9)	0.26 (0.12-0.56)
sVCAM-1 (103700 pg/ml)	66.7 (44.7-84.4)	95.5 (91.9-97.8)	61.5 (40.6-79.8)	96.4 (93.0-98.4)	15 (7.6-29)	0.35 (0.20-0.62)

PPV, Positive Predictive Value, NPV, Negative Predictive Value, +LR, Positive likelihood ratio, -LR, Negative likelihood ratio.

Our "Rule in ALN" Criteria Operate Better for Proliferative ALN

To corroborate the sensitivity of our "rule in ALN" criteria established in the cross-sectional cohort and determine if they operate similarly for proliferative and non-proliferative LN classes, we measured urinary Adiponectin, MCP-1, sVCAM and PF4 in a biopsy-proven ALN cohort. A total of 53 patients were included, of whom 35 had proliferative LN and 18 non-proliferative class (4 with class V and chronic proliferative LN and 14 with pure II or V LN classes). **Supplementary Table 2** shows their demographic and clinical characteristics at the time of the urine collection. As seen in **Table 5**, the sensitivity of our "rule in ALN" criteria was similar for the group of proliferative ALN, 91.4% for the presence of 2 or more elevated biomarkers (higher sVCAM cut-off of 103,700) and 77.1% for the presence of 3 or more elevated biomarkers (sVCAM cut-off of 46,000). However, the sensitivities were much lower for the non-proliferative classes, suggesting that our "rule in ALN" criteria work better for proliferative LN.

TABLE 4 | Operating characteristics for different combinations and number of elevated urinary biomarkers to accurately detect ALN patients.

Biomarkers	Operating characteristics calculated using sVCAM cut-off of 46000					
	Sensitivity (95% CI)	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**+LR (95% CI)**	**-LR (95% CI)**
2 Elevated biomarkers* Different combinations	91.7 (73.0-99.0)	90.1 (85.4-93.7)	50.0 (34.6-65.4)	99.0 (96.5-99.9)	9.25 (6.11-14)	0.09 (0.02-0.35)
Adiponectin-MCP-1	33.3 (15.6-55.3)	100 (98.4-100)	100 (63.1-100)	93.3 (89.3-96.1)	NA	0.67 (0.50-0.88)
Adiponectin-PF4	75.0 (53.3-90.2)	97.3 (94.2-99.0)	75.0 (53.3-90.2)	97.3 (94.2-99.0)	28 (12-63)	0.26 (0.13-0.51)
MCP-1-PF4	37.5 (18.8-59.4)	100 (98.4-100)	100 (66.4-100)	93.7 (89.8-96.4)	NA	0.62 (0.46-0.84)
sVCAM-1- Adiponectin	70.8 (48.9-87.4)	93.2 (89.1-96.2)	53.1 (34.7-70.9)	96.7 (93.4-98.7)	10 (6.04-4.18)	0.31 (0.17-0.58)
sVCAM-1- MCP-1	33.3 (15.6-55.3)	99.1 (96.8-99.9)	80.0 (44.4-97.5)	93.2 (89.2-96.1)	37 (8.33-164)	0.67 (0.51-0.89)
sVCAM-PF4	70.8 48.9-87.4)	96.9 (93.6-98.7)	70.8 (48.9-97.4)	96.9 (93.6-98.7)	22 (10-49)	0.30 (0.16-0.56)
3 Elevated biomarkers# Different combinations	70.8 (48.9-87.4)	98.2 (95.5-99.5)	81.0 (58.1-94.6)	96.9 (93.7-98.7)	39 (14-107)	0.30 (0.16-0.55)
Adiponectin-MCP-1-sVCAM-1	29.2 (12.6-51.1)	100 (98.4-100)	100 (59.0-100)	92.9 (88.9-95.8)	NA	0.71 (0.54-0.91)
Adiponectin-MCP-1-PF4	33.3 (15.6-55.3)	100 (98.4-100)	100 (63.1-100)	93.3 (89.3-96.1)	NA	0.67 (0.50-0.88)
MCP-1-sVCAM-1-PF4	33.3 (15.6-55.3)	100 (98.4-100)	100 (63.1-100)	93.3 (89.3-96.1)	NA	0.67 (0.50-0.88)
	Operating characteristics calculated using sVCAM cut-off of 103700					
Biomarkers	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**+LR (95% CI)**	**-LR (95% CI)**
2 Elevated biomarkers* Different combinations	87.5 (67.6-97.3)	94.6 (90.8-97.2)	63.6 (45.1-79.6)	98.6 (95.9-99.7)	16.2 (9.15-29)	0.13 (0.05-0.38)
sVCAM-1-Adiponectin	58.3 (36.6-44.9)	97.8 (94.8-99.3)	73.7 (48.8-90.9)	95.6 (92.1-97.9)	26 (10-66)	0.43 (0.27-0.68)
sVCAM-1-MCP-1	33.3 (15.6-55.3)	99.1 (96.8-99.9)	80.0 (44.4-97.5)	93.2 (89.2-96.1)	37 (8.33-164)	0.67 (0.51-0.89)
sVCAM-1-PF4	62.5 (40.6-81.2)	98.7 (96.1-99.7)	89.3 (59.6-96.4)	96.1 (92.6-98.2)	46 (14-148)	0.38 (0.23-0.64)
3 Elevated biomarkers# Different combinations	62.5 (40.6-81.2)	99.1 (96.8-99.9)	88.2 (63.6-98.5)	96.1 (92.7-98.2)	69 (17-285)	0.38 (0.23-0.63)
Adiponectin-MCP-1-sVCAM-1	29.2 (12.6-51.1)	100 (98.4-100)	100 (59.1-100)	92.9 (88.9-95.8)	NA	0.71 (0.54-0.91)
MCP-1-sVCAM-1-PF4	33.3 (15.6-55.3)	100 (98.4-100)	100 (63.1-100)	93.3 (89.3-96.1)	NA	0.67 (0.50-0.88)

**Any 2 of the 4 biomarkers elevated, #Any 3 of the 4 biomarkers elevated. PPV, Positive Predictive Value; NPV, Negative Predictive Value; +LR, Positive likelihood ratio; -LR, Negative likelihood ratio; NA, Not Applicable due to perfect specificity.*

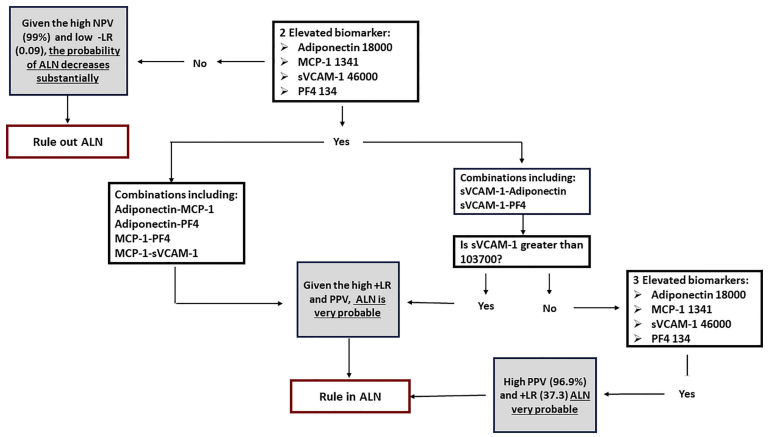

FIGURE 3 | Proposed 2 step approach for the detection of ALN patients.

DISCUSSION

Given the invasive nature of the KB, the current gold standard for LN diagnosis, and the known drawbacks of proteinuria, the most commonly used parameter for LN surveillance, there is a tremendous need for biomarkers that accurately identify active LN cases. In this study we identified 4 urinary biomarkers that not only discriminate between ALN and non-ALN patients, but also have the capacity to reflect clinical improvement, and we have proposed "rule out" and "rule in" criteria that accurately detect proliferative ALN patients. Importantly, this can be accomplished relatively inexpensively using conventional ELISAs at an approximate cost of only 6.90 CAD per sample to perform all 4 urinary biomarkers. All 4 biomarkers, Adiponectin, MCP-1, sVCAM-1 and PF4, have been previously proposed by our group and others as potential biomarkers for ALN (15–24). In addition, prior studies have shown that all 4 analytes correlate with the activity index in the kidney biopsy (16, 29, 30), suggesting that they play a role in LN pathogenesis.

Higher levels of serum Adiponectin have been found in ALN (15, 31). While the pathogenic role of Adiponectin in LN is still unclear, several studies support an anti-inflammatory (32) and even reno-protective (33) action. However, it has been shown that the low molecular weight isoform of Adiponectin, has pro-inflammatory properties (34–37). This finding suggests that under inflammatory conditions the predominant isoform could shift converting Adiponectin's action from anti-inflammatory to proinflammatory.

MCP-1 is induced by type I interferons and multiple pro-inflammatory cytokines (38). MCP-1 has potent chemiotactic activity, especially for macrophages and neutrophils (39–41), and thus may act to promote leucocyte recruitment to the kidney. Consistent with this possibility, it was shown to increase prior to proteinuria in LN flares (42) and higher levels are associated with worse clinical outcomes (20, 42–45).

Serum and urinary levels of sVCAM-1, a surrogate marker for endothelial expression of VCAM and endothelial activation (46, 47), have been shown to be elevated in SLE and to correlate with overall disease activity and the presence and severity of LN (29, 30, 48–51). sVCAM-1 can also serve as a chemotactic stimulus for monocytes (52) and T lymphocytes (53). Hence, sVCAM-1 may be responsible for the recruitment, adhesion and transmigration of multiple phagocyte cells to the kidney (54).

PF4 has been implicated as a possible urinary biomarker for LN in several studies (16, 21). PF4 is mainly released by activated platelets and is an inflammatory response mediator with potent chemotactic, especially for monocytes and neutrophils (55–57)

In the first stage of our study, 4 urinary biomarkers were chosen from 15 studied analytes based on their property to decrease with therapy and accurately discriminate between complete responders and non-responders to therapy. These

TABLE 5 | Sensitivity of our "rule in ALN" criteria in a biopsy-proven ALN cohort.

	Sensitivity (95%CI) using sVCAM-1 cut-off of 46000 pg/ml		
	Whole cohort (N = 53)	**Proliferative LN (N = 35)**	**Non-Proliferative LN (N = 18)**
2 Elevated biomarkers	81.1 (68.0-90.6)	91.4 (76.9-98.2)	61.1 (35.8 – 82.7)
3 Elevated biomarkers	66.0 (51.3-78.8)	77.1 (59.9-89.6)	55.6 (30.8-78.5)
	Sensitivity (95%CI) using sVCAM-1 cut-off of 103,700 pg/ml		
2 Elevated biomarkers	79.3 (65.9-89.2)	91.4 (76.9-98.2)	55.6 (30.8-78.5)
3 Elevated biomarkers	56.6 (42.3-70.2)	62.9 (44.9 – 78.5)	55.6 (30.8-78.5)

results were further validated when we analyzed the subpopulation of 22 patients from the cross-sectional cohort who were diagnosed with ALN 12 months prior to the urine collection, where most of the patients who achieved CR at 24 months had < 2 elevated biomarkers (our "rule out" ALN criteria). Our results are in accordance with the study by Brunner et al, where they showed that decreased levels of several urinary biomarkers, including Adiponectin and MCP-1, could predict treatment responses (58). However, in contrast to our study they found that several of these could predict responses, as early as 3 months following initiation of therapy. There were 2 key differences between that study and ours. Firstly, Brunner et al. studied LN in children and young adults, and secondly, many of their patients were treated with cyclophosphamide, whereas the majority of our patients were treated with mycophenolate. It is likely that these treatment differences explain the differences in time course for the response of the biomarkers to therapy, as shown in a sub-analysis of their data in which they contrasted patients treated with cyclophosphamide and mycophenolate, where the mycophenolate data showed similar delayed responses to those seen with our patients.

In the second stage of the study, based on the operating characteristics from our established cut-offs and the number of elevated biomarkers needed to accurately detect ALN cases, we propose a 2-step approach for the classification of ALN. Our "rule out" ALN criteria had an excellent NPV and -LR. The operating characteristics for the "rule in ALN" criteria were also very good. Even though the CI for the PPV´s were relatively wide, which could be a consequence of the small sample size of ALN patients, the lower limit of the CI of the +LR´s were all close to or above 10, indicating that the presence of LN is very likely.

The "rule out ALN" criteria included 2 ALN patients, both of whom started their flare 12 months prior to the sample collection and achieved CR at year 2. As was shown in **Figure 1**, most of the patients that demonstrated a CR at year 2, had normalized their urinary biomarker levels at year 1, thus, it is not surprising that these 2 ALN patients were not detected by our test. Furthermore, it has been demonstrated that up to 60% of patients who are still clinically active (proteinuria) have no histologic activity in repeat kidney biopsies, hence it's possible that these 2 patients that were included in our ALN group based on our ALN criteria were instead inactive, although without a kidney biopsy we can only speculate.

Most of the false positives for the "rule in ALN" criteria were patients with RLN who had a relatively recent LN flare (2 years prior), subsequent flares, or chronic proteinuria that was interpreted by their physician as secondary to damage. Prior studies have demonstrated that up to 30% of the patients who achieve clinical remission after induction therapy will continue with histologic activity on repeat kidney biopsies (14) and that active histologic findings may continue for up to 2 years or longer after a LN flare, even in the absence of significant proteinuria (59). Hence, it is possible that these 'false positive' patients had ongoing kidney inflammation. It is notable that our cohort included 9 patients with chronic proteinuria and 2 more with CKD (with no proteinuria), and only 3 (27%) of these met the "rule in criteria", suggesting that in general, elevated levels of our urinary biomarkers do not simply reflect kidney damage and may add relevant information to proteinuria for the detection of active LN. Given the lack of a KB at the time of the urine collection, we cannot definitively determine if the elevated biomarkers in these cases reflect kidney inflammation or are true false positives.

Recent data indicate that a KB performed two years after the LN flare can provide important clinical information, with residual kidney inflammatory activity forecasting subsequent LN flares (59). Our findings indicate that a subset of patients with RLN have elevated levels of inflammatory factors and that some of these will develop a subsequent flare. It will be important to correlate our urinary biomarkers with repeat KBs to determine if these biomarkers could serve as potential surrogates for ongoing kidney activity, which could eliminate the need for a repeat KB.

In addition to RLN, 3 patients with NLN met the "rule in ALN" criteria. Of these, 2 had high levels of sVCAM-1, and had high titers of antiphospholipid antibodies, one with antiphospholipid syndrome (APS) and recurrent thrombosis, and the other with relapsing episodes of vasculitis. Increased expression of soluble adhesion molecules, including sVCAM-1, has been demonstrated in patients with APS (60). Furthermore, sVCAM-1 has been suggested as a prognostic marker of clinical complications in APS including abortions, repeated thrombosis and kidney involvement (60, 61), which could account for the elevated levels of this urinary biomarker in these patients. Another important aspect to consider when using the "rule in criteria" is the presence of urinary infections that could certainly cause false positives and should be ruled out.

As the cohorts for stage 1 and 2 of the study included predominately proliferative ALN patients, we further validated the sensitivity of our "rule in ALN" criteria in a biopsy-proven ALN cohort, of whom around 30% had non-proliferative classes. The sensitivity of our criteria was similar in the proliferative group but substantially lower for the non-proliferative classes. These results are not surprising considering that the pathogenic role of all 4 biomarkers is more in keeping with proliferative LN. In addition, we and other groups have shown that all 4 analytes correlate with the activity index in the kidney biopsy (16, 29, 30). A strength of our study is that we validated the proposed biomarkers in three independent cohorts and by 2 different methods of detection, demonstrating the reproducibility of their discriminatory and predictive abilities. In addition, the unbiased patient recruitment of the cross-sectional cohort reflected a real-life scenario, with LN patients at different stages of their flare, which allowed us to create cut-offs that may be more sensitive to detect ongoing lower grade activity.

This study has several limitations. The sample size of ALN patients in our second cross-sectional cohort was small and the majority of the ALN patients in both cohorts had proliferative LN. A further limitation is the lack of KBs at the time of the urine collection for many of the LN patients, making it difficult to conclude that false negative patients lacked renal inflammation and conversely that false positive patients were truly false positives. To account for these limitations, we further validated

the sensitivity of our "rule in ALN" criteria in a biopsy-proven ALN cohort that included non-proliferative ALN patients. We recognize that our cut-offs and criteria to detect ALN need to be externally validated in an independent cohort.

In summary our results provide further evidence to support the role of Adiponectin, MCP-1, sVCAM-1 and PF4 in the detection of proliferative ALN cases. In addition, we show the clinical utility of measuring multiple rather than a single biomarker. Finally, we propose a novel "rule in" and "rule out" approach for the detection of proliferative ALN with excellent operating characteristics which may provide additional information beyond proteinuria for the detection of proliferative ALN patients and monitoring the response to treatment.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Board of the University Health Network. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PF, ZT, KG, LW-G, and JW were responsible for study conception and design. LW-G, KG, MK, DB, DG, MU, ZT, PF, and JW were responsible for the acquisition of data. EA, LW-G, and JW performed the data analysis and interpretation. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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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.2022.889931/full#supplementary-material

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Screening Biomarkers for Systemic Lupus Erythematosus Based on Machine Learning and Exploring Their Expression Correlations With the Ratios of Various Immune Cells

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Background: Systemic lupus erythematosus (SLE) is an autoimmune illness caused by a malfunctioning immunomodulatory system. China has the second highest prevalence of SLE in the world, from 0.03% to 0.07%. SLE is diagnosed using a combination of immunological markers, clinical symptoms, and even invasive biopsy. As a result, genetic diagnostic biomarkers for SLE diagnosis are desperately needed.

Method: From the Gene Expression Omnibus (GEO) database, we downloaded three array data sets of SLE patients' and healthy people's peripheral blood mononuclear cells (PBMC) (GSE65391, GSE121239 and GSE61635) as the discovery metadata (n_{SLE} = 1315, n_{normal} = 122), and pooled four data sets (GSE4588, GSE50772, GSE99967, and GSE24706) as the validate data set (n_{SLE} = 146, n_{normal} = 76). We screened the differentially expressed genes (DEGs) between the SLE and control samples, and employed the least absolute shrinkage and selection operator (LASSO) regression, and support vector machine recursive feature elimination (SVM-RFE) analyze to discover possible diagnostic biomarkers. The candidate markers' diagnostic efficacy was assessed using the receiver operating characteristic (ROC) curve. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was utilized to confirm the expression of the putative biomarkers using our own Chinese cohort (n_{SLE} = 13, n_{normal} = 10). Finally, the proportion of 22 immune cells in SLE patients was determined using the CIBERSORT algorithm, and the correlations between the biomarkers' expression and immune cell ratios were also investigated.

Results: We obtained a total of 284 DEGs and uncovered that they were largely involved in several immune relevant pathways, such as type I interferon signaling pathway, defense response to virus, and inflammatory response. Following that, six candidate diagnostic

biomarkers for SLE were selected, namely ABCB1, EIF2AK2, HERC6, ID3, IFI27, and PLSCR1, whose expression levels were validated by the discovery and validation cohort data sets. As a signature, the area under curve (AUC) values of these six genes reached to 0.96 and 0.913, respectively, in the discovery and validation data sets. After that, we checked to see if the expression of ABCB1, IFI27, and PLSCR1 in our own Chinese cohort matched that of the discovery and validation sets. Subsequently, we revealed the potentially disturbed immune cell types in SLE patients using the CIBERSORT analysis, and uncovered the most relevant immune cells with the expression of ABCB1, IFI27, and PLSCR1.

Conclusion: Our study identified ABCB1, IFI27, and PLSCR1 as potential diagnostic genes for Chinese SLE patients, and uncovered their most relevant immune cells. The findings in this paper provide possible biomarkers for diagnosing Chinese SLE patients.

Keywords: machine learning, diagnostic biomarker, systemic lupus erythematosus, immune cell disturbance, CIBERSORT

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease that primarily affects the adolescent and menopausal women (1). The clinical manifestations of SLE are heterogeneous, involving the blood, kidney, nerve and some other systems, making the treatment and management difficult and complicated (2). Currently, the Chinese hospitals often adopt European and American (EULAR/ACR) diagnostic guidelines for SLE diagnosis (3, 4), whereas the molecular profiling of SLE is highly heterogeneous among different races and locations (5, 6). Consequently, the specificity and sensitivity of diagnostic indicators recommended by the EULAR/ACR need to be validated further in Chinese population.

Nowadays, the diagnosis of SLE is primarily based on a series of clinical manifestations and laboratory indicators, such as skin erythema, arthralgia, complement C3, C4, and anti-dsDNA antibodies, etc. (7–11). SLE must be diagnosed based on clinical signs along with multiple immunological indicators. When patients were diagnosed as SLE, they often had occurred a certain degree of systemic involvements (12). Even an invasive biopsy is required to diagnose lupus nephritis (13). Because of the complexity, lag, and invasiveness of current SLE diagnostic methods, researchers are looking for new genetic biomarkers in the hopes of developing a simpler, faster, and more objective diagnostic "gold standard" than current markers/tests, without the need for clinical symptoms, especially in the Chinese population.

Until now, immune cells such as lymphocytes, dendritic cells, macrophages, basophils, and neutrophils have been identified to be disrupted in the course of SLE (14–17). There are differences in the disturbed cell types and proportions between SLE individuals (18–21). The identification of altered immune cells in SLE aids us in understanding the cellular results of the disease and establishing an appropriate diagnostic or treatment strategy.

In this study, we used the Gene Expression Omnibus (GEO) database to obtain three expression microarray datasets of SLE patients' and healthy people's peripheral blood mononuclear cells (PBMC). Then, we pooled the three datasets as a metadata cohort (n_{SLE} = 1315, n_{normal} = 122) and looked for the differentially expressed genes (DEGs) between SLE and controls. Next, we identified the diagnostic biomarkers of SLE using different machine learning algorithms. Following that, we merged four GEO data sets as the validation data (GSE4588, GSE50772, GSE99967, and GSE24706; n_{SLE} = 146, n_{normal} = 76) and confirmed the expression of the identified diagnostic biomarkers, then used logistic regression to develop a diagnostic prediction model. Furthermore, we validated the expression of the candidate biomarkers using our own Chinese cohort (n_{SLE} = 13, n_{normal} = 10). Moreover, we used the CIBERSORT algorithm to quantify the proportion of 22 immune cells in PBMC of SLE patients and healthy people. Finally, we explored the relationship between the expression of the identified biomarkers and the ratios of immune cells in PBMC of SLE patients.

MATERIALS AND METHODS

Data Download and Processing

Seven expression matrix files of SLE PBMC samples were downloaded from the GEO database, namely GSE65391, GSE121239, GSE61635, GSE4588, GSE50772, GSE99967, and GSE24706. The GSE65391 dataset contained 924 SLE and 72 control samples, the GSE121239 dataset contained 292 SLE and 20 control samples, GSE61635 dataset contained 99 SLE and 30 control samples, the GSE4588 dataset contained 15 SLE and 19 control samples, the GSE50772 dataset contained 61 SLE and 20 control samples, the GSE99967 dataset contained 42 SLE and 17 control samples, and GSE24706 dataset contained 28 SLE and 20 control samples. Then, the three files GSE65391, GSE121239, and GSE61635 were pooled into a metadata cohort for the following analysis, after the batch effects were removed using the R package "SVA". In addition, the four data sets (GSE4588, GSE50772, GSE99967, and GSE24706) were also merged as the verification data after normalization.

DEGs Screening and Functional Analysis

The DEGs were screened out using the R package "limma" based on the metadata cohort's data set. The heat map showing the

expression of DEGs was depicted using the R package. Next, the DAVID database was used to analysis the functions of the DEGs based on gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. In addition, the R package "clusterProfiler" and "DOSE" were used for the Disease ontology (DO) enrichment analyses. The gene set enrichment analysis (GSEA) was used to distinguish the most significant functional items between SLE and controls. The gene set "c2.cp.kegg.v7.0.symbols.gmt" from the Molecular Signatures Database (MSigDB) (22) was selected as the reference gene set.

Identification and Verification of the Diagnostic Markers

Two algorithms, namely least absolute shrinkage and selection operator (LASSO) logistic regression and support vector machine-recursive feature elimination (SVM-RFE), were used to screen the potential SLE diagnostic markers. The "glmnet" R package was used to perform the LASSO analysis to identify the optimal diagnostic markers in SLE, and the SVM-RFE was used to find the optimal variables. The candidate diagnostic markers were picked by intersecting the markers identified by these two algorithms. Then, the expression of the candidate diagnostic biomarkers were verified based on the merged dataset containing GSE4588, GSE50772, GSE99967, and GSE24706.

The Diagnostic Efficacy of the Biomarkers in SLE

To examine the diagnostic efficacy of the candidate markers, the receiver operating characteristic (ROC) curves were drawn based on the discovery metadata and the validation data set.

Patients

A total of 23 whole blood samples (including 13 SLE samples and 10 control samples) were collected from Shenzhen People's Hospital. Patients diagnosed as SLE, with a SLE disease activity index (SLEDAI) score more than 5 were included. The clinical manifestations of all patients were shown in **Table 1**. All participants volunteered to participate in this research. This work was approved by the Ethics Committee of Shenzhen People's Hospital (LL-KY-2021393). After collecting all of the whole blood samples, the PBMCs were isolated, and dissolved in Trizol (Beyotime, R-0016), and then stored in -80 °C.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The total RNA was isolated from the PBMC according to the manufacturer's instructions. The transScript all-in-one first-strand cDNA synthesis superMix for qPCR (One-step gDNA removal) kit (TransGen Biotech, AT341-01) was used for the reverse transcription of mRNA. After that, the qPCR assays were conducted out with the PerfectStart Green qPCR SuperMix kit (TransGen Biotech, AQ601-02), with the following primers: GAPDH (Forward: 5-TGACTTCAACAGCGACACCCA-3. Reverse: 5-CACCCTGTTGCTGTAGCCAAA-3). ABCB1 (Forward: 5-GTCTGGACAAGCACTGAAAGATAAGA-3. Reverse: 5- CAACGGTTCGGAAGTTTTCTATTGC-3). EIF2AK2 (Forward: 5- AGAAGGCGGAGCGTGAAGTAAAAG-3. Reverse: 5- ATCCATCCCAACAGCCATTGTAGTG-3). HERC6 (Forward: 5- CCTGCCAAGCCTAAACCTGAGAAG-3. Reverse: 5- TACAGAGCCAGTGGGAAAGGAAGG-3). ID3 (Forward: 5-GGACGACATGAACCACTGCT-3. Reverse: 5-TCAGTGGCAAAAGCTCCTTT-3). IFI27 (Forward: 5-TCTG CAGTCACTGGGAGCAA-3. Reverse: 5-CCCAATGGAGC CCAGGAT-3), PLSCR1 (Forward: 5-CCTCAGTATCCACCG ACAGCATTC-3. Reverse: 5-ACTGGCTGATTTGGGACAGG AAAG-3). All the primers were synthesized by the Sangon Biotech Company. Among them, GAPDH was an internal reference gene. The gene relative expressions were calculated by the 2-ΔΔCT method. A p value < 0.05 was considered statistically significant.

Immune Cell Composition

The CIBERSORT algorithm (23) was used to calculate the ratios of various immune cells in PBMC of SLE patients and healthy people based on the expression matrix, and the R package "vioplot" was used to visualize the proportions of 22 immune cells in SLE and control groups. The "corrplot" package was used to create the heat map displaying the quantitative correlation between distinct immune cells. In addition, the "ggplot2" R package was utilized to investigate the association between the expression of the diagnostic markers and the ratios of immune cells.

RESULTS

Screening the DEGs Between the SLE PBMC Versus Control PBMC

After batch effects were removed using the R package "SVA," we combined three expression array data sets collected from the GEO database (GSE65391, GSE121239, and GSE61635) into one discovery data set, which included data from 1315 SLE patients and 122 healthy people. We used principal component analysis (PCA) to cluster all of the samples and discovered that each sample's point was randomly distributed, indicating that the normalization was done correctly (**Supplementary Figure S1**). As a consequence, 284 genes were discovered to be differentially expressed in the PBMC of SLE patients versus healthy people (p value < 0.0001, fold change > 1.5) (**Figure 1A**). We used the heat map showing the expression of the DEGs in each healthy people and SLE patient (**Figure 1B**). Through an enrichment analysis of the DEGs, we found that these DEGs were enriched in some typical autoimmune-disease-relevant pathways, such as type I interferon signaling pathway, innate immune response, inflammatory response, and systemic lupus erythematosus (**Figures 1C–F**). In addition, we conducted the DO enrichment analyses of the DEGs, and discovered that the DEGs were primarily involved in several immune related diseases, namely bacterial infectious disease, hematopoietic system disease, human immunodeficiency virus infectious disease, and rheumatic disease, etc. (**Figure 1G**). Furthermore, the GSEA results showed that the DEGs in SLE patients were mainly involved with chemokine signaling pathway, complement and coagulation cascades, cytoplasmic DNA sensing

TABLE 1 | The clinical information of the 13 SLE patients and 10 healthy people in our study.

	SLE (n = 13)	Healthy people (n = 10)
Gender (female/male)	11/2	9/1
Age (year), median (range)	38 (15-57)	31 (26-37)
C3(g/L), median (range)	0.76 (0.21-0.85)	N/A
C4(g/L), median (range)	0.14 (0.03-0.28)	N/A
ANA(+) (percentage)	13 (100%)	N/A
Anti-dsDNA(+) (percentage)	10 (76.9%)	N/A
ANuA(+) (percentage)	9 (69.2%)	N/A
Proteinuria (g/24 h), median (range)	0.363 (0.045-10.98)	N/A
SLEDAI-2K, median (range)	9 (6-21)	N/A
Rash (percentage)	3 (23.1%)	N/A
Alopecia (percentage)	2 (15.4%)	N/A
Fever (percentage)	4 (30.8%)	N/A
Pleurisy (percentage)	3 (23.1%)	N/A
Leukopenia (percentage)	4 (30.8%)	N/A
Pericarditis (percentage)	3 (23.1%)	N/A
Arthritis (percentage)	2 (15.4%)	N/A
Organic encephalopathy syndrome (percentage)	1 (7.7%)	N/A
Hematuria (percentage)	1 (7.7%)	N/A
Thrombocytopenia (percentage)	2 (15.4%)	N/A
Lupus headache (percentage)	1 (7.7%)	N/A
Vasculitis (percentage)	2 (15.4%)	N/A

SLE, systemic lupus erythematosus; ANA, anti-nuclear antibody; Anti-dsDNA, anti-dsDNA antibodies; ANuA, anti-nucleosome antibodies; C3, Complement C3; C4, Complement C4; N/A, not applicable; SLEDAI-2K, SLE disease activity index 2000.

pathway, RIG-I-like receptor signaling pathway, and Toll-like receptor signaling pathway (**Figures 1H, I**).

Identification of the Potential Diagnostic Biomarkers for SLE Based on Machine Learning

Then, we adopted two machine learning algorithms to screen the biomarkers for SLE, namely LASSO regression and SVM-RFE algorithm. As a result, the LASSO regression algorithm revealed 70 probable biomarkers, while the SVM-RFE approach identified 37 (**Figures 2A, B**). After that, we made an intersection of the 70 and 37 probable biomarkers to arrive at 14 common biomarkers (**Figure 2C**; **Supplementary Table S1**). To further confirm the reliability of these biomarkers, we verified their expressions based on the validation data set (n_{SLE} = 146, n_{normal} = 76). The result showed that 10 genes harbored the similar expression trend with statistical significance in both discovery and validation data sets, including ABCB1, EIF2AK2, HERC6, PLSCR1, ID3, IFI27, SCRN1, CD160, HSP90AB1, and PCYOX1. Among these 10 genes, we selected ABCB1, EIF2AK2, HERC6, PLSCR1, ID3, and IFI27 for the following study because the statistical differences of their expression was most significant in both the discovery and validation sets (**Figures 2D–I**).

The Diagnostic Efficacy of the Six Candidate Biomarkers for SLE

Subsequently, we plotted the ROC curves for the six candidate biomarkers, and found that ABCB1, EIF2AK2, HERC6, ID3, IFI27, and PLSCR1 all had a good diagnostic effect in the discovery data set, with an AUC value 0.839, 0.912, 0.894, 0.891, 0.902, and 0.907, respectively. When we used the six markers as one single signature, the AUC value reached 0.96 (**Figure 3A**). On the other hand, we also verified the diagnostic effect of the six genes in the validation data set, and discovered that the AUC values of the six biomarkers, including ABCB1, EIF2AK2, HERC6, ID3, IFI27, and PLSCR1, were 0.754, 0.854, 0.81, 0.731, 0.875, and 0.851, respectively. When the six markers were combined as one single signature, the AUC value reached 0.913 (**Figure 3B**). The result suggests that the six genes have a good diagnostic power for SLE, and the power is higher when they are used together.

The Validation of the Potential Diagnostic Markers Using Our Own Chinese Cohort Revealed that ABCB1, IFI27 and PLSCR1 Were More Likely to be SLE Biomarkers for the Chinese Population

As mentioned above, SLE is highly heterogeneous among different races and regions (24). To ensure that the biomarkers we screened could be applied in the Chinese population, we further performed the RT-qPCR assays on the expression of these six markers in the PBMC of 13 Chinese SLE patients and 10 Chinese healthy controls (**Supplementary Table S1**). Consistently, the mRNA expression level of ABCB1 in PBMC of SLE patients was significantly lower than that of healthy controls (**Figure 4A**). SLE patients had considerably greater levels of IFI27 and PLSCR1 expression than healthy controls (**Figures 4B, C**). Meanwhile, we found that the expression differences of EIF2AK2, HERC6 and ID3 in SLE patients and healthy controls followed the same pattern as the discovery and validation cohorts (though the difference was not statistically significant) (**Figures 4D–F**). Thus, these results indicate that our findings are reproducible, and ABCB1, IFI27 and PLSCR1 are more likely to be SLE biomarkers in the Chinese population. Notably, ABCB1 is a novel found biomarker for SLE that has not yet to be published, to the best of our knowledge.

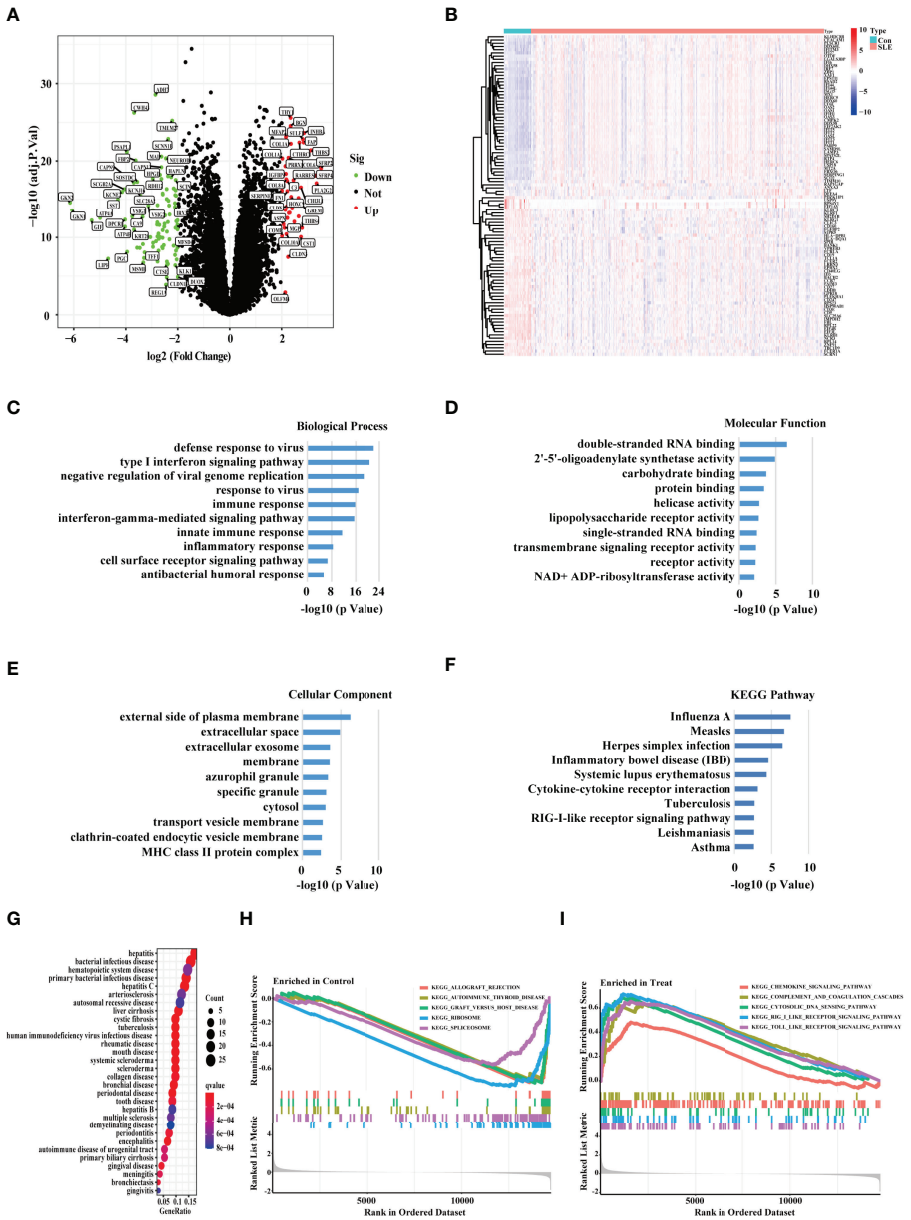

FIGURE 1 | Screening the DEGs between the SLE PBMC versus Control PBMC. **(A)** Volcano plot showing the DEGs between the PBMC of SLE and control samples based on the metadata set including GSE65391, GSE121239, and GSE61635. Green represented down-regulation, and red represented up-regulation (SLE/Control). **(B)** The heat map showing the DEGs in each sample of SLE patients and healthy people. "Con" represented control samples, and "SLE" represented SLE patients. **(C–E)** The GO, **(F)** KEGG, and **(G)** DO enrichment analysis of the DEGs. The size of the dots represented the number of genes in each enriched module. The color of the dots represented the q value. **(H–I)** The GSEA analysis revealing the enriched pathways in the PBMC of SLE and control samples.

The Ratio Changes of Immune Cells in SLE Patients, and Their Correlation With the Expression of ABCB1, IFI27 and PLSCR1

The onset of SLE causes changes in the proportion and function of a series of immune cells. To find the biomarkers whose expression were correlated with the proportions of immune cells, we first analyzed the ratio changes of 22 immune cells in 1315 SLE patients and 122 healthy people using the CIBERSORT algorithm. The results showed that compared with the control group, the proportions of naive B cells, CD8 T cells, CD4 memory resting T cells, CD4 memory activated T cells, and resting natural killer (NK) cells in SLE were significantly lower, while monocytes, macrophages M0, activated dendritic cells, neutrophils were higher (**Figure 5A**). Consistently, a number of previous studies have also demonstrated that the ratio of

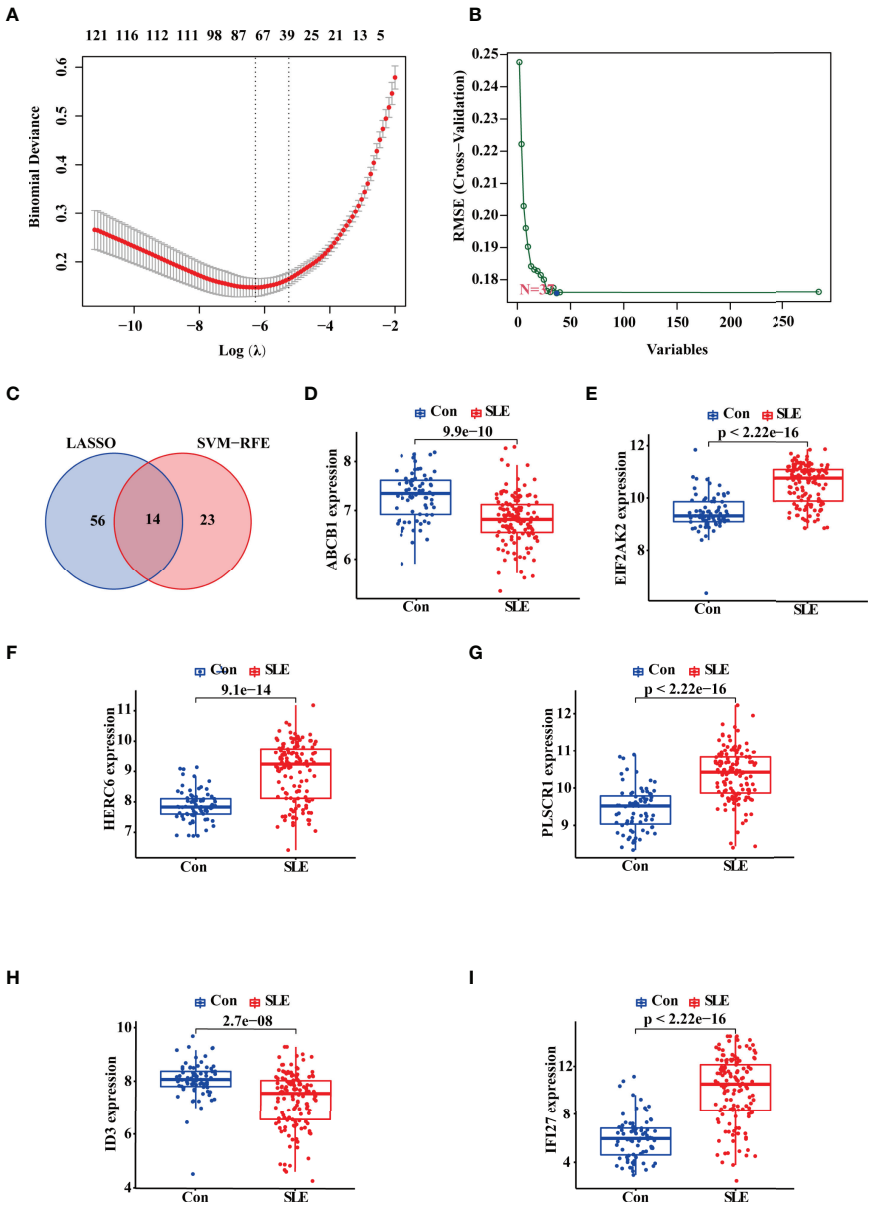

FIGURE 2 | Identification and Validation of the Diagnostic Biomarkers for SLE. **(A, B)** LASSO logistic regression and SVM-RFE algorithm screening diagnostic biomarkers for SLE. **(C)** The Venn diagram showing the intersection of the diagnostic biomarkers screened by the two algorithms. **(D–I)** Validation of the expression of diagnostic biomarkers based on the validate data set including GSE4588, GSE50772, GSE99967, and GSE24706. "Con" represented the control samples, and "SLE" represented the SLE patients.

resting NK cells was decreased in SLE patients and the ratios of monocytes, neutrophils were increased in SLE patients (25–27).

Following that, we investigated the correlation between the ratios of the 22 types of immune cells in SLE patients, and discovered that the degrees of memory B cells and activated dendritic cells, the levels of regulatory T cells (Tregs) and memory B cells, the levels of Tregs and activated dendritic cells all had a strong positive link, respectively. Furthermore, the ratio of CD8 T cells was adversely linked with that of neutrophils, memory B cells, and monocytes (**Figure 5B**).

Finally, we looked at the relationship between the immune cell ratios and the expression of ABCB1, IFI27 and PLSCR1 in SLE patients. As a result, the ABCB1 expression was positively correlated with the levels of CD8 T cells, resting NK cells, CD4 memory resting T cells, naive B cells, CD4 memory activated T cells, and macrophages M2, and negatively correlated with the ratios of activated mast cells, resting mast cells, CD4 naive T cells, Tregs, memory B cells, plasma cells, macrophages M0, activated dendritic cells, neutrophils, and monocytes (**Figure 5C**). The IFI27 expression was positively linked with the degrees of

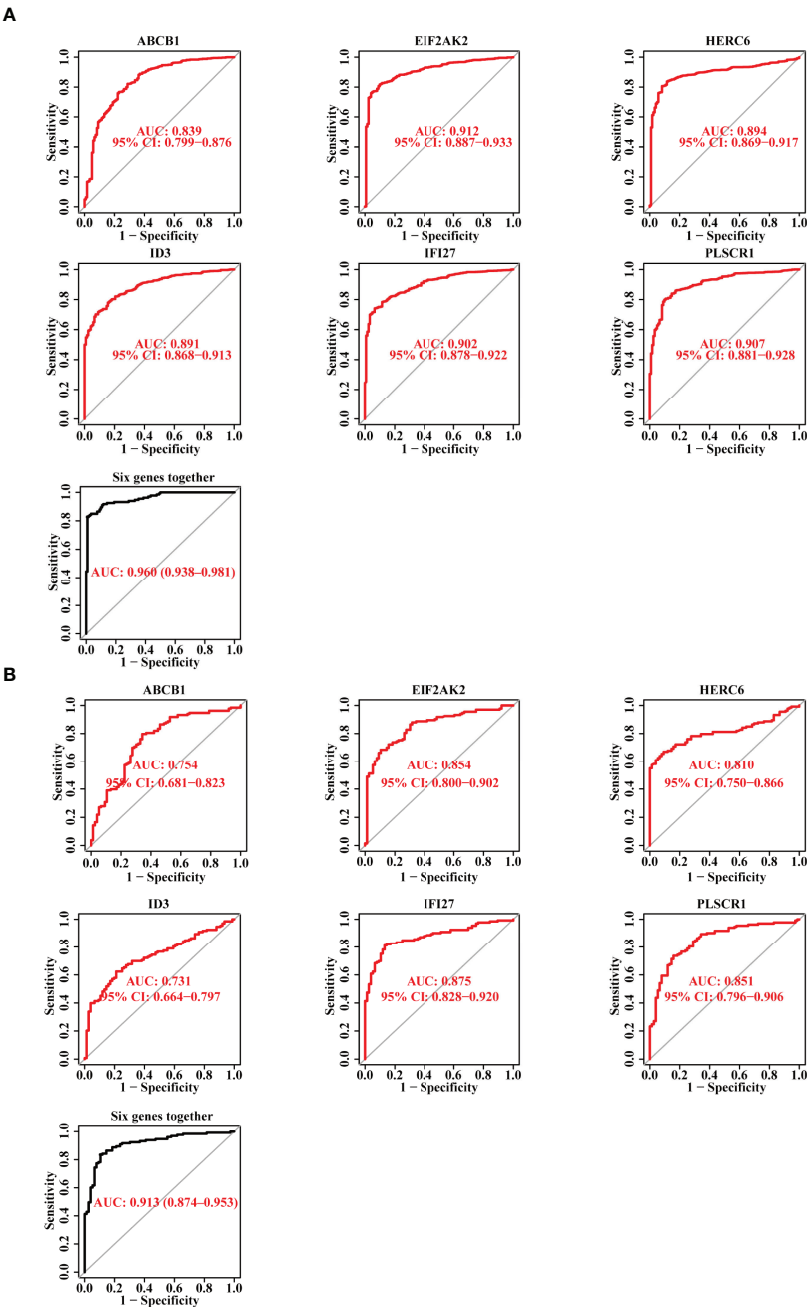

FIGURE 3 | The Diagnostic Efficacy of the Selected Diagnostic Markers for SLE. **(A)** The ROC curve showing the AUC value of ABCB1, EIF2AK2, HERC6, ID3, IFI27, and PLSCR1 based on the data set of the discovery cohort. **(B)** The ROC curve showing the AUC value of ABCB1, EIF2AK2, HERC6, ID3, IFI27, and PLSCR1 based on the data set of the validation cohort.

activated dendritic cells, plasma cells, resting mast cells, activated NK cells, neutrophils, macrophages M0, memory activated cells CD4, and macrophages M1, and negatively linked with CD8 T cells, resting dendritic cells, naive B cells, and CD4 memory resting T cells (**Figure 5D**). The PLSCR1 expression was positively correlated with the ratios of neutrophils, activated dendritic cells, resting mast cells, macrophages M0, plasma cells, monocytes, and activated NK cells, and negatively correlated with eosinophils, CD4 naive T cells, resting dendritic cells, resting NK cells, CD8 T cells, naive B cells, CD4 memory resting T cells (**Figure 5E**). All in all, the ABCB1 expression was most closely linked to the ratios of CD8 T cells and monocytes, the IFI27 expression to the levels of activated dendritic cells and CD4 memory resting T cells, and the

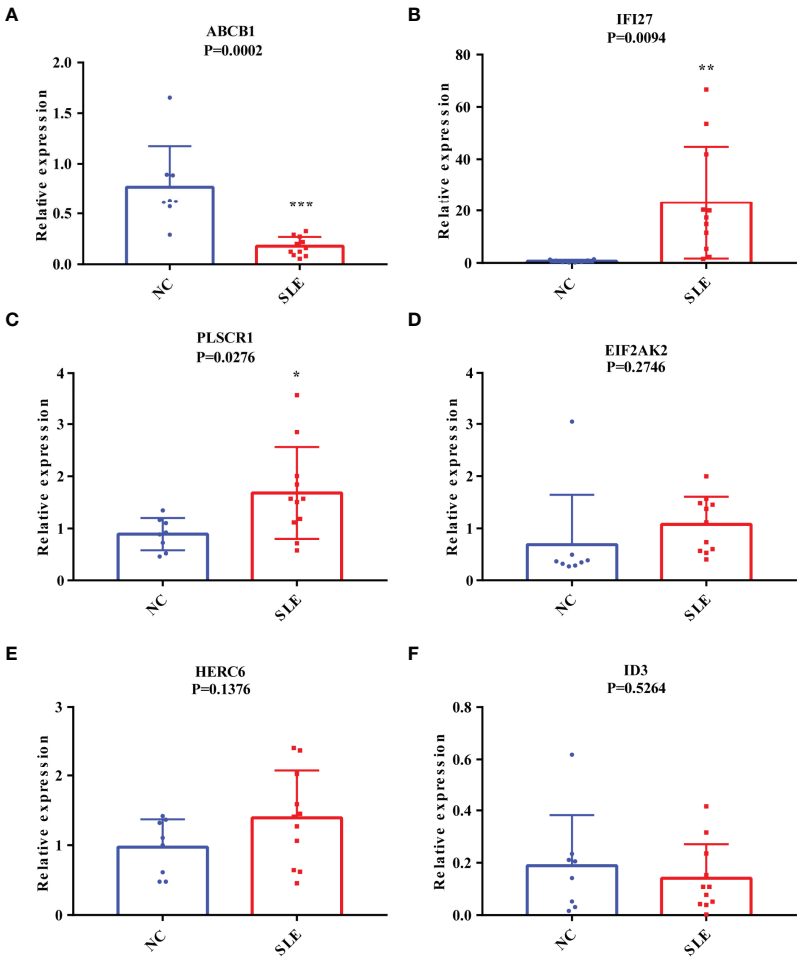

FIGURE 4 | The Validation of the Putative Diagnostic Markers by RT-qPCR in Chinese population using Our Own Cohort. **(A-F)** The relative mRNA expression levels of ABCB1, IFI27, PLSCR1, EIF2AK2, HERC6, and ID3 in the PBMC of SLE patients and healthy people. "*" represented P <0.05, "**" represented P <0.01, and "***" represented P <0.001.

PLSCR1 expression to the degrees of neutrophils and CD4 memory resting T cells.

DISCUSSION

SLE is a type of multi-system autoimmune disease, the pathophysiological mechanisms of which have not been thoroughly elucidated (27). In clinic, SLE is diagnosed according to the clinical features, such as injures of skin, joints, kidneys, nervous system, as well as the serologic parameters, such as autoimmune antibody. Furthermore, the diagnosis of SLE must be based on the clinical manifestations (such as organ damages) together with multiple immunological indicators (12, 28). Some types of organ damage need to be diagnosed in an invasive way, causing great pain to the patients. Therefore, the diagnosis of SLE is a tedious, complex and time-consuming process, and it is of great significance to search for genetic markers for SLE diagnosis.

In this study, we screened the putative diagnostic biomarkers of SLE using machine learning. We all know that the conclusions drawn from a single data set are typically limited, unrepeatable, or inconsistent. To ensure the correctness of our findings, we retrieved three cohorts from the GEO database (n_{SLE} = 1315, n_{normal} =122). Among the six putative biomarkers, we confirmed their expression using our own Chinese cohort (n_{SLE} =13, n_{normal} =10), and three of them were statistically significant, while the other three genes showed the same expression trend to the machine learning results. The reason for this phenomenon could be due to the small sample size and the racial heterogeneity. Thus, a bigger cohort may be necessary for the further validation in future research.

In 2021, Zhao X's team has revealed several potential SLE biomarkers based on a comprehensive bioinformatics analysis (27). Compared with this paper, we have the following advancements, extensions and deeper studies: First, we established a larger discovery cohort (n_{SLE} = 1315, n_{normal} =122) to ensure that the markers screened out are more universal and accurate. Second,

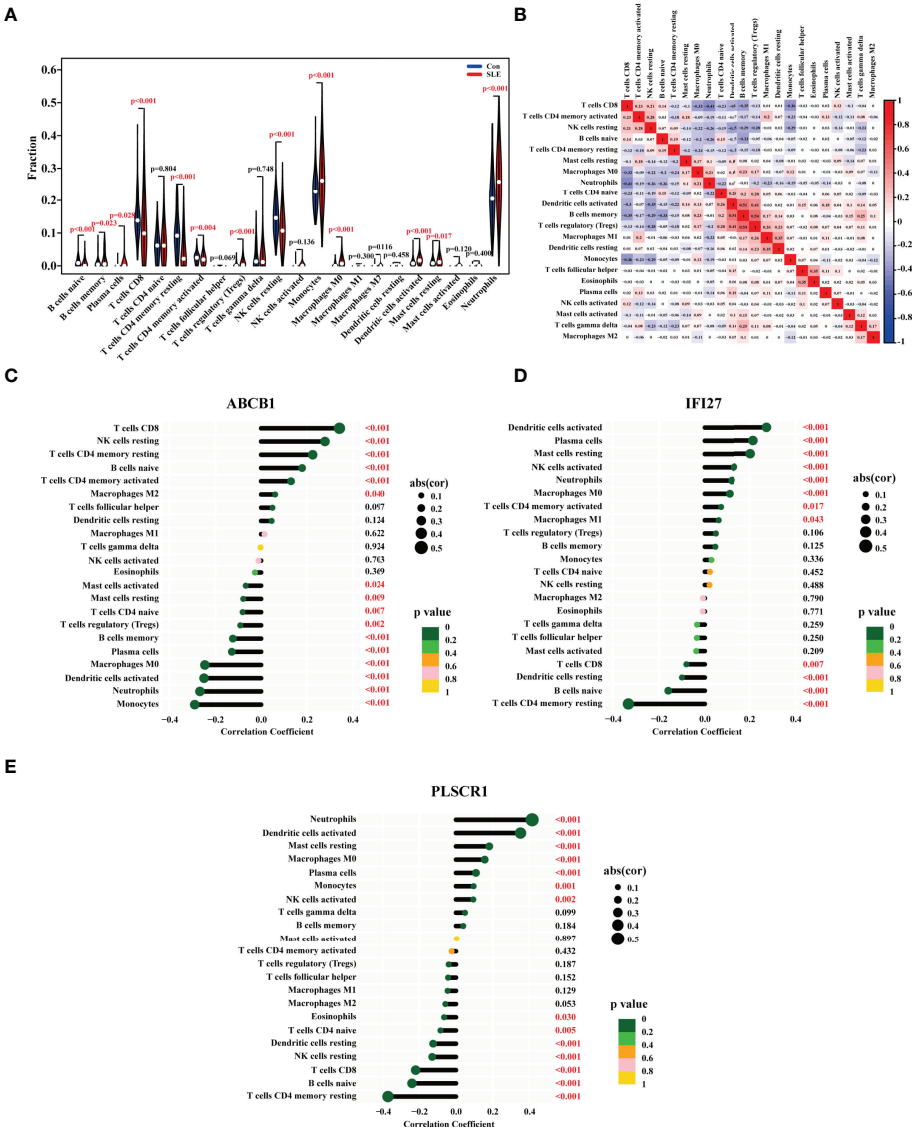

FIGURE 5 | The Ratios of Immune Cells in the PBMC of SLE Patients and their Correlations with the Biomarkers' Expression. **(A)** Violin diagram showing the proportion of 22 types of immune cells in the PBMC of SLE patients and healthy people. The red marks represented $p < 0.05$. **(B)** The heat map showing the proportion correlation between the 22 types of immune cells in SLE patients. The size of the colored squares represented the strength of the correlation, and red represented a positive correlation, and blue represented a negative correlation. The correlation between **(C)** ABCB1, **(D)** IFI27, **(E)** PLSCR1 expression with the degrees of immune cells in SLE. The size of the dots represented the strength of the correlation. The color of the dots represented the p value. A $p < 0.05$ was considered statistically significant.

we used two machine learning methods, LASSO and SVM, to screen diagnostic markers, whereas Zhao X's work screened biomarkers through overlapping the DEGs of six independent data sets. Compared with the intersection method, LASSO and SVM have stronger power in identifying biomarkers with higher distinguishing efficacy. Third, we validated the screened markers using our own Chinese cohort, thereby the markers had potential application for Chinese patients.

We also observed a link between the infiltration of certain immune cells and the expression of biomarker genes. However, more evidence is needed to support the link in a wider range of situations, and the relevance of this correlation is unknown at this time. Furthermore, immune cell infiltration and marker gene expression may not be causally related. The systemic inflammation that occurs in lupus may cause changes in immune cell ratio, and the marker gene expression may be linked to that inflammation.

Among the six SLE candidate markers, ABCB1, HERC6 and ID3 are identified for the first time. Furthermore, we confirmed the expression of ABCB1 in the Chinese population using our own cohort. ABCB1, also known as P-glycoprotein (P-GP), is a

multidrug resistance protein mainly expressing in barrier organs such as brain, liver, kidney, and skin (29–32). Recently, ABCB1 has been reported being expressed in a variety of immune cells, such as monocytes, antigen presenting dendritic cells, T cells and B cells, and is involved in the efflux of inflammatory molecules (33). Therefore, it's possible that ABCB1 plays a role in SLE by participating in the inflammatory pathways. Besides, HERC6 has been revealed as a key determinant of cellular antiviral activity, and is also one constituent gene of type I interferon signaling pathway (34, 35), which was regarded as a central pathogenic pathway in SLE (36). Additionally, several studies have proclaimed that ID3 is a key regulator of IL-5 production and the homeostasis of B-1a B cells (37). ID3 helps the tuberculosis subunit vaccine to induce long-term immune memory, providing immune protection against M tuberculosis infection (38).

Machine learning, on the other hand, detected three recognized SLE biomarkers, including EIF2AK2, IFI27, and PLSCR1, confirming the algorithms' accuracy. The protein encoded by EIF2AK2, as one key component of the innate immune system, is increasingly expressed in T cells of SLE patients, and is likely to be associated with cellular translation and proliferation (39). Besides, EIF2AK2 selectively regulates the transcription of genes functioning in immune response in SLE (39). In proteasome-associated autoinflammatory cell models, the activation of EIF2AK2 is discovered responding to the decreased proteasome function (40). Numerous studies have demonstrated the indispensable role of IFI27 (Interferon (IFN)-α-inducible protein 27) in SLE (41–43). IFI27 is strongly correlated with the levels of T helper type 1 (Th1) cells, T helper type 2 (Th2) cells and activated dendritic cells (aDC), and the up-regulation of IFI27 is highly related to many inflammatory events induced by these immune cells (44, 45). Ultimately, PLSCR1 has been found an increased expression in multiple systemic autoimmune diseases, such as primary antiphospholipid syndrome, rheumatoid arthritis, idiopathic inflammatory myopathies and SLE (46, 47). Meanwhile, the PLSCR1 expression was discovered to be associated with type I interferon-stimulated genes (48), and was higher in neutrophils, dendritic cells, and macrophages (43), which is consistent with our results.

CONCLUSIONS

Generally speaking, we identified six genes as prospective SLE biomarkers (n_{SLE} = 1315, n_{normal} =122), including ABCB1, EIF2AK2, HERC6, ID3, IFI27, and PLSCR1, and demonstrated that ABCB1, EIFI27, and PLSCR1 might be suitable for the Chinese population. Meanwhile, we discovered the quantitative changes of 13 types of immune cells in SLE patients, as well as the link between the expression of ABCB1, IFI27, and PLSCR1 and the ratios of different immune cells. Our findings provide potential biomarkers for Chinese SLE patients, and give the insight into the relationship between gene expression and immune cell ratios.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shenzhen People's Hospital (LL-KY-2021393). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

WZ conceived and designed the project. YZ wrote the manuscript. SL provided data analysis support. XH and DL contributed to clinical sample collection. YC, ZZ, and WC performed the experiments. YX and GW provided conceptual guidance. DT and YD supervised the study. All authors read, revised and approved the final 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.2022.873787/full#supplementary-material

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Comprehensive Urinomic Identification of Protein Alternatives to Creatinine Normalization for Diagnostic Assessment of Lupus Nephritis

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Introduction: The current gold standard used for urine biomarker normalization, creatinine, poses a challenge to translate to the point of care because antibodies to creatinine are difficult to develop and currently available ligands to creatinine are sub-optimal for this purpose. Hence, protein alternatives to creatinine are clearly needed. To address this need, lupus nephritis was selected as a model disease where urine protein assessment is required for diagnosis.

Methods: A comprehensive proteomic screen of 1129 proteins in healthy and lupus nephritis urine was executed to identify protein alternatives to creatinine for the normalization of urine biomarkers. Urinary proteins that correlated well with creatinine but did not vary with disease were further validated by ELISA in an independent cohort of lupus nephritis subjects.

Results: The comprehensive proteomic screen identified 14 urine proteins that correlated significantly with urine creatinine but did not differ significantly between SLE and controls. Of the top five proteins selected for ELISA validation, urine HVEM and RELT once again showed significant correlation with urine creatinine in independent cohorts. Normalizing a lupus nephritis biomarker candidate ALCAM using urinary HVEM demonstrated comparable diagnostic ability to creatinine normalization when distinguishing active lupus nephritis from inactive SLE patients.

Conclusions: The discovery of urine HVEM as a protein alternative to creatinine for biomarker normalization has applications in the engineering of antibody-based point of care diagnostics for monitoring lupus nephritis progression.

Keywords: urine, creatinine, biomarker, SLE, lupus nephritis, point-of-care

INTRODUCTION

The advent of personalized medicine and the development of large-scale OMICs technologies have accelerated the discovery of noninvasive biomarkers for diagnostic, prognostic, and therapeutic applications. For diseases affecting the renal system, urine represents a promising body fluid that is potentially enriched for disease biomarkers.

Excessive protein in the urine is an indication of the glomerular filtration barrier becoming compromised, and hence, this is a commonly used marker of renal disease. In addition to assaying total urine protein, specific urine proteins (and metabolites) are interrogated for countless diseases including bladder, prostate, and renal cancer (1–4), drug screening for addiction and therapeutic monitoring, acute kidney injury (5), chronic kidney disease (6), and lupus nephritis (7) and other genitourinary and gynecological conditions. To correctly interpret urine biomarker data, one needs to account for the hydration status of the patient. This is currently achieved by normalizing the biomarker level to urinary creatinine. Creatinine, a waste product of muscle metabolism, is currently the gold standard for urinary glomerular filtration rate normalization (8). Thus, for example, urine albumin creatinine ratio ("ACR") is a routine diagnostic test for the evaluation of renal diseases, both inflammatory and non-inflammatory in origin.

However, translation of creatinine normalization to point of care diagnostics is a challenge. The most common assay used at the point of care is a sandwich lateral flow assay using antibodies to the target biomarker, best exemplified by the pregnancy test strip employing a sandwich assay for detecting human chorionic gonadotropin (hCG). Translating creatinine normalization to lateral flow point of care assay format has been challenging in that the small size of the creatinine metabolite makes it difficult to generate good antibodies to this molecule, thus limiting the translation of disease-specific urine protein biomarkers to antibody-based point of care applications, including applications in lupus nephritis.

To address this bottleneck, we wondered if there could be a protein alternative to creatinine. A proteomic strategy was devised to screen for such molecules, using lupus nephritis as a model of renal disease where proteinuria is common. Indeed, besides the ACR, a multitude or urine proteins are being systematically evaluated to ascertain if they have disease diagnostic potential for this disease. Specifically, a comprehensive proteomic screen of 1129 human proteins, as outlined in **Figure 1**, was undertaken using diseased (lupus nephritis) and healthy urine samples to identify urinary proteins that correlate with urinary creatinine (but not with disease) and can thus be used for normalization of urine biomarker levels. Out of 14 urine proteins that met all selection criteria, five were further validated by ELISA in an independent patient cohort, resulting in the identification of HVEM as the most promising marker for urine biomarker normalization. Having such protein alternatives to urinary creatinine would greatly facilitate urine biomarker monitoring as well as the design of point of care lateral flow tests for routine monitoring of LN, as well other renal diseases.

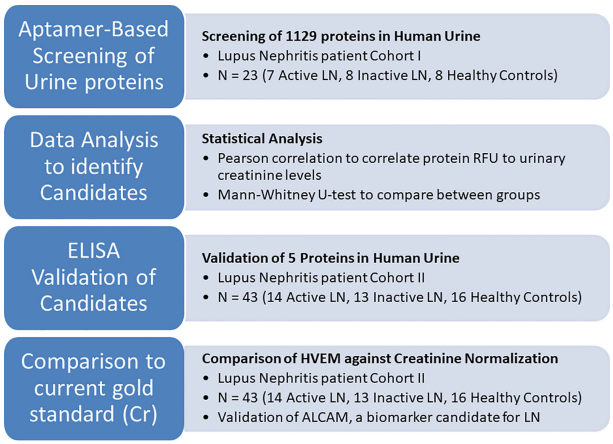

FIGURE 1 | An overview of the current study to identify protein alternatives to creatinine for urine biomarker normalization. First, the urine of 23 human subjects was comprehensively interrogated for 1129 proteins using an aptamer-based proteomic screen. Proteins that correlated well with creatinine and did not differ among patient groups and healthy controls were identified using Pearson correlation and the Mann-Whitney test, respectively. Of these, 5 proteins were chosen for ELISA validation in an independent cohort of 43 subjects. HVEM, the most promising molecule for urine biomarker normalization, was used to normalize the lupus nephritis biomarker candidate urine ALCAM.

METHODS

Human Urine Samples

For the initial aptamer-based targeted proteomic screening, 23 human urine samples were obtained from the University of Texas Southwestern Medical Center (UTSW) consisting of seven active lupus nephritis (LN), eight inactive SLE, and eight healthy controls (HC). Samples were obtained after informed consent at UTSW with Institutional Review Board approvals from UTSW and the University of Houston.

For ELISA validation of the hits from the proteomic screen, 43 human urine samples were obtained from the Johns Hopkins Medical Center (JHMC) and BioreclamationIVT consisting of 14 active LN, 13 inactive disease, and 16 healthy controls. Detailed clinical information pertaining to these subjects is provided in **Table 1**. The patient samples were obtained after informed consent at JHMC with Institutional Review Board approvals from both JHMC and the University of Houston. Active LN patients had biopsy proven LN with the renal component of SLEDAI > 8 (i.e., rSLEDAI > 8), while inactive disease was defined as the rSLEDAI = 0 and SLEDAI < 4. Inactive patients with SLICC (Systemic Lupus Collaborating Clinics) renal activity scores > 4were excluded from the study (9). SLEDAI was determined following the ACR disease guidelines (10).

TABLE 1 | Demographic and clinical characteristic of the primary validation cohort.

Variable	Healthy Controls n=16	Inactive SLE n=13	Active LN n=14
Race			
Caucasian	7	7	7
African American	9	6	7
Age (yr)			
Mean	40 ± 10.7	48 ± 17.6	39 ± 12.5
Range	27–57	24–70	21–60
SLEDAI			
Mean	N/A	0 ± 0.6	11 ± 2.9
Range	N/A	0–2	8–18
rSLEDAI			
Mean	N/A	0 ± 0	9 ± 1.5
Range	N/A	0–0	8–12

Means are expressed with standard deviation.
NA, Not Available.

Matched healthy control urine samples were purchased from BioreclamationIVT (Westbury, NY).

Aptamer-Based Targeted Proteomic Screen of 1129 Proteins

An aptamer-based proteomic screen of 1129 proteins was conducted as described (11). This proteomic platform from Somalogic was used because of its comprehensive coverage (>1000 proteins), high specificity and high sensitivity, allowing for the detection of proteins up to the femtomolar range. The specificity of the assay is derived from the use of a panel of 1129 unique aptamers, which are modified DNA oligonucleotides selected because they were specific to one protein each, with minimal cross-reactivity (12). Urine was diluted 20% in diluent buffer and added to aptamer-coated beads. After incubation for 3.5 hours, the sample was removed and the beads were washed to remove unbound protein. Proteins in the sample that had bound to the aptamer coated beads were then biotinylated. The protein-aptamer complexes were photocleaved, collected, and immobilized on streptavidin coated magnetic beads, where a series of washes ensured specific binding of the aptamers to the proteins. The aptamers were uncoupled from the proteins using a high salt buffer, hybridized onto a DNA microarray, and the results were reported as relative fluorescence units. Proteomic studies were carried out at the Houston Omics Collaborative (https://hoc.bme.uh.edu/).

Statistical Analysis

The relative fluorescence unit readout from the hybridization array for each aptamer (corresponding to individual protein biomarkers) was normalized across the samples to correct for any variations due to the hybridization procedure, using control samples and probes. R Version 1.0.136 with the readxl, stats (13), and Hmisc, packages were used to carry out further data analysis. Mann-Whitney U-test and Student t-test were used to compare between groups to identify proteins that were significantly different between subject groups. Pearson correlation was used to correlate the relative fluorescent units of each protein in the sample to the urinary creatinine of the subject (Cayman Chemical, Ann Arbor, MI, USA) to identify proteins that correlated well with creatinine.

ELISA Validation

ELISA validation was carried out for five proteins: Herpesvirus entry mediator (HVEM), bone morphogenetic protein receptor type 2 (BMPRII), Dectin-1, Serine Peptidase Inhibitor Kunitz Type 2 (SPINT2), and Receptor Expressed In Lymphoid Tissues (RELT). Kits were purchased for HVEM (Cat. No. EK1226, Boster Biological Technology, Pleasanton, CA, USA), BMPRII (Cat. No. ELH-BMPR2-1, RayBiotech, Inc., Norcross, GA, USA), Dectin-1 (Cat. No. ELH-DECTIN1-1, RayBiotech, Inc., Norcross, GA, USA), SPINT2 (Cat. No. DY1106, R&D Systems, Inc., Minneapolis, MN, USA), and RELT (Cat. No. SEK10530, Sino Biological Inc., Beijing, China). The samples were also assayed for ALCAM (Cat. No. DY656, R&D Systems, Inc., Minneapolis, MN, USA), a potential urinary biomarker for lupus nephritis (14). Validation data were analyzed and graphed in GraphPad Version 6.05 using the Mann Whitney U-test, receiver operator curves (ROC), and area under the ROC curve (AUC).

RESULTS

1129-Plex Proteomic Screening Results

23 human urine samples were screened for the levels of 1129 proteins using a comprehensive targeted proteomic screen. Of the urine proteins interrogated, several were significantly elevated in the urine of patients with active LN (14). As opposed to the previous study that was designed to identify novel urine biomarkers for LN (14), the focus of this study was to identify urine proteins (out of the 1129 interrogated) that correlated best with urine creatinine, and did not vary with disease status. Using Pearson correlation, we identified 62 urine proteins that were positively correlated with creatinine ($r > 0.5$, $P < 0.05$), as depicted in **Figure 2**. Of these 62 proteins, 48 were removed from further consideration as they were significantly

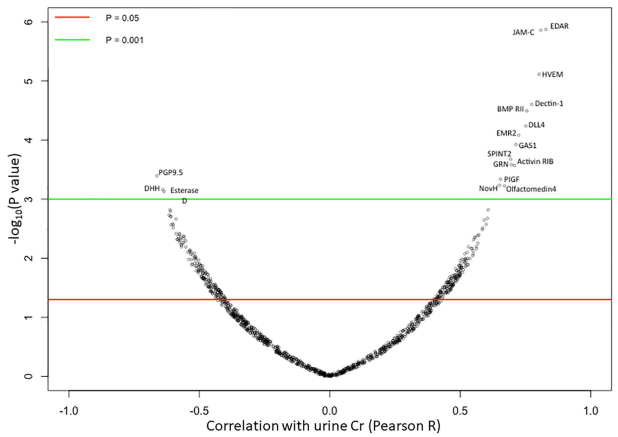

FIGURE 2 | Correlation of 1129 Urine Proteins with Urine Creatinine. Each of the 1129 urine proteins assayed on the aptamer-based screen was correlated to urinary creatinine (using Pearson correlation) in 15 SLE patients and 8 healthy controls, and the results are displayed using a volcano plot. Horizontal red and green lines show threshold P value of the Pearson correlation at $P < 0.05$ and $P < 0.01$ respectively. 14 urine proteins were identified to be significantly positively correlated to urine creatinine while 3 urine proteins were identified to be significantly negatively correlated with urine creatinine.

different between at least two subject groups using Student t-test or Mann Whitney U-test at $P < 0.1$. Of the remaining 14 proteins, listed in **Table 2**, the top five proteins ranked based on Pearson correlation were HVEM, BMPRII, Dectin-1, SPINT2, and RELT. The correlation of these urine proteins with urine creatinine from the screening assay is summarized in **Figures 3A–E**). These five proteins were chosen for further validation using an orthogonal assay platform (ELISA) in an independent cohort of urine samples.

TABLE 2 | Urine proteins that were positively correlated with urinary creatinine but were invariant between LN patients and healthy controls.

UrineProtein	Correlation with Creatinine†
HVEM	0.78
BMP RII	0.76
Dectin-1	0.75
SPINT2	0.69
RELT	0.62
CLM6	0.60
JNK2	0.60
PAPP-A	0.58
HSP70 protein 8	0.56
PTN	0.54
Elafin	0.51
IL-1Rrp2	0.51
RASA1	0.50
APP	0.50

†*Pearson r*

For this analysis, the indicated urine proteins and creatinine were assayed in 7 active LN, 8 inactive LN, and 8 healthy control urine samples using an aptamer-based screening platform.

ELISA Validation of Candidate Proteins

An independent cohort of 43 urine samples was used for ELISA validation, comprised of 16 HC, 13 inactive SLE, and 14 active LN urine samples. ELISA kits for all five target proteins were pre-tested for their detection sensitivity in urine. Urine BMPRII and SPINT2 were too low in concentration to be detected by ELISA. HVEM, Dectin-1, and RELT were validated further in a total of 43 urine samples. A correlation of these molecules as assayed by ELISA with urinary creatinine is shown in **Figure 4**. Once again,

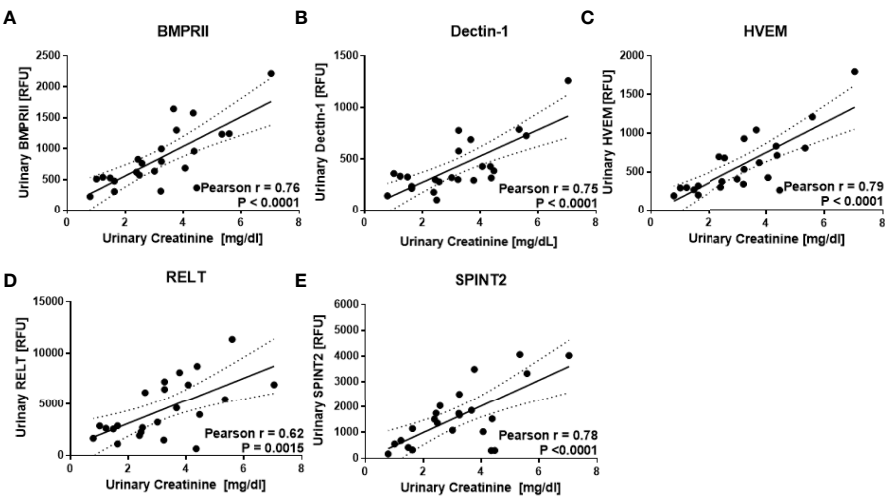

FIGURE 3 | Urine proteins that correlate best with urine creatinine. The aptamer-based screen identified five urine proteins that correlated best with urine creatinine but not differ between subject groups. As described in **Figure 1**, the levels of 1129 urine proteins were assayed in 7 active LN, 8 inactive LN, 8 healthy controls using an aptamer-based proteomic screen. Of these, urine BMPRII, Dectin-1, HVEM, RELT, and SPINT2 **(A–E)** exhibited the highest Pearson R correlation with creatinine but did not differ significantly between the subject groups.

urinary HVEM and RELT were noted to have a significant positive correlation with urinary creatinine (Pearson r = 0.61, P < 0.0001 and r = 0.58, P < 0.0001, respectively) in this independent validation cohort (**Figures 4A, B**). In contrast, urinary Dectin-1 did not show a positive correlation with urinary creatinine in these validation samples (Pearson r = 0.48, P = 0.0012). For the most promising of these proteins, HVEM, the impact of ethnicity was evaluated further. Urinary HVEM correlated with urinary creatinine in both Caucasian and African American subjects (Pearson r = 0.70, P = 0.0004 and Pearson r = 0.58, P = 0.0047, respectively) as shown in **Figures 4D, E**. These findings were extended to an larger independent cohort of lupus patients, again comprised of Caucasian and African American subjects. Again, as shown in **Figures 5A, B**), urinary HVEM correlated with urinary creatinine in both ethnic groups.

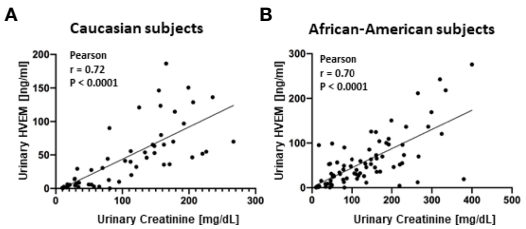

FIGURE 5 | Extended ELISA validation of urine HVEM in an independent cohort of subjects. Urine HVEM, the most promising biomarker for urine biomarker normalization, was assayed in an additional cohort of Caucasian **(A)** and African American **(B)** subjects, again showing a significant positive correlation with urine creatinine across both races. In **(A)**, 23 SLE and 30 healthy urine samples were interrogated. In **(B)**, 49 SLE and 30 healthy urine samples were interrogated.

Testing the Ability of Urine HVEM to Normalize Urine Biomarkers in LN

Given that urinary HVEM correlated consistently with urinary creatinine, we next assessed whether urinary HVEM could be used to normalize urine biomarker levels, just as urinary creatinine is currently used. The same validation cohort of 43 urine samples used above to assay urinary HVEM and creatinine was interrogated for the levels of urinary ALCAM, a biomarker candidate for LN (14). Urine ALCAM normalized by creatinine, the current gold standard, showed a fold change of 4.06 (Mann Whitney U-Test P = 0.0040) between active and inactive lupus nephritis patients, while normalization of urine ALCAM with urine HVEM showed a similar fold change of 4.41 (Mann Whitney U-Test P = 0.0369) between active and inactive lupus nephritis, as shown in **Figures 6A, B**). ROC curves in **Figures 6C, D**) illustrate the diagnostic ability of urine ALCAM for distinguishing active lupus nephritis using the two normalization methods (urine creatinine versus urine HVEM), showing comparable performance characteristics with AUC values of 0.79 and 0.71 respectively.

DISCUSSION

This study is the first to screen over 1,100 human proteins for markers that could be used to normalize urine biomarkers, using a highly specific and sensitive targeted proteomic platform. By applying statistical criteria and identifying proteins that correlate

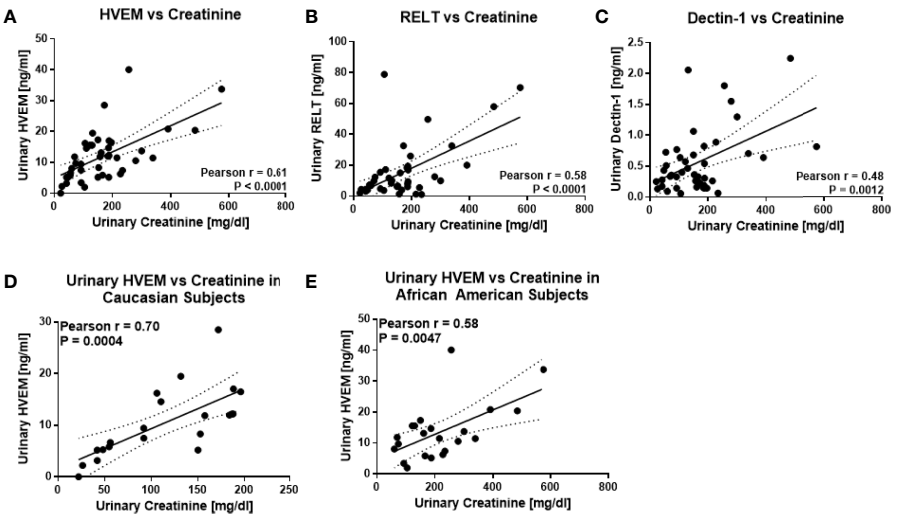

FIGURE 4 | ELISA validation of the top urine protein candidates in an independent cohort of 14 active LN, 13 inactive LN, 16 healthy control subjects. Urine samples were pretested for dilution and assayed for urinary HVEM **(A)**, urinary RELT **(B)**, and urinary Dectin-1 **(C)** in these 43 urine samples. A significant positive correlation was noted between urine HVEM and RELT with urinary creatinine. Urine HVEM, the most promising biomarker for urine biomarker normalization, was assayed in 21 Caucasian **(D)** and 22 African American **(E)** subjects, again showing a significant positive correlation with urine creatinine across both races.

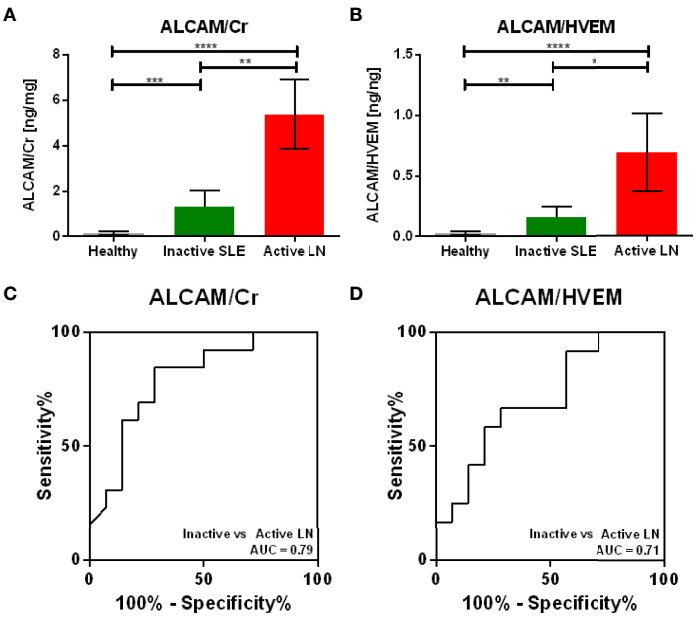

FIGURE 6 | Normalizing Urine ALCAM using Urine HVEM versus Urine Creatinine. As urine HVEM emerged as the most promising marker for urine biomarker normalization, the diagnostic utility of HVEM normalization was compared to the gold standard, creatinine normalization. Normalization of urine ALCAM, a proposed biomarker for diagnosing lupus nephritis, using urinary creatinine **(A)** and urinary HVEM **(B)** shows comparable active versus inactive lupus nephritis ALCAM fold changes of 4.06 and 4.41, respectively. The diagnostic ability of ALCAM normalized with creatinine **(C)** and ALCAM normalized with HVEM **(D)** shows comparable ROC AUC values of 0.79 and 0.71, respectively. Bars show mean ± standard error of the mean. One sample was removed from the plots, as the HVEM concentration was too low to be detected by ELISA. *, **, *** and **** represent $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

with creatinine, but not with disease, we have identified 14 urine proteins that could potentially be used for urine biomarker normalization, either in standard laboratory tests, or in point of care applications. This is of practical importance because the metabolite creatinine does not readily lend itself for antibody-based diagnostics.

Urine validation of these candidates by ELISA further supports the need for easily detectable urine markers for biomarker normalization, as two of the five proteins chosen for validation were too low in concentration for ELISA to detect, which will make it even harder to detect these molecules at the point of care. Of the five urinary protein candidates chosen for validation, three, urinary HVEM, Dectin-1, and RELT, were detectable by ELISA. However, urinary Dectin-1 did not correlate with urine creatinine in the larger validation cohort. Hence, urinary HVEM and urinary RELT emerged as the leading urine protein candidates for urine concentration normalization. In this study, urine concentrations of HVEM ranged from 5 ng/ml to 34 ng/ml in healthy subjects and 5 ng/ml to 41 ng/ml in patients with active LN. Urine concentrations of RELT ranged from 1 ng/ml to 71 ng/ml in healthy subjects and 4 ng/ml to 79 ng/ml in patients with active LN. In pursuing urine HVEM further, it exhibited good correlation with urine creatinine in both Caucasian and African American subjects. When using urinary HVEM as a normalization marker for the LN urinary biomarker candidate ALCAM, both HVEM and creatinine normalization exhibited comparable fold changes and ROC AUC values. Unlike creatinine, antibodies to HVEM are readily available, thus rendering it attractive for antibody-based diagnostic assays, of particular relevance in point of care applications.

HVEM is a member of the tumor necrosis factor receptor superfamily and is a cell surface receptor that is used by the herpes simplex virus for cellular entry. It is also involved in the regulation of T-cell responses by inflammatory and inhibitory signaling pathways (15). HVEM is widely expressed in the gallbladder, appendix, lymph nodes, tonsils, spleen, adrenal glands, stomach, rectum, kidney, bladder, and endometrium (16). Expression of HVEM has been documented to be increased in ovarian serous adenocarcinoma tissue (17), colorectal cancer epithelium (18), esophageal squamous cell carcinoma (19), and breast cancer (20). Soluble HVEM has also been implicated in the serum of patients with hepatocellular carcinoma (21), gastric cancer (22), allergic asthma, atopic dermatitis and rheumatoid arthritis (23). HVEM has also been implicated into innate mucosal defense against bacteria by promoting genes associated with immunity in the colon of a mouse model for Escherichia coli infection (24). Interestingly, one report shows that active SLE patients had a significantly higher proportion of circulating HVEM-expressing CD4+T-cells than healthy individuals (25). However, urinary HVEM does not appear to be elevated in renal diseases or in autoimmunity.

This study represents the first comprehensive proteomic screen for urine proteins that can potentially be used as a

substitute for urine creatinine, for normalizing urine biomarker levels. We find urine HVEM is not altered in patients with active LN and that urine HVEM correlates well with urine creatinine. The utility of having such a normalizer protein for calibration of urine biomarkers extends beyond lupus nephritis. Urine biomarker testing is widely used for assessing cancers (1–4), multiple renal diseases (5–7), and other diseases (26) as well as for drug testing (27). Even when total protein in urine is assayed in clinical diagnostics, it is normalized to creatinine, in the form of the ACR test. Urinary HVEM can certainly be used for normalization in all of the above scenarios, readily extending these tests to encompass potential point of care assays.

Further studies are warranted in which urinary HVEM and urinary creatinine are compared head-to-head in larger, independent cohorts of lupus nephritis patients as well as in other diseases where urinary biomarkers have diagnostic potential. Renal micropuncture studies are also warranted to detail how HVEM is handled in the nephron to assess if it is neither secreted nor absorbed. Studies are also warranted to assess if HVEM can be used to estimate the glomerular filtration rate. Finally, some of the other urine protein candidates described in this work (e.g., BMPRII, SPINT2) warrant further investigation, with comparisons to urinary creatinine and urinary HVEM.

CONCLUSION

The current gold standard used for urine biomarker normalization, creatinine, poses a challenge to translate to point of care applications because antibodies to creatinine are difficult to develop and currently available ligands to creatinine are sub-optimal for this purpose. This comprehensive screen of >1000 proteins has identified urine HVEM as an alternative to creatinine for normalizing the concentrations of urine biomarkers. The discovery of urine HVEM as well as other normalizing proteins paves the way towards accurate monitoring of disease specific urine biomarkers, with unique implications for antibody-based point of care diagnostics.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The proteomic profiling data comparing lupus nephritis and controls has previously been published (PMID: 32366845).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB Boards at University of Houston, John Hopkins Medical University and UT Southwestern Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SSo and CM designed the study; RS and MP provided the human samples for the study. SSo, SSt, and RL carried out the experiments; SSo analyzed the data; SSo and CM drafted and revised the paper; all authors read and approved the final version of the manuscript.

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