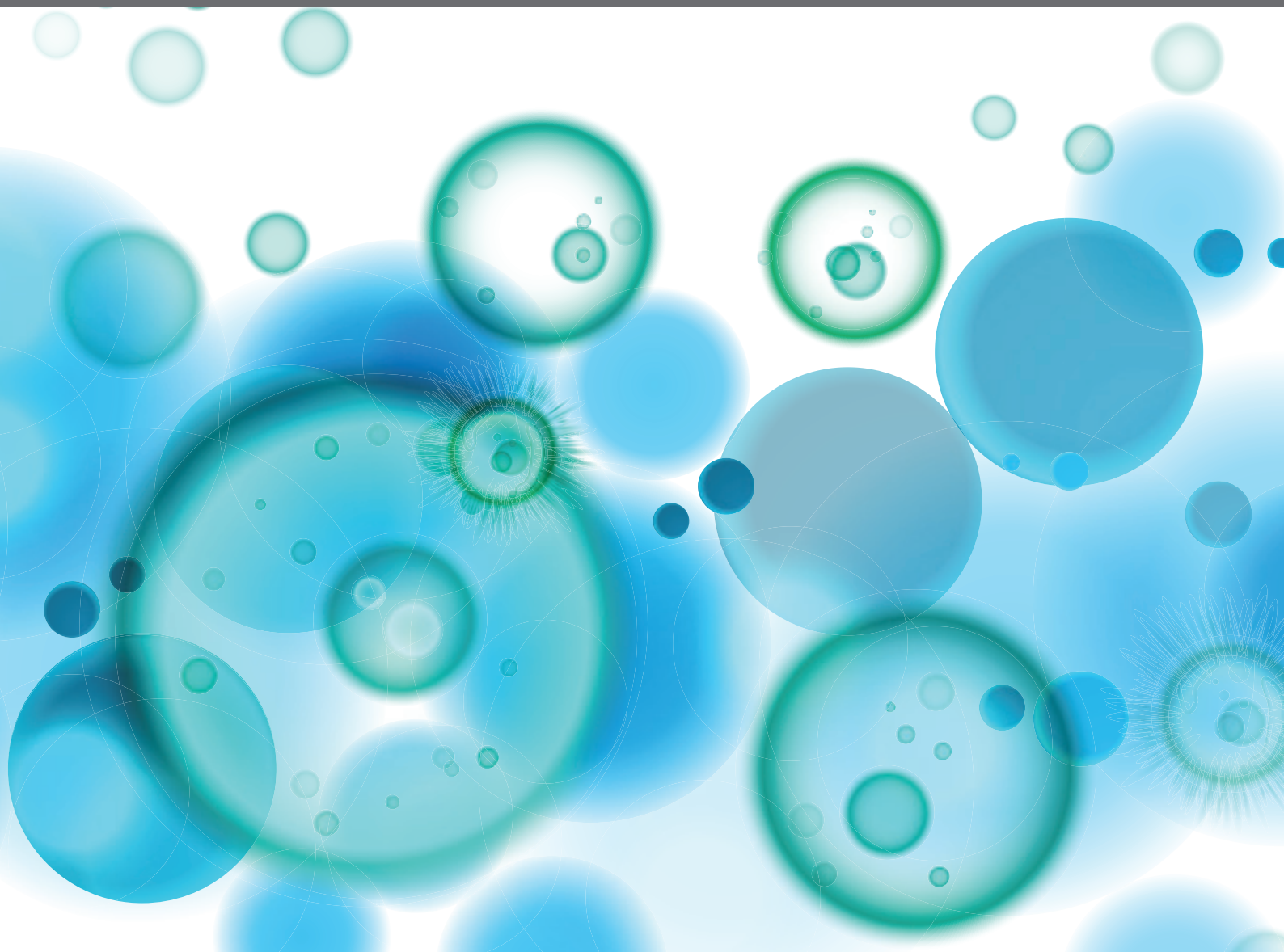


MONITORING IMMUNE RESPONSES IN RENAL AUTOIMMUNE AND AUTOINFLAMMATORY DISEASES

EDITED BY: Y. K. O. Teng, Charles Dickson Pusey, Augusto Vaglio,
Cees Van Kooten and Chi Chiu Mok
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MONITORING IMMUNE RESPONSES IN RENAL AUTOIMMUNE AND AUTOINFLAMMATORY DISEASES

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Table of Contents

- 06 Editorial: Immune Monitoring Responses in Renal Autoimmune Diseases**
Y. K. Onno Teng, Charles Dickson Pusey, Augusto Vaglio, Chi Chui Mok and Cees van Kooten
- 09 Association of Urine sCD163 With Proliferative Lupus Nephritis, Fibrinoid Necrosis, Cellular Crescents and Intrarenal M2 Macrophages**
Ting Zhang, Hao Li, Kamala Vanarsa, Gabriel Gidley, Chi Chiu Mok, Michelle Petri, Ramesh Saxena and Chandra Mohan
- 19 Phospholipase A2 Receptor Autoantibodies as a Novel Serological Biomarker for Autoimmune Thyroid Disease Associated Nephropathy**
Biao Huang, Yi Zhang, Liang Wang, Qingqing Wu, Ting Li, Jue Zhang, Qiuhua Zhang, Huiming Sheng, Jiandong Bao and Zhigang Hu
- 26 C1q A08 is a Half-Cryptic Epitope of Anti-C1q A08 Antibodies in Lupus Nephritis and Important for the Activation of Complement Classical Pathway**
Wen-Jun Wu, Ying Tan, Xiao-Ling Liu, Feng Yu and Ming-Hui Zhao
- 37 High-Throughput Sequencing-Based Analysis of T Cell Repertoire in Lupus Nephritis**
Xiaolan Ye, Zhe Wang, Qiang Ye, Jing Zhang, Ping Huang, Jingying Song, Yiwen Li, Hongjuan Zhang, Feifeng Song, Zixue Xuan and Kejian Wang
- 44 The Complement C3a and C3a Receptor Pathway in Kidney Diseases**
Shuang Gao, Zhao Cui and Ming-hui Zhao
- 53 Clinical Utility of Serial Measurements of Antineutrophil Cytoplasmic Antibodies Targeting Proteinase 3 in ANCA-Associated Vasculitis**
Gwen E. Thompson, Lynn A. Fussner, Amber M. Hummel, Darrell R. Schroeder, Francisco Silva, Melissa R. Snyder, Carol A. Langford, Peter A. Merkel, Paul A. Monach, Philip Seo, Robert F. Spiera, E. William St. Clair, John H. Stone and Ulrich Specks
- 65 B Cell Subsets and Cellular Signatures and Disease Relapse in Lupus Nephritis**
Desmond Y. H. Yap, Susan Yung, Paul Lee, Irene Y. L. Yam, Cheryl Tam, Colin Tang and Tak Mao Chan
- 76 Idiopathic Membranous Nephropathy: Glomerular Pathological Pattern Caused by Extrarenal Immunity Activity**
Wenbin Liu, Chang Gao, Zhiyuan Liu, Haoran Dai, Zhendong Feng, Zhaocheng Dong, Yang Zheng, Yu Gao, Xuefei Tian and Baoli Liu
- 89 Biomarkers and Diagnostic Testing for Renal Disease in Sjogren's Syndrome**
Giacomo Ramponi, Marco Folci, Salvatore Badalamenti, Claudio Angelini and Enrico Brunetta
- 96 The Emerging Role of Renal Tubular Epithelial Cells in the Immunological Pathophysiology of Lupus Nephritis**
Seokchan Hong, Helen Healy and Andrew J. Kassianos

- 104 4C3 Human Monoclonal Antibody: A Proof of Concept for Non-pathogenic Proteinase 3 Anti-neutrophil Cytoplasmic Antibodies in Granulomatosis With Polyangiitis**
 Jérôme Granel, Roxane Lemoine, Eric Morello, Yann Gallais, Julie Mariot, Marion Drapeau, Astrid Musnier, Anne Poupon, Martine Pugnière, Seda Seren, Dalila Nouar, Valérie Gouilleux-Gruart, Hervé Watier, Brice Korkmaz and Cyrille Hoarau
- 121 Monitoring Immune Responses in IgA Nephropathy: Biomarkers to Guide Management**
 Haresh Selvaskandan, Sufang Shi, Sara Twaij, Chee Kay Cheung and Jonathan Barratt
- 142 Autoimmunity and SLE: Factual and Semantic Evidence-Based Critical Analyses of Definitions, Etiology, and Pathogenesis**
 Ole Petter Rekvig
- 160 Th17-Immune Response in Patients With Membranous Nephropathy is Associated With Thrombosis and Relapses**
 Marion Cremoni, Vesna Brglez, Sandra Perez, Fabrice Decoupigny, Kévin Zorzi, Marine Andreani, Alexandre Gérard, Sonia Boyer-Suavet, Caroline Ruetsch, Sylvia Benzaken, Vincent Esnault and Barbara Seitz-Polski
- 171 Vasculature-Associated Lymphoid Tissue: A Unique Tertiary Lymphoid Tissue Correlates With Renal Lesions in Lupus Nephritis Mouse Model**
 Md. Abdul Masum, Osamu Ichii, Yaser Hosny Ali Elewa, Yuki Otani, Takashi Namba and Yasuhiro Kon
- 188 Highly Sensitive Flow Cytometric Detection of Residual B-Cells After Rituximab in Anti-Neutrophil Cytoplasmic Antibodies-Associated Vasculitis Patients**
 Laura S. van Dam, Jelle M. Oskam, Sylvia W. A. Kamerling, Eline J. Arends, O. W. Bredewold, Magdalena A. Berkowska, Jacques J. M. van Dongen, Ton J. Rabelink, Cees van Kooten and Y. K. Onno Teng
- 200 Rituximab Induces Complete Remission of Proteinuria in a Patient With Minimal Change Disease and No Detectable B Cells**
 Maximilian Webendörfer, Linda Reinhard, Rolf A. K. Stahl, Thorsten Wiech, Hans-Willi Mittrücker, Sigrid Harendza and Elion Hoxha
- 209 Pathogenicity of Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody in Granulomatosis With Polyangiitis: Implications as Biomarker and Future Therapies**
 Jérôme Granel, Brice Korkmaz, Dalila Nouar, Stefanie A. I. Weiss, Dieter E. Jenne, Roxane Lemoine and Cyrille Hoarau
- 223 Autoantibodies Targeting Intracellular and Extracellular Proteins in Autoimmunity**
 Peter D. Burbelo, Michael J. Iadarola, Jason M. Keller and Blake M. Warner
- 236 Autoantibodies in the Diagnosis, Monitoring, and Treatment of Membranous Nephropathy**
 Vladimir Tesar and Zdenka Hruskova

246 Case Report: Expanding Clinical, Immunological and Genetic Findings in Sideroblastic Anemia With Immunodeficiency, Fevers and Development Delay (SIFD) Syndrome

Leonardo Oliveira Mendonca, Alex Isidoro Prado, Izelda Maria Carvalho Costa, Marcia Bandeira, Rafael Dyer, Samar Freschi Barros, Karen Francine Khöler, Luiz Augusto Marcondes Fonseca, Jorge Kalil, Fabio Morato Castro and Myrthes Anna Maragna Toledo-Barros



Editorial: Immune Monitoring Responses in Renal Autoimmune Diseases

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Keywords: Glomerulonephritis, renal autoimmune diseases, Lupus Nephritis, ANCA - associated vasculitis, membranoproliferative glomerulonephritis (MPGN)

Editorial on the Research Topic

Monitoring Immune Responses in Renal Autoimmune Disease

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Exciting times for the field of renal autoimmune diseases have begun. In 2021, for the first time, two new drugs belimumab (1) and voclosporin (2) are approved for the treatment of lupus nephritis (LN) (1, 2). Other novel targeted therapies demonstrate clinical efficacy in large, randomized trials, such as avacopan for ANCA-associated vasculitis (AAV) (3), imlifidase for Goodpasture’s disease and iptacopan for IgA nephropathy (IgAN). Pathogenic molecules are specifically targeted by new drugs that help to uncover novel aspects of disease mechanisms leading to glomerulonephritis. Simultaneously, the field is boosted by novel big data technologies on the single cell levels such as high-sensitive multi-color flow cytometry, single-cell genomics (single-cell RNA sequencing - scRNAseq), single cell metabolomics and proteomics. The novel treatment options in renal autoimmune diseases almost simultaneously require new immunomonitoring tools. ‘Immunomonitoring’ includes the wide range of approaches to monitoring immune responses by the cellular immune system (e.g. T-cells, B-cells, plasma cells, dendritic cells, neutrophils etc.), or by the humoral immune system (e.g. cytokines, (auto-)antibodies, urinary markers, etc.). Monitoring relevant immune responses in patients with renal autoimmune diseases helps us to better understand a) the underpinning immunological pathophysiology of these diseases; b) the beneficial effects of novel treatments on autoimmunity; and c) can potentially help doctors and patients guide a personalized treatment strategy, adding information on immunological (non-)response to a clinical (non-) response treatment and on disease prognosis. In the present Research Topic, we have been able to collect for you a vast amount of research addressing novel ways and the role of immunomonitoring in the broad range of renal autoimmune disease including LN, AAV, IgAN, idiopathic membranous nephropathy (iMN) and complement-mediated disease (CMD).

IMMUNOMONITORING IN LUPUS NEPHRITIS

A review by professor Rekvig sets the stage for this Research Topic by eluding to the fundamentals of immunomonitoring in SLE/LN. It addresses the issue whether, from a principal point of view,

anti-dsDNA antibodies can be accepted as a clinical biomarker for SLE, without clarifying what we define as an anti-dsDNA antibody, and in which biologic contexts these antibodies appear.

The group of Wu et al. addressed the epitope spreading of anti-C1q autoantibodies in LN with regard to the “anti-C1q A08 antibodies”. These are part of the anti-C1q antibody family, which recognize nearly complete cryptic epitope in ELISA. Of note, these anti-C1q antibodies recognize the exposed epitope of C1q coated on an ELISA plate. C1q A08 was demonstrated to be important for activation of classical complement pathway and is an important contributor to pathologically relevant anti-C1q antibodies in LN.

The group of Hong et al. provided an overview of the recent advances in our understanding of renal tubular epithelial cells in LN, and the potential role of tubular epithelial cells as biomarkers in the diagnosis, prognosis, and treatment of LN, as well as the future therapeutic potential of targeting the tubulointerstitium for the treatment of patients with LN. In line with this review, several newly identified urine biomarkers, including monocyte chemoattractant protein-1 (MCP-1), neutrophil gelatinase associated lipocalin (NGAL), TNF-like WEAK inducer of apoptosis (TWEAK), and vascular cell adhesion molecule-1 (VCAM), are proteins that may arise directly from inflamed kidneys and have been promising in monitoring LN disease status. In this Research Topic, the group of Zhang et al. demonstrated that the urinary biomarker sCD163 outperformed C3, C4, urinary protein-to-creatinine ratio, or anti-dsDNA antibody in discriminating non-proliferative LN class II or V from proliferative LN class III or IV. A promising finding that can help identifying active LN patients in whom a renal biopsy could be avoided.

Lastly, two studies in this Research Topic address cell-directed immunomonitoring in LN. With respect to T-cells, the group of Ye et al. demonstrated significant differences in TCR diversity and usage of TRBV/TRBJ genes between SLE patients and healthy controls, identifying a set of signature V–J combinations characteristic for their SLE cohort. Further research in this field may facilitate the development of novel immune biomarkers. With respect to B-cells, the group of Yap et al. explored B-cell subsets demonstrating an exhaustion of the naïve B-cell subset in the most active and relapsing LN patients. This observation relates to the phenomenon of ‘B-cell hyperactivation’ in LN and the group demonstrated that microRNA-148a is an important mediator and might be a potential therapeutic target in LN.

IMMUNOMONITORING IN IMN

The group of Liu et al. reviews the striking low quantity of inflammatory cells in the kidney of iMN patients, related to the immune responses leading to anti-podocyte IgG4 autoantibodies in iMN. The expanding spectrum of anti-podocyte antibodies identified in iMN patients is reviewed by the group of Tesar and Hruskova.

Both reviews set the stage for the findings by the group of Cremoni et al. who demonstrated a dysregulated cytokine balance skewed towards Th17 immune responses in iMN, with worse prognosis with more relapses and thromboembolic events.

Lastly, the group of Huang et al. demonstrated anti-PLA2R autoantibodies in patients with autoimmune thyroid disease, notably Hashimoto hypothyroiditis, which is associated with proteinuric Autoimmune Thyroid Disease (AITD) associated nephropathy in 10% of the cases. Given the commonality of anti-PLA2R autoantibodies in these overlapping disease, this common pathologic substrate may help identifying those patients that could profit from immunosuppressive or B-cell targeted therapies.

IMMUNOMONITORING IN ANCA-ASSOCIATED VASCULITIS

Monitoring the immune responses in AAV patients is very much directed at dissecting the underpinning mechanisms of anti-neutrophil cytoplasmic autoantibodies (ANCA). The group of Granel et al. reflects on the Kurlander effect of PR3-ANCA autoantibodies and the arguments supporting the existence of pathogenic as well as non-pathogenic PR3-ANCAs, which is a relevant concept when employing immunomonitoring in AAV. Subsequently, the group of Thompson et al. investigated serial measurements of PR3-ANCA in AAV patients and their relation to non-response to induction treatment (or vice versa, achieving remission to treatment) and prediction of future relapses. In conjunction, the group of van Dam et al. demonstrated a strong reduction in circulating B-cells after treatment of AAV patients with the B-cell depleting agent rituximab (RTX). However, when employing high-sensitivity flow cytometry as an immunomonitoring tool, RTX treatment never resulted in complete B-cell depletion and residual ANCA-specific memory B-cells remained detectable which opens a whole, new field of immunomonitoring in AAV patients undoubtedly warranting further research.

IMMUNOMONITORING IN COMPLEMENT-MEDIATED DISEASES, IGA NEPHROPATHY AND OTHER RENAL AUTOIMMUNE DISEASES

With respect to IgAN, the group of Selvaskandan et al. provides a truly complete overview of the immense progress made in identifying and validating new biomarkers to facilitate personalization of prognostication and treatment of IgAN.

With respect to CMDs, the group of Gao et al. reviewed the dual roles of the C3a/C3aR interactions, that can exert anti-inflammatory or pro-inflammatory effects depending on the type of kidney disease, with the aim of understanding in-depth its controversial roles and its potential therapeutic value.

Lastly, the group of Ramponi et al. reviewed the current literature to provide guidance on serum biomarkers that could support further testing for kidney involvement in primary Sjögren syndrome (pSS). Patients with pSS typically present with proximal renal tubular acidosis, distal renal tubular acidosis and/or acute (on chronic) kidney impairment for which the most classical kidney lesions are

tubulointerstitial nephritis (TIN) and membranoproliferative glomerulonephritis (MPGN).

renal autoimmune diseases will lead to useful intervention to influence disease progression with effective targeted drugs.

CONCLUSION

The present Research Topic aims to foster knowledge and discussion on investigating and understanding relevant pathological mechanisms underpinning renal autoimmune diseases. As such, these collective research efforts strengthen our Immuno-nephrology community's capabilities to translating the wealth of data, mostly derived from recent technological advances, into useful discoveries for clinical applicability. Ultimately, the monitoring of the relevant immune phenomena in patients with

AUTHOR CONTRIBUTIONS

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Association of Urine sCD163 With Proliferative Lupus Nephritis, Fibrinoid Necrosis, Cellular Crescents and Intrarenal M2 Macrophages

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CD163 is a marker for alternatively activated macrophages, which have been implicated in the pathogenesis of lupus nephritis (LN). In our preliminary screening of urine proteins in LN, urine soluble CD163 (sCD163) was significantly elevated in patients with active LN. To evaluate the potential of sCD163 as a biomarker in LN, urine sCD163 was assayed in patients with active LN, active non-renal lupus patients (ANR), inactive SLE and healthy controls (HC), using ELISA and normalized to urine creatinine. The correlation of urine sCD163 with clinical parameters and renal pathological attributes was further investigated in LN patients with concurrent renal biopsies. A total of 228 SLE patients and 56 HC were included from three cohorts. Results demonstrated that urine sCD163 was significantly elevated in active LN when compared with HC, inactive SLE, or ANR in African-American, Caucasian and Asian subjects (all $P < 0.001$). In LN patients with concurrent renal biopsies, urine sCD163 was significantly increased in patients with proliferative LN when compared with non-proliferative LN ($P < 0.001$). Urine sCD163 strongly correlated with SLEDAI, rSLEDAI, activity index (AI) of renal pathology, fibrinoid necrosis, cellular crescents, and interstitial inflammation on biopsies (all $P < 0.01$). Macrophages, particularly M2 macrophages, the predominant cells expressing CD163 within LN kidneys, represented a potential source of elevated urine sCD163, based on single-cell RNA sequencing analysis. To conclude, urine sCD163 discriminated patients with active LN from other SLE patients and was significantly elevated in proliferative LN. It strongly correlated with concurrent AI and several specific pathological attributes, demonstrating its potential in predicting renal pathology.

Keywords: CD163, lupus nephritis, urine biomarker, renal pathology, activity index

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies and involvement of multiple organ systems. One of the leading causes of morbidity and mortality in SLE is lupus nephritis (LN), which is clinically evident in more than half of all SLE patients. Approximately 10–17% of LN patients will progress to end-stage renal disease (ESRD) (1–3). LN is classified by the International Society of Nephrology/Renal Pathology Society (ISN/RPS) into six categories based on pathological findings (4, 5). Class III and class IV, categorized as proliferative LN, are the most severe forms of LN, carrying the highest risk of progression to ESRD and thus requiring intensive immunosuppressive therapy (2, 6, 7).

Renal biopsy is the current gold standard for the diagnosis and classification of LN. However, the limited tissue obtained each time may not accurately reflect the complete spectrum of renal lesions in a given patient's kidneys due to sampling error; moreover, its invasiveness and attendant complications discourage repeated biopsy at patient follow-up. In contrast, urine samples can be easily obtained and are ideal for frequent monitoring. Non-invasive urinary biomarkers may emerge as an alternative method for LN assessment, as these markers are more convenient to assay, one day even at home, and allow repeated examinations (8). Specific urinary biomarkers which discriminate active LN and predict concurrent underlying LN pathology could be instrumental in guiding the management of LN.

In our preliminary aptamer-based targeted proteomic screen of >1000 urine proteins in LN, urine CD163 was noted to be significantly higher in patients with active LN (**Supplementary Figure 1**). Here, we pursue this initial observation further, given that CD163 is a marker for alternatively activated macrophages, which have been implicated in the pathogenesis of LN (9). While CD163⁺ macrophages can only be observed and analyzed on tissue biopsies, soluble CD163 (sCD163), derived from the extracellular portion of CD163 when cleaved by metalloproteinases, can easily be measured in diverse body fluids, including serum, urine, synovial fluid and cerebrospinal fluid (9, 10). The shedding of CD163 is enhanced by various pathological conditions including infections, liver diseases, malignancies, and autoimmune diseases. Indeed, sCD163 has been used as a biomarker for macrophage activation in several inflammatory diseases (9, 11).

To evaluate the potential of urine sCD163 as a biomarker in LN, and to investigate its correlation with clinical indices and pathological attributes, we assayed urine sCD163 in SLE patients with diverse disease activities from multiple ethnicities.

MATERIALS AND METHODS

Patients, Sample Collection, and Preparation

Urine samples from three cohorts of patients with SLE were included in this study. The primary cohort was comprised of 123 patients with SLE from the Division of Rheumatology,

Johns Hopkins University (JHU) School of Medicine, Baltimore, MD, United States. The validation cohort included 60 patients with SLE from Tuen Mun Hospital, Hong Kong (HK). An independent cohort of 45 LN patients with concurrent renal biopsies was drawn from the University of Texas Southwestern (UTSW) Medical Center's Renal Clinic, Dallas, TX, United States. Gender and ethnicity matched healthy subjects were recruited as controls. Informed consent was obtained from all patients, and this study was approved by the Institutional Review Board of the University of Houston, JHU School of Medicine, Tuen Mun Hospital, and UTSW. All patients met the 2012 Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE (12). For all patients, hybrid SLE disease activity index (SLEDAI) was used, where proteinuria was scored if > 0.5 g/24 h. The renal SLEDAI (rSLEDAI) summated the renal domains of SLEDAI, including hematuria (>5 red blood cells/high-power field), pyuria (>5 white blood cells/high-power field), proteinuria (>0.5 g/24h), and urinary casts. SLE patients were classified into three groups. Active LN (AR) was defined as biopsy-proven LN with rSLEDAI \geq 4. None of the active LN patients in this study had isolated hematuria or pyuria. Active non-renal SLE patients (ANR) had SLEDAI \geq 5 and rSLEDAI = 0. The Inactive (or low disease activity) group included SLE patients with SLEDAI \leq 4, and clinical SLEDAI (omitting anti-dsDNA and complement) \leq 2. Clean-catch midstream urine samples were collected and refrigerated within 1 hour of sample collection. The samples were then aliquoted and stored at -80°C . SLEDAI, rSLEDAI, physician global assessment (PGA), complete blood count, serum creatinine, urinalysis, urine protein to creatinine ratio, C3, C4, and anti-dsDNA were recorded.

Renal Histology

In the UTSW biopsy-concurrent cohort of 45 LN patients, renal biopsies were performed within 30 days of urine procurement. All 45 renal biopsies were documented for LN classes, and 42 biopsies had been scored for activity index (AI), chronicity index (CI), and their component attributes. AI was scored based on 6 components including endocapillary hypercellularity, glomerular leukocyte infiltration, wire loop deposits, fibrinoid necrosis, cellular crescents, and interstitial inflammation. The CI was scored based on glomerulosclerosis, fibrous crescents, tubular atrophy and interstitial fibrosis. The maximum score was 24 points for the AI and 12 for CI (13).

Assay of Urine sCD163 and Urine Creatinine

Urine sCD163 was assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech), following manufacturer instructions. Briefly, diluted urine samples were added to anti-CD163 pre-coated 96-well microplates, followed by biotin-conjugated anti-CD163 detection antibody, streptavidin-HRP, and substrate. Optical densities were read using a microplate reader at 490 nm (ELX808 from BioTek Instruments, Winooski, VT, United States). The levels of urine creatinine were assayed using the Creatinine Parameter Assay

Kit (R&D Systems). Urine sCD163 values were normalized to urine creatinine before further analysis.

Statistical Analysis

Data were analyzed using GraphPad Prism 7. The Mann Whitney *U* test was used for comparisons between two groups, and the analysis of variance (ANOVA) test with subsequent post-test pairwise comparisons was used for comparison of multiple groups. Chi-square test or Fisher's exact test was used to compare percentages. Non-parametric Spearman correlation was performed for correlation analysis. Receiver operating characteristic (ROC) curve was used to compare the performance of urine sCD163 versus other parameters and to determine the optimal cut-off values. A two-tail *P* value less than 0.05 was considered significant.

RESULTS

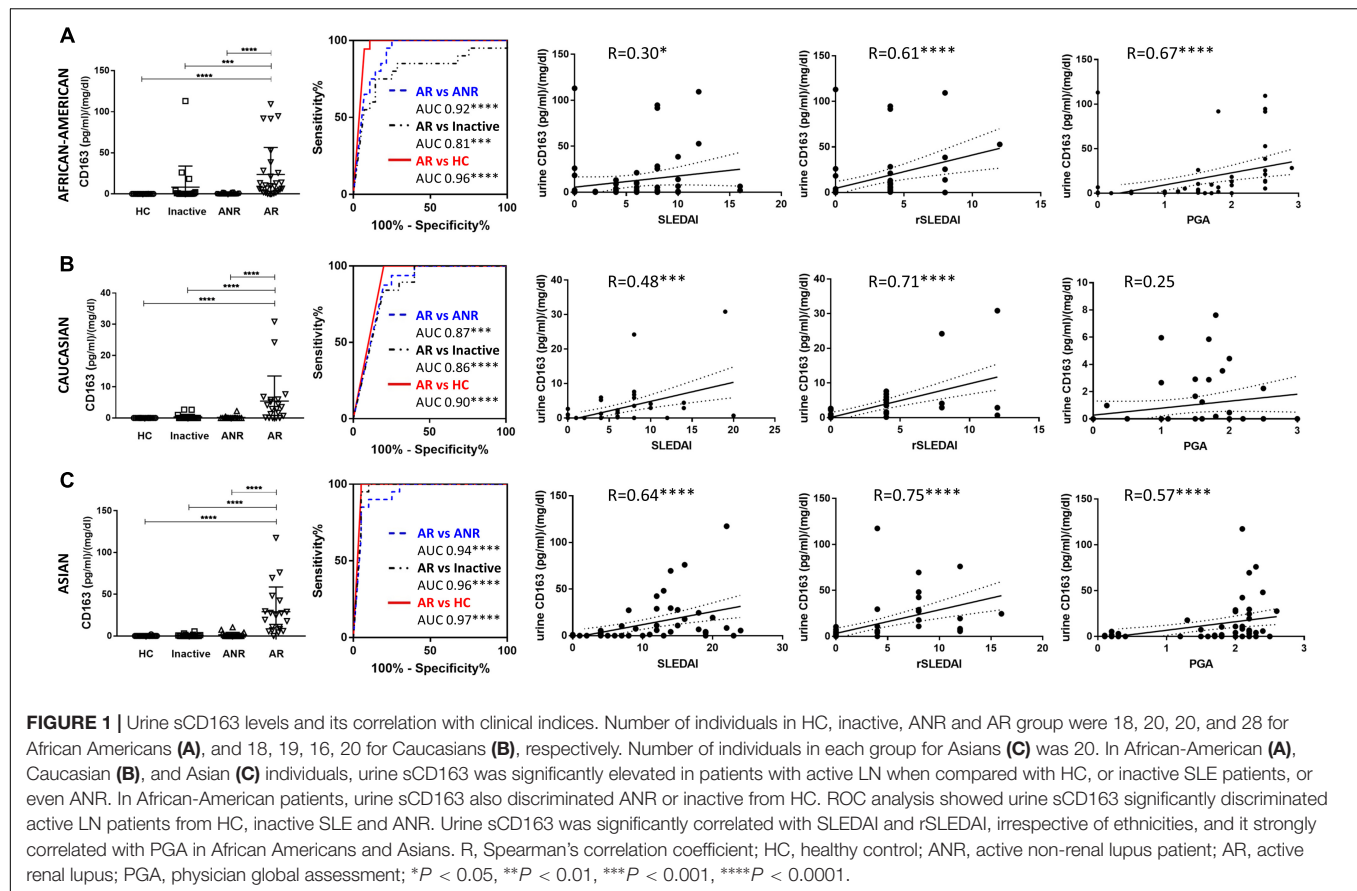
Urine sCD163 Was Significantly Elevated in Active LN in African Americans and Caucasians

The primary cohort from JHU was comprised of 48 active LN, 36 ANR, 39 inactive SLE patients, and 36 healthy controls (Table 1). Analyses were done separately for African Americans and Caucasians. Results demonstrated that in both African Americans and Caucasians, Cr normalized urine sCD163 was significantly elevated in patients with active LN when compared with healthy controls, or inactive SLE, or ANR patients (all *P* < 0.001). In African American patients, urine sCD163 could further discriminate ANR or inactive patients from healthy controls (both *P* < 0.05). Importantly, urine sCD163 significantly

TABLE 1 | Characteristics of primary cohort with African American and Caucasian patients.

	HC <i>n</i> = 36	Inactive SLE <i>n</i> = 39	Active non-renal <i>n</i> = 36	Active LN <i>n</i> = 48
Ethnicity				
African American, <i>n</i> (%)	18 (50.00%)	20 (51.28%)	20 (55.56%)	28 (58.33%)
Caucasian, <i>n</i> (%)	18 (50.00%)	19 (48.72%)	16 (44.44%)	20 (41.67%)
Female, <i>n</i> (%)	32 (88.89%)	35 (89.74%)	32 (88.89%)	44 (91.67%)
Age (years)	30.93 ± 5.34	38.96 ± 12.10	39.86 ± 11.88	34.08 ± 10.60
Clinical assessment				
SLEDAI	N/A	0.64 ± 0.93	7.00 ± 2.16	8.02 ± 3.86
rSLEDAI	N/A	0	0	5.42 ± 2.54
PGA	N/A	0.70 ± 0.73	1.17 ± 0.68	1.79 ± 0.58
System involvement				
Mucocutaneous, <i>n</i> (%)	N/A	6 (15.38%)	32 (88.89%)	12 (25.00%)
Joints, <i>n</i> (%)	N/A	5 (12.82%)	11 (30.56%)	5 (10.42%)
Neurological, <i>n</i> (%)	N/A	0 (0.00%)	2 (5.56%)	0 (0.00%)
Hematological, <i>n</i> (%)	N/A	7 (17.95%)	5 (13.89%)	2 (4.17%)
Laboratory measurement				
uPr/Cr (mg/mg)	N/A	0.17 ± 0.13	0.11 ± 0.09	2.01 ± 2.19
ESR (mm/h)	N/A	28.60 ± 29.88	41.26 ± 24.09	39.50 ± 28.93
SCr (mg/dl)	N/A	0.84 ± 0.30	0.82 ± 0.19	0.90 ± 0.30
anti-dsDNA positivity, <i>n</i> (%)	N/A	4 (10.26%)	29 (80.56%)	25 (52.08%)
anti-dsDNA titer (IU/ml)	N/A	12.84 ± 49.18	143.11 ± 209.05	95.44 ± 170.15
C3 (mg/dl)	N/A	115.44 ± 26.51	80.72 ± 29.53	93.79 ± 31.62
C4 (mg/dl)	N/A	20.88 ± 7.54	13.56 ± 7.74	18.77 ± 10.61
Medications				
Prednisone, <i>n</i> (%) [*]	N/A	15 (38.46%)	19 (52.78%)	32 (66.67%)
Hydroxychloroquine, <i>n</i> (%)	N/A	32 (82.05%)	31 (86.11%)	38 (79.17%)
Mycophenolate mofetil, <i>n</i> (%)	N/A	17 (43.59%)	20 (55.56%)	29 (60.42%)
Azathioprine, <i>n</i> (%)	N/A	3 (7.69%)	3 (8.33%)	6 (12.50%)
Tacrolimus, <i>n</i> (%)	N/A	0 (0.00%)	2 (5.56%)	2 (4.17%)
Cyclophosphamide, <i>n</i> (%)	N/A	0 (0.00%)	0 (0.00%)	1 (2.08%)
Methotrexate, <i>n</i> (%)	N/A	1 (2.56%)	3 (8.33%)	1 (2.08%)
Urine sCD163 (pg/ml)/(mg/dl)				
	0 (0, 0)	0 (0, 0.88)	0 (0, 0.04)	5.56 (1.50, 13.41)
African American	0 (0, 0)	0 (0, 1.28)	0 (0, 0.61)	9.23 (2.96, 26.22)
Caucasian	0 (0, 0)	0 (0, 0)	0 (0, 0)	3.22 (0.55, 5.88)

Data were presented as numbers (percentage), average ± standard deviation, or median (Q1, Q3). ^{*}Percentage of patients taking prednisone was significantly different between inactive SLE and active LN. *P* < 0.05.



correlated with SLEDAI and rSLEDAI in both the African American and Caucasian cohorts, and correlated strongly with PGA in African American subjects (Figures 1A,B).

Urine sCD163 Was Also Significantly Elevated in Active LN in Asian Patients

We further validated urine sCD163 in another cohort of patients, comprised of 20 active LN, 20 ANR, 20 inactive SLE, and 20 healthy controls, all of Asian origin (Table 2). In Asian patients, urine sCD163 was also significantly elevated in active LN compared with other SLE patients or healthy controls, and it was strongly correlated with SLEDAI, rSLEDAI, and PGA (Figure 1C). Notably, in patients with active LN, when three ethnic groups were compared, urine sCD163 was higher in African-American and Asian subjects than in Caucasian patients.

Urine sCD163 Was Significantly Elevated in the Presence of Concurrent Proliferative LN

Given that urine sCD163 was elevated in active LN patients, we further explored whether the elevation of urine sCD163 differed between LN classes. Urine sCD163 was assayed in 45 LN patients with concurrent renal biopsies (Table 3). This cohort yielded matched urine/renal-tissue specimens from the same patients, allowing one to examine if urine proteins could predict

concurrent renal pathology changes. Patients were dichotomized as proliferative LN (class III or IV, $n = 37$) or non-proliferative LN (class II or V, $n = 8$), based on pathology analysis of the biopsy. Among the renal biopsies from patients with Class III or IV LN, the frequencies of AI-related pathological changes were as follows: endocapillary hypercellularity, 77.14%; glomerular leukocyte infiltration, 57.14%; wire loop deposits, 42.86%; fibrinoid necrosis, 48.57%; cellular crescents, 62.86%; interstitial inflammation, 62.86%. Likewise, in these biopsies, the frequencies of CI-related pathological attributes were as follows: glomerulosclerosis, 62.86%; fibrous crescents, 11.43%; tubular atrophy and interstitial fibrosis, 62.86%. Urine sCD163 was significantly elevated in patients with proliferative LN, especially in LN IV, and it outperformed conventional parameters including C3, C4, and anti-dsDNA antibody in differentiating proliferative LN from non-proliferative diseases (Figures 2A–E).

Urine sCD163 Significantly Correlated With Renal Pathological Activity Indices

Given that urine sCD163 was significantly elevated in patients with concurrent proliferative LN when compared with non-proliferative LN, we then investigated whether it correlated with particular pathological attributes. Importantly, urine sCD163 significantly correlated with concurrent AI of renal pathology (Figure 2F), particularly with fibrinoid necrosis, cellular

TABLE 2 | Characteristics of secondary validation cohort with Asian patients.

	HC <i>n</i> = 20	Inactive SLE <i>n</i> = 20	Active non-renal <i>n</i> = 20	Active LN <i>n</i> = 20
Female, <i>n</i> (%)	20 (100.00%)	19 (95.00%)	19 (95.00%)	19 (95.00%)
Age (years)	24.58 ± 3.78	45.10 ± 11.25	33.5 ± 14.28	38.30 ± 12.28
Clinical assessment				
SLEDAI	N/A	2.45 ± 1.61	9.25 ± 6.63	15.05 ± 4.80
rSLEDAI	N/A	0	0	8.20 ± 3.55
PGA	N/A	0.17 ± 0.088	1.84 ± 0.32	2.14 ± 0.25
System involvement				
Mucocutaneous, <i>n</i> (%)	N/A	0 (0.00%)	14 (70.00%)	8 (40.00%)
Joints, <i>n</i> (%)	N/A	0 (0.00%)	7 (35.00%)	7 (35.00%)
Neurological, <i>n</i> (%)	N/A	0 (0.00%)	1 (5.00%)	0 (0.00%)
Hematological, <i>n</i> (%)	N/A	1 (5.00%)	10 (50.00%)	3 (15.00%)
Laboratory measurement				
uPr/Cr (mg/mg)	N/A	N/A	N/A	2.73 ± 2.28
anti-dsDNA positivity, <i>n</i> (%)	N/A	11 (55.00%)	14 (70.00%)	17 (85.00%)
anti-dsDNA titer (IU/ml)	N/A	101.00 ± 82.98	159.05 ± 122.17	222.25 ± 99.06
C3 (mg/dl)	N/A	89.45 ± 28.03	75.90 ± 27.00	47.75 ± 23.14
C4 (mg/dl)	N/A	16.95 ± 8.16	12.35 ± 5.73	8.90 ± 8.39
Medications				
Prednisone, <i>n</i> (%)	N/A	18 (90.00%)	20 (100.00%)	20 (100.00%)
Hydroxychloroquine, <i>n</i> (%)*	N/A	12 (60.00%)	19 (95.00%)	14 (70.00%)
Mycophenolate mofetil, <i>n</i> (%)	N/A	4 (20.00%)	4 (20.00%)	15 (75.00%)
Azathioprine, <i>n</i> (%)	N/A	17 (85.00%)	12 (60.00%)	17 (85.00%)
Tacrolimus, <i>n</i> (%)	N/A	2 (10.00%)	3 (15.00%)	6 (30.00%)
Cyclophosphamide, <i>n</i> (%)	N/A	6 (30.00%)	4 (20.00%)	7 (35.00%)
Cyclosporin, <i>n</i> (%)	N/A	3 (15.00%)	2 (10.00%)	2 (10.00%)
Urine sCD163 (pg/ml)/(mg/dl)	0 (0, 0)	0 (0, 0.76)	0 (0, 1.41)	22.02 (7.97, 32.78)

Data were presented as numbers (percentage), average ± standard deviation, or median (Q1, Q3). *Percentage of patients taking hydroxychloroquine was significantly higher in active non-renal group; percentage of patients taking mycophenolate mofetil was significantly higher in active LN group. *P* < 0.05.

crescents, and interstitial inflammation. In contrast, conventional parameters including C3, C4, and anti-dsDNA antibody did not correlate with AI or its component attributes. However, urine sCD163 did not correlate with renal pathology chronicity index (CI) or its component histological attributes (Table 4).

Urine sCD163 could potentially be derived from intra-renal infiltrating immune cells, given that myeloid cells are known to express this surface molecule. Single cell RNA-sequencing data (scRNA-seq) of renal infiltrating immune cells have recently been reported (14). Interrogation of this publicly deposited database indicated that macrophages were the predominant source of surface CD163 within LN kidneys, particularly M2 macrophages (Figures 3A–C), although CD163 was also expressed on other intra-renal macrophage populations including phagocytic CD16⁺ macrophages and tissue-resident macrophages. These findings suggest that intra-renal macrophages, particularly the M2 macrophages, may be the dominant source of sCD163 in the urine of LN patients.

DISCUSSION

Conventional biomarkers, including C3, C4, and anti-dsDNA antibody, have been classically used to evaluate general disease

activity in SLE. However, they do not predict or correlate well with LN or disease flares (8, 15). Anti-C1q is associated with renal involvement in SLE patients, particularly proliferative glomerulonephritis (16). Urinary biomarkers have now emerged as a potential tool for evaluating LN and potential treatment targets, as these proteins may arise directly from the inflamed kidneys (8, 15). Several newly identified urine biomarkers including monocyte chemoattractant protein-1 (MCP-1), neutrophil gelatinase -B associated lipocalin (NGAL), TNF-like WEAK inducer of apoptosis (TWEAK), and vascular cell adhesion molecule-1 (VCAM), have been promising in monitoring disease status (8). The identification of better biomarkers which significantly discriminate active LN and strongly correlate with renal biopsy or underlying mechanisms has been a priority.

We found that urine sCD163 significantly correlated with conventional parameters including urine protein to creatinine ratio, anti-dsDNA and serum C3 (all *P* < 0.05, Supplementary Table 1), and outperformed C3, C4, urine protein to creatinine ratio, and anti-dsDNA antibody in discriminating proliferative LN from non-proliferative LN (Figure 2E). Its strong correlation with concurrent renal pathology activity further supports the further study of urine sCD163 as a promising biomarker of LN.

TABLE 3 | Characteristics of LN patients with concurrent renal biopsies.

	All LN <i>n</i> = 45	LN II or V <i>n</i> = 8	LN III or IV <i>n</i> = 37	<i>P</i> value [‡]
Race				
African American, <i>n</i> (%)	17 (37.78%)	3 (37.50%)	14 (37.84%)	>0.9999
Caucasian, <i>n</i> (%)	24 (53.33%)	5 (62.50%)	19 (51.35%)	0.705
Asian, <i>n</i> (%)	4 (8.89%)	0 (0.00%)	4 (10.81%)	>0.9999
Female, <i>n</i> (%)	41 (91.11%)	6 (75.00%)	35 (94.59%)	0.1395
Age (years)	31.58 ± 8.82	30.63 ± 6.37	31.78 ± 9.32	0.8787
Clinical assessment				
SLEDAI	12.71 ± 4.85	10.25 ± 5.92	13.24 ± 4.50	0.1299
rSLEDAI	8.53 ± 4.14	6.00 ± 4.28	9.08 ± 3.96	0.0728
Laboratory measurement				
uPr/Cr (mg/mg)	3.27 ± 2.65	2.05 ± 2.18	3.54 ± 2.69	0.0842
Scr (mg/dl)	1.68 ± 1.61	1.78 ± 1.76	1.66 ± 1.60	0.3971
anti-dsDNA positivity, <i>n</i> (%)	30 (66.67%)	4 (50.00%)	26 (70.27%)	0.2019
anti-dsDNA titer (IU/ml)	853.00 ± 1013.62	172.50 ± 311.70	957.69 ± 1046.26	0.1638
C3 (mg/dl)	76.24 ± 35.74	77.63 ± 38.40	75.95 ± 35.69	0.8884
C4 (mg/dl)	11.60 ± 10.79	10.13 ± 7.04	11.92 ± 11.49	0.9666
Renal pathology[§]				
Activity index	8.90 ± 5.83	3.57 ± 3.05	9.97 ± 5.69	0.0048**
Endocapillary hypercellularity, <i>n</i> (%)	28 (66.67%)	1 (14.29%)	27 (77.14%)	0.0032**
Glomerular leukocyte infiltration, <i>n</i> (%)	21 (50.00%)	1 (14.29%)	20 (57.14%)	0.0931
Wire loop deposits, <i>n</i> (%)	20 (47.62%)	5 (71.43%)	15 (42.86%)	0.2289
Fibrinoid necrosis, <i>n</i> (%)	17 (40.48%)	0 (0.00%)	17 (48.57%)	0.03*
Cellular crescents, <i>n</i> (%)	22 (52.38%)	0 (0.00%)	22 (62.86%)	0.0029**
Interstitial inflammation, <i>n</i> (%)	23 (54.76%)	1 (14.29%)	22 (62.86%)	0.0341*
Chronicity index	3.93 ± 3.01	4.57 ± 3.21	3.80 ± 3.00	0.5975
Glomerulosclerosis, <i>n</i> (%)	27 (64.29%)	5 (71.43%)	22 (62.86%)	>0.9999
Fibrous crescents, <i>n</i> (%)	5 (11.90%)	1 (14.29%)	4 (11.43%)	>0.9999
Tubular atrophy and interstitial fibrosis, <i>n</i> (%)	26 (61.90%)	4 (57.14%)	22 (62.86%)	>0.9999
Medications				
Prednisone, <i>n</i> (%)	45 (100.00%)	8 (100.00%)	37 (100.00%)	>0.9999
Hydroxychloroquine, <i>n</i> (%)	40 (88.89%)	7 (87.50%)	33 (89.19%)	>0.9999
Mycophenolate mofetil, <i>n</i> (%)	35 (77.78%)	4 (50.00%)	31 (83.78%)	0.1383
Azathioprine, <i>n</i> (%)	4 (8.89%)	0 (0.00%)	4 (10.81%)	>0.9999
Cyclosporin, <i>n</i> (%)	1 (2.22%)	0 (0.00%)	1 (2.70%)	>0.9999
Cyclophosphamide, <i>n</i> (%)	1 (2.22%)	0 (0.00%)	1 (2.70%)	>0.9999
Urine sCD163 (pg/ml)/(mg/dl)	26.34 (9.42, 73.42)	7.43 (2.70, 8.85)	50.77 (14.54, 91.71)	0.0002

Data were presented as numbers (percentage), average ± standard deviation, or median (Q1, Q3). [‡]*P* values were for comparisons between LN II or V and LN III or IV. [§]In the renal pathology section, the percentages were calculated based on the number of patients who had complete information for AI and CI (all LN, *n* = 42; LN II or V, *n* = 7; LN III or IV, *n* = 35).

This predictive potential of sCD163 may be attributed to the close connection of urine sCD163 with disease pathogenesis. CD163, a transmembrane scavenger receptor, is exclusively expressed on macrophages and monocytes (17). Macrophages have been implicated in pathogenesis of SLE in many studies, with the spectrum of activation phenotypes ranging from classically activated inflammatory M1 to alternatively activated M2 macrophages (18–20). M2 macrophages can be further classified as M2a, M2b and M2c, which display distinct functions of pro-fibrosis, immunity-regulation, and remodeling or anti-inflammation, respectively (19). CD163 has been recognized

as a marker of M2 macrophages, especially M2c (21). These findings are clearly consistent with recent scRNA-seq analysis, revealing a predominant CD163^{high} M2 macrophages in LN kidneys (Figures 3A–C) (14).

It has been reported that M1 macrophages are dominant in SLE, as suggested by elevated markers of M1 macrophages, the cytokine milieu favoring M1 macrophages, and predisposing genetic factors, while M2a and M2c subpopulations were reduced in SLE, potentially contributing to defective anti-inflammatory balance (18–20). However, the predominant intra-renal subpopulations of macrophages in SLE have been

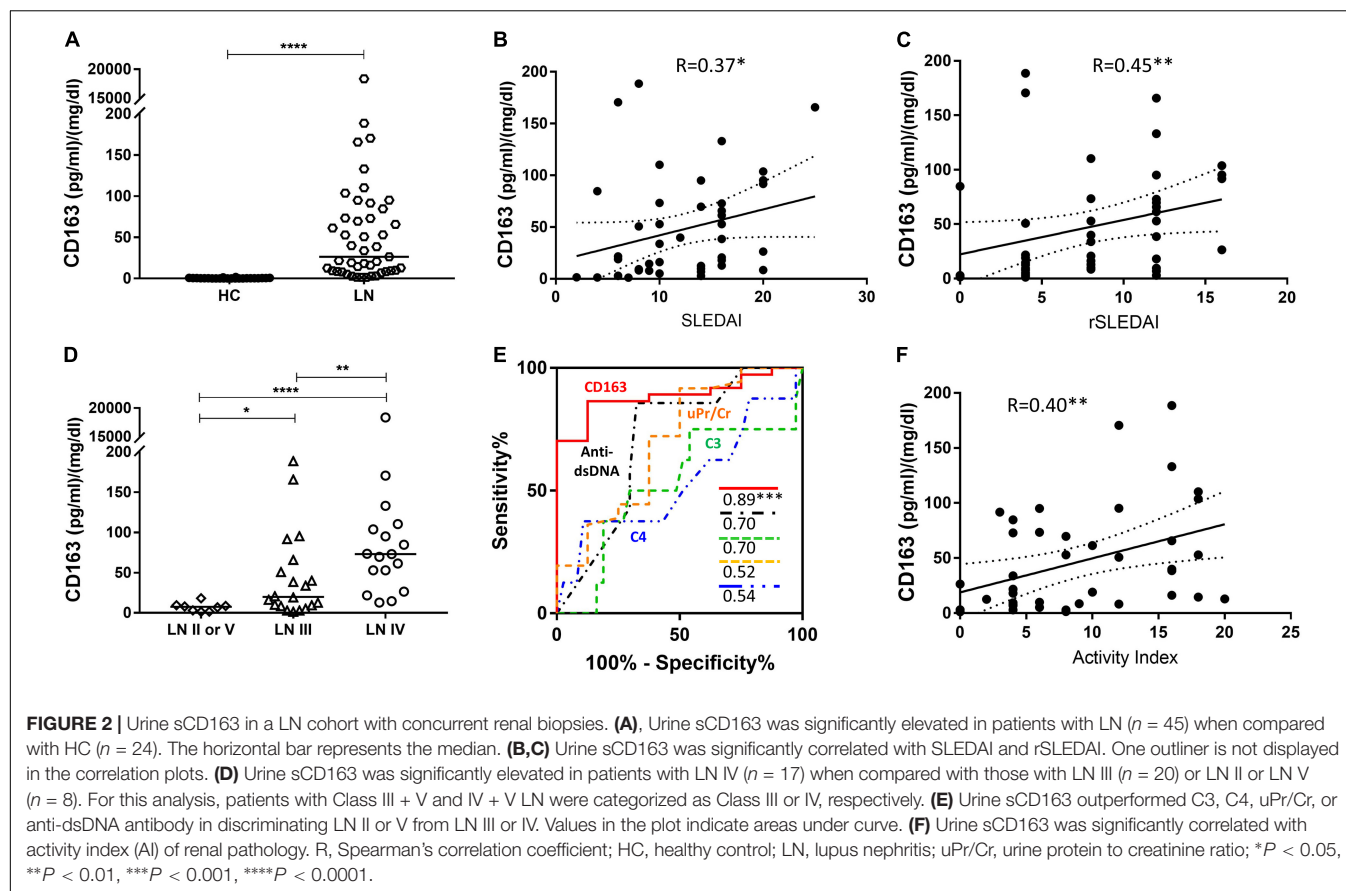


TABLE 4 | Correlation of urine sCD163 and conventional metrics with renal pathology activity and chronicity indices in biopsy-concurrent LN patients.

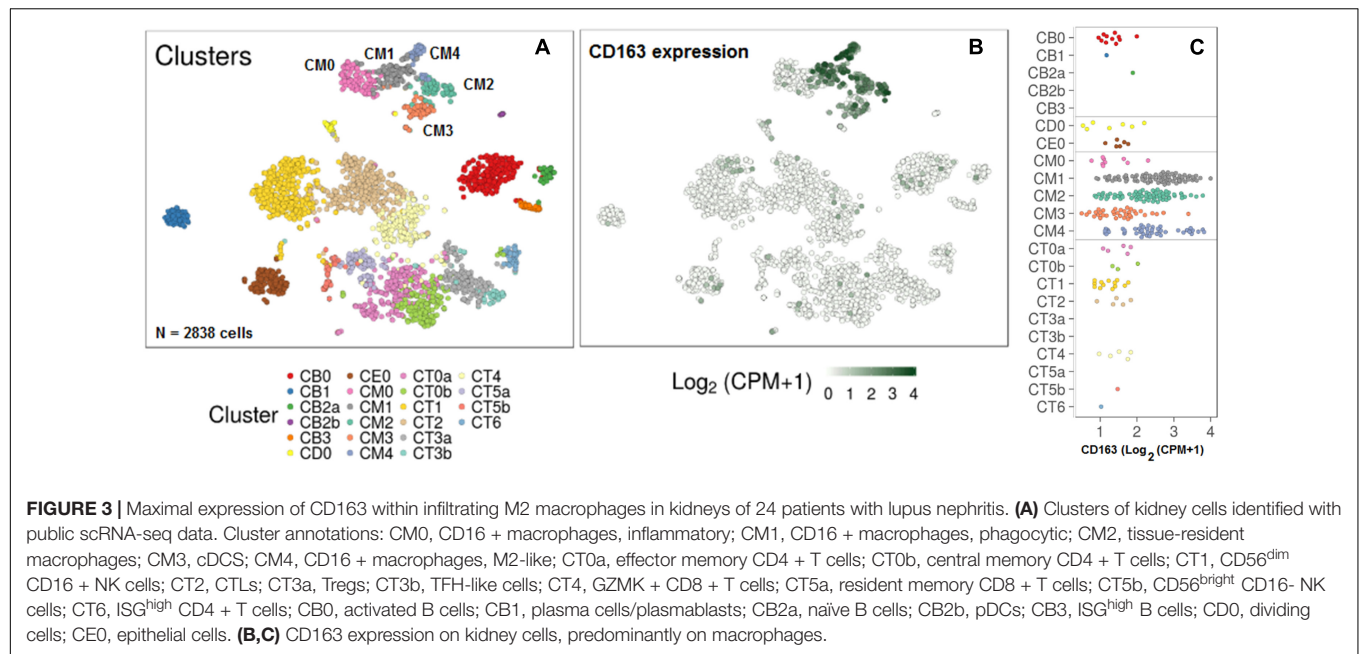
	AI	Endocapillary hypercellularity	Glomerular leukocyte infiltration	Wire loop deposits	Fibrinoid necrosis	Cellular crescents	Interstitial inflammation	CI	Glomerulo-sclerosis	Fibrous crescents	Tubular atrophy and interstitial fibrosis
CD163	0.40**	0.18	0.20	-0.22	0.45**	0.48**	0.39*	-0.14	-0.08	-0.02	0.05
C3	-0.19	-0.22	-0.10	-0.06	-0.13	-0.05	-0.21	0.28	0.46**	0.32*	-0.07
C4	-0.08	-0.11	-0.08	-0.02	0.004	-0.004	-0.25	0.28	0.41**	0.13	-0.01
anti-dsDNA	0.094	0.19	-0.078	-0.11	0.12	0.060	-0.071	-0.29	-0.41**	-0.26	-0.12

Values in the table indicate R values by Spearman correlation analysis. AI, activity index; CI, chronicity index; * $P < 0.05$; ** $P < 0.01$.

controversial and vary across studies, partly due to the markers used for identification of subpopulations. In one study, immunohistochemistry (IHC) demonstrated that infiltration of macrophages was dominated by CD163⁺ M2c-like macrophages in both glomerular and tubulointerstitial compartments (22). CD163⁺ M2c-like macrophages were significantly elevated in LN III and LN IV when compared with LN V, and correlated with AI of renal pathology (22). While tubulointerstitial CD163⁺ macrophages significantly correlated with serum creatinine, serum urea and creatinine clearance, glomerular CD163⁺ macrophages negatively correlated with plasma C3 and C4 (22, 23). Additionally, CD163 was found mainly expressed in active crescentic glomerulonephritis, proliferative glomerular lesions and areas of tubulointerstitial injury (21–24). In another study

including patients with pauci-immune necrotizing glomerulitis, CD68⁺ and CD163⁺ macrophages predominated at sites of fibrinoid necrosis (25). These literature reports, taken together with our findings in concurrent renal biopsies, suggest that M2 macrophages may play an important role in driving or modulating interstitial inflammation, cellular crescent formation, and fibrinoid necrosis. Furthermore, the predominant expression of CD163 by M2 macrophages rather than other renal cells supports the use of urine sCD163 as an easily measurable yardstick of renal macrophage infiltration.

CD163 expression on macrophages has been reported to be influenced by several medications including glucocorticoids, mycophenolate mofetil (MMF), tacrolimus, rituximab, and cyclophosphamide (22, 26–29). In the primary cohort with



African American and Caucasian patients, the percentage of patients taking prednisone was significantly higher in active LN compared to the inactive group (Table 1). In the validation cohort with Asian patients, the percentage of patients taking MMF was significantly higher in active LN group compared to inactive or ANR group (Table 2). However, in each ethnic group, when patients who took a certain medication were compared with patients who did not take the same medication, no significant difference in urine sCD163 was noted (Supplementary Table 2). Thus, the observed elevation in urine sCD163 in active LN and proliferative LN could not be attributed to medications. Indeed, it has been reported that urine sCD163 levels were comparable between glucocorticoid treated and untreated patients with LN IV, and was not related to the dosage of glucocorticoids (23).

Whether other organ involvement in SLE could influence the level of urine sCD163 has been questioned. Of relevance, serum/plasma level of sCD163 was significantly higher in SLE compared to healthy controls, and it correlated with anti-dsDNA antibodies, anti-chromatin antibodies, leukopenia, and SLEDAI (9, 30, 31). However, elevated plasma sCD163 levels in LN were not associated with ISN/RPS class (23), suggesting that increased urine sCD163 is unlikely to be the consequence of enhanced leakage of circulating sCD163 or systemic activation of macrophages, although this needs to be examined more systematically.

Given that urine sCD163 is predictive of concurrent proliferative LN in patients with clinically active renal disease, and given its association with active crescent formation and interstitial inflammation, monitoring urine sCD163 might represent a convenient, non-invasive method to track underlying renal disease in patients with LN. Hence, urine sCD163 may potentially be useful to guide management of LN. Since proliferative LN and elevated renal pathology AI are associated with worse patient and renal outcome, aggressive management is

warranted in these patients (32, 33). Further studies are clearly imperative to ascertain how urine sCD163 varies over time in the same patients, when serially monitored. Longitudinal studies are needed to assess if urine sCD163 can be used to predict renal flares or track response to treatment.

Elevated interleukin IL-6, IL-10, and macrophage colony-stimulating factor (M-CSF) in the glomerular microenvironment of LN have been reported to promote macrophage differentiation into CD163⁺ cells (23, 34). Nevertheless, the origin of increased macrophages in LN is not clear. One study suggested that tissue-resident macrophages and circulating monocytes were independently maintained, and monocytes did not contribute significantly to tissue macrophages in the steady state (35). However, others have proposed that resident macrophages are dominant in kidneys and are distinct from infiltrating macrophages that originate and renew from bone marrow, and that resident and infiltrating inflammatory macrophages both contribute to ongoing renal damage (36, 37). Moreover, the role of macrophages as well as CD163⁺ cells in LN has not been fully elucidated. In several mouse LN models, systemic depletion of macrophages or inhibition of macrophage recruitment ameliorated nephritis (20), while in others, depletion of macrophages slowed resolution and promoted fibrosis (37, 38). M2c macrophages are considered to have remodeling or anti-inflammatory roles (19, 22). Polarization of macrophages to a M2c-like phenotype is essential for efficient clearance of apoptotic cells, which when defective contribute to initiation and perpetuation of SLE, making induction of M2 macrophages an attractive therapy for SLE (39, 40). However, given the increased infiltration of CD163⁺ macrophages in active crescents, proliferative LN and acute tubulointerstitial lesions, it has also been suggested that CD163⁺ macrophages are involved in disease progression in kidney injury (24). Targeting macrophages has been suggested as a promising SLE therapeutic

strategy (20), but whether CD163 would serve as a more precise target in modulating macrophages needs to be investigated.

To conclude, urine sCD163 was significantly elevated in active LN. It accurately discriminated proliferative LN from non-proliferative LN and strongly correlated with concurrent renal pathological AI as well as fibrinoid necrosis, cellular crescents, and interstitial inflammation. These features support the use of urine sCD163 as a measurable biomarker for evaluating renal disease progression and for guiding management of LN. Prospective studies in larger cohorts to evaluate the performance of urine sCD163 as well as in-depth mechanistic studies unraveling the role of CD163 in LN pathogenesis are warranted.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of the University of Houston, JHU School of Medicine, Tuen Mun Hospital, and UTSW.

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AUTHOR CONTRIBUTIONS

CM designed the study. CCM, MP, and RS collected the samples used in this study. TZ, HL, and KV performed the experiment. TZ, GG, and CM analyzed the data. TZ, CCM, MP, RS, and CM drafted and revised the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00671/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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Phospholipase A2 Receptor Autoantibodies as a Novel Serological Biomarker for Autoimmune Thyroid Disease Associated Nephropathy

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Aims: To develop a highly sensitive immunoassay for PLA2R autoantibodies and study the relationship between PLA2R autoantibodies and autoimmune thyroid disease-associated nephropathy.

Methods: We applied a highly sensitive time-resolved fluoroimmunoassay to quantitatively detect the concentration of phospholipase A2 receptor (PLA2R) antibodies in the serum of patients with Graves' disease, Hashimoto's thyroiditis (HT), nephrotic patients with idiopathic membranous nephropathy (IMN), and normal controls. We immunohistochemically analyzed the existing PLA2R target antigen in the thyroid tissue of patients with Graves' disease and HT, as well as the nephridial tissue of nephrotic patients with IMN.

Results: PLA2R antibody concentrations in the serum of normal controls, patients with nodular goiter, Graves' disease, and HT, as well as patients with IMN were 1.13 ± 0.43 , 1.07 ± 0.22 , 2.12 ± 2.11 , 8.07 ± 4.74 , and 15.91 ± 19.50 mg/L, respectively. PLA2R antibody concentration in the serum and the area under the receiver operating characteristic curve in patients with HT and IMN were increased significantly. Immunohistochemistry revealed obvious staining of PLA2R in tissues from patients with HT, with a positive rate of 66.67%.

Conclusions: PLA2R is a potential pathogenic target antigen for HT, and the production of PLA2R antibodies may cause autoimmune thyroid disease-associated nephropathy.

Keywords: phospholipase A2 receptor, autoantibodies, thyroid disease, autoimmune, nephropathy

INTRODUCTION

Autoimmune thyroid disease (AITD) includes Graves' disease and Hashimoto's thyroiditis (HT). AITD has a morbidity of approximately 2% and is named as AITD-associated nephropathy when accompanied by kidney disease. Some patients with thyroid disease experience proteinuria for several months or years after confirmation of the disease (1). HT combined with membranous nephropathy was first reported in 1976 by O'Regan et al. (2); membranous nephropathy can be

classified as idiopathic and secondary membranous nephropathy according to the disease etiology. Recent studies showed that idiopathic membranous nephropathy (IMN) is generally associated with production of phospholipase A2 receptor (PLA2R) antibodies, indicating that PLA2R is a major target antigen of the disease. Serum levels of PLA2R autoantibodies may be useful for the diagnosis of IMN (3–5). Reports of AITD-associated nephropathy have increased gradually over the past 40 years; however, how membranous nephropathy is caused by AITD remains poorly understood. Because PLA2R serves as the target antigen of IMN, and IMN is associated with AITD, the relationship between PLA2R in AITD should be determined. Therefore, in the present study, we used a highly sensitive time-resolved fluoroimmunoassay (TRFIA) technique to establish a quantitative method for detecting and analyzing PLA2R antibodies in the serum of patients with several common thyroid diseases and IMN. Moreover, we performed immunohistochemical analysis of pathological samples obtained from patients to investigate the clinical significance of AITD in relation to PLA2R.

MATERIALS AND METHODS

Chemicals and Instrumentation

Goat anti-human IgG antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Recombinant PLA2Rs, DAB solution, hematoxylin, were provided by the Wuxi Jiangyuan Industrial Technology and Trade Corporation (Jiangsu, China). Goat anti-PLA2R antibodies were purchased from Abcam (Cambridge, UK). A europium-labeling kit (1244-302), including N^1 -(*p*-isothiocyanatobenzyl)-diethylenetriamine- N^1, N^2, N^3, N^4 -tetraacetic acid (DTTA) and enhancement solution, was purchased from Perkin Elmer (Waltham, MA, USA). Diethylenetriaminepentaacetate (DTPA), bovine serum albumin, Tris, and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Sepharose CL-6B columns were obtained from Pharmacia Co. (Uppsala, Sweden). The 96-well polystyrene microtiter plates were obtained from Nunc International (Roskilde, Denmark). All other reagents were of analytical grade and acquired from domestic manufacturers.

A model DU-650 spectrometer from Beckman (Brea, CA, USA) was used to detect proteins during antibody collection throughout the purification process. An AutoDELFIA₁₂₃₅ from Perkin Elmer was used to measure the level of Eu^{3+} fluorescence in the microtiter wells. A model AUTION MAX AX-4030 from Arkray (Edina, MN, USA) was applied as the urine automatic analyzer and a Cobas c702 autobiochemical analyzer was obtained from Roche (Basel, Switzerland). A Roche electrochemistry luminescence instrument (E170) was used to detect various thyroid serological indicators. An Olympus fluorescence microscope (Tokyo, Japan) was used for immunohistochemical analyses.

Research Methods and Subjects

Preparation of Eu^{3+} -Goat Anti-human IgG Antibodies

Eu^{3+} -goat anti-human IgG antibodies were prepared as described previously (6). Briefly, 1 mg of goat anti-human

IgG antibody was added to a small bottle containing 0.2 mg Eu^{3+} -DTTA and then incubated at room temperature for 20 h. Chromatographic separation was performed on the reaction solution, and protein peaks were evaluated.

Preparation of the Coated Plate (6)

A volume of 100 μ L of recombinant PLA2R antigen was diluted to 5 mg/L with 0.05 M carbonate at a pH of 9.6 and coated overnight. On the following day, the coating buffer was discarded, and the plate was blocked with a buffer containing 2% bovine serum albumin for 2 h. The buffer was discarded, after which the plate was evacuated under vacuum and stored at -20°C .

Preparation of the Standard Antibody Product

A serum from patients with IMN was added to an affinity chromatographic column connected with PLA2R recombinant antigens. The column was cleaned with TBS buffer, eluted with glycine with a pH of 2.7, monitored using an ultraviolet spectrophotometer (A280), and the first elution peak was collected to obtain a pure stock solution of the anti-PLA2R antibody. The solution was then further diluted with the reaction buffers to obtain working standard products with different concentrations.

Determination of the Anti-PLA2R-IgG Concentration in the Serum

The working standard product or serum (100 μ L) was diluted with reaction buffer (1:100) and added to a microwell plate, which was incubated at 25°C for 1 h with agitation. After four rinses, 100 μ L of Eu^{3+} -goat anti-human IgG antibodies was diluted with reaction buffer and incubated at 25°C for 1 h. After six rinses, 200 μ L of the enhancement solution was added followed by shaking for 5 min.

Assessment of the Anti-PLA2R-IgG TRFIA Method

Sensitivity

Mean and standard deviations (SD) were calculated for count values at zero concentration points on the standard curves of 10 groups. The sensitivity was calculated as the concentration corresponding to the value of mean+2SD determined via the standard curve.

Precision

The intra- and inter batch coefficients of variation were determined from measurements obtained from quality-controlled samples of three different concentrations.

Recovery Rate

After the background response from non-specific binding was determined for the TRFIA, two anti-PLA2R-IgG standard concentrations were added to the samples. The ratio of each measured value to its theoretical value was then calculated.

Research Subjects

Patients from Jiangsu Jiangyuan Hospital and the Wuxi People's Hospital affiliated with Nanjing Medical University, were enrolled in the study. The study protocol was approved by

the medical ethics committee of Jiangsu Jiangyuan Hospital and Affiliated Wuxi People's Hospital of Nanjing Medical University, the patients provided informed consent for the use of their samples. We assessed the thyroid color Doppler ultrasound report in combination with the patients' medical history, clinical manifestation, and laboratory examination in reference to the Diagnostic Standard for HT, Graves' disease, and Hypothyroidism in Clinical Endocrinology. There were 40 patients with HT, 47 patients with Graves' disease, and 10 patients with nodular goiter. Some patients were subjected to immunohistochemical analysis of the thyroid tissue. In addition, 46 patients were confirmed to have IMN (without cancer, lupus nephropathy, diabetic nephropathy, hepatitis B virus-associated nephritis, purpura nephritis), and 64 controls were included as healthy physical controls without thyroid disease, nephropathy, gastroduodenal disorders, or liver disease.

Indirect Immunofluorescence

An indirect immunofluorescence technique was applied to the thyroid surgical tissue and renal biopsy tissue. Rabbit anti-PLA2R (Sigma) was diluted (1:500) and applied to the frozen tissue. Next, horseradish peroxidase-labeled goat anti-rabbit secondary antibodies were added. The tissues were dyed using DAB solution and counterstained with hematoxylin.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, Inc., La Jolla, CA, USA) and SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The values are expressed as the mean \pm standard deviation. Independent samples *t*-test, Wilcoxon test, and Mann-Whitney test were used to compare groups. A two-sided $P < 0.05$ was considered as statistically significant.

RESULTS

Examination of Anti-PLA₂R-IgG-TRFIA

Figure 1 presents the anti-PLA₂R-IgG-TRFIA standard curve obtained from a Log-LogB functional data processing program. The sensitivity of the method was 0.07 mg/L. The intra- and inter-batch coefficients of variation were 4.7 and 9.2%, respectively. The average recovery was 93.2%.

Analysis of the Sample Results

The samples were divided into five groups. The concentration of PLA₂R antibodies in each serum sample was detected using the TRFIA method, and the results are shown in **Figure 2**.

Table 1 presents the level of urine protein, hematuria, serum creatinine (Scr), glomerular filtration rate, and anti-PLA₂R-IgG in the serum of different patients with thyroid disease and IMN. The results showed that the positive rates of urine protein and hematuria in patients with IMN were high, and some patients tested positive for thyroid disease. The glomerular filtration rate increased in patients with graves' disease. The concentrations of serum PLA₂R antibodies in patients with HT and IMN were increased significantly. The positive rate of patients with HT and IMN were 97.50% and 82.61%, respectively.

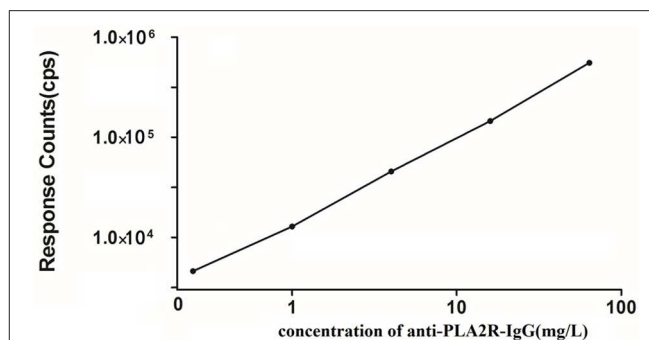


FIGURE 1 | Standard curve of anti-PLA₂R-IgG-TRFIA.

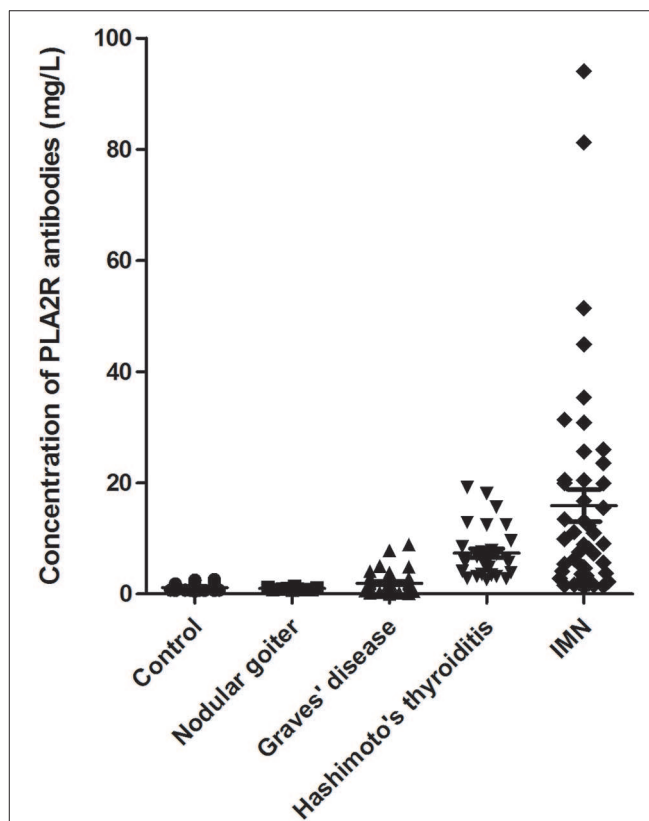


FIGURE 2 | Anti-PLA₂R-IgG detection in the serum of patients. Sera from 46 patients with IMN, 40 patients with HT, 47 patients with Graves' disease, 10 patients with nodular goiter, and 64 healthy controls were examined.

Receiver Operating Characteristic (ROC) Curve Analysis

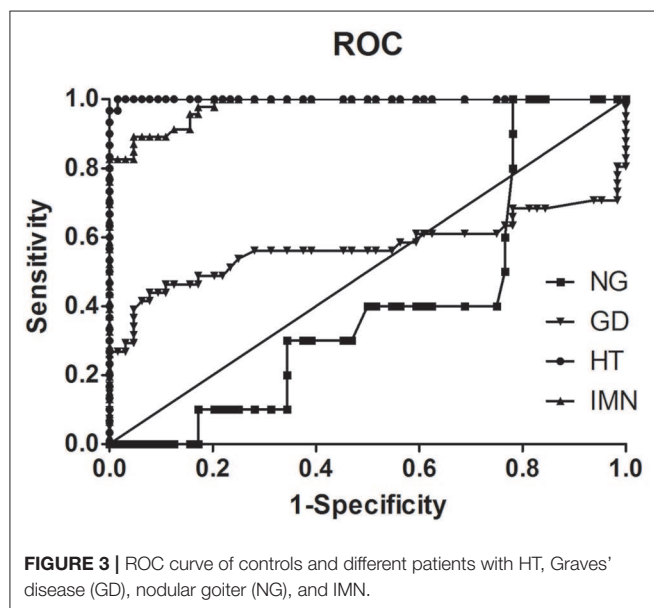
Figure 3 shows the ROC curve for the level of serum anti-PLA₂R-IgG in controls and patients with HT, Graves' disease, nodular goiter, and IMN; the areas under the curve for the analysis of sensitivity for HT, Graves' disease, nodular goiter, and IMN were 1.000 ± 0.001 , 0.569 ± 0.069 , 0.402 ± 0.084 , and 0.926 ± 0.027 , respectively. The differences between HT, Graves' disease, or IMN and healthy volunteers were all significant ($P < 0.01$).

TABLE 1 | Characteristics of healthy volunteers and different patients with thyroid disease or IMN.

	Healthy volunteers (n = 64)	Graves' disease (n = 47)	Nodular goiter (n = 10)	HT (n = 40)	IMN (n = 46)
Age, years (mean \pm SD)	39.1 \pm 11.4	40.2 \pm 11.4	38.2 \pm 15.6	42.1 \pm 13.9	44.6 \pm 12.1
Male (n)/female (n)	34/30	13/34	7/3	9/31	25/21
Positive rate of Urine protein (%)	0	2.1	10	2.5	97.8
Positive rate of hematuria (%)	0	12.33	20	16.67	100
Scr (μ mol/L)	63.81 \pm 12.82	45.45 \pm 14.17*	45.80 \pm 13.97*	57.54 \pm 10.27*	75.58 \pm 35.63*
GFR (ml/min)	109.12 \pm 11.87	126.79 \pm 18.23*	109.12 \pm 12.63	103.12 \pm 16.46	101.78 \pm 22.04
Anti-PLA2R IgG (mg/L)	1.13 \pm 0.43	2.12 \pm 2.11	1.07 \pm 0.22	8.07 \pm 4.74*	15.91 \pm 19.50*
Range of anti-PLA2R IgG concentrations (mg/L)	0.50–2.60	0.12–8.97	0.77–1.40	2.59–21.76	1.29–94.05

GFR (CKD-EPI) = $a \times (\text{serum creatinine}/b)^c \times (0.993)^{\text{age}}$, PLA2R, phospholipase A2 receptor; Scr, serum, creatinine; IMN, idiopathic membranous nephropathy; HT, Hashimoto's thyroiditis.

* $P < 0.05$, patient groups were compared to the healthy volunteer group.



Immunohistochemical Results

Immunohistochemical analysis was performed on pathological sections of thyroid tissue obtained from in-patients undergoing thyroidectomy with HT and nodular goiter, as well as the nephridial tissue from patients with IMN using goat antibodies against PLA2R (Figure 4). The membranes of both sides of the thyrocytes from patients with HT and glomerular podocytes from patients with IMN were obviously stained, indicating that both patient groups contained the same PLA2R target antigens. The positive rate of tissues from patients with HT was 66.67% and tissues from patients with IMN was 84.78%; the staining in the thyroid tissues of patients with HT was weaker than that in the glomeruli of patients with IMN, indicating that the content of PLA2R in thyroid tissues was lower than that in the glomeruli of patients with IMN, and the concentration of antibodies against thyroid tissues was also

lower than that of PLA2R antibodies in the serum of patients with IMN.

DISCUSSION

The incidence of thyroid disease and nephropathy is high, with 4.6% of the US population suffering from hypothyroidism and 1.3% from hyperthyroidism. Additionally, 10% of American adults suffer from some level of chronic kidney disease (CKD). Thyroid disorders are risk factors for CKD (7). As critical human organs, the thyroid and kidney are very closely related. Studies have shown that the thyroid can promote kidney growth and development and maintain kidney functions, and the kidney can function as a metabolic and eliminating organ for the thyroid. Therefore, AITD-associated nephropathy has attracted increased attention (8). Early in 1952, some scholars showed that 11% of patients with AITD had symptoms of proteinuria (9, 10). Patients with AITD may develop secondary renal damage, which may represent nephrotic syndrome and or less frequently renal function damage (11). Renal damage may occur prior to or simultaneously with thyroid disease symptoms. Some reports have suggested that higher thyroid-stimulating hormone levels were associated with a greater risk of subsequent CKD. Individuals with subclinical hypothyroidism and those with overt hypothyroidism are more likely to have CKD than those with euthyroid (12). The pathogenesis of AITD combined with renal damage is not completely understood and has been suggested to be associated with the formation of *in situ* immune complexes resulting from deposition of thyroglobulin and thyroid microsomal antigens in the glomerulus (13, 14). Thyroid diseases including both hypo- and hyperthyroidism are associated with several types of glomerulonephritis. The most common renal diseases observed in AITD are MN, membranoproliferative glomerulonephritis, minimal change disease, IgA nephropathy, focal segmental glomerulosclerosis, antineutrophil cytoplasmic autoantibody vasculitis, and amyloidosis. Different hypotheses have been proposed regarding the relationship between AITD and

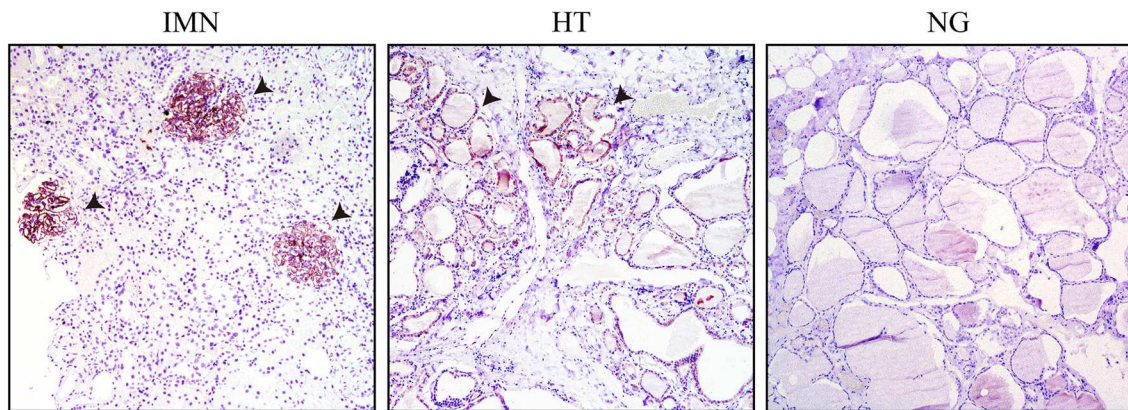


FIGURE 4 | Immunohistochemical results of patients with IMN, HT, and NG, with the papillary micro-magnified by 100-fold (the arrow pointing to the positive regions indicate the glomerulus of IMN or thyroid cell members of HT).

glomerulopathies, and several potential mechanisms for this association have been considered (15). The pathophysiology links between thyroid dysfunction and glomerulonephritis involve proteinuria and formation of immune complexes. This association is extremely common in autoimmune thyroiditis. These complexes are mainly responsible for alterations in renal function by depositing on the basement membrane of the glomeruli (8). However, why some renal damage occurs prior to thyroid disease symptoms is unclear. Presently, AITD-associated nephropathy is primarily diagnosed by thyroglobulin immunohistochemical staining, which shows an extremely low positive rate. Li et al. (16) conducted immunohistochemical examination of 4 patients with AITD-associated nephropathy and found that only 1 patient had thyroglobulin deposited under capillary basement membrane epithelial cells, and other 3 patients tested negative. Thus, other generic antigens may be present, and our study showed that the concentration of anti-PLA2R-IgG in the serum of patients with IMN was significantly increased. The same results were observed in patients with HT, which is also known as chronic lymphocytic thyroiditis and an AITD disease. HT, which generally occurs in middle-aged people, begins imperceptibly and slowly, and the patients would inadvertently show an enlarged thyroid while most thyroid functions remain normal; however, some patients may have symptoms accompanied by transient hyperthyreosis, typically referred to as Hashitoxicosis, and have hypothyroidism in the late stage of the disease. This disease was characterized in that high-titer antithyroid antibodies could be detected, large amount of plasmocytes and lymphocytes in thyroid tissue of patients were infiltrated to form lymphoid follicles; lymphocytes would form lymphoblasts after contact with thyroid antigens, and further produce migration inhibitory factors and lymphocytotoxins to warn the patients had T cells and corresponding antigens thereof served as thyrocyte components; it has been reported that a HT, upon initiation of treatment with levothyroxine, he had progressive deterioration in renal function and proteinuria. A renal biopsy revealed coexistent necrotizing and crescentic glomerulonephritis and membranous

nephropathy. Induction treatment with oral cyclophosphamide and prednisone, at the end of 6 months of treatment, there was improvement in renal function and proteinuria (17), indicating that HT and membranous nephropath would have same pathogenesis. Anti-PLA2R-IgG has been confirmed as an autoantibody of IMN; therefore, in this study, a highly sensitive method for detecting anti-PLA2R-IgG in the serum was established with a sensitivity up to 0.07 mg/L. In a previous study, we compared in-house TRFIA and Euroimmun ELISA. Within the measurable range of the two methods, the correlation coefficient was $R^2 = 0.925$. Because of the higher sensitivity of the TRFIA method among patients clinically diagnosed has having IMN, the ELISA positive rate was 66.7% and the in-house TRFIA positive rate was 89.7%. Thus, the results of TRFIA were better than those of ELISA (6, 18, 19). In addition, IMN can be distinguished from secondary membranous nephropathy and IgA nephropathy by setting different thresholds, which is more conducive for the differential diagnosis of IMN in the clinic (20, 21). The concentration of anti-PLA2R-IgG in the serum of patients with IMN was high, whereas its concentration in the serum of patients with HT was only slightly increased. The concentration of anti-PLA2R-IgG in the serum of patients with Graves' disease and thyroid tumor was mostly consistent with that in healthy people. Immunohistochemical analysis was further conducted on nephridial tissues from patients with IMN and thyroid tissues from patients with HT. The results showed that the PLA2R positive rate was far greater than that in thyroid tissues from patients with Graves' disease and nodular goiter patients, indicating that PLA2R is not only the target antigen of IMN but also of HT, and anti-PLA2R-IgG is not only an autoantibody of IMN, but also of HT. This may be one cause of AITD-associated nephropathy. This helps to explain why renal damage occurred before, after, or simultaneously with thyroid disease. As HT shares some symptoms with hyperthyroidism and hypothyroidism, and hypothyroidism occurs at a later stage, the generic target antigen PLA2R explains why hyperthyroidism and hypothyroidism symptoms were sometimes accompanied by nephropathy, and patients with

hypothyroidism nephropathy were in a more serious condition. Therefore, PLA2R autoantibodies may be a novel serological biomarker for AITD-associated nephropathy.

CONCLUSION

We developed a highly sensitive immunoassay for anti-PLA₂R-IgG and found that the concentration of anti-PLA₂R-IgG in the serum of patients with HT was increased. This is the first study to show that patients with HT express PLA2R antigens. Our results provide a foundation for studies of the nosogenesis and treatment of AITD-associated nephropathy.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

This study protocol was approved by the medical ethics committee of Jiangsu Jiangyuan Hospital and Affiliated Wuxi People's Hospital of Nanjing Medical University (KYL2016001). All enrolled subjects provided their written informed consent

for study participation, and all methods were performed in accordance with the relevant guidelines and regulations.

AUTHOR CONTRIBUTIONS

BH, LW, and ZH contributed conception and design of the study. YZ, QW, TL, JZ, and JB performed the experiments. QZ, HS, and ZH performed the clinical analysis. All authors contributed to manuscript revision, read, 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.

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C1q A08 Is a Half-Cryptic Epitope of Anti-C1q A08 Antibodies in Lupus Nephritis and Important for the Activation of Complement Classical Pathway

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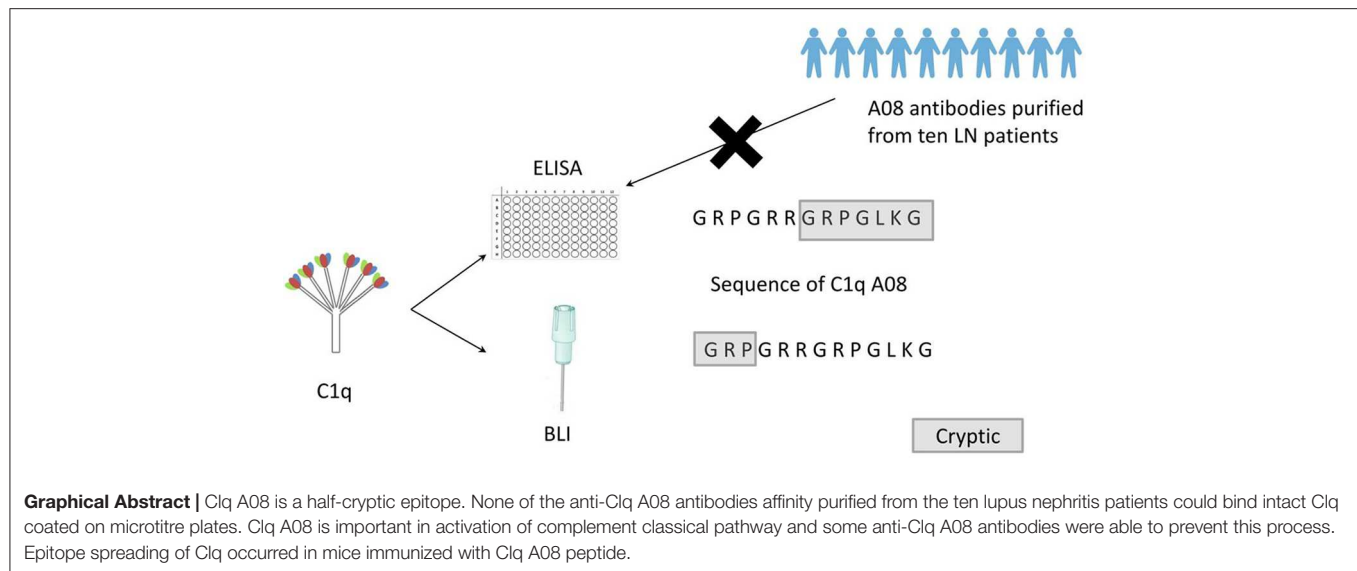
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To investigate the fine epitope(s) of anti-C1q A08 antibodies and their roles in complement activation in lupus nephritis, C1q A08 and related peptides with various amino acid sequences around A08 were synthesized. Anti-C1q A08 antibodies from 10 lupus nephritis patients were purified from plasmapheresis samples, and four monoclonal antibodies against C1q A08 were screened and identified from mouse hybridoma cells, to study the fine epitope(s) of C1q A08 using ELISA and Biolayer Interferometry (BLI). The biofunction of anti-C1q A08 antibodies for complement classical pathway activation was investigated by C3 activation assay. Anti-C1q A08 antibodies and anti-C1q antibodies were also detected in the sera of female BALB/C mice immunized by C1q A08 peptides. None of the anti-C1q A08 antibodies, which were affinity purified from the 10 lupus nephritis patients, could bind intact C1q coated on microtitre plates, neither could the anti-C1q antibodies bind to C1q A08 peptides coupled on resin, indicating that the human anti-C1q antibodies and anti-C1q A08 antibodies may recognize different epitopes of C1q. One of the four C1q A08 mAbs (32-4) bound to the six amino acids of N-terminus of C1q A08, while another C1q A08 mAb (17-9) bound to eight or 10 amino acids of C-terminus of A08. The third and fourth C1q A08 mAb (1A12 and 4B11) bound to the whole sequence of A08. Only 32-4 mAb bound to the intact C1q coating on an ELISA plate, whereas 17-9 mAb, 1A12 mAb, and 4B11 mAb could not. However, using a BLI assay, 17-9 mAb, 1A12 mAb, and 4B11 mAb, but not 32-4 mAb, could bind to intact C1q. Furthermore, 1A12 mAb and 4B11 mAb, but not 32-4 and 17-9 mAb, could inhibit the activation of complement classical pathway. Anti-C1q A08 antibodies were detected in all the female BALB/C mice in the experimental group but not in the control group. Two out of six in the experimental group developed anti-C1q antibodies. C1q A08 is a half-cryptic epitope of C1q involving N-terminal six amino acids of C1q A08, and this is important to the activation of a complement classical pathway, and some anti-C1q A08 antibodies were able to prevent this process. Epitope spreading of C1q occurred in the mice immunized with C1q A08 peptides.

Keywords: lupus nephritis, C1q A08, half-cryptic epitope, complement classical pathway, epitope mapping



INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by breach of immune tolerance with an overproduction of various autoantibodies, such as anti-dsDNA antibodies, anti-Smith antibodies, and anti-C1q antibodies (1). SLE patients can develop several complications and target organ inflammation, of which lupus nephritis is the most crucial risk factor of morbidity and mortality. Almost all SLE patients exhibit deposition of immune complexes in glomeruli, 40–60% of which develop clinical lupus nephritis (2). The complement system plays an important role in the clearance of apoptotic cells, which release numerous cellular substances, such as dsDNA and proteins (3). The complement classical pathway is of major interest in lupus nephritis research, and C1q is the first protein in this classical pathway whose deficiency indicates a much higher risk of developing SLE. Interestingly, few patients exhibit C1q gene mutation, but anti-C1q antibodies have been found in more than 50% of lupus nephritis patients. Several studies have found an association between anti-C1q antibodies and disease activity in lupus nephritis (4–6), but its role in the pathogenesis of lupus nephritis still remains to be elucidated.

C1q, an ultra large protein with a molecular weight of about 460 kDa, is composed of six A chains, six B chains, and six C chains. The A chain is covalently linked to the B chain, while the C chain is non-covalently linked to the AB dimer in one ABC strand and is also covalently linked to the C chain of another ABC strand to form an ABC-CBA doublet (7). Each chain includes an N-terminal collagen-like region (CLR), which is responsible for mediating immune mechanisms, and a C-terminal globular head region, which is responsible for recognizing target ligands, such as an immune complex and bacterial and viral surface proteins (8). C1q is an important linker protein between the innate immune system and adaptive immune system through binding to antigen–antibody immune complexes to activate a complement classical pathway. In SLE, the generation of autoantibodies along with the deposition of immune complexes causes chronic

inflammation and tissue injury, where C1q acts a significant role in clearing such immune complexes and apoptotic cells (9–11).

Importantly, Vanhecke et al. used anti-C1q antibodies derived from SLE patients in a microarray-based scan to identify the B-cell epitope of C1q, and found that C1q A08 (C1q A15-27: GRPGRRGRPGLKG) is the most important epitope of C1q (12). It was also found that binding of C1q A08 is correlated to bindings of C1q for the same sera, where C1q A08 seemed to be an exposed epitope on C1q. Our recent study, based on a large Chinese cohort, further confirmed that C1q A08 antibodies are better than antibodies against intact C1q in correlating with lupus nephritis activity as well as predicting renal prognosis (13).

The sequence of C1q A08 contains four arginine residues and one lysine residue, which is strongly positively charged. Prior studies have shown that C1q A14-26, with only one amino acid shift as compared to C1q A08 peptides, can bind some ligands, such as DNA (14), CRP (15, 16), fibronectin (17), LPS (18, 19), vWF (20), and amyloid P (21) as well as advanced glycan end products (22), indicating that C1q A08 may be an important functional sequence. It was believed that characterizing C1q A08 in greater detail would help better understand the biology of C1q in physiological and pathological conditions, particularly in the pathogenesis of lupus nephritis.

Therefore, anti-C1q A08 antibodies from lupus nephritis patients were purified, and four monoclonal mouse anti-human C1q A08 antibodies were screened. Various conformational epitopes and biofunctions of the anti-C1q A08 antibodies were further investigated *in vitro*.

RESULTS

Binding of C1q to Anti-C1q A08 Antibodies Purified From Plasma Samples of Lupus Nephritis

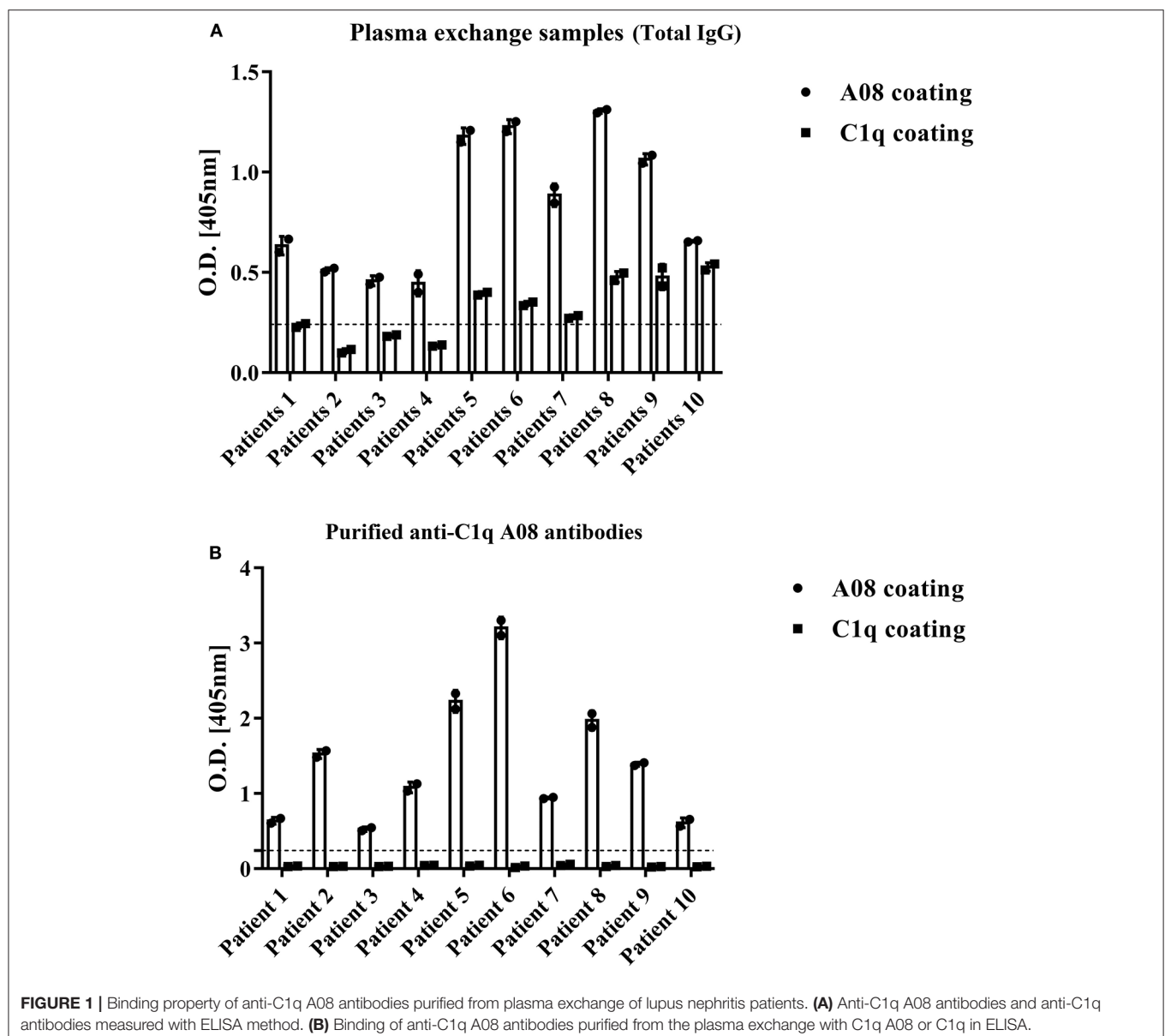
To study the fine epitope(s) of anti-C1q A08 antibodies, plasma exchange samples from 10 lupus nephritis patients, who were

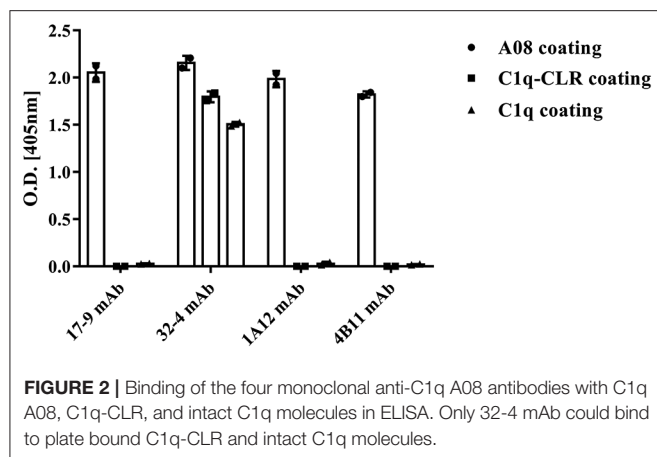
positive for anti-C1q A08 antibodies, were used to isolate and purify anti-C1q A08 antibodies (Figure 1A). Total IgG was purified using a Protein G column, and anti-C1q A08 antibodies were isolated from total IgG using Pabpur SulfoLink beads coupling with GRPGRRRPGLKGC (C1q A08 peptides fused with cysteine). All the anti-C1q A08 antibodies from the 10 lupus nephritis patients were bound to A08 but not intact C1q (Figure 1B).

Mapping of Binding Site of C1q Using Monoclonal A08 Antibodies Derived From Hybridoma Cells

All the four monoclonal C1q A08 antibodies could bind to C1q A08 peptides using the ELISA (enzyme linked immunosorbent assay) method, while only 32-4 mAb could bind to C1q CLR

and intact C1q molecule. Similarly, with anti-C1q A08 antibodies purified from plasma samples of the 10 lupus nephritis patients, 17-9 mAb, 1A12 mAb, and 4B11 mAb did not bind to C1q coated on Costar polystyrene microtiter plates (Figure 2). As illustrated in Figure 3, a group of C1q A08-related peptides were used to map the binding site of the four anti-C1q A08 mAbs on C1q coated on Costar polystyrene microtiter plates. The binding site of 32-4 mAb and 17-9 mAb was on the six N-terminal amino acids and the 10 C-terminal amino acids, respectively, whereas the binding properties of 1A12 and 4B11 were dependent on the entire A08 sequence (Figure 3). The results showed that, when C1q was coated on Costar polystyrene microtiter plates, the six N-terminal amino acids were exposed, but the other seven C-terminal acids were not completely exposed, indicating that A08 was a half-cryptic-half-exposed epitope. A summary diagram of





epitope and binding activity for different monoclonal antibodies is shown in **Supplementary Figure 1**.

Evaluation of Binding Affinity of C1q and Anti-C1q A08 Antibodies Using BLI

The binding property of human IgG with C1q was studied to validate if C1q could keep its natural conformation when coupled on an AR2G sensor. *In vivo*, C1q bound to surface bound IgM pentamer or IgG but not free IgM or IgG, which was arranged to clear immune complexes. When human IgG was coupled on an AR2G sensor, C1q could bind to IgG. However, when C1q was coupled on an AR2G sensor, mouse and human total IgG could not bind to C1q at all (**Figure 4C**).

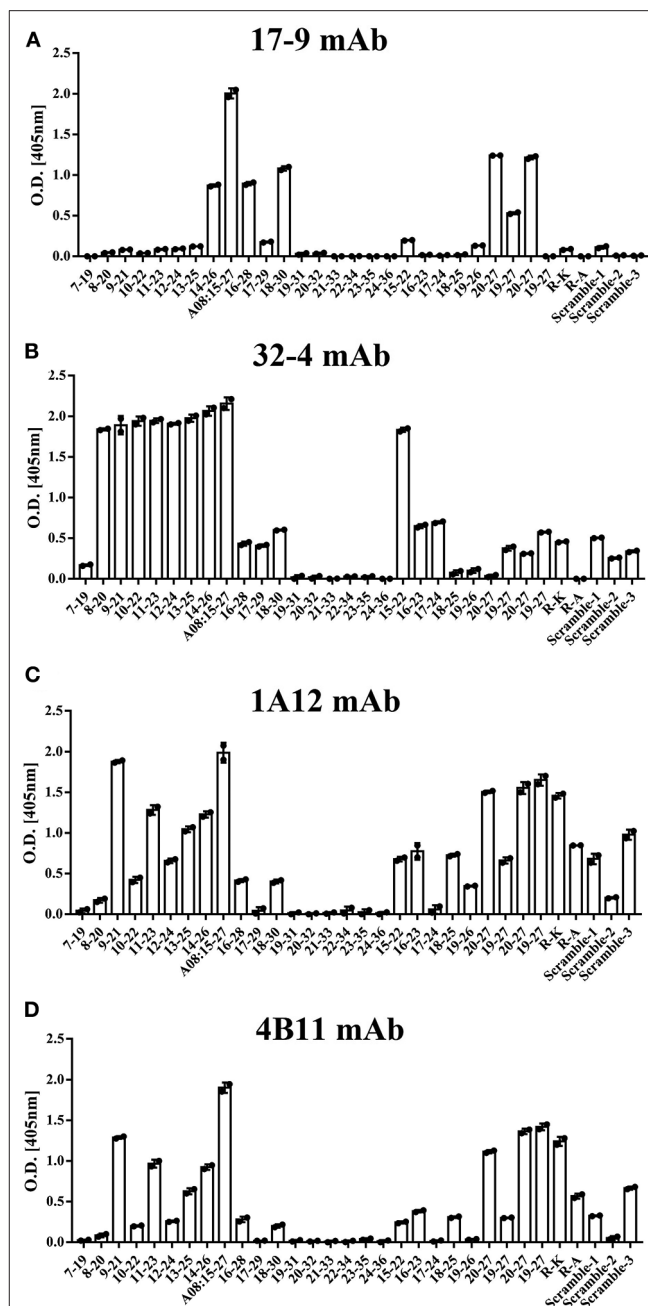
In the case of the four monoclonal A08 antibodies, 17-9, 1A12, and 4B11, but not 32-4, could bind to C1q and C1qCLR coupled on an AR2G sensor, which was quite opposite to the results in ELISA (**Figure 4**). The results showed that about 10 C-terminal amino acids were completely exposed but the other three N-terminal acids were not when C1q or C1q CLR was coated on AR2G sensor. Here, A08 was also a half-cryptic epitope but was different from that in ELISA.

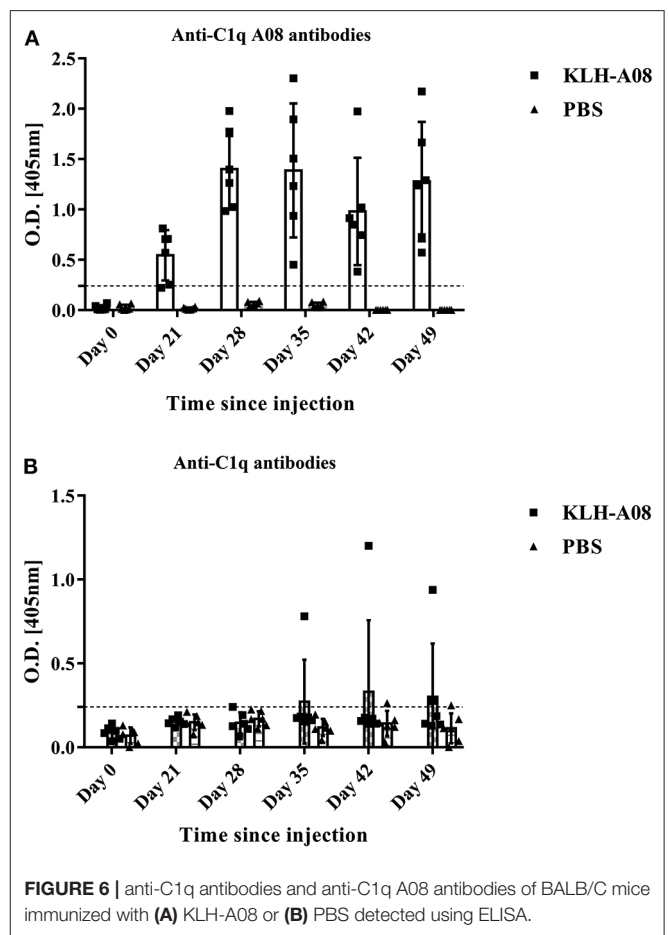
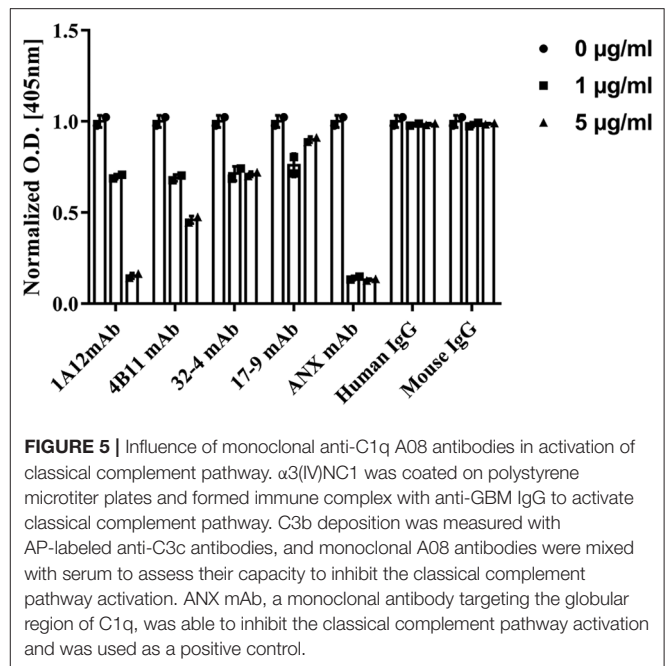
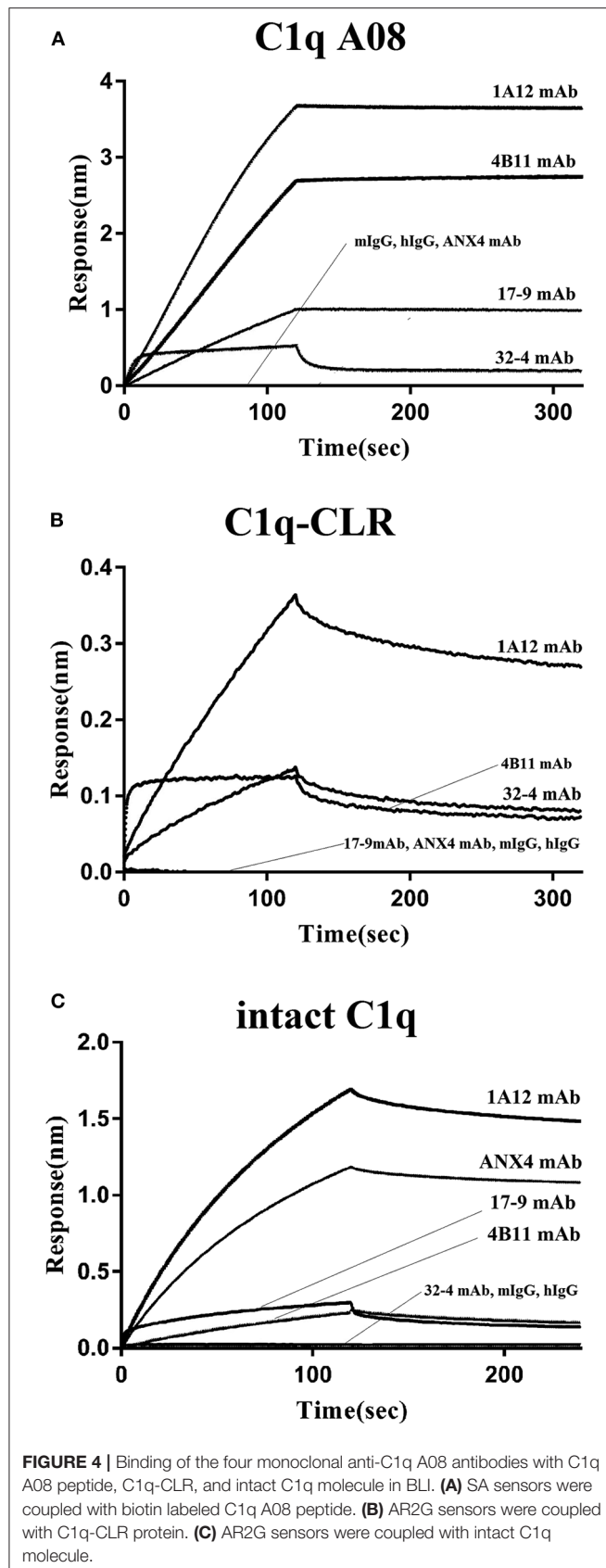
Influence of Monoclonal A08 Antibodies on Activation of Classical Complement Pathway

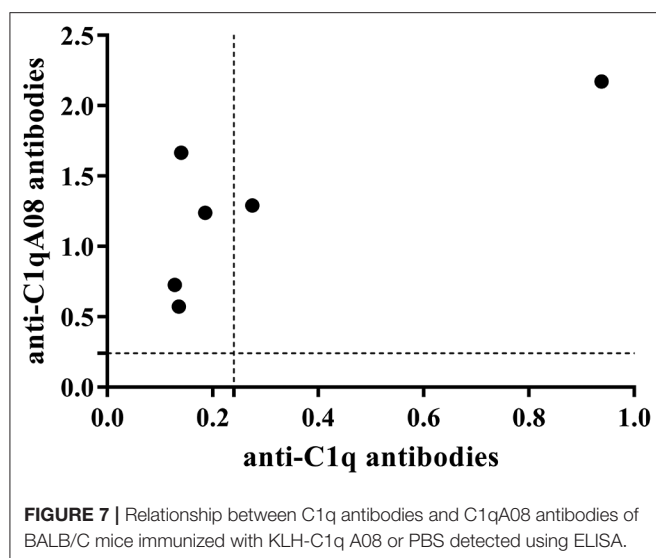
The influences of the four monoclonal A08 antibodies on the activation of a classical complement pathway are illustrated in **Figure 5**. Both 1A12 and 4B11, rather than 17-9 and 32-4, could inhibit the activation of complement classical pathway. The characterization of the four monoclonal anti-C1q A08 antibodies is listed in **Table 1**. Neither mouse total IgG nor human total IgG influenced the activation of classical complement pathway.

Epitope Spreading of C1q After Immunization With C1q A08 *in vivo*

Anti-C1q A08 antibodies appeared on day 49 in all the six mice of the experimental group but not in the control group (**Figure 6A**).







Furthermore, for the two mice in the experimental group that developed anti-C1q antibodies, one was strongly positive and the other was weakly positive (**Figure 6B**). The two mice that developed anti-C1q antibodies were also positive for anti-C1q A08 antibodies (**Figure 7**). The results indicated that epitope spreading of C1q may occur in the two mice with positive anti-C1q antibodies, as anti-C1q A08 antibodies in the other four mice of experimental group did not bind intact C1q by ELISA.

DISCUSSION

Most of clinical studies indicated that anti-C1q antibodies may be pathogenic in lupus nephritis (23, 24). C1q plays an important role in the clearance of immune complexes and apoptotic cells, whereas anti-C1q antibodies can interfere with the biofunction of C1q. A08 was found to be the most important B cell epitope (12), and the clinical importance of anti-C1q A08 antibodies has been validated in a large Chinese cohort (13). However, the conformational change of C1q A08 in C1q as well as the biofunction of anti-C1q A08 was still unclear.

This study firstly found that all 10 samples from lupus nephritis were positive for anti-C1q A08 antibodies, and seven of them were positive for C1q antibodies. All the anti-C1q A08 antibodies purified from the 10 lupus nephritis patients bound to A08 but not intact C1q coated on ELISA (**Figure 1B**). The results suggested that A08 peptide amino acids are cryptic or half-cryptic; however, as only 10 samples were included, the possibility cannot be ruled out that some A08 antibodies from lupus patients can bind to intact C1q coated on ELISA. Schaller et al. (25) found that the C1q antibodies of SLE patients are mostly collagen region antibodies, which are merely exposed in the plate-bound C1q. By conducting epitope mapping that utilized one monoclonal antibody of the aforementioned Fabs, Vanhecke et al. (12) identified C1q A08 as the major epitope. When competing with plasma samples to bind to C1q-bound plate, C1q A08 peptides could merely inhibit 10% of binding;

but after C1q was degenerated, the inhibition rate of C1q A08 peptides surpassed 30%, suggesting that C1q A08 is a nearly fully-cryptic epitope to which most C1q antibodies did not bind. A BLI assay in which intact C1q was coupled on an AR2G sensor was employed to study the binding property of the four monoclonal A08 antibodies with C1q. It was found that about 10 C-terminal amino acids of the A08 amino acid sequence were completely exposed, but only three N-terminal amino acids were not exposed. Thus, the 17-9 mAb, 1A12 mAb, and 4B11 mAb, but not the 32-4 mAb, could bind to C1q or C1q CLR in BLI. When C1q was coated on Costar polystyrene microtiter plates, the six N-terminal amino acids were completely exposed, whereas the seven C-terminal amino acids were not exposed. Only the 32-4 mAb, rather than 17-9, 1A12, or 4B11, could bind to C1q in ELISA. Interestingly, all the human anti-A08 antibodies affinity purified from plasma samples of lupus nephritis patients did not bind to the intact C1q by ELISA, implying that the binding property of most A08 autoantibodies from patients are dependent on entire A08 sequences like 1A12 mAb or 4B11 mAb. The sequence of A08 is special with four arginine residues and one lysine residue, which endows C1q A08 with strongly positive charge. It makes sense that such sequences of C1q A08 has strong immunogenicity and nearly all amino acids of A08 are exposed *in vivo*, which makes C1q A08 the most important B-cell epitope. However, when C1q was coated on microtitre plates, the conformation of C1q changed, and several C-terminal amino acids turned cryptic. As a result, nearly all anti-C1q A08 antibodies were unable to bind to C1q in ELISA; 32-4 mAb, however, only recognized the six N-terminal amino acids. Thus, the majority of the anti-C1q A08 antibodies from patients with lupus nephritis could not bind to C1q coated on ELISA plate. That is why anti-C1q A08 antibodies and anti-C1q antibodies of lupus nephritis patients were not all overlapped. Our data showed that the so-called “anti-C1q A08 antibodies” and “anti-C1q antibodies” defined by ELISA were in fact both anti-C1q antibodies but not completely overlapped, which means the so-called “anti-C1q A08 antibodies” are a part of the anti-C1q antibody family, which recognized nearly complete cryptic epitope in ELISA, while the so-called “anti-C1q antibodies” are anti-C1q antibodies that recognized exposed epitope of C1q coated on ELISA plate.

Furthermore, our study showed that C1q A08 is significant in the activation of C1, and, thus, some anti-C1q A08 antibodies can inhibit the complement activation. Though 17-9 mAb, 1A12 mAb, and 4B11 mAb all can bind to C1q or C1q CLR in BLI, only the 1A12 mAb and 4B11 mAb can inhibit the activation of complement classical pathway. The isotypes of 1A12 and 4B11 were mouse IgG3, which can activate the classical complement pathway by Fc, while 32-4 was human IgG4, which cannot activate classical complement pathway. The isotype of 17-9 was mouse mIgG2a, which can activate complement classical pathways. Thus, the isotypes of C1q A08 mAbs seemed to have no influence on activation of complement classical pathway. The influence of anti-C1q A08 antibodies on the activation of C1 depends on the binding site of anti-A08 antibodies on A08. It seemed that the conformation of C1q experiences change during activation, and some anti-C1q A08 antibodies may prevent the

process through a stereo-hindrance effect. Those results may provide a novel insight into the process of C1 activation. The complement system plays an important role in the clearance of immune complexes in tissues, and the important pathogenesis involved in lupus nephritis is the dysfunction for the clearance of immune complexes and apoptotic cells. Based on our prior study, this *in vitro* study showed that anti-C1q A08 antibodies may inhibit the activation of complement classical pathway, which may in turn interfere with the clearance of immune complex or apoptotic cells. Moreover, prior studies showed that A08 can bind to the von Willebrand factor (vWF) as well as some other proteins and can activate the complement classical pathway. Thus, anti-C1q A08 antibodies may also interfere with the binding between C1q A08 and other ligands to block the activation of complement classical pathway.

The limitation of the current study mainly lies in the lack of the precise conformational structure of C1q on different surfaces. Antibodies against plate-bound C1q from SLE patients were not isolated and used as controls. The amount of the mouse antibody was too small, and the evidence of epitope spreading was not so solid. Furthermore, anti-C1q A08 antibodies cannot be isolated from the mouse and purified, since the serum sample was not sufficient. Thus, the structural study of C1q is still needed to provide more insights into the role of anti-C1q A08 antibodies in lupus nephritis.

In conclusion, C1q A08, one important but half-cryptic epitope involving six N-terminal amino acids, is important in activation of complement classical pathway, and some anti-A08 antibodies can prevent this process. The epitope spreading of C1q in BALB/C mice immunized with C1q A08 peptide occurred, indicating that C1q A08 is important for development of anti-C1q antibodies detected by ELISA.

MATERIALS AND METHODS

Reagents

Female BALB/C mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. In the experiments outlined below, avidin from egg white (Aladdin), C1q (EMD chemicals), alkaline phosphatase (AP) substrate P-nitrophenyl phosphate (Sigma-Aldrich), Mouse total IgG (Sigma-Aldrich), and human total IgG (Sigma-Aldrich) were used. Furthermore, the following antibodies were adopted: AP-conjugated polyclonal goat anti-human IgG (γ -chain specific) (Sigma Aldrich), AP-conjugated polyclonal goat anti-mouse IgG (whole molecule, Sigma-Aldrich), AP-conjugated polyclonal goat anti-rabbit IgG (whole molecule, Sigma-Aldrich), and polyclonal rabbit anti-human C3c (Dako). C1q CLR was produced by partial pepsin digestion of C1q as previously described (13).

Plasma Exchange Samples

Plasma exchange fluids were obtained from anti-C1q A08 antibodies positive lupus nephritis patients during the treatment with plasmapheresis.

Informed consent was obtained for blood and plasma exchange samples. The research was in compliance with the Declaration of Helsinki, and the design of this work was approved by the local ethical committees.

A08 Monoclonal Antibodies From Hybridoma Cells

Three female BALB/c mice were injected subcutaneously with 0.5 mg A08 peptide coupled with Keyholelimpet hemocyanin (KLH) in complete Freund's adjuvant on day 0, and boosts were performed with 0.5 mg A08 peptide coupled with KLH in incomplete Freund's adjuvant on both day 7 and 14. Splenocytes were fused with the myeloma cell line SP2/0. Fused cells were grown on hypoxanthine-aminopterin-thymidine (HAT) selective semi-solid media for 15 days, and the resulted hybridomas clones were transferred to 96-well tissue culture plates. The supernatants were isolated and tested in an ELISA assay for reactivity against A08 peptides. Positive clones were isotyped and cultured for 30 days to identify stable expressing clones. Four A08 monoclonal antibodies were screened and named as 17-9, 32-4, 1A12, and 4B11, respectively. They were used for binding assays, epitope mapping, BLI, and the complement activation study.

It should be noted that hybridoma cells expressing 32-4 mAb were difficult to culture, and the total mRNA was extracted. After RT-PCR to get cDNA, redundant primers hybridizing to the leader sequence (5' primer) and to the C region immediately downstream of the V-J region (3' primer) were used to clone the V regions (26). Cloned V regions of 32-4 mAb were then expressed as joined to the constant region of human IgG4/kappa. The other three monoclonal A08 antibodies 17-9, 1A12, and 4B11 were identified as mouse IgG2a/kappa, mouse IgG3/lambda, and mouse IgG3/lambda, respectively.

Recombinant Human $\alpha 3(IV)NC1$

The recombinant 6*His-tagged human $\alpha 3(IV)NC1$ with signal peptide was cloned into a pcDNA3.1 vector, which was transiently transfected with HEK-293T cells. After being cultured for 7 days, the supernatant was collected and applied to HisTrap HP column to isolate human $\alpha 3(IV)NC1$ protein.

Purification of Total IgG From Plasma Exchange of Anti-GBM Antibody Positive Patients

Total IgG containing antibodies against $\alpha 3(IV)NC1$ was isolated from plasma exchange samples of anti-GBM antibody positive patients using protein G column. Total IgG was eluted with citric acid/sodium citrate buffer (20 mM, pH = 2.7). After adjusting pH to 7.3, the buffer of total IgG was changed to a phosphate-buffered saline (PBS) buffer with 10 kDa ultrafiltration.

The recombinant human $\alpha 3(IV)NC1$ and anti-GBM antibodies were used to form immune complex and to evaluate complement activation via the classic pathway.

Synthesis of C1q A08 Related Peptides

Biotinylated peptides, non-biotinylated peptides, and KLH-conjugated peptides with >95% purity were synthesized by GenScript, as described previously (13). Peptide A08 (GRPGRRRPGLKG) is derived from the C1q A chain with sequence from the 15 to 27th amino acids. Eighteen 13-er peptides ranging from the 7 to 24th amino acids and six 8-er peptides ranging from the 15 to 27th amino acids were synthesized for epitope mapping of different A08 monoclonal antibodies. The sequence of all C1q A08 related

TABLE 1 | Characterization of monoclonal anti-C1q A08 antibodies.

Antibody	Biotinylated -C1q A08	C1q-CLR	C1q	Inhibit of C1 activation
17-9 mAb (mIgG2a)	+	-	-	-
32-4 mAb (hlgG4)	+	+	+	-
1A12 mAb (mIgG3)	+	-	-	+
4B11 mAb (mIgG3)	+	-	-	+
ANX4 mAb (hlgG4)	-	-	+	+
Mouse total IgG	-	-	-	-
Human total IgG	-	-	-	-

TABLE 2 | The sequence of C1q A08-related peptides.

Sequence
7-19: PDGKKGEAGRPGR
8-20: DGKKGEAGRPGR
9-20: GKKGGEAGRPGRG
10-22: KKGEAGRPGRRG
11-23: KGEAGRPGRRG
12-24: GEAGRPGRRG
13-25: EAGRPGRRG
14-26: AGRPGRRG
A08: GRPGRRG
16-28: RPGRRG
17-29: PRRGRG
28-30: RRRGRG
19-31: RRRGRG
20-32: RRRGRG
21-33: GRPGRG
22-34: RPGRG
23-35: PGRG
24-36: GLKGEQGEPA
15-22: GRPGRG
16-23: RPGRG
17-24: PRRGRG
18-25: GRRGRG
19-26: RRRGRG
20-27: RRRGRG
19-27: RRRGRG
R-K: GRPGKKGPGLK
R-A: GRPGAAGAPGLK
Scramble 1: KGGAPRRGGLPRR
Scramble 2: RRGPRLRGPKGGG
Scramble 3: RPRGLRGPRGGK

peptides are listed in Table 2. In parallel, other related peptides (first, scrambled A08, KGGAPRRGGLPRR; second, scrambled A08, RRGPRLRGPKGGG; third, scrambled A08, RPRGLRGPRGGK; A08 [R→K], GRPGKKGPGLK; and A08 [R→A], GRPGAAGAPGLK) were used as controls (13).

Isolation and Purification of Anti-C1q A08 Antibodies From Plasma Exchange of Lupus Nephritis Patients

Peptides with >95% purity were synthesized by GenScript, and A08-Cys was synthesized where the cysteine was added for conjugation with Pabpur Sulfolink Beads supplied by SMART lificiences. A carboxyl group of iodoacetic acid was immobilized on resin, and iodine ions are good leaving groups. Thiol of cysteine with low pKa reacts easily with iodoacetic acid to immobilize peptide or protein on resin. About 50 μ l of plasma samples from lupus nephritis patients were diluted in a 1:1 ratio with PBS buffer and applied to Protein G column to isolate total IgG, which was eluted with citric acid/sodium citrate buffer (20 mM, pH = 2.7). After adjusting pH to 7.3, total IgG was applied to A08 affinity column to isolate anti-A08 IgG, which was eluted with citric acid/sodium citrate buffer (20 mM, pH = 2.7). After adjusting pH to 7.3, the buffer of anti-A08 IgG was changed to a PBS buffer with 10 kDa ultrafiltration. The purified antibodies were used for testing binding property to plate-bound C1q and C1q A08.

Detection of Anti-C1q Antibodies, Anti-C1q CLR Antibodies, and Anti-C1q A08 Antibodies With an ELISA Assay

As described previously, (27) human C1q (Sigma-Aldrich), C1q CLR, and neutravidin (Pierce Biotechnology) proteins diluted at previously determined concentrations of 5, 1 μ g/ml in 0.05 M bicarbonate buffer (pH = 9.6), and 5 μ g/ml in carbonate buffer (0.1 M sodium carbonate, pH = 9.6) were coated on the wells of one half of polystyrene microtiter plates (Costar, Corning) at 4°C overnight, respectively. The wells in the other half were coated with bicarbonate or carbonate buffers alone to act as antigen-free wells. Free binding sites were blocked with 0.01 M PBS containing 0.1% Tween 20 (PBST) and 1% (10 mg/ml) bovine serum albumin except for the neutravidin plates at 37°C for 1 h, which were incubated with biotinylated peptides at 5 mg/ml in PBS at room temperature for 2 h. Sera were diluted to 1:200 in PBST/0.5 M NaCl to detect anti-C1q and anti-C1q CLR antibodies, and 1:200 in 0.1% PBST to detect anti-A08 related peptides antibodies. The volumes for both this step and subsequent steps were 100 μ l, and all incubations were carried out at 37°C for 1 h. The plates were washed three times with PBST. Alkaline phosphatase-conjugated anti-human IgG (Calbiochem), diluted at 1/3,000, was used as a detection antibody. The P-nitrophenyl phosphate (1 mg/ml; Sigma-Aldrich) was used in substrate buffer (1.0 M diethanolamine and 0.5 mM MgCl₂, pH = 9.8). Optical density was measured at 405 nm. Samples were considered positive if they exceeded the mean plus 2 SD from 100 healthy blood donors.

Bi-layer Interferometry (BLI) Assays

Octet Binding Assay of C1q, C1q A08, and C1q CLR to Anti-C1q A08 Antibodies

SA sensors were used to load biotinylated A08 peptides at a concentration of 20 μ g/mL, and the loading level was about 1.5 nm. The sensors were then moved to HEPES

(hydroxyethyl piperazine ethanesulfonic acid) buffer wells for baseline generation and subsequently to anti-C1q A08 antibodies wells (100 nM) for 120 s. Then they were dipped to HEPES buffer wells for dissociation for 200 s. The sensors were generated with glycine-HCl buffer (100 mM, pH = 2.5). ANX4 mAb, normal mouse and human total IgG were used as controls.

AR2G sensors were used to load C1q CLR and C1q at a concentration of 10 µg/mL, and the loading level was about 2.5 nm. The sensors were then moved to HEPES buffer wells for baseline generation and subsequently to anti-C1q A08 antibodies wells (100 nM) for 120 s. They were then dipped into HEPES buffer wells for dissociation for 200 s. The sensors were generated with glycine-HCl buffer (100 mM, pH = 2.5). ANX4 mAb, normal mouse, and human total IgG were used as controls.

C3 Activation Assay

Detection of C3 activation was carried out as previously described with brief modification (22). Polystyrene microtiter plate (Costar) was coated with 100 µl of 10 µg/mL recombinant human α3(IV)NC1 in 0.05 M bicarbonate buffer. After overnight incubation, the wells were blocked with 0.2% (w/v) gelatin in PBST and then washed with PBST. IgG from anti-GBM antibody-positive patients was diluted to 200 µg/ml in PBST and added to the plates. ANX4 mAb, which is an antibody targeting the globular region of C1q that can also inhibit activation of the complement classical pathway, was used as positive control (28), while normal mouse and human total IgG were used as negative controls. After incubation and washing, the mixture of serum samples and anti-A08 antibodies samples was diluted in VBS [5 mM barbitol, 145 mM NaCl, 0.15 mM CaCl₂, 1 mM MgCl₂, and 0.1% Tween 20, pH = 7.4], added to the plates, and incubated at 37°C for 1 h. The plates were washed with VBS, and the bound C3b was detected using rabbit anti-human C3c (Dako), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Aldrich); this was then followed by the colorimetric substrate, P-nitrophenyl phosphate (1 mg/ml; Sigma-Aldrich). The results were recorded as the net optical absorbance (average value of antigen wells minus average value of antigen-free wells) at 405 nm in an ELISA reader (Bio-Rad 550).

Immunization With C1q A08 Peptide

Twelve 5-week-old female BALB/C mice were divided into two groups. For experimental group, six mice were immunized subcutaneously with 0.5 mg KLH-conjugated C1q A08 peptide in incomplete Freund's adjuvant (IFA, Difco Laboratories) on day 0, followed by subcutaneous booster injections of 0.5 mg KLH-conjugated C1q A08 peptide in complete Freund's adjuvant (CFA, Difco Laboratories) on days 7 and 14. For control group, six mice were immunized with PBS buffer only in adjuvant using the identical schedule. Sera was obtained on days 0, 21, 28, 35, 42, and 49, respectively. Anti-A08 antibodies and anti-C1q antibodies were detected by ELISA, and alkaline phosphatase-conjugated goat F(ab')₂ anti-mouse IgG (Abcam) was used as secondary antibody.

Statistical Analyses

Differences of quantitative parameters between groups were assessed using the *t*-test for data normally distributed or the non-parametric test for data not normally distributed. Differences of semi-quantitative data were tested using the Mann-Whitney *U*-test. Differences of qualitative data were compared using the χ^2 test. The Spearman Correlation was used to analyze the correlation. Analyses were performed with statistical software SPSS 21.0. *p* < 0.05 was considered as significant.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical committee of Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethical committee of Peking University First Hospital.

AUTHOR'S NOTE

C1q plays an essential role in the adaptive and innate immune system, and C1q A08 is an important epitope of C1q in lupus nephritis patients. In this paper, it was confirmed that C1q A08 is a half-cryptic epitope using the ELISA method, indicating that most anti-C1q A08 antibodies from lupus nephritis patients could not bind to C1q with ELISA. Further, it was found that C1q A08 plays an important role in activation of complement classical pathway. Moreover, it was revealed that epitope spreading of C1q occurred in the mice immunized with C1q A08 peptides. Our findings demonstrated the relationship between anti-C1q antibodies and anti-C1q A08 antibodies, implying that C1q A08 may play an important role in pathogenesis of lupus nephritis.

AUTHOR CONTRIBUTIONS

M-HZ, FY, W-JW, and YT designed the study, analyzed the data, and wrote the manuscript. X-LL collected the samples.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00848/full#supplementary-material>

Supplementary Figure 1 | 32–4 mAb bond to the six amino acids of N-terminus of C1q A08, while 17–9 mAb bond to eight or 10 amino acids of C-terminus of C1q A08. The binding of 1A12 and 4B11 mAb seemed to depend on the entire sequence of C1q A08. 1A12 mAb and 4B11 mAb could bind to the 10 amino acids of C-terminal of C1q A08, while the three amino acids of N-terminal of C1q A08 also contributed to the binding.

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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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High-Throughput Sequencing-Based Analysis of T Cell Repertoire in Lupus Nephritis

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T cell receptor (TCR)-mediated immune functions are closely related to autoimmune diseases, such as systemic lupus erythematosus (SLE). However, technical challenges used to limit the accurate profiling of TCR diversity in SLE and the characteristics of SLE patients remain largely unknown. In this study, we collected peripheral blood samples from 10 SLE patients with lupus nephritis (LN) who were confirmed by renal biopsy, as well as 10 healthy controls. The TCR repertoire of each sample was assessed by high-throughput sequencing to examine the distinction between SLE subjects and healthy controls. Our results showed statistically significant differences in TCR diversity and usage of TRBV/TRBJ genes between the two groups. A set of signature V–J combinations enabled efficient identification of SLE cases, yielding an area under the curve (AUC) of 0.89 (95% CI: 0.74–1.00). Taken together, our results revealed the potential correlation between the TCR repertoire and SLE status, which may facilitate the development of novel immune biomarkers.

Keywords: T cell receptor, lupus nephritis, systemic lupus erythematosus, immune repertoire, next-generation sequencing

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disorder. As one of the most common and severe complications in SLE, lupus nephritis (LN) is a major cause of SLE-related morbidity and mortality (1, 2). LN requires confirmation by renal biopsy, which is an invasive procedure (3, 4). Without early diagnosis and treatment, LN can usually progress to end-stage renal disease (ESRD) (5). Since it is impractical to perform renal biopsy repeatedly, a non-invasive method for diagnosis and prognosis surveillance of LN is urgently needed (6).

It has been reported that highly diversified T cell receptors (TCRs) are crucial for adaptive immunity in health and disease (7, 8). TCRs are generated by genomic rearrangement of the variable (V), diversity (D), and joining (J) regions, along with palindromic and random nucleotide additions (9). Recently, a series of studies have demonstrated substantial changes in the TCR repertoire of SLE patients (10–13). For instance, Liu et al. (11) found significant differences in V, J, and V–J pairs in SLE patients. And 198 SLE-associated TCR clones were identified for correlation with clinical features (11). However, the changes of TCR repertoire in SLE patients with LN have yet to be described.

In this study, we performed high-throughput sequencing to characterize the TCR repertoire in peripheral blood samples from SLE patients with LN and healthy controls. The results may help understand the property and alteration of T cell immunity in the occurrence and development of SLE.

MATERIALS AND METHODS

Study Participants

A total of 10 SLE patients with LN and 10 healthy controls were recruited from the Zhejiang Provincial People's Hospital, Hangzhou, China. The pathological status of LN patients was confirmed by renal biopsy. The controls were confirmed with no autoimmune disorders or kidney complications. Written informed consents were obtained from all participants. This study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital.

The baseline characteristics of SLE and control groups were presented in **Table 1**. Following professional guidelines, the diagnosis of LN was confirmed with histopathological examination of renal biopsy. The SLE cases belong to Class-II, Class-IV, and Class-V, respectively. Although the range of age was larger in the SLE group than in the control group (20–68 vs. 35–52), the average age was not significantly different between the two groups (45.9 vs. 45.8). In the current study, we used European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) classification criteria for SLE, which is a combination of multiple disciplines and international recognition, thus displayed great sensitivity and specificity. The subjects included in our study have multi-organ injury, including hematologic, mucocutaneous, serosal, and renal. In addition, renal biopsy score of class II or V LN is 8 and class II or IV is 10. Therefore, the SLE disease activity index score was 17.40 ± 4.74 .

Whole Blood Sample Processing

Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood with Ficoll[®] to get the highest concentration of lymphocytes. Each type of lymphocyte cell was isolated with monoclonal antibodies specific for the particular lymphocyte cell subset. All cell samples were resuspended in RNeasyProtect[®] and stored at 4°C until ready to extract RNA. For low cell counts ($<5 \times 10^5$), total RNA was extracted from the Qiagen[®] RNeasy[®] Micro kit (catalog #74004). For higher cell counts, RNA was extracted from the Qiagen[®] RNeasy[®] Mini kit (catalog #74104).

Library Construction and Sequencing

RT-PCR multiplex primer sets (iRepertoire, Inc., Huntsville, AL, USA) were used to amplify the CDR3 region of the TCR β chain. The whole library construction process was automatically operated in the iR-Proprocessor[™] and iR-Cassette (iRepertoire, Inc., Huntsville, AL, USA). Then library products with different bar codes were pooled and paired-end sequenced by Illumina MiSeq v2 300-cycle Kit (Illumina Inc.), average read depth of 1M reads each sample (14, 15).

Raw Data Analysis

Sequences were aligned to TCR β germline V-, D-, and J-genes according to IMGT/GENE-DB database. Analyzed by the Smith–Waterman algorithm using iR-map pipeline and visualized in iRweb (iRepertoire, Inc., AL, USA). Data analysis included peptide sequences, uCDR3, shared CDR3s, and V- and J-gene usage. Detailed method has been described by Wang et al. (16). The statistics of sequencing quality has been presented in **Table S1**. The sequencing quality of one SLE sample and one control sample was double-checked and shown in **Figure S2**. The raw data can be freely downloaded online at: <https://figshare.com/search?q=DIO%3A10.6084%2Fm9.figshare.11911284&searchMode=1>.

Statistical Analysis

All statistical analysis was performed using R software (version 3.6.1). Indexes of normal distribution were expressed by mean \pm standard deviation. *T*-test for independent samples was

TABLE 1 | Basic characteristics of study subjects.

Basic characteristics	SLE group (n = 10)	Control group (n = 10)
Age (year, mean \pm SD)	45.9 \pm 16.5	45.8 \pm 5.2
Female/Male	9/1	3/7
Low C3 or low C4, No. (%)	9 (90%)	NA
ANA positive, No. (%)	10 (100%)	NA
Anti-dsDNA positive, No. (%)	1 (10%)	NA
Anti-Sm, No. (%)	1 (10%)	NA
Serum creatinine (Scr, μ mol/L, the range of normal: 44.0~133)	209.97 \pm 277.76	NA
Systemic lupus erythematosus disease activity index score	17.40 \pm 4.74	NA
Proteinuria (mg)	1431.35 \pm 2076.84	NA
Renal biopsy classification, No. (%)	Class-II: 3 (30%); Class-IV: 5 (50%); Class-V: 2 (20%)	NA NA NA
Clinical domains	Hematologic: 3 (30%) Mucocutaneous: 5 (50%) Serosal: 3 (30%) Renal: 10 (100%)	NA NA NA NA

Low C3 or low C4, below the lower limit of normal level; ANA, antinuclear antibody; anti-dsDNA, antibodies to double-stranded DNA; anti-Sm, anti-Smith; renal biopsy classification, according to International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003; Class II, mesangial proliferative lupus nephritis; Class IV, diffuse lupus nephritis; Class V, membranous lupus nephritis.

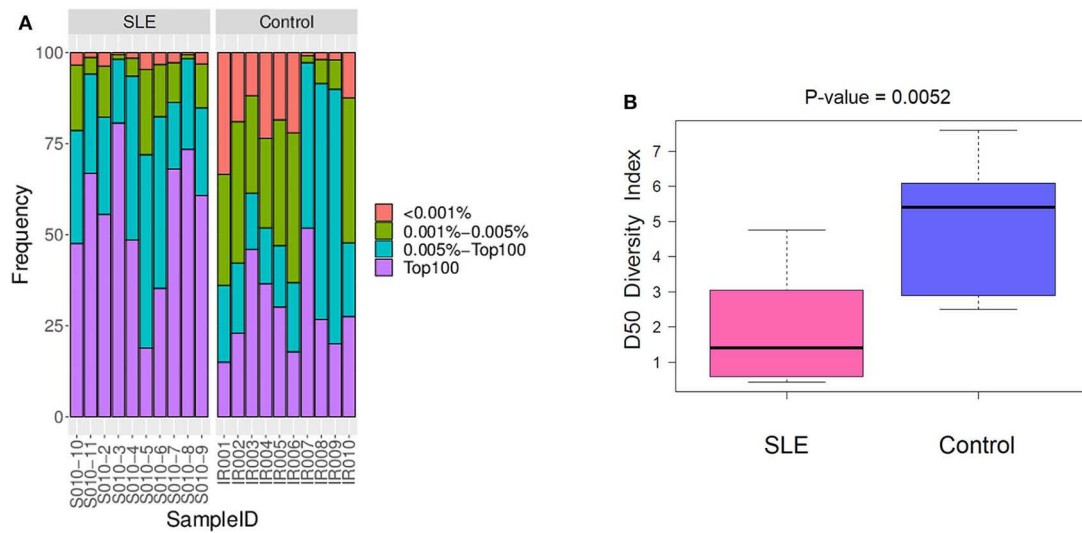


FIGURE 1 | The abundance and diversity of T cell receptor (TCR) clonotype. **(A)** The frequency distribution of different clonotypes. **(B)** The TCR diversity of each group was measured by the D50 index at the level of the V-J combination.

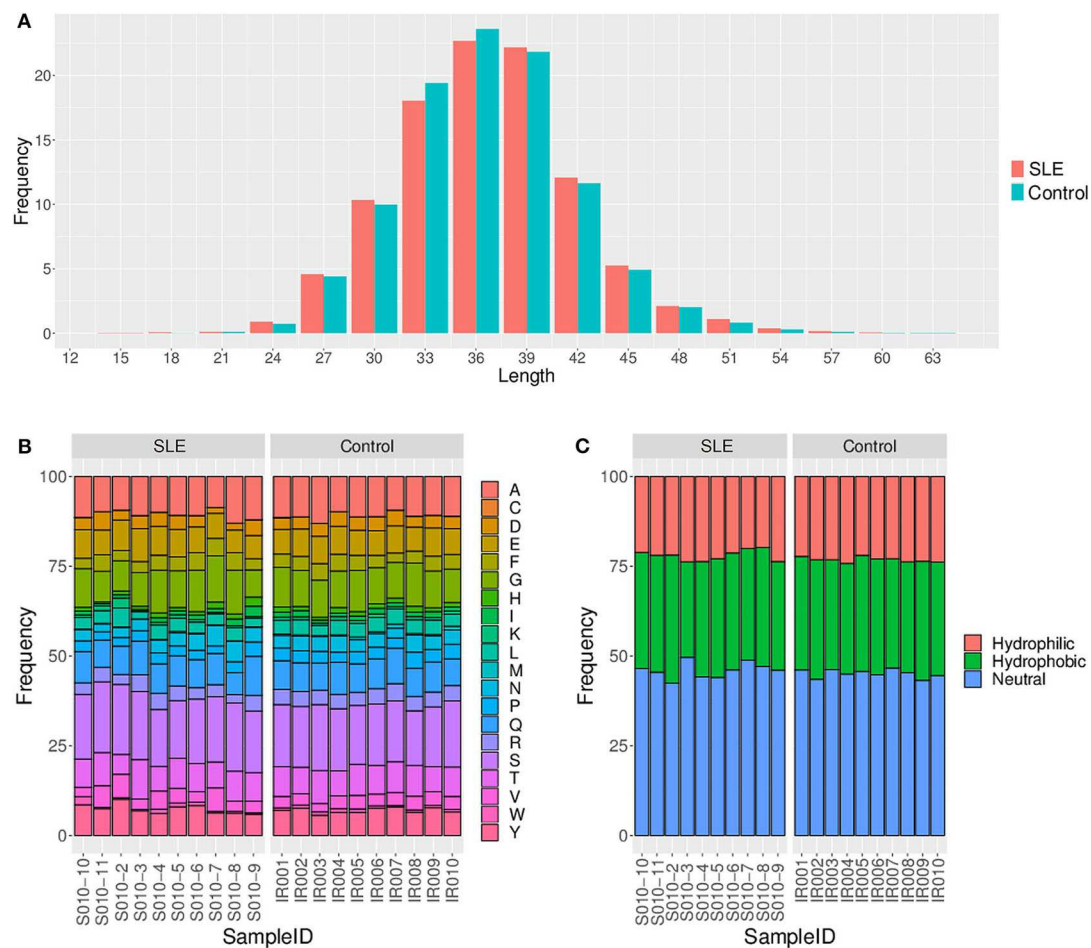
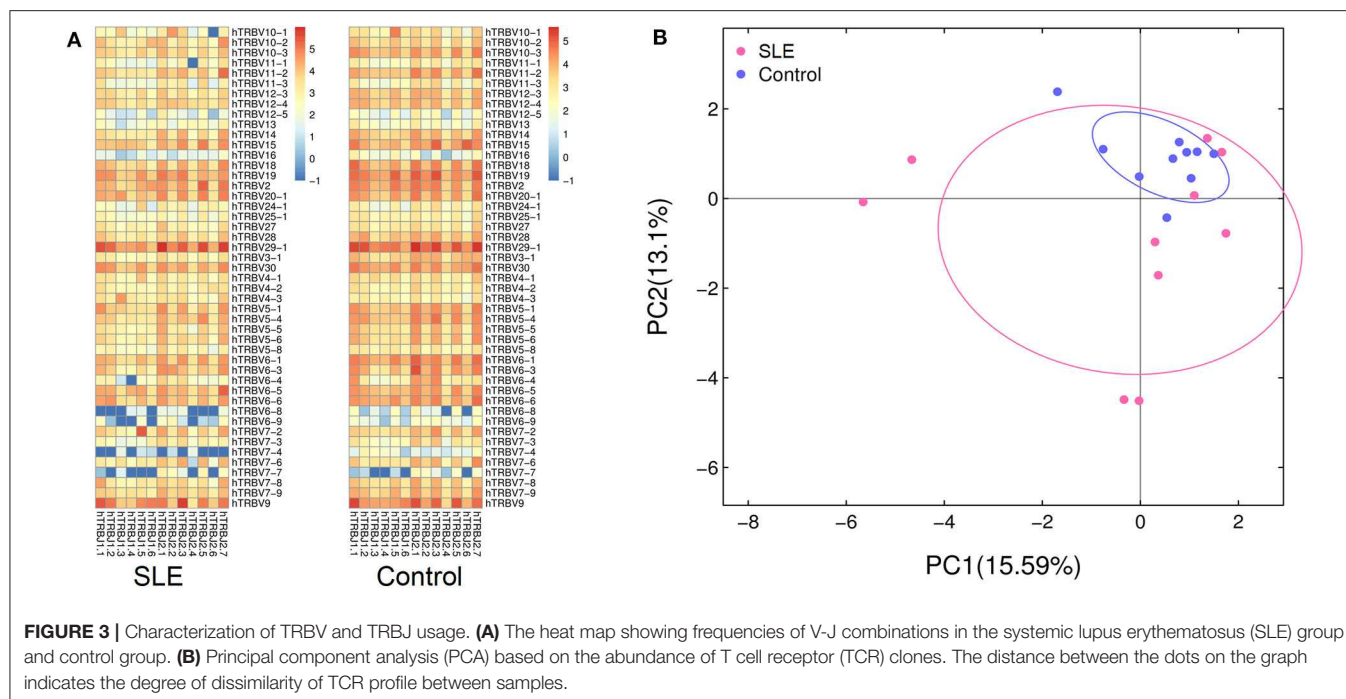


FIGURE 2 | Comparison of the CDR3 length **(A)**, amino acid composition **(B)**, and amino acid hydrophilicity **(C)** between the systemic lupus erythematosus (SLE) group and control group.



performed on comparison between groups. Indexes of non-normal distribution were expressed by median (interquartile interval). Chi-square test was used to compare the counting indexes between groups. Logistic regression was used to analyze the relationship between the specific clone expression level and the clinical outcome. To identify the signature clonotypes, Random Forest analysis (“randomForest” package in R software) together with leave-one-out cross validation was performed to estimate the area under the receiver operating characteristics (ROC) curve and the importance of individual variables.

RESULTS

Repertoire Diversity in Systemic Lupus Erythematosus

We primarily analyzed the abundance and diversity of different TCR clonotypes. The CDR3 sequences were divided into four groups (<0.001, 0.001–0.005, 0.005–top 101, and top 100, respectively) based on their frequency in our samples. The results showed that low abundance clones (i.e., frequency <0.001 and 0.001–0.005%) were less abundant, while top 100 clones were more frequent in SLE individuals (Figure 1A; Table S2), suggesting putatively decreased TCR diversity. The significantly lower D50 diversity index in the SLE group as compared to control group (Figure 1B) further confirmed that the TCR diversity is evidently impaired in SLE. On the other hand, we observed no substantial differences in CDR3 length and amino acid composition between SLE and control groups (Figure 2; Tables S3, S4).

Characteristics of TRBV and TRBJ Gene Usage in Lupus Nephritis

We then evaluated the gene usage of TRBV and TRBJ in SLE cases and control subjects (Figure 3A). A series of V–J combinations were identified for differential abundance in the two groups (Table 2). We further performed Principal Component Analysis (PCA) on the V–J combination frequency profile. As shown in the PCA plot (Figure 3B), a significant difference was found between SLE and control groups (PERMANOVA $P < 0.05$), as the samples from the control subjects were highly clustered in the upper right quarter of the graph. On the other hand, no obvious difference in sex or renal biopsy classification was detected on the PCA plot (Figure S1).

We also trained a random forest model (see *Materials and Methods*) to evaluate whether the TCR profile could help discriminate between SLE and normal subjects. In the ROC curve, a set of signature clones showed efficient performance in identifying SLE cases. The leave-one-out cross validation yielded an area under the curve (AUC) of 0.89 (95% CI: 0.74–1.00; Figure 4). Such distinction between SLE and control groups promised the possibility of developing TCR biomarkers for early diagnosis of SLE and possibly LN (see *Discussion* below).

DISCUSSION

To summarize, the results suggested that (1) the SLE status could substantially influence the immune system by impairing the TCR diversity of patients; (2) clear differences in particular V–J combinations could arise between SLE patients and healthy controls; (3) machine learning models were trained to effectively discriminate SLE individuals from control subjects, which

TABLE 2 | V–J combinations with asymmetric expression in the two groups.

V Gene	J Gene	Normalized expression in SLE group	Normalized expression in control group	No. of positive control samples	No. of positive SLE samples
TRBV11-1	TRBJ1-1	88.2 ± 15.6	–	0	3
TRBV12-5	TRBJ2-1	128.6 ± 295.1	–	0	3
TRBV25-1	TRBJ2-3	100.9 ± 8.2	–	0	4
TRBV27	TRBJ1-1	300.7 ± 7.6	–	0	3
TRBV4-1	TRBJ2-5	184.1 ± 41.2	–	0	3
TRBV5-5	TRBJ1-6	56.2 ± 7.2	–	0	3
TRBV6-9	TRBJ2-7	35.9 ± 2.6	–	0	3
TRBV7-6	TRBJ1-5	146.9 ± 24.2	–	0	4
TRBV7-8	TRBJ2-2	163.4 ± 14.0	–	0	3
TRBV10-1	TRBJ1-1	–	58.6 ± 6.6	3	0
TRBV10-3	TRBJ2-6	–	77.4 ± 5.4	5	0
TRBV12-3	TRBJ1-4	–	117.6 ± 3.4	3	0
TRBV12-3	TRBJ2-2	–	567.3 ± 3.7	5	0
TRBV12-4	TRBJ1-4	–	50.4 ± 6.6	3	0
TRBV13	TRBJ2-1	–	43.5 ± 5.3	3	0
TRBV14	TRBJ2-2	–	132.4 ± 5.7	4	0
TRBV28	TRBJ1-6	–	246.1 ± 6.9	3	0
TRBV3-1	TRBJ2-2	–	698.9 ± 2.8	3	0
TRBV3-1	TRBJ2-5	–	34.0 ± 3.5	3	0
TRBV5-5	TRBJ1-4	–	75.1 ± 6.0	3	0
TRBV6-4	TRBJ1-1	–	2163.4 ± 7.3	6	0

The V–J combinations listed above are widely expressed ($n \geq 3$) in one group while not expressed at all in the other group.

may allow the development of diagnostic techniques for early detection of SLE (and possibly LN) risks.

A series of studies have characterized specific signatures of T cell repertoires in patients with various autoimmune diseases (17–19). For instance, Thapa et al. (13) used next-generation sequencing to assess T cell repertoire in peripheral blood (PB) of SLE patients. The results showed a significant decrease in TCR diversity of SLE patients compared to healthy controls (13). In particular, there was evidence that the TCR repertoire profile might serve as a potential biomarker of SLE (11, 12, 20, 21). In addition, Liu et al. (11) reported significant differences in V–J segment usage between the SLE and control groups. However, these studies did not examine the changes of TCR repertoire in LN status. Therefore, some of the differentially expressed clones in our study were not found in previous publications (e.g., TRBV12-5/TRBJ2-1, TRBV6-9/TRBJ2-7, TRBV10-1/TRBJ1-1, TRBV3-1/TRBJ2-2, etc.).

Here we clearly demonstrated that partial expansion of T cells could be observed in SLE patients with LN, which was characterized by decreased TCR diversity and the enrichment or reduction of specific V–J combinations. Due to the altered TCR profile, a series of clonotypes were used as a signature to a trained prediction model. In spite of a limited sample size, our model efficiently discriminated SLE (and possibly LN) individuals from healthy controls, which is worth further validation in larger cohorts. Our pilot study will inspire the subsequent research on the complicated immune environment in SLE and LN.

Of note, our findings are consistent with previously reported results that infiltrating T cells within renal tissue may be targeted toward nephritogenic antigens by the function of TCR β genes.

For example, Massengill et al. (20) found intrarenal lymphocytes in LN showing striking oligoclonal expansion. Our finding also suggested impaired TCR diversity in SLE and possibly LN. Moreover, Sui et al. (12) found the distributions of CDR3, VD indel, and DJ indel lengths to be comparable between the SLE and healthy controls, even though the degree of clonal expansion in the SLE group was significantly greater than in the healthy controls. Likewise, no significant differences in CDR3 length and amino acid composition were detected in our samples. The above evidences corroborated the reliability of our results.

The present study also has several important limitations. First of all, the sample size was relatively small, which impaired the statistical power. Considering potential factors that may confound the TCR characteristics, further studies with larger cohorts and long-term outcome measurements in both SLE patients and matched controls are required to better understand the immunological significance of TCR changes (22, 23). It would be more enlightening if a large sample enables to identify particular LN-specific TCR sequences. Secondly, the current sample did not include those from SLE patients without LN. Since a clinically important biomarker should predict which SLE individuals will develop LN later, subsequent research should make a comparison between SLE patients with and without LN. In addition, the human leukocyte antigen (HLA) gene profiles of the studied subjects are not assessed (24, 25), which may restrict the generalizability of our results.

In summary, we demonstrated a sequencing-based method to present the T cell repertoire characteristics of SLE patients with LN. As T cells play a pivotal role in the etiology of SLE, this study provided a better understanding of TCR-mediated adaptive

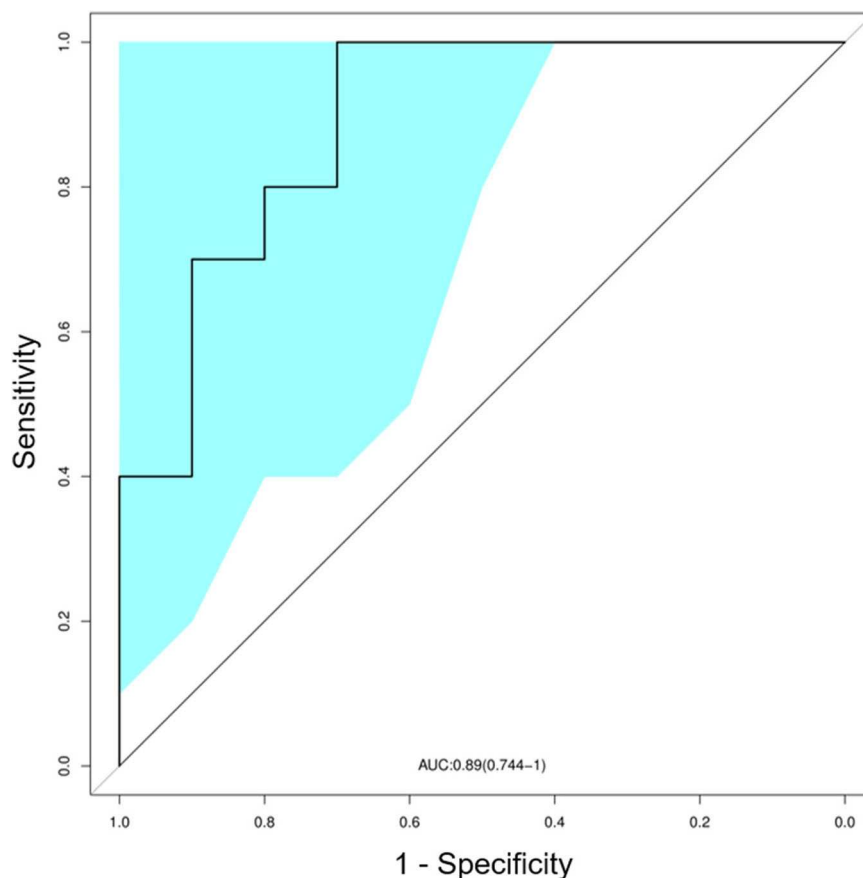


FIGURE 4 | Classification of systemic lupus erythematosus (SLE) by random forest model with a receiver operating characteristics (ROC) curve evaluating the performance. The colored area showed the 95% confidence interval (CI) of the curve.

immunity in SLE. More importantly, our results suggested the potential of developing non-invasive diagnostic solutions for SLE and possibly LN with TCR-based biomarkers.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Zhejiang Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ZX and ZW contributed to the conception and design. XY, HZ, YL, JS, and QY acquired the samples. XY, JZ, and FS contributed

to the execution of the experiments. PH and KW performed the analysis of the data. ZX, ZW, and KW drafted the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01618/full#supplementary-material>

Figure S1 | Principal component analysis (PCA) plot illustrating sex category (A) and renal biopsy classification (class III to class V) of LN patients (B).

Figure S2 | As shown in **Table S1**, subject S010-7, and IR010 seem to be different from other samples with lower rate of reads passing bioinformatics filters.

However, removal of these two samples did not lead to substantial changes in the pattern of PCA plot (PERMANOVA $P = 0.02$).

Table S1 | Sequence quality control statistics.

Table S2 | The frequency of different types of clones for each study subject.

Table S3 | The amino acid composition for each study subject.

Table S4 | The composition of amino acid hydrophilicity for each study subject.

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Conflict of Interest: ZW and JZ are employed by the company GS Medical (Beijing) Technology Development LLC.

The remaining 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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The Complement C3a and C3a Receptor Pathway in Kidney Diseases

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The pathogenesis of some kidney diseases is closely associated with complement activation, where the C3a/C3a receptor (C3aR) might play a crucial role. C3a/C3aR has dual roles and may exert anti-inflammatory or pro-inflammatory effects depending on different cell types and diseases. In the kidneys, C3aR is primarily expressed on the tubular epithelium and less in glomerular podocytes. C3aR expression is enhanced and the levels of C3a in the plasma and urine are increased in kidney diseases of several types, and are associated with disease progression and severity. The C3a/C3aR pathway facilitates the progression of glomerular and tubulointerstitial diseases, while it has opposite effects on urinary tract infections. Clinical trials targeting C3a/C3aR in kidney diseases are lacking. Here, we reviewed the studies on the C3a/C3aR pathway in kidney disease, with the aim of understanding in-depth its controversial roles and its potential therapeutic value.

Keywords: complement, C3a, C3a receptor, kidney disease, inflammation

INTRODUCTION

Complement activation participates in the pathogenesis of a variety of diseases and induces tissue damage. The complement system is activated through three pathways (**Figure 1**). The classical pathway is initiated by the interaction of C1 with IgG or IgM antibodies, and then by cleavage of C2 and C4 to form the C3 convertase, C4b2a. In the lectin pathway, mannose-binding lectin (MBL) binds to the bacterial polysaccharide surface to form a complex, which also cleaves C2 and C4 to generate C4b2a. The alternative pathway involves spontaneous activation, C3 is cleaved to generate C3b, then C3b constitutes another C3 convertase, C3bBb, with factor B (1).

The three pathways converge on C3 convertase, C4b2a or C3bBb, which cleaves C3 and begins the common pathway. C3 is split between arginine (Arg) 77 and serine (Ser) 78 into two fragments, the smaller one as C3a and the larger one as C3b (2, 3). C3b is an opsonin that can lead to destruction of microbes by coating and decorating them. It also participates in the common pathway by forming the C5 convertase, C4b2aC3b (in classical and lectin pathways) and (C3b)₂Bb (in alternative pathway). C5 convertase cleaves C5 into C5a and C5b. C5b binds to C6 and C7 to form a trimeric complex. Then, C8 and C9 bind to form the membrane attack complex (MAC). The lipophilic protein C7 makes the complex attached to the cell membrane by inserting into the lipid bilayer (2).

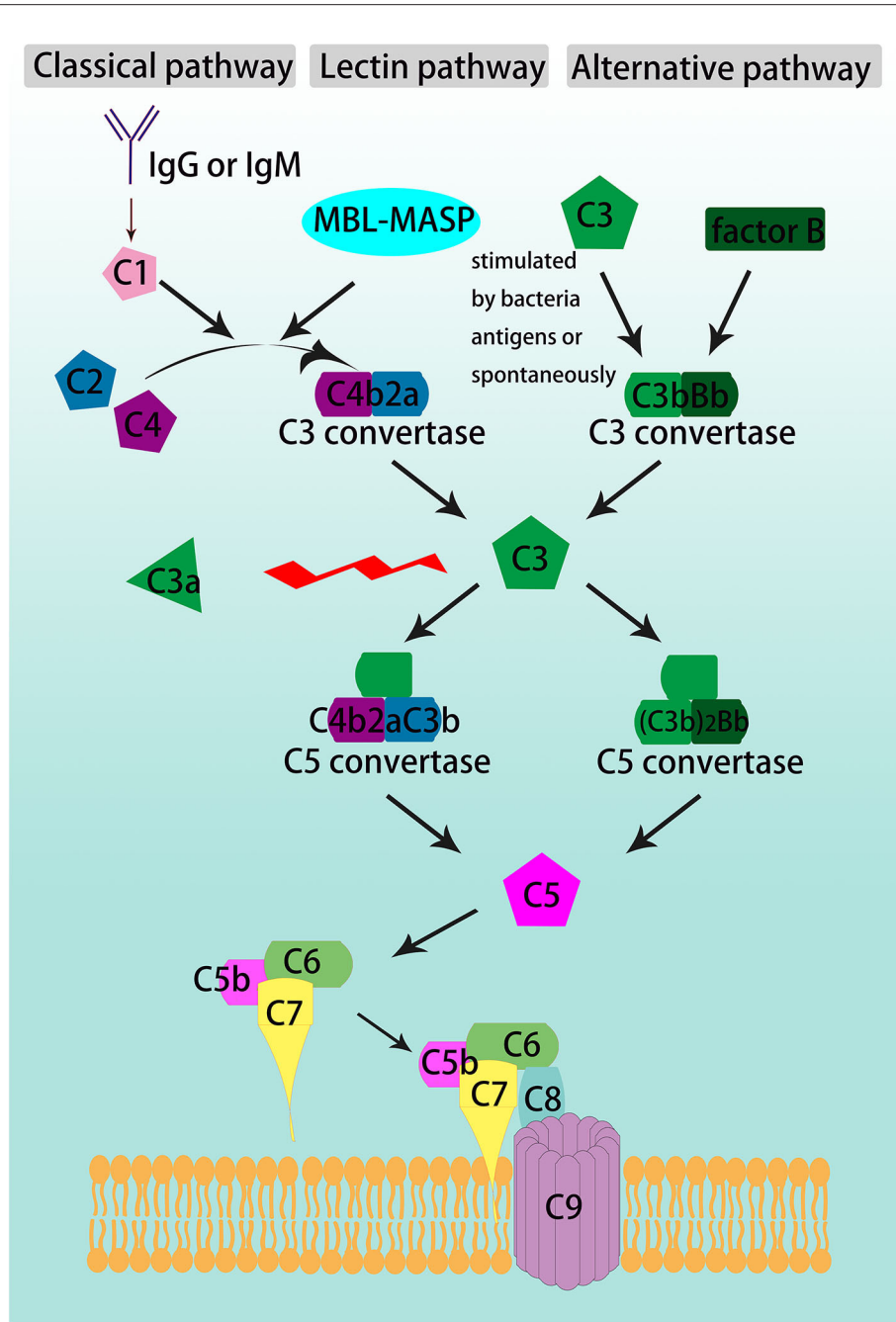


FIGURE 1 | Schematic overview of complement cascade. The complement system is activated through the classical, lectin, and alternative pathways, converge at the formation of C3 convertases. The classical pathway is activated by either IgG or IgM, the lectin pathway is triggered by the binding of mannose-binding lectin (MBL) to the pathogens' polysaccharide surface, and the alternative pathway begins with C3 spontaneous cleavage. C3 is the beginner and the core member of common pathway which is formed by the convergence of the three initiating pathways and activate C3, C5-C9 through sequence to form the terminal complement cascade, namely membrane attack complex (MAC).

C3a is an anaphylatoxin that exerts various effects after binding to the C3a receptor (C3aR). In kidneys, C3aR is expressed at high levels on tubular epithelial cells (4). In glomeruli, the staining for C3aR is restricted to glomerular epithelial cells (podocytes) and is much weaker than that

in the tubule (4). There are controversies about the roles of C3a/C3aR in kidney disease onset and tissue damage. In this review, we focused on the inflammatory effects after C3a binding with C3aR and its roles in the development of kidney diseases.

C3a and C3aR

Human C3a is a small 9 kDa peptide that comprises 77 amino acids and has four anti-parallel helical structures that are trapped by three disulfide bridges (5). The binding site of C3a to C3aR includes the C-terminal amino acids leucine-glycine-leucine-alanine-Arg (3, 5). When C3a is cleaved to C3a-desArg, it loses the ability to bind to C3aR, but binds to the second receptor of C5a, named C5aR2 (5). The antibodies currently used to detect C3a can distinguish C3 from its cleavage product; however, they fail to discriminate C3a from C3a-desArg, and may even detect C3a-desArg with higher sensitivity (5). Thus, it is necessary to develop antibodies that specifically recognize C3a. In healthy individuals, the plasma concentration of C3a is 119 ng/ml, and is derived from the degradation of C3 in the alternative pathway (5). However, the C3a concentration varies widely in different studies, from 20 to 156 ng/ml, due to the protocol and sample characteristics. The levels of C3a are increased in the sera due to removal of proteases that hydrolyzes C3a in the process of plasma clotting. Studies have reported that C3a levels in the circulation were elevated in pregnant women and may reach 182.5 ± 150.0 ng/ml in the plasma in the first trimester (5, 6).

Human C3aR is a 55 kDa protein comprising 482 amino acids and is a seven-transmembrane domain receptor that belongs to the G-protein coupled receptor family (4, 7–9). C3aR was first cloned in 1996 and was isolated from a cDNA expression library from U-937 cells (8). The human C3aR gene is located on chromosome 12p13.2-3 as a single copy. C3aR mRNA expression has been detected in several major organs such as the kidney (4), brain (10), lung (11), intestine (12), subcutaneous adipose tissue

(13), and other (9). There are mainly four post-translational modifications (14): C3aR is highly glycosylated; tyrosine sulfated (9, 15) and especially Tyr174 plays a critical role in high-affinity binding to C3a; phosphorylated and S-acylated (5). After binding to C3a, C3aR is activated and triggers intracellular signaling. The principle signaling pathway is mediated by the pertussis toxin (PT)-sensitive G protein $G\alpha_i$ in immune cells (16) and the PT-insensitive $G\alpha_{12/13}$ that leads to the activation of the ERK1/2 pathway and cytoskeletal changes (17). C3aR activation also causes an increase in intracellular Ca^{2+} , while C5aR activation has a stronger effect on promoting Ca^{2+} elevation (5). Another essential mediator is arrestin that can terminate the influence of C3aR to Ca^{2+} . A C3aR mutant failed to bind to β -arrestin 2. Thus, in mast cells, C3aR desensitization and internalization were inhibited by silencing β -arrestin 2, which leads to a prolonged release of Ca^{2+} (18).

The Function of C3a/C3aR in the Immune System

C3a is a critical chemotactic mediator in the immune system, and C3a/C3aR pathway has a dual, anti-inflammatory and pro-inflammatory roles in different cells and diseases [Figure 2; (3)]. In general, C3a/C3aR participates in the response of the immune system in three ways, acting on resident innate immune cells to up-regulate or down-regulate different cytokines, activate dendritic cells (DCs), and regulate T cell signaling between lymphocytes and antigen presenting cells (APCs) (19).

C3a acts anti-inflammatory effects in pathogens, i.e., bacteria/fungi, neutrophils in the bone marrow reservoir, and

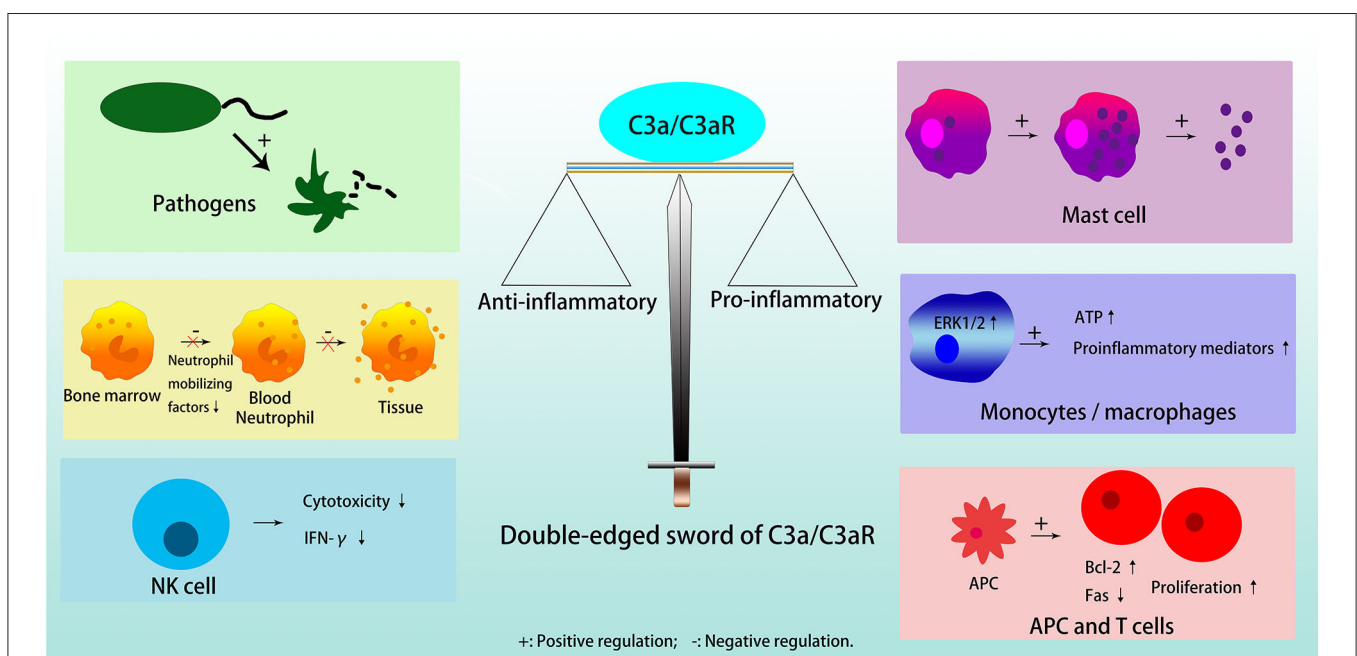


FIGURE 2 | Double edged sword of C3a/C3aR. C3a/C3aR plays anti-inflammatory effects in inducing pathogens elimination, inhibiting neutrophil migration from bone marrow and degranulation in tissue, and reducing cytotoxicity and expression of IFN- γ in NK cell. C3a/C3aR plays pro-inflammatory effects in inducing mast cells to generate and secrete small granule spherical particles, upregulating the expression of proinflammatory mediators by activating ERK1/2 and releasing extracellular ATP in monocytes or macrophages, and promoting T cell proliferation and differentiation directly or indirectly through APC.

natural killer (NK) cells. Furthermore, although there is a high expression of functional C3aR on the neutrophil cell membrane, C3a does not stimulate neutrophil degranulation to activate inflammatory response (3). C3a can also inhibit the migration of neutrophils from the bone marrow into the circulation by directly inhibiting neutrophil mobilizing factors, for example, G-CSF (3, 18). In NK cells, C3a is a negative regulator because it is able to not only inhibit NK cell cytotoxicity *in vivo*, but also to down-regulate the expression of IFN- γ (20).

C3a exerts pro-inflammatory functions in mast cells, macrophages/monocytes, T cells, and APC. Mast cells participate in allergic reactions, in which anaphylatoxin C3a induces them to produce and secrete small granule spherical particles, in a process named IgE-independent degranulation, through a rapid rise of intracellular calcium levels (21, 22). In monocytes or macrophages, C3a induces the release of proinflammatory mediators after binding to C3aR, activation of ERK1/2, and release of extracellular ATP, which in turn induces P2X7 that cooperates with NF- κ B and enhances the production of IL-1 β (23). When the activity of monocytes prevails over neutrophils, C3a plays a catalytic role in inflammation in general (3). In T cells, C3a/C3aR activates phosphoinositide-3-kinase- γ and induces phosphorylation of AKT, while, it up-regulates the antiapoptotic protein Bcl-2 and down-regulates the proapoptotic molecule Fas, to decrease T cell apoptosis and enhance their proliferation (24). In addition, C3a/C3aR can promote T cell proliferation through its effect on APC (23). The absence of C3, C3a or C3aR on APC results in a reduction in MHC II expression, which limits T cell proliferation and differentiation by insufficient antigen presentation (25, 26). C3aR blockade or deficiency results in decreased secretion of inflammatory factors, such as IL-2 and IFN- γ in T cells, and IL-1, IL-12, and IL-23 in APC (26).

In the studies of C3a and C3aR, the C3aR agonists and antagonists play pivotal roles. The peptide ligand WWGKKYRASKLGL, also called “super agonist,” is the most effective C3aR agonist, 15-fold more potent than C3a. There are several new potent and selective agonists validated by the calcium release assay, that is, FLPLAR 26/24 and FWTLAR 54/55. The most common and effective C3aR antagonist SB290157 is a trifluoroacetate salt, with an effective IC₅₀ of 27.7, 7.0, and 12.5 nM in human, mouse, and guinea pig RBL-2H3 cells, respectively (27). Nevertheless, SB290157 treatment had off-target activity resulting in rapid neutropenia and transient hypertension (28). There are even reports suggesting that SB290157 functions as an agonist. C3a binds to C3aR to induce calcium mobilization, a marker of C3aR activation, whereas SB290157 also induced calcium mobilization in a dose-dependent manner (28). SB290157 showed antagonist effects on cells with low levels of C3aR expression, while it acted as an agonist on cells with high levels of C3aR (29).

C3aR knockout animals have been employed in numerous experiments, and questions arise regarding the choice of complete knockout or conditional knockout. In complete knockout animals, both local organs studied and circulating immune cells lack C3aR expression. Considering the different functions of C3aR in different cells, its role in circulating

cells might interfere with the effects on local organs. The conditional knockout animals can restrict C3aR deficiency in specific organs, but the circulating immune cells that participate in the pathogenesis still possess normal expression of C3aR, which may weaken the effects. Thus, cautious interpretations should be given when there is a difference between the results from knockout animals and those with the C3aR antagonist.

C3a/C3aR participates in the pathogenesis of various diseases. In Alzheimer's disease, the C3a/C3aR pathway mediated microtubule-associated protein tau modulation by targeting STAT3, and the expression of C3aR was negatively correlated with cognitive function and positively correlated with Braak stages (10). In asthma, C3a/C3aR promoted smooth muscle contraction, mucus secretion, and recruitment of inflammatory cells. Deficiency of C3aR was protective to the lungs in a murine model of allergic airway disease (30, 31). In coronary artery disease, the expression of C3aR was positively correlated with activated glycoprotein IIb/IIIa in platelets, and the incidence of stroke and myocardial infarction was reduced in C3aR^{-/-} mice (32). C3a/C3aR played a protective role in intestinal ischemia-reperfusion (IR) injury by inhibiting neutrophil mobilization (33).

C3a/C3aR in Kidney Diseases

C3a/C3aR plays crucial roles in various kidney diseases (Figure 3).

Primary Glomerular Diseases

IgA nephropathy (IgAN)

IgAN is characterized by mesangial IgA and C3 deposition, and C3a/C3aR contributes to its pathogenesis. In 1992, Abou-Ragheb et al. found a positive correlation between plasma C3a levels and plasma creatinine levels in patients with IgAN, and suggested the measurement of plasma anaphylatoxins as a possible indicator of disease activity and prognosis (34). However, Janssen et al. found that the C3a levels could not predict renal prognosis or reflect the status of neoantigens that develop after C3 activation, which is an indicator of disease activity and outcome (35). In 2014, Liu et al. observed elevated staining of C3aR and C3a in glomeruli, and high levels of C3a in the sera and urine from IgAN patients (36). Both urinary C3a levels and glomerular C3aR and C3a staining correlated positively with proteinuria, serum creatinine levels, and histopathological injuries (30). In 2015, Zhu et al. found associations of variants of genes encoding complement factor H (CFH), CFH-related protein 3 (CFHR3), CFH-related protein 1 (CFHR1) with high levels of circulating C3 and low-levels of serum C3a in IgAN patients. CFHR1 protein is a competitive antagonist of CFH; therefore, higher levels of CFH inhibit complement activation, leading to the inhibition of C3 cleavage to C3a. Thus, in patients with low CFH, CFHR1, or CFHR3, there is excess cleavage of C3 and a buildup of C3a in the plasma (37). In 2016, a study of a cohort of patients with IgAN revealed that plasmapheresis is effective in reducing urinary C3a levels and the probability of dialysis-dependence (38). In 2017, Zhang et al. showed that C3aR knockout mice had lower levels of proteinuria and reduced IgA and C3 deposition in the kidney as well as reduced histological injury when IgAN was induced by

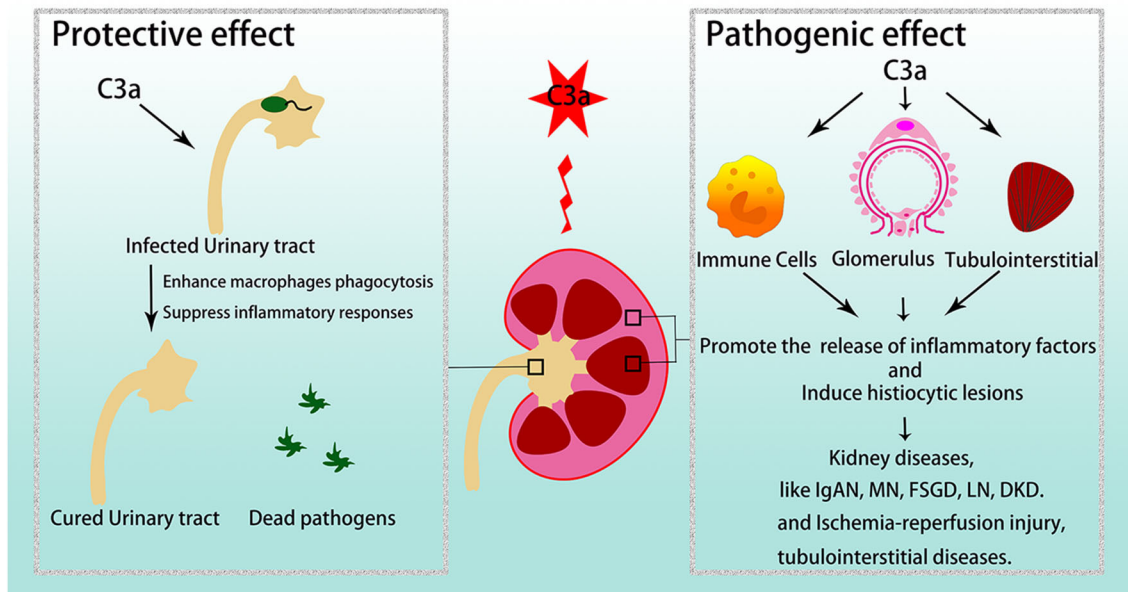


FIGURE 3 | C3a/C3aR in kidney diseases. C3a/C3aR is related to disease development and severity in various glomerular diseases and tubulointerstitial injuries. However, in urinary tract infections, C3a/C3aR acts protective effect. IgAN, IgA nephropathy; MN, membranous nephropathy; FSGS, focal segmental glomerulosclerosis; LN, lupus nephritis; DKD, diabetic kidney disease.

Sendai virus. They also detected a decrease in $\text{TNF-}\alpha$, $\text{TGF-}\beta$, $\text{IL-1}\beta$, IL-6 , and monocyte chemoattractant protein-1 (MCP-1) in mouse kidneys (39). In 2019, Zhang et al. found that plasma C3a in patients with IgAN was positively correlated with plasma and mesangial galactose-deficient IgA1 molecules (Gd-IgA1) (38, 40).

These studies suggest a direct role of C3a/C3aR in IgAN, although it is unclear whether its involvement is at the level of the kidney. A conditional knockout mouse, where C3aR is deleted from the kidney, will be a useful tool to elucidate this.

Membranous nephropathy (MN)

Primary MN is currently identified as an autoimmune disorder of M-type phospholipase A2 receptor (PLA2R), which is expressed on podocyte membrane. Cross-sectional studies have found that C3a levels were remarkably high in the plasma and urine of MN patients, and they were even higher in the circulation of patients with positive total and IgG4 anti-PLA2R antibodies than those without the antibody. Patients who achieved complete remission had lower levels of serum C3a (41, 42). The deposition of IgG4 anti-PLA2R is a reliable predictor of MN that activates the complement cascade by binding to MASP-1/2/MBL complex (42). In hepatitis B virus-associated MN, the significant reduction in plasma C3a levels was also related to remission during treatment (43).

We can speculate that C3a/C3aR is involved in MN pathogenesis. Previous studies have only shown that circulating C3a levels increase and much work is needed to confirm the role of the C3a/C3aR pathway. For example, we can further investigate C3aR expression in podocytes, and can clarify the

pathogenic role of C3a/C3aR by inducing disease in C3a/C3aR deficient animals or by blocking C3aR.

Focal segmental glomerulosclerosis (FSGS)

FSGS is caused by the loss or injury of podocytes. In 2016, Morigi et al. found that C3a/C3aR participated in podocyte depletion and glomerulosclerosis. In FSGS patients with progressive proteinuria, protein-overload mice and *in vitro* podocyte culture, C3a/C3aR caused podocyte damage by activating glial cell line-derived neurotrophic factor (GDNF)/c-Ret (the receptor of GDNF) pathway, which is a critical adaptive response when podocytes are exposed to toxic injury. C3a/C3aR also induced parietal epithelial cells to up-regulate CXCR4, which resulted in parietal epithelial cell proliferation and migration, and glomerulosclerosis (44). In adriamycin (ADR)-treated mice, Liu et al. found that C3aR expression was increased in the kidneys. Furthermore, it has been found that resveratrol suppressed inflammatory response, glomerulosclerosis, and renal interstitial fibrosis through down-regulation of the C3aR/C5aR-Sphk1 pathway (33).

C3a/C3aR also plays a role in tubulointerstitial fibrosis in FSGS. In 2018, Han et al. detected low-levels of expression of versican in C3aR^{-/-} ADR mice. They suggested that C3a promoted the transcription of versican. The β -catenin/TCF transcription factor complex was indispensable for the expression of versican. C3a induced the phosphorylation of AKT, which promotes β -catenin/T-cell factor (TCF) expression by inhibiting GSK-3 β to directly phosphorylate β -catenin and indirectly stabilize it (45). They treated cultured tubular cells with sera from FSGS patients, which contained high levels of C3a and found

that the expression of long non-coding RNA LOC105375913 was increased in a time- and dose-dependent manner, which resulted in the up-regulations of collagen I and fibronectin levels in tubular cells. This expression could be inhibited by a C3aR blocker (46).

Previous studies have shown that C3a/C3aR participates in the pathogenesis of FSGS by regulating various signaling pathways both in glomeruli and tubules, and mentioned the protective function of the C3aR blocker. Furthermore, C3a/C3aR targeted therapy may be applied to FSGS patients.

Secondary Glomerular Diseases

Lupus nephritis (LN)

LN is mediated by the deposition of immune complexes and complement activation products in the kidney tissue. Studies have shown that both C3a and C3aR are increased in patients with LN and related to disease severity and activity. In 2007, Mizuno et al. found that C3aR staining was positive in 42.9% of all LN kidney specimens and in 81.3% of sections classified as WHO IV LN. The intensity of C3aR staining was positively correlated with LN histological activity score (14). In 2017, Song et al. found that the plasma levels of C3a were elevated, especially in patients with active LN, while they were much lower in patients in remission and in SLE patients without clinical renal involvement (47). Animal experiments have validated the pathogenic effects of C3a/C3aR. As early as in 2005, Bao showed that in the kidneys of MRL/lpr mice, the expression of C3aR was significantly elevated. C3aR antagonist treatment could reduce the expression of IL-1 β and RANTES in the kidney, relieve pathological injuries, and prolong survival (48). However, in 2008, Wenderfer found a protective effect of C3aR in the early stage of LN. In C3aR^{-/-}MRL/lpr mice at 8 weeks, various pathogenic chemokine receptors were found to be increased, except for MCP-1, and the mice showed an earlier onset of renal injury compared to the controls. However, C3aR expression in glomeruli had no effect on long-term prognosis (49).

Data show high levels of C3a and C3aR expression in LN, but the direct role of C3a/C3aR in the kidney remains to be elucidated. The immune system is over-activated in LN, which reminds us of the role of C3a/C3aR in the immune system mentioned above.

Diabetic kidney disease (DKD)

In patients with DKD, C3a/C3aR is remarkably activated and is involved in its pathogenesis. Both the levels of C3a in the plasma and urine were significantly elevated in DKD patients compared to diabetic patients without kidney injury (50, 51). Urinary C3a was positively correlated with urinary protein as well as with the estimated glomerular filtration rate (eGFR). C3a was also correlated with glomerular lesion classification of DKD and the progression of disease (50, 52). C3aR expression was enhanced in early and advanced DKD (53). Li et al. have reported that C3aR antagonist treatment could alleviate kidney damage in DKD rats induced by a high-fat diet and streptozotocin by inhibiting Wnt/ β -catenin, TGF- β /smad3 signaling pathways, IKB α phosphorylation, and IL-6 release, to reduce inflammation and fibrosis in glomerular endothelial cells (53, 54). Li et al. have

also reported that C3aR-deficiency reduced kidney damage in diabetic rats. The possible mechanism was that the absence of C3aR suppressed T-cell activation by inhibiting the release of cytokines such as IL-4, IL-23, and IL-27 from macrophages (55).

The above studies have clearly demonstrated the pathogenic role of C3a in DKD and suggested its mechanism. The next research direction is to use C3aR as a therapeutic target to treat DKD.

Tubulointerstitial Diseases

Ischemia-reperfusion (IR) injury

IR injury results in acute tubular necrosis, during which oxygen and nutrients needed to maintain normal metabolism are deprived, where the cells die through necrosis and release abundant endogenous ligands. After restoration of perfusion, endogenous ligands activate innate immune responses by stimulating inflammatory cell recruitment and activation (56, 57). In both tubular epithelial cells and infiltrating neutrophils, monocytes, and macrophages, C3a/C3aR stimulated the production of cytokines and chemokines, such as TNF- α , IFN- γ , MIP-1, MCP-1, IL-1 β , IL-6, IL-8, and IL-17, which are thought to be involved in IR kidney injury (56–58). C3a/C3aR also promoted the expression of KIM-1, which is a functional and specific marker for acute tubular necrosis (56, 57). Simone et al. have demonstrated that C3a participated in IR injury by enhancing NADPH oxidase activity and promoting α -SMA protein expression (59). Curci et al. have reported that IR injury promoted the process of C3a-induced epithelial to mesenchymal transition (EMT), which leads to fibrosis through the AKT pathway (60).

The C3a/C3aR pathway promotes ischemia-reperfusion injury through an excess of cytokines and oxidative stress. However, there are few reports on the changes in C3a/C3aR expression. We need to examine whether the expression of C3 is abnormal during the injury. In addition, the direct effect of C3a/C3aR in the kidney remains unresolved and requires further investigation.

Chronic tubulointerstitial diseases

Chronic tubulointerstitial inflammation and fibrosis occur in most chronic kidney diseases. Studies have shown that C3a/C3aR can induce tubulointerstitial inflammation and fibrosis by mediating EMT in proximal tubular epithelial cells through the TGF- β 1/CTGF signaling pathway (61, 62). The C3aR antagonist effectively inhibited EMT induced by C3a, and ameliorated the pathology of ADR mice and preserved renal function and limited interstitial fibrosis (62). Bao et al. have induced complement activation by transplanting Crry^{-/-}C3^{-/-} kidney to C3aR^{-/-} host, where circulating C3 from the host acted on donor kidneys deficient in Crry (a membrane protein that inhibits C3 convertase, equivalent to human CD55) to induce complement activation. The results showed that deficiency in C3aR reduced the kidney tubulointerstitial inflammation and fibrosis (63). C3a could induce T-cells to release IL-17A by ERK, STAT3/5, and NF- κ B resulting in an inflammatory response and fibrosis (64).

Although studies have shown the pathogenic effect of C3a/C3aR in chronic tubulointerstitial diseases, its direct

renal effect is still unknown. Meanwhile, we cannot confirm the fatal role of the C3a/C3aR pathway in the chronic inflammatory fibrosis process because almost all complement and inflammatory factors are involved in this process. It is worth choosing several important factors for comparison.

Urinary Tract Infections

C3a/C3aR exerts a protective effect during infections. For example, in a mouse model of *Listeria monocytogenes*, C3a/C3aR up-regulated Bcl-2 while down-regulated Fas, caspase-3, and IFN- γ , which ameliorate organism-induced apoptosis (65, 66). Similarly, in uropathogenic *Escherichia coli* (UPEC)-induced renal injury, C3a/C3aR also had a protective role by enhancing macrophage phagocytosis induced by LPS despite suppression of inflammatory responses (67, 68). Here, C3a agonism may offer an interesting new therapeutic option along with standard antibiotic care.

SUMMARY AND PROSPECTION

Despite the large number of clinical trials targeting C5, C5a, C5aR1 on AAV, IgAN, aHUS, PNH, and other diseases, and the approved clinical use of eculizumab for PNH, aHUS, and

myasthenia gravis, clinical trials targeting C3 are less and mostly on phase I, and no clinical trials targeting C3a or C3aR are registered. Initial discussions primarily relied on theoretical considerations of primary complement deficiencies, whereas C3 deficiency often leads to a broader range of susceptibilities to infections, which is mostly attributed to the opsonic activity of C3b. However, it can be effectively avoided by developing therapies targeting C3a/C3aR, which participates in inflammatory responses but not opsonization. The major impediment derives from the controversies over the C3a/C3aR functions in different cell types and in different diseases, and the complexity of its intercellular signaling pathways. In-depth investigations and awareness of the roles of C3a/C3aR in kidney diseases are needed that will lead to a further expansion of potential indications for complement treatments in the future.

AUTHOR CONTRIBUTIONS

SG contributed to analysis and manuscript preparation and wrote the manuscript. ZC helped perform the analysis with constructive discussions. MZ contributed to the conception of the study. All authors contributed to the article and approved the submitted version.

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Clinical Utility of Serial Measurements of Antineutrophil Cytoplasmic Antibodies Targeting Proteinase 3 in ANCA-Associated Vasculitis

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Background: The utility of ANCA testing as an indicator of disease activity in ANCA-associated vasculitis (AAV) remains controversial. This study aimed to determine the association of ANCA testing by various methods and subsequent remission and examine the utility of a widely used automated addressable laser-bead immunoassay (ALBIA) to predict disease relapses.

Methods: Data from the Rituximab vs. Cyclophosphamide for ANCA-Associated Vasculitis (RAVE) trial were used. ANCA testing was performed by direct ELISA, capture ELISA, and ALBIA. Cox proportional hazards regression models were used to evaluate the association of PR3-ANCA level and subsequent remission or relapse. The ALBIA results are routinely reported as >8 when the value is high. For this study, samples were further titrated. A decrease and increase in PR3-ANCA were defined as a halving or doubling in value, respectively.

Results: A decrease in ANCA by ALBIA at 2 months was associated with shorter time to sustained remission (HR 4.52, $p = 0.035$). A decrease in ANCA by direct ELISA at 4 months was associated with decreased time to sustained remission (HR 1.77, $p = 0.050$). There were no other associations between ANCA decreases or negativity and time to remission. An increase in PR3-ANCA by ALBIA was found in 78 of 93 subjects (84%). Eleven (14%) had a PR3-ANCA value which required titration for detection of an increase. An increase of ANCA by ALBIA was associated with severe relapse across various subgroups.

Conclusions: A decrease in ANCA by ALBIA at 2 months and by direct ELISA at 4 months may be predictive of subsequent remission. These results should be confirmed in a separate cohort with similarly protocolized sample and clinical data collection. A routinely used automated ALBIA for PR3-ANCA measurement is comparable to direct ELISA in predicting relapse in PR3-AAV. Without titration, 14% of the increases detected by ALBIA would have been missed. Titration is recommended when this assay is used for disease monitoring. The association of an increase in PR3-ANCA with the risk of subsequent relapse remains complex and is affected by disease phenotype and remission induction agent.

Keywords: ANCA-associated vasculitis, PR3-ANCA, remission, relapse activity, biomarker

INTRODUCTION

The antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are characterized by necrotizing inflammation affecting predominantly small vessels (1). Three conditions comprise AAV: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA). GPA and MPA have many clinical similarities and have been studied in the same clinical trials, whereas EGPA has been excluded from these studies (2–6).

Historically, the course of AAV was inevitably fatal. The advent of treatment with immunosuppressive regimens such as cyclophosphamide (CYC) or rituximab (RTX), in combination with glucocorticoids (GCS), changed this course to one of a chronic relapsing disease. Remission is achievable with induction therapy in 70–90% of patients but more than half of patients in remission are at risk for relapse, particularly if they have ANCA reacting with proteinase 3 (PR3-ANCA) (7). Morbidity and mortality in AAV not only occurs from the disease process itself but also from complications secondary to immunosuppression. Therefore, balancing the risks of immunosuppression with the need for disease control is imperative, and accurate prediction of relapses is an important contributor to this balance (8–10).

Ever since the discovery of ANCA, the utility of ANCA testing as an indicator of disease activity or prediction tool of relapse has been investigated with conflicting results (11–34). To date, there has not been evidence that decreases in ANCA levels indicate subsequent remission (27). More recent studies demonstrating an association between rising PR3-ANCA levels and risk of relapse showed such associations to be dependent on ANCA-detection methodology, disease phenotype, and treatment regimen (34, 35). The current study aimed to determine the association of ANCA testing by various methods and subsequent remission. As ANCA-test methodologies are evolving, and automated addressable laser-bead immunoassay (ALBIA) ANCA testing platforms are more widely used in high volume laboratories, this study also aimed to examine the utility of an ALBIA for relapse prediction in AAV patients in comparison to methods previously reported.

METHODS

Patients

Serum samples and clinical data from the Rituximab vs. Cyclophosphamide for ANCA-Associated Vasculitis (RAVE) trial were used (3). All patients had provided consent for the use of both serum samples and clinical data collected during the RAVE trial for subsequent ancillary studies. The RAVE trial was approved by the Institutional Review Boards of each participating center.

Details of the RAVE trial are described elsewhere (3, 36). Briefly, RAVE was a multicenter, randomized, double-blind, double placebo-controlled trial design that included 197 patients with severe, ANCA-positive GPA or MPA. Of the 197 patients, 131 were PR3-ANCA positive and 66 were MPO-ANCA positive. The patients were randomized 1:1 to either RTX with 4 weekly treatments of 375 mg/m² or oral CYC of 2 mg/kg/day for 3–6 months followed by Azathioprine (AZA) to month 18. All patients received GCS consisting of intravenous methylprednisolone followed by a prednisone taper. The follow-up protocol consisted of visits at baseline; then weekly for 4 weeks, then monthly until 6 months, then every 3 months until month 18. Subsequently, patients were seen every 6 months until trial closeout which occurred 18 months after the last patient was enrolled. Additional study visits occurred at the patients' and providers' discretion, usually in the case of disease relapse or serious adverse event (3, 36).

It has been established that patients with PR3-ANCA are at increased risk of relapse compared to MPO-ANCA positive patients (7, 33, 36–41). The combination of a lower number of MPO-ANCA patients enrolled in the RAVE trial with less frequent relapses in this population resulted in a small sample of relapsing MPO-ANCA patients (3, 36). For this reason, PR3-ANCA positive patients were the population of interest for the current study.

Disease Activity and Phenotype

Assessment of disease activity was completed at each study visit using the BVAS/WG instrument with active disease defined as a score of ≥ 1 and a score of 0 reflecting remission (42). Complete remission was defined as a BVAS/WG of 0 with a prednisone dose of 0 mg. Sustained remission was defined as a BVAS/WG

of 0 with a prednisone dose of 0 mg for 6 months. A patient was considered to relapse if there was an increase of BVAS/WG of ≥ 1 after achievement of complete remission. A severe relapse was defined as an increase in BVAS/WG of >3 , new major item on BVAS/WG, or if induction therapy was reinitiated per clinician discretion (43).

Organ manifestations were recorded at enrollment and at each study visit with the BVAS/WG. The disease phenotype used for these analyses are based on that present at the time of enrollment. Patients were categorized into 1 or more of 5 groups: granulomatous disease only, any granulomatous disease, any capillaritis, renal involvement, and alveolar hemorrhage. These partially overlapping categories are described in detail elsewhere (35).

ANCA Testing

ANCA testing was performed using standardized direct enzyme-linked immunosorbent assays (ELISAs) for PR3-ANCA and MPO-ANCA (supplied by Euroimmun) on all baseline serum samples (28). If found to be ANCA positive, serial samples were tested using multiple methods including direct ELISA, capture ELISA (44), and an automated addressable laser-bead immunoassay (ALBIA) (BioPlex 2200, Biorad) (45). Serum samples for each patient were run together at a single laboratory from the second thaw cycle of each sample for each visit.

A PR3-ANCA test result obtained by ALBIA is considered equivocal if a value of 0.4–0.9. In this study, if a value was within the equivocal range the presence of a cANCA pattern was confirmed by immunofluorescence. This method increases the sensitivity of the ALBIA assay without compromising specificity. Results of the ALBIA assay are routinely reported to a value of 8 units, after which it is reported as >8 units. For this study, samples were additionally titrated 1:1, 1:4, 1:10, and 1:100 with the highest value recorded. If a value was not titrated, then the first titrated value was used. An increased PR3-ANCA level was defined as doubling in value compared to the lowest visit in the last 6 months. A decreased ANCA level was defined as halving of value compared to the highest visit in the last 6 months. All changes were outside the intra-assay and inter-assay coefficients of variation.

Statistical Analyses

Statistical analyses were completed using SAS, version 9.3 (SAS Institute). Descriptive data are reported as mean (SD), median and percentages.

PR3-ANCA Levels and Remission

This analysis was based on all patients who achieved complete remission following remission induction therapy ($n = 108$) whether achieved on originally assigned treatment or after cross-over. Cox proportional hazards models were used to assess whether ANCA level decreases were associated with subsequent remission. Analysis was completed looking at ANCA decrease for the event of interest of complete remission or sustained remission. To examine whether a decrease in ANCA was associated with remission, the times of interest were 2 and 4 months after enrollment in the study (time 0). Patients were

classified according to their ANCA as “decrease” or “no decrease” Patients classified as “no decrease” included all patients that did not meet criteria for a decrease as defined as halving of ANCA value including those who had an increase in ANCA, had stable ANCA levels and had a decrease in ANCA that did not meet criteria. Time to complete remission and sustained remission were then estimated by using the Kaplan-Meier method.

All analyses were performed for the entire cohort and for patient subsets defined according to disease phenotype and treatment groups. $P < 0.05$ were considered statistically significant.

PR3-ANCA Levels and Relapse

This analysis was based on patients who achieved complete remission with the originally assigned remission induction treatment only ($n = 93$). Cox proportional hazards models were also used to assess whether PR3-ANCA level increases were associated with subsequent relapse. This analysis was completed looking at a rise in PR3-ANCA for the event of interest of “any” relapse and “severe” relapse. Hazard ratios with corresponding 95% confidence intervals were used to quantify the increase in risk of relapse within 12 months after a PR3-ANCA increase. In order to determine the accuracy of the model to discriminate patients at increased risk of relapse, c-indices were calculated as was completed in a previous study (35). A c-index of 0.5 indicates no discrimination, and a value of 0.7–0.8 indicates adequate discrimination (46).

Increase in PR3-ANCA was examined using a binary time-varying covariate with the variable having a value of “0” from the time of complete remission to the date that an increase in PR3-ANCA occurred and a value of “1” following an increase in PR3-ANCA. With this method, if an increase occurred at the time of a relapse it would not be detected. Kaplan-Meier analysis that included patients with an increase in PR3-ANCA was completed with time 0 defined as the time of increase.

RESULTS

Correlation of Direct ELISA and Automated ALBIA Results

Results obtained with the direct ELISA and the automated ALBIA are positively correlated (Spearman 0.8741, $p < 0.0001$); therefore, with an increase in one an increase in the other is expected, and the opposite is true (Figure 1). As the tests do not have a correlation coefficient of 1.0, a change by a certain increment in one will not result in that same degree of change in the other test.

PR3-ANCA Titer Decreases and Subsequent Remission

The baseline characteristics of the 131 PR3-ANCA positive participants of the RAVE trial have been described elsewhere (47). The median time from enrollment to remission, complete remission, and sustained remission were 2, 6, and 9 months, respectively. There was a decrease in PR3-ANCA at 2 months in 93 (71%), 50 (38%) and 120 (92%) of patients measured by direct ELISA, capture ELISA, and ALBIA, respectively. Of

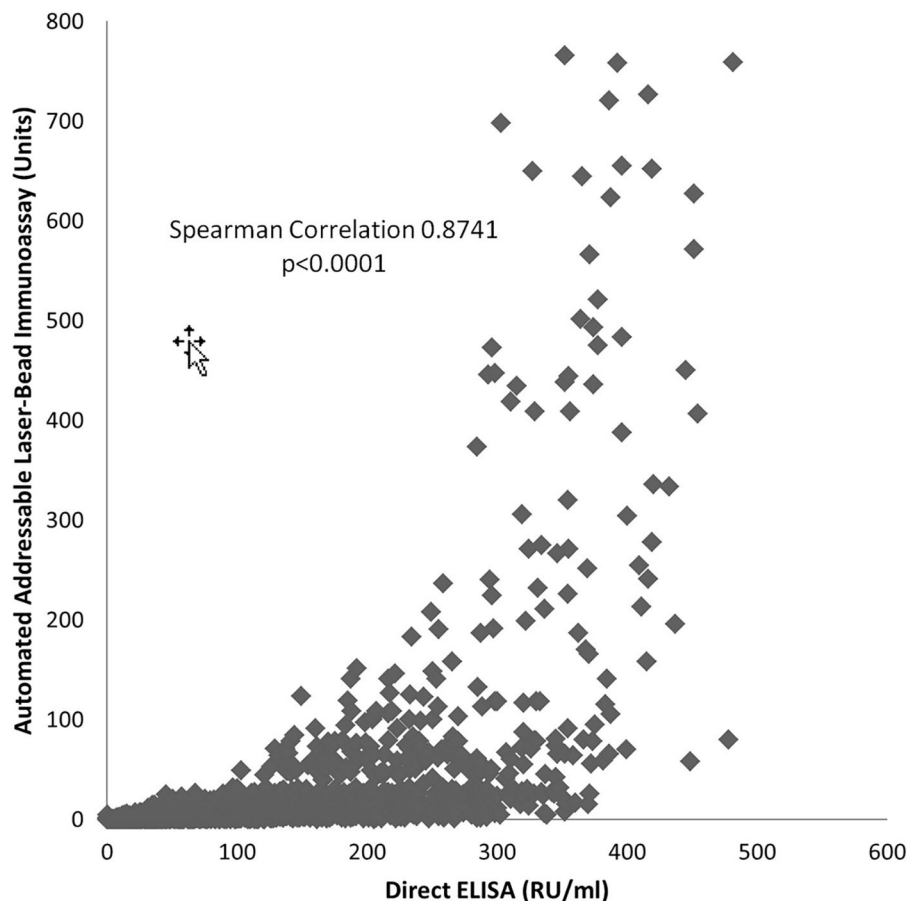


FIGURE 1 | Relationship of PR3-ANCA Measurement by Automated Addressable Laser-Bead Immunoassay and Direct ELISA.

the 131 participants, 108 met criteria for complete remission at some point during follow-up and 92 met criteria for sustained remission. Those patients who had a decrease in PR3-ANCA by ALBIA at 2 months had a shorter time to sustained remission (HR 4.52, 95% CI 1.11, 18.42, $p = 0.035$) (**Table 1**). In patients who had a decrease in PR3-ANCA by ALBIA at 2 months, the median time to sustained remission was 12 months (9 and 12 months for RTX and CYC, respectively). Among the 11 patients (4 RTX, 7 CYC) who did not have a decrease in PR3-ANCA by ALBIA at 2 months, only 2 patients (both RTX) achieved sustained remission during follow-up.

A decrease in PR3-ANCA at 4 months occurred in 101 (77%), 68 (52%), and 121 (92%) of patients by direct ELISA, capture ELISA, and ALBIA, respectively. A decrease in PR3-ANCA by direct ELISA at 4 months was associated with decreased time to sustained remission (HR 1.77, 95% CI 1.03, 3.28) (**Table 1**) with a median time to sustained remission of 9 months in those patients who had a decrease in PR3-ANCA by direct ELISA at 4 months compared to 12 months in those who did not.

PR3-ANCA negativity occurred at 4 months in 74 (56%), 41 (31%), and 55 (42%) patients by direct ELISA, capture ELISA and ALBIA, respectively. There was no association between

PR3-ANCA negativity at 4 months by any PR3-ANCA assay and time to complete or sustained remission (**Table 1**).

When stratified by treatment group there was an association between a decrease in PR3-ANCA by ALBIA at 2 and 4 months and time to sustained remission in patients treated with CYC/AZA (**Table 2**).

A decrease in PR3-ANCA by direct or capture ELISA at 4 months was associated with decreased time to complete remission in patients with granulomatous disease (**Table 3**). There was no other association between PR3-ANCA decrease or negativity and time to complete or sustained remission when stratified by treatment group or disease phenotype (**Tables 2, 3**).

PR3-ANCA Titer Increases Determined by ALBIA and Subsequent Relapse

The baseline characteristics of the 93 PR3-ANCA positive patients who achieved complete remission have been reported elsewhere (35). Relapses occurred in 55 of the 93 subjects (59%). An increase in PR3-ANCA by ALBIA was found in 78 of 93 subjects (84%). Of these patients 11 (14%) had a PR3-ANCA value >4 units which subsequently increased to a value >8

TABLE 1 | Association between a decrease in PR3-ANCA and time to remission.

	Complete remission [†]			Sustained remission [†]		
	HR	95% C.I.	p	HR	95% C.I.	p
Overall (N* = 131)						
Decrease by 2 months						
Direct (n [†] = 93)	1.51	(0.95, 2.40)	0.079	1.45	(0.90, 2.42)	0.139
Capture (n [†] = 50)	1.26	(0.86, 1.84)	0.245	1.14	(0.75, 1.72)	0.541
ALBIA (n [†] = 120)	2.11	(0.86, 5.22)	0.104	4.52	(1.11, 18.42)	0.035
Decrease by 4 months						
Direct (n [†] = 101)	1.75	(1.03, 2.97)	0.038	1.77	(1.03, 3.28)	0.050
Capture (n [†] = 68)	1.39	(0.94, 2.05)	0.102	1.29	(0.86, 1.98)	0.225
ALBIA (n [†] = 121)	1.73	(0.70, 4.26)	0.234	3.81	(0.94, 15.53)	0.062
Negative by 4 months						
Direct (n [†] = 74)	1.07	(0.73, 1.58)	0.729	1.00	(0.66, 1.51)	0.996
Capture (n [†] = 41)	1.18	(0.80, 1.75)	0.413	0.97	(0.62, 1.50)	0.881
ALBIA (n [†] = 55)	1.08	(0.73, 1.57)	0.721	0.98	(0.65, 1.49)	0.939

*Total number of patients.

[†]Number of patients who experienced the given decrease in ANCA by the stated time period.

[‡]Separate analyses were performed for each remission definition and ANCA decrease definition using proportional hazards regression. Findings are summarized using the hazard ratio (HR) and corresponding 95% confidence interval. A hazard ratio significantly > 1.0 indicates that experiencing the given ANCA decrease by the given time period is associated with a shorter time to achieving the given remission endpoint.

units and therefore required titration for the detection of the increase. Four of these 11 patients experienced a subsequent relapse. Relapse occurred concurrently or after a rise in PR3-ANCA by ALBIA in 47 of the 55 relapses (85%) and within 1 year of an increase in 29 of the 55 relapses (53%). The median time to any relapse after PR3-ANCA increase was 15.4 months. Kaplan-Meier estimates for time to relapse following an increase in PR3-ANCA level stratified by disease phenotype and treatment arms are shown in **Table 4**. The number of patients with rise in PR3-ANCA followed by a relapse and time to relapse for the entire cohort with categorization by severity of relapse, disease phenotype, and treatment received are shown in the **Supplementary Figure**.

Of the 15 patients who did not experience an increase in PR3-ANCA, 8 (53%) developed a subsequent relapse (**Supplementary Figure**). Additionally, of the 78 patients who had an increase in PR3-ANCA, 31 (40%) did not experience a relapse. Five of these 31 had <1 year of follow-up after PR3-ANCA increase. Twenty patients had a PR3-ANCA increase but then had a subsequent decrease. Of these patients who had an initial increase but then a decrease, 3 experienced a relapse.

An increase of PR3-ANCA levels determined by ALBIA was associated with subsequent severe relapse ($p = 0.002$). This association was true for the subgroups of patients with renal involvement, capillaritis, diffuse alveolar hemorrhage, and those treated with RTX (**Table 5**). These results are comparable to the previously reported data on the utility of direct ELISA to predict relapse. Of the 42 severe relapses that occurred in this patient cohort, 39 (93%) had a preceding increase in PR3-ANCA (**Supplementary Figure**).

The effect of disease phenotype on PR3-ANCA increase and relapse association was also investigated. For the association

of PR3-ANCA increase to severe relapse, the c-indices ranged from 0.61 in patients with granulomatous disease only to 0.74 in patients with DAH, the subgroup in which the most significant association was observed (**Table 5**). The median time to any relapse following an increase in PR3-ANCA in patients with DAH was 10.2 months (**Table 4**). In this subgroup, there were no relapse that occurred without a preceding PR3-ANCA increase (**Supplementary Figure**).

There was also a difference amongst treatment groups in the association between PR3-ANCA increase and relapse. This association was stronger among patients treated with RTX compared to those treated with CYC/AZA (**Table 5**). In the patients who were treated with RTX, 46% (23 of 50) experienced a severe relapse. Ninety-six percent (22 of 23) of these relapses were preceded by a rise in PR3-ANCA in this group. In comparison, 44% (19 of 43) patients treated with CYC/AZA experienced a severe relapse and 89% (17 of 19) were preceded by an increase in PR3-ANCA (**Supplementary Figure**).

DISCUSSION

The diagnostic utility of ANCA testing for vasculitis is well-established, and the revised 2017 international consensus statement of ANCA testing in GPA and MPA has summarized results obtained with various state-of-the-art antigen-specific ANCA test methodologies including ALBIA, and recommends their use as primary diagnostic ANCA tests for GPA and MPA (48). Automated ALBIA platforms use glass, latex or magnetic beads to immobilize the antigen or antibody of interest. Light scatter and fluorescence are then used to obtain antibody measurements (45). Vasculitis specific commercial automated

TABLE 2 | Association between a decrease in PR3-ANCA and time to remission—by Treatment.

	Complete remission [†]			Sustained remission [†]		
	HR	95% C.I.	p	HR	95% C.I.	p
RTX (N* = 66)						
Decrease by 2 months						
Direct (n [‡] = 50)	1.41	(0.67, 2.97)	0.360	1.41	(0.66, 3.02)	0.381
Capture (n [‡] = 26)	1.17	(0.68, 1.99)	0.575	1.06	(0.59, 1.89)	0.853
ALBIA (n [‡] = 62)	2.05	(0.49, 8.53)	0.322	1.28	(0.31, 5.27)	0.735
Decrease by 4 months						
Direct (n [‡] = 55)	1.62	(0.62, 4.21)	0.323	1.85	(0.66, 5.17)	0.241
Capture (n [‡] = 38)	1.25	(0.71, 2.21)	0.442	1.19	(0.65, 2.19)	0.567
ALBIA (n [‡] = 63)	1.16	(0.28, 4.77)	0.841	0.71	(0.17, 2.95)	0.642
Negative by 4 months						
Direct (n [‡] = 39)	1.10	(0.62, 1.92)	0.752	0.97	(0.53, 1.76)	0.910
Capture (n [‡] = 19)	1.19	(0.67, 2.08)	0.555	1.04	(0.55, 1.94)	0.912
ALBIA (n [‡] = 29)	1.04	(0.61, 1.76)	0.893	0.96	(0.54, 1.71)	0.883
CYC (N* = 65)						
Decrease by 2 months						
Direct (n [‡] = 43)	1.42	(0.78, 2.60)	0.255	1.46	(0.77, 2.78)	0.250
Capture (n [‡] = 24)	1.29	(0.74, 2.23)	0.373	1.24	(0.69, 2.23)	0.475
ALBIA (n [‡] = 58)	2.06	(0.64, 6.63)	0.228	∞	(3.84, ∞)	<0.001
Decrease by 4 months						
Direct (n [‡] = 46)	1.63	(0.85, 3.13)	0.140	1.75	(0.86, 3.54)	0.121
Capture (n [‡] = 30)	1.37	(0.79, 2.36)	0.265	1.38	(0.77, 2.46)	0.282
ALBIA (n [‡] = 58)	2.06	(0.64, 6.63)	0.228	∞	(3.84, ∞)	<0.001
Negative by 4 months						
Direct (n [‡] = 35)	0.96	(0.56, 1.66)	0.894	1.02	(0.57, 1.81)	0.960
Capture (n [‡] = 22)	1.16	(0.66, 2.03)	0.599	0.89	(0.48, 1.65)	0.710
ALBIA (n [‡] = 26)	1.04	(0.60, 1.82)	0.880	0.98	(0.54, 1.79)	0.948

*Total number of patients in the given treatment group.

[‡]Number of patients in the given treatment group who experienced the given decrease in ANCA by the stated time period.

[†]Separate analyses were performed for each remission definition and ANCA decrease definition using proportional hazards regression. Findings are summarized using the hazard ratio (HR) and corresponding 95% confidence interval. A hazard ratio significantly > 1.0 indicates that experiencing the given ANCA decrease by the given time period is associated with a shorter time to achieving the given remission endpoint.

ALBIAs can measure multiple antibodies of interest from the same serum sample in a single tube including PR3-ANCA, MPO-ANCA, and anti-glomerular basement membrane (anti-GBM) antibody. The agreement between automated ALBIAs with immunofluorescence and commercially available ELISA kits have been shown to be high (45). Given the relative ease of this assay without compromising analytic sensitivity or specificity, many high volume clinical laboratories now utilize automated ALBIAs for detection of ANCA.

The 2017 consensus statement does not address the clinical utility of serial ANCA testing as a biological marker of AAV disease activity (48). This has been controversial for years (11–34). Using mostly indirect immunofluorescence to determine ANCA titers or ELISA methods, several studies have suggested that a decrease in ANCA titer during induction therapy may be indicative of disease response (15, 20, 26) while others using capture ELISA did not find a clear association

(27). The present study demonstrated a decrease in PR3-ANCA by ALBIA at 2 months was associated with decreased time to sustained remission. This association was strongest in patients treated with CYC/AZA. In patients treated with CYC/AZA who did not have a decrease in PR3-ANCA by ALBIA, none achieved sustained remission. The reason no significant association was observed for patients treated with RTX may be explained by the fact that most of these patients had a decrease (62 and 63 of the 66 patients by 2 and 4 months, respectively). Hence, a lack of PR3-ANCA decrease by ALBIA at 2 months may be predictive of refractory disease. Clinicians should closely monitor these patients and be prepared to change therapy if the clinical response is delayed or incomplete.

A prior study demonstrated a possible association of PR3-ANCA increases determined by direct ELISA with subsequent relapse during serial follow-up of patients (35). This association

TABLE 3 | Association between a decrease in PR3 ANCA and time to remission—by Phenotype.

	Complete remission [†]			Sustained remission [‡]		
	HR	95% C.I.	p	HR	95% C.I.	p
Renal Involvement (N* = 78)						
Decrease by 2 months						
Direct (n [†] = 56)	1.08	(0.61, 1.91)	0.801	1.20	(0.65, 2.19)	0.563
Capture (n [†] = 30)	1.09	(0.67, 1.77)	0.735	0.94	(0.55, 1.59)	0.809
ALBIA (n [†] = 72)	1.80	(0.56, 5.78)	0.323	2.08	(0.51, 8.56)	0.310
Decrease by 4 months						
Direct (n [†] = 62)	1.40	(0.70, 2.79)	0.346	1.65	(0.78, 3.50)	0.191
Capture (n [†] = 39)	1.27	(0.78, 2.08)	0.341	1.00	(0.60, 1.69)	0.992
ALBIA (n [†] = 73)	1.12	(0.35, 3.59)	0.848	1.39	(0.34, 5.70)	0.647
Negative by 4 months						
Direct (n [†] = 41)	1.03	(0.63, 1.69)	0.900	1.05	(0.62, 1.77)	0.849
Capture (n [†] = 24)	1.05	(0.63, 1.75)	0.846	0.83	(0.47, 1.46)	0.513
ALBIA (n [†] = 29)	1.11	(0.68, 1.83)	0.678	1.04	(0.61, 1.79)	0.878
Capillaritis (N* = 105)						
Decrease by 2 months						
Direct (n [†] = 76)	1.13	(0.69, 1.85)	0.631	1.15	(0.67, 1.95)	0.617
Capture (n [†] = 41)	1.06	(0.69, 1.61)	0.792	0.98	(0.62, 1.55)	0.922
ALBIA (n [†] = 95)	1.90	(0.76, 4.71)	0.169	3.96	(0.97, 16.23)	0.056
Decrease by 4 months						
Direct (n [†] = 83)	1.39	(0.78, 2.48)	0.270	1.51	(0.80, 2.87)	0.208
Capture (n [†] = 54)	1.24	(0.81, 1.90)	0.330	1.13	(0.71, 1.79)	0.604
ALBIA (n [†] = 96)	1.49	(0.60, 3.69)	0.391	3.24	(0.79, 13.26)	0.102
Negative by 4 months						
Direct (n [†] = 59)	1.01	(0.66, 1.54)	0.979	0.97	(0.61, 1.54)	0.896
Capture (n [†] = 34)	1.03	(0.66, 1.58)	0.911	0.84	(0.51, 1.37)	0.478
ALBIA (n [†] = 42)	1.04	(0.68, 1.59)	0.862	0.99	(0.62, 1.58)	0.975
DAH (N* = 38)						
Decrease by 2 months						
Direct (n [†] = 30)	1.65	(0.62, 4.35)	0.313	1.34	(0.46, 3.92)	0.596
Capture (n [†] = 12)	0.81	(0.38, 1.73)	0.582	0.86	(0.37, 2.01)	0.727
ALBIA (n [†] = 37)	1.46	(0.19, 10.98)	0.713	∞	(0.49, ∞)	0.164
Decrease by 4 months						
Direct (n [†] = 33)	3.02	(0.70, 13.05)	0.139	3.85	(0.52, 28.69)	0.189
Capture (n [†] = 18)	0.95	(0.46, 1.94)	0.885	0.97	(0.43, 2.15)	0.935
ALBIA (n [†] = 37)	1.46	(0.19, 10.98)	0.713	∞	(0.49, ∞)	0.164
Negative by 4 months						
Direct (n [†] = 21)	1.33	(0.63, 2.80)	0.459	0.91	(0.40, 2.05)	0.820
Capture (n [†] = 11)	0.80	(0.37, 1.75)	0.574	0.48	(0.18, 1.29)	0.145
ALBIA (n [†] = 17)	1.27	(0.62, 2.61)	0.518	1.12	(0.50, 2.51)	0.778
Granulomatous (N* = 102)						
Decrease by 2 months						
Direct (n [†] = 75)	1.57	(0.91, 2.70)	0.108	1.44	(0.81, 2.55)	0.212
Capture (n [†] = 42)	1.41	(0.91, 2.17)	0.124	1.35	(0.84, 2.15)	0.213
ALBIA (n [†] = 94)	3.10	(0.97, 9.95)	0.057	3.22	(0.79, 13.15)	0.104
Decrease by 4 months						
Direct (n [†] = 79)	1.95	(1.06, 3.58)	0.033	1.80	(0.94, 3.43)	0.076
Capture (n [†] = 55)	1.68	(1.07, 2.64)	0.026	1.57	(0.97, 2.54)	0.068
ALBIA (n [†] = 95)	2.41	(0.75, 7.71)	0.138	2.53	(0.62, 10.32)	0.197
Negative by 4 months						
Direct (n [†] = 63)	0.97	(0.62, 1.50)	0.874	0.86	(0.53, 1.37)	0.518
Capture (n [†] = 34)	1.29	(0.83, 2.00)	0.260	1.10	(0.68, 1.80)	0.694
ALBIA (n [†] = 47)	1.02	(0.66, 1.56)	0.936	0.89	(0.56, 1.42)	0.629

*Total number of patients with the given phenotype.

[†]Number of patients with the given phenotype who experienced the given decrease in ANCA by the stated time period.[‡]Separate analyses were performed for each remission definition and ANCA decrease definition using proportional hazards regression. Findings are summarized using the hazard ratio (HR) and corresponding 95% confidence interval. A hazard ratio significantly > 1.0 indicates that experiencing the given ANCA decrease by the given time period is associated with a shorter time to achieving the given remission endpoint.

TABLE 4 | Kaplan-Meier estimates for relapse following a rise in PR3-ANCA*.

	ALBIA				
	N*	Median months to relapse	Cumulative Relapse, % (95% C.I.)		
			6-months	12 months	18 months
Overall					
Any relapse	72	15.4	26 (15, 35)	42 (29, 52)	52 (38, 62)
Severe relapse	80	22.1	22 (12, 30)	35 (23, 45)	42 (30, 52)
Baseline capillaritis					
Any relapse	58	22.1	23 (11, 33)	36 (22, 48)	44 (29, 56)
Severe relapse	66	24.2	19 (8, 28)	32 (19, 42)	37 (24, 48)
Baseline renal					
Any relapse	44	–	26 (11, 38)	31 (15, 44)	39 (22, 53)
Severe relapse	51	37.0	20 (8, 31)	29 (15, 41)	36 (20, 52)
Baseline DAH					
Any relapse	20	10.2	43 (15, 61)	54 (24, 72)	67 (35, 83)
Severe relapse	22	14.3	34 (10, 51)	49 (22, 67)	60 (31, 77)
Rituximab group					
Any relapse	37	14.6	22 (7, 35)	42 (24, 57)	55 (34, 69)
Severe relapse	44	21.7	21 (8, 32)	35 (19, 48)	45 (28, 59)
Cyclophosphamide					
Any relapse	35	18.2	29 (12, 42)	41 (22, 56)	48 (28, 63)
Severe relapse	36	24.2	22 (7, 35)	34 (16, 48)	38 (19, 52)

*Analyses include individuals who experienced a rise in PR3-ANCA during follow-up while at risk for the given relapse endpoint. Individuals who experienced an ANCA increase concurrent with the given relapse event are not included. Time zero corresponds to the date of the ANCA increase.

was not found when the capture ELISA was used for PR3-ANCA detection (35). The present study examined the utility of an automated ALBIA in predicting relapse in AAV patients in comparison to methods previously reported. The present study replicates the previous findings obtained by direct ELISA that PR3-ANCA may have clinical utility in relapse prediction with limitations (35). It was found that an increase in PR3-ANCA has the strongest association with relapses of AAV in patients who: experience a severe relapse, have severe disease manifestations caused by capillaritis such as diffuse alveolar hemorrhage or renal involvement, and in patients treated with rituximab. In the present study, there was no association between a PR3-ANCA increase and relapse in patients with isolated necrotizing granulomatous inflammation at baseline, further supporting the stronger correlation between PR3-ANCA and capillaritis compared to PR3-ANCA and granulomatous inflammation seen in past studies (34, 35). *In vitro* and *in vivo* studies also provide support for this observation (49–52). ANCA has been shown to induce neutrophil activation that leads to capillaritis manifestations *in vitro* (49, 50). In some proposed animal models of PR3-ANCA disease, capillaritis also develops but convincing evidence of granulomatous inflammation has not been reported to date (51–53). The association of PR3-ANCA increase and subsequent relapse was consistent in all patients with capillaritis at disease presentation, regardless of treatment regimen received.

There was a stronger association of PR3-ANCA increase and relapse in patients treated with RTX compared to those treated with CYC/AZA. This may have several reasons. First, PR3-ANCA

positive patients are more likely to turn ANCA negative when treated with RTX than when treated with CYC for remission induction (3). Second, per study protocol, patients in the RTX arm did not receive additional maintenance agents such as AZA or methotrexate after induction with RTX with four weekly treatments of 375 mg/m² (3). Thus, B cell depleting therapy seems to be suppressing ANCA production more effectively than therapy that merely suppresses the B cell numbers. Conversely, when ANCA production resumes as B cells reconstitute a PR3-ANCA level increase may be more vigorous and more clearly identify patients at risk for relapse.

It is important to note that not all patients who had a rise in PR3-ANCA had a subsequent relapse. This was true even amongst patients with phenotypes and treatment regimens where PR3-ANCA and relapse were strongly associated with subsequent relapse such as those with capillaritis and those treated with RTX induction therapy. The risk of relapse therefore needs to be weighed against the side effects of treatment with individual patient factors considered. It is also interesting to note that among 12 patients who did not have an increase in PR3-ANCA, 9 (75%) did not experience a severe relapse during follow-up.

This study reconfirms that different ANCA detection assays perform differently when applied in clinical practice. This is why it is important for clinicians to know how each assay performs. The differences between assays are most likely the result of the PR3 antigen being presented differently in the solid phase in the different assays. Since ANCA are polyclonal antibodies it is not surprising that these antibodies recognizing different epitopes

TABLE 5 | Proportional hazards regression assessing whether an increase in ANCA is associated with relapse (Truncated after 1-year).

	HR	95% C.I.	p	c-index
All subjects				
Any flare	1.57	0.81, 3.05	0.156	0.55
Severe flare	5.45	1.83, 16.19	0.002	0.65
According to treatment				
Cyclophosphamide				
Any flare	1.64	0.50, 5.41	0.418	0.52
Severe flare	8.04	0.97, 66.47	0.053	0.62
Rituximab				
Any flare	1.59	0.65, 3.86	0.307	0.54
Severe flare	5.58	1.11, 28.04	0.037	0.64
According to renal involvement				
Without renal involvement				
Any flare	2.67	0.83, 8.56	0.100	0.60
Severe flare	3.04	0.80, 11.54	0.103	0.63
With renal involvement				
Any flare	1.09	0.47, 2.54	0.844	0.50
Severe flare	10.32	1.32, 80.97	0.026	0.67
According to baseline capillaritis				
Without capillaritis				
Any flare	4.74	0.53, 42.52	0.165	0.63
Severe flare	4.38	0.47, 40.42	0.193	0.64
With capillaritis				
Any flare	1.23	0.60, 2.54	0.573	0.52
Severe flare	5.43	1.55, 19.11	0.008	0.66
According to baseline DAH				
Without DAH				
Any flare	0.96	0.45, 2.06	0.918	0.50
Severe flare	2.87	0.91, 9.06	0.072	0.61
With DAH				
Any flare	5.24	1.13, 24.26	0.034	0.68
Severe flare	∞	4.21, ∞	<0.001	0.74
According to new vs. relapsing				
Relapsing disease				
Any flare	1.24	0.54, 2.83	0.613	0.51
Severe flare	6.62	1.43, 30.79	0.016	0.65
New disease				
Any flare	2.51	0.68, 9.27	0.166	0.59
Severe flare	7.18	0.89, 58.03	0.065	0.66
Any granulomatous disease				
Any flare	1.89	0.81, 4.24	0.143	0.56
Severe flare	7.22	1.64, 31.75	0.009	0.64
Granulomatous disease only				
Any flare	2.88	0.28, 30.00	0.375	0.59
Severe flare	2.88	0.28, 30.00	0.375	0.61

bind differently in the various assays which present the antigen in different fashions. This is why it is important to know what assays can be used clinically. The current findings expand the acceptable PR3-ANCA testing methods for disease monitoring to include the more convenient, routinely used automated ALBIA technology. It is important to note that without titration of the ALBIA values, 14% of the increases detected would have been missed. It is therefore strongly suggested that the serum samples be titrated when the automated ALBIA is used for AAV disease monitoring. In this study values considered equivocal in the

ALBIA assay (0.4–0.9) had the cANCA pattern confirmed by immunofluorescence. Confirmation by immunofluorescence or another ANCA assay should be completed for equivocal values if using the ALBIA assay as a high-volume screening assay to increase the sensitivity without compromising specificity.

There are several limitations to this study. Classifications of disease phenotype and severity of relapse were based on BVAS/WG forms completed during the RAVE trial by expert clinicians. This data was unable to be verified at the time of this study. Disease phenotype was classified based on disease activity

at baseline and not adjusted for changes in phenotype at relapse. The intervals of ANCA measurement were in accordance with the RAVE study protocol (3). For the time period following the first 6 months of remission induction therapy, this consisted of ANCA measurement every 3 months until month 18 from enrollment, followed by ANCA measurement every 6 months until completion of the study. With these set intervals it is possible that changes in ANCA titer may have been missed as it has been suggested that more frequent ANCA testing is better associated with disease activity prediction (30). Only PR3-ANCA positive patients were analyzed for this study. This was due to the established increased risk of relapse in this population compared to MPO-ANCA positive patients (7, 33, 36–41). A much higher number of MPO-ANCA positive patients would have to be followed in order to study the relationship between ANCA levels and disease relapse in MPO-ANCA positive patients. The current study involved a large set of analyses of the association of changes in ANCA levels determined by different detection methods with various subsequent outcomes, in two treatment groups. Thus, some of the statistically significant findings, both positive and negative, may be due to chance. Prior to incorporating any of these findings into clinical management strategies, the results of this study should be confirmed in an independent cohort using similar techniques.

CONCLUSIONS

A PR3-ANCA decrease by ALBIA at 2 months was associated with decreased time to sustained remission and may be predictive of refractory disease. Measurement of PR3-ANCA by ALBIA at 2 months may help clinicians identify those patients who will not respond to the current therapy. Clinicians should consider changing to an alternative therapy or close monitoring of patients without a decrease in PR3-ANCA by ALBIA at 2 months. ALBIA for PR3-ANCA measurement is comparable to direct ELISA in predicting relapse in PR3-AAV. Therefore, ALBIA which is a widely used ANCA assay in many high-volume laboratories can be used for serial ANCA testing for disease monitoring with no need to change to direct ELISA for disease monitoring. It is important to note without titration, 14% of the increases detected by ALBIA would have been missed. Consequently, titration is recommended when this assay is used for disease monitoring in AAV. Our study has limitations, and the results should be confirmed in a separate cohort. The association of an increase in PR3-ANCA with the risk of subsequent relapse remains complex and is affected by disease phenotype and remission induction agent. Individual patient factors need to be considered when applying this information clinically.

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 Institutional Review Board, Mayo Clinic, Rochester, MN. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GT completed data organization and analysis, writing of manuscript. AH, FS, and MS generated the ANCA data. DS completed statistical analysis, generation of tables. LE, CL, PMe, PMo, PS, RS, ES, JS, and US were involved in the RAVE trial design and completed the clinical data collection. US designed the present project, completed data collection, writing of manuscript. All authors were involved with editing the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.02053/full#supplementary-material>

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B Cell Subsets and Cellular Signatures and Disease Relapse in Lupus Nephritis

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Introduction: Renal relapses adversely affect the long-term outcomes of patients with lupus nephritis (LN), but the pathogenic mechanisms remain elusive. B cell signatures of miR-148a, BACH1, BACH2, and PAX5 expression are relevant to the regulation of B lymphocyte homeostasis. It is unknown whether B cell signature is related to the relapse of LN.

Methods: We compared B lymphocyte subsets and cellular signatures during disease quiescence between LN patients with multiple relapses (MR, ≥ 3 LN relapses within 36 months) and those with no relapse (NR). Also, circulating B lymphocytes were isolated from treatment-naïve patients with active LN and treated with antagomir-148a *in vitro* to investigate the relationship between miR-148a, BACH1, BACH2, and PAX5.

Results: MR patients ($n = 19$), when compared with NR ($n = 14$), showed significantly lower percentage of circulating naïve B cells and higher memory B cell-to-naïve B cell ratio. MR patients also showed higher miR-148a levels in sera and B cells, and lower BACH1, BACH2, and PAX5 expression in naïve and memory B cells. Antagomir-148a upregulated BACH1, BACH2, and PAX5 expression, and reduced B cell proliferation upon stimulation, in naïve and memory B cells isolated from treatment-naïve active LN patients.

Conclusion: Altered B cell subsets and cellular signatures of miR-148a, BACH1, BACH2, and PAX5 may be associated with distinct patient phenotypes related to the risk of LN relapse.

Keywords: B cells, subsets, B cell signatures, lupus nephritis, disease relapse

INTRODUCTION

Lupus nephritis (LN) is a common and severe organ involvement in patients with systemic lupus erythematosus (SLE). Although the use of effective immunosuppressive treatments have markedly improved the clinical outcomes of LN patients (1, 2), disease relapse still constitutes a clinically important issue in the management of LN patients. Repeated LN relapses will lead to

attrition of nephrons and increased cumulative toxicities of immunosuppressive medications, thus jeopardizing the long-term patient and renal outcomes (3–7).

The mechanisms leading to disease relapse in LN remain elusive, which renders the prediction and prevention of relapse clinically challenging. Aberrant lymphocyte response, breach of B cell tolerance, and hyper-reactivity of B lymphocytes all contribute to the pathogenesis of LN (8, 9). Both B and T cells exhibit immunological memory, a property which allows lymphocytes to react efficiently to autoantigens that they have been exposed to previously. Previous studies also demonstrated that memory B cells and plasma cells are less affected by conventional immunosuppressive treatments and hence are more readily reactivated resulting in disease relapse (10, 11). While both B and T lymphocytes serve as crucial effector immune-reactive cells, the B cell repertoire is believed to play an important role in LN relapse, as renal relapse is often correlated with or preceded by, a rise in autoantibodies such as anti-dsDNA. Indeed, perturbations in B lymphocyte subpopulations have been noted in lupus patients during different disease activity states (10, 12).

The B cell repertoire is regulated by various microRNAs and B cell transcription factors. In this context, microRNA-148a (miR-148a) is highly abundant in B cells and plasma cells, and has been shown to control important B cell transcription factors (e.g., BACH2) in promoting plasma cell differentiation and regulating B cell tolerance (13, 14). Upregulation of miR-148a can decrease *Gadd45a*, *Pten*, and *Bcl2l1* expressions, and thereby inhibit the apoptosis of immature B lymphocytes upon B cell receptor engagement (14). Moreover, the maturation and proliferation of B lymphocytes and plasma cells are orchestrated by important B cell transcription factors such as BACH1, BACH2, and PAX5 (15, 16). BACH2, with BACH1 serving as an auxiliary, shows critical functions in various stages of B cell development. BACH2 together with BACH1 suppresses the “myeloid genes” in pre-pro-B cells by binding to their putative regulatory regions, and promotes early B cell development (15). BACH2 also helps to determine B cell subpopulations within germinal centers, and can interact with BCL-6 to inhibit Blimp-1 transcription and thus plasma cell differentiation (17). Previous studies also reported that murine splenic B lymphocytes, in the absence of BACH2, showed increased differentiation into plasma cells via both Blimp-1-dependent and -independent pathways (18). PAX5 is a pivotal regulator in B cell development as the differentiation and functions of all mature B lymphocytes are highly dependent on PAX5 expression. PAX5 directs lymphoid progenitor cells to commit to the B cell lineage, promotes B lymphocytes maturation, and also regulates V(H)-DJ(H) recombination during antibody synthesis (16). Taken together, downregulation of transcription repressors BACH2, BACH1, and PAX5 are instrumental for normal homeostasis of B lymphocytes and plasma cells, and aberrant expression of these transcription factors have been implicated in the development of autoimmune and hematological disorders (15, 16). Furthermore, the homeostasis and function of lymphocytes are also influenced by the cytokine milieu. In this context, BAFF, IL-6, and IL-21 affect B cell survival and differentiation

while IL-2, IL-4, IL-6, IL-10, IL-18, IFN- α , IFN- γ , IL-17, IL-21, and IL-23 can modulate Th1/Th2 and Th17/Treg balance, and elevated levels of these cytokines have been observed in SLE, including patients with LN (19–27). While these B cell signatures and cytokines have important regulatory effects on B lymphocyte biology, their roles and changes in LN relapse have not been fully elucidated.

Based on these backgrounds, we hypothesize that altered B cell subsets and related cellular signatures may be associated with differences in the risk of disease relapse in LN. In this study we examined B lymphocyte subsets, levels of related cytokines, and B cell signatures in LN patients during disease quiescence, and compared two distinct clinical phenotypes characterized by multiple relapses (MR) or no relapse (NR) after initial presentation. We also performed *in vitro* studies with B cells isolated from treatment-naïve active LN patients to investigate the effect of miR-148a inhibition on BACH1, BACH2, and PAX5 expression and cell proliferation.

MATERIALS AND METHODS

Patients

The study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (Approval number: UW 12-389). All experiments in this study followed the general requirements specified by the work safety regulations approved by the University of Hong Kong, which was in accordance with good practices and standards along the lines of CEN15793:2011 and WHO guidelines in biosafety and biosecurity. To compare the lymphocyte subsets, serum cytokines, and B cell signatures in MR and NR patients, blood samples (30 ml) were obtained from biopsy-proven Class III/IV \pm V LN patients with the following inclusion criteria: (1) patients who had multiple relapses (defined as ≥ 3 LN relapses within 36 months, unrelated to treatment non-compliance) (MR group) or those with no relapse (defined as never relapsed after the first episode of nephritis) (NR group); and (2) patients with quiescent disease (SLEDAI score < 4 with no points in the renal domain), and on a stable dose of prednisolone (5–7.5 mg/day for ≥ 4 months) alone or in combination with mycophenolate (1–1.5 g/day) or azathioprine (50–100 mg/day) as maintenance treatment. LN relapse was defined as proteinuria > 1 g/day, presence of urinary red blood cells (RBC) > 30 /hpf or RBC casts, a 15% increase in serum creatinine compared with baseline, and anti-dsDNA level > 30 IU/ml. LN relapse was confirmed with a kidney biopsy. Exclusion criteria were: (1) patients who received calcineurin inhibitors or mammalian target of rapamycin inhibitors as maintenance immunosuppression, or biologics (e.g., rituximab, belimumab, or abatacept) in the preceding 12 months; (2) patients who relapsed due to treatment non-compliance.

For the antagomir studies, we obtained blood samples (30 ml) from treatment-naïve patients with biopsy-proven Class III/IV \pm V LN and active renal disease (denoted by proteinuria > 1 g/day, presence of urinary RBC > 30 /hpf or RBC casts, a

15% increase in serum creatinine compared with baseline, and anti-dsDNA level >30 IU/ml). Blood samples (30 ml) were also obtained from healthy subjects as control.

Analysis of Lymphocyte Subsets

Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep™, then washed with PBS and resuspended in 1 ml RPMI 1640 medium supplemented with 10% FCS, 10% DMSO, and 1% penicillin/streptomycin. PBMC were washed with PBS and stained with Zombie NIR fixable viability dye (diluted 1:100) for 30 min at room temperature. Non-specific Fc binding was blocked with Human TruStain FcX solution for 15 min on ice. For analysis of B lymphocyte subsets, cells were stained with monoclonal antibodies against human CD20 (1:40), together with CD27 (1:40), and CD138 (1:5) followed by incubation on ice for 30 min. For the assessment of T lymphocyte subsets, aliquots of PBMC were activated with phorbol myristate acetate (PMA, 50 ng/ml) and calcium ionophore (0.5 µg/ml) for 4 h in a 37°C tissue culture incubator. Brefeldin A (5 µg/ml) was added to samples during the last 3 h of incubation with PMA and calcium ionophore. PBMC were washed with staining buffer (PBS with 5% EDTA and 0.5% BSA) and stained with Zombie NIR fixable viability dye (diluted 1:100) for 30 min at room temperature. Non-specific Fc binding was blocked as above. Cells were incubated with monoclonal antibodies against CD25 (1:40) for 30 min on ice, then fixed and permeabilized. Cells were then labeled with monoclonal antibodies against CD3 (1:40) and/or CD4 (1:20), CD8 (1:40), IFN-γ (1:200), IL-4 (1:250), IL-17 (1:10), and FoxP3 (1:40) for 50 min on ice. Stained lymphocytes were washed twice with staining buffer prior to analysis using a BD LSRFortessa™ flow cytometer (BD Biosciences) and 50,000 cells counted. Data were analyzed using FlowJo software Version 10 (Tree Star Inc., Ashland, OR, United States). The percentage of naïve B cells (CD20⁺CD27⁻), memory B cells (CD20⁺CD27⁺), plasma cells (CD20⁻CD27⁺CD138⁺), cytotoxic T cells (CD3⁺CD8⁺), Th1 cells (CD3⁺CD4⁺IFN-γ⁺), Th2 cells (CD3⁺CD4⁺IL-4⁺), Th17 cells (CD3⁺CD4⁺IL-17⁺), and Treg (CD3⁺CD4⁺CD25⁺FOXP3⁺) were analyzed and compared between MR and NR patients.

Measurements of Serum Cytokine Levels

Serum levels of cytokines were determined by commercially available ELISA kits according to manufacturers' instructions (BAFF, IL-10, and IL-18 by Quantikine™ ELISA kits, R&D Systems Inc., Bio-Techne H.K. Limited, Hong Kong; IL-2, IL-4, IL-6, IL-17, and IFN-γ by MiniABTS ELISA Development kits, PeproTech, Dakewe BioTech (H.K.), Hong Kong; IL-21 and IL-23 by Ready-Set-Go!™ ELISA kit, ThermoFisher Scientific, Life Technologies Limited, Hong Kong; and IFN-α by ELISA^{PRO} kit, Mabtech, Dakewe BioTech (H.K.), Hong Kong). The detection limits for BAFF, IL-2, IL-4, IL-6, IL-10, IL-17, IL-18, IL-21, IL-23, IFN-α, and IFN-γ were 62.5–4,000 pg/ml, 62.5–4,000 pg/ml, 15.6–1,000 pg/ml, 23.0–1,500 pg/ml, 0.78–50 pg/ml, 15.6–1,000 pg/ml, 25.6–1,000 pg/ml, 8.0–1,000 pg/ml, 15.0–2,000 pg/ml, 3.16–316 pg/ml, and 23.0–1,500 pg/ml respectively.

Isolation of Memory and Naïve B Cells for *in vitro* Studies

PBMC were first separated from whole blood (30 ml) obtained from LN patients or healthy subjects using Lymphoprep™, and naïve and memory B cells were further isolated by the EasySep Human Memory B Cell Isolation Kit (STEMCELL™ Technologies, Lokco Technology Limited, Hong Kong) according to manufacturer's instructions using 1×10^8 cells/ml in a 1 ml suspension. The purity of isolated B cells was 96.7–98.0% as confirmed by flow cytometry.

In vitro Antagomir-148a Studies

To determine the effect of miR-148a on cell proliferation and BACH1, BACH2, and PAX5 gene expression, isolated B cells from treatment-naïve LN patients with active disease were plated at a density of 10^5 cell/ml in 96-well plates (100 µl/well) to assess changes in cell proliferation or in 24-well plate (1 ml/well) for total RNA extraction. For cell proliferation studies, B lymphocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE, 5 µM in PBS final concentration) for 30 min prior to stimulation. B lymphocytes were pre-incubated with or without, scrambled antagomir (SCr-antagomir, as control) (1 µM) or antagomir-148a (1 µM) for 2 h, and then stimulated with CpG (2.5 µg/ml) in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin for up to 3 days (28). Synthetic antagomirs (2'-O-methyl RNA oligo) targeting miR-148a (sequence: 5-mA(*)mC(*)mAmAmGmUmUmCmUmGmUmAmGmUmGmCmAmC(*)mU(*)mG(*)mA(*)-3-Chol) and scramble control (sequence: 5-mU(*)mC(*)mAmCmGmCmA mGmAmUmUmCmAmUmAmA(*)mC(*)mG(*)mU(*)-3-Chol) were custom-synthesized by Applied Biological Materials Inc. (Lokco Technology Limited, Hong Kong). All ribonucleotides are 2'-O-methyl modified (mN) and (*) represent a phosphorothioate modification of the backbone. At the 3'-end of the oligonucleotides, a cholesterol molecule was added. Cell proliferation was assessed every 24 h for up to 72 h, and total RNA was extracted after 3 days of stimulation. The cell proliferation of naïve and memory B cells was determined by CFSE signals using BD LSRFortessa™ flow cytometer (BD Bioscience) (Figure 1), and expressed as a percentage of total cells counted (50,000 cells).

Determination of miR-148a, BACH1, BACH2, and PAX5 in Sera and B Cells

Total RNA was extracted from B lymphocytes isolated from LN patients and healthy controls, and also from cultured B cells following incubation with antagomir-148a as stated above. microRNAs were extracted from sera using the miRVANA™ PARIS™ kit (Applied Biosystems, Life Technologies Limited, Hong Kong) according to the manufacturer's instructions. The levels of miR-148a, BACH1, BACH2, and PAX5 were determined by quantitative qPCR using standard methods. miR-148a expression was normalized to U6 expression (both measured by Taqman miRNA assay; Assay 000470 for miR-148a and Assay 001973 for U6). BACH1, BACH2, and PAX5 expressions were normalized to GAPDH expression. The primer

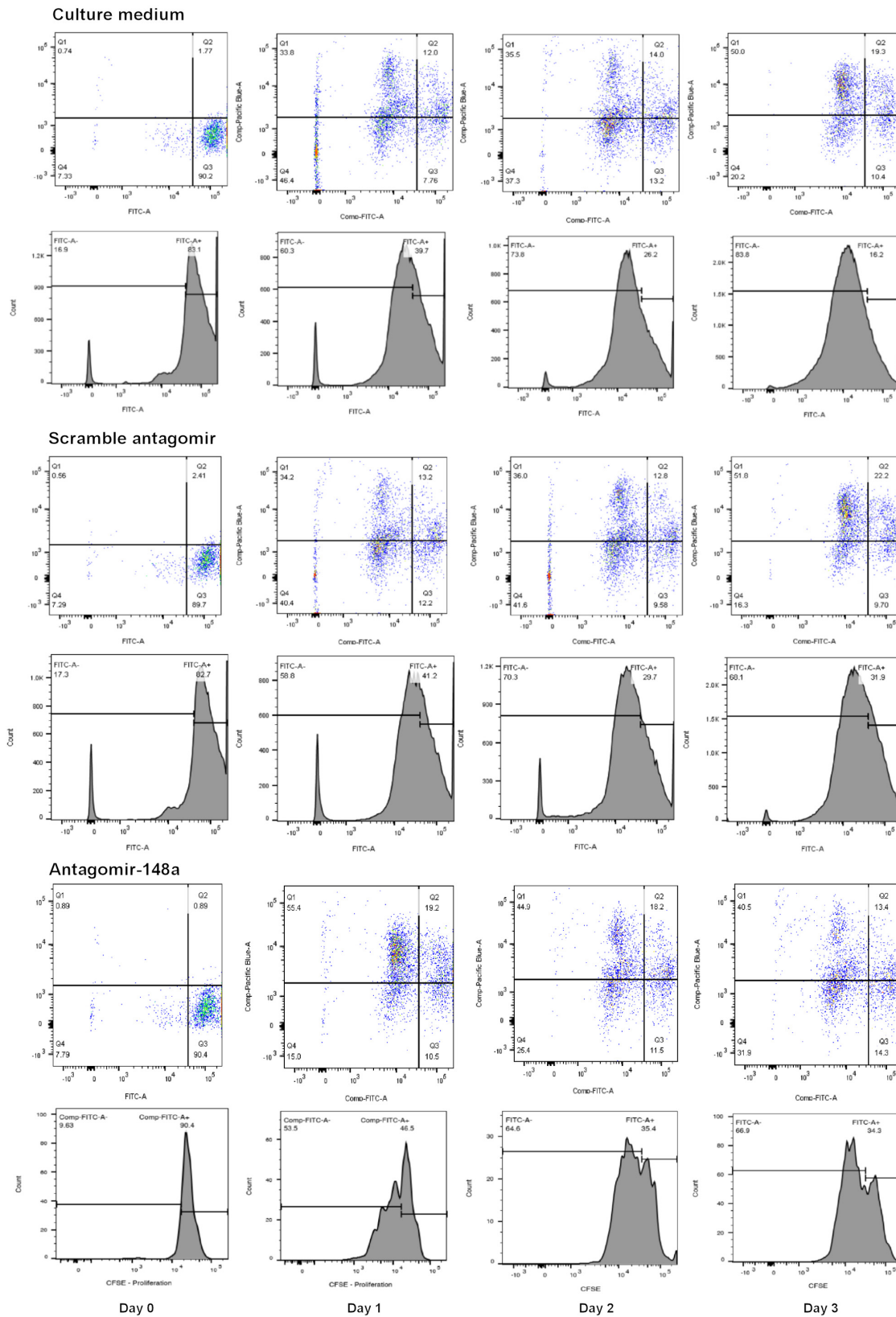


FIGURE 1 | A representative flow cytometric analysis of the CFSE proliferation assay for B cells treated with scramble antagonist or antagomir-148a.

sequences of BACH1, BACH2, PAX5, and GAPDH were listed as follows:

Gene	Direction	Sequence (5' to 3')
BACH1	Forward	5'-TAG TGT GGA GCG AGA AGT GG-3'
	Reverse	5'-ACC TAA CCA CGG ACA CTC AG-3'
BACH2	Forward	5'-CCA GCA ATG ACT CAG GCA TC
	Reverse	5'-TCA TGA GTC TTG TCG CTG GT-3'
PAX5	Forward	5'-GGG TGG AGT GGG AGA AAT CA-3'
	Reverse	5'-CCA TGT TCT CTG GTT CCC CT-3'
GAPDH	Forward	5'-TGA CCT TTC TGT AGC TGG GG-3'
	Reverse	5'-CAA GCC CAC CCC TTG TCT AA-3'

Statistical Analysis

Categorical variables were expressed as frequencies (percentages), and analyzed by the Chi-square or Fisher-Exact test where appropriate. Continuous variables were expressed as mean \pm SD or median (range), and compared by the Mann-Whitney test. Inter-group comparisons were analyzed by ANOVA, followed by a *post hoc* Dunnett's test. Associations between different clinical and immunological parameters were assessed by Spearman rank's correlation coefficient. All statistical analyses were performed using a Graphpad Prism 7.0 (San Diego, CA, United States) and *p* values of less than 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

Circulating lymphocyte subsets, serum cytokine levels, and B cell signatures were analyzed in 33 LN patients (19 patients in the MR group and 14 patients in the NR group, respectively) during disease quiescence. Patient characteristics are presented in **Table 1**.

Lymphocyte Subset Profiles in Lupus Nephritis Patients With Multiple Relapses or Those With No Relapse

Circulating naïve B cells were significantly lower in MR patients (median 0.7%, range 0.1–14.1%) compared with NR patients (median 4.0%, range 0.4–24.6%) ($p = 0.017$) (**Table 2**). The percentage of circulating memory B cells and plasma cells were comparable between the two groups. MR patients showed significantly higher memory-to-naïve B cell ratio [median (range): 0.8 (0.1–9.0), vs 0.2 (0.1–2.0) in NR patients, $p = 0.024$], and also numerically higher plasma cell-to-naïve B cell ratio [median (range): 0.3 (0.1–7.5), vs 0.1 (0.1–2.7) in NR patients, $p = 0.098$]. The percentage of circulating T cell subsets were similar between both groups ($p > 0.05$, for all) (**Table 2**).

Relationship Between Lymphocyte Subsets, and Anti-dsDNA Antibodies and C3 Levels in Lupus Nephritis Patients With Multiple Relapses or Those With No Relapse

Lymphocyte subsets showed no relationship with serum anti-dsDNA antibodies and C3 levels during disease quiescence in MR and NR patients ($p > 0.05$, for all).

Serum Cytokine Profiles in Lupus Nephritis Patients With Multiple Relapses or Those With No Relapse

The MR and NR patients did not differ in their serum levels of BAFF, IL-2, IL-4, IL-6, IL-10, IL-17, IL-18, IL-21, IL-23, IFN- α , and IFN- γ during disease quiescence ($p > 0.05$, for all) (**Table 2**).

Serum miR-148a Expression in Lupus Nephritis Patients With Multiple Relapses or Those With No Relapse

The MR group showed significantly higher serum miR-148a expression than the NR group (1.0 ± 0.0 , 0.7 ± 0.2 , and 9.4 ± 6.9 fold difference for healthy controls (HC) and NR and MR patients respectively; $p < 0.001$, MR vs NR or HC).

miR-148a, BACH1, BACH2, and PAX5 Expression in Naïve B Cells From Lupus Nephritis Patients With Multiple Relapses or Those With No Relapse

The expression of miR-148a, BACH1, BACH2, and PAX5 was determined in naïve B cells isolated from MR and NR patients. The miR-148a expression was significantly higher in the naïve B cells from MR patients compared with NR patients (1.0 ± 0.0 , 0.7 ± 0.3 , and 5.8 ± 2.4 fold difference for HC, NR, and MR respectively; $p < 0.001$, MR vs NR or HC) (**Figure 2A**). Naïve B cells from MR patients showed lower BACH1 (1.0 ± 0.0 , 2.9 ± 0.8 , and 0.8 ± 0.3 fold difference for HC, NR, and MR respectively), BACH2 (1.0 ± 0.0 , 3.4 ± 0.9 , and 0.9 ± 0.3 fold difference for HC, NR, and MR respectively), and PAX5 (1.0 ± 0.0 , 2.7 ± 0.8 , and 1.0 ± 0.8 fold difference for HC, NR, and MR respectively) expression compared with NR patients ($p < 0.001$, MR vs NR, for all) (**Figure 2C**).

miR-148a, BACH1, BACH2, and PAX5 Expression in Memory B Cells From Lupus Nephritis Patients With Multiple Relapses or Those With No Relapse

The expression of miR-148a, BACH1, BACH2, and PAX5 was also determined in memory B cells from MR and NR patients. Memory B cells from MR patients showed significantly higher miRNA-148a expression than NR patients and HC (1.0 ± 0.0 , 0.8 ± 0.3 , and 5.8 ± 1.7 fold difference for HC, NR, and MR respectively; $p < 0.001$, MR vs NR or HC) (**Figure 2B**).

TABLE 1 | Clinical characteristics of lupus nephritis patients with multiple relapses or those with no relapse and those with active nephritis.

	MR (n = 19)	NR (n = 14)	Active LN (n = 8)	p value [†]
Sex (F/M)	16/3	14/0	5/3	0.244
Age (years)	48.0 ± 6.3	52.1 ± 9.6	38.0 ± 19.5	0.240
Class of LN				
III	4	6	0	0.260
IV	5	7	6	0.270
III + V	4	0	1	0.120
IV + V	6	1	1	0.090
Duration of follow-up from last nephritic episodes (months)	81.8 ± 46.7	110.9 ± 21.3	25.4 ± 17.7	0.091
Maintenance treatment				
PRED alone (n, %)	4 (21.0%)	6 (42.8%)	2 (25.0%)	0.257
PRED + MMF (n, %)	11 (57.9%)	4 (28.6%)	6 (75.0%)	0.158
PRED + AZA (n, %)	4 (21.1%)	4 (28.6%)	0 (0%)	0.442
Clinical parameters				
White cell count (×10 ⁹ /ml)	5.4 ± 1.9	4.9 ± 1.6	5.2 ± 2.4	0.480
Lymphocyte count (×10 ⁹ /ml)	1.3 ± 0.5	1.4 ± 0.7	0.9 ± 0.4	0.951
Serum C3 (mg/dl)	94.9 ± 31.7	80.0 ± 19.9	43.5 ± 22.8	0.329
Anti-dsDNA (IU/ml)	34.8 ± 38.8	23.9 ± 21.5	245.5 ± 101.4	0.910
eGFR (ml/min/1.73 m ²)	59.4 ± 27.9	74.3 ± 21.2	61.3 ± 31.2	0.107
Serum albumin (g/l)	39.8 ± 5.5	43.1 ± 2.1	27.4 ± 5.8	0.055
Urine protein excretion (g/D)	0.28 ± 0.38	0.06 ± 0.11	3.2 ± 2.8	0.238

AZA, azathioprine; eGFR, estimated glomerular filtration rate; MMF, mycophenolate mofetil; MR, multiple relapses; NR, no relapse; PRED, prednisolone. Categorical variables were expressed as frequency (percentage), and analyzed by the Chi square test. Continuous variables were expressed as mean ± SD, and analyzed with the Mann-Whitney test. [†]Comparison between MR and NR.

TABLE 2 | Circulating lymphocyte subset and cytokine profiles in lupus nephritis patients with multiple relapses or those with no relapse.

Circulating lymphocyte subsets	MR (n = 19)	NR (n = 14)	p value
B lymphocyte subsets			
Naïve B cell (%)	0.7% (0.1–14.1%)	4.0% (0.4–24.6%)	0.017
Memory B cell (%)	0.6% (0.2–6.3%)	1.0% (0.1–3.2%)	0.123
Plasma cells (%)	0.2% (0.1–1.1%)	0.3% (0.1–2.9%)	0.382
Memory B/Naïve B ratio	0.8 (0.1–9.0)	0.2 (0.1–2.0)	0.024
Plasma cell/Naïve B cell ratio	0.3 (0.1–7.5)	0.1 (0.1–2.7)	0.098
T lymphocyte subsets			
CD8 + cytotoxic T cells (%)	25.8% (5.2–52.7%)	23.8% (8.2–48.9%)	0.808
Th1 cells (%)	3.3% (0.2–11.2%)	3.8% (0.1–6.4%)	1.000
Th2 cells (%)	3.9% (0.1–31.6%)	11.3% (0.2–47.7%)	0.087
Th17 cells (%)	0.3% (0.1–3.3%)	0.4% (0.1–2.2%)	0.545
Treg (%)	0.7% (0.1–3.0%)	0.7% (0.1–12.2%)	0.662
Serum cytokine levels			
BAFF (pg/ml)	1534.8 ± 674.3	1989.5 ± 2442.8	0.465
IL-6 (pg/ml)	445.1 ± 525.2	545.7 ± 740.6	0.708
IL-21 (pg/ml)	16.5 ± 22.7	19.2 ± 23.3	0.796
IFN-α (pg/ml)	29.4 ± 17.2	35.7 ± 27.7	0.781
IFN-γ (pg/ml)	125.8 ± 190.8	95.3 ± 106.1	0.679
IL-2 (pg/ml)	3240.4 ± 3786.5	7050.4 ± 11487.4	0.464
IL-4 (pg/ml)	36.4 ± 10.6	39.5 ± 19.2	0.760
IL-10 (pg/ml)	8.5 ± 7.9	32.5 ± 83.1	0.707
IL-17 (pg/ml)	232.7 ± 246.2	238.5 ± 301.9	0.679
IL-18 (pg/ml)	230.3 ± 127.1	238.0 ± 166.8	0.674
IL-23 (pg/ml)	13.3 ± 3.4	25.3 ± 41.1	0.947

MR, multiple relapses; NR, no relapse; Th, T helper cells; Treg, regulatory T cells; BAFF, B cell activating factor; IFN, interferon; IL, interleukin. Results expressed as median (range) or mean ± SD, and analyzed with the Mann-Whitney test.

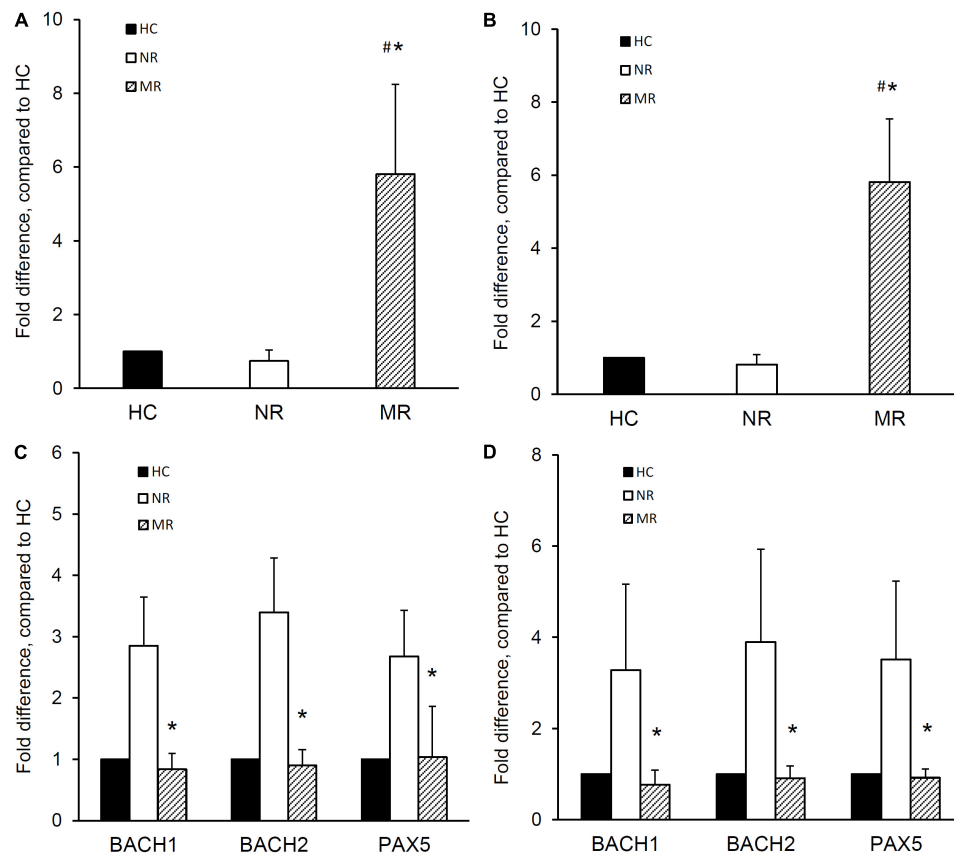


FIGURE 2 | miR-148a, BACH1, BACH2, and PAX5 expression in naïve and memory B cells isolated from lupus nephritis patients with multiple relapses or those with no relapse. Naïve and memory B cells were isolated from lupus nephritis patients with multiple relapses (MR, \square) ($n = 19$) or those with no relapse (NR, \square) ($n = 14$). miR-148a and BACH1, BACH2, and PAX5 expressions were determined by qPCR. miR-148a expression in panel (A) naïve B cells and (B) memory B cells was normalized to U6 expression and BACH1, BACH2, and PAX5 expressions in panel (C) naïve B cells and (D) memory B cells were normalized to GAPDH expression, and expressed as fold difference compared to healthy controls (HC, \blacksquare) ($n = 10$). Data expressed as mean \pm SD and analyzed by ANOVA followed by a *post hoc* Dunnett's test. * $p < 0.001$, MR vs NR; # $p < 0.001$, MR vs HC.

Memory B cells from MR patients also showed significantly lower BACH1 expression than NR patients (1.0 ± 0.0 , 3.3 ± 1.9 , and 0.8 ± 0.3 fold difference for HC, NR, and MR respectively; $p < 0.001$, MR vs NR). BACH2 and PAX5 expression was also lower in MR patients compared to NR patients (BACH2: 1.0 ± 0.0 , 3.9 ± 2.0 , and 0.9 ± 0.3 fold difference; PAX5: 1.0 ± 0.0 , 3.5 ± 1.7 , and 0.9 ± 0.2 fold difference for HC, NR, and MR respectively; $p < 0.001$, MR compared with NR, for both) (Figure 2D).

Effect of Antagomir-148a Treatment on BACH1, BACH2, and PAX5 Expression and Cell Proliferation in Naïve and Memory B Cells

The effect of miR-148a on the BACH1, BACH2, and PAX5 expression and cell proliferation was next investigated in naïve and memory B cells isolated from eight treatment-naïve active LN patients (Table 1).

The incubation of naïve B cells with antagomir-148a mitigated miR-148a expression after 24 h, and this inhibition was sustained

for 3 days ($p < 0.001$, antagomir-148a vs Scr-antagomir or no treatment) (Figure 3A). After 3 days, the inhibition of miR-148a expression was accompanied by a 3.7 ± 0.3 , 5.6 ± 2.6 , and 3.3 ± 1.1 fold increase in BACH1, BACH2, and PAX5 expression respectively compared to control cells ($p < 0.01$ for all, antagomir-148a vs Scr-antagomir) (Figure 3C). Incubation of naïve B cells with antagomir-148a for 3 days significantly suppressed cell proliferation compared to cells incubated with Scr-antagomir ($4.5 \pm 2.8\%$ vs $16.3 \pm 8.0\%$, antagomir-148a vs Scr-antagomir, $p < 0.01$) (Figure 3E).

Similarly, incubation of memory B cells with antagomir-148a abrogated miR-148a expression after 24 h, and the inhibition was sustained for 3 days (Figure 3B). miRNA-148a inhibition was accompanied by a 3.8 ± 0.4 , 4.5 ± 1.4 , and 4.1 ± 1.3 fold increase in BACH1, BACH2, and PAX5 expression respectively compared to control cells ($p < 0.01$ for all, antagomir-148a vs Scr-antagomir) (Figure 3D). Inhibition of miR-148a expression significantly reduced cell proliferation compared to control cells ($31.7 \pm 6.2\%$ vs $57.4 \pm 3.4\%$, antagomir-148a vs Scr-antagomir, $p < 0.001$) (Figure 3F).

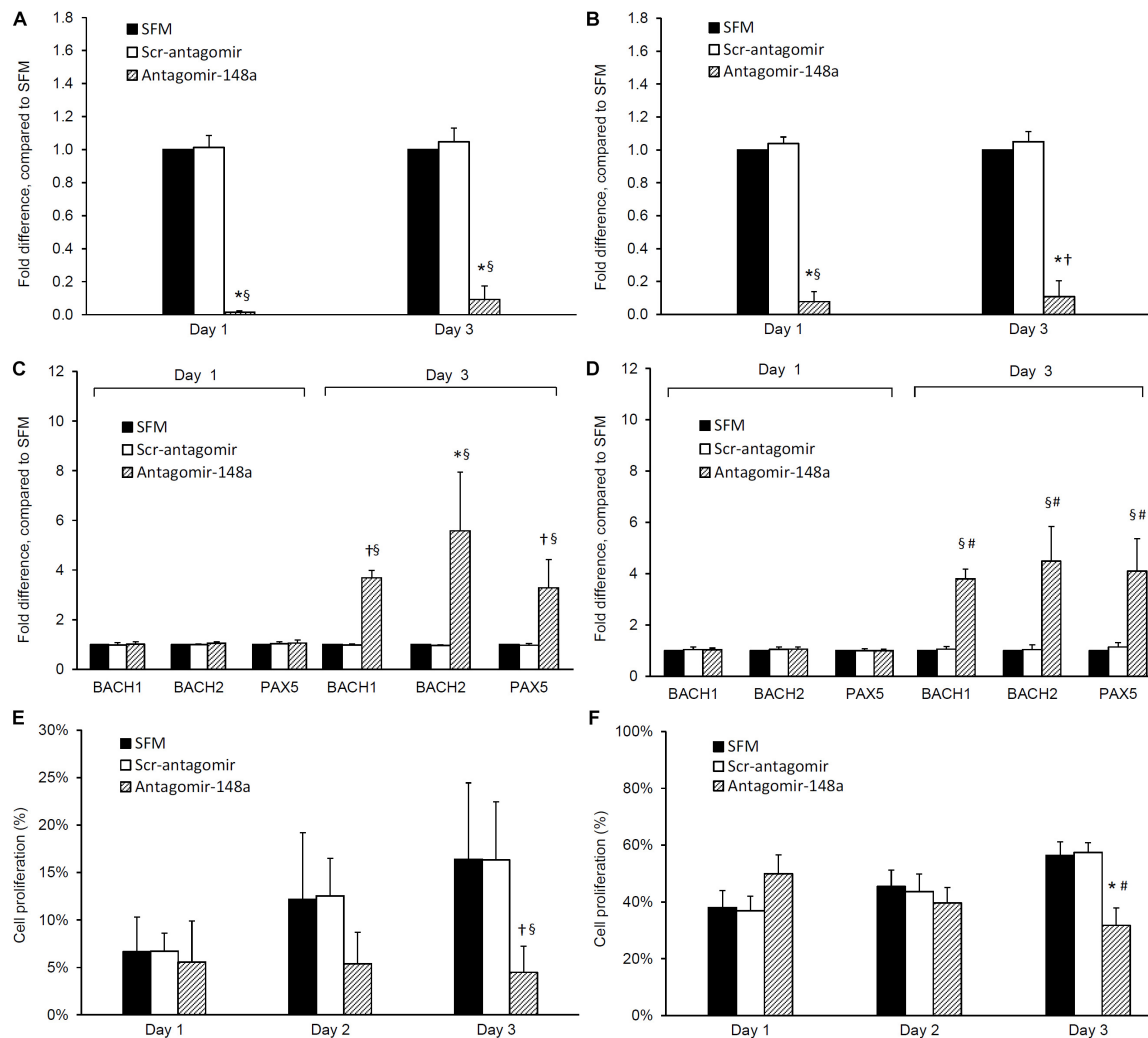


FIGURE 3 | Effect of antagomir-148a on miRNA-148a, BACH1, BACH2, and PAX5 expression and cell proliferation in naïve and memory B cells from treatment-naïve active lupus nephritis patients. Naïve and memory B cells were isolated from eight treatment-naïve active lupus nephritis patients and cultured with serum free medium (SFM) (■) in the presence or absence of scrambled antagomir sequence (Scr-antagomir) (□) or antagomir-148a (▨) for 2 h. Naïve and memory B cells were then stimulated with CpG (in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin) for 3 days, total RNA extracted and miR-148a, BACH1, BACH2, and PAX5 expressions determined by qPCR. miR-148a expression in panels (A) naïve and (B) memory B cells were normalized to U6 expression, and BACH1, BACH2, and PAX5 expression in panels (C) naïve B cells and (D) memory B cells were normalized to GAPDH expression respectively, and expressed as fold difference compared to SFM. Cell proliferation in panels (E) naïve B cells and (F) memory B cells was determined by CFSE proliferation assay after 1, 2, and 3 days. Data expressed as mean \pm SD and analyzed by ANOVA followed by a *post hoc* Dunnett's test. * $p < 0.001$, § $p < 0.05$, antagomir-148a vs Scr-Antagomir; # $p < 0.001$, † $p < 0.05$ antagomir-148a vs SFM.

DISCUSSION

The B cell repertoire has crucial pathogenic roles in LN which include synthesis of autoantibodies, presentation of autoantigens, and secretion of pro-inflammatory and anti-inflammatory mediators that regulate the immune response (29–36). Accumulating evidence suggests that SLE is associated with perturbations in B lymphocyte subpopulations, epitomized by an expansion of class-switched memory B cells relative to naïve cells in active lupus patients compared to patients during disease quiescence (10, 37, 38). Changes in B cell subtypes pertaining to LN relapse, however, remain to be characterized.

Our current data revealed that, during disease remission, MR patients exhibited a higher memory-to-naïve B cell ratio and decreased circulating naïve B cells compared with NR patients. Alterations in the B cell subset profile in MR patients may be related to repeated exposure to autoantigens, which stimulates naïve B cells to differentiate into more mature B cell subtypes. Memory B cells have multiple properties pertinent to disease relapse in SLE and LN patients. B lymphocytes which differentiate into memory B cells typically remain dormant and are therefore less susceptible to conventional induction agents which show cell-cycle dependent anti-proliferative effects (38, 39). These memory B cells, nevertheless, possess long-lasting immunological

memory and can generate potent and efficient immune response following stimulation with previously encountered antigens (39). Memory B cells from lupus patients also show reduced FcγRIIb expression, which leads to an augmented influx of calcium ion and diminished inhibitory signals for memory B cell activation (10, 40).

Whilst plasma cells are the key pathogenic cells responsible for autoantibody production in LN (10, 38), we did not find any significant difference in the number of circulating plasma cells between MR and NR patients. Detecting a difference in circulating plasma cells can be difficult because plasma cells predominantly reside in the bone marrow and are only present in very low frequencies in the circulation. Emerging data suggests the pathogenic roles of T lymphocytes in LN, with the CD4⁺ T helper subset being a major driver for B cell differentiation. Aberrant signaling, proliferation, and cytokine production have been observed in T cells from SLE patients (25, 41–45). Here we detect no significant difference in the T cell subsets profile between MR and NR patients, and it remains possible that functional rather than quantitative differences in T lymphocytes occur in MR and NR patients although such postulations need verification in further studies.

The homeostasis and immunological functions of B and T lymphocytes are regulated by various pro-inflammatory and anti-inflammatory cytokines such as BAFF, IL-2, IL-4, IL-6, IL-10, IL-17, IL-18, IL-21, IL-23, IFN-α, and IFN-γ (19–27). In this study, we also measured the levels of these cytokines to investigate whether their changes could affect the B cell subset profiles in MR and NR patients. We did not detect any change in the serum levels of these cytokines, and this may be attributed to the time of sample collection, that is, during disease remission.

miR-148a plays an instrumental role in B cell development, and its increased expression has been observed in B and T lymphocytes and also tissue pathologies in SLE patients and murine lupus models (46–49). Upregulated miR-148a expression renders B lymphocytes more resilient to B cell receptor-induced apoptosis, resulting in improved survival of immature B cells (14). BACH1 and BACH2 are essential transcription factors in B lymphocytes that are regulated by miR-148a, and together they exert suppressive effects on the maturation and homeostasis of B lymphocytes (13–15, 17, 18). PAX5 has also been reported to inhibit the development and proliferation of B lymphocytes but its relationship with miR-148a remains poorly understood (16). Based on our findings in the B cell subset profile, we next evaluated the blood miR-148a level and its expressions in naïve and memory B cells, and also investigated the effect of miR-148a on BACH1, BACH2, and PAX5 expression in B lymphocytes. Our present data showed that, even during disease remission, MR patients had a significantly higher serum miR-148a level compared to NR patients. MR patients also exhibited higher miR-148a expression in naïve and memory B cells, and this was accompanied by lower BACH1, BACH2, and PAX5 expressions in B lymphocytes. To further elucidate the effect of miR-148a on BACH1, BACH2, and PAX5 expression and cell proliferation in naïve and memory B cells, we treated B cells isolated from active LN patients with antagomir-148a, an oligonucleotide that inhibits miR-148a expression. Inhibition of miR-148a resulted in an upregulation of BACH1, BACH2, and

PAX5 expressed and decreased cell proliferation in naïve and memory B cells, suggesting that miR-148a exerts an inhibitory effect on transcription factors which negatively modulates B lymphocyte differentiation and proliferation.

It has been shown that increased miR-148a expression can downregulate *Gadd45a*, *Bim*, and *PTEN* and inhibit apoptosis of immature B cells, thus leading to enhanced B cell autoreactivity (14). Reinstitution of miR-148a-transfected hematopoietic stem cells to lethally irradiated MRL/lpr mice restored lupus phenotypes and decreased the survival rates compared to control mice (14). Other investigators have demonstrated that miR-148a could downregulate BACH2 expression in B lymphocytes isolated from C57BL/6 mice, resulting in enhanced differentiation and survival of plasma cells (13). Over-expression of BACH2 would inhibit “myeloid genes” in pre- and pro-B cells, and thus divert them from committing to the lymphoid lineage (15). BACH2 can also interact with BCL-6 to suppress Blimp-1 expression during germinal center reaction, leading to reduced plasma cell differentiation (17). Independent researchers also reported that mouse splenic B cells deficient in BACH2 showed a more ready differentiation into plasma cells (18). Diminished BACH2 expression was observed in B lymphocytes from lupus patients, and transfection of BACH2 into these B cells resulted in increased apoptosis and suppressed proliferation (50). Furthermore, one recent GWAS meta-analysis also identified BACH2 as a susceptibility locus in Chinese lupus patients (51).

Data regarding the relationship between miR-148a and PAX5 expression is limited. Our results suggest that inhibition of miR-148a will upregulate PAX5 expression and suppresses B cell proliferation in LN patients. PAX5 is a master regulator in the development and function of B lymphocytes. During early B lymphocyte development, PAX5 can direct lymphoid progenitors to commit a B cell destiny by repressing “B-lineage-inappropriate” genes and simultaneously activating B-cell-specific genes (16). PAX5 can also induce V(H)-DJ(H) recombination during immunoglobulin production to enhance the antibody repertoire (16). In this study, we did not explore the interaction between BACH1, BACH2, and PAX5 in B lymphocytes. Notwithstanding, our findings suggest that elevated miR-148a expression in B lymphocytes from MR patients may decrease expression of BACH1, BACH2, and PAX5 and hence promote their differentiation into more mature B cell subpopulations. Such alterations in cellular signatures may account for the increased memory-to-naïve B cell ratio and decreased circulating naïve B cells as observed in MR patients, which thereby may increase propensity for disease relapse in LN patients. The mechanisms by which miR-148a downregulates BACH1, BACH2, and PAX5 expression remains obscure and putative mechanisms include base pairing to sequence motifs in the 3′UTR of mRNA with exact or closely analogous complementarities or epigenetic modifications of downstream target genes (46, 52, 53).

While it is important to conduct *in vivo* studies to verify our current *in vitro* findings, one should appreciate that the commonly used murine lupus models are not optimal for investigating disease relapse. Due to the limited clinical samples, we only measured the level of BACH1, BACH2, and PAX5

transcripts but not the protein levels, which remains an important shortcoming of this study. Another limitation of our study was that we did not examine the other immune-reactive cells and the complement cascades which are also highly relevant in LN pathogenesis. Also, clinical materials were only obtained during disease remission and were not compared with samples collected during active LN flares. Nevertheless, one should appreciate that patients in the MR and NR groups were carefully matched for patient characteristics and background immunosuppressive therapies, and thus the observed differences in B cell subsets and related cellular signatures is more likely due to intrinsic immunological abnormalities rather than patient heterogeneity and effect of treatments.

CONCLUSION

The results suggest that altered B cell subsets and cellular signatures of miR-148a, BACH1, BACH2, and PAX5 are associated with distinct phenotypes related to the risk of renal relapse in patients with LN.

DATA AVAILABILITY STATEMENT

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

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ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (Approval number: UW 12-389). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DY, SY, and TC contributed to conception and conduct of the study, analysis of data and preparation of the manuscript. PL, IY, CTam, and CTang contributed to technical support and analysis of data. All authors critically revised and approved the final version of the manuscript and agreed to be accountable for all aspects of ensuring the accuracy and integrity of the work.

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Idiopathic Membranous Nephropathy: Glomerular Pathological Pattern Caused by Extrarenal Immunity Activity

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Idiopathic membranous nephropathy (IMN) is a pathological pattern of glomerular damage caused by an autoimmune response. Immune complex deposition, thickness of glomerular basement membrane, and changes in the podocyte morphology are responsible for the development of proteinuria, which is caused by the targeted binding of auto-antibodies to podocytes. Several auto-antigens have recently been identified in IMN, including M-type receptor for secretory phospholipase A2 (PLA2R1), thrombospondin type-1 domain-containing 7A (THSD7A), and neural epidermal growth factor-like 1 protein (NELL-1). The measurement of peripheral circulating antibodies has become an important clinical reference index. However, some clinical features of IMN remain elusive and need to be further investigated, such as the autoimmunity initiation, IgG4 predominance, spontaneous remission, and the unique glomerular lesion. As these unresolved issues are closely related to clinical practice, we have proposed a hypothetical pathogenesis model of IMN. Induced by environmental stimuli or other causes, the PLA2R1 antigen and/or THSD7A antigen exposed to extrarenal tissues, such as lungs, then produce the auto-antibodies that target and cause damage to the podocytes in circulation. In this review, we highlighted the potential association between environmental stimuli, immune activity, and glomerular lesions, the underlying basis for spontaneous immune and proteinuria remission.

Keywords: autoimmune response, inflammation, pathogenesis model, podocyte, spontaneous remission, environmental stimuli

INTRODUCTION

Membranous nephropathy (MN) is one of the major glomerular diseases in adults, accounting for 20–30% of glomerular disease cases (1, 2). MN is pathologically characterized by the accumulation of immune complex deposits outside the glomerular basement membrane (GBM), which is adjacent to podocytes. The thickening GBM appears to have a “spike” to enfold the deposits (3). Immune complex deposits contain the antigens *in situ*, immunoglobulins (Ig) G binding to antigens, and membrane attack complexes (MAC) formed by complement activation, which are the traces left by the antibody-dependent immune response and the major basis for the current

understanding of the pathogenesis of MN (4). This unique glomerular lesion is thought to be associated with many causes. According to patient histories and clinical manifestations, about 20% of MN can be attributed to clinical diseases, such as hepatitis B infection, systemic lupus erythematosus, cancer, or drug side-effects, which are known as secondary MN. In addition, about 80% of MN are clinically unable to be identified by the secondary factors, which are known as idiopathic or primary MN (IMN or PMN) (4). IMN is thought to be caused by IgG that target podocytes; the predominant form of IgG is considered to be IgG4 (5, 6). The podocyte auto-antigens identified in adult IMN include M-type receptor for secretory phospholipase A2 (PLA2R1) (7), thrombospondin type-1 domain-containing 7A (THSD7A) (8), and neural epidermal growth factor-like 1 protein (NELL-1) (9), which accounts for 70–80, 3–5, and 5–10% of IMN, respectively. Moreover, the measurement of antibodies against PLA2R1 (aPLA2R1ab) in peripheral blood has been widely used in clinical diagnosis (10), prediction, and treatment guidance (11). The natural history of the untreated MN is variable; spontaneous remission occurs in 40–50% of patients and the remaining patients progress slowly to end-stage renal disease (ESRD) or decease due to complications or any other unrelated underlying diseases after 5–15 years (12). IMN recurs in about half of kidney allograft recipients, which leads to graft dysfunction and failure (13).

The understanding of the pathogenesis has been improving in the last two decades (14). However, many factors in IMN still need to be further clarified. For instance, how does the immune response initiate the produce of the auto-antibodies against podocytes? Why are the auto-antibodies predominately IgG4? And how does the spontaneous remission occur in some patients? This review attempts to propose a hypothetical pathogenesis model to answer these issues while summarizing the main findings from the relevant available literature.

EXTRARENAL AUTOIMMUNE RESPONSE CAN CAUSE IMN

IMN is mainly considered to be an organ-restricted autoimmune glomerular disease due to the discovery of podocyte auto-antigens such as PLA2R1 (3). However, there is currently a lack of evidence that IMN is an autoimmune disease induced by *in situ* podocyte auto-antigens. In addition, Xu et al., van de Logt et al., and our team have recently proposed the hypothesis of IMN pathogenesis induced by an extrarenal autoimmune response (mainly in the lungs) (14–16). Despite the current lack of direct evidence, we can speculate on the feasibility of this hypothesis from the following topics.

Circulating Antibodies Against Podocyte Exteriors of the Kidney Cause IMN Development: Hints From Previous Studies

Heymann nephritis, the first experimental MN induced in rats, was described in 1959, and was then known as active Heymann nephritis (AHN), which was immunized by an insoluble subcomponent of the brush border of the proximal tubule, called

fraction 1A (Fx1A). Subsequently, it was found that injection of anti-Fx1A IgG in rats could induce the analogous lesion more rapidly, which was named as passive Heymann nephritis (PHN) (4). In 1978, two different teams from Couser and Hoedemaeker infused anti-Fx1A IgG into isolated rat's kidneys *ex vivo* to study pathological changes of IMN; the idea that extrarenal circulating antibodies bind podocyte auto-antigens to form *in situ* immune complexes was proposed first (17, 18). Later, the major antigenic proteins in Fx1A were identified as megalin, which is a podocyte membrane receptor of low-density lipoproteins (19, 20). IgG passes through the glomerular endothelial cells and GBM freely, thus binding to megalin on the surface of podocytes, which in turn forms PHN (21). Other experimental MN induced by injection of exogenous anti-podocyte antibodies were carried out, such as a mice model of anti-podocyte glomerulonephritis, THSD7A homologous rodent MN, and transgenic mice MN with PLA2R1, which are similar to that of PHN (22–24). These findings suggest that extrarenal circulating antibodies against podocytes can induce MN-like pathological changes in normal kidneys. And, this phenomenon is not only limited to experimental MN.

In 2002, the neutral endopeptidase (NEP) was identified by Ronco's team in neonatal MN (25); it supports the idea that the circulating anti-podocyte antibodies can be produced without the involvement of the kidney. Mothers with NEP deficiency were exposed to NEP during a previous spontaneous abortion or pregnancy, and their immune systems produced anti-NEP antibodies afterwards, which crossed the placenta barrier and bound to the fetal glomerular podocytes during pregnancy (26). Similar to PHN and other above-mentioned experimental MN models, nephritogenic antibodies of neonatal MN are produced not by the person themselves but by the immune system of nonego, which can be classified as “passive MN.” These previous studies suggest that IMN can be induced by an autoimmune response outside the kidney.

Potential Relationship Between Extrarenal Disease, Environment, and Pathogenesis of IMN

After the identification of auto-antigens such as PLA2R1, the pathogenesis of IMN was considered as primary MN (3). However, when idiopathic and secondary MN are distinguished using circulatory aPLA2R1ab, its diagnostic specificity is relatively inadequate. It was reported that 9 out of 32 patients with secondary MN presented with positive anti-PLA2R1, including 7 cancer patients, 1 Crohn's disease patient, and 1 patient with scleroderma (27). Furthermore, the result from another cohort showed that, among the 24 patients with psoria-associated MN, 7(29.2%) patients had positive anti-PLA2R1 (28). And, in 39 patients of hepatitis B virus-associated MN, 25(64.1%) patients with aPLA2R1ab positive were found (29). Also, among the 37 patients with membranous lupus nephritis, 7(18.9%) patients were found to be anti-PLA2R1. More interestingly, 3 patients with non-renal lupus disease were found to be anti-PLA2R1 positive (30). There is a possibility that PLA2R1-associated MN coexists with these extrarenal diseases,

as the causal relationship between them cannot be completely excluded (31).

The causal relationship in THSD7A-associated MN is more obvious. Although the association between THSD7A and cancer is disputed (32, 33), THSD7A can be detected in the tumor, metastatic lymph node cells, and dendritic cells of lymph nodes (34, 35). In a cohort of breast and colorectal tumor patients, THSD7A is also expressed in breast tumor tissues (20/20, 100% patients positive) and colorectal tumor tissues (79/81, 97.5% patients positive). More importantly, in 3 of the 4 tumor patients with proteinuria who were followed up, proteinuria was in complete remission within 6 months after tumor resection without any other interventions (36). However, the kidney disease of these 4 patients were undiagnosed, even though THSD7A is currently considered only associated with IMN. A patient who had repeated renal biopsy showed negative expression of THSD7A in their kidney tissue in the first biopsy, however the second renal biopsy showed the MN lesion with positive THSD7A and manifested malignant tumor after 17 months. After 4 months, the serum THSD7A auto-antibody showed negative after surgical resection and chemotherapy with paclitaxel liposome carboplatin (37). More interestingly, inspired by two cases of THSD7A-associated MN accompanied by angiolymphoid hyperplasia with eosinophilia (ALHE), the researchers assumed that vascular endothelial growth factor A (VEGF-A) up-regulated the expression of THSD7A in endothelial cells *in vitro* (38). The tumor growth is closely related to angiogenesis; VEGF is secreted by cancer cells and stromal cells stimulate the proliferation and survival of endothelial cells, leading to the formation of new blood vessels (39). This may be one of the ways for THSD7A to establish the relationship between tumor and IMN. Extrarenal diseases may induce IMN by affecting these auto-antigens expressed outside the kidney. The autoimmune response of IMN (or primary MN) may not be limited to the kidneys, when precisely observed.

The pathogenesis of IMN has been associated with environmental stimuli (40). The morbidity of MN in China has been gradually increasing, which could be possibly related to the long-term exposure to air pollution. Each 10 mg/m³ increase in PM_{2.5} concentration over 70 mg/m³ is related with 14% higher odds for MN (2). It is speculated that air pollution can be involved in the pathogenesis of IMN by inducing inflammation and oxidative stress in the lungs (14–16), it can also activate antigen presenting (APC) cells and autoreactive T cells in the inflammatory microenvironment. In addition, helicobacter pylori (HP) is closely related to chronic mucosal inflammation in the stomach and duodenum (41), and MN patients are also closely related to gastric HP infection (42–44). Consistent with these speculations, two risk alleles for primary MN have been recently identified in *NFKB1* and *IRF4*, and both of them were involved in defense against common pathogens. It is worth mentioning that all four genome-wide significant risk loci (*PLA2R1*, *IRF4*, *NFKB1*, and *HLA*) with highly pleiotropic effects identified in this extensive genetic study have a concordant effect on the risk of inflammatory bowel disease (IBD); this fact suggests that there is a shared pathogenic mechanism between IBD and IMN (45). Thus, inflammation can be one of the

pathways for environmental stimuli and extrarenal diseases, which links to the pathogenesis of IMN.

Non-inflammatory Glomerular Lesion of IMN Might Not Be Consistent With *in situ* Induced Organ-Specific Autoimmune Diseases

Unlikely most organ-specific autoimmune diseases induced by *in situ* auto-antigen exposure, such as diabetes mellitus type 1, inflammatory bowel disease (IBD), multiple sclerosis, and psoriasis, in which affected organs are infiltrated with immune cells (46–49), the kidney pathological manifestations of IMN shows almost no inflammatory cells' infiltration except for complement activation (4). As mentioned above, the pathogenesis of IMN is probably associated with inflammation, and it is difficult to imagine an autoimmune response induced by auto-antigen exposure in glomerulus without any involvement of immune cells. There is a hypothesis that a soluble form of podocyte auto-antigen, such as soluble PLA2R1, sheds and induces an autoimmune response (4), which could partly explain the non-inflammatory lesion to the glomerulus. However, direct evidence of an autoimmune response induced by soluble PLA2R1 is still lacking, and this hypothesis is poor. Firstly, it is unlikely that podocytes will release large amounts of soluble PLA2R1 without being affected. If so, the presence of proteinuria should predate the autoimmune response. Secondly, soluble PLA2R1 should be distributed in the blood vessels according to the concentration gradient; the closer one goes to the podocytes, the higher the concentration, which also possibly induces the immune cells to approach the glomerulus and deposit circulating immune complexes at multiple sites in the glomerulus. IMN caused by an extrarenal autoimmune response can be a better explanation for these kidney pathological manifestations. In addition, the deposition of extrarenal IgG4 also further inhibits the inflammation in the glomerulus (detailed below).

Auto-Antigens Associated With IMN Pathogenesis Expresses in Many Tissues Including the Kidneys

Besides the kidney podocyte, PLA2R1 also express in the lungs, placenta, liver, and skeletal muscles (50). PM_{2.5} induces inflammatory responses in the lungs, causing infiltration of inflammatory cells (51, 52), such as neutrophils and alveolar macrophages, which also express PLA2R1 (53, 54). The extrarenal expression of PLA2R1 provides the basis for extrarenal aPLA2R1ab production. Interestingly, the production of aPLA2R1ab does predict significant kidney damage. Recently, Burbelo et al. reported that aPLA2R1ab were detectable at a median of 274 days before renal biopsy diagnosis (interquartile range, 71–821 days) and aPLA2R1ab seropositivity occurred closely or before the pre-diagnostic non-nephrotic range proteinuria in the majority of cases (55). The aPLA2R1ab is considered directly pathogenic and the kidneys can just be a target. The direct evidence of humoral immunity induced by exposure to the lung's PLA2R1 is required for further studies.

THSD7A was initially characterized as an endothelial protein that is expressed in the placental vasculature, which can be involved in promoting endothelial cell migration during angiogenesis (56–58). Angiogenesis mostly occurs in tissue repair or tumor metastasis, and in this phenomenon, both inflammation and active immune response are down-regulated, which is also consistent with the immunosuppressive effect of VEGF (39). The immune induction phenomena of THSD7A seems to be different from that of PLA2R1.

NELL-1 is a newly discovered third auto-antigen of IMN podocytes (9), which is highly expressed in osteoblasts and promotes bone regeneration. The C-terminal region of NELL-1 mediates osteoblastic cell adhesion through integrin $\alpha 3 \beta 2$ (59, 60). NELL-1 is overexpressed in patients with craniosynostosis, one of the most common congenital craniofacial deformities, where it is specifically up-regulated within prematurely fusing sutures (61, 62). It indicates that different auto-antigens in IMN have different expression phenomena, and these different phenomena that affect the exposure of auto-antigens are potentially mutually exclusive. IMN may be the same pathological pattern of different diseases. Thus, it is necessary to figure out that different phenomena of different auto-antigens initiating extrarenal autoimmune response leads to IMN.

AUTO-ANTIGENS AND EXTRARENAL AUTOIMMUNE-INDUCING PHENOMENA

The autoimmune response is the result of a combination of factors, and the fundamental question regards how self-tolerance fails and how self-reactive lymphocytes are activated. Despite the lack of research on these topics in IMN, we can still try to figure out some important questions on the basis of our current understanding.

Characteristics of Auto-Antigens

One of the key preconditions for an extrarenal autoimmune response that involves the kidney is that these antigenic proteins must be expressed in the kidney and, more precisely, in the podocytes. Virtually all structures in the glomerulus and all domains and micro-domains of the endothelium and podocyte are accessible to the circulating antibodies. More importantly, the fate of immune complexes formed by binding to glomerular components varies with the location of the glomerular antigens, the basal domain, and/or the slit diaphragm, which persists longer than the others (63). This finding may suggest that the location of auto-antigenic proteins may determine their ability to cause damage to podocytes. Recently, using a variety of microscopes, it was found that THSD7A localizes at the basal aspect of foot processes, closely following the meanders of the slit diaphragm in human and mice, and the anti-THSD7A antibodies bind THSD7A expressed on the slit diaphragm (64). In human podocytes, THSD7A expression is accentuated at filopodia and thin arborized protrusions, an expression pattern associated with the decreased membrane activity of cytoskeletal regulators. Phenotypically, THSD7A expression in human podocytes is associated with an increase in cell size and enhanced adhesion

to collagen type IV-coated plates (64). Again, due to the lack of evidence on the precise localization of PLA2R1, it is currently reasonable to believe in renal pathology that PLA2R1 could also express at the basal domain or slit diaphragm of podocytes and can disrupt the function of podocytes, such as their adhesion ability to collagen type IV (GBM) (65). The uniformity of NELL-1 staining along the GBM and correlation with the subepithelial deposit suggests that this protein is shed from the podocytes (9). It is unlikely that NELL-1 is shed from mesangial cells or endothelial cells, since there was no mesangial or subendothelial staining in the NELL-1 positive MN. Nevertheless, NELL-1 is also likely present as an extracellular component and can deposit in the GBM (9). These antigens are restricted to express in the space between podocytes and GBM, which could also be the reason for the limitation of pathological manifestations.

Another key precondition is that the autoimmune-inducing phenomena outside the kidney must specifically induce exposure of these antigens, such as conformational changes, molecular simulations, or up-regulated expression. From the mutual exclusion of these antigenic proteins in IMN, it seems that the immune-inducing environment of different antigens is also mutually exclusive. Interestingly, the subclass of IgG deposition of NELL-1-associated MN is mainly IgG1, which is inconsistent with PLA2R1- and THSD7A-associated MN with the IgG4 as the principal antibody (9). It suggests that there is heterogeneity in the autoimmune process of IMN, and needs to be further subdivided according to different immune processes in future studies. In other words, the variety of auto-antigens can be selected by different immune-induced phenomena.

PLA2R1-Autoimmune Response, Inflammation, and Genetic Susceptibility

PLA2R1 belongs to the mannose receptor family and is a type I transmembrane glycoprotein with 185kDa, whose extracellular part is composed of N-terminal cysteine-rich (CysR, or ricin B) domain, a single fibronectin type II (FnII) domain, and 8 C-type lectin-like domains (CTLDs) (66). The four identified domains containing major B-cell epitopes are CysR, CTLD1, CTLD7, and CTLD8, and epitope antigenicity in these domains is all determined by spatial conformation (67, 68). The N-terminal region of PLA2R1 is the predominant target of autoimmunity, as is THSD7A, and epitope recognition evolves over time and as the disease worsens (69–71). Patients with epitope spreading usually have more severe disease and worse prognosis (**Figure 1**). In general, PLA2R1 acts as the receptor mediating sPLA2 endocytosis, continuously trafficking between the cell membrane and the endosome to transport sPLA2 (50, 66, 72). Beyond mediating sPLA2, the current understanding of the biological function of human PLA2R1 is quite limited. It is speculated that human PLA2R1 is closely related to inflammation.

Either through ovalbumin-induced lung inflammation or murine α -myosin heavy chain-induced autoimmune myocarditis, the affected tissues in the PLA2R1-deficiency mice show more inflammatory cell infiltration and more severe inflammatory response (73, 74). Furthermore, rat PLA2R1 expression was up-regulated in an inflammatory

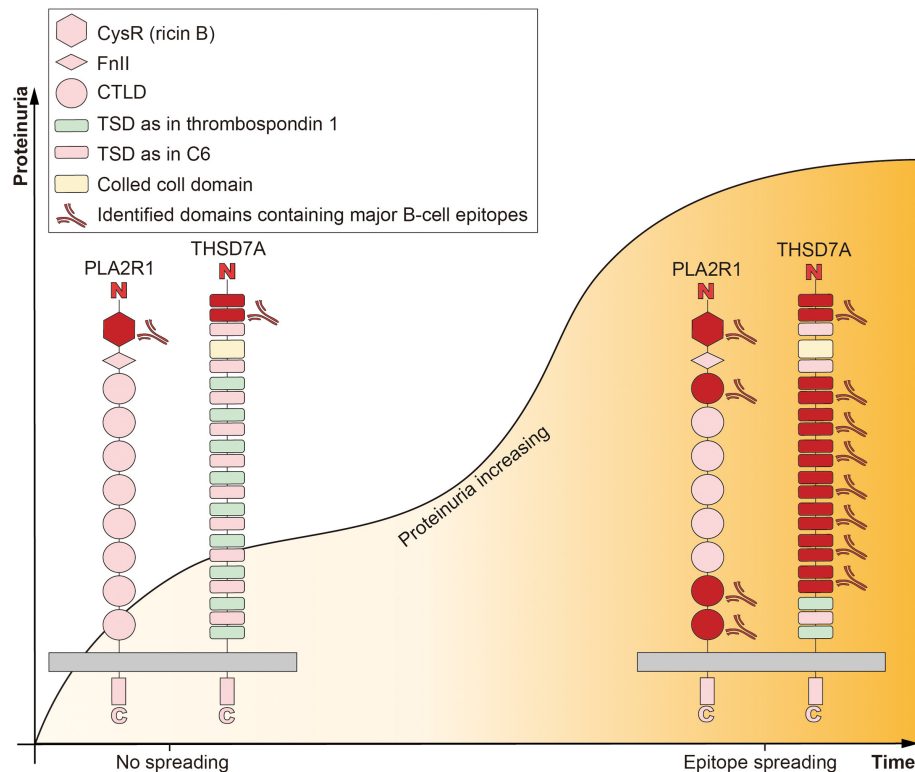


FIGURE 1 | Epitope spreading accompanied with diseases progression in idiopathic membranous nephropathy. The extracellular part of M-type receptor for secretory phospholipase A2 (PLA2R1) is composed of N-terminal cysteine-rich (CysR, or ricin B) domain, a single fibronectin type II (FnII) domain, and 8 C-type lectin-like domains (CTLDS). The four identified domains containing major B-cell epitopes are CysR, CTLD1, CTLD7, and CTLD8. CysR is the predominant target of autoimmunity. The extracellular part of thrombospondin type-1 domain-containing 7A (THSD7A) is composed of a colled coll domain and 21 thrombospondin type 1 domains (TSDs). These TSDs show high structural homology either to the TSDs of thrombospondin 1 or complement component 6 (C6). The predominant target of autoimmunity is exists in the N-terminal region. Epitope spreading is defined as the diversification of epitope specificity from the initial focused, dominant epitope-specific immune response, to subdominant and/or cryptic epitopes, which accompanied by maturation of the immune response and progression of the disease (proteinuria increasing).

environment (for example, PLA2R1 expressed on lymphocytes and granulocytes can be up-regulated by interleukin (IL)-1b *in vitro*) (75). However, these studies have limitations since the rodent kidney does not express PLA2R1. Human PLA2R1 can also be involved in inflammatory processes, such as the potential function of PLA2R1 expressed on neutrophils and alveolar macrophages (53, 54). Human PLA2R1 is also closely related to asthma. PLA2R1 is differentially overexpressed in bronchial epithelial brushings of children with atopic asthma (76); it is also believed that there could be a potential involvement of risk alleles on *PLA2R1* in adults with asthma who have occupational exposure, which plays an important role in NF- κ B pathway, leading to inflammation (77). We hypothesized that human PLA2R1 can be up-regulated in an inflammatory environment, which can be related to the autoimmune-induced environment of PLA2R1 in IMN.

Genome-wide association studies (GWASs) clarified the understanding of IMN genetics. The two risk alleles in *HLA-DQA1* and *PLA2R1* are closely related to IMN development, and the same results have been obtained in multiple interracial genetic studies (45). Recently, *NFKB1* and *IRF4*, the novel genome-wide significant risk loci for MN have been identified,

with large effects encoding two transcriptional master regulators of inflammation. Due to the bi-ethnic composition of the cohort, the ethnicity-specific effects at the HLA locus can be further defined. *DRB1*1501* is a major risk allele in East Asians, *DQA1*0501* in Europeans, and *DRB1*0301* in both ethnicities, which suggests that different epitopes are likely presented to T cells to initiate the anti-PLA2R1 response in East Asians and Europeans. In line with previous studies, a single haplotype at the *PLA2R1* locus exhibits genetic interactions with HLA-*DRB1* risk alleles in both ethnicities, the three amino acid residues encoded by *DRB1*1501* and *DRB1*0301* in the *DR β 1* chain of major histocompatibility complex (MHC) class II promoting epitope presentation to T cells, which could be the cause of IMN susceptibility (45, 78).

Under the activation of antigenic peptides and MHC class II molecules, CD4⁺ T cells differentiate into several subtypes. This process is regulated by many cytokines produced by innate immune cells. Previous studies have observed that IMN is dominated by Th2 type immune response with increased IL-4 production of peripheral Th cells, and increased IL-13 mRNA expression of peripheral blood monocytes in patients (79, 80). Rosenzwaig et al. and Roccatello et al. described a low level of

regulatory T (Treg) cells in two cohorts of IMN ($n = 25$ and $n = 17$, respectively) at diagnosis and an increasing level of these cells after remission induced by rituximab, associated with low levels of a Treg cytokine IL-35 that also increases with remission (81, 82). Although the effector cells that induce T cell differentiation in IMN and their immune-induced microenvironment are not yet clear, the above data also suggest an inflammation tendency in IMN.

IgG4 DOMINATE AND THE ROLE OF IgG4

IMN is considered an IgG4-dominant disease (7, 8). In three IMN cohorts of detected peripheral circulating IgG subclasses, all the positive rates of IgG4 were the highest, with the proportions of 89% (83), 94% (84), and 100% (85), respectively, and the titer of IgG4 was also the highest. Among other IgG subclasses, IgG1 also accounts for a higher percentage in IMN patients, but the titer is relatively insufficient (84). Interestingly, the deposited IgG subclass in the glomerulus indicates the Ig subclass switch as the disease progresses. In the early stage (electron microscopy stage I), IgG1 is the major IgG subclass, and IgG4 dominates in all later stages (electron microscopy stage II-IV) (by pathological criteria according to Ehrenreich and Churg, IMN is divided into four stages) (86). Correspondingly, IgG1, IgG2, IgG3, and IgG4 coexist in most patients at the first stage of the disease, while at relapse, IgG4 was the predominant subclass (87). When comparing with other IgG subclasses, IgG4 is usually unable to activate complement and has a higher affinity for antigens (88, 89). Exostosin 1 (EXT1)/exostosin 2 (EXT2)-associated MN represent a secondary form of MN and the antibody in EXT1/EXT2-associated MN is IgG1 (90). Ravindran et al. described a high spectral count of complement proteins C3, C4, C5, C6, C7, C8, and C9 in glomerular that both exist in PLA2R-associated and EXT1/EXT2-associated MN by using mass spectrometry. However, complement protein C1 was present in low spectral counts in EXT1/EXT2-associated MN, which appeared higher than in PLA2R-associated MN (91). Each of the three complement pathways seem to be involved in the glomerular complement activation, while classical pathways may not be evident when IgG4 is dominant (87, 91), which suggest that the respective antibody titers and ratios between the different IgG subclasses will determine the involvement of different pathways of complement activation. Thus, it is necessary to discuss the potential causes of Ig class switching of antibodies in IMN.

Reasons for IgG4 Domination: Anti-inflammatory and Increased Affinity

The diversity of the human antibody repertoire is generated by V(D)J gene rearrangement that gives antibodies their complex array of effector functions, and accumulation of VDJ somatic point mutations are generally considered to boost higher affinity antibodies through selection within the germinal centers (88). IgM > IgG3 > IgG1 > IgG2 > IgG4 is considered to be the temporal model sequence of Ig class switching in the germinal center reaction. Differences in epitopes' affinity, complement

fixation ability, and Fc receptor (FcR) binding ability between these subclasses could help to coordinate the inflammation and humoral defenses over the time course of a response (88). At the early stage of germinal center reaction, IgM⁺ B cells switch to IgG3, which recruits FcγR-mediated functions in the early response. IgG1 then emerges as the major effector of antigen clearance, and subsequently IgG2 competes with IgG1 to produce immune complexes which slows the inflammatory drive. Persisting antigens can finally stimulate high affinity IgG4 that outcompetes other isotypes (88, 92). IgG4 may inhibit the binding of other IgG subclasses to antigens by its high affinity, reaching a certain proportion in the formed immune complex and blocking FcγR-mediated processes, such as phagocytosis and release of pro-inflammatory cytokines (88). IgG4 response is often formed by following repeated or long-term exposure to antigen (89, 93). For instance, IgG4 plays a protective role in allergies, with prolonged or repeated allergen exposure and increased production of IL-10, and IgG4 gradually increases and blocks IgE to inhibit excessive allergic reactions (94). And, in asymptomatic filarial infection, elevations in IgG4 are also often associated with high worm loads and with high plasma levels of IL-10 (95). As a key cytokine for immune regulation, IL-10 plays an important role in inhibiting the overreaction to antigens, including inflammation and adaptive immune responses (96). IL-10 is also considered to specifically modulate the differentiation of B cells into IgG4-responsive types (97, 98). Up-regulation of IL-10 mRNA expression was observed in peripheral blood monocytes in IMN patients (80). During the early stage of IMN, patients have higher counts of M2-like monocytes, higher levels of serum IL-10, and the IL-10⁺ M2 counts were positively correlated with disease activity (99), which could suggest that antibody class switching is driven by innate immunity in the early stage of IMN. In general, the anti-inflammatory properties of IgG4 and its high affinity to antigens are responsible for its delay appearance and dominant role in the active immune process (88, 89, 93).

Hypothetical Model: Ig Class Switching of Antibody and IMN Disease Progression

As mentioned above, IMN is closely related to inflammation, environmental stimuli, and genetic susceptibility. Pathogens or PM2.5, as the continuous stimulus, often induce constant local chronic inflammation. It also leads to the accumulation and continuous activation of macrophages at the inflammatory site, so as to cause bystander damage (100–102). In inflammatory conditions, auto-antigenic proteins can induce autoimmune diseases by altering conformation and epitopes, dissociating from tissues, up-regulating expression, or further modifying auto-antigens, while exposed to the immune system (103–105). Even the inflammatory cells themselves can be the sources of auto-antigens (both neutrophils and alveolar macrophages expressing PLA2R1) in IMN (14). The exposure of autoantigens then aggravates the local inflammation, which leads to the formation of a vicious circle (105). Under the stimulation of environmental factors, the function of immune cells will also be affected, such as the auto-reactive T cells and the antigen presenting cells, thus

increasing the risk of loss of self-tolerance abilities (106, 107). This will subsequently induce the humoral immunity targeted to the extrarenal auto-antigen (PLA2R1 or THSD7A), which is programmed to control the local inflammation and tissue damage.

Based on this understanding, we have further speculated about the development of IMN in this section, although it remains just a hypothesis. In the very early stages of IMN, anti-PLA2R1/THSD7A IgM or IgG3 could have been deposited in glomeruli in very small quantities. Since the source of the immune response is not present in the kidney, only very small quantities of IgM or IgG3 and the subsequent potential complement activation could cause previous damage to the podocytes. However, their low affinity makes it difficult for them to bind to the antigens which are naturally expressed in podocytes for a long time. Separate from IgM and IgG3; IgG1 has a high affinity for antigens, when the antibody subclass switch to IgG1 predominantly, the IgG1 in plasma can deposit in the glomerulus in large quantities, inducing early IMN (stage I). The original purpose of IgG1 production is to drive the inflammation and antigen clearance in the extrarenal site, while the immune system also aims to block the excessive inflammatory response simultaneously, resulting in the production of IgG2 and IgG4 (88, 89). IgG4 achieves bi-specificity through its Fab exchange, which can make a high-efficiency, high-affinity blocking antibody, inhibiting the excessive inflammation (108). Moreover, IgG4 can competitively inhibit IgG1 binding to antigens with its higher affinity, thus achieving a major proportion in the immune complex of IMN. With the deposition of IgG4, the glomerulus lesion aggravates. Although the complement activation is significantly reduced, the high affinity of IgG4 also can affect the normal function of podocytes, causing significant destruction (Figure 2).

Potential Role of IgG4: Non-inflammatory and Organ-Limited Renal Manifestations

The anti-inflammatory properties of the autoimmune response that targets extrarenal sites could be the potential cause for the non-inflammatory lesions of IMN. Driven by extrarenal autoimmunity, IgG4 deposition is predominately found in the glomerulus of most IMN patients with its high production and affinity, which suppresses the inflammatory response. In addition, the immune complex of IMN is mainly subepithelial deposited, without direct contact with the blood. Even with the activation of complement to produce anaphylatoxins, such as C3a and C5a, it is difficult to cross the GBM and endothelial cells in reverse to induce a local inflammatory response. The elevated plasma levels of C3a and C5a in IMN patients (109) are more likely to be induced by extrarenal autoimmunity. Hence, neither plasma C5a and C3a are associated with IMN disease activity, however urinary C5a has a positive correlation with between plasma aPLA2R1ab levels and proteinuria (109). Thus, the products of subepithelial complement activation of glomeruli are more released into urine and do not play a role in inducing inflammatory cell infiltration.

A specific time period is required for extrarenal inflammation to induce autoimmunity and antibody affinity maturation.

Included, repeated, or long-term exposure to antigens during this period can affect time duration. Without a certain affinity of antibodies, it is difficult for large amounts of IgG to be deposited due to the glomerular charge barrier. It will take time for the kidneys to present significant symptoms. Thus, the development of extrarenal tissue damage is prior to IMN progression and when large amounts of IgG4 are produced, the excessive extrarenal inflammation should also be suppressed, which can result in a time lag of symptoms in the extrarenal tissue, as well as in the kidney (Figure 3). More importantly, auto-antigens may function differently in different tissues (their function in extrarenal tissue is not as prominent as it is in the podocytes) and the symptoms of loss of function are not as specific as in the kidney, and it is difficult to establish a clear link clinically. The reported relationship between sarcoidosis and MN seems to support this argument; a high prevalence of PLA2R1-associated MN among patients with MN associated with active sarcoidosis has been described (31).

THE SPONTANEOUS REMISSION OF IMN

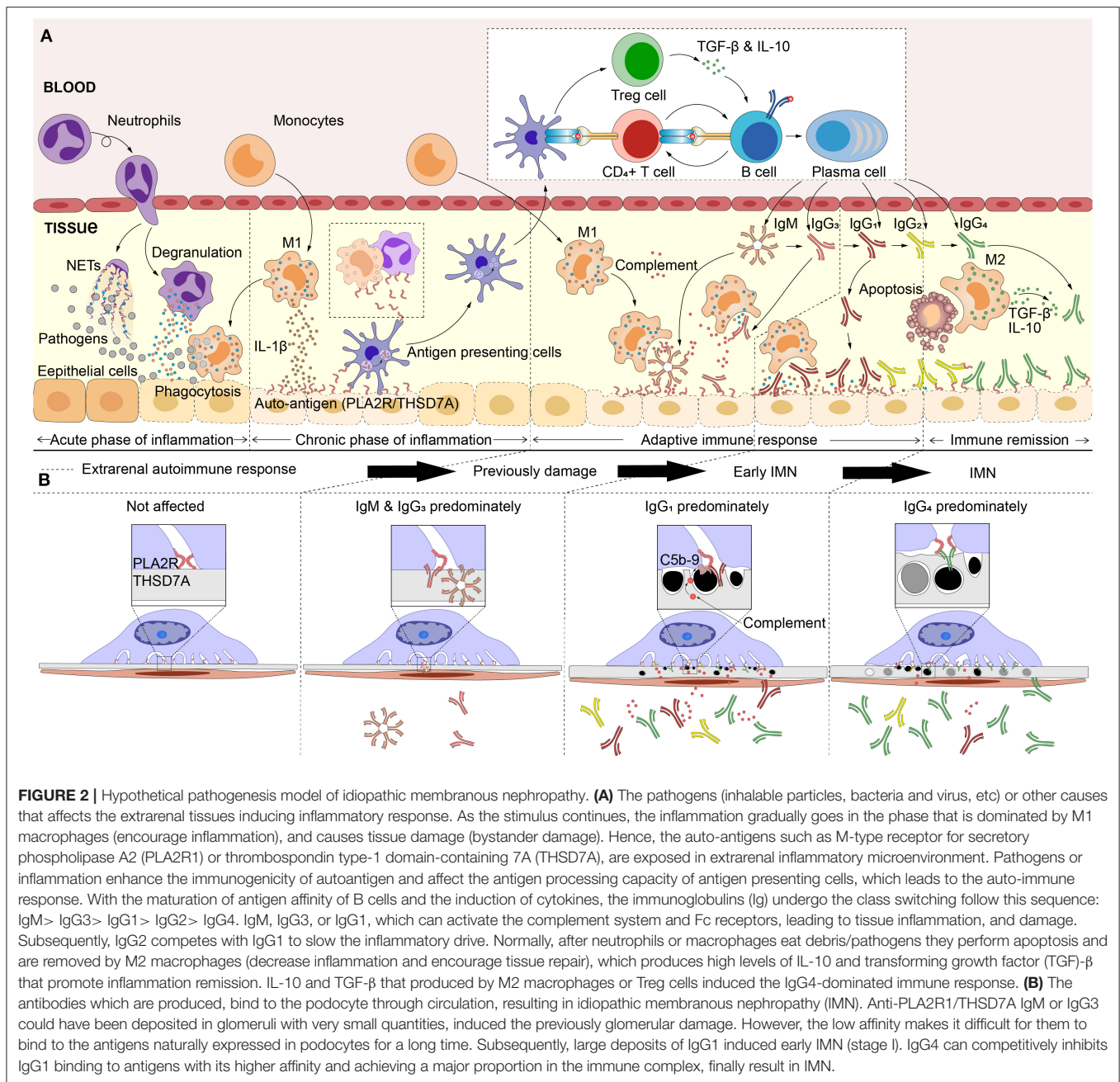
In the natural course, 40–50% of MN patients achieved spontaneous remission (12). Although spontaneous remission was more common in IMN patients with less baseline proteinuria, spontaneous remission also occurred in 22% of patients among those with baseline proteinuria >12 g/24 h (110). Immune remission predicts the remission of proteinuria, such as the decrease of aPLA2R1ab titer is earlier than that of proteinuria (111).

Self-Termination of the Extrarenal Autoimmune Response: Immune Remission

The high affinity and anti-inflammatory properties of IgG4 can contribute to shaping the pathology of IMN glomeruli and inhibit the activity of the extrarenal autoimmune response. IgG4 binds to antigens in large quantities by its high affinity, blocks the immunogenicity of antigens, and competitively inhibits the proinflammatory activity of other IgG subclasses (88). In the case of antigen blocking and inflammation suppression, the immune response tends to be alleviated. The extent to which extrarenal auto-antigens are exposed to the immune system gradually decreases with IgG4 shows a dominant type of response. The immune remission of extrarenal sites is affected by multiple factors that are related to the degree of environmental stimulation and immune regulation, and the achieved degree of remission of individuals. With the gradual remission of the autoimmune response, the production of anti-podocyte antibodies decreases, and the deposited immune complexes are gradually degraded with the glomerular self-repair.

Reversibility of Podocytes: Proteinuria Remission

The podocyte slit-diaphragm between adjacent foot processes is the final filtration barrier of the glomerulus (112). Condensation of the actin cytoskeleton at the base of effaced podocyte



foot processes is a prominent feature of both human and experimental MN, which is accompanied by various alterations in the intervening filtration slits, including widening, formation of occluding-type junctions, and displacement and disruption of slit-diaphragms (113). These changes are accompanied by a reduction in the amount and alteration in the distribution of nephrin and podocin, both of which are essential for slit-diaphragm integrity (114, 115). The slit-diaphragm is a multicomponent structure that includes heterophilic and homophilic interactions between nephrin, and neph 1, and possibly cadherins (112). Podocin is associated with the cytoplasmic tail of nephrin, and likely stabilizes nephrin and the

slit-diaphragm in a lipid raft domain in the podocyte plasma membrane (116). As nephrin dissociates from actin and podocin, its ability to tether the cytoskeleton and plasma membrane gradually diminishes, which could be the cause for the dislocation of the slit diaphragm and proteinuria in rat PHN (115). The sublethal injury mediated by anti-Fx1A and complement *in vitro* was accompanied by the dissolution of F-actin microfilaments and loss of the focal adhesion complexes in rat podocytes. The cytoskeleton is dissociated from the matrix-attached integrins due to the loss of the focal adhesion complexes that anchors the cytoskeleton to the integrin. However, podocytes retained stromal integrins even in the case of injury (117). This finding

corresponds to human's MN, and the distribution of $\beta 1$ -integrin in renal biopsies in patients with various glomerular diseases (including membranous and other forms of glomerulonephritis, minimal change disease) are similar to that in normal glomeruli (118). These findings can be related to the foot process fusion

or effacement in MN. It is worth noting that, after the removal of anti-Fx1A and complement for 18 h, podocytes recover back to normal cellular morphology, indicating that the injury was reversible and podocytes have a certain level of self-repair ability (117). The glomerulus does have a self-healing ability depending upon the pressure applied; if the pressure remains within the limits of the self-repairing ability, the glomerulus gradually goes toward the self-healing process with the gradual relief of pressure, recovering its original function, and finally achieving the remission of proteinuria (119). In a pathological state, the specific marker proteins of podocytes gradually disappear, while macrophage-related markers, such as CD68, are gradually expressed (120). Podocytes mainly perform the important function of acting as a filtering barrier under normal circumstances; while undergoing phenotypic changes, podocytes can protect themselves under pressure. When the adverse factors are removed gradually, the podocytes transformed into normal phenotypes and resume a normal morphological structure, which could be the potential cause of proteinuria remission (Figure 4).

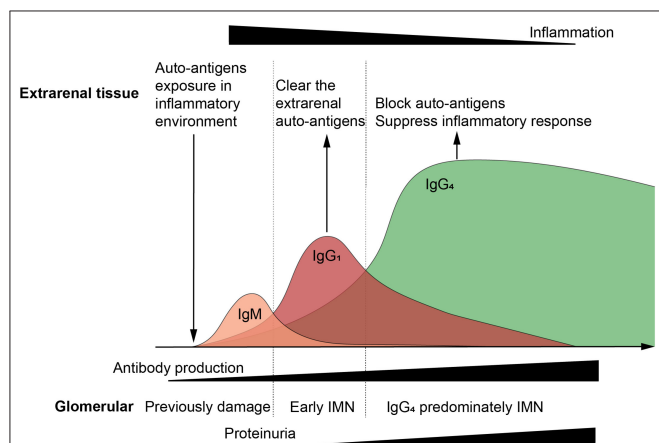


FIGURE 3 | Relationship between extrarenal tissue, immune response, and glomerular damage. Auto-antigens exposure in inflammatory environment induce the auto-immune response, and subsequently immunoglobulins (Ig) undergo class switching. IgG1 mediates the most auto-antigen clearance as the primary effector antibody, and IgG4 blocks auto-antigens as the high affinity anti-inflammatory antibody. When pathogens and auto-antigens are effectively eliminated, the extrarenal inflammatory, and immune responses tend to be alleviated. However, large amounts of antibodies are deposited in the glomeruli, especially IgG4, leading to filtration barrier damage and proteinuria. Extrarenal remission and glomerular damage aggravation were observed, which can be the reason that why idiopathic membranous nephropathy is having trouble finding the primary foci.

CONCLUSIONS

In this review, we proposed a hypothetical model of IMN pathogenesis that extrarenal immune activity causes additional kidney damage, which could to some extent explain some clinical features of IMN. Environmental stimuli or other factors induce the exposure of auto-antigens to extrarenal tissues, and initiate the autoimmune response targeted podocytes. Most IMN patients have a common immune pathway characterized by IgG4 response, which may be the result of an extrarenal autoimmune response transition to anti-inflammatory. The anti-inflammatory properties of autoimmune responses may decorate the unique

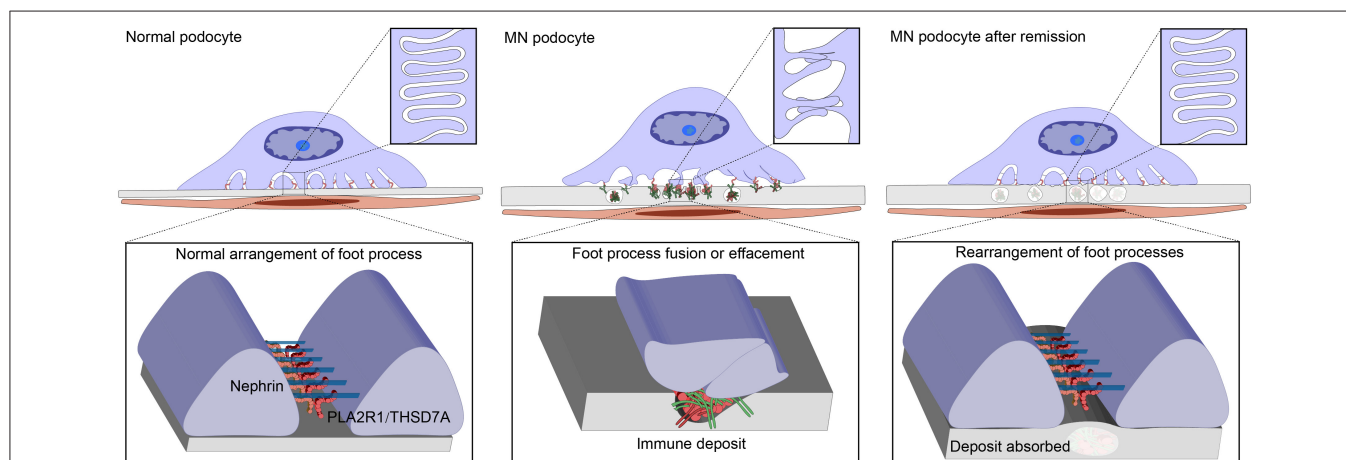


FIGURE 4 | Changes of Podocyte morphology under various conditions. M-type receptor for secretory phospholipase A2 (PLA2R1) or thrombospondin type-1 domain-containing 7A (THSD7A) potentially maintain the slit diaphragm or the adherence of podocytes to glomerular basement membrane (GBM), involved in the normal arrangement of the foot processes. Anti-PLA2R1/THSD7A antibodies bind podocytes forming the immune deposits and resulted in thickness of GBM and morphological changes of podocytes, including cell body enlargement and foot process effacement. Under the damage degree that does not exceed the self-repairing ability threshold, the podocytes gradually goes toward self-healing with the gradual relief of pressure, recovering the original structure, and function. Foot processes rearrange and immune deposits are absorbed by GBM, and finally is the restoration of normal filtration capacity and proteinuria remission.

pathological pattern and renal limitation of IMN. The production of IgG4 outside the kidney promoted immune remission, and the reversibility of podocytes is a necessary condition for proteinuria remission, both of which jointly promoted spontaneous remission. **Figures 3, 4** are simplified illustrations of this understanding. We believe that our proposed hypothesis is beneficial to the further understanding of MN and future study in this field.

AUTHOR CONTRIBUTIONS

WL, CG, XT, and BL contributed to the conception and design of the review study. WL, CG, and HD wrote the first draft of

the manuscript. YG, ZD, YZ, ZL, and ZF wrote sections of the manuscript. XT and BL discussed and revised the content of the review article. All authors contributed to manuscript revision, read and approved the submitted version.

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Biomarkers and Diagnostic Testing for Renal Disease in Sjogren's Syndrome

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Primary Sjogren's syndrome (pSS) is an autoimmune disorder in which lymphocytic infiltration leads to lacrimal and salivary glands dysfunction, which results in symptoms of dryness (xerophthalmia and xerostomia). Extraglandular features are common and may affect several organs. Renal involvement has long been known as one of the systemic complications of pSS. The most classical lesion observed in pSS is tubulointerstitial nephritis (TIN) and less frequently membranoproliferative glomerulonephritis (MPGN), which is related to cryoglobulinemia. In some cases, renal biopsy is necessary for the definitive diagnosis of kidney involvement. Patients may present with proximal renal tubular acidosis, distal renal tubular acidosis and chronic kidney disease. Response to treatment is usually favorable. However, occasionally severe and rarely lethal outcomes have been described. Recently, several case series and cross-sectional studies have been published which investigated the factors associated with renal involvement in pSS and the most accurate screening tests for early detection. The presence of xerophthalmia, anti-SSA and rheumatoid factor positivity, low C3 levels and other features have all shown either positive or inverse associations with the development of renal complications. Serum creatinine, alpha-1-microglobulin, cystatin-C have been evaluated as early detection biomarkers with variable accuracy. More advanced techniques may be necessary to confirm proximal and distal renal tubular acidosis, along with nephrogenic diabetes insipidus. The aim of the current paper is to summarize and critically examine these findings in order to provide updated guidance on serum biomarkers and further testing for kidney involvement in pSS.

Keywords: biomarkers, renal disease, diagnostic test, autoimmunity, Sjogren's syndrome

INTRODUCTION

Sjögren's syndrome (SS) is a systemic autoimmune disease which primarily causes dysfunction of exocrine glands. This leads to dryness of the ocular and oral mucosa (xerophthalmia and xerostomia), along with possible involvement of the pharynx, larynx, and vagina (1).

Furthermore, SS can also be complicated by severe manifestations such as multiorgan involvement and hematologic malignancies (2). A number of genetic and environmental factors may intertwine in the etiology of SS. The disease predominantly affects females (in a 9/1 ratio) in their middle age, but it can also affect different populations (1).

When SS affects a previously healthy individual, it is defined primary SS (pSS). When it affects patients suffering from another connective tissue disease, such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), it is considered to be secondary SS (sSS) (1).

The vast majority of patients present with a combination of xerostomia and xerophthalmia (sicca syndrome). These may include the sensation of sand or gravel in the eye or the need to drink liquids frequently to contrast oral dryness (1).

Systemic manifestations of SS include cryoglobulinemic purpura, non-erosive symmetrical arthritis, interstitial lung disease, Raynaud's phenomenon, pericarditis, autoimmune hepatitis and primary biliary cirrhosis, polyneuropathy, thyroiditis, B cell lymphoma, autoimmune haemolytic anemia, and eventually renal disease, ranging from glomerulonephritis to tubulointerstitial nephritis (1, 3, 4).

Renal involvement has long been known to be multifaceted and possibly underestimated in SS. This review aims to shed light on the most recent developments in the field, with a special focus on biomarkers and diagnostic testing necessary to elicit subclinical renal damage (5, 6).

RENAL DISEASE IN SJOGREN'S SYNDROME: GLOMERULAR AND TUBULOINTERSTITIAL INVOLVEMENT

A variety of renal manifestations has been described in pSS, with chronic and acute tubulointerstitial nephritis being the most common ones. Although less common, glomerular involvement has also been described, often in the setting of cryoglobulinemia (5–7). Most common presentations of kidney disease in pSS and their corresponding histological features have been depicted in **Figure 1**. The prevalence of renal involvement in pSS has been difficult to assess reliably, mostly due to changes in diagnostic criteria in the last 20 years and the presence of numerous studies with small number of patients or mixed cohorts of pSS and sSS (7).

According to some retrospective registries, kidneys would be affected in about 1% of pSS patients (8). However, according to several European studies, this may be as high as 5–14% (7, 9, 10). Interestingly, the prevalence of renal involvement in pSS was found to be more than 30% in a cohort of 573 Chinese patients (11). This variation in estimates may be attributed to use of different diagnostic criteria or underdiagnosis of subclinical tubular disease, which cannot be detected with standard kidney function screening tests (7). A different hypothesis, which is considered further in the article, is that ethnicity may play a role in the predisposition to renal disease in pSS (12). This would explain the wide variation in prevalence among studies performed in different world regions.

Symptomatic manifestations of renal dysfunction usually affect middle-aged patients, several years after the onset of pSS (7, 10, 13). These are included within the ESSDAI score, which tracks the activity of the disease. Renal activity of disease can range from absent to high, with low and moderate levels in-between. The considered factors are presence of haematuria and proteinuria

(indicative of glomerular disease), renal failure, tubular acidosis (detected by hyperchloremic metabolic acidosis) and clearly histological evidence of active renal lesions (glomerulonephritis or interstitial lymphoid infiltrate) (9).

In histopathological studies, chronic tubulointerstitial nephritis was the most common finding (5, 13, 14). This is characterized by the presence of small lymphocytes (mixed T cells and B cells), plasma cells, and monocytes infiltrating the renal interstitium, together with atrophy of the tubules and fibrosis (5). These alterations imply a mixture of active inflammation and residual damage.

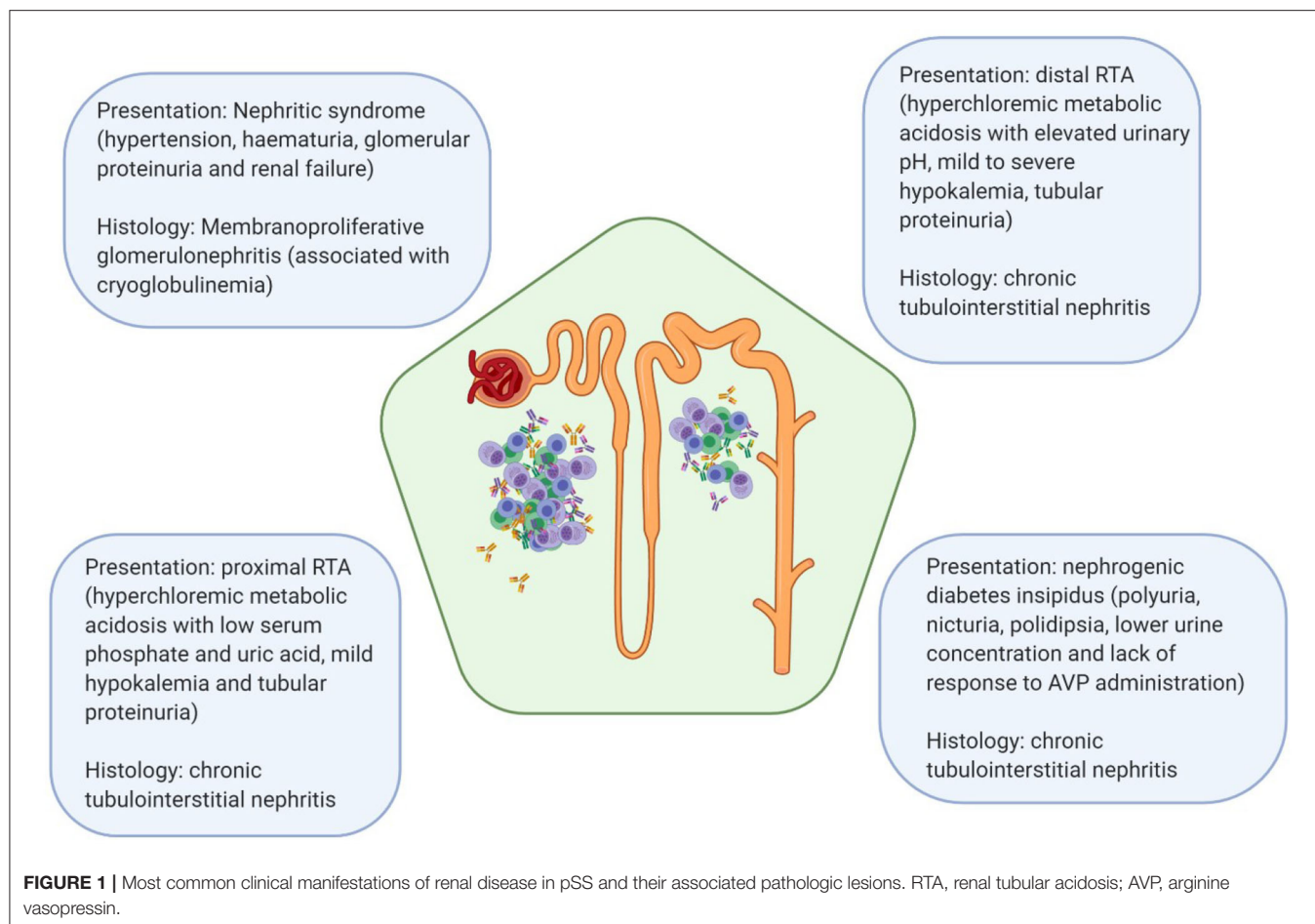
Tubulointerstitial nephritis usually presents insidiously, with tubular proteinuria (which cannot be readily identified by urine testing sticks due to the absence of albumin), renal tubular acidosis (RTA) and electrolytes disturbances (7, 15). A characteristic finding of TIN in pSS is the presence of distal RTA (dRTA, or type I RTA), which presents with an elevated urinary pH (>5.5) in the setting of an hyperchloremic metabolic acidosis (6, 16).

Unfortunately, the incomplete variant of dRTA may be more subtle and requires urinary acidification testing to be elicited (urinary pH persistently elevated after ammonium chloride administration) (6). Among these patients, hypokalemia due to dysfunction of the distal tubules H⁺/K⁺-ATPase is very common (almost 50%, according to one study) (13). When hypokalemia is associated with metabolic acidosis, an unusual finding, it is strongly suggestive of RTA.

Hypokalemia can also cause hypokalemic muscle paralysis, with generalized muscle weakness. Respiratory muscles can be affected as well. In one case, a patient died of cardiac arrest due to hypokalemia caused by dRTA in pSS (13). More commonly, dRTA can present with nephrolithiasis (7). This is caused by hypercalciuria and hypocitraturia. Consequently, renal stones in pSS may raise the suspicion of TIN.

Although less frequent, nephrogenic diabetes insipidus (nDI) and proximal RTA (pRTA, or type II RTA) can also be manifestations of chronic TIN in Sjogren's syndrome. In one study, testing with vasopressin unmasked a urinary concentration defect in ~20% of the participants (6). In a different cohort, nocturia, and lower urine concentration were observed in more than 80% of the patients (13). Proximal RTA is caused by ineffective bicarbonate resorption in the proximal tubule and can be distinguished from dRTA mostly due to low serum levels of phosphate and uric acid, which are similarly involved in its pathogenesis (7, 16).

Glomerular disease in pSS primarily takes the form of membranoproliferative glomerulonephritis (MPGN) and was observed in a substantial minority of patients in several bioptic studies (5, 8, 13, 14). MPGN is most commonly induced by cryoglobulinemia and manifests with features of nephritic syndrome (hypertension, haematuria, acute renal failure) or rarely rapidly progressive glomerulonephritis (when a rapid decline in glomerular filtration rate, GFR, is present) (7). In MPGN, the mesangial cells proliferate abnormally and the glomerulus is infiltrated with macrophages, with a consequent increase in the amount of mesangial matrix and a thickening



of the basement membrane (5). In some cases, focal segmental glomerular sclerosis (FSGS) and membranous nephropathy (MN) have been observed in kidney biopsies of pSS patients (8, 14).

Patients who suffer from renal complications of Sjogren's disease are treated with corticosteroids, immunosuppressants, or a combination of both. Immunosuppressants described in literature include cyclophosphamide and, more recently, rituximab. Regardless of the treatment protocol and the underlying lesion, immunosuppressant treatment is uniformly regarded as effective and tolerably safe. According to researchers, most patients responded to treatment with an improvement in renal function and a reduction in proteinuria. Nevertheless, a small number of patients progressed to end-stage kidney disease despite immunosuppression (8, 13, 14).

TESTING FOR RENAL DYSFUNCTION IN SJOGREN'S SYNDROME: FROM SCREENING TO CONFIRMATION

In order to approach this topic, it is important to highlight how several disorders may mimic the systemic involvement and renal manifestations observed in pSS, which are not specific to it. Among these, the most important to keep in mind for the

clinician are IgG4-associated disease, sarcoidosis, RA, and SLE (in which sSS may be present) (7). Therefore, diagnosis of pSS should be validated with the 2016 ACR/EULAR classification criteria [labial salivary gland biopsy with lymphocytic sialoadenitis (LSGB+), anti-SSA+, elevated ocular staining score, positive Schirmer test, and reduced salivary flow rate] (17).

Due to the progressive nature of renal disease in pSS and the overall excellent response to treatment, timely diagnosis is essential. It is suggested that screening for all patients should include yearly urinalysis and serum creatinine when manifestations of systemic disease are present (7, 8).

Furthermore, serum electrolytes should be measured in all patients at least yearly in order to detect disturbances due to TIN presenting as RTA. These should not be limited to measurements of sodium and potassium but should include chloride and bicarbonate. This will allow detection of hyperchloremic metabolic acidosis and potential hypokalemia (7). Although these recommendations are solely based on the opinion of experts opinions, with the lack of strong evidence supporting them, it is reasonable to aim for an early recognition of TIN, in order to administer steroids before irreversible fibrosis ensues.

In that subset of patients in whom abnormalities of renal function are detected, testing should be performed twice a year, with further examinations. Phosphate and uric acid, relevant to diagnosis of pRTA, should be included in the serum panel.

Furthermore, urinary analysis of pH, osmolality, proteinuria, creatinuria, calciuria, citraturia, urinary sediment, and culture should be performed (7). As it was mentioned before, testing for proteinuria should not be specific for albumin, so that tubular proteinuria can be reliably diagnosed. Renal echography should be similarly performed twice a year if hypercalciuria is present, to rule out nephrolithiasis (7). In this case, a nephrologist may be consulted and kidney biopsy taken into consideration.

When hyperchloremic acidosis is detected in the patient's serum, the following diagnostic approach may be helpful in evaluating the etiology (16, 18, 19). Firstly, the serum anion gap (AG) should be calculated to confirm the presence of an hyperchloremic, or normal AG, metabolic acidosis. This is performed by subtracting Cl^- and HCO_3^- to Na^+ concentration. A normal AG is usually considered to be 8–12. Hypoalbuminemia may lead to pseudonormalization of the AG, so that 2.5 mEq/L should be added to the AG measurement for each 1 g/dL decrease in albumin levels from 4.5 g/dL.

The urine AG should then be measured (urinary $\text{Na}^+ + \text{urinary K}^+ - \text{urinary Cl}^-$). When this is abnormally elevated, the suspicion of a dRTA ensues. On the contrary, if the urinary AG is normal, the cause of the acidosis is likely extrarenal (e.g., gastrointestinal fluid losses) or proximal tubular bicarbonate loss. Urinary pH should then be measured. Even though it is abnormally elevated in all forms of renal acidosis, it can rise above 5.3 only in distal RTA (19). If doubts persist, ammonium chloride, and IV sodium bicarbonate testing may be performed, although their execution and interpretation may require the aid of a nephrologist (19).

Hypophosphatemia can be observed in both dRTA and pRTA. Hypophosphatemia may lead to acquired hypophosphatemic osteomalacia, a disease of bone metabolism which presents with bone pain, weakness and increased susceptibility to fractures. In one review, 38 cases of pSS presenting with osteomalacia were reported (20). Most of these patients developed osteomalacia in the setting of dRTA, although few suffered from Fanconi's syndrome. In a different case report, a patient with pSS was reported to suffer from osteomalacia in the setting of Fanconi's syndrome, which led to hypophosphatemia and metabolic acidosis (21). Interestingly, hypocalcaemia was absent due to a secondary increase in parathyroid hormone levels.

PREDISPOSING FACTORS AND EMERGING BIOMARKERS

Recently, several original studies were published by Chinese researchers (Jing Luo and colleagues) which focused upon factors associated with renal disease and plausible biomarkers of subclinical renal inflammation in pSS (22–25).

In 2015, Zhao et al. evaluated in a cross-sectional study which factors were associated with systemic involvement in pSS (23). Although researchers did not focus specifically on renal disease, they found higher rates of systemic involvement in patients with anti-SSB and parotid enlargement or purpura at presentation. Interestingly, they evaluated the correlation of flaccid paralysis due to hypokalemia with the main immunological markers

of pSS (anti-SSA, antinuclear antibodies, rheumatoid factor, low complement, and hypergammaglobulinemia). Due to the strict theoretical correlation of hypokalemic flaccid paralysis with dRTA due to chronic TIN, hypokalemic paralysis may be assumed to be a surrogate of renal damage. Flaccid paralysis was associated with anti-SSA positivity and rheumatoid factor (RF) positivity, while it did not show any association with antinuclear antibodies (ANA) and low complement (C3/C4) or hypergammaglobulinemia (hyperIgG) (23).

Three years later, a retrospective study conducted on 103 patients who had undergone kidney biopsy was published by Yang et al. (22). When patients with biopsy-proven renal disease were compared with a control group of pSS patients by means of univariate analysis (which may affect the reliability of results), several associations emerged. Patients with renal disease had significantly lower rates of interstitial lung disease and generally lung involvement, along with lower rates of leukopenia, objective xerostomia, xerophthalmia and hypergammaglobulinemia. In contrast, they had strikingly higher rates of corticosteroids treatment. Indeed, 96.1% of renal cases were treated with corticosteroids in comparison with 23.3% of non-renal cases (22). Clearly, it is difficult to draw any meaningful conclusion on pSS subsets when such an asymmetry in the studied populations, capable of explaining many of the previous differences, is present. Though, it is notable how renal involvement appears to be a strong driver of corticosteroids treatment in pSS, at least in this cohort.

A group of researchers led by Luo published two retrospective studies, in 2019, addressing the specific issue of renal involvement in pSS (24, 25). In one of these, 434 pSS patients (217 of which suffering from renal involvement) are compared in a multivariate analysis to detect clinical, serological and immunological factors associated with renal disease (25). Xerophthalmia and anti-SSA/Ro52 were found to be negatively associated with renal involvement while histological positivity of LSGB, reduced C3, hypoalbuminemia and anemia were all significantly more common in the renal disease group. Treatment with steroids or other immunosuppressants was not considered by the investigators.

In the other study, 1,002 patients were investigated with a similar approach (24). However, researchers extended the number of clinical features considered and also included possible biomarkers of early renal damage within the studied variables. Patients with renal disease were found to have higher serum levels of prealbumin, anti-scl-70, RF, anti-extractable nuclear antigen (ENA), anti-SSB, anti-SM, urea, creatinine, cystatin C, α_1 -microglobulin (α_1 -MG), serum β_2 -microglobulin, and other molecules. Reduced hemoglobin and C3 levels were also more common in the renal cases. On the contrary, anti-SSA were more common in patients without renal disease.

In order to evaluate the sensitivity of serum biomarkers for renal dysfunction in patients with known renal disease, receiver operating characteristic (ROC) curve was drawn for creatinine, cystatin C, α_1 -MG and various combinations of these. Area under the curve was best for the combination of creatinine and α_1 -MG, with a significant difference compared to creatinine alone (0.824 vs. 0.777) (24).

TABLE 1 | Summary of most relevant findings from recent studies on kidney disease in pSS.

Author	Patient number	Country	Year	Factors positively associated with kidney disease	Factors inversely associated with kidney disease	Serum and urine biomarkers
Zhao et al.	483	China	2015	Anti-SSA, RF		
Yang et al.	103	China	2018	Steroids treatment	ILD, xerostomia, xerophthalmia, hyperIgG	
James et al.	839	United Kingdom	2018			Serum free light chains, β 2-microglobulin
Zeron et al.	10007	Worldwide (7,289 Europeans)	2019	Asian ethnicity, southern countries, young age at diagnosis	Whites, Hispanics and African Americans; Northern countries, older age at diagnosis	
Luo et al.	434	China	2019	LSGB+, low C3, hypoalbuminemia, anemia	Xerophthalmia, anti-SSA	
Luo et al.	1002	China	2019	Prealbumin, anti-scl-70, RF, ENA, anti-SSB, anti-SM, urea, creatinine, cystatin C, α ₁ -MG, serum β ₂ -microglobulin, anemia, low C3	Anti-SSA	Combination of serum creatinine and urine α ₁ -MG

Zhao, Yang, and Luo considered in their analyses the most common clinical and laboratory features which can be altered in pSS. James et al. considered markers of B-cell activation (BAFF, FLC, and β 2M). Zeron et al. considered epidemiological factors and latitude in their study.

In 2018, a study was published in which B-cell activity markers and organ involvement in pSS were considered (26). Levels of serum free light chains (FLC) and β 2-microglobulin (β 2M) were found to be associated with renal disease. While renal impairment may itself lead to increased levels of FLC and β 2M, authors corrected for glomerular function and results remained significant.

In 2019, another retrospective study was published which evaluated epidemiological factors such as age, ethnicity, gender, and latitude and their association with organ involvement in pSS (12). The study was based upon an existing registry of pSS patients (Sjogren Big Data Consortium) and included 10,007 patients from all over the world (although European patients were more represented than others). Renal involvement was significantly associated with younger age at diagnosis. Besides, individuals of Asian ethnicity were also at considerably higher risk of developing renal disease (10.2% compared with 3.8% in Whites, 2.2% in Hispanics and 1.4% in African Americans) (12).

For what concerns the latitude at which study participants lived, or North-South gradient, patients who lived in southern regions were significantly more affected by systemic involvement of pSS, including renal disease. Remarkably, this applied to Europe and Asia while it was not observed in America. This was the first time a North-South gradient of disease severity was observed in pSS.

Recently, the possible association of anti-SSA positivity with increased renal involvement was partially supported by a new experiment. Indeed, expression of two micro ribonucleic acids (miRNA), molecules involved in the regulation of gene expression, was found to be elevated in patients with pSS and

anti-SSA (27). Patients with high expression of miR-146a and miR-4484 were found to have higher rates of renal disease and to be associated with anti-SSA positive pSS. Unfortunately, the control group was composed of patients not suffering from pSS and it is therefore difficult to assess the role of these molecules within the inflammatory process.

In a different study, it was noted that increased tubulointerstitial complement deposition (C4d in the absence of C1q) could be observed in most patients suffering from pSS renal disease (28). Investigators hypothesized a role for the mannose binding lectin pathway of complement activation. If this were confirmed by other studies, more extensive complement testing than C3 and C4 alone may become indicated in pSS. Relevant predisposition factors and exploratory biomarkers of kidney damage were summarized in **Table 1**.

DISCUSSION

It was long debated whether increased prevalence of kidney disease in Asian patients was due to methodological issues or a true difference in the underlying numbers. Apparently, Asian patients are indeed more at risk of renal disease, which seems to be one of the main pSS's complications in this subset of patients (7, 12). This may have some genetic reasons, although environmental factors are also conceivable (29). Along with Asian ethnicity, young age at diagnosis and residence in southern countries seem to be predisposing factors (12). While younger age at diagnosis may simply be the consequence of a more

aggressive disease course, it is completely unclear why latitude is associated with systemic involvement.

Whether anti-SSA are associated with renal disease is still unknown. While two studies found a negative correlation of anti-SSA with renal disease, in one study a positive correlation was noted (with flaccid paralysis due to hypokalemia) (23–25).

In two studies, levels of C3 were found to be reduced more commonly in patients with renal disease than in the other group (24, 25). While this is contradicted by another study, it may further support the notion that complement activation plays a role in Sjogren's nephritis (23, 28). As complement pathways are multiple, it is unknown which of those is most involved and through which mechanisms.

A promising combination of biomarkers may be the utilization of combined serum creatinine and urinary α_1 -MG testing. Although the addition of urinary α_1 -MG would increase the cost of testing, it would allow to detect early tubular dysfunction with a better accuracy than creatinine alone (24). As α_1 -MG is not an acute phase protein, variations in its urinary levels can be safely inferred not to be caused by a transient state of systemic inflammation (30). While FLC and β_2 M testing may also be associated with renal damage, their role as diagnostic biomarkers was not considered yet (26).

Limitations of these discoveries should be considered. While one study was probably flawed by the huge difference in corticosteroids treatment between the patients and control group, it is even more remarkable that this issue was not considered in the following ones (23–25). Steroids act on most organ systems with deep immune and hematological implications. The same applies to other immunosuppressants. It would therefore be fundamental to consider the impact of treatment on proposed associations.

Furthermore, little is known about the relevance of biomarkers and predisposing factors in predicting the response to treatment and the overall outcome. Creatinine levels and proteinuria, when present, are known to improve with treatment of the underlying renal inflammation, as it may be expected (8, 13, 14). However, no study has been yet performed with the aim of quantifying how these molecules levels (e.g., creatinine, α_1 -MG, FLC, β_2 M) may affect treatment outcome or prognosis.

Another issue is the low quality of the studies reviewed, which are mostly cross-sectional retrospective studies. This study design does not allow to draw meaningful cause-effect relationships and is also subject to all the possible bias of retrospective studies. Eventually, in one study it was proposed that patients with anti-dsDNA in pSS are at higher risk of developing renal disease. Due to the strong association of anti-dsDNA with SLE, it is plausible that these patients suffered from sSS in the setting of SLE (31).

All in all, it is evident that an increase in interest for pSS-associated renal disease took place in the last few years. This was mostly driven by Chinese researchers, possibly because of the higher incidence of the disease in their population. Although findings may still be difficult to implement in clinical practice, new and intriguing possibilities emerged with these which certainly deserve further investigations.

AUTHOR CONTRIBUTIONS

GR, MF, and CA contributed substantially to the conception and design of the review. EB and SB supervised and provided critical revision of the article. GR and MF wrote the manuscript with support from EB. All authors contributed to the article and approved the submitted version.

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The Emerging Role of Renal Tubular Epithelial Cells in the Immunological Pathophysiology of Lupus Nephritis

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Systemic lupus erythematosus (SLE) is a systemic, autoimmune disease that can involve virtually any organ of the body. Lupus nephritis (LN), the clinical manifestation of this disease in the kidney, is one of the most common and severe outcomes of SLE. Although a key pathological hallmark of LN is glomerular inflammation and damage, tubulointerstitial lesions have been recognized as an important component in the pathology of LN. Renal tubular epithelial cells are resident cells in the tubulointerstitium that have been shown to play crucial roles in various acute and chronic kidney diseases. In this context, recent progress has been made in examining the functional role of tubular epithelial cells in LN pathogenesis. This review summarizes recent advances in our understanding of renal tubular epithelial cells in LN, the potential role of tubular epithelial cells as biomarkers in the diagnosis, prognosis, and treatment of LN, and the future therapeutic potential of targeting the tubulointerstitium for the treatment of patients with LN.

Keywords: lupus nephritis, renal tubular epithelial cells, tubulointerstitial lesions, kidney fibrosis, kidney inflammation

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic, systemic autoimmune disease that can affect and cause damage in various organs (1). The incidence and prevalence of SLE varies according to geographic and ethnic backgrounds, with the overall prevalence ranging from 3.2 to over 500 per 100,000 individuals (2). In terms of ethnic differences, the incidence and prevalence of SLE in African Americans, Hispanics, and Asians are ~2–5 times greater than in Caucasians (3). Further, females are predominantly affected during their childbearing years, with SLE identified as one of the leading causes of death in the young female population (4).

Lupus nephritis (LN), the involvement of SLE in the kidney, is one of the most common and severe manifestations of this autoimmune disease. The current International Society of Nephrology/Renal Pathology Society (ISN/RPS) pathological classification of LN is exclusively based on glomerular lesions (5). Tubulointerstitial inflammation, however, is frequently observed in LN, and several recent studies show that tubulointerstitial damage is a potent predictor for poor long-term renal outcomes in LN (6–9). Renal tubular epithelial cells (RTECs) are actively involved in the immune response in the kidney through the production of pro-inflammatory cytokines/chemokines and *via* interactions with immune cells (10). In this context, several

studies have begun to examine the functional role of tubular epithelial cells in this autoimmune disorder. Here, we review the current knowledge on the role of RTECs in the pathogenesis of LN (**Figure 1**), relating the functional evidence provided from studies of experimental animal models to observations made in humans.

INCIDENCE AND PREVALENCE OF LN

LN occurs in up to 50% of SLE cases and is associated with increased morbidity and mortality compared with non-LN SLE patients. Although advances in diagnosis and treatment have been made, LN remains a significant cause of end-stage renal disease (ESRD), with more than 20% of patients with LN progressing to ESRD within 15 years of initial diagnosis (11). Indeed, the rates of developing ESRD have not improved and even tended to increase in recent decades. Given that the most common demographic affected by SLE is women of childbearing age, this has significant deleterious health and socioeconomic impacts (2). Further compounding this issue, clinical trials for therapeutics targeting LN have shown disappointing results thus far (12). This is, in part, due to our limited understanding of the cellular and molecular pathways driving the pathogenesis of LN.

INITIATION AND PATHOGENESIS OF LN

Intracellular material [e.g., chromatin, double-stranded DNA (dsDNA)] released during cell death plays a central role in the pathogenesis of SLE. The defective clearance of this cellular debris and loss of self-tolerance drives the production of antinuclear antibodies (e.g., anti-dsDNA antibodies) and formation of immune complexes (ICs) of self-nuclear antigens and its autoantibodies. Glomerular deposition of these ICs is considered the initiating step in the development of LN (13). In turn, this triggers a pro-inflammatory response characterized by complement activation and immune cell infiltration that drives the glomerular pathology of LN. However, a significant proportion (up to 97.6%) of SLE patients without overt proteinuria and/or renal dysfunction have glomerular lesions associated with histopathological deposition of ICs in the mesangium (14–16). Interestingly, these patients do not develop significant impairment in kidney function during long-term follow-up periods (15). These studies indicate that glomerular IC deposition alone is insufficient for the development of clinically significant LN, leading to recent research interest to examine the tubulointerstitial compartment in the pathogenesis of this disease.

TUBULOINTERSTITIAL DAMAGE IN LN

Although current histopathological classifications of LN are exclusively determined by the features and extent of glomerular lesions (5), other components of the kidney are also participants in the disease process (17). Tubulointerstitial damage is identified as one of the pathological features of the lupus kidney. Tubulointerstitial lesions are often associated with

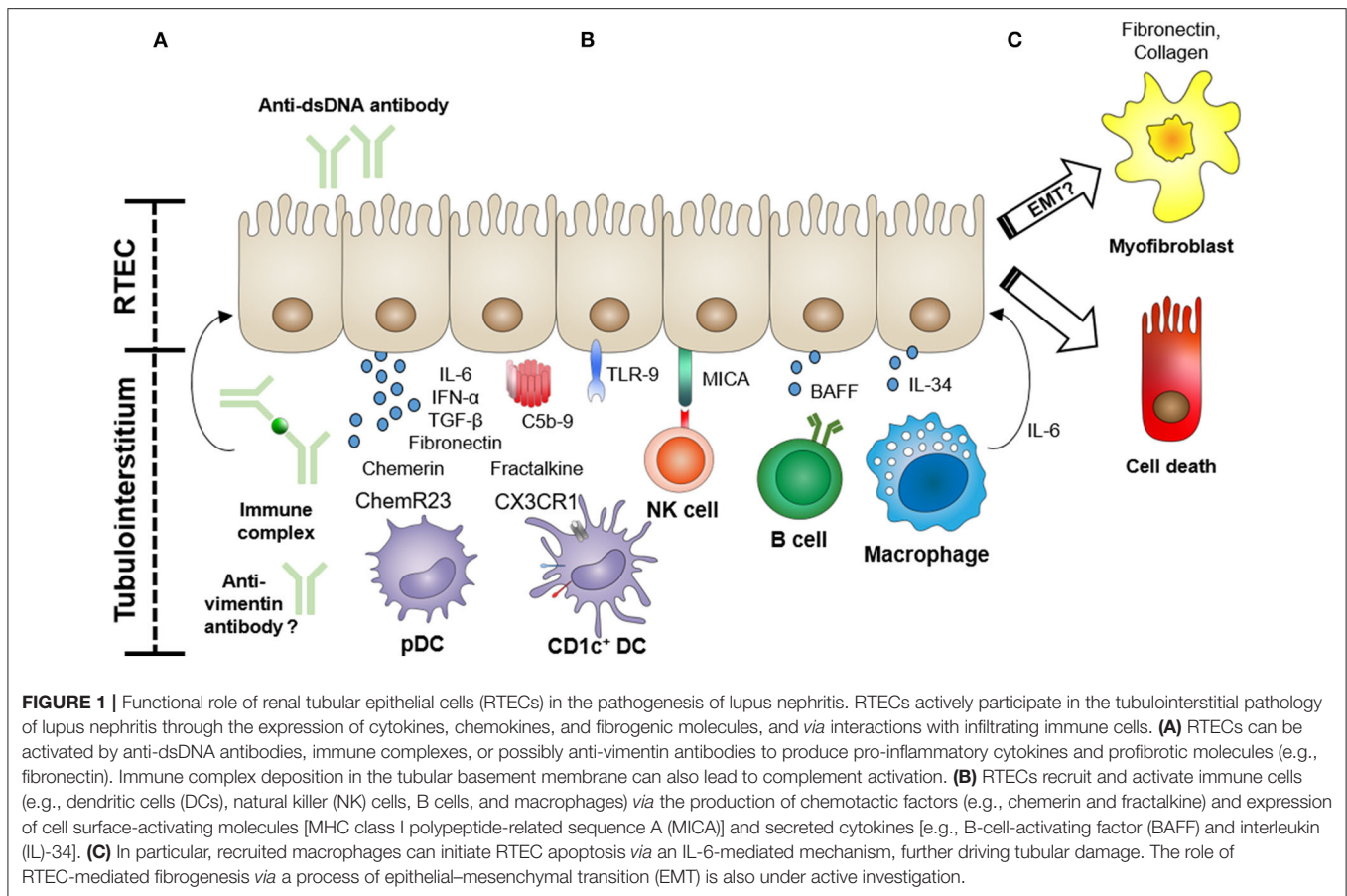
more severe (proliferative and sclerosing) forms of glomerular injury in LN (18, 19), with tubular atrophy and interstitial inflammation/fibrosis incorporated in assessments of active and chronic changes in LN (5). Recent research has also revealed the importance of tubulointerstitial damage in the prognosis of LN (6–9). In particular, tubulointerstitial inflammation and scarring are shown to be a more accurate predictor of long-term renal outcomes in LN than the glomerular-based ISN/RPS classification (7, 8). In a multivariate analysis of 105 patients with LN, tubulointerstitial lesions were significantly associated with the development of ESRD [hazard ratio (HR) 3.89, 95% confidence interval (CI) 1.25–12.14; $p = 0.02$], whereas glomerular histology based on ISN/RPS classes was not ($p = 0.72$) (8). Thus, understanding the mechanisms underlying tubulointerstitial inflammation is of importance in the study and treatment of LN.

In human LN, inflammatory cells have been shown to infiltrate the tubulointerstitium and form T:B cell aggregates or germinal center-like structures containing follicular dendritic cells (DCs) (20). In this study, Chang et al. analyzed the *in situ* immunoglobulin (Ig) repertoire, concluding that intrarenal B-cell clonal expansion was occurring within these tubulointerstitial germinal centers (20). Similarly, in lupus mice with nephritis, plasma cells secreting anti-dsDNA antibodies were primarily found in the kidney tubulointerstitium rather than the spleen or bone marrow (21). These collective findings identify the tubulointerstitial compartment as an important site of autoreactive B-cell immunity in LN.

IC deposits are identified along the tubular basement membrane (TBM) in LN patients. Wang et al. associated the presence of TBM deposits with more active disease, including higher serum creatinine levels and poorer prognosis in non-proliferative LN (22). Indeed, IC deposition in the TBM can lead to activation of the complement system, which, in turn, is associated with more severe tubulointerstitial pathology (interstitial fibrosis and tubular atrophy) (23). Interestingly, in a study of LN biopsies, the antibody subclass composition of TBM deposits was shown to differ from vascular and glomerular deposits, suggesting that tubular ICs are formed independently of ICs from the circulation and glomeruli (24). Examining the cellular and molecular pathways triggered by these tubular deposits and their utility as prognostic biomarkers is an emerging field of research focus.

RENAL TUBULAR EPITHELIAL CELLS IN LN

RTECs are a kidney-resident population that responds and contributes to pathological processes in acute and chronic kidney diseases. RTECs can actively modulate tubulointerstitial immune cell responses (e.g., DCs, T cells, and B cells) through the production of soluble factors (e.g., pro-inflammatory cytokines and chemokines) and expression of cell-surface costimulatory and coinhibitory molecules (25–30). RTECs are, therefore, studied as a plausible dominant target of anti-dsDNA antibodies in the pathogenesis of LN.



ANTI-dsDNA ANTIBODY AND IC TRIGGERING OF RTECs

Early studies examining anti-dsDNA antibody triggering of RTECs used monoclonal antibodies (mAbs) derived from nephritic mice or SLE patients with clinically active LN. These seminal investigations showed that mouse and human antibodies to dsDNA, which also cross-react with small nuclear ribonucleoproteins (SnRNPs) A and D polypeptides, cause direct RTEC injury (31, 32). Interestingly, mouse mAb BWds3 was shown to bind to the cell surface of porcine RTEC line (PK-15) without penetration into the intracellular space, resulting in significant cell lysis. In contrast, mouse mAb BWdsl, which was internalized into the cytoplasm and nuclei of RTECs, displayed only a modest lytic effect (31). These findings suggest that anti-dsDNA antibodies that bind to the surface of RTECs, but without cellular penetration and cytoplasmic/nuclear translocation, have more pathogenic potential. A subsequent mechanistic study using mutants of mouse anti-dsDNA mAb 3E10 showed that penetration required only the F(ab) (antigen-binding fragment) portion, demonstrating a process of antibody internalization independent of Fc receptor-mediated binding (32).

The pathogenic interactions of anti-dsDNA antibodies with RTECs have also been investigated using polyclonal antibodies (pAbs) from sera of patients with LN. Compared with control

IgG or non-anti-DNA IgG, binding of anti-dsDNA pAb from active LN patients to human RTECs induced secretion of pro-inflammatory cytokines [e.g., interleukin (IL)-6] (33). Another study by Yung et al. investigated the contribution of anti-dsDNA antibodies on fibrogenesis in RTECs (34). The excessive accumulation of extracellular matrix (ECM) proteins (e.g., collagen and fibronectin) is considered a histopathological hallmark of tubulointerstitial fibrosis (35). In their study, Yung et al. showed fibronectin to be highly expressed in the TBM of LN renal biopsies and colocalizing with antibody deposition. The group subsequently reported that anti-dsDNA pAb from LN patients triggered a significant increase in soluble and cell-associated fibronectin expression in human RTECs—a process dependent, in part, on the secretion of the profibrotic molecule transforming growth factor (TGF)- β by RTECs (34). This data suggests that fibrosis development in LN is initiated and amplified *via* complex signaling pathways involving anti-dsDNA antibodies, fibronectin, and TGF- β in RTECs.

It must also be noted that a characterization of *in situ*-expressed immunoglobulins from LN biopsy specimens identified vimentin, but not dsDNA, as the dominant target of humoral immunity in human lupus tubulointerstitial nephritis (36). In addition, previous studies have reported that titers of anti-dsDNA antibodies are not significantly associated with the degree of tubulointerstitial damage in patients with LN

(7, 19). Thus, future studies addressing the effects of alternate autoantibodies (other than anti-dsDNA antibodies) on RTECs is required to better comprehend the *in vivo* tubulointerstitial pathophysiology of human LN.

TLR EXPRESSION IN RTECS

Emerging evidence reports that Toll-like receptors (TLRs) are actively involved in the pathogenesis of SLE and LN. In particular, nucleic acid-sensing TLRs such as TLR-3 (recognizing double-stranded RNA), TLR-7 (recognizing single-stranded RNA), and TLR-9 (recognizing dsDNA) have been implicated in the dysregulated immunity of LN, either responding to self-nucleic acids alone or in ICs (37). Initially described in innate immune cells (e.g., macrophages and DCs), TLRs are also expressed in non-immune cells. Expression of TLR-9 has been detected in the tubulointerstitium of patients with LN (38). Furthermore, significant correlations between the levels of TLR-9 expression in RTECs and tubulointerstitial damage have been reported in NZB/NZW lupus mice and LN patients (39). In this study, sera or ICs from SLE patients were shown to significantly induce TLR-9 in human RTECs (HK-2 cells) compared with those from healthy controls or undifferentiated connective tissue disease patients, although the difference in sera between SLE patients with and without LN was not addressed (39). This increased RTEC expression of TLR-9 was inhibited with short synthetic oligodeoxynucleotides, supporting an important stimulatory role for the DNA component within ICs in LN.

The role of other TLR classes in the tubulointerstitial pathology of LN is unclear and often complicated by findings in other renal diseases. For instance, TLR-4 signaling inhibits tubular damage, but also promotes fibrosis in a model of obstructive nephropathy (40). Given the importance of other TLRs (TLR-2 and TLR-4) in the development of glomerular injury in LN (41–43), functional evaluations of these TLRs in mediating the tubulointerstitial pathology of LN is now required.

RTEC CYTOKINE PRODUCTION IN LN

Cytokines and chemokines contribute to LN immunopathogenesis, with the active role of RTECs as a source of these soluble factors of particular interest. Type I interferons (IFN- α/β) are considered to be pivotal in the pathogenesis of LN, with diverse effects on innate and adaptive immune cells (44). While plasmacytoid DCs (pDCs) are the major producer of type I IFN, expression in renal parenchymal cells has also been reported. In human biopsies with severe LN, RTECs have been identified as a key producer of IFN- α (45). In addition, a type I IFN-regulated signature was detected in RTECs, but not in the glomeruli, indicating a potential autocrine effect (45). Subsequent *in vitro* stimulation of RTECs with IFN- α was shown to induce expression of low-molecular mass protein-7 (LMP-7), a proteolytic subunit of the immunoproteasome that shapes the repertoire of antigenic peptides presented on major histocompatibility complex (MHC) class I molecules. Indeed, LMP7 was also highly expressed in renal tubules within

biopsies from patients with severe LN (45). Interestingly, immunoproteasome inhibitors are emerging as promising therapeutic agents in the treatment of lupus (46).

B-cell-activating factor (BAFF) is another key cytokine in LN, essential for B-cell survival and maturation (47). RTECs have recently been identified as an important source of BAFF, with tubular expression in lupus-prone MRL-Fas^{lpr} mice and biopsies of patients with LN correlating with disease activity (48). In this study, *in vitro* functional assays with human RTECs revealed an autoamplification loop in which ligation of BAFF with its binding receptor (BAFF-R) induced colony-stimulating factor (CSF)-1 that, in turn, triggered further BAFF expression. In addition, BAFF stimulation was shown to augment cellular cytotoxicity in CSF-1-primed RTECs (48). These complex BAFF-dependent signaling pathways in RTECs may thus contribute to the established cell death and tubular atrophy observed in LN (49). Interestingly, belimumab, a mAb to BAFF, has shown promising results in the treatment of LN, although patients with severe LN were excluded from clinical trials (50).

The diagnostic and therapeutic potential of macrophage growth factor IL-34 in LN has also been examined. Serum IL-34 levels have been shown to correlate with SLE Disease Activity Index (SLEDAI) scores and distinguish between different histological classes of LN in patients with insignificant proteinuria, indicating its utility as a surrogate biomarker for subclinical LN (51). Wada et al. recently demonstrated robust RTEC expression of IL-34 in biopsies from LN patients and MRL-Fas^{lpr} lupus mice, with significant associations between expression levels and disease activity. Further mechanistic investigations using this lupus mouse model showed that IL-34 enhances intrarenal macrophage accumulation/proliferation, leading to macrophage-mediated RTEC apoptosis (52). These findings identify IL-34 as a novel therapeutic target of RTEC-mediated immunopathogenesis in LN.

RTEC–IMMUNE CELL INTERACTIONS IN LN

Chemotactic and activatory signals between RTECs and tubulointerstitial immune cells are promising therapeutic targets in LN. RTEC recruitment of pDCs into the renal tubulointerstitium in human LN has been proposed *via* a chemerin–ChemR23 axis (53). In this study, De Palma et al. demonstrated human RTEC production of functionally active chemerin in response to pro-inflammatory cytokine tumor necrosis factor (TNF)- α , resulting in efficient recruitment of ChemR23⁺ pDC in transendothelial migration assays (53).

A dysregulated natural killer (NK) cell profile has been associated with the development of SLE. Reduced peripheral NK cell numbers and impaired cytotoxic functions have been reported in SLE patients, with NK cell deficiencies particularly prominent in patients with LN (54). However, circulating NK cells from patients with active SLE also have an activated phenotype, producing large amounts of pro-inflammatory cytokine IFN- γ (55). Evidence in LN patients of strong RTEC expression of MHC class I polypeptide-related sequence A

(MICA), the activating ligand for NK receptor NKG2D, provides a possible mechanistic pathway for this human NK cell activation (56). In SLE-prone (MRL/MpJ and MRL/lpr) mice undergoing a lupus nephritic process, kidney NK cells similarly have an activated phenotype as demonstrated by IFN- γ production and signal transducer and activator of transcription 5 (STAT5) phosphorylation (56). STAT5 is a member of the STAT family of proteins, which signal *via* the Janus kinase (JAK)/STAT pathway, supporting the proposed application of JAK inhibitors for the treatment of LN (57).

Myeloid cells (e.g., DCs, monocytes/macrophages) also contribute to LN pathology via their specialized phagocytic and antigen-presenting functions, leading to inflammation and tubulointerstitial fibrosis (58). We have reported that TGF- β -expressing human CD11c⁺ myeloid DCs are recruited and retained in the renal tubulointerstitium *via* RTEC-derived fractalkine, providing evidence of a profibrotic RTEC–DC interaction (30). We have also recently proposed a pathogenic role for CD11c⁺ macrophages in the tubulointerstitial damage of LN (59). In the urine of LN patients, we identified a population of CD11c⁺ macrophages with an activated and pro-inflammatory phenotype, as defined by expression of costimulatory molecules (CD80, ICOSL, and OX40L) and cytokines (IL-6, IL-1 β) (59). Furthermore, in this study, peripheral monocytes treated with sera from SLE patients acquired the identical phenotypic characteristics of these urinary CD11c⁺ macrophages and were shown in functional experiments to trigger IL-6-mediated fibronectin expression and apoptosis in human RTECs (59). This investigation supports the concept of a pathogenic role for RTEC–myeloid cell interactions in LN fibrogenesis.

RTECs IN LN FIBROGENESIS

As stated in preceding sections, RTECs can secrete profibrotic molecules (e.g., collagen, fibronectin) in response to anti-dsDNA antibody and inflammatory immune cells. A comprehensive study applying an unbiased single-cell RNA sequencing approach to kidney tissue from LN patients associated a fibrotic gene signature in tubular cells (upregulation of genes encoding ECM-related proteins—COL1A1, COL1A2, COL14A1, and COL5A2) with failure to respond to treatment (60). Of note, the fibrotic gene signature was detectable in a proportion of biopsies without tubulointerstitial fibrosis (as measured by standard histopathological assessment), suggesting that this identified signature may be of diagnostic utility for predicting tubulointerstitial damage prior to the development of overt fibrosis. Follow-up longitudinal studies with repeat biopsies are required to establish whether the presence of this fibrotic gene signature in tubular cells can indeed predict the development of renal fibrosis.

Tubular ECM expression has also been linked to the process of renal epithelial–mesenchymal transition (EMT), a mechanism of fibrogenesis during which RTECs differentiate into myofibroblasts and secrete ECM proteins (61, 62). While there is some controversy regarding tubular EMT, Liu et al. recently reported that oncostatin M, a member of the IL-6 cytokine family,

could induce tubular EMT and fibrotic lesions in a murine model of LN (63). Further evaluation of this mechanism and tubular EMT in human LN is required.

Hypoxia has been proposed as one of the pathological drivers of injury in LN (64), with tubulointerstitial expression levels of hypoxia-inducible factor (HIF)-1 α correlating with histopathological activity in patients with LN (65). Given that HIF-1 α expression in RTECs can promote renal tubulointerstitial fibrosis (66), assessing the role of hypoxic RTECs in LN fibrogenesis will also be an important research area for future investigation.

THERAPEUTIC APPROACHES TARGETING RTECs IN LN

Given the pathogenic functions ascribed to RTECs in LN, therapeutic targeting of this tubular cell population (and its overexpressed molecules) has been an area of intense preclinical investigation. Fractalkine is one such molecule proposed for clinical targeting in LN. In addition to a previously highlighted function in kidney DC recruitment (30), Fu et al. provided evidence of a fractalkine–Wnt/ β -catenin axis that promotes EMT progression and tubulointerstitial fibrosis in the kidneys of MRL/lpr mice and human RTECs (HK-2 cells) (67). Interestingly, the profibrotic Wnt/ β -catenin signaling pathway is also increased in the tubular compartment of lupus-prone mice (proteinuric NZB/NZW mice), accompanied by elevated serum and renal levels of proapoptotic factor dickkopf-1 (Dkk-1) (68). It is proposed that the proapoptotic effects of Dkk-1 may perpetuate autoimmunity *via* release of chromatin-containing ICs (68). Thus, the development of novel treatments or repurposing of approved drugs targeting fractalkine [e.g., E6011, a humanized antifractalkine monoclonal antibody assessed in clinical trials for rheumatoid arthritis (69)] may be of therapeutic benefit in LN.

Kallikreins are a subgroup of serine proteases that exert multiple biological functions under normal and pathological conditions (e.g., hypertension, cancer, and inflammation) (70). Of particular relevance to LN, kallikreins and their end product, bradykinin, suppress type I IFN responses (71). Tissue kallikrein-1 (KLK1) is expressed in human RTECs under *in vitro* diseased conditions (72). The inducible expression of KLK1 in RTECs has also been shown to downmodulate local pathological reactions and confer renoprotection in mice with spontaneous LN (73). Further preclinical studies of KLK1 may support the evaluation of DM199, a recombinant form of human KLK1 currently in acute ischemic stroke clinical trials (74), for the treatment of LN.

CONCLUSION

The collective findings from experimental mouse models and human clinical studies highlight the importance of tubulointerstitial damage in LN. In particular, RTECs are central effector cells within this local microenvironment, mediating renal pathology *via* the expression of pro-inflammatory/profibrotic molecules and through complex interactions with tubulointerstitial immune cells. This

accumulating evidence provides important insights in understanding the mechanisms of RTEC-mediated pathology in LN, a potent predictor of longer-term renal outcomes. The broader application of these findings introduces novel approaches with the potential for greater prognostic and therapeutic specificity in targeting the tubulointerstitial expression of immunostimulatory molecules and EMT progression in human LN.

AUTHOR CONTRIBUTIONS

SH, HH, and AK drafted, revised, and approved the final version of the manuscript. All authors have participated

sufficiently in the work to take public responsibility for the content.

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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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4C3 Human Monoclonal Antibody: A Proof of Concept for Non-pathogenic Proteinase 3 Anti-neutrophil Cytoplasmic Antibodies in Granulomatosis With Polyangiitis

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Granulomatosis with polyangiitis (GPA) is a severe autoimmune vasculitis associated with the presence of anti-neutrophil cytoplasmic antibodies (ANCA) mainly targeting proteinase 3 (PR3), a neutrophilic serine proteinase. PR3-ANCA binding to membrane-bound PR3 on neutrophils induce their auto-immune activation responsible for vascular lesions. However, the correlation between PR3-ANCA level and disease activity remains inconsistent, suggesting the existence of non-pathogenic PR3-ANCA. In order to prove their existence, we immortalized B lymphocytes from blood samples of GPA patients in remission having persistent PR3-ANCA to isolate non-activating PR3-ANCA. We obtained for the first time a non-activating human IgG1κ anti-PR3 monoclonal antibody (mAb) named 4C3. This new mAb binds soluble PR3 with a high affinity and membrane-bound PR3 on an epitope close to the PR3 hydrophobic patch and in the vicinity of the active site. 4C3 is able to bind FcγRIIA and FcγRIIIB and has a G2F glycosylation profile on asparagine 297. 4C3 did not induce activation of neutrophils and could inhibit human polyclonal PR3-ANCA-induced activation suggesting that 4C3 is non-pathogenic. This characteristic relies on the recognized epitope on PR3 rather than to the Fc portion properties. The existence of non-pathogenic PR3-ANCA, which do not activate neutrophils, could explain the persistence of high PR3-ANCA levels in some GPA patients in remission and why PR3-ANCA would not predict relapse. Finally, these results offer promising perspectives particularly regarding the understanding of PR3-ANCA pathogenicity and the development of new diagnostic and therapeutic strategies in GPA.

Keywords: anti-neutrophil cytoplasmic antibodies, proteinase 3, granulomatosis with polyangiitis, epitope, human neutrophils

INTRODUCTION

Granulomatosis with polyangiitis (GPA) is a relatively rare necrotizing autoimmune systemic vasculitis affecting mainly small to medium vessels, with histological inflammatory lesions and granulomas (1, 2). Despite current therapies, this vasculitis can be severe and lethal (3). In vasculitis nomenclature, it is part of the group of anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV): GPA is associated with cytoplasmic ANCA (cANCA), detected by immunofluorescence (IF) on fixed neutrophils (1). These cANCA mainly target proteinase 3 (PR3) and are thus called PR3-ANCA. PR3 is a 29 kDa serine protease contained in neutrophils granules which can also be located at the membrane (mbPR3) (4). PR3-ANCA are very specific to GPA when detected by immunoassays (over 90%) (5). They are found in about three quarters of GPA patients, however, 10% of patients have MPO-ANCA and less than 10% have no detectable ANCA (5, 6).

Proteinase 3-ANCA have a central role in GPA pathophysiology: they bind neutrophils previously primed by tumor necrosis factor alpha (TNF α ; called primed-neutrophils) and cause their auto-immune activation, which is responsible for vasculitis lesions (7, 8). Interaction between PR3-ANCA and neutrophils is of two types: on the one hand, a link between PR3-ANCA fragment antigen binding (Fab) and mbPR3 on primed neutrophils; and on the other hand, a link between PR3-ANCA fragment crystallisable (Fc) and Fc gamma receptors (Fc γ R); Fc γ RIIA (CD32a); and Fc γ RIIIB (CD16b) (9). Neutrophil activation by PR3-ANCA results in an adherence phenotype with increased expression of CD11b/CD18 (Mac-1) (10), induction of NETosis (11), production of reactive oxygen species (ROS) (12), protease release by degranulation (13) and production of proinflammatory cytokines (14).

Despite the central role of PR3-ANCA in GPA physiology, the correlation between PR3-ANCA level and disease activity is inconsistent in the literature (15, 16) except to predict relapse in patients with renal involvement (17, 18). Furthermore, PR3-ANCA can persist in GPA patients during remission without predicting relapse (16, 19), can be found in healthy people (20), and in other conditions than AAV (21). In a recent study, 15% of patients in complete remission had persistently positive PR3-ANCA > 12 months (16). Indeed, several factors involved in PR3-ANCA interaction with neutrophils (level of mbPR3, epitopes on PR3, subclass, and glycosylation of PR3-ANCA) influence auto-immune activation of neutrophils *in vitro* and disease activity. The level of mbPR3 correlates with neutrophil activation induced *in vitro* by PR3-ANCA and disease activity in GPA patients (9). PR3-ANCA targets different epitopes on PR3 with proportions differing between patients and with a different evolution in the same patient depending on disease activity (21, 22). PR3-ANCA found in the active phase of the disease mostly recognized a region close to the active site, corresponding to epitope 1 determined by using murine anti-human PR3 antibodies (23–25), and inhibit PR3 enzymatic activity *in vitro* (26–28). Furthermore, alpha 1-antitrypsin (α 1AT), a natural inhibitor of PR3, removes induced-mbPR3 from the neutrophil membrane and through a conformational modification, impairs

the binding of PR3 to PR3-ANCA-recognizing epitope 1 (29). Concerning isotypes of PR3-ANCA, IgG appears to be the most involved in PR3-ANCA pathogenicity, essentially IgG1 and IgG3 subclasses (14, 29, 30). Finally, glycosylation of PR3-ANCA linked to asparagine 297 is also involved in PR3-ANCA pathogenicity (31–33).

Therefore, these clinical and experimental data suggest the existence of non-pathogenic PR3-ANCA especially in patients in remission who have persistent PR3-ANCA and in healthy people. In this context, we aimed to obtain and characterize non-pathogenic PR3-ANCA from GPA patients in remission. In this study, after B cell immortalization, we presented a human monoclonal antibody (mAb) specific to PR3 (4C3) with a non-pathogenic function *in vitro*. Our results offer promising perspectives particularly concerning the understanding of PR3-ANCA pathogenicity in GPA and the development of new diagnostic and therapeutic strategies targeting non-pathogenic and pathogenic PR3-ANCA.

MATERIALS AND METHODS

Obtaining PR3-ANCA From Immortalized B Cells of GPA Patients

Immortalization of B Cells From GPA Patients

Blood samples were obtained from GPA patients treated at the Regional University Hospital Center of Tours after informed consent and from healthy donors of the French Blood Establishment Centre-Atlantique. The samples were registered in a collection of human biological samples reported to the Ministry of Research (DC-2012-1636) in accordance with Decree N°2007-1120 of August 2007. Briefly, blood samples from GPA patients in remission with a persistent PR3-ANCA level (Table 1) were collected on Acid-Citrate-Dextrose tubes. Peripheral Blood Mononuclear Cells (PBMC) were then isolated using density gradient centrifugation (Lymphosep, Biowest, France) and enriched with memory B lymphocytes after isolation of total B

TABLE 1 | Patient's main characteristics.

	Patient P1	Patient P2	Patient P3
Sex	Female	Male	Male
Age at diagnosis	49 y/o	56 y/o	75 y/o
Age at blood collection	60 y/o	61 y/o	81 y/o
Organ impairment	Kidney, articular and ENT	Pulmonary and ENT	Kidney, pulmonary and neurological
Time since obtaining remission	2 years	2 years	1 year
Treatment	Prednisolone 5 mg/day	Prednisolone 5 mg/day and methotrexate 10 mg/week	Prednisolone 5 mg/day
ANCA type	cANCA	cANCA	cANCA
PR3-ANCA level	23 IU/ml	> 177 IU/ml	67 IU/ml
Total B cells CD19+	1/mm ³	72/mm ³	36/mm ³

lymphocytes with the «B-cell isolation kit» (Miltenyi Biotech, Germany) followed by cell sorting of memory B cells (CD19⁺ CD27⁺) with FACS Melody (BD Biosciences, United States). Cells were immortalized using DDXK-HuBBBTM kit (Eurobio, France) by adapting the manufacturer's instructions. During the first 10 days of culture, formation of clusters was evaluated by microscopic observation and the cells were stimulated again at day 9 of culture.

Detection of PR3 Selective IgG by ELISA

After 20 days of expansion, cell culture supernatants were harvested and tested for the presence of IgG by ELISA. The IgG positive wells were selected and retested, and negative clones were eliminated. The presence of anti-PR3 IgG was then tested on positive clones by ELISA using native purified human PR3 (Athens Research, United States) for capture (2 µg/ml) and horseradish peroxidase-labeled goat anti-human IgG (1 µg/ml) for detection. Wells with at least three consecutive positive supernatants in ELISA were amplified to provide enough cells for further testing.

Purification of 4C3 Antibody

After confirmation of PR3 specificity, the 4C3 clone was amplified through successive passages from a 24-well plate to a 6-well plate and then into high density flasks (CELLLineTM, WheatonTM) consisting of two compartments separated by a 10 kDa semi-permeable, cellulose acetate membrane. Monoclonality of 4C3 was confirmed using PCR with IgG heavy chain gene rearrangements (data not shown). Cells and supernatants were harvested every three to 4 days over a period of 70 days. The viability, number of cells and level of production of anti-PR3 IgG were checked during the total culture period. All supernatants collected from cell culture were filtered through a 0.2 µm filter after centrifugation for 10 min at 500 g and antibodies contained in supernatants were purified by affinity chromatography (HitrapTM Protein A 1 mL, GE HealthCare) on an AKTATM device (GE HealthCare, United States). The purity of the different fractions was checked using SDS-PAGE and coloration with Coomassie Blue. The final 4C3 antibody concentration, obtained with a BCA assay, was 5.974 mg/ml. Specificity of 4C3 in the presence of PR3 (1 µg/ml) was confirmed by ELISA.

Recombinant Form of 4C3

RNA was isolated from the B cell clone 4C3 and cDNA was synthesized using the Superscript IV first-strand synthesis system (Invitrogen, United States). Sequences corresponding to both the VH and VL regions of the 4C3 clone immunoglobulin were PCR amplified using High-fidelity Taq polymerase Platinum SuperFi II (Invitrogen, United States) and primers were set according to Tiller et al. (34). Briefly, forward primers matched consensus sequences on human κ and γ1 IgG signal regions, reverse primers matched sequences in the constant regions. The PCR products were gel purified (PCR gel extraction kit, Qiagen, Germany) and cloned into pCR4-Blunt-TOPO vector following the manufacturer's instructions (Invitrogen, United States). DNA from positive clones was purified and analyzed for sequencing.

Sequences corresponding to VH and VL domains were PCR amplified, purified and subcloned into pFUSEss-CHlg and pFUSEss-CLlg vectors (Invivogen, France) that enabled the secreted form of both heavy and light IgG chains to be produced. Restriction digests were performed and products ligated using T4 DNA ligase as recommended by the manufacturer. Mammalian HEK-293 cells (ATCC[®] CRL-1573TM) were cultured in Free Style 293F medium (Gibco) at 37°C 8% CO₂ at 135 rpm. A total of 37.5 µg of plasmid was mixed with 37.5 µl of Free Style Transfection Reagent and incubated for 10 min at room temperature (RT) before addition to cells. Supernatant was collected at day 7 of transfection and r4C3 was purified as for 4C3. The final concentration of r4C3 was 3.5 mg/ml. The specificity of the r4C3 was confirmed by ELISA.

Characterization of 4C3 and PR3 Interaction

Immunofluorescence

2 × 10⁵ purified neutrophils were seeded on a coverslip previously coated with Poly-L-lysine for 20 min at 37°C. After washing with PBS, neutrophils were fixed with either PFA 4% for 10 min or with methanol 100% for 5 min at RT and then permeabilized with PBS-saponin 0.5% for 10 min at RT. Coupling of 4C3 with Alexa Fluor 488 (AF488) was performed with the Protein Labeling Kit (Invitrogen, United States) in accordance with the manufacturer's instructions. Neutrophils were incubated overnight at 4°C with either 4C3-AF488 (60 µg/ml) or IgG from patient P2 (1/100^e). Rabbit anti-human Fc-AF488 (Life technologies) was used as a secondary antibody for IgG P2 condition. Nuclei from cells were stained with DAPI (4',6-diamidino-2-phenylindole). Observations were performed using a fluorescence microscope (Nikon Eclipse Ti microscope) and analysis was carried out with ImageJ software.

Western Blotting Analysis of PR3 by 4C3

To confirm recognition of native antigen, neutrophils from healthy donors or HeLa cells (ATCC[®] CCL-2TM) were lysed in radioimmunoprecipitation (RIPA) / glycerol buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, and 1% sodium Deoxycholate) supplemented with phosphatase inhibitor (Sigma-Aldrich, United States). Whole-cell lysates, native and reduced PR3 (Athens Research), elastase and cathepsin G (CatG; 5 µg) were separated using SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked, then incubated with purified 4C3 overnight at 4°C. Hsc70 was used as a reference protein. After staining with horseradish peroxidase-labeled goat anti-human IgG secondary antibody, blots were revealed with an Enhanced Chemiluminescence Detection Kit (GE HealthCare, United States).

Flow Cytometry

Purified neutrophils or blood from healthy donors were first incubated with 2.5 µg of human BD Fc block (BD Pharmingen) 10 min at room temperature followed by staining with 4C3-AF488 (2 to 100 µg/ml) or with a commercial murine anti-human PR3 antibody (WGM2-FITC at 10 µg/ml from Hycult Biotech) for 30 min at 4°C before FACS analysis on a MACS Quant

(Miltenyi Biotech, Germany). Surface staining with CD45-APC H7/CD3-BV786/CD14-VioBlue/CD15-PE was also performed on blood samples. Data were analyzed with FlowJo software.

Affinity Determination of 4C3 to PR3

Affinity measurements of 4C3 and r4C3 for human PR3 were assessed by surface plasmon resonance (SPR) using a T200 apparatus (GE HealthCare). All experiments were performed at 25°C on CM5S dextran sensor chips in PBS flow buffer containing 0.005% of P20 surfactant. 4C3 or r4C3 were captured (200–300 RU) on anti-human IgG Fc-antibody immobilized on CM5S using the anti-human Fc capture kit (GE HealthCare). Increasing concentrations of PR3 protein (Athens Research, Georgia, United States) were injected at 50 µl/min on 4C3 or r4C3 coated flowcells. Phenoxy methane sulphonyl fluoride was added in the injection buffer to minimize the proteinase activity of the PR3. The K_D were evaluated with a stoichiometric Langmuir fitting model using BiacoreT200 evaluation software.

Enzymatic Activity of PR3

Enzymatic activity of PR3 was measured after incubation of 4C3 with 10 nM PR3 at different ratios (10:1, 5:1, and 2:1) for 30 min before adding the fluorescent substrate of PR3 (ABZ-VADnVADYQ-YN02 from GeneCust) (35). 50 nM α 1AT was used to inhibit PR3 activity. The fluorescence was measured through absorbance reading at a wavelength of 420 nm using spectrofluorimeter (SPECTRAMax GeminiTM XPS).

In silico Epitope Mapping of 4C3 on PR3

The 4C3 epitope was predicted using the MAbTope method (36). Briefly, MAbTope is a docking-based method that generates 5×10^7 poses for the antibody-antigen complex and filters these poses in order to obtain the 30 best solutions, using several scoring functions. The interface analysis in these 30 top-ranked solutions allowed identification of residues that exhibit the highest probability of being involved in the interaction. From the prediction, four peptides were designed from the highly probable residues and ordered with an N-terminally-linked biotin (GeneCust, Luxembourg). Each peptide was synthesized in three versions: the actual predicted peptide (numbered.2), and 2 others shifted 3 amino-acids upstream (numbered.1) or downstream (numbered.3). The interaction between the biotinylated peptides and 4C3 or Eculizumab (an anti-C5 mAb), used as a negative control, was assessed using HTRF[®] (Homogeneous Time Resolved Fluorescence). Briefly, HTRF is a TR-FRET (Time Resolved – Fluorescence Resonance Energy Transfer)-based technology allowing observing protein-protein interaction when these latter ones are coupled to donor (here a rare earth cryptates) and acceptor fluorophores and close enough so that the light emitted by the donor can activate the acceptor (37, 38). Five random peptides were also used as negative controls (CTL1 to 5). All experiments were performed in PPI-Terbium detection buffer (CisBio Bioassays, France). Peptides (2 mM) were first incubated with 8 ng 4C3 for 1 h at RT before adding 5 µl streptavidin-conjugated anti-IgG Fc antibody with HTRF compatible fluorophores, Terbium cryptate and d2, respectively, (CisBio Bioassays, France). 1 h later, the fluorescence emissions

at 620 nm and 665 nm were measured on a TriStar² LB 942 Modular microplate reader (Berthold Technologies GmbH & Co. Wildbad, Germany). Data are represented as specific FRET signals calculated as the ratio of the emission at 665 nm divided by the emission at 620 nm subtracted from the binding on the non-relevant Ab (i.e., Eculizumab).

Functional Characterization of Neutrophil Activation by 4C3

Purification of IgG From Serum

For the functional tests, we selected sera of GPA patients with active disease according to clinics, treatment and level of PR3-ANCA. GPA patients with active disease were consecutively diagnosed at the University Hospital Centre of Tours since 2017 to 2019. We purified IgG from five independent GPA patient sera during active phase of the disease after diagnosis (IgG GPA; **Table 2**) or during remission for patient P2 (IgG P2) and from two independent healthy donors with a protein G SepharoseTM 4 fast flow kit (GE HealthCare, United States). Briefly, non-pooled sera were incubated with protein G for 1 h at RT before elution. Filtration of samples was conducted using a Spin-X[®] UF Concentrator ultrafiltration system. Purified IgG from non-pooled sera underwent electrophoresis on a 4-12% Bis-tris Gel before staining with Coomassie Blue. The presence of PR3-ANCA in separate IgG preparations from active GPA patients and from patient P2 was confirmed by ELISA using an anti-PR3 EuroImmun kit, as previously described (39), whereas separate purified IgG preparations from healthy donors did not contain any PR3-ANCA.

Neutrophil Purification and TNF α Priming

Human neutrophils from independent healthy donors were purified using negative magnetic selection with the commercial kit “EasySep Direct Human Neutrophil Isolation Kit” (StemCells, Canada) following the manufacturer’s instructions. At the end of isolation, neutrophils were suspended in HBSS solution without calcium and magnesium. The purity of isolated neutrophils was around >95%, as assessed by flow cytometry (CD15-PE and Live dead, Miltenyi Biotech, Germany). Cells were primed with TNF α (Sigma-Aldrich, United States) at a final concentration of

TABLE 2 | Main characteristics of active GPA patients, consecutively diagnosed from 2017 to 2019, from which IgG were purified to induce auto-immune activation of neutrophils.

	GPA 1	GPA 2	GPA 3	GPA 4	GPA 5
Sex	Male	Male	Male	Male	Male
Age at diagnosis	68 y/o	71 y/o	79 y/o	77 y/o	53 y/o
Organ impairment	Kidney, neurological and ENT	Articular and ENT	Kidney and articular	Pulmonary and ENT	Kidney and pulmonary
ANCA type	cANCA	cANCA	cANCA	cANCA	cANCA
PR3-ANCA level	83 IU/ml	36 IU/ml	43 IU/ml	112 IU/ml	58 IU/ml

2 ng/ml for 15 min at 37°C in a water bath (primed-neutrophils) as previously described (13, 40–43).

Assessment of ROS Production by Neutrophils

The activation of neutrophils from independent healthy donors was evaluated through ROS production using a dihydrorhodamine 123 (DHR 123) assay as previously described (39, 40, 42). Purified neutrophils were suspended in HBSS with 1 mM Ca^{2+} and 1 mM Mg^{2+} and incubated with 5 $\mu\text{g/ml}$ cytochalasin B (Cayman Chemical, United States) to enhance oxygen radical production, for 5 min at 37°C. Cells were then loaded with 2 μM DHR 123 and 2 mM Sodium Azide (NaN_3) for 5 min at 37°C under agitation. Primed-neutrophils were incubated with 4C3 (2 to 100 $\mu\text{g/ml}$), r4C3 or separate IgG preparations from two healthy donors and from four active GPA patients (200 $\mu\text{g/ml}$) for 45 min at 37°C. A non-relevant antibody (6H4, IgG1 κ , anti-ovalbumin) was used as a negative control. A combination of phorbol myristate acetate (PMA, 50 ng/ml) and calcium ionophore (ICa, 10 μM), both powerful neutrophil activators (44, 45), was used as a positive control of neutrophil activation. The reaction was stopped with ice-cold PBS-EDTA (1 mM) before measurement of DHR 123 fluorescence using flow cytometry. For neutralization experiments, pre-treated primed-neutrophils were first incubated with 4C3 (20 $\mu\text{g/ml}$) for 15 min at 37°C and then separate IgG preparations from five active GPA patients at diagnosis (IgG GPA, 200 $\mu\text{g/ml}$) were added for 45 additional minutes. To determine the influence of heterogeneous neutrophils from healthy donors, the level of membrane PR3 expression was assessed for each donor using flow cytometry.

Degranulation and Adhesion of Neutrophils

Neutrophils were primed with $\text{TNF}\alpha$ and stimulated with 4C3 (2 and 20 $\mu\text{g/ml}$), r4C3 (2 and 20 $\mu\text{g/ml}$), IgG from GPA patients (200 $\mu\text{g/ml}$) or PMA-ICa for 45 min at 37°C. After incubation, cells were washed and stained with CD63-FITC (degranulation) or double stained with CD11b VioBlue/CD18 FITC (BD Biosciences, United States; adhesion phenotype) for 20 min at 4°C before analysis using flow cytometry. The percentage of positive cells and MFI were determined using FlowJo software. The degranulation of neutrophils was also assessed by CatG release. Supernatants were harvested and incubated with fluorescent substrate of cathepsin G (ABZ-TPFSGQ-YNO2 from GeneCust) (46) for 30 min before fluorescence reading by spectrofluorimetry at 420 nm. Results are expressed as a ratio of ΔRFU compared to the normalized $\text{TNF}\alpha$ condition (basal degranulation).

Properties of 4C3 Fc Portion Glycosylation Study

The glycoform profile of the 4C3 Fc domain was assessed through high-resolution mass spectrometry using a Vion IMS-QT mass spectrometer hyphenated to an Acquity UPLC H-Class chromatography (Waters, United Kingdom). 800 ng of 4C3 was injected onto an XBridge BEH300 C4 column heated to 90°C. A desalting step was conducted using an isocratic gradient with 95 % solvent A (H_2O + 0,1 % formic acid) and 5 % solvent B (acetonitrile + 0,1 % formic acid) for 2 min at 0.5 ml/min.

Then, 4C3 was eluted with a flow rate of 0.4 ml/min. Data were acquired on positive mode with an ESI source over a 500 to 4,000 m/z range with a scan rate of 1 Hz and analyzed using UNIFI 1.9.4 software and MaxEnt algorithm for deconvolution (Waters, United Kingdom).

Affinity Determination of 4C3 Binding to Fc γ RIIIB

The interaction between 4C3 and human Fc γ RIIIB was performed using poly-histidine Fc γ R (R&D Systems, United States) captured on an immobilized anti-poly-histidine mAb (GE HealthCare, United States). Affinity measurements were assessed using SPR as described above for 4C3 and PR3 and evaluated through a steady-state equilibrium fitting model. Rituximab (an anti-CD20 mAb) was used as a control for Fc γ RIIIB binding.

Statistical Analysis

Results are expressed as the means \pm SD. Normality of sample distribution was tested prior to conduct any comparison between groups. Non-parametric statistical tests were performed as normality failed, and/or equal variance test failed. Statistical significance was determined using Mann–Whitney rank sum test for unpaired data or using Wilcoxon matched-pairs signed rank test for paired data, depending on the analysis. When necessary, Kruskal Wallis was used to compare groups pairwise. Graphs were produced using GraphPad Prism 7 software (La Jolla, CA, United States).

RESULTS

4C3 Is an IgG1 κ PR3-ANCA Able to Recognize Both Soluble PR3 With a High Affinity and Membrane Bound PR3

Existence of non-pathogenic PR3-ANCA is suggested by the existence of PR3-ANCA in healthy donors (20) and the inconsistent correlation between PR3-ANCA level and disease activity in GPA (15). Moreover, a high level of PR3-ANCA can persist during remission without predicting relapse (16). Therefore, we decided to immortalize B lymphocytes from three GPA patients (P1, P2, and P3) using an EBV-derivative immortalization procedure in order to access the entire B cell repertoire in few weeks and to obtain clones producing fully human Abs. Clinical characteristics of the three GPA patients (P1, P2, and P3) are summarized in **Table 1**. Blood samples from the GPA patients in remission having a persistent PR3-ANCA level (**Table 1**) were collected before B cell immortalization but mAbs were successfully obtained for only P2. Indeed, P1 had a too low level of total B lymphocytes and P3 had a high number of granulocytes during culture. P2 was diagnosed with GPA in 2011 following ENT (Ear, Nose and Throat) and pulmonary disorders with alveolar hemorrhage. This patient was first treated with cyclophosphamide for 6 months followed by treatment with corticosteroids and methotrexate. The last biological examination at the time of collection indicated a circulating B-cell count of $72/\text{mm}^3$ and a PR3-ANCA level greater than 177 IU/ml. For this patient, we obtained approximately 1000 wells containing

immortalized B lymphocytes and about 50% of these clones produced detectable IgG. From these, three clones (4C3, 4C5, and 5D11) were selected taking into account their capacity to produce antibodies able to recognize PR3 (**Figure 1A** left panel). Clone 4C5 spontaneously stopped producing Abs. Finally, 4C3 was the only mAb specific to PR3 (Patent n°19/11722). Indeed, 4C3 did not recognize non-relevant antigens such as ovalbumin, peanut and alpha-gal, whereas 5D11 did (**Figure 1A** right panel). Using ELISA, we determined that 4C3 was an IgG1 (**Figure 1B** left panel) with a light chain kappa (IgG1κ; **Figure 1B** right panel) whereas the serum from P2 contained different subclasses of IgG. By sequencing, we revealed that 4C3 had a G1m3,1 allotype (data not shown).

To further characterize 4C3 properties, we investigated its ability to bind human neutrophils using IF microscopy. A diffuse cytoplasmic fluorescence was observed, which is the typical cANCA appearance to diagnose GPA patients (**Figure 1C**). The same staining was observed with the IgG of patient P2 from which 4C3 clone derived confirming the presence of cANCA in this serum (**Supplementary Figure 1**). To confirm the anti-PR3 specificity, 4C3 was used as the primary antibody in a western blotting analysis performed on protein lysates of primed-neutrophils from healthy donors as well as HeLa cells and human PR3 used as negative and positive controls, respectively. 4C3 recognized human native PR3 with the expected molecular weight (**Figure 1D** upper panel). By contrast, no staining was observed in HeLa cells, which do not express PR3 (**Figure 1D** upper panel). In addition, a single band around 28 kDa appeared in the condition of primed neutrophil lysate (**Figure 1D** upper panel). Finally, we showed that 4C3 did not recognize other neutrophil proteases such as elastase and CatG, which have the same molecular weight as PR3 (**Figure 1D** lower panel). Taken together, these results show that 4C3 specifically binds PR3.

After demonstrating the recognition of the intracellular neutrophilic PR3, we investigated the ability of 4C3 to bind mbPR3 using flow cytometry of immune cells from human blood. A high level of staining on CD15⁺ neutrophils could be observed. No significant staining could be observed on CD3⁺ T lymphocytes, which do not expose any PR3 and a moderate staining on CD14⁺ monocytes could also be observed. By contrast, a high level of staining on CD15⁺ neutrophils could be observed with a complete right shift of the histogram (**Figure 1E**). Moreover, we also obtained a bimodal expression of PR3 for two independent healthy donors and no expression of PR3 was found for five healthy donors (data not shown). The observed preferential binding to neutrophils was confirmed through flow cytometry analysis using 4C3 on activated or non-activated purified human neutrophils with different stimuli (**Figure 1F**). We observed a MFI increase when 4C3 was incubated with TNFα-primed-neutrophils. It should be noted that TNFα has previously been shown to induce translocation of intracellular PR3 to the membrane (47). In contrast, when incubated with α1AT, a natural inhibitor which modifies hydrophobic patch of PR3 and removes the induced mbPR3 from the membrane of primed-neutrophils (29), there was a significant decrease in the MFI. On the other hand, PMA-ICa, strong activators of neutrophils (44, 45), induced a marked increase in the MFI with

4C3 (**Figure 1F**). Furthermore, this staining was comparable to that obtained with WGM2 mAb, a commercial murine anti-human PR3 antibody, on human neutrophils tested under the same conditions (**Supplementary Figure 2**). These results confirm that 4C3 is able to bind mbPR3. Finally, using SPR, we showed that 4C3 binds PR3 with a high affinity of 7.4×10^{-10} M (**Supplementary Table 1**). 4C3 from immortalized P2 B cells is a typical IgG1κ PR3-ANCA able to recognize soluble PR3 with a high affinity and mbPR3.

4C3 Binds to a New Conformational Epitope on PR3

Depending on the patient and the stage of the disease, ANCA recognize conformational rather than sequential epitopes (23, 48). In order to study the role of PR3 conformation in the interaction with 4C3, we performed western blotting analysis using both native and denatured conditions and 4C3 as the primary antibody. PR3 binding was significantly impaired under denatured conditions compared to native conditions, suggesting that 4C3 recognizes a conformational epitope on PR3 (**Figure 2A**). In order to map such a conformational epitope on PR3, *in silico* docking analyses were performed based on the publically available PR3 structure (49) and structure modeling of VH and VL regions from 4C3 (Patent n°19/11722). The 30 top-ranked solutions obtained from the docking procedure for the 4C3-PR3 complex are well grouped on the 3D structure of PR3 and are represented in **Figure 2B** (**Figure 2B** upper panel). Amino acid residues of PR3 involved in these top-ranked solutions were divided into four categories according to their raw probability of belonging to the epitope, from purple for the highest probability to light blue for the lowest but still significant probability (**Figure 2B** middle panel) and on the linear sequence of PR3 (**Supplementary Figure 3A**). From the predictions of residues belonging to the epitope, four groups of validation peptides, each constituted of 15 residues, were then designed and represented on the 3D structure of PR3 (**Figure 2B** lower panel). For each of the 4 targeted regions of 15 residues, two more derivative 15-residue long peptides were designed: the first starting and ending 3 residues downstream and the second starting and ending 3 residues upstream (**Table 3**). Among those peptides, 4C3_3.2 (a.a 152–166) and 4C3_4.1 (a.a 122–136) significantly induced the highest HTRF ratios (**Figure 2C**) suggesting that these amino acid sequences correspond to the epitope recognized by 4C3 (**Supplementary Figure 3A**). Therefore, the conformational epitope recognized by 4C3 contains some residues of peptides 3.2 (in orange) and 4.1 (in yellow) exposed on the PR3 surface and located around the hydrophobic patch (in green) and in close vicinity to the active site (in purple) of PR3 as shown on **Figure 2D** (**Figure 2D**). Two possible orientations of 4C3 (in blue) among the 30 top-ranked solutions obtained from the docking procedure are also shown in **Figure 2D**. The first shows that 4C3 masks the hydrophobic patch without preventing access to the active site (**Figure 2D**, upper panel) while the second represents 4C3 masking the hydrophobic patch with an inaccessible active site (**Figure 2D**, lower panel). In

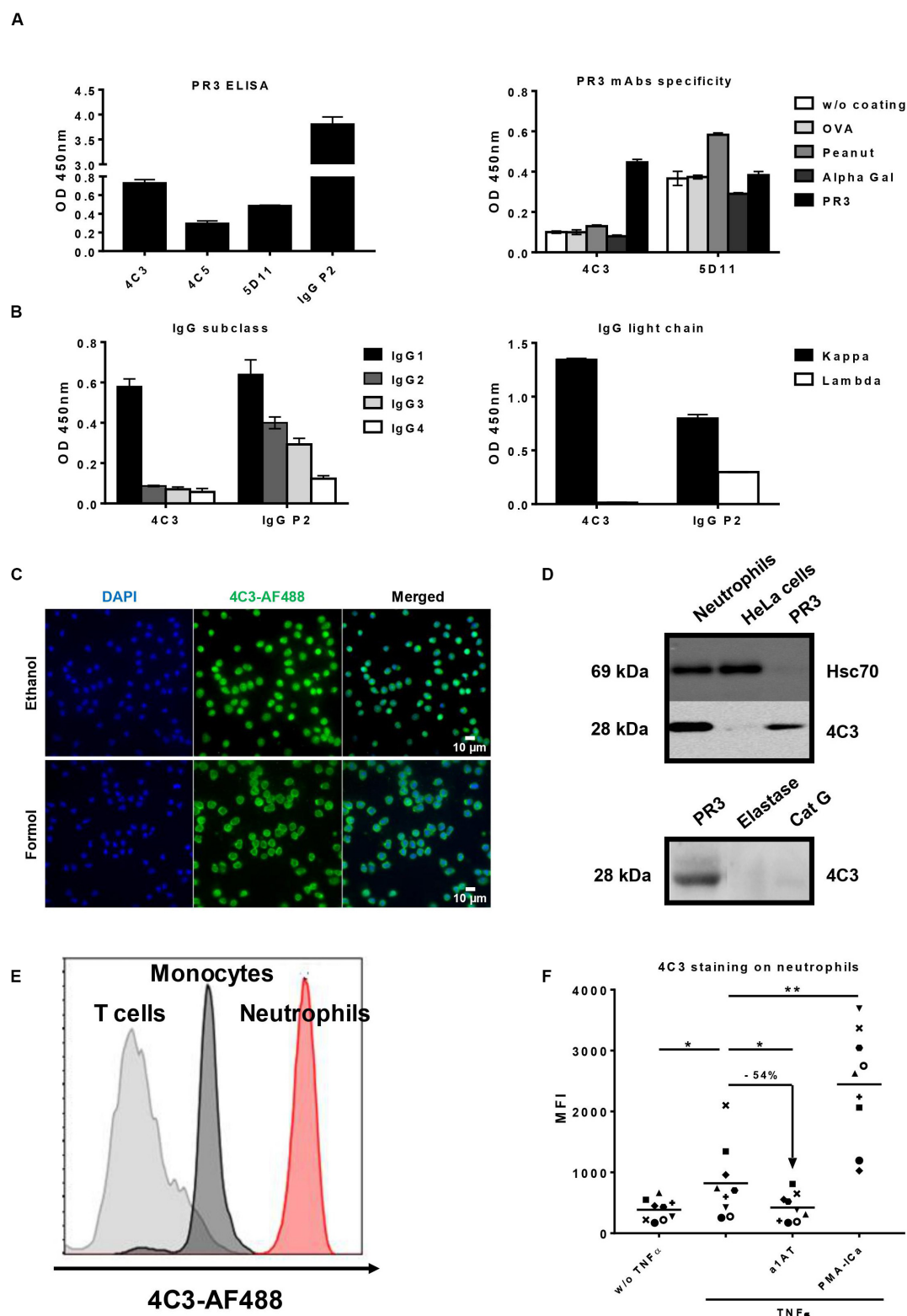


FIGURE 1 | Identification and characterization of an anti-proteinase 3 (PR3) monoclonal human antibody 4C3. **(A)** Identification of a monoclonal human antibody specific to PR3. Binding of native PR3 by ELISA (left panel) in the presence of three antibodies (4C3, 4C5, and 5D11) obtained by immortalization of P2 B cells or serum from P2 containing PR3-ANCA used as a positive control (IgG P2). Similar results were obtained in four independent experiments. Specificity of 4C3 and 5D11 was checked by ELISA (right panel) in the presence of different antigens (PR3, OVA, Peanut, Alpha Gal). $n = 5$. **(B)** 4C3 is an IgG1 κ . Subclass (left panel) and (Continued)

FIGURE 1 | Continued

nature of light chain (right panel) of 4C3 were determined by ELISA compared to IgG P2. Similar results were obtained in three independent experiments. **(C)** 4C3 has a PR3-ANCA immunofluorescence pattern. Human neutrophils from healthy donors were purified and then fixed with ethanol (upper line) or formal (lower line) before staining with DAPI and 4C3-AF488 (1/100^e). Objective x60. Superposition of fluorescence with ImageJ (Merged). Results from one of three experiments are presented. **(D)** 4C3 specifically recognizes intracellular PR3. Protein lysates (10 µg) from human neutrophils and HeLa cells were loaded before incubation with anti-Hsc70 or 4C3 antibodies for western blotting analysis. PR3 was used as a positive control, elastase and CatG (5 µg) as negative controls. Similar results were obtained in three independent experiments. **(E)** 4C3 has a membrane staining compatible with mbPR3 staining. Blood from a healthy donor was stained with a mixture containing CD45-APC H7/CD3-BV786/CD14-VioBlue/CD15-PE/4C3-AF488. Analysis of 4C3 staining at the surface of T cells (gated on CD3 positive cells, gray histogram), of monocytes (gated on CD14 positive cells, black histogram), and of neutrophils (gated on CD15 positive cells, red histogram). Similar results were obtained in five independent experiments. **(F)** 4C3 binds mbPR3 on quiescent and activated neutrophils. Human purified neutrophils from nine independent healthy donors were primed (TNFα) or not (w/o TNFα) with TNFα at 2 ng/ml for 15 min before staining with 4C3-AF488 (20 µg/ml). Alpha 1 anti-trypsin (α1AT) was used to solubilize mbPR3 by modifying its hydrophobic patch which is involved in its interaction with the membrane. PMA-ICa was used as a positive control of neutrophil activation. Each symbol represents one healthy donor. *n* = 9. NS: Non-significant; **p* < 0.05 and ***p* < 0.005.

addition, the binding of 4C3 to PR3 observed using an ELISA was impaired by 44% in the presence of α1AT, inducing a conformational change of PR3 and preventing the binding of the PR3-ANCA-recognizing epitope 1 to PR3 (29) (**Supplementary Figure 3B**). This confirms that the epitope recognized by 4C3 is partially masked by α1AT and that some amino acid residues involved in this epitope are localized in the epitope 1, close to the active site of PR3. Moreover, we demonstrated that incubation of PR3 with molar excess of 4C3 did not result in a decrease in PR3 enzymatic activity, unlike with α1AT, which induced an inhibition (**Supplementary Figure 3C**), thus suggesting that the active site is still accessible in the presence of 4C3.

To conclude, 4C3 recognizes a conformational epitope on PR3 localized close to the active site and the hydrophobic patch on a region overlapping that of epitope 1 without affecting PR3 enzymatic activity.

4C3 Inhibits Neutrophil Activation Induced by Polyclonal PR3-ANCA

Proteinase 3-ANCA have a central role in GPA, by cross-linking mbPR3 and Fc gamma receptors at the surface of neutrophils, causing auto-immune activation of the latter (7, 8). *In vitro* stimulation of TNFα-primed-neutrophils by chimeric anti-human PR3 mAbs or purified IgG from GPA patients (IgG GPA) induces their auto-immune activation leading to ROS production, protease release by degranulation and adhesion molecules upregulation (10, 12, 13).

To characterize 4C3 further, we investigated the *in vitro* functionality of 4C3 on human primed-neutrophils (**Figure 3**). First, we assessed ROS production induced by primed-neutrophils after 4C3 stimulation. 4C3 was not able to induce ROS production whereas separate IgG preparations from active GPA patients at diagnosis (IgG GPA) led to marked production. On the contrary, separate IgG preparations from healthy donors (IgG HD), obtained and used under the same conditions, did not allow ROS production by primed-neutrophils (**Figure 3A**). Interestingly, neutrophil stimulation by non-pooled purified IgG from the patient P2 did not induce significant ROS production compared to IgG GPA purified from GPA patients at diagnosis (**Figure 3A**). We checked that neutrophil activation induced by purified IgG GPA (from non-pooled preparations) was specific to PR3

activation as pre-incubating neutrophils with α1AT strongly decreased ROS production induced by IgG GPA (data not shown). Primed-neutrophil stimulation with lower (2 µg/ml) and higher (100 µg/ml) concentrations of 4C3 showed no increase in ROS production (**Supplementary Figure 4A**). The capacity of neutrophils to degranulate was measured by CD63 expression at the neutrophil surface and also by proteinase release in supernatants after activation. Similar results were obtained with no difference between 4C3 stimulation and TNFα condition or IgG from patient P2 on CD63 staining and CatG activity (**Figure 3B** and **Supplementary Figure 4B**). Moreover, we analyzed the expression of CD11b and CD18 (Mac1 complex) to explore the adhesion phenotype of neutrophils following 4C3 stimulation. The presence of 4C3 did not induce any increased surface expression of CD11b/CD18 whereas non-pooled IgG preparations from healthy donors or GPA patients at diagnosis significantly induced upregulation of these two adhesion markers (**Figures 3C,D**). It should be noted that IgG from patient P2 induced an intermediate adhesion phenotype (**Figures 3C,D**).

We then studied the capacity of 4C3 to inhibit neutrophil activation induced by polyclonal PR3-ANCA. Neutrophils were pre-incubated with 4C3 or with 6H4, a human anti-ovalbumin IgG1κ strictly produced and stored under the same conditions as 4C3. Purified IgG was obtained from five distinct GPA patients at diagnosis (IgG GPA) and then added to the two neutrophil preparations and ROS production analyzed as previously described. Interestingly, 4C3 was able to reduce the ROS production induced by IgG GPA stimulation with an inhibition mean of 58% on the five different patients (percentages of inhibition from 36 to 71%; **Figure 3E**). On the contrary, 6H4, a non-relevant antibody, was not able to inhibit the effect of IgG GPA (**Figure 3E**). Taken together, these results indicate that 4C3 did not activate neutrophils and was able to inhibit neutrophil activation induced by IgG GPA.

Properties of 4C3 Fc Portion do Not Explain the Non-activation of Neutrophils

We previously demonstrated that 4C3 was not able to induce primed-neutrophil activation. Therefore, we studied the properties of its Fc portion in order to investigate whether this unexpected feature could result from a defect in the 4C3 Fc

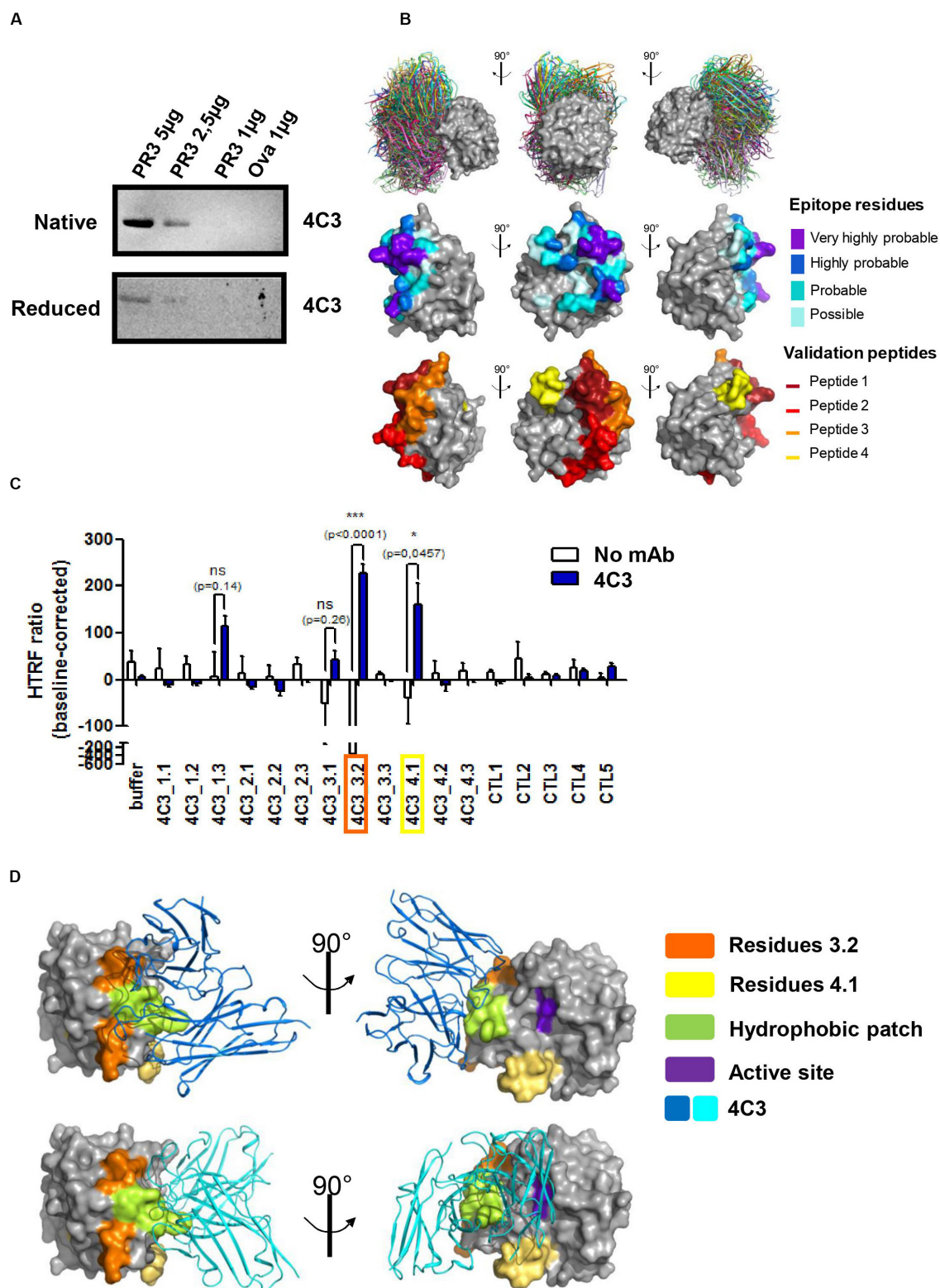


FIGURE 2 | 4C3 binds PR3 on a new epitope near the hydrophobic patch and close to the active site. **(A)** 4C3 preferentially recognizes a conformational epitope. Recognition of native (upper gel) or reduced PR3 (lower gel) used at different quantities in western blots after incubation of the membrane with 4C3. Ovalbumin was used as a negative control of binding. **(B)** Prediction of the epitope recognized by 4C3 on PR3. View of the top 30 ranked predicted conformations for the complex between 4C3 and PR3. The target is shown as a gray surface and the antibody in color (first line). The residues that probably belong to the epitope are shown on the

(Continued)

FIGURE 2 | Continued

structure of PR3 in four categories from purple for the highest probability to light blue for the lowest but still significant probability (second line). Four groups of validation peptides were designed and for each group, the targeted region of 15 residues is presented (third line). **(C)** 4C3 preferentially binds peptides 3.2 and 4.1 of PR3. Analysis by HTRF of the binding of 4C3 with PR3 predicted peptides. Results are expressed as means \pm SEM of three independent experiments and each condition was tested in triplicates. Statistical differences are indicated on the graph (* $p < 0.05$; *** $p < 0.0005$). **(D)** 4C3 recognizes an epitope near the hydrophobic patch and close to the active site of PR3. The amino acids corresponding to the positive peptides (3.2 in orange and 4.1 in yellow) are indicated on the surface of PR3 (in gray). The catalytic triad (active site) of PR3 is shown in purple while the hydrophobic patch is represented in green. Two possible predicted conformations for the complex 4C3 (in blue) and PR3 (in gray) among the top 30 conformations are presented.

TABLE 3 | Validation peptide sequences.

Peptide	Start	Sequence	End
4C3_1.1	188	GIDSFVIWGCATRLF	202
4C3_1.2	191	SFVIWGCATRLFPDF	205
4C3_1.3	194	IWGCATRLFPDFFTR	208
4C3_2.1	72	HFSVAQVFLNNYDAE	86
4C3_2.2	75	VAQVFLNNYDAENKL	89
4C3_2.3	78	VFLNNYDAENKLNDI	92
4C3_3.1	149	TWTFRCRPHNICTF	163
4C3_3.2	152	TFFCRPHNICTFVPR	166
4C3_3.3	155	CRPHNICTFVPRRKA	169
4C3_4.1	122	GTQCLAMGWGRVGAH	136
4C3_4.2	125	CLAMGWGRVGAHDPP	139
4C3_4.3	128	GWGRVGAHDPPAQV	142

portion. 4C3 was able to bind Fc γ RIIB (CD16b) with an affinity of 2.7×10^{-6} M (**Supplementary Figure 5**) and prevented the binding of an anti-Fc γ RIIA (anti-CD32), similarly to rituximab used as a control, on the THP-1 human monocytic cell line (data not shown). These results suggest that 4C3 is able to bind the two main Fc gamma receptors at the neutrophil surface.

Moreover, mass-spectrometry profiling of the glycosylation pattern of 4C3 demonstrated an increase in galactose residues revealing a G2F profile compared to the classical asparagine 297-linked glycosylation of an IgG1 (**Figure 4A**).

In order to confirm that the non-activation of primed-neutrophils induced by 4C3 was not due to the properties of its Fc portion, we engineered a recombinant form of 4C3 (r4C3) with a different Fc portion. After 4C3 sequencing, an IgG1 recombinant form was produced with a different allotype (G1m17,1) in a different cell culture system (HEK). Therefore, 4C3 and r4C3 had the same variable heavy and light chain region but a slightly different Fc portion. We first showed that 4C3 and r4C3 had the same ability to recognize PR3 using ELISA (**Figure 4B**). This result was confirmed by SPR as r4C3 had a similar affinity for PR3 to 4C3 (**Figure 4C** and **Supplementary Table 1**). Finally, primed-neutrophil stimulation with r4C3 did not cause significant ROS production as the negative control and 4C3 contrary to the powerful activator PMA-ICa (**Figure 4D**) and no CatG release was observed in the presence of r4C3 (**Figure 4E**).

To conclude, 4C3 does not induce primed-neutrophils activation despite having a functional Fc portion. r4C3, with a different but functional Fc portion, does not induce primed-neutrophils activation either. These results suggest that non-activation of primed-neutrophils by 4C3 is not due to the Fc

portion properties of 4C3, but rather to its Fab domain and therefore to the epitope recognized on PR3.

DISCUSSION

Clinical observations suggest the existence of non-pathogenic PR3-ANCA in GPA: 1/correlation between PR3-ANCA level and disease activity is inconsistent (15), 2/a high level of PR3-ANCA can persist during remission without predicting relapse (16), and 3/PR3-ANCA can be detected in healthy donors (20). Furthermore, the existence of non-pathogenic PR3-ANCA could be rational to scavenge neutrophils debris. Indeed, a negative correlation between circulating PR3 and ANCA in AAV patients in remission has been observed and is an indirect argument supporting this hypothesis (50). To our knowledge the existence of non-pathogenic PR3-ANCA has never been demonstrated. Indeed, PR3-ANCA pathogenicity, i.e., their potential to induce auto-immune activation of neutrophils, is influenced by several factors including the level of mbPR3 on neutrophils (9), their epitope on PR3 (23), their type of Ig (14, 30, 51) and their glycosylation (31–33). All these characteristics have been studied with chimeric or murine anti-human PR3 antibodies or with human anti-PR3 polyclonal IgG but never with a human monoclonal anti-PR3 antibody.

In this study, we obtained and sequenced for the first time a fully human PR3-ANCA mAb (4C3) by immortalization of B lymphocytes from a GPA patient in remission who had a persistently high level of PR3-ANCA. Most of monoclonal antibodies are produced by transfection of genes encoding human immunoglobulin from cell lines or hybridomas after immunization. These antibodies are not fully human. In this study, we chose B cell immortalization, starting directly from human B lymphocytes, in order to obtain a fully human mAb closer to those produced in GPA patients. The main interests with this method are that the fully human monoclonal antibodies obtained had relevant PR3 recognition, Fc functionality and glycosylation similar or near to that of the PR3-ANCA from P2 patient (a patient in remission).

4C3 has all the characteristics of a classic PR3-ANCA: a cANCA IF pattern and a specificity toward PR3 (1). Furthermore, 4C3 is an IgG1k, the most frequent subclass of PR3-ANCA able to induce neutrophil activation (14, 30, 41). Nevertheless, 4C3 appears to be non-pathogenic as it was not able to induce *in vitro* human neutrophil activation contrary to human polyclonal PR3-ANCA from the acute phase of GPA. A limitation of this presented study is the investigation of only one mAb anti-PR3 without any activating mAb obtained from a GPA patient

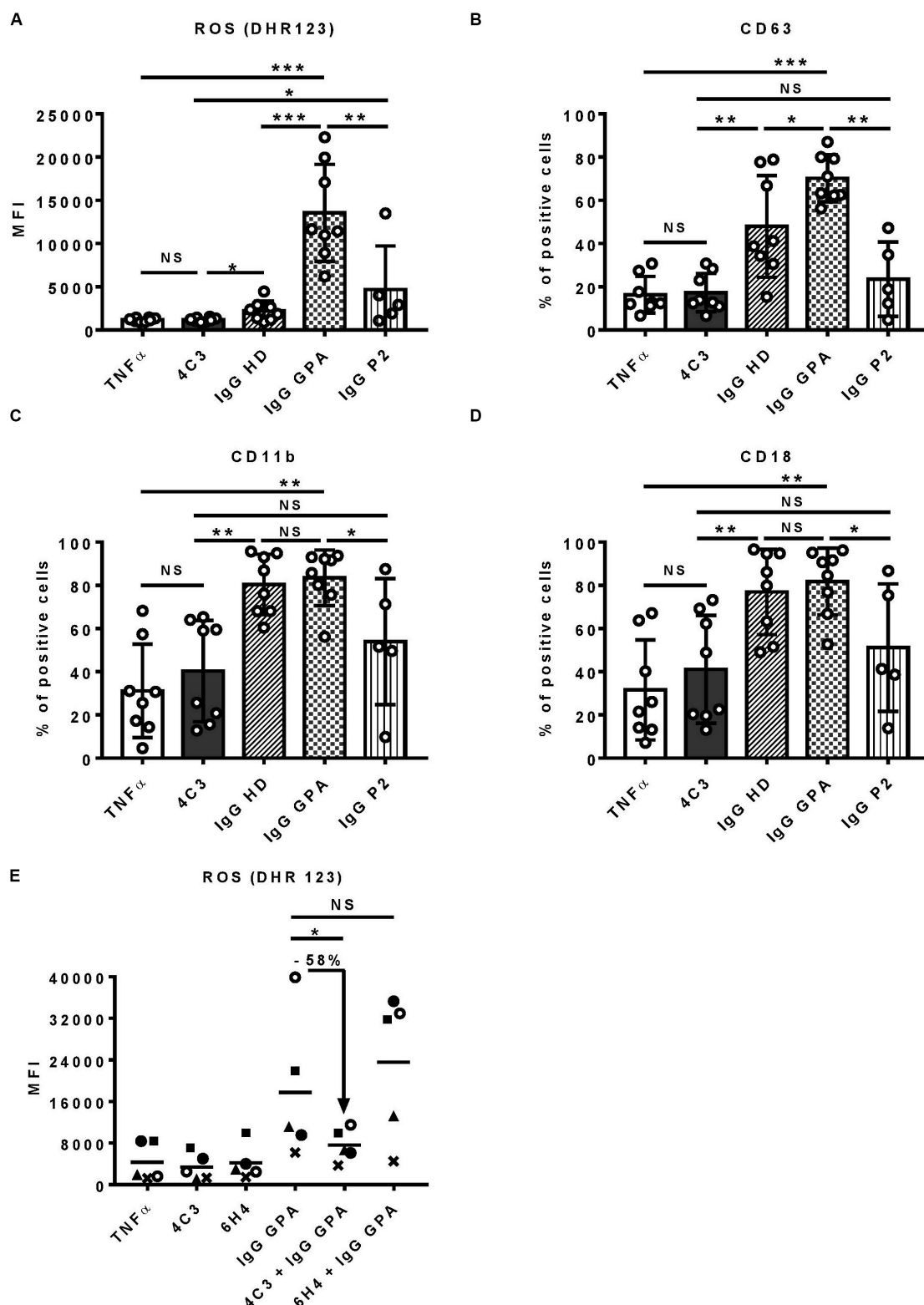


FIGURE 3 | 4C3 does not allow activation of human neutrophils and inhibits PR3-ANCA-induced activation. Purified neutrophils from eight independent healthy donors were primed with TNF α (2 ng/ml) for 15 min at 37°C (white columns) before incubation for 45 min with 4C3 (gray columns), separate (non-pooled) IgG preparations from two healthy donors (hatched columns) or from four active GPA patients at diagnosis (checkered columns) or IgG from patient P2 (vertical lines).

(A) 4C3 does not induce production of reactive oxygen species (ROS) by neutrophils. ROS production was assessed by measuring the fluorescence (MFI) of DHR

(Continued)

FIGURE 3 | Continued

123 by flow cytometry. **(B)** 4C3 does not induce degranulation. The degranulation of neutrophils was assessed by CD63 expression represented in percentage of positive cells. **(C, D)** 4C3 does not increase the adhesion phenotype of neutrophils. The adhesion criteria of neutrophils was assessed by measuring CD11b **(C)** and CD18 **(D)** surface expressions by flow cytometry. Results are expressed as mean \pm SEM obtained in eight independent experiments with a circle representing one experiment. NS: Non-significant; * $p < 0.05$; ** $p < 0.005$; and *** $p < 0.0005$. **(E)** 4C3 is able to inhibit ROS production induced by polyclonal PR3-ANCA. After priming, neutrophils from five independent healthy donors were first incubated for 45 min with 4C3 (gray column) or 6H4 (non-relevant mAb obtained under the same conditions as 4C3; black column) before addition of separate IgG preparations purified from five independent active GPA patients (IgG GPA at 200 μ g/ml). Each symbol represents one healthy donor. ROS production was assessed by measuring the fluorescence (MFI) of DHR 123 by flow cytometry. The percentage of inhibition induced by 4C3 in ROS production is indicated on the graph. NS: Non-significant; * $p < 0.05$. $n = 5$.

at diagnosis. However, the existence of non-pathogenic PR3-ANCA, as 4C3, could explain why PR3-ANCA can persist during remission without predicting relapse and why they can be found in healthy donors.

Using the original MABTope method and HTRF assay, we were able to demonstrate that 4C3 recognized a conformational epitope on PR3 and amino acid residues involved in this epitope are contained in linear peptides 4C3_3.2 (a.a 152–166) and 4C3_4.1 (a.a 122–136). Based on a 3D structure, the conformational epitope recognized by 4C3 is localized around the hydrophobic patch that allows the PR3 to be anchored to the neutrophil membrane (29). However, this particular location did not prevent 4C3 binding to the neutrophil surface. Indeed, some commercial antibodies targeting epitope 5, situated in the hydrophobic patch region, are not able to bind mbPR3 (29). Moreover, α 1AT complexation with PR3 partially inhibits 4C3 binding on its epitope suggesting that some amino acid residues involved in this epitope are localized on epitope 1. Indeed, the binding of PR3-ANCA recognizing epitope 1 is impaired by α 1AT inducing a conformational modification of PR3 (29). The main epitope region recognized by 4C3 could be confirmed by inhibition ELISA experiments using murine anti-PR3 monoclonal antibodies as previously performed with patient sera containing PR3-ANCA (52). We also confirmed the binding of 4C3 to mbPR3 on monocytes and neutrophils compared to a commercial murine anti-human PR3 mAb recognizing epitope 3 on PR3 (25). The capacity of 4C3 to bind mbPR3 after the priming of neutrophils was a necessary prerequisite to study neutrophil activation (9). Finally, it should be noted that it is the first time that the affinity of a human PR3-ANCA to PR3 has been evaluated. 4C3 binds PR3 with a high affinity (K_D : 7.4×10^{-10} M). This affinity is a thousand-fold higher than that of IgG to Fc γ R (53) and supports that the binding of PR3-ANCA to mbPR3 is a fundamental step in fixing PR3-ANCA to the neutrophil surface.

Despite being a human IgG1 PR3-ANCA with high binding affinity to mbPR3, 4C3 was not able to induce the activation of primed-neutrophils. Indeed, primed-neutrophil stimulation with 4C3 did not induce any ROS production, degranulation or CD11b/CD18 upregulation by human neutrophils. Furthermore, neutrophil pre-incubation with 4C3 significantly reduced by 58% the ROS production induced by pathogenic IgG GPA stimulation. GPA is still a serious disease despite current therapies used (54), with a mortality rate of 25% at 5 years (3), significant morbidity related to the disease and its management (55) and a high risk of relapse (56). Thus, 4C3 ability to inhibit the fundamental implication of PR3-ANCA binding to PR3 in the autoimmune

activation of neutrophils seems an attractive way to develop new therapies. This result is in line with results obtained with α 1AT, a serine protease inhibitor, which clears PR3 from the membrane and thus prevents its binding to PR3-ANCA. Indeed, it has already been demonstrated that pre-incubating neutrophil with α 1AT decreases ROS production induced by PR3-ANCA (40, 57). In our study, 4C3 did not clear mbPR3, as α 1AT did, but may act in competition with pathogenic PR3-ANCA. Different hypotheses could be raised: the first suggests that the interaction of 4C3 with PR3 changes the conformation of the latter and consequently changes the major epitope of pathogenic PR3-ANCA. The second and third possibilities are that when 4C3 binds mbPR3, the major epitope of pathogenic PR3-ANCA is masked or 4C3 Fc domain limits their binding to Fc γ Rs. In all cases, this prevents the interaction of pathogenic PR3-ANCA with neutrophils. Therefore, the 4C3 capacity to inhibit auto-immune activation of neutrophils induced by polyclonal IgG PR3-ANCA, although tested by neutrophil pre-incubation with 4C3, could be a promising therapeutic strategy at the active phase of the disease. Indeed, 4C3 has the advantage to have a high-affinity to PR3 and has a long half-life of an IgG1. 4C3 could compete on neutrophils fixation, especially on young neutrophils, before pathogenic PR3-ANCA binding. However, the non-pathogenic characteristic of 4C3 needs further investigation but is an interesting avenue to develop regarding the pathogenicity of PR3-ANCA.

The non-activation of neutrophils by 4C3 could not be explained by the nature of the mAb because chimeric anti-human PR3 mAb was able to induce neutrophil activation in the literature (14, 58). Furthermore, polyclonal stimulation of neutrophils with purified IgG from remission patient P2 (from which 4C3 was obtained and containing a high PR3-ANCA level), did not cause significant neutrophil activation compared to IgG GPA. The correlation between neutrophils activation induced by IgG fractions from GPA patients and disease activity of these patients has already been observed (51). This interesting result highlights that a polyclonal solution of PR3-ANCA is not sufficient to induce neutrophil activation probably because the proportion of pathogenic and non-pathogenic PR3-ANCA is variable from one patient to another. We can therefore deduce that the original patient P2 had non-pathogenic PR3-ANCA. Finally, it must be underlined that auto-immune activation of neutrophils by IgG ANCA was recently challenged by Popat and Robson. Indeed, purified IgG from AAV patient sera, even in active disease, did not induce neutrophil activation from two different healthy donors (59). Interestingly, the same IgG preparations obtained from MPO-ANCA positive patients

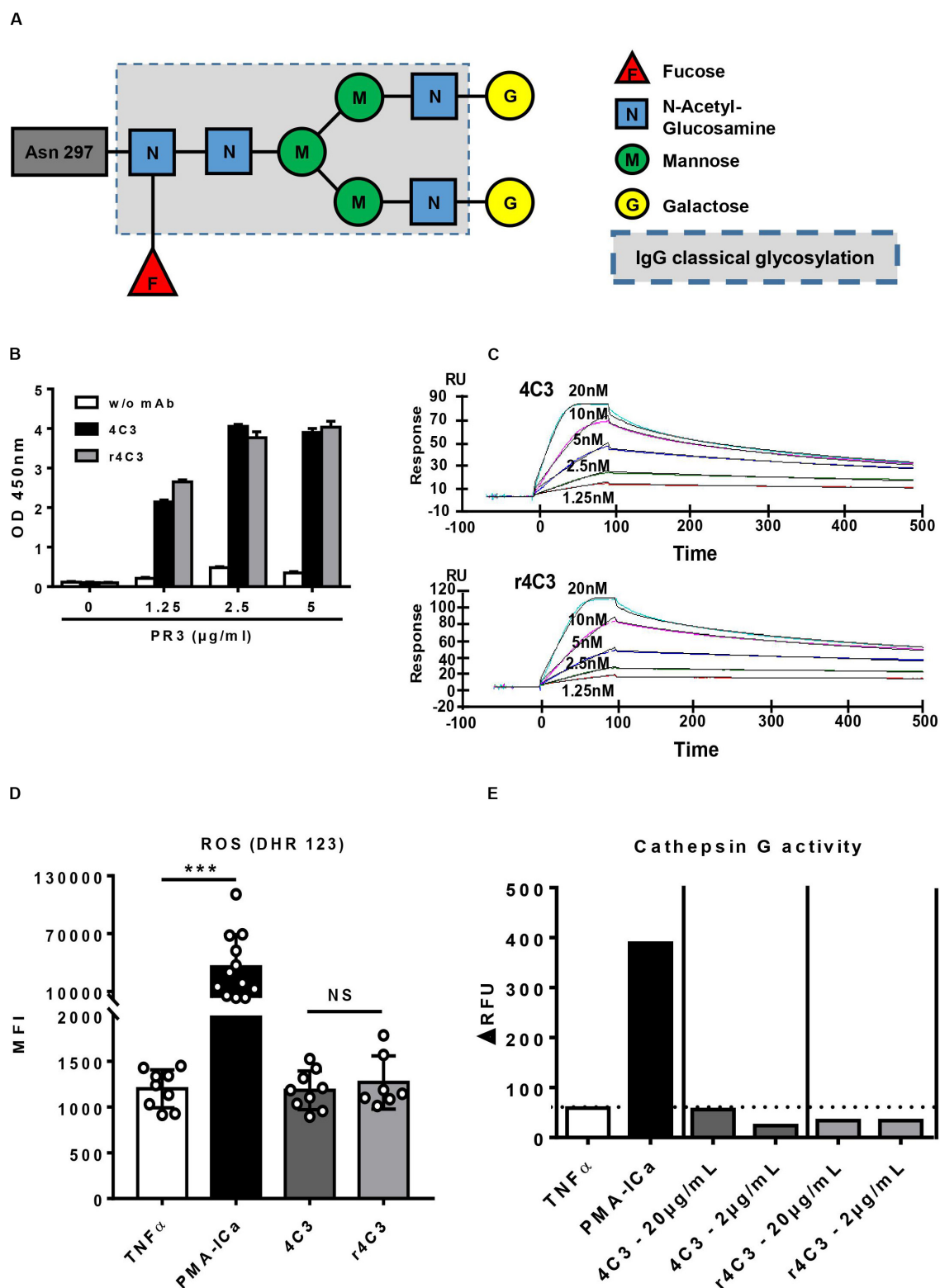


FIGURE 4 | Non-activation of neutrophils by 4C3 is independent of a defect in the Fc portion. **(A)** 4C3 has a G2F profile of glycosylation with an increase in galactose residues. Glycosylation of 4C3 in asparagine 297, obtained by mass spectrometry, is presented compared to classical glycosylation of IgG (dotted line). **(B)** The recombinant form of 4C3 (r4C3) has the same ability to bind PR3 as native 4C3. Binding of 4C3 (black columns) and recombinant 4C3 (r4C3; gray columns) (Continued)

FIGURE 4 | Continued

to PR3 by ELISA. BSA (0 μ g of PR3) was used as a negative control of binding. Results are expressed in Optical Density. The results of one experiment out of three are presented. **(C)** r4C3 has an affinity for PR3 comparable to that of 4C3. Increasing concentrations of PR3 (1.25 to 20 nM) were injected on 4C3 and r4C3 coated flow cells for 500 min. Binding was monitored as an increase in SPR signal expressed in resonance units (RU). The results of one experiment out of three are presented. **(D)** r4C3 does not induce ROS production by neutrophils. Purified neutrophils from nine independent healthy donors were primed with TNF α (2 ng/ml) for 15 min at 37°C (white histogram) before incubation for 45 min with PMA-ICa (black column), 4C3 (dark gray column) or r4C3 (light gray column). ROS production was assessed by measuring the fluorescence (MFI) of DHR 123 by flow cytometry. $n = 9$. NS: Non-significant. *** $p < 0.0005$. **(E)** r4C3 does not increase cathepsin G activity. Purified neutrophils from three independent healthy donors were primed with TNF α (2 ng/ml; white histogram) for 15 min at 37°C before incubation for 45 min with 4C3 (dark gray columns) or r4C3 (light gray columns) at 2 and 20 μ g/ml or PMA-ICa (black column). Cathepsin G activity was assessed after adding the substrate to the supernatants of neutrophils and reading the fluorescence (Δ RFU) by spectrofluorimetry. The results of one experiment out of three are presented.

but not from PR3-ANCA positive patients were used by the same group, in another study, and promoted inflammation through monocyte stimulation (60). Another study suggested that neutrophil activation induced by IgG ANCA, mostly observed in the literature, could be related to the persistence, after purification, of elements from patient sera and not to IgG ANCA (61). Regardless, in our study, the purity of IgG was verified by SDS-PAGE and only one band was visible.

To investigate further the non-pathogenic characteristic of 4C3, we studied its Fc portion properties and developed a recombinant form to eliminate the presence of a defect in the Fc portion of 4C3. We demonstrated that the 4C3 Fc domain was functional with a capacity to bind both Fc γ RIIA and Fc γ RIIIB with an expected affinity for an IgG1 (53). Fc γ RIIA and Fc γ RIIIB, the two main Fc γ R present at the neutrophil surface (62), are involved in ROS production, NETosis and degranulation induced by PR3-ANCA stimulation (7, 9). Furthermore, deglycosylation of the PR3-ANCA Fc portion attenuates neutrophil activation (63), but 4C3 has a glycosylated Fc portion. However, 4C3 glycosylation is with an elevated level of galactose residues, i.e., a G2F profile. Modifications in glycosylation of IgG linked with asparagine 297 are known to be involved in autoimmune diseases (31). IgG from GPA patients show low levels of bisection, sialylation and galactosylation in the active phase of the disease (32, 33, 64, 65) and, in one study, clinical remission was associated with complete glycan normalization of total IgG1 (33). Moreover, hyposialylation of IgG PR3-ANCA is correlated with disease activity and with ROS production by neutrophils (65). Contrary to IgG found in the active phase of the disease with low levels of galactosylation, 4C3 has an elevated level of galactose residues. This “anti-inflammatory” profile could also explain its non-pathogenic characteristic.

Results obtained with the recombinant form of 4C3 (r4C3) are in agreement with the fact that the non-activation property of 4C3 could not be due to a defect in the Fc portion. r4C3 has a different allotype, G1m17,1, which is one of the main allelic form of IgG1 (66) and is functional as it is classically used in therapy, e.g., rituximab (67). Therefore, the non-activation of neutrophils by 4C3 is not due to a particularity of its Fc portion because r4C3 with a functional Fc portion did not induce neutrophil activation either. Furthermore, we can hypothesize that IgG1 allotype is not implicated in neutrophil activation by PR3-ANCA as it has been suggested for other immune mechanisms (68).

The non-pathogenic character of 4C3 seems to be related to its epitope targeted on PR3. In the literature, ANCA mainly recognize conformational epitopes (23, 48) with variations

between patients and in the same patient during the course of the disease according to disease activity (22, 69). PR3-ANCA found in the active phase of the disease have been shown mainly to recognize a region close to the active site, called epitope 1 (23–25), and to inhibit *in vitro* PR3 enzymatic activity (26–28). Based on the linear representation of known PR3 epitopes in GPA from van der Geld et al. (23), we can conclude that amino acid residues corresponding to the conformational epitope recognized by 4C3 are localized on the C-terminal region in the sequence of the complementary peptide of PR3 (a.a 87–172) which is hypothesized to initiate autoimmunity through idiotype–anti-idiotype response (23). This new epitope is different from any epitope already described in the literature (23) and 4C3 binding on this epitope does not affect *in vitro* PR3 enzymatic activity. This last result strengthens the non-pathogenic character of 4C3 because, in contrast, pathogenic PR3-ANCA found in the active phase of the disease generally inhibit *in vitro* PR3 enzymatic activity (26–28). The non-pathogenic characteristic of 4C3 could be explained by its binding to the particular epitope of PR3 and by a particular Fc domain orientation preventing its binding to Fc γ R. Several epitopes are associated with PR3-ANCA but, unlike MPO-ANCA, none is specifically associated with the active phase of the disease or remission (23, 48). Indeed, Roth et al. have described MPO-ANCA, directed against a linear epitope on MPO (AA 447–459), exclusive to disease activity (48). The level of those MPO-ANCA correlates extremely well with disease activity whereas the total MPO-ANCA level did not (48). Furthermore, epitopes of asymptomatic or natural auto-antibodies were localized close to epitopes related to active disease (48). The hypothesis of discovering a specific epitope associated with remission in PR3-AAV, e.g., the one targeted by 4C3, seems relevant as specific epitopes associated with active disease or remission in MPO-AAV have been already described. As shown for MPO, the epitope recognized by 4C3 is close to epitopes recognized by pathogenic PR3-ANCA, i.e., close to the active site and to the epitope 1 region.

To conclude, non-pathogenic PR3-ANCA, which are not able to activate neutrophils, exist and can be isolated in GPA patients in remission. This could explain why PR3-ANCA can persist during remission without predicting relapse and can also be found in healthy donors. The non-pathogenic characteristic of 4C3 could be related to its ability to recognize a particular conformational epitope on PR3. This newly described epitope could be linked to remission and might be used as a remission biomarker within the framework of “personalized medicine approach” in PR3-AAV management

suggested by Osman et al. (70). Improved understanding of differences between non-pathogenic and pathogenic PR3-ANCA is necessary to develop these new biomarkers in GPA. Finally, our human mAb 4C3, which has an inhibitory property, could be interesting in a therapy to inhibit auto-immune activation of neutrophils induced by PR3-ANCA of GPA patients in the active phase of the disease.

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 at: <http://www.wwpdb.org/>, PDB:4ODX, <http://www.wwpdb.org/>, PDB:3SKJ, and <http://www.wwpdb.org/>, PDB:1FUJ.

ETHICS STATEMENT

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

CH and BK conceived the study. RL, CH, and JG conceived and designed the experiments while JG, EM, YG, JM, MD, SS, and RL performed them. JG and RL were involved in data analysis. MP performed SPR experiments. AP and AM did epitope mapping.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.573040/full#supplementary-material>

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Monitoring Immune Responses in IgA Nephropathy: Biomarkers to Guide Management

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IgA nephropathy (IgAN) is the commonest biopsy-reported primary glomerulonephritis worldwide. It has an incidence which peaks among young adults, and 30 to 40% of patients' progress to end stage kidney disease within twenty years of diagnosis. Ten-year kidney survival rates have been reported to be as low as 35% in some parts of the world. The successful management of IgAN is limited by an incomplete understanding of the pathophysiology of IgAN and a poor understanding of how pathophysiology may vary both from patient to patient and between patient groups, particularly across races. This is compounded by a lack of rigorously designed and delivered clinical trials in IgAN. This is slowly changing, with a number of Phase 2 and 3 clinical trials of novel therapies targeting a number of different putative pathogenic pathways in IgAN due to report in the next 5 years. From our current, albeit limited, understanding of the pathophysiology of IgAN it is unlikely a single therapy will be effective in all patients with IgAN. The successful management of IgAN in the future is, therefore, likely to be reliant on targeted therapies, carefully selected based on an individualized understanding of a patient's risk of progression and underlying pathophysiology. The potential role of biomarkers to facilitate personalization of prognostication and treatment of IgAN is immense. Here we review the progress made over the past decade in identifying and validating new biomarkers, with a particular focus on those that reflect immunological responses in IgAN.

Keywords: IgA nephropathy, biomarkers, treatment, Berger's disease, management biomarkers in IgA nephropathy

INTRODUCTION

IgA nephropathy (IgAN) is the most common biopsy reported cause of primary glomerulonephritis worldwide (1–4). It is characterized by IgA deposition in the glomerular mesangium, which variably triggers a series of inflammatory and fibrotic cascades leading to a spectrum of clinical presentations, ranging from asymptomatic non-visible hematuria to rapidly progressive glomerulonephritis (5–7). The incidence of IgAN peaks among young adults and runs a progressive course to end stage kidney disease (ESKD) in up to 40% of patients within twenty years of diagnosis (8, 9). Until recently, predicting which patients were most at risk of progression at the point of diagnosis remained a clinical challenge. The development of the International IgAN Prediction Tool in 2019 has in part addressed this issue, facilitating the provision of timely counseling (10).

Despite this development, there remain two key barriers to the provision of safe, effective care to those diagnosed with IgAN:

1. An incomplete understanding of the pathophysiology of IgAN and a poor understanding of how pathophysiology may vary both from patient to patient and between patient groups, particularly across races.
2. A lack of rigorously designed and delivered clinical trials in IgAN. This is slowly changing, however, with a number of Phase 2 and 3 clinical trials of novel therapies due to report over the next 5 years.

From our current, albeit limited, understanding of the pathophysiology of IgAN it is unlikely a single therapy will be effective in all patients with IgAN. The successful management of IgAN in the future is, therefore, likely to be reliant on targeted therapies, carefully selected based on an individualized understanding of a patient's risk of progression and underlying pathophysiology. The potential role of biomarkers to facilitate personalization of prognostication and treatment of IgAN is therefore garnering much interest. New biomarkers in IgAN may in the future inform:

1. **Diagnosis:** Kidney biopsy is currently the only diagnostic test of IgAN; however, it is invasive and associated with discomfort, short term restrictions on activity and lifestyle, and some morbidity.
2. **Prognostication:** The International IgAN Prediction Tool provides individualized risk up to 5 years from diagnosis but relies on a number of non-specific markers of kidney damage and can only be used within 6 months of the diagnostic kidney biopsy.
3. **Treatment selection:** At present, kidney biopsy features at time of diagnosis are used by some clinicians to guide some treatment decisions but this approach has not been formally tested or validated outside single centers and is not recommended in international guidelines.
4. **Monitoring response to treatment:** This is currently limited in IgAN to assessment of non-specific measures including urine protein excretion and serum creatinine/estimated glomerular filtration rate (eGFR).

Before any new biomarker is introduced into clinical practice, we must consider whether it is:

1. **Biologically plausible:** the biomarker should be relevant to our understanding of IgAN pathophysiology.
2. **Sensitive:** the biomarker should accurately measure the proportion (ideally 100%) of actual positives that are correctly identified as such (e.g., the percentage of IgAN cases who are correctly identified as having IgAN using the novel biomarker).
3. **Specific:** the biomarker should accurately measure the proportion (ideally 100%) of actual negatives that are correctly identified as such (e.g., the percentage of healthy people who are correctly identified as not having IgAN using the novel biomarker).

4. **Validated:** measurement of the biomarker must be technically validated both within and across laboratories with a single assay system.
5. **Generalizable:** the biomarker should ideally be informative across ethnic groups and geographical regions.
6. **Minimally invasive to collect:** the biomarker should be measurable in easy to collect biological samples such as blood, urine, sputum, and feces.
7. **Relatively resistant to degradation:** the biomarker should be stable under reasonable storage conditions, to allow for the delay between collection of the sample in clinical practice and analysis in a laboratory.
8. **Easy to measure:** the biomarker should be measured using readily available technologies such that most laboratories in the world will have the facilities and expertise to undertake measurement.
9. **Inexpensive:** for rapid integration into clinical pathways the biomarker should be relatively inexpensive to measure using existing technology.

AN EXEMPLAR OF TRANSLATING BIOMARKER DISCOVERY FROM THE BENCH TO THE BEDSIDE IN IGAN

The Oxford Classification of IgA Nephropathy

In 2004, the International IgAN Network and the Renal Pathology Society agreed to develop a histopathological scoring system that would “have **clear definitions**, be **simple to use** in clinical practice, be **reproducible** and have a **prognostic value** independent of the clinical parameters at the time of biopsy” (11). Eighteen kidney pathologists, from 10 different countries spanning 4 continents, first undertook extensive iterative work to define pathologic variables with acceptable inter-observer reproducibility. All lesions were identifiable with light microscopy alone, minimizing the requirement for other more expensive and less widely available testing methods. Where groups of such features closely correlated, variables were further selected on the basis of least susceptibility to sampling error and ease of scoring in routine practice. This process identified six pathologic variables that were then used to interrogate prognostic significance independent of clinical data. In a retrospective analysis, sequential clinical data were obtained on 265 adults and children with IgAN from eight countries across four continents who were followed for a median of 5 years. Kidney biopsies from all patients were independently scored for these six lesions by five pathologists blinded to the clinical data. Four lesions, mesangial hypercellularity (M), endocapillary hypercellularity (E), segmental glomerulosclerosis (S), and tubulointerstitial fibrosis/tubular atrophy (T) were finally selected based on simplicity of assessment, independence from other lesions, inter-observer reproducibility and sufficient independence from clinical parameters in their predictive value. Crescents were not included in the initial classification due to a low prevalence, however, following a similar analytical process and separate scoring of 3,096 kidney biopsies the fraction of crescent-containing

glomeruli was shown to associate with survival from either a $\geq 50\%$ decline in eGFR or ESKD adjusting for the covariates used in the original Oxford study and the C score was added to complete the MEST-C score (12, 13). Critical to the translation of the Oxford Classification into routine international clinical practice was the extensive number of independent validation studies, to date, there have been 21 published validation studies, including more than 7,000 patients: 12 Asian, 5 European, 2 North American, and 2 comprising more than one continent (14).

The Oxford Classification and its component biomarkers fulfil all of the requirements of a good prognostic biomarker set (**Figure 1**): assessment utilizes an existing stained kidney biopsy section which is universally available in routine clinical care and therefore incurs no additional expense to clinical services, biomarkers were selected based on acceptable inter-observer reproducibility, included cases represented different ages, genders and races to ensure generalizability, and there has been extensive independent cross-continental validation.

As a direct consequence of the international collaboration that delivered the Oxford Classification, research teams from across the world have subsequently worked together to develop the International IgAN Prediction Tool, which for the first time

provides patients with an individualized risk of a 50% reduction in eGFR or ESKD (defined as an eGFR < 15 ml/min/1.73 m², transplantation or dialysis) up to 5 years following kidney biopsy. It uses biomarkers that are widely available in all parts of the world, are cheap to measure, are already embedded in routine clinical care and have been individually validated as prognostic biomarkers (**Table 1**). Like the Oxford Classification, the patient population used to derive the tool was ethnically diverse, including 3,067 patients from Europe, China and Japan optimizing generalizability. A further 1564 patients from Europe, Asia, North America, South America, China, and Japan were used to externally validate the model. The tool has recently been validated in a separate study of 1,275 patients and other validation studies will be published in the near future (15).

In our view, both the Oxford Classification and the International IgAN Prediction Tool have set the benchmark that all novel biomarkers need to reach to justify translation into clinical practice in IgAN. A large number of biomolecules associated with the immune system have been reported in small discovery studies as candidate biomarkers in IgAN; however, at present none have come close to meeting this benchmark (examples are given in **Tables 2, 3**) and consequently to date

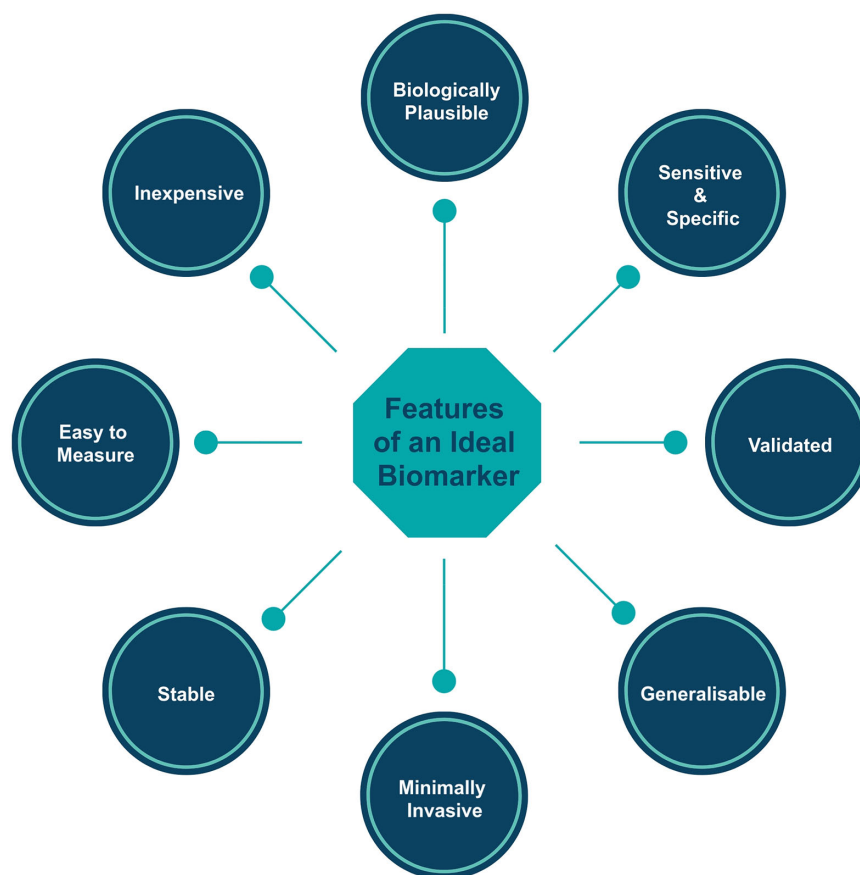


FIGURE 1 | Features of the ideal biomarker.

TABLE 1 | The data elements included in the International IgAN Prediction Tool*.

Estimated GFR at biopsy	ml/min/1.73m ²
Systolic blood pressure	mmHg
Diastolic blood pressure	mmHg
Proteinuria at biopsy	g/day
Age at biopsy	years
Race	
Caucasian	
Chinese	
Japanese	
Other	
Use of ACE inhibitor or ARB at the time of biopsy	
No	
Yes	
MEST M-score	
0	
1	
MEST E-score	
0	
1	
MEST S-score	
0	
1	
MEST T-score	
0	
1	
2	
Immunosuppression use at or prior to biopsy	
No	
Yes	

*Using clinical and histologic data at biopsy users can determine a 50% decline in eGFR or kidney failure at selected time intervals. The tool is not validated for use with data obtained remotely from the time of biopsy.

none have entered routine clinical practice. Proposed diagnostic and prognostic biomarkers linked to the immune response in IgAN include immunoglobulin A (IgA) and its associated complexes, components of the complement system, serum, urine and kidney-specific microRNAs and soluble CD89 (76–78). Additional biomarkers have been identified from genome wide association studies, peripheral blood mononuclear cell phenotyping, microbiome profiling, and metabolomic- and proteomic-based interrogation of serum, plasma, urine, and kidney tissue (76). In this review, we have deliberately focussed on those biomarkers that inform clinical decision making in IgAN currently and those most mentioned in the general IgAN literature (Figure 2), rather than those biomarkers that might suggest a likely underlying disease mechanism and therefore be potentially useful for drug discovery. We, therefore, discuss the most widely published biomarkers associated with the dysregulated immune response in IgAN, and (i) whether they might inform future clinical care and (ii) how well their performance has been validated and (iii) how likely they are to add value to the traditional biomarkers currently used in clinical practice (Table 4).

TRADITIONAL BIOMARKERS CURRENTLY USED IN THE MANAGEMENT OF IgAN

Diagnosis and prognostication in IgAN have changed little over the past few decades. A kidney biopsy remains the only way to

diagnose IgAN. Current strategies for predicting the likelihood of progression in IgAN and defining an “at risk” population suitable for a trial of immunomodulating therapy are based on a panel of biomarkers collected as part of routine clinical practice that combine non-IgAN specific measures [proteinuria, blood pressure (BP), eGFR], and IgAN-related measures from the diagnostic kidney biopsy. Proteinuria, eGFR, BP, and histopathological features of the kidney biopsy are by far the most valuable biomarkers we currently have in IgAN. All have been thoroughly and independently validated in cohorts from across the globe and provide unrivalled diagnostic and prognostic information. Any new diagnostic or prognostic biomarker must show additional value above these measures (Figure 1), and to date none have achieved this.

Kidney Biopsy and Histomorphometry Diagnosis

Since the initial description in 1968, a kidney biopsy demonstrating dominant or co-dominant mesangial IgA deposition is the only way to diagnose IgAN (79).

Prognosis

As already discussed, the Oxford Classification defines five histopathological lesions, MEST-C, that independently predict prognosis in IgAN, has been adopted as the international standard for kidney biopsy reporting in IgAN and forms a key component of the International IgAN Prediction Tool (10). Furthermore, there is compelling experimental evidence that each of these lesions associates with known pathogenic pathways triggered by mesangial deposition of IgA immune complexes in IgAN (80).

Treatment Selection

Currently, there are no validated features in the kidney biopsy that can be used to guide treatment selection in IgAN, including the MEST-C score. Retrospective and uncontrolled clinical trial data have suggested that endocapillary hypercellularity, a measure of glomerular macrophage accumulation, may be modifiable with systemic immunosuppression, leading to an improved outcome in Chinese and Caucasian patients (13, 81, 82). However, there have been no studies that have randomized patients on the basis of this histopathological feature and therefore international guidelines do not currently recommend basing treatment decisions on kidney biopsy features.

Monitoring Response to Treatment

Repeat kidney biopsy is rarely performed in IgAN, however, over the past 5 years there have been a few small repeat kidney biopsy studies published, both single center retrospective cohort studies and prospective clinical studies of immunosuppression (81, 82). At present, however, there is too little data to draw any firm conclusions on the value of repeat biopsy in IgAN.

Despite offering diagnostic, prognostic and possibly treatment relevant information the kidney biopsy is invasive and associated with discomfort, short term restrictions on activity and lifestyle, and appreciable morbidity. Furthermore, due to sampling error,

TABLE 2 | Reported putative biomarkers in IgAN (serum, plasma, and whole blood).

Biomarker	Proposed Predictivity Parameter	Sample Size/Controls	Study design	Country of Study	Conclusion	Reference
25-hydroxy-vitamin D	Prognosis	105 IgAN Patients	Prospective cohort study	China	25-hydroxy-vitamin D deficiency was associated with IgAN progression and poorer histological features (MEST-C score)	(16)
Copeptin	Prognosis	59 IgAN patients	Prospective cohort study	Netherlands	Copeptin was associated with disease severity and progression in IgAN (doubling of creatinine, ESKD or commencement of immunosuppressive therapy)	(17)
Fibroblast growth factor 23	Prognosis	180 IgAN patients	Prospective cohort study	Sweden	Serum FGF23 predicted ESKD or \geq 25% reduction in eGFR within 10 years, independent of age, sex, albumin, PTH and bone minerals.	(18)
Matrix Metalloproteinase 7	Prognosis	244 IgAN patients	Retrospective cohort study	China	Elevated MMP7 was independently associated with kidney fibrosis and kidney function decline.	(19)
Neutrophil to lymphocyte ratio	Response to treatment	66 IgAN patients in remission, 33 persistent disease activity	Retrospective cohort study	China	Patients with an NLR of <2.43 were more likely to achieve remission with corticosteroids.	(20)
NGAL (urine and serum)	Prognosis	121 IgAN patients	Prospective cohort study	Korea	Elevated Urine and serum NGAL combined was associated with a greater decline in eGFR.	(21)
Oxylipins and arachidonic acid metabolites	Response to treatment	96 IgAN patients	Randomized, placebo controlled, double blind clinical trial	United States of America	Oxylipins and arachidonic acid metabolites changes may predict remission in response to fish oil in IgAN	(22)
Platelet derived growth factor	Diagnosis	33 IgAN patients, 48 disease controls	Prospective case control study	Germany	PDGF were elevated in patients with IgAN compared to disease controls	(23)
Plasma CRP	Prognosis	174 IgAN patients	Retrospective cohort study	Finland	CRP, albumin and white blood cell count elevations are associated with IgAN progression	(24, 25)
White blood cell count						
Plasma insulin level						
Plasma alpha defensins	Diagnosis	169 IgAN patients, 83 healthy controls	Retrospective case control study	China	Plasma alpha defensins are higher in IgAN patients compared to healthy controls.	(26)
Plasma acylcarnitines	Prognosis	81 IgAN patients	Retrospective cohort study	China	Elevated plasma acylcarnitines were associated with IgAN progression	(27)
Soluble ectodomain of vascular cell adhesion marker 1	Diagnosis and Prognosis	327 IgAN patients, 55 healthy controls	Retrospective case control study	China	sVCAM-1 is elevated in IgAN patients compared to healthy controls and was associated with proteinuria, low eGFR and histological severity (MEST-C score)	(28)
Soluble interleukin 2 receptor alfa	Prognosis	194 IgAN patients, 84 healthy controls	Prospective case control study	Sweden	sIL-2Ra predicted progression of kidney disease in IgAN patients.	(29)
Soluble ST2	Diagnosis and Prognosis	74 IgAN patients, 34 healthy controls	Prospective case control study	China	Soluble ST2 was higher in IgAN patients, and correlated positively with worsening histology (WHO histological classification), negatively with eGFR and positively with proteinuria.	(30)
Transforming growth factor β 1	Diagnosis and Prognosis	100 IgAN patients, 56 health controls.	Retrospective case control study	China	TGF β 1 was elevated in patients with IgAN compared to healthy controls, and was more elevated in patients with more severe histological lesions (WHO classification)	(31)
Tumour Necrosis Factor β	Diagnosis and Prognosis	147 IgAN patients, 126 healthy controls	Retrospective case control study	China	TNFA is higher in patients with IgAN compared to healthy controls, and positively correlated with proteinuria and histological severity. TNFa negatively correlated with eGFR.	(32)
Tumour Necrosis Factor receptor	Prognosis	347 IgAN patients	Prospective cohort study	Korea	Elevated TNFR concentrations were associated with IgAN histological severity (local grading system) and disease progression	(33)
	Prognosis	106 IgAN patients, 34 healthy controls	Retrospective case control study	Japan	TNFR correlated with urinary protein creatinine ratio and degree of interstitial fibrosis	(34)
Uric Acid	Prognosis	623 IgAN patients	Retrospective cohort study	China	Uric acid levels correlated with severity of tubulointerstitial damage (MEST-C score and Beijing classification system).	(35)
	Prognosis	226 IgAN patients	Prospective cohort study	Japan	Elevated uric acid is a risk factor of IgAN progression.	(36)
	Prognosis	611 IgAN patients	Retrospective cohort study	Japan	Elevated uric acid is a risk factor of IgAN progression, but only in those with CKD3a	(37)

(Continued)

TABLE 2 | Continued

Biomarker	Proposed Predictivity Parameter	Sample Size/Controls	Study design	Country of Study	Conclusion	Reference
	Prognosis	202 IgAN patients	Retrospective cohort study	Finland	Uric acid levels correlated with severity of tubulointerstitial damage (Local grading system).	(38)
	Prognosis	111 IgAN patients	Prospective cohort study	Turkey	Elevated uric acid is a risk factor of IgAN progression.	(39)
	Prognosis	93 IgAN patients	Retrospective cohort study	Turkey	Elevated uric acid is a risk factor of IgAN progression.	(40)

kidney biopsies can provide misleading information and occasionally be uninformative.

Proteinuria

Prognosis

The presence and the amount of proteinuria have long been recognized as risk factors for kidney function decline in kidney disease (83), and proteinuria is an important prognostic biomarker in IgAN (84, 85). In epidemiological studies time averaged proteinuria (TAP) is the strongest independent predictor of IgAN progression and ESKD. The 10-year risk of ESKD is only 5% if TAP is <1 g/day, increasing to 60% with TAP >3 g/day (86). This finding has been validated in three large studies in Korea (Islan Hospital, 500 patients) Europe (VALIGA, 1,147 patients), and China (Nanjing Registry, 1,155 patients), the latter two retrospective studies also demonstrated a kidney survival benefit in those maintaining TAP <0.5 g/day (87–89). Proteinuria is a key component of the International IgAN Prediction Tool.

Treatment Selection

Persistent, elevated proteinuria >1 g/24 h, despite maximal supportive care, is currently used to define an “at risk” IgAN population whose risk of progression is sufficiently high to warrant a trial of immunosuppression, with the attendant risk of side effects. Likewise, persistent proteinuria >1 g/24 h is also used as an entry criteria for all currently recruiting Phase 2 and 3 trials of immunomodulatory therapies in IgAN.

Monitoring Response to Treatment

An early reduction in proteinuria with a range of interventions has been associated with improved long term kidney survival in IgAN (86, 90). In 2019, the FDA (Food and Drug Administration) accepted an early change in proteinuria as a reasonably likely surrogate end point for a treatment’s effect on progression to ESKD in IgAN. An early change in proteinuria is now being used to monitor the response to a range of novel and repurposed therapies currently being evaluated in IgAN (NCT03841448, NCT03762850, and NCT01738035).

Measurement of proteinuria offers a non-invasive and inexpensive way to risk stratify patients with IgAN and monitor the response to new therapies. There is extensive evidence that proteinuria is not only a biomarker of glomerular injury but also a contributor to downstream kidney inflammation through a number of established mechanisms, including tubular

chemokine and complement activation (91, 92) and therefore is a biologically plausible biomarker for risk of progression. A major challenge, however, is that proteinuria can vary within an individual and is affected by exercise, dietary sodium excretion and the method of assessment. The gold standard in clinical trials is to measure a protein-to-creatinine ratio (UPCR) on an aliquot of a 24-h urine collection. For most in routine clinical practice, a 24-h urine collection is impractical and instead either a first morning or randomly timed urine is used to measure a UPCR, both of which are prone to significant variability (93).

While albuminuria and microalbuminuria offer value as an early biomarker of disease progression in other kidney conditions, there is at present minimal data to support albuminuria as a superior biomarker to proteinuria in IgAN (94, 95). Microalbuminuria has been shown to co-present with hematuria more often in those with IgAN; however, its sensitivity and specificity are too low to be of any value in clinical practice (96). Furthermore, the albumin-to-creatinine ratio (ACR) yields little benefit over UPCR in IgAN (97, 98).

Blood Pressure

Prognosis

Hypertension at diagnosis is an established risk factor for kidney function loss in all forms of kidney disease, including IgAN (10, 84, 86, 99). The BP at diagnosis is included in the International IgAN Prediction Tool. BP control improves outcomes at 20 years, with the incidence of dialysis or death in a cohort of French patients being 5% for normotensives, 19% for controlled hypertensives ($\leq 130/80$ mmHg), and 42% in uncontrolled hypertensives (100).

BP measurement is a non-invasive, inexpensive, and extensively validated prognostic biomarker in IgAN.

Estimated Glomerular Filtration Rate

Prognosis

The eGFR at time of diagnosis is a well-established predictor of future risk of kidney function decline and ESKD in IgAN. In a study of 2,269 Japanese patients, the incidence of ESKD was 90% in those who presented with a serum creatinine ≥ 220 $\mu\text{mol/L}$ (101), and similar findings have been reported in multi-ethnic cohorts from the USA, Europe and Asia (100, 102–105). eGFR is included in the International IgAN Prediction Tool.

Treatment Selection

The current KDIGO Clinical Practice Guidelines for Glomerulonephritis suggest that immunomodulatory therapy

TABLE 3 | Reported putative biomarkers in IgAN (urine).

Biomarker	Proposed Predictivity Parameter	Sample Size/Controls	Study design	Country of Study	Conclusion	Reference
Adiponectin	Diagnosis and Prognosis	12 IgAN patients, 10 disease controls and 24 healthy controls	Retrospective case control study	Japan	Adiponectin correlated with urine albumin creatine ratio in both IgAN patients and disease controls.	(41)
α -1-microglobulin	Diagnosis	17 IgAN patients, 16 disease controls, 10 healthy volunteers	Retrospective case control study	Japan	Urinary α -1-microglobulin was lower in patients with IgAN compared to healthy volunteers and disease controls.	(42)
Angiotensinogen	Prognosis	36 IgAN patients, 14 disease controls and 15 healthy controls	Retrospective case control study	Korea	Urinary angiotensinogen correlated with urinary protein creatine ratio, but was not specific for IgAN.	(43)
Aquaporin 2	Diagnosis	44 IgAN patients, 21 disease controls, 40 healthy controls	Retrospective case control study	Italy	Urinary aquaporin 2 was higher in patients with IgAN compared to disease and healthy controls. Higher aquaporin 2 levels were associated with proteinuria and hypertension.	(44)
β -2-microglobulin	Prognosis	51 IgAN patients	Retrospective cohort study	Korea	β -2-microglobulin levels correlated with kidney function, proteinuria and was a predictor of disease progression	(45)
Calprotectin & TIMP2*IGFBP7	Prognosis	113 IgAN patients	Prospective cohort study	Germany	Calprotectin and TIMP2*IGFBP7 were not useful in discriminating between patients at risk of progressive disease and those who or not, or those who achieved remission and those who did not.	(46)
CD89-Transglutaminase-2 Product	Prognosis	160 IgAN/HSP patients	Prospective cohort study	Belgium, Poland, Italy	CD89 -Transglutaminase product was associated with proteinuria in IgAN and HSP patients	(47)
C-megalin	Prognosis	73 IgAN patients, 5 disease controls	Retrospective case control/cohort study	Japan	C-megalin was associated with mesangial hypercellularity and chronic extra capillary abnormalities in IgAN patients.	(48)
Collagen type III neo-epitope fragment (C3M)	Prognosis	48 IgAN patients	Prospective cohort study	Greece	Urine C3M was lower in IgAN patients who subsequently developed progressive kidney disease	(49)
Collagen type 4	Prognosis	34 IgAN patients	Prospective cohort study	Japan	Elevated urinary collagen 4 was associated with histologically severe lesions on biopsy	(50)
CXCL1	Prognosis	425 IgAN patients, 160 disease controls and 74 healthy controls	Retrospective case control study	China	Urinary CXCL1 correlated with proteinuria, tubular atrophy and interstitial fibrosis and was independently associated with a greater risk of kidney function decline.	(51)
Epidermal growth factor	Prognosis	33 IgAN Patients	Retrospective cohort study	Greece	EFG negatively correlated with the extent of fibrosis in kidney biopsy	(52)
	Prognosis	58 IgAN patients	Retrospective cohort study	Spain		(53)
Epidermal growth factor to monocyte chemotactic peptide 1 ratio	Prognosis	132 IgAN patients	Prospective cohort study	Italy	A low EGF:MCP1 ratio was associated with a greater risk of kidney function decline, and was found to be an independent risk factor for kidney function decline.	(54)
Exosomes	Diagnosis and Prognosis	55 IgAN patients, 24 healthy controls, 25 disease controls	Retrospective case control study	China	Urinary exosomes were compared to healthy and disease controls, and correlated with histological severity.	(55)
Exosomal CCL2 mRNA	Diagnosis and Prognosis				Exosomal CCL2 was compared to healthy and disease controls, and correlated with tubulointerstitial inflammation and kidney function deterioration	
Free kappa light chains	Diagnosis and Prognosis	49 IgAN patients, 42 disease controls, 40 healthy controls	Retrospective case control study	Italy	Free kappa light chains were reduced in IgAN compared to healthy and disease controls. Concentration was inversely correlated with histological severity (MEST-C)	(56)
IgA-Uromodulin complex	Diagnosis	126 IgAN patients, 94 disease controls	Retrospective case control study	Japan	Urinary IgA-Uromodulin complex had a sensitivity 81.7%, specificity of 73.4% and a diagnosis efficiency of 78.2% for IgAN	(57)
IL1 β	Diagnosis	13 IgAN, 3 Henoch-Schönlein purpura, 11 disease controls, 5 healthy controls	Retrospective case control study	China	IL1 β was elevated in the urine if IgAN patients compared to healthy and disease controls	(58)
IL6	Prognosis	33 IgAN Patients	Retrospective cohort study	Greece	IL6 was significantly elevated in IgAN patients with more kidney fibrosis. IL6 was also shown to be associated with histological progression in a Japanese cohort.	(52)
		58 IgAN patients	Retrospective cohort study	Spain		(53, 59)

(Continued)

TABLE 3 | Continued

Biomarker	Proposed Predictivity Parameter	Sample Size/Controls	Study design	Country of Study	Conclusion	Reference
Kidney injury molecule 1	Prognosis	113 IgAN patients	Prospective cohort study	Germany	KIM1 was not useful in discriminating between patients at risk of progressive disease and those who or not, or those who achieved remission and those who did not.	(46)
	Response to Treatment	37 IgAN patients	Retrospective cohort study	Korea	KIM1 was reduced in response to treatment, which included immunosuppression. The study was not set up to predict which patients would respond to treatment	(60)
	Prognosis	51 IgAN patients	Retrospective cohort study	China	KIM1 correlated with severity of tubulointerstitial fibrosis on biopsy (MEST-C score).	(61)
	Diagnosis and Prognosis	202 IgAN patients, 46 disease controls, 60 healthy controls	Retrospective case control study	China	KIM1 was elevated in IgAN patients compared to disease and healthy controls, and correlated with proteinuria, creatinine and tubulointerstitial injury. KIM 1 predicted kidney function decline	(62)
	Diagnosis and Prognosis	40 IgAN patients, 10 healthy controls	Retrospective case control study	Korea	KIM1 was elevated in IgAN patients compared to healthy controls and correlated with histological severity (Lee's grades).	(63)
	Prognosis	65 IgAN patients, 65 healthy controls	Retrospective case control study	Netherlands	KIM1 correlated with degree of proteinuria and predicted kidney function decline	(64)
Laminin G like 3	Diagnosis	43 IgAN patients, 65 disease controls, 30 healthy controls	Retrospective case control study	France	Laminin G like 3 was reduced compared to healthy and disease controls, but only in those with conserved kidney function.	(65)
	Diagnosis and Prognosis	49 IgAN patients, 42 disease controls, 40 healthy controls	Retrospective case control study	Italy	Laminin G like 3 was reduced in IgAN compared to healthy and disease controls. Concentration was inversely correlated with histological severity (MEST-C)	(56)
Low molecular weight proteins	Prognosis	70 IgAN patients	Prospective cohort study	Netherlands	Urinary low molecular weight proteins offered no benefit over traditional risk factors in a multivariate model.	(66)
Mannose binding lectin	Prognosis	162 IgAN patients, 50 healthy controls	Retrospective case control study	China	Urinary MBL correlated with proteinuria, creatinine, blood pressure, histological severity and predicted kidney disease progression.	(67)
Matrix metalloproteinase 7	Prognosis	946 IgAN patients	Prospective cohort study	China	MMP7 was an independent risk factor for disease progression, and improved the risk prediction of the MEST-C score	(68)
MCP1	Prognosis	33 IgAN Patients	Retrospective cohort study	Greece	MCP1 was elevated in IgAN patients with more kidney fibrosis.	(52)
		58 IgAN patients	Retrospective cohort study	Spain		(53)
Neutrophil Gelatinase associated lipocalin	Prognosis	121 IgAN patients	Retrospective cohort study	Korea	Elevated urinary NGAL predicts kidney disease progression.	(21)
	Diagnosis and Prognosis	70 IgAN patients, 40 healthy controls	Retrospective case control study	China	NGAL was elevated in patients with IgAN compared to healthy controls, and correlated with histological severity (Lee's Grades)	(69)
	Prognosis	113 IgAN patients	Prospective cohort study	Germany	NGAL was not useful in discriminating between patients at risk of progressive disease and those who or not, or those who achieved remission and those who did not.	(46)
Periostin	Prognosis	345 IgAN patients, 56 disease controls	Prospective cohort study	Korea	Urinary periostin/creatinine values were elevated in patients with fibrosis and tubular atrophy, and in those who developed progressive kidney disease.	(70)
Podocalyxin	Prognosis	51 IgAN patients	Retrospective cohort study	Japan	Podocalyxin and podocyte count correlated with histological severity (Shigematsu classification)	(71)
Podocytes serum-and-glucocorticoid inducible kinase 1	Diagnosis and Prognosis	76 IgAN patients, 33 healthy volunteers	Retrospective case control study	China	Urinary SGK1 was elevated in patients with IgAN compared to healthy controls, correlated with the degree of tubulointerstitial damage in IgAN (MEST-C score), proteinuria and kidney insufficiency	(72)
Soluble transferrin receptor	Diagnosis and Prognosis	71 IgAN/HSP patients, 61 disease controls	Retrospective case control study	Belgium	soluble transferrin receptor concentrations were higher compared to disease controls, and correlated with proteinuria	(73)
TGFβ	Prognosis	58 IgAN patients	Retrospective cohort study	Spain	TGFβ was elevated in IgAN patients with more kidney fibrosis.	(53)

(Continued)

TABLE 3 | Continued

Biomarker	Proposed Predictivity Parameter	Sample Size/Controls	Study design	Country of Study	Conclusion	Reference
TNF α	Diagnosis	13 IgAN, 3 Henoch-Schönlein purpura, 11 disease controls, 5 healthy controls	Retrospective case control study	China	TNF α was elevated in the urine if IgAN patients compared to healthy and disease controls	(58)
Uromodulin (fragment)	Diagnosis	32 IgAN patients, 36 disease controls, 30 healthy controls	Prospective case control study	China	Uromodulin (fragment) distinguished IgAN patients from disease and healthy controls.	(74)
Vitamin D Binding Protein	Response to Treatment	80 IgAN patients	Prospective cohort study	China	Urinary vitamin D binding proteins were elevated in IgAN patients whose proteinuria failed to settle with irbesartan.	(75)

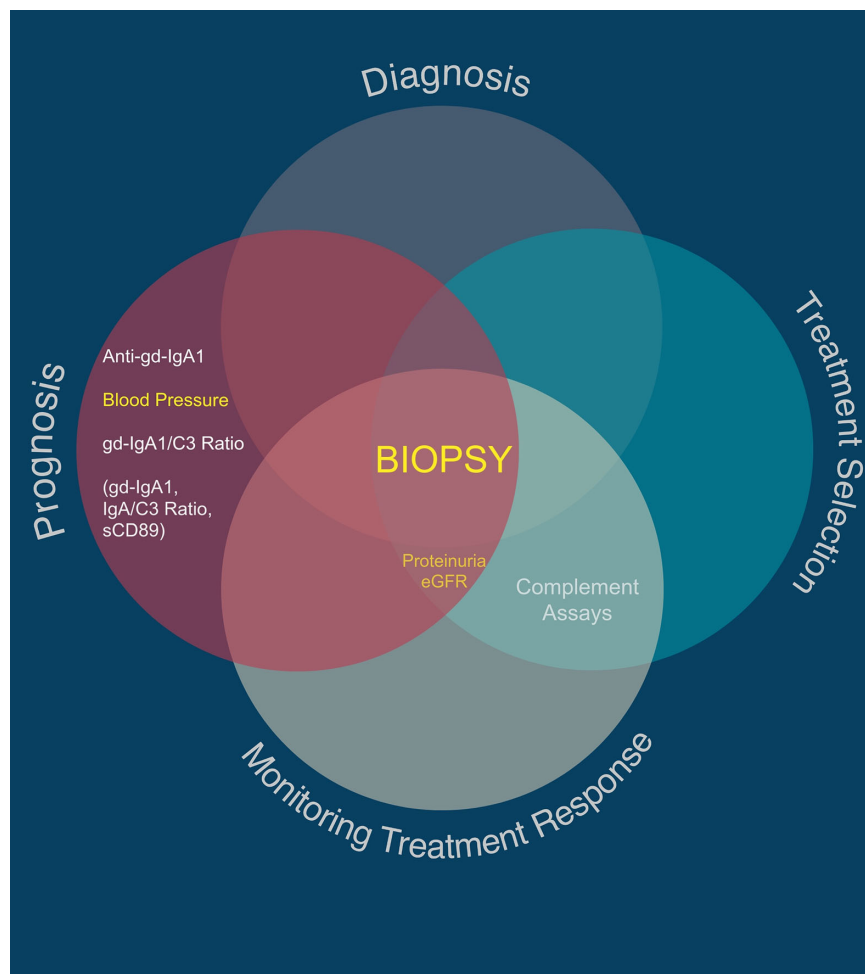
**FIGURE 2** | Utility of traditional and suggested utility for novel IgAN-specific biomarkers. Traditional biomarkers are shown in yellow. Proposed novel IgAN-specific biomarkers with the greatest number of associated publications are shown in white, with those in parentheses being regularly reported as useful biomarkers in the literature but with very low quality evidence for sensitivity and specificity in IgAN.

TABLE 4 | Comparison of the clinical utility of traditional and proposed novel IgAN-specific biomarkers.

Traditional Biomarkers	Affordable and accessible	Minimally Invasive	Biologically Plausible	Diagnostic	Prognostic	Treatment Selection	Monitor Treatment Response	Widely Validated
Proteinuria								
Hypertension								
eGFR								
Histology								
Proposed Novel Biomarkers in blood and urine								
serum IgA								
IgA/C3								
Polymeric IgA								
Secretory IgA								
Galactose-deficient IgA1								
Urinary galactose-deficient IgA1								
GalNAc content of IgA1 hinge region								
Anti-Gd-IgA1								
Anti-Gd-IgA1 – Gd-IgA1 complexes								
Soluble CD89								
Complement Assays								
microRNAs								
PBMC Phenotyping								

Green: sufficient evidence present to justify use; Yellow: Some evidence present to justify use; Red: Insufficient evidence present to justify use.

should not be used in patients with an eGFR < 50 ml/min/1.73 m², and all currently recruiting clinical trials in IgAN have a lower level of eGFR (between 30 and 45 ml/min/1.73 m²) below which patients are excluded from the study. This is for two principal reasons. Firstly, at lower eGFR levels it is predicted there will be less immunological activity and more fibrotic remodeling in the kidneys which means the disease will be unresponsive to immunosuppression. Secondly, at lower levels of eGFR the risk of treatment emergent adverse events increases and, therefore, the risk to benefit ratio becomes unacceptably high.

Monitoring Response to Treatment

Fundamentally, any therapy for IgAN should preserve eGFR. Measuring meaningful changes in eGFR in a slowly progressive disease such as IgAN has, however, presented significant challenges to researchers and regulators and, until recently, severely impacted on the attractiveness of studying new therapies in IgAN. The rate of eGFR decline, or eGFR slope, has been proposed as a valid end point in clinical trials to facilitate the recruitment of early disease stage patients, in whom treatment benefits may otherwise be either missed or would require costly, prolonged studies to capture end points seen in late stage kidney disease (106–108). Two- and three-year eGFR-based end points are now being used in all currently recruiting phase 3 studies of immunotherapies in IgAN to confirm response to treatment.

While proteinuria, BP, and eGFR are all minimally invasive, easily measurable, biologically plausible, relatively inexpensive and extensively validated, they each have their own limitations and all are non-specific and, therefore, tell us nothing about the pathophysiology operating in an individual. In order to be adopted into clinical practice any new IgAN-specific biomarker must, at the very least, provide insights into patient specific

disease mechanisms to enable a tailored approach to treatment selection, which at present traditional biomarkers cannot deliver.

PROPOSED NOVEL BIOMARKERS THAT MAY HELP IN MONITORING THE IMMUNE RESPONSE IN IgAN

Immunoglobulin A

Biological Plausibility

As the diagnosis of IgAN is based on the presence of mesangial IgA deposition it is not surprising that IgA was one of the first potential biomarkers studied in IgAN.

Diagnosis

Total serum IgA is elevated in approximately 50% of all cases of IgAN, however, this is neither sensitive or specific enough to be diagnostic of IgAN (109). Van der Boog et al. demonstrated that levels of the high molecular weight fraction of serum IgA, polymeric IgA (containing the circulating IgA-immune complexes), were elevated in 51 Dutch patients with IgAN compared to healthy subjects, but again sensitivity and specificity were insufficient to be of diagnostic value in IgAN (110). Secretory IgA (sIgA) is the major immunoglobulin secreted at mucosal surfaces but can also be found in the serum. Levels of sIgA are elevated in a proportion of patients with IgAN compared to healthy subjects (111, 112), but again sIgA is not sensitive or specific for IgAN (levels are also raised in other disease states including other primary glomerulonephritides and gut disorders) (111, 113).

In an effort to enhance the diagnostic power of serum IgA levels some investigators have promoted the potential value of

measuring the serum IgA/C3 ratio in IgAN. Tomino et al. reported that the IgA/C3 ratio was significantly elevated in Japanese IgAN patients compared to disease controls (114–116), and this has been reproduced in separate Japanese and Chinese IgAN cohorts (117, 118). These observations have not, however, been confirmed in Caucasian cohorts and while the IgA/C3 ratio is elevated in IgAN it still does not meet the sensitivity and specificity for a diagnostic test.

Prognosis, Treatment Selection, and Monitoring Response to Treatment

A small number of studies have examined the relationship of total serum IgA levels, serum IgA/C3 ratio, serum levels of polymeric IgA, and levels of urinary sIgA with clinical disease parameters (hematuria, proteinuria, eGFR, and severity of histopathological injury) and found inconsistent associations (110). Tan et al. measured urinary sIgA in 202 Chinese patients and found higher sIgA concentrations in patients with more severe histopathological findings, defined as Haas-IV or V lesions (119). However, these patients also had higher serum creatinine levels, higher BPs and higher levels of proteinuria. There seemed to be little additive value of measuring urinary sIgA concentrations over traditional biomarkers (120).

THE FOUR HIT HYPOTHESIS OF IgAN

A widely reported paradigm to explain the pathophysiology of IgAN is the “four hit” hypothesis, which postulates four separate pathophysiological processes are needed in order for IgAN to develop (Figure 3) (121). The first hit is the appearance in the circulation of increased levels of poorly O-galactosylated IgA1

(gd-IgA1). This IgA1 may also exhibit reduced O-linked sialylation and a reduction in N-acetylgalactosamine (GalNAc) residues at the hinge region of IgA1 and is believed to be derived from mucosally primed plasma cells (122). The second hit is the generation of IgG and/or IgA autoantibodies directed against the poorly O-galactosylated hinge region of gd-IgA1, with the third hit being the formation of anti-gd-IgA1-gd-IgA1 immune complexes. The fourth and final hit is the variable development of inflammatory and fibrotic processes in the kidney, triggered by the deposition of anti-gd-IgA1-gd-IgA1 immune complexes in the mesangium (121). We will deal with each of these hits and their potential to act as biomarkers of the immune response in IgAN.

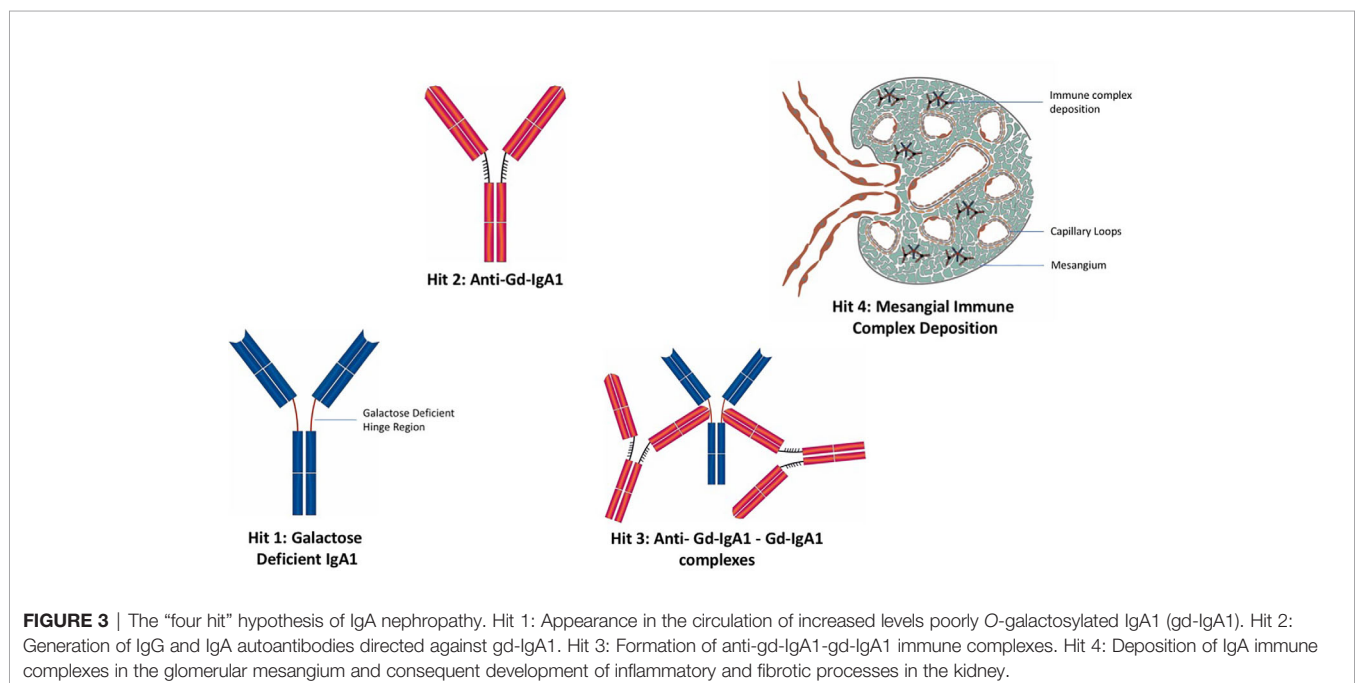
Galactose-Deficient IgA1 (HIT 1)

Biological Plausibility

The generation of gd-IgA1 is widely considered to be the first hit of the “four hit” hypothesis in IgAN (Figure 3), and its potential as a biomarker has been explored by a number of groups.

Diagnosis

Serum levels of gd-IgA1 have been reported as elevated, compared to healthy subjects and kidney disease controls, in multiple IgAN cohorts from across the globe including cohorts from Japan, Italy, China, UK, and US (123–127). Similarly, urinary gd-IgA1 has been reported to be elevated in IgAN, compared to disease controls and healthy subjects and also to correlate with proteinuria (128). Despite these associations, gd-IgA1 has no current value as a diagnostic test of IgAN as gd-IgA1 levels lack sensitivity due to the highly variable levels within IgAN cohorts, making a standardized threshold for diagnosis impossible, particularly as there is no standardized and accepted method to measure gd-IgA1 (125). Perhaps, more importantly,



gd-IgA1 lacks specificity for IgAN, most notably, gd-IgA1 serum levels are equally elevated in unaffected relatives of IgAN cases (129–131).

Prognosis

Zhao et al. reported an association between higher serum gd-IgA1 levels and a greater risk of progressive kidney disease, defined as 50% decline in eGFR, ESKD or death, in a Chinese cohort with a median follow up of 4 years. This association remained after adjustment for immunosuppression use, angiotensin converting enzyme inhibitor use, proteinuria, histopathological severity (based on the Haas classification), and eGFR at the time of presentation (132). However, at least two other groups have found no association between serum gd-IgA1 levels and disease severity (124, 133). Indeed, a meta-analysis of 22 studies (1,657 patients) found no association between serum gd-IgA1 levels and IgAN severity, suggesting that the serum gd-IgA1 level offers little in terms of a prognostic biomarker at present (134).

More recently, the plasma gd-IgA1/C3 ratio has been reported as an independent risk factor for IgAN progression in a large prospective study of 1,210 Chinese patients (135). There is no similar data available for Caucasian patients. We do not know yet whether addition of the plasma gd-IgA1/C3 ratio will improve the prognostic performance of the International IgAN Prediction Tool and this needs to be formally tested.

Treatment Selection and Monitoring

Response to Treatment

Similarly, there is little information to support the use of serum gd-IgA1 levels in guiding treatment choices. There have been a small number of studies from Japan reporting a reduction in the serum levels of gd-IgA1 following tonsillectomy and pulsed steroid treatment, and that this reduction is associated with an improved kidney outcome (136, 137). Iwatani et al. reported a small case series of adult Japanese IgAN cases ($n = 7$, four demonstrating remission) where the GalNAc content of serum IgA1 hinge region glycopeptides was marginally increased following tonsillectomy and pulsed steroid treatment, and this increase correlated with remission (138). However, the major challenge interpreting such data based on treatment effects of tonsillectomy and pulsed steroid therapy is that there remains significant controversy over whether this regimen actually alters IgAN outcome.

In addition to measuring serum levels of gd-IgA1, and the “Hit 2” autoantibodies discussed below, a number of investigators have examined peripheral blood lymphocyte and mononuclear cell subsets in an effort to identify changes in the key immune cells responsible for gd-IgA1 and autoantibody synthesis, as well as those mediating inflammation in IgAN (139–143). Results of these studies are inconsistent and none have been validated and therefore their utility as biomarkers in IgAN is uncertain. Observations in IgAN include a higher frequency of circulating CXCR5⁺CD4⁺ T cells (127), expansion of the non-classical monocyte subset (144), increase in the number of CD23⁺ and CD19⁺CD5⁺ B-cells, and increased expression of CD62L

by lymphocytes (145–148). In a study by Cox et al., a microarray analysis of peripheral blood mononuclear cells (PBMC) identified dysregulation in the PI2K/Akt and WNT-beta-catenin signaling pathways along with dysregulation in apoptotic pathways (142). A single study has examined PBMC in urine in IgAN and this demonstrated a correlation between CD14⁺ and CD56⁺ cells in the urine and the presence of crescents on biopsy (149). In summary, results of peripheral blood immunophenotyping by flow cytometry have been disappointing and have failed to identify consistent changes in IgAN that could be used as viable clinical biomarkers, although it is fair to say that the studies performed thus far are limited in scope and numbers. It is highly likely that immunophenotyping will be extensively re-visited with the commencement of a number of Phase 2 clinical trials of B cell directed therapies targeting BAFF {B-cell activating factor [BLyS (B-lymphocyte stimulator)]} and APRIL (A Proliferation-inducing ligand), two factors critical for T cell independent IgA class switch recombination and B cell proliferation and survival.

Autoantibodies Against gd-IgA1 (HIT 2)

Biological Plausibility

While generation of autoantibodies against gd-IgA1 constitutes the second hit of the “four hit hypothesis” (Figure 3), there remains significant debate over the absolute requirement for IgG (and IgA) autoantibody production in IgAN. In many IgAN kidney biopsy series, a significant number of cases do not demonstrate mesangial IgG, classical pathway complement activation is rare and drugs commonly and effectively employed in autoimmune diseases such as rituximab, cyclophosphamide, and mycophenolate mofetil have been shown to be ineffective in IgAN. It seems more likely that IgA-immune complexes form through direct interaction of polymeric IgA molecules, while the development of autoantibodies, when they occur, may amplify immune complex formation but are not an absolute requirement for immune complex formation in IgAN.

Diagnosis

Yanagawa et al. found that gd-IgA1 specific IgG and IgA levels were elevated in a Japanese IgAN cohort compared to disease controls and healthy subjects (123). Similar findings were reported by Berthou et al. in a French cohort (150). However, up to 25% of non-IgAN CKD patients also have elevated levels of these autoantibodies meaning that they are not suitable to be used as a diagnostic test in IgAN, even when combined with serum levels of gd-IgA1 (123).

Prognosis

In a study by Berthou et al. looking at outcomes in French IgAN cohort serum levels of gd-IgA1-specific IgG and IgA autoantibodies were associated with the risk of disease progression (150). It is not known whether inclusion of these autoantibody levels will improve the prognostic performance of the International IgAN Prediction Tool and this needs to be formally tested before any recommendation can be made concerning their prognostic value in IgAN.

Treatment Selection and Monitoring Response to Treatment

One might imagine that if IgAN were a classical autoimmune disease that immunosuppression would reduce autoantibody levels and induce remission of disease. This is not the case in IgAN. As an example, rituximab treatment is associated with peripheral CD20 depletion, reduction in autoantibody levels (PLA2R, PR3- and MPO ANCA, and ds-DNA) and clinical remission in a number of kidney autoimmune diseases (151–153). By contrast, in IgAN rituximab treatment is associated with a similar level of CD20 depletion but has no measurable effect on serum gd-IgA1 or IgG autoantibody levels (154). This difference in response in IgAN suggests that the simple measurement of the putative autoantigen (gd-IgA1) and autoantibody may well not be sufficient to monitor response to therapy in IgAN, or indeed inform therapeutic options, as conventional immunotherapies successfully employed to treat autoimmune disease (often based on the presence of an autoantibody) have shown very little efficacy in IgAN.

Anti-gd-IgA1–gd-IgA1 Immune Complexes (HIT 3)

Anti-gd-IgA1–gd-IgA1 complexes constitute the third hit of the “four hit” hypothesis (**Figure 3**). In a small study of 50 Japanese patients, Suzuki et al. found that levels of serum gd-IgA1 and IgG-gd-IgA1 immune complexes were associated with the extent of hematuria and proteinuria (155). Other than this association no other studies have been performed to allow evaluation of whether measurement of anti-gd-IgA1–gd-IgA1 complexes could be used as a biomarker in IgAN.

Soluble CD89 (an Alternative HIT 3) Biological Plausibility

CD89 (myeloid Fc α R1-receptor) is a transmembrane glycoprotein found mainly on myeloid cells, which functions as a receptor for the Fc component of human IgA (156). Interaction with IgA can induce shedding of CD89 from myeloid cell surfaces, generating a soluble form of the receptor (sCD89) (157). It has been hypothesized that sCD89 may play a role in circulating immune complex formation in IgAN (158). In mouse models it has been demonstrated that sCD89-IgA complexes can deposit in the mesangium and through interaction with the transferrin receptor (CD71) and tissue transglutaminase-2 (TG-2) produce an IgAN-like disease with haematoproteinuria and kidney function decline (159). Soluble CD89 has been detected in both the urine and serum of patients with IgAN (47, 160, 161).

Diagnosis

In a small French cohort of 30 patients, Launay et al. found levels of sCD89 in PEG (polyethylene glycol) precipitated serum were elevated compared to levels in 30 healthy subjects and 45 disease controls (162). However, there was considerable variation in the sCD89 levels in the IgAN cohort, preventing its development as a diagnostic test. Furthermore, a Dutch group subsequently reported a lack of specificity, with sCD89 being detected in equal amounts in IgAN and healthy subjects (163).

Prognosis

A low serum sCD89-IgA immune complex level at the time of diagnosis (presumed due to consumption in the kidney) has been associated with a worse prognosis in a cohort of Swedish IgAN patients (160). This could not, however, be replicated in a patient cohort from Korea (160, 161). In a multi-center study of 160 Caucasian patients with IgAN or IgA vasculitis that measured urinary levels of sCD89 and TG-2, Moresco et al. found that both sCD89 and TG-2 levels were lower in patients with active disease compared to those in remission. However, both markers correlated closely with proteinuria suggesting that for prognostication there is little added benefit of measuring sCD89 and TG2 in addition to proteinuria (47).

Treatment Selection and Monitoring Response to Treatment

There is no data to support the use of sCD89 or sCD89-IgA immune complexes in guiding therapy decisions in IgAN.

Inflammation, Remodeling, and Fibrosis in the Kidney (HIT 4)

Current evaluation of the kidney biopsy in IgAN includes light microscopy of normally 4–5 differently stained kidney sections, immunostaining for a limited panel of immune components and, when available, electron microscopy, alongside scoring of the PAS-stained section to generate the Oxford MEST-C score. The techniques employed have changed little over the past 50 years. Apart from the Oxford score, there have been many studies that have reported specific features in the routine evaluation of the biopsy that are part of the immunological response and may aid prognostication in IgAN including intensity and distribution of glomerular IgA immune complexes both using immunostaining and electron microscopy, co-deposition of IgG, and intensity of Complement C3 deposition. These studies have not been validated in large populations and currently it is unclear whether any of these features add value above the traditional biomarkers used in clinical practice. Future work mirroring the methodologies employed by the International IgAN Network and Renal Pathology Society to formulate the Oxford Classification may identify and validate features, currently routinely assessed in all IgAN kidney biopsies, that could add value in risk prediction and treatment decision making. In addition, with the introduction of machine learning in nephropathology it may be possible in the future to extract novel morphological biomarkers from routinely stained kidney sections that can contribute to improving prognostic accuracy of the MEST-C score and inform treatment decisions (164).

It will not be surprising that there are many hundreds of individual, non-validated, studies reporting over or under expression of specific protein biomarkers in small cohort studies in kidney biopsies in IgAN and it is impossible to cover all of these in this review. It is also impossible to comment on whether measurement of any of these proteins adds value to prognostication and/or treatment decisions in IgAN. However, as the number of novel and repurposed immunomodulatory therapies being evaluated in IgAN steadily grows, the need to be

able identify biomarkers of specific intrarenal pathomechanisms is becoming increasingly important as this will ultimately aid appropriate treatment selection. In our view the immediate priority for biomarker development is to identify biomarkers that will aid with the selection of patients and monitoring of response to the increasing number of new drugs that inhibit different components of the complement system. A number of which, are currently being evaluated in IgAN, including Cemdisiran (NCT03841448: a small interfering RNA that inhibits hepatic C5 synthesis), LNP023 (NCT03373461: small molecule factor B inhibitor), IONIS-FB-LRx (NCT04014335: anti-sense inhibitor of factor B), and Narsoplimab (NCT03608033: human monoclonal antibody directed against mannose-binding lectin-associated serine protease 2).

The Complement System: A Major Driver of Inflammation and Remodeling in IgAN

The complement system comprises more than 30 proteins and protein fragments that are part of the innate arm of the immune system and function as a cascade to amplify local inflammatory responses to foreign pathogens, or host injury signals. The common pathway of the complement system can be triggered by one of three routes; the lectin pathway, the alternative pathway or the classical pathway (165).

Biological Plausibility

Complement components C3 and properdin are present in 80%–90% of all kidney biopsies in IgAN. Complement components C4 or C4d, mannose-binding lectin (MBL), and the terminal complement complex (C5b–C9) are frequently detected, whereas the typical absence of C1q suggests that the classical pathway is not activated. By contrast, there is an increasing body of evidence supporting a role for the lectin pathway activating complement in IgAN. *In vitro* studies have demonstrated that polymeric IgA purified from patients with IgAN can strongly activate the lectin complement pathway and this may be amplified further by the O-glycosylation changes that define gd-IgA1 (166, 167). This activation is likely amplified rapidly by the alternative pathway, which is dysregulated in many IgAN cases associated with mutations in alternative pathway genes.

Importantly, when assessing complement activation in IgAN while changes in serum levels of complement components including C3, C4, CFHR5 and mannose binding lectin (MBL) have been variably reported these changes are inconsistent, not validated and at present clinically uninformative, making evaluation of complement activation *in situ* in the kidney and in the urine an essential focus for future biomarker studies in IgAN. There have been few published studies of complement component excretion in the urine and in our view this is a missed opportunity to identify novel biomarkers of kidney complement activation and response to therapy, which we are sure will be the focus of discovery and validation studies by those investigators currently examining complement therapies in IgAN.

Prognosis

There are multiple lines of evidence to support an association between the extent of glomerular complement activation and

severity of disease in IgAN, although at present there is no standardized and validated “complement staining panel” for routine use in clinical practice in IgAN. Glomerular C3 deposition and presence of the terminal complement complex (C5b-9) have been correlated with severity of histological damage and reported as independent risk factors for kidney function decline (168). Glomerular CFHR5 deposition, a key regulator of the alternative pathway, is similarly associated with IgAN progression (169). Likewise, glomerular deposition of MBL, a key component of the lectin pathway, is associated with more severe proteinuria and histological injury (167). Further supporting the importance of lectin pathway activation, Espinosa et al. found C4d deposition (in the absence of C1q, C4d deposition signifies lectin pathway activation) was associated with more severe histological damage, and was an independent risk factor for disease progression (170). In each of these studies immunostaining was carried out in isolation of other components of the complement pathway, and therefore, it is impossible to determine the relative contribution of lectin and alternative pathway activation to progression of IgAN. There have also been no analyses to evaluate the value of adding complement immunostaining to the IgAN risk prediction score and so we must await these analyses before we are able to conclude on the importance of *in situ* complement activation in predicting prognosis in IgAN.

Treatment Selection and Monitoring Response to Treatment

Ahead of using a complement-directed therapy in IgAN it would be desirable to know the extent of complement activation occurring in the kidneys and the dominant pathway driving glomerular inflammation. As already mentioned studies of complement activation in the kidney have thus far studied pathways in isolation and what we need going forward is a validated “complement panel” for staining kidney biopsies that (1) confirms complement activation, (2) assesses the amount of complement activation, and (3) determines the relative contribution of lectin and alternative pathways- both to justify the use of a complement therapy and direct therapy toward alternative pathway (e.g., LNP023), lectin pathway (e.g., Narsoplimab), or common pathway (e.g., Cemdisiran) inhibition. These studies will hopefully be undertaken in the near future.

MOVING EVALUATION OF THE KIDNEY BIOPSY IN IgAN INTO THE 21ST CENTURY: MULTI-OMICS-BASED BIOMARKER DISCOVERY AND THE KIDNEY BIOPSY

With the development of novel molecular techniques it is now possible to generate highly detailed transcriptomic landscapes not only of whole kidney tissue but also microdissected glomerular and tubulointerstitial compartments. Techniques available include bulk RNA-sequencing (RNA-seq), single

nuclei RNA sequencing (snRNA-seq), and single nuclei Assay for Transposase-Accessible Chromatin sequencing (snATAC-seq). From this data, it is possible using bioinformatic tools to estimate both the composition of cell types within the whole biopsy and the expression of genes in those cell types. Early transcriptomic analysis of kidney tissue in IgAN has focussed on bulk RNA-seq and has identified specific transcriptomic signatures associated with specific histopathological lesions (171). These studies are at an early stage but it is likely that over the next decade transcriptomic analysis of the kidney biopsy in IgAN will provide important prognostic information and aid treatment decisions.

In addition to conventional transcriptomics, there have also been a small number of miRnomic studies performed in kidney tissue in IgAN. MicroRNAs (miRs) are short, non-coding oligonucleotides that regulate gene expression by disrupting translation. Since their discovery in 1993 multiple pathophysiological roles of miRs have been reported in a wide variety of conditions including IgAN (78). A small number of reports have identified miRs that are associated with components of the “four hit” hypothesis, including generation of gd-IgA1 (172, 173) and inflammation, remodeling and fibrosis in the kidney. A cluster of miRs (miRs 21-5p, 155, 199a-5p, 205, and 214-3p) have been associated with development of fibrosis and interstitial scarring in IgAN (174–177); miRs 21-5p, 214-3p, and 199a-5p are associated with kidney function decline, while others are associated specifically with mesangial inflammation (178, 179) and the modulation of endocapillary hypercellularity (180). In parallel with changes in miR expression in the kidney, changes in urinary miR excretion have also been reported in IgAN. While all of these studies are small and currently not validated we feel there is a potential for future development of miR biomarkers in IgAN.

In addition to molecular techniques, proof of concept studies are now emerging for advanced proteomic and metabolomic analysis of the kidney biopsy and urine to identify novel biomarkers, although these studies are very much in their infancy and their findings are unlikely to be integrated into clinical practice for the foreseeable future (181–184).

EMERGING MODIFIERS OF THE “FOUR HITS” AND THEIR POTENTIAL ROLES AS BIOMARKERS IN IgAN

Genomic Biomarkers in IgAN

A number of genome wide association studies (GWAS) have identified risk alleles associated both with the development of IgAN and the risk of progression to ESKD. A number of these risk alleles are associated with genes directly involved in modulating the immune response. With the advent of next-generation sequencing (NGS), whole exome sequencing (WES), and whole genome sequencing (WGS) the depth of genomic knowledge of IgAN will increase exponentially over the next decade. To date integrating genomic data into risk scores for prognostication and treatment decision making has not been

performed in IgAN. As an example of the possible utility of the currently available genomic data we generated an IgAN-Genetic Risk Score (GRS) using 14 single-nucleotide polymorphisms (SNPs) drawn from the largest European GWAS and calculated the IgAN-GRS in 464 biopsy proven IgAN Caucasian cases from the UK Glomerulonephritis DNA Bank and in 379 767 Europeans in the United Kingdom BioBank (UKBB) (185). We used the mean of IgAN-GRS to calculate the proportion of potential IgAN cases in subjects with hematuria and other non-specific kidney phenotypes in the UKBB. We estimated that IgAN accounted for 19% of hematuria cases and 28% of cases with hematuria, hypertension, and microalbuminuria in the UKBB. In this study, we used an IgAN-GRS to estimate the prevalence of IgAN contributing to common phenotypes that would not normally be biopsied. Further work is needed to assess if an IgAN-GRS may be useful for individual diagnosis and aid prognostication in IgAN.

Microbiomic Biomarkers in IgAN

There has been increasing interest in the role of the microbiome in IgAN, in particular as part of the gut-kidney axis and as a stimulus for gd-IgA1 synthesis and release into the circulation. IgAN may flare during mucosal inflammation and infection (186) and development of glomerular IgA deposits in at least two murine models of IgAN is dependent upon the presence of commensal gut bacteria (162, 187). Furthermore, GWAS have identified risk alleles associated with genes involved in maintaining the integrity of the gut mucosal barrier in IgAN (188). Reduced gut microbial diversity has been reported to be associated with progressive IgAN compared to non-progressors and healthy subjects (189). Exploration of the gut microbiome to identify non-invasive biomarkers is therefore of great interest, although again we are at the start of the biomarker discovery and validation journey. With the potential for the introduction of a targeted-release formulation of budesonide (NEFECON®) for the treatment of IgAN, which is designed to deliver budesonide to the Peyer's patches in the terminal ileum, identifying biomarkers that help us monitor the impact of gut-directed therapies is likely to be important in monitoring local response to therapy.

BIOMARKERS TO PREDICT RECURRENCE OF IgAN IN THE KIDNEY TRANSPLANT

In those patients who develop ESKD, transplantation is the preferred method of kidney replacement therapy. Recurrence of IgAN in the transplant is a significant issue, occurring in up to 60% of patients (190–193). Predicting which patients are most likely to develop recurrent disease would facilitate more effective pre-transplantation counseling and may in the future allow a personalized approach to the use of pre-emptive treatments, when these therapies become available.

In a small study of 38 transplant recipients Berthelot et al. measured IgA-sCD89 complexes, gd-IgA1 and IgG-anti-IgA

antibodies pre-transplant and correlated these levels to risk of IgAN recurrence (194). Pre-transplant gd-IgA1 and IgG-anti-IgA antibody levels were higher and IgA-sCD89 complexes lower in patients who went on to develop disease recurrence compared to those who did not develop recurrent disease. While these results are of interest, a number of confounding factors that may have influenced risk of recurrence were identified including use of basiliximab or anti-thymocyte globulin (ATG) induction regimens.

CONCLUSION

We are in desperate need of validated IgAN-specific biomarkers to support treatment decision making and prognostication. At present, we heavily rely on traditional, largely non-specific, biomarkers of generic kidney disease to guide our care of patients with IgAN. While there have been many studies reporting novel biomarkers in IgAN, at present, none of these have translated into standard clinical care, largely due to the small nature of individual studies and the almost uniform absence of properly designed validation studies. If all of the new therapies currently being evaluated in clinical trials, which include endothelin receptor antagonists, B cell directed therapies, mucosal steroids, and complement inhibitors, are shown to be safe and effective clinicians are going to need robust biomarkers

to both help guide their choice of treatment and monitor response to therapy in their patients with IgAN. Our hope is that the bioresources generated as part of these large global trials will facilitate robust biomarker discovery and validation studies, which in the fullness of time may result in new biomarkers translating into the clinic.

AUTHOR CONTRIBUTIONS

ST authored the CD89 section of this work. SS authored the section on complement. CC authored the section on the microbiome. HS and JB contributed equally to the remainder of the manuscript. HS, CC, and JB were involved in editing the manuscript and JB was the supervising author. All authors contributed to the article and approved the submitted version.

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Autoimmunity and SLE: Factual and Semantic Evidence-Based Critical Analyses of Definitions, Etiology, and Pathogenesis

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One cannot discuss anti-dsDNA antibodies and lupus nephritis without discussing the nature of Systemic lupus erythematosus (SLE). SLE is insistently described as a prototype autoimmune syndrome, with anti-dsDNA antibodies as a central biomarker and a pathogenic factor. The two entities, “SLE” and “The Anti-dsDNA Antibody,” have been linked in previous and contemporary studies although serious criticism to this mutual linkage have been raised: Anti-dsDNA antibodies were first described in bacterial infections and not in SLE; later *in* SLE, viral and parasitic infections and in malignancies. An increasing number of studies on classification criteria for SLE have been published in the aftermath of the canonical 1982 American College of Rheumatology SLE classification sets of criteria. Considering these studies, it is surprising to observe a nearby complete absence of fundamental critical/theoretical discussions aimed to explain how and why the classification criteria are linked in context of etiology, pathogenicity, or biology. This study is an attempt to prioritize critical comments on the contemporary definition and classification of SLE and of anti-dsDNA antibodies in context of lupus nephritis. Epidemiology, etiology, pathogenesis, and measures of therapy efficacy are implemented as problems in the present discussion. In order to understand whether or not disparate clinical SLE phenotypes are useful to determine its basic biological processes accounting for the syndrome is problematic. A central problem is discussed on whether the clinical role of anti-dsDNA antibodies from principal reasons can be accepted as a biomarker for SLE without clarifying what we define as an anti-dsDNA antibody, and in which biologic contexts the antibodies appear. In sum, this study is an attempt to bring to the forum critical comments on the contemporary definition and classification of SLE, lupus nephritis and anti-dsDNA antibodies. Four concise hypotheses are suggested for future science at the end of this analytical study.

Keywords: systemic lupus erythematosus, anti-dsDNA antibodies, lupus nephritis, syndrome, semantics

INTRODUCTION

SLE, lupus nephritis and anti-dsDNA antibodies represent cores of this, in principal eclectic study. The narrative is in its nature a critical view on definition of lupus nephritis as part of the syndrome SLE, and its classification, etiology and pathogenesis. In particular, the interrelationship between numerous classification criteria has not been given priority in the literature, notably not in the original manuscripts presenting the 1982 American College of Rheumatology [ACR (1)] and the 2012 Systemic Lupus Erythematosus International Collaborating Clinics [SLICC (2)] sets of classification criteria. In the introduction to the revised SLICC SLE classification criteria it is stated “*To ensure that there is a consistent definition of SLE for the purposes of research and surveillance, classification criteria for SLE are needed*” (2). This statement indicates that the ACR or SLICC classification criteria are valid as reliable approaches to define SLE, even though they do not define SLE as a homogenous disease since the classification criteria by definition provides hundreds of clinical phenotypes [discussed in (3)]. **Figure 1** principally demonstrates the clinical phenotype variability problem. One basic problem is that the SLE study objects—the patients—are included based on selected heterogeneous clusters of classification criteria as defined in the 1982 ACR (1), the 1997 revised ACR (4), the 2012 SLICC criteria (2) and recently the 2019 EULAR/ACR classification criteria for SLE (5) instead of selecting cohorts of patients with a homogenous phenotype like lupus nephritis and anti-dsDNA antibodies as selection parameters.

This critical argumentation is not equally relevant to studies on elements of systemic autoimmunity, like autoimmunity to dsDNA in SLE [see e.g., (3, 6–16)]. Such studies are focused on distinct autoimmune processes that are unlinked from a solitary SLE context, as is indicated by the triangular¹ link of anti-dsDNA antibodies to SLE, infections and malignancies (**Figure 2A**). Autoimmunity to chromatin structures is, however, *relevant* for SLE (11, 13, 14, 35–38), and for pathogenesis of organ manifestations like lupus nephritis, dermatitis and cerebral affections, as discussed below.

Paradoxically, we are not able to explain *why* the classification criteria by any combinations *define* SLE. The criteria are neither etiologically nor pathogenetically linked to each other, a problem that has not been seriously discussed [see published discussions in (1, 2, 5)]. In the context, lupus nephritis may robustly stand on own feet as a unique and identifiable disease, as unintentionally (?) indicated in the SLICC criteria, as this set of criteria says that a person may have SLE if positive for anti-dsDNA antibodies and demonstrating proteinuria. Thus, we are not able to provide a concise definition of SLE and lupus nephritis, but we identify SLE when we encounter patients. This is based on inconsistent rather than coherent classification criteria, intuition, and on experience.

¹Triangular in this context: Relating to, or involving three elements (Merriam-Webster).

SYSTEMIC LUPUS ERYTHEMATOSUS—THE SYNDROME

SLE is an enigmatic disease, in which little of its pathogenesis and less of its etiology is understood. In the history of SLE, it is not possible to recognize penetrating studies that focus on an autoimmune origin (in sense of etiology) of SLE, but autoimmunity is recognized as a *disease-modifying* factor (in sense of pathogenesis) that promote disease progression (3, 39–41). Rather, genetics in humans (42–44) and in mice (45, 46), infections (13, 15, 47–53), or cancers (13, 54–57) may be relevant research foci to study molecular processes accounting for etiology. The transformation of etiology into pathogenic autoimmune processes are regarded central to understand the imaginative syndrome SLE.

SLE: Syndrome, Etiology, and Pathogenicity—Clarifying the Terms (Lexical and Logic Semantics and Simplifications)

Three terms are used to describe SLE: Syndrome, etiology, pathogenesis. The term *syndrome* means concurrence—symptoms appearing simultaneously. Etiology comes from etymologic: the study of causation, origination from the Greek αἰτιολογία, aitiología, “giving a reason for” (αἰτία, aitia, “cause”; and -λογία, -logia) (58). Etiology means the predisposition of a disease or syndrome, and therefore something that promotes pathophysiologic processes. Pathophysiology means the origination and development of a clinical disease. If etiology means the basic initiator, pathogenesis means the effector of the disease. These terms are important to consider if we aim to understand how to categorize hypotheses, basic and clinical science on SLE—and how to probe hypotheses aimed to understand the impact of classification criteria. There exists no evidence that SLE is promoted by an autoimmune etiology, because the 11 ACR or 17 SLICC criteria are definitively not connected to a common etiology. The criteria may per statements appear cumulatively in the body at different time points as specified (1, 2) and interpreted in the study of Arbuckle et al. (59). Considering the highly diverse nature of individual classification criteria, the criteria may in fact rely on different etiologies, and consequently on different pathogenic processes. The autoimmune pathogenesis involved in evolution of the syndrome SLE may therefore be set in motion not by a uniform underlying etiology, but by etiologies promoting individual classification criteria. A definition of SLE as a syndrome (3, 12, 39, 60) is therefore etymologically and theoretically unjustified. There are few discussions related to this problem, but are tangentially approached by Touma et al. (61).

Furthermore, accepting that criteria like “The anti-dsDNA antibody” may appear timely unlinked from a clinically overt pathogenic process challenges the Witebsky postulates attempting to define a disorder as autoimmune and pertained by a specific autoimmune response (62, 63) in analogy to the Koch’s postulates to define a causative relationship between a defined

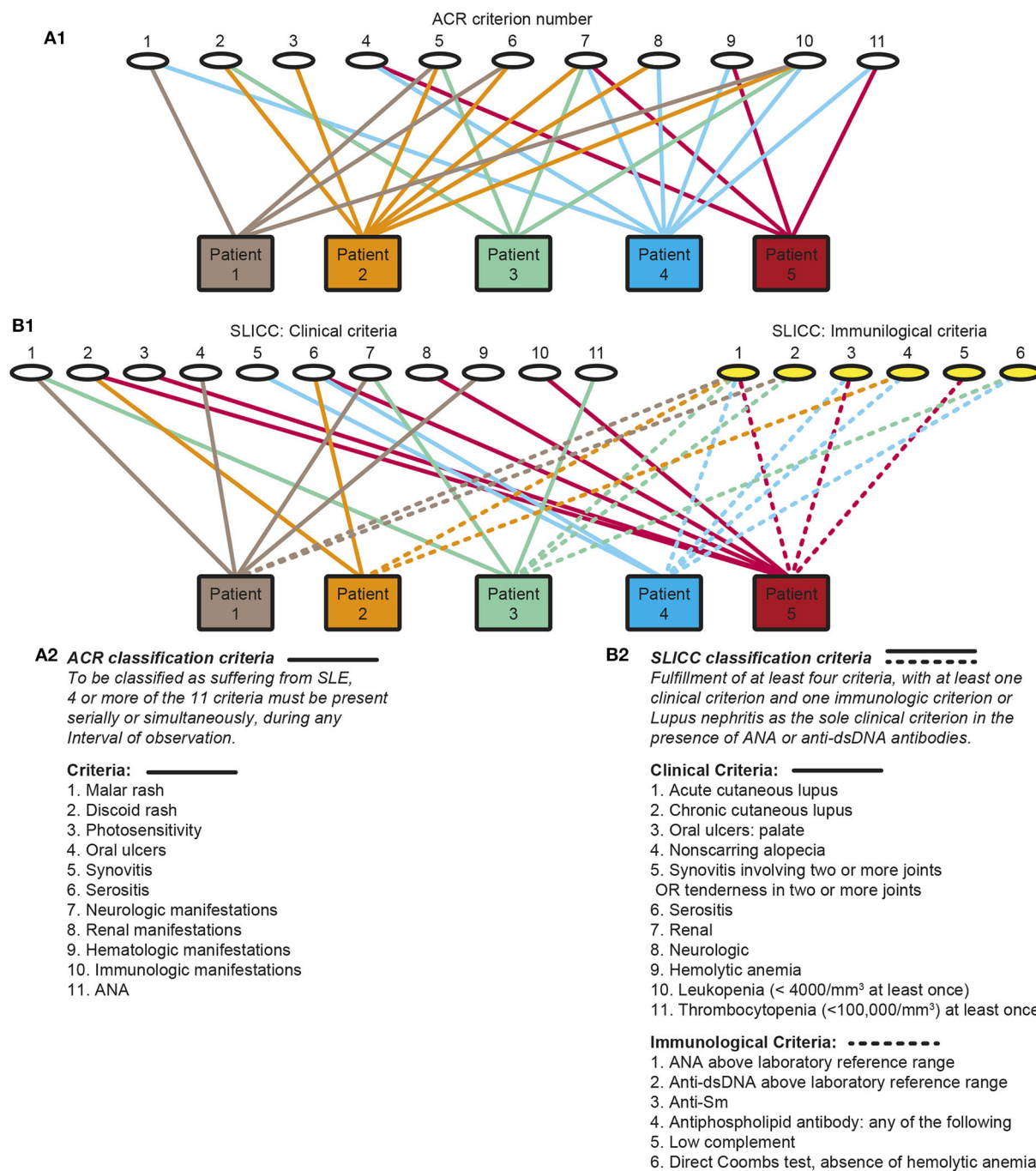


FIGURE 1 | Principal problems linked to classification of systemic lupus erythematosus (SLE). Classification of SLE patients according to The American College of Rheumatology (ACR) (**A1,A2**) or by The Systemic Lupus International Collaborating Clinics Criteria (SLICC) (**B1,B2**) classification criteria are descriptively problematized. Each of the classification systems identify a substantial diversity of clinical phenotypes. The 11 ACR criteria is presented by numbers (**A1**, the classification criteria are presented as a focused table in **A2**). Five patients are demonstrated. The patients share some criteria, but diverge with respect to others, and their clinical phenotypes differ individually. Similarly, each of 11 clinical and 6 immunological SLICC criteria are presented by numbers (**B1**, the classification criteria are presented as a focused table in **B2**). These chaotic figures (**A1**, **B1**) demonstrate that the use of the ACR and the SLICC criteria is problematic as bases for scientific analyses covering genetics, etiology, pathogenesis, and response to experimental therapy in patient cohorts as the study objects do not represent a homogenous group of patients. The patients in these figures are fictive but they reflect problems with the ACR and SLICC criteria in real life (Part of this figure (A) is a reprint with permission of Figure 1 in Rekvig (3)).

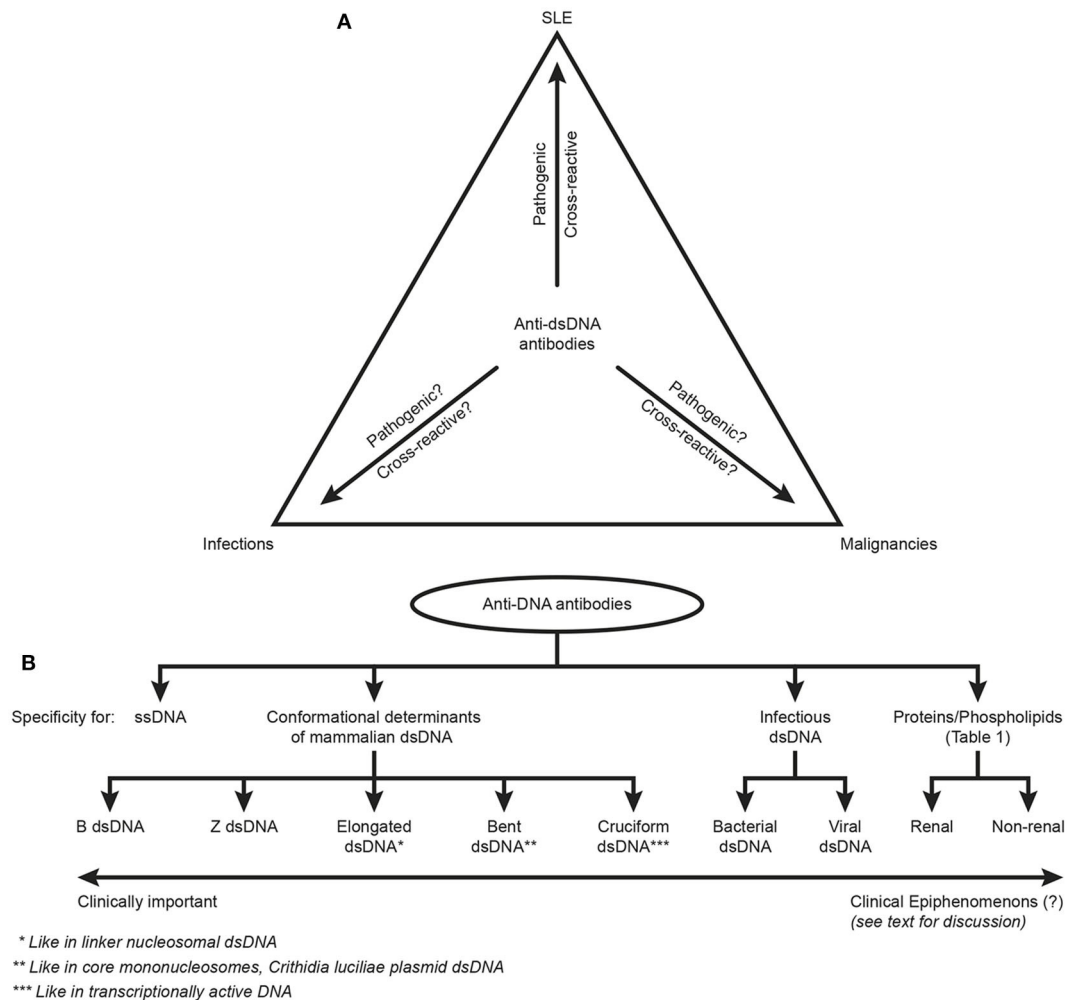


FIGURE 2 | Principal problems linked to the ACR and SLICC classification criterion “The anti-dsDNA antibody” (criterion 11 in ACR) or “Anti-dsDNA” (criterion 6, Immunological criteria, SLICC). Principal simplified problems are linked to the inadequate terminology of the anti-dsDNA antibodies. One problem [demonstrated in (A)] illustrates that “The anti-dsDNA antibody” is not unique for SLE, but appears regularly in context of infections and malignancies (see text for details). Little is examined whether anti-dsDNA antibodies are pathogenic and cross-reactive in the latter two categories of conditions (question marks in A) as they are in SLE. This triangular nature of anti-dsDNA antibodies has not been considered in the classification criteria, and poorly in the relevant literature on SLE. The second dominant problem considered for the “Anti-dsDNA antibody” is that the antibodies are presented as if “it” is monospecific for dsDNA. 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” is categorized in 4 main categories and cross-categories for mammalian dsDNA, 2 for infectious dsDNA and 2 for cross-reaction of anti-dsDNA for renal and non-renal proteins/phospholipids (see Table 1 for details on the latter category). Antibodies for all these dsDNA structures have been identified by conventional assay systems, like ELISA in physiological salt (elongated/bent B dsDNA), in high salt (Z dsDNA), cruciform dsDNA, bacterial and viral 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 in (B). Thus, data in this figure require that assay systems for anti-dsDNA antibodies relates to categorized structural dsDNA 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.

microbe and a consequently defined disease². The essence of the Witebsky postulates is that an autoantibody account for a given tissue damage, and the characteristic pathological changes can be reproduced upon transfer of the autoantibody (or suspected T cells) into experimental animals. This is discussed below.

²“Koch”. Random House Webster’s Unabridged Dictionary.

SLE: A Short History of Non-linear Periodic Paradigm Shifts Leading to Our Times Syndrome

The syndrome originally being described as a skin disease in antiquity has evolved into a complex disease through milestones defined as non-linear paradigm shifts (64–66). What can we learn from this still ongoing evolution of SLE, and how can we include the term SLE into a scientifically insinuated disease entity? In

this context, the complexity of SLE and patients suffering from SLE has been thoughtfully and eruditely presented by David Isenberg (67).

The transformation of SLE from a serious monosymptomatic skin disease into a syndrome has in Ludvik Fleck's (68) and Thomas Kuhn's (69) sense not evolved linearly, but through radical paradigm shifts. The central milestones appeared after studies in the 1850ies and their aftermaths. Central perceptions derive from paradigms settled by Cazenave in 1850ies (70), and later by Kaposi (71), Osler (72, 73), and Jadassohn [(74), reviewed in (64, 65, 70, 75)]. Through these paradigm shifts, the definition of SLE has evolved into that of a syndrome including systemic affection of viscera (71, 73) and later also comprising immunological, biochemical and hematological parameters (1, 2, 5). This has resulted in new authoritarian descriptions of SLE as in the canonical ACR (**Figure 1A**) or SLICC (**Figure 1B**) sets of criteria. With still ongoing expansion of classification criteria we have not reached a consensus on what SLE is, or what its etiology or pathogenesis are. Thus, the paradigm shifts resulting in the modern perception of SLE has not been very helpful to understand what SLE basically is, but they provided an understanding of its complex and systemic nature. An intriguing question we can raise in this context is if SLE of today at all is the disease known in antiquity as a skin disease.

When SLE should be interpreted from principals as those observed, implemented and decoded in the classification criteria, the *positivists*³ would (and indeed do so!) describe the syndrome through collection of facts/criteria. The elements that exert this collection of facts have, however, not reached a logic description based on firm scientific data beyond statistical co-appearance - cumulatively or simultaneously. By this, the syndrome SLE can, as it is understood today, be classified by a hermeneutic⁴ approach to understand its nature.

SLE: A Primary or Secondary Autoimmune Syndrome; Etiology vs. Pathogenesis

A classical statement promotes SLE as a prototype complex autoimmune syndrome (1, 2, 5, 61, 76, 77). However, this statement is standing in a certain contextual, but contrafactual paradigm hampered by one central logical problem: We do not understand an etiological origin of the classification criteria, or what the link between the current criteria are. We have till now not determined if they at all emerge from an inner biological coherence. Theoretically, they might be determined by a common etiology, or by diverse pathogenic processes that account for the apparently non-coherent classification criteria. If we aim our studies to understand the meaning of all the classification criteria for SLE, we need to distinguish the syndrom's etiology from the (secondary) pathogenicity that account for the manifold of the syndrome's classification criteria.

³Positivism: A philosophical system founded by Auguste Comte, concerned with positive facts and phenomena, and excluding speculation upon ultimate causes or origins.

⁴Hermeneutics is the theory and methodology of interpretation, especially the interpretation of biblical texts, wisdom literature, and philosophical texts.

The latter statement has not been profoundly discussed in the literature. Only few exceptions from this offensive comment on the classification criteria have been discussed. One obvious exception is expressed in the SLICC criteria; a patient is said to have SLE if having two criteria fulfilled: nephritis (proteinuria) concurrently with anti-dsDNA antibodies (2). Here, the antibody is strategically and logically linked to renal inflammation in a causal relationship: The antibody as inducer of de facto renal inflammation in accordance with the Witebsky proposals. The other comes from a study published by Pisetsky et al. (77) where they introduce a principal system for categorization of SLE phenotypes; i.e., defining phenotypes of SLE in groups according to interrelated criteria to define subgroups of SLE. In fact, Pisetsky's suggestion resembles data from Isenberg et al. where they upon longitudinal studies of 988 SLE patients identified different clusters of phenotypes (76). The newly suggested revision of the criteria published by Aringer et al. (5) and Touma et al. (61) do not help much here, as these revised criteria cement non-interrelated affections into an enigmatic disease entity! This is recently critically analyzed and discussed by Petri et al. (78).

SLE: A Cumulative Model for the Classification of SLE Raises Problems Linked to the Terms Etiology and Pathogenesis

Relevant in the present context is to understand what ties the evolving number of classification criteria together aimed to classify the *syndrome* SLE—a common etiological or a common pathogenetical mechanism? Or are they tied together as a result of a domino effect of pathogenic events: one leads to other events that are not initiated by the primary etiology? And what is the rationale behind the statements in the classification criteria that any events (processes, clinical criteria, deviating laboratory parameters) counts over the timeline of the syndrome. Classification criteria that appear disparate in time count cumulatively. According to the definition of the term syndrome—concurrence—this term does not harmonize with the statement that the criteria may appear simultaneously or at any time point in the history of a patient. If the criteria are related to each other as inducers (autoimmunity?) or responders (organ affections?) then how can the one or the other appear disparate over years? This is an accepted, although contrafactual, statement in the classification criteria which is not in agreement with the Witebsky postulates to define a disease as caused by a specific autoimmune antibody or an autoimmune T cell.

On the other hand, an autoimmune pathogenesis of SLE may be a valid term for some of the criteria (like lupus nephritis or lupus-related skin and cerebral affections) characterizing SLE. In harmony with this, data demonstrate that the kidney disease evolves and is maintained (*pathogenesis*), but not proven to be initiated (*etiology*) by autoimmune responses with anti-dsDNA antibodies as the central pathogenic factor (11, 16, 33, 79–81). However, other criteria than lupus nephritis, lupus dermatitis (82), and certain cerebral affections (83), have pathogenic origins that are beyond the impact of anti-dsDNA antibodies. It may be wise to probe the term autoimmune pathogenesis with the

Witebsky postulates (62, 63) to establish a causative relationship between a specific autoimmune response and a subsequent autoimmune disease.

Another principal problem related to the use of classification criteria is based on epidemiological studies and studies on the effect of experimental therapeutic modalities. A critical question must therefore be if patients implemented in multicenter-based ACR or SLICC defined cohorts are homogenous to a degree that allow us to validate results related to basic aspects of SLE, like its etiology, pathogenesis, epidemiology and effect of experimental therapy. This somewhat pedantic discussion is important since SLE is regarded as an integrated and unified syndrome—however without parameters that justify this assumption.

CURRENT APPROACHES TO STUDY THE NATURE OF SLE

The contemporary ACR or SLICC criteria-related definitions of SLE and its canonical link to autoantibodies against dsDNA (10, 13, 14) can be confronted by argumentations at different theoretical levels;

- i. Do we have clear evidence-based definitions of the syndrome and its marker antibodies;

Our contemporary insight into the syndrome SLE derives from three mainstream types of scientific approaches. One is based on identifying basic hypotheses related to separate processes accounting for individual classification criteria. The second approach is aimed to analyse why a wide diversity of clinical, biological, and biochemical parameters in SLE cohorts are implemented as diagnostic and classification measures. The third is a neglected approach; lack of studies to elucidate why the diverse classification criteria are appearing clustered in SLE. These approaches have not guided us into evidence-based definitions of SLE and its canonical marker antibodies. If we are going further into these problems, we do not need to generate more classification criteria, we need to select conservative and uniform selection criteria in order to implement homogenous patient cohorts, like those positive for proteinuria and anti-dsDNA antibodies. By this, we can analyse whether these two selection criteria define SLE and classification criteria that are pathogenetically linked to nephritis and anti-dsDNA antibodies. To select cohorts based on all combinations of classification criteria, as demonstrated in **Figure 1**, may yield some statistically significant combination of symptoms/parameters, but not information on pathogenesis and even less on etiology of each criterium or SLE itself.

- ii. Do we perform sound theoretical considerations applied to etiology and to pathogenesis of the syndrome itself as opposed to its individual criteria;

Such requested studies are difficult to identify in historical or contemporary studies. One possible approach could be to identify analytically or through studies of relevant literature etiological and pathogenetic processes accounting for individual classification criteria.

- iii. Can we implement open-minded reservations in this argumentation, or is this approach dominated by dogmatic conclusions deriving from statistical data (the positivistic approach)?

In my opinion we have to generate clear reservations when implementing newly and previously defined classification criteria. If statistically significant associations of criteria should be weighted, then biological and pathogenetical studies must be performed to promote information as to why these criteria tell us something about SLE.

Therefore, prevailing limitations of contemporary cohort studies are founded on analyses of highly heterogenic groups of SLE patients [discussed in (3)]. This simple fact makes studies of SLE difficult without clearly defined and reflected hypotheses (see suggested hypotheses in the conclusion section).

WHAT MAY EMERGE FROM THESE THEORETICAL TRIBULATIONS AND CONSIDERATIONS?

A conclusion of these reflections and concerns is that we need to reconsider how we classify SLE. We also need to generate new testable hypotheses, and accordingly to perform studies on clinically homogenous patient cohorts, and to define biomarkers relevant for such homogenous cohorts of SLE patients. Basically, we need to determine whether revised or contemporary classification criteria for SLE are etiologically or pathogenetically logic and understandable. In sum, we must prioritize, or categorize according to Pisetsky's definition (77), the criteria to approach a more uniform and homogenous definition of the syndrome SLE. For example, a homogenous cohort could be patients demonstrating anti-dsDNA antibodies concurrently with proteinuria, taking only these two criteria into account. In that context it would be intriguing to observe which of clinical or laboratory parameters would deviate from normal values.

“THE ANTI-DSDNA ANTIBODY” - AN ACCOUNT TO ITS NATURE AND STRUCTURAL DNA SPECIFICITIES

This heading indicates a problem. “The anti-DNA antibody,” as defined in ACR or SLICC classification criteria, is just that, and does not reflect anti-dsDNA antibodies specific for various dsDNA structures (see **Figure 2B**). This statement underscores the problems defined in the following proclamations. Anti-dsDNA antibodies occur in SLE, are a classification criterion for SLE, exist in autoimmune syndromes other than SLE (13), in bacterial (48, 53, 84), viral (49, 85–87), and parasitic infections (88), and in cancers [(89), see **Figure 2A**]. Importantly, these sets of anti-dsDNA antibodies have multiple specificities for unique DNA structures (**Figure 2B**). They have a pathogenic impact in SLE (but not in infections or in cancers?), and they may even be detected in healthy individuals [see general discussions in (10, 13, 14)].

Anti-dsDNA Antibodies: Appearing in Principally Different Clinical Conditions

The annexation of “The anti-DNA antibody” as a criterion for SLE does not communicate its pertinent clinical impact aside from simply being claimed to be involved in lupus pathogenesis [although how is still disputed (11)] or in which circumstances the antibodies are clinical epiphenomena distinctively separated from their assumedly pathogenic effects or their status as biomarker. See in this context a concise discussion of the term biomarker by Califf (90). Thus, rather of being a unique biomarker antibody for SLE, the antibodies demonstrate clinical associations with SLE, infections and malignancies, aside from appearing sporadic in other disorders (13).

In SLE, anti-dsDNA antibodies are pathogenic in context of lupus nephritis (11, 79), dermatitis (82, 91), and in certain forms of cerebral lupus (27, 83, 92). Whether these pathogenic pathways are determined by cross-reaction with non-DNA structures (see Table 1 for examples) or by homologous recognition of chromatin/dsDNA exposed in e.g., glomeruli (11, 93, 94), skin basement membranes (82), or in the brain (27) is still not firmly established. These clinical origins of anti-dsDNA antibodies has not been seriously considered in the classification criteria, nor in the relevant literature on anti-dsDNA antibodies and SLE [discussed in (1, 2, 5, 13, 14, 39)].

Anti-dsDNA Antibodies: Recognition of Disparate Unique dsDNA Structures and Not Simply dsDNA (a Review of Relevant Literature)

In the modern history of DNA discoveries, different forms of DNA structures have been described in highly focused research projects directed at describing what DNA is, which DNA structures exist, and their role in facilitating and regulating transcription of genes. Therefore, the second principal problem to be considered is that the antibodies are presented as if they constitute one specificity for dsDNA— “The anti-dsDNA antibody” (1, 2, 5). “The anti-dsDNA antibody” is not an unambiguous term, and the antibody reflects specificities to a variety of structures far beyond the canonical double helix structure. These structures represent the contexts in which dsDNA is presented to the immune system. The term “The anti-dsDNA antibody” comprises specificities toward ssDNA (95), Z DNA [left-handed dsDNA (96–99)], bent and elongated B DNA [right-handed dsDNA (100, 101)], diverse ss- and ds-RNA sequences and RNA-DNA double-strand hybrids (102, 103), folded and unfolded cruciform DNA structures (104, 105), bacterial DNA (106, 107), and finally different forms of viral dsDNA (108–110) that differ from mammalian dsDNA structurally and serologically (85, 110). Among these individual DNA structures, the most enigmatic in an auto-immunogenic context is the mammalian B form of dsDNA, since many of the other DNA structures were proven immunogenic, but this was not the case with mammalian B DNA. Therefore, over decades B DNA was considered non-immunogenic [(99, 111, 112), reviewed in (13)]. Anti-dsDNA antibodies further cross-react with a large panel of proteins and phospholipids.

TABLE 1 | Examples of anti-dsDNA antibodies that cross-react with non-DNA structures.

Anti-dsDNA antibody cross-react with	References
α -actinin	(17, 18)
Laminin	(19, 20)
C1q	(21)
Several cross-reactive activities presented at “Fifth International Workshop on anti-DNA anti-bodies in London 2002 to highlight relevant properties of pathogenic anti-DNA antibodies”	(22)
Phospholipids	(23)
Nucleosomes	(24)
Platelet integrin GPIIb 49–66	(25)
TLR 4	(26)
NR2 glutamate receptor	(27)
Cell surface proteins***	(28)
Ribosomal P protein	(29)
Collagen IV	(30)
Pneumococcal antigen	(31)
EBNA	(32)
Entactin	(33)
Entactin*	(34)

*Mono-specific anti-Entactin antibody is included to suggest a control non-cross-reactive antibody to determine if dsDNA as a cross-reactive specificity is required to gain pathogenic potential.

This heterogenous group of antigens targeted by anti-dsDNA antibodies are exemplified in Figure 2B and Table 1. The referred antibody specificities have been detected in natural situations (13), while antibodies have at least been raised experimentally to cruciform DNA structures (105).

One relevant question in this regard is whether fine molecular DNA antibody-specificities differ between their appearance in infections, malignancies and in SLE, as some antibodies may appear depending on the clinical situation, as is demonstrated for experimental induction of Z dsDNA but not B dsDNA specific antibodies in mice (99) although both appear in SLE. A similar observation relates to the fact that the frequency of antibodies to elongated mammalian dsDNA, as nucleosomal linker dsDNA, is higher than antibodies to bent dsDNA as in the core mononucleosome, both present on the same chromatin structure (101, 113, 114) or to bent dsDNA as in the plasmid of *Crithidia luciliae* (100).

ANTI-dsDNA ANTIBODIES: ASSAY CONDITIONS DO NOT *PER SE* DETERMINE LEVELS OF ANTIBODY AVIDITIES, BUT REFLECT DISPARATE UNIQUE dsDNA SPECIFICITIES

The term “The anti-dsDNA antibody” has over decades shaped the concept that different assay systems detect antibodies possessing different avidities to dsDNA, but not different molecular or structural dsDNA specificities. This problem has

not been considered when discussing binding of antibodies to dsDNA in principally different antibody assay systems. This conflict is demonstrated in **Figure 2B**. Antibodies binding the “ssDNA/dsDNA” structures are in the figure classified into 4 main categories with 5 subcategories for mammalian dsDNA, 2 for infectious dsDNA and 2 for cross-reaction of anti-dsDNA antibodies with renal and non-renal proteins and phospholipids (**Figure 2B**, see **Table 1** for details on the latter categories). These categories and subcategories are examples of pertinent diversity of dsDNA structures recognized by this family of antibodies [see e.g., (115–117), all specific for functional DNA structures or infectious-derived chromatin/DNA].

Antibodies for these dsDNA structures have been identified by conventional assay systems, like ELISA-based detection of anti-dsDNA antibodies against dsDNA in physiological salt; in high salt (Z dsDNA); cruziform dsDNA; bacterial and viral dsDNA [summarized in (10, 13)]. The idiom that anti-dsDNA antibodies are presented in a singular form (“The dsDNA”) must be challenged by the comprehensive structural recognition diversity. This clearly opens for individual specificities generated by different functional/structural states of dsDNA rather than individual avidities (either low or high) linked to different assay conditions. For example, if an antibody binds dsDNA in 2M NaCl, it binds to a structure shaped in 2M NaCl; the Z dsDNA (99, 118–121), and not because it has a high avidity over-winning the strength of the high salt concentration as in the Farr assay (122–124). This is also relevant for binding of other proteins to dsDNA structures in various salt concentrations (125–127). This difference is also evident from the fact that it is easier to experimentally induce antibodies to Z dsDNA than to B dsDNA (99). Similarly, antibodies that bind elongated dsDNA as in ELISA and antibodies that bind bent dsDNA as in *Crithidia luciliae* or in the core nucleosome may possess the same level of avidities, but the antibodies recognize different structures, elongated vs. bent dsDNA (128).

Thus, interpretation of data in **Figure 2B** demonstrate that assay systems for anti-dsDNA antibodies detect specificities that may have no or high potential impact as biomarker for SLE. Their individual impact as classification criteria for SLE has, however, not been investigated. This problem needs to be solved in order to select the proper assays for clinically relevant anti-dsDNA antibodies.

Anti-dsDNA Antibodies: Immunogenic Origin—Facts and Controversial Hypotheses

The third principal problem considered for the anti-dsDNA antibodies relates to its biological origin—what imposes production of these antibodies. Normally, mammalian dsDNA is non-immunogenic (111, 129, 130). Tolerance in a normal homeostatic situation is maintained at several biological check-point levels. B cells specific for dsDNA above a certain level of affinity are deleted in the bone marrow (131, 132); their antigen receptors are edited, with loss of affinity for dsDNA [(133–135), see a general discussion in (136)]; or they may appear anergic and non-functional (137, 138). Tolerance is also

controlled by T helper cells. CD 4+ T cell deletion prevents autoimmunity, and CD 4+ T cells targeting chromatin-derived peptides are normally anergic (139). This is evident from experiments demonstrating that anergic CD 4+ T cells can be rendered functional in response to IL-2 (140, 141), thus helping B cells to transform into antibody-producing plasma cells. If such cells are deleted (142, 143), this will prevent B cells to receive competent CD4+ T cell signals to be transformed into antibody-producing plasma cells. This situation is presented in a simplified version in **Figure 3A**, where tolerant (anergic or deleted) T cells are indicated.

In SLE, however, autologous chromatin may gain immunogenic power because e.g., histone-specific CD 4+ T cell tolerance is truly terminated [**Figure 3B**, as described in (144–146, 148, 149)]. Termination of histone-specific CD4+ T cell tolerance is also easily achieved in experimental contexts (140, 141, 150), thus demonstrating that CD4+ T cell autoimmunity to chromatin-derived peptides is a latent property of the normal immune system (141).

Data have been demonstrated that infections may provide chromatin-binding proteins allowing cognate interaction of chromatin-specific B cells and non-tolerant helper T cell specific for the infectious chromatin-associated proteins [**Figure 3C**, see e.g., (13, 15, 49, 50, 86, 147)]. This model is denoted the hapten-carrier model for *in vivo*-induction of anti-chromatin/anti-dsDNA antibodies.

Anti-dsDNA antibodies can also be produced through a mechanism known as molecular mimicry (151–154), and the central and important study by Lafer et al. published in 1981 opened this topic for further studies of molecular mimicry as a potential driving force for appearance of anti-dsDNA antibodies (23).

Which of these models (described in **Figures 3B,C**) are operational in malignancies have not been investigated in depth. Since malignancies are complicated by viral and bacterial infections (155–157), this apprehension may hypothetically authorize the hapten-carrier model for infection-induced anti-dsDNA antibodies also in malignant diseases [as indicated in **Figure 3C**, exemplified by the role of e.g., the viral dsDNA-binding polyomavirus T antigen (50, 147)]. It is therefore tempting to assume that infections in malignant diseases may encompass a model in analogy to the one presented in **Figure 3C**—the hapten-carrier model, as explained in the next section.

Anti-dsDNA Antibodies: *In vivo* Expression of Virus-Derived, DNA-Binding Proteins Render Chromatin Immunogenic—Evidence for the Hapten-Carrier Model

In **Table 2**, data are presented providing evidences that *in vivo* expression of single dsDNA/chromatin-binding viral proteins instigate the production of anti-dsDNA, anti-histone, and anti-transcription factor antibodies like anti-TATA-binding protein (TBP), anti-cAMP-response element binding protein (CREB) antibodies in accordance with the hapten (dsDNA) -carrier (viral dsDNA-binding protein) model. Molecular mimicry is less

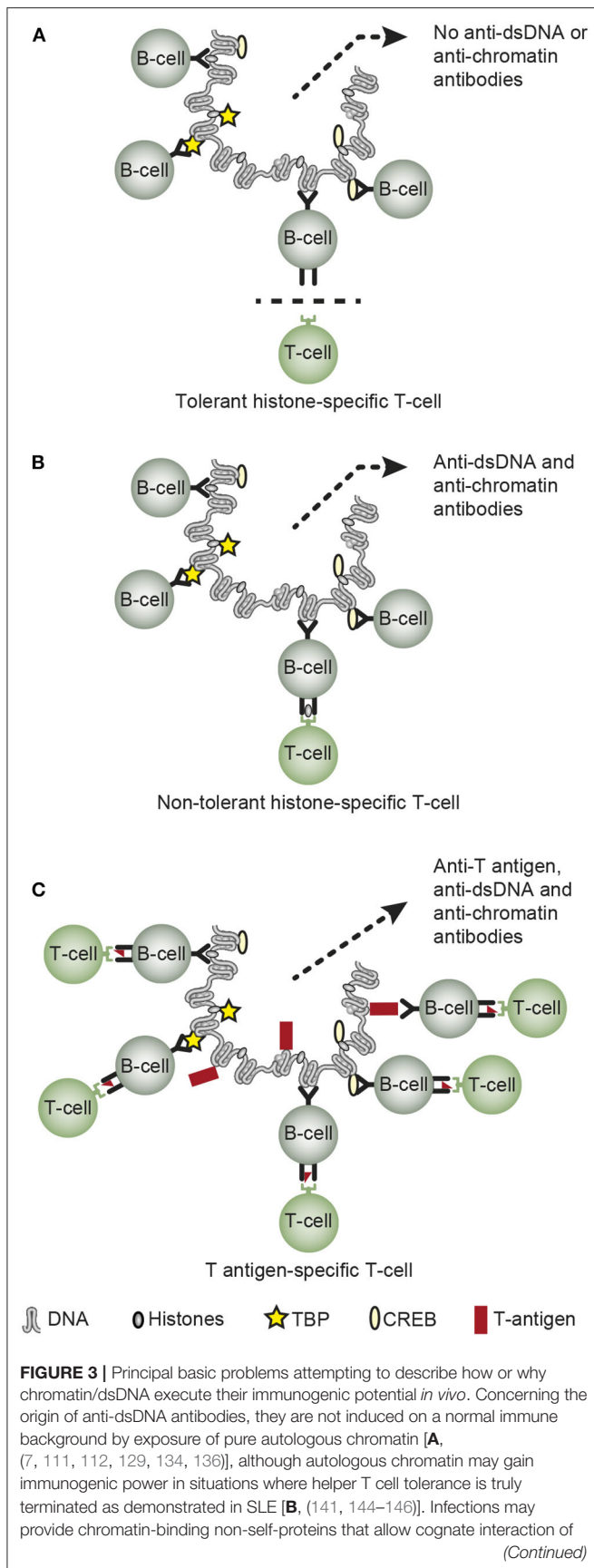


FIGURE 3 | chromatin/dsDNA-specific B cells and non-tolerant helper T cells specific for the infectious chromatin-associated protein [C, see Table 2 for examples (15, 49, 50, 86, 147)]. However, the possible immunogenicity of chromatin in malignancies has not been explored in depth, although infections as are predisposed for in cancers may encompass a model that is analogous to the one presented in (C) (C is reprinted with permission from Figure 5 in Rekvig (3)).

probable as explanation for production of anti-dsDNA in context of these experiments, since *in vivo*-expression of a mutated SV40 T antigen, the SLT155_{T>S}, rendered the SV40 T antigen non-dsDNA-binding and did not elicit production of anti-dsDNA or anti-histone antibodies [Table 2, (147)]. Expression of this mutant protein resulted, however, in antibodies to T antigen (Table 2). Summarizing the models described in Figures 3A–C and Table 2, tolerance is maintained in a normal individual, terminated in SLE patients and lupus-prone mice, and tolerance is also terminated in context of certain (complicating) infections.

Anti-dsDNA Antibodies: Tolerance to Chromatin and the Role of Autologous Chromatin-HMGB1 Complex and of DNase 1L3 Gene Deficiency in Promoting Anti-dsDNA Antibody Responses

There is still, aside from generation of hypotheses (158–161), no consensus as to whether pure autologous chromatin is rendered immunogenic in context of reduced clearance of apoptotic chromatin from dead and dying cells. Furthermore, no firm evidence has been provided that exposure of neutrophil extracellular traps (NETs) induce antibodies to dsDNA [see e.g., an insightful and still relevant discussion by Pieterse and van der Vlag (162)]. These hypotheses have been discussed over the last 2 decades, although sound experimental biologically relevant evidences are still lacking [see a discussion in (3, 13, 161, 162)].

Two independent observations may, however support the view that autologous chromatin possesses auto-immunogenic potential. Urbanaviciute et al. demonstrated that anti-dsDNA/chromatin antibodies are induced upon exposure of the high mobility group box protein 1 (HMGB1) tightly attached to chromatin in apoptotic cells (150). In another study, a null mutation in the DNase1L3 gene was described in SLE patients with lupus nephritis (163). This deficiency correlated with production of anti-dsDNA antibodies and lupus nephritis. In agreement with the observational study in familiar SLE, mice with experimentally deficient expression of the DNase 1L3 gene developed analogous anti-dsDNA antibodies and lupus nephritis (164). Thus, in DNase 1L3 gene deficient individuals extracellular degradation of chromatin is reduced and this deficiency correlates with promotion of anti-dsDNA antibodies. Thus, clearance deficiency of chromatin due to DNase 1L3 deficiency or release of extracellular chromatin in complex with HMGB1 from apoptotic cells, are two potential sources of complex autologous immunogens in both mice and humans.

TABLE 2 | Experimental expression of single viral DNA-binding proteins in context of plasmid injections, promote production of anti-dsDNA antibodies and to a variety of chromatin proteins.

Plasmid	promoter	Expressed antigen	Anti-T antigen antibodies	Anti-dsDNA antibodies	Anti-Histone antibodies	Anti-TBP*	Anti-CREB**	References
pRSV-BKT	RSV LTRt	Pyv T ag	681 ± 40 ***	715 ± 69	557 ± 112	318 ± 99	402 ± 91	(50, 147)
pRcCMV-BLT	HCMV ie-1	Pyv T ag	1282 ± 186	871 ± 53	763 ± 87	468 ± 254	552 ± 186	(50, 147)
pBS-BLT	None	Not expressed	Not detected	Not detected	Not detected	Not detected	Not detected	(50, 147)
pRcCMV-SLT155	HCM ie-1	SV 40 T ag	573 ± 34	360 ± 46	259 ± 52	Not tested	Not tested	(50, 147)
pRcCMV-SLT155 _{T>S}	HCM-ie-1	SV 40 T ag Mutant non-dsDNA binding	442 ± 44	29% ± 2, 5****	Not detected	Not tested	Not tested	(50, 147)
pRA 17	SCM-ie 1	EBNA 1 (69.7 kDa)	Not tested	EBNA-1, dsDNA, Sm	Not tested	Not tested	Not tested	(49)

All reported experiments are performed in BALB/c mice.

*TATA-binding protein.

**cAMP-response element binding protein.

***The results are given as titers. These were determined by ELISA analyses of the induced serum antibodies aimed to quantify autoantibodies in mice injected by various T antigen expressing/non-expressing plasmids. The titers were determined from 2-fold dilution curves starting from dilution 1/100. The titers were defined as the reciprocal value of the dilution giving 50% of maximal binding to wells, as determined by individual reference sera (147).

****These sera gave only marginal binding values as their binding at 1/100 dilution gave only 29% of the binding of a reference serum included in these assays.

ANTI-dsDNA ANTIBODIES AND LUPUS NEPHRITIS

In the next section new aspects and interpretive problems will be discussed in attempts to understand the link between anti-dsDNA antibodies, lupus nephritis and SLE. This is on one hand easy to do when considering the enormous amount of studies accepting this linkage, but on the other hand difficult if basic scientific data are considered critically.

SLE and “The Anti-dsDNA Antibody” — Clinical and Biological Contexts

Anti-dsDNA antibodies were first described in an infectious context in 1938–1939 (165–167), while in an autoimmune context in 1957 (168–171). Despite considerable scientific efforts we have not reached consensus on four fundamental aspects of anti-dsDNA antibodies in SLE. These aspects are comprised by four dogmatic areas: Their (i). Origin, (ii). Structural DNA specificities, (iii). Pathogenic impact, and *iv*. Assumed link to SLE [see discussions above and in (3, 11, 13, 14, 172)].

Anti-dsDNA Antibodies—Specificity Critically Determines Nephrogenicity and May Also Affect Alveolitis and Dermatitis?

Consensus has been established that anti-dsDNA antibodies promote lupus nephritis. How they do so are still controversial. The schisms divide scientists into two main interpretive groups. One assumes that the antibodies bind chromatin exposed in the kidneys (11, 173–176). This model is presented in **Figure 4A**. The other mainstream model implies that nephritogenic anti-dsDNA antibodies cross-react with intrinsic glomerulus basement membrane (GBM) constituents (illustrated in **Figures 4B, 5**). Which of the many autoantibodies described in SLE (183) involved in promotion of lupus nephritis remain, however, elusive. The cross-reacting antibodies assumed to be implicated

in lupus nephritis recognize among many ligands laminin (19, 184, 185), α -actinin (17, 18, 186), C1q (21), and entactin [(30, 33), **Table 1**].

There is, however, one central problem in these studies. Each of them focuses on one cross-reactive pattern and conclude that the actual cross-reaction correlates with lupus nephritis. Surprisingly, no discussion is presented that require a mutual multicenter study that compare the different cross-reactions in one cohort of lupus nephritis patients. This approach is awaited because nephritogenic-prone cross-reactions can be identified, and those that do not correlate with nephritis can be separated as unrelated epiphenomena. It would be important also to test if mono-specific antibodies not recognizing dsDNA, like those monospecific for e.g., laminin, or α -actinin, have the potential to promote lupus-like nephritis, as is suggested for the mono-specific anti-entactin antibody presented in **Table 2**.

To understand the basis for these problems, it is necessary to understand the unique processes of the two dominating models for lupus nephritis. In **Figure 4A**, the chromatin model is presented. On top, a principal presentation of the architecture of a glomerulus is illustrated and in Line 1 a principal transition of the mesangial matrix into the GBM is indicated.

In a classical progression of lupus nephritis, as described in (NZBxNZW)F1 mice (177, 178) chromatin-IgG complexes deposit in the mesangial matrix and form the early mesangial nephritis (**Figure 4A**, line 2). One consequence of this limited inflammation is silencing of renal DNase 1, reduced fragmentation of chromatin from dead cells, and subsequent accumulation of large chromatin fragments in complex with IgG in the GBM [line 3, (178, 179)]. This process forms the basis for a systemic glomerular inflammation and progression of lupus nephritis into end stage renal disease. Silencing of DNase 1 expression in this situation is unique to the kidney and does not occur in other organs (179). Notably, the mesangial matrix and the GBM share constituents like laminins, collagens and entactin. As chromatin-IgG complexes bind laminins and

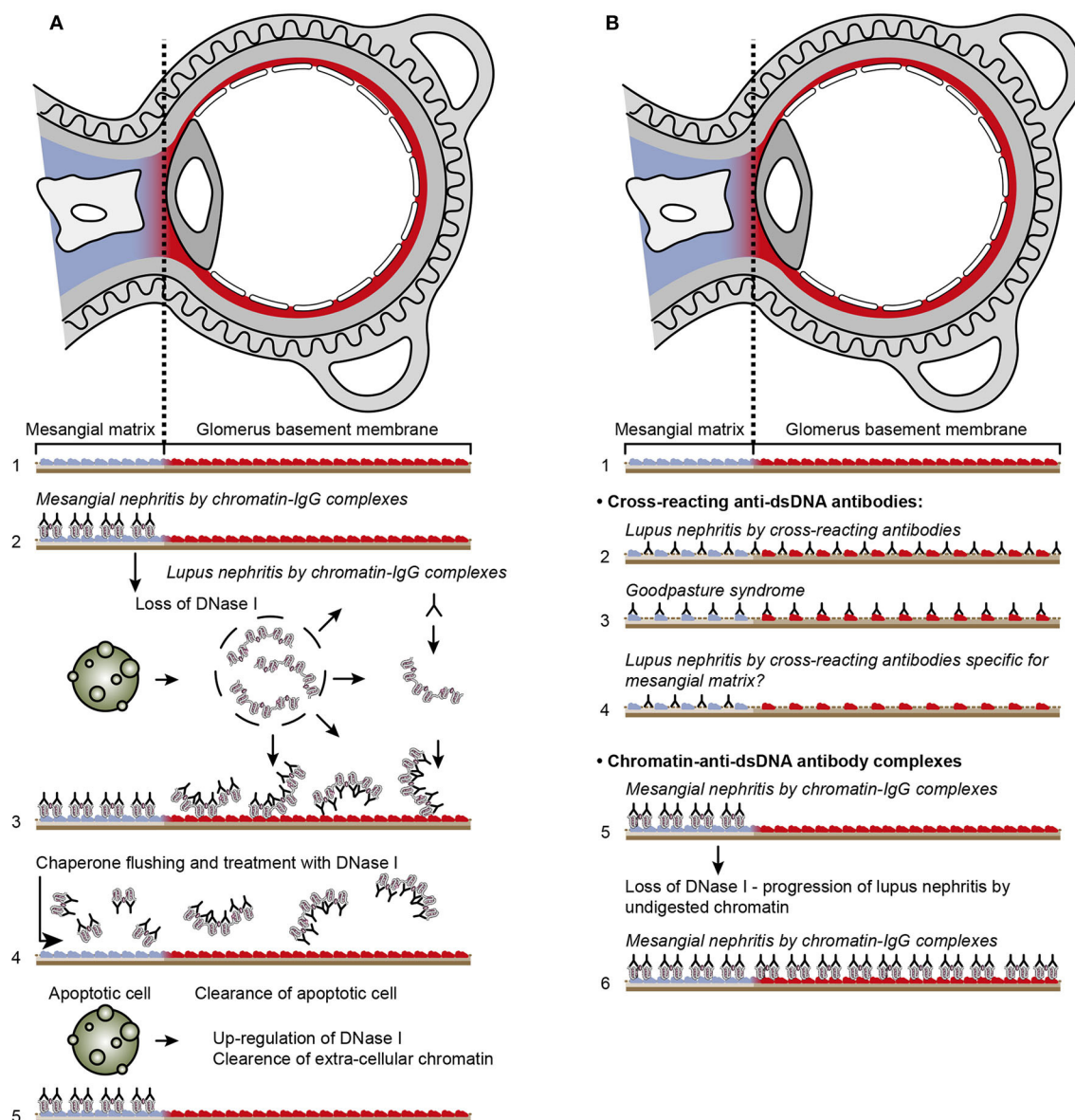
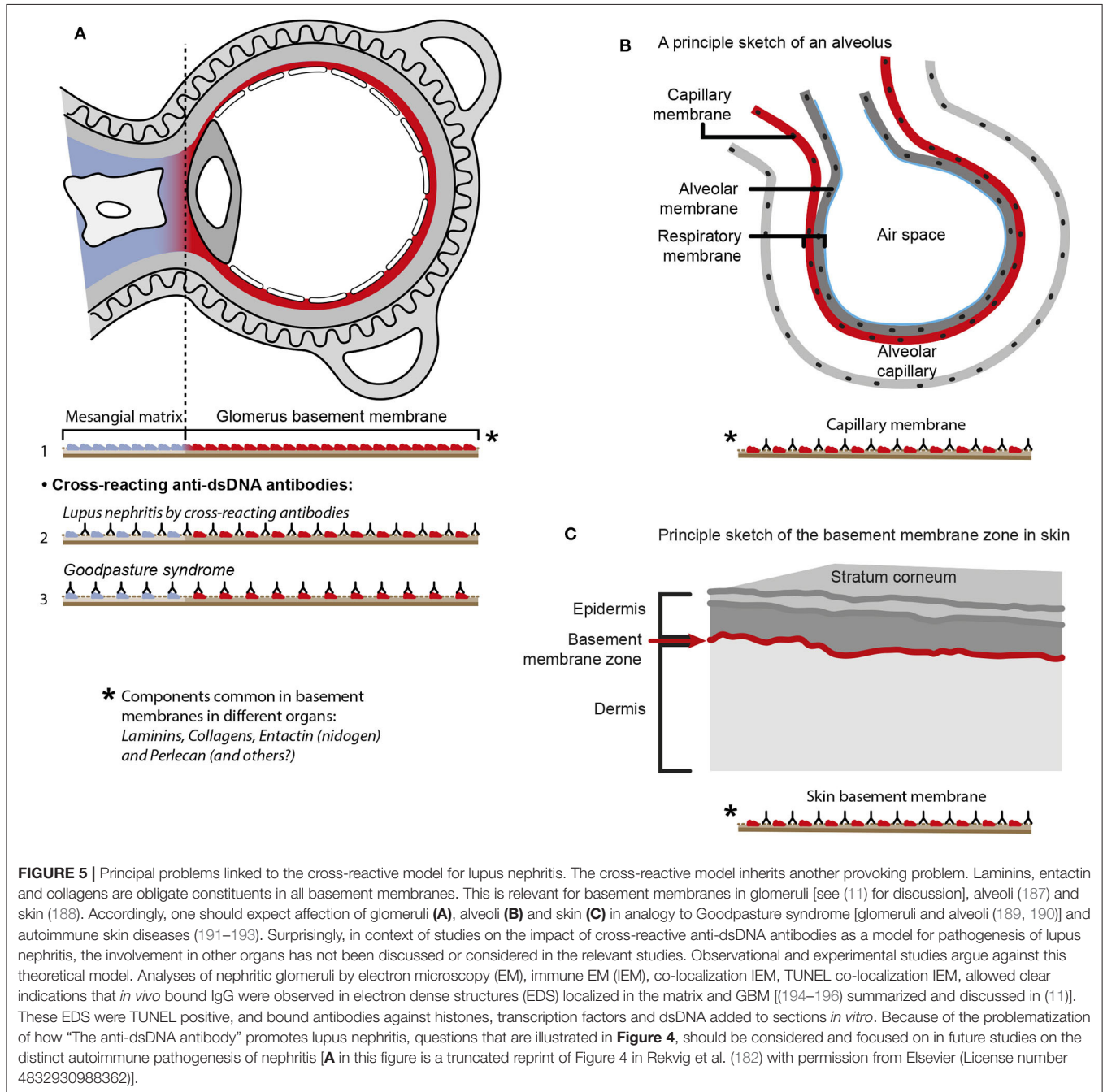


FIGURE 4 | Principal problems to be solved before the chromatin or the cross-reactive model for lupus nephritis can be settled. In **(A)**, the chromatin model is presented. On top, a principal presentation of the architecture of a glomerulus is described. In line 1, the mesangial matrix (blue) and its transition into the GBM (red) is principally demonstrated. In a classical progression of lupus nephritis (177, 178), chromatin-IgG complexes deposit in the mesangial matrix and form the early mesangial nephritis (line 2). One consequence of this limited inflammation is silencing of the renal endonuclease DNase 1, a consequent reduced fragmentation of chromatin from dead cells, and a subsequent deposition of large chromatin fragments in complex with IgG within the GBM [line 3, (178, 179)]. This forms the process that promote glomerular inflammation and progression of lupus nephritis into end stage disease [discussed in (11)]. Silencing of DNase 1 expression in this situation is unique to the kidney and does not occur in other organs (179). Since chromatin-IgG complexes bind laminins and collagens in the GBM with relatively high affinity (180), and are released locally in the glomerulus, these observations may explain the canonical progression of lupus nephritis as described by Weening et al. (177). This process may have therapeutic consequences, since chromatin prone to be deposited in GBM may be removed by flushing kidneys with the negatively charged heparin or other analogous chaperone molecules [line 4, (181)⁵], and theoretically, the process may be interrupted upon upregulation of renal DNase 1 expression [line 5, (181)]. In **(B)**, the glomerulus architecture is organized as in **(A)**, and the matrix-GBM transition is principally illustrated (line 1). In the cross-reacting model, cross-reacting anti-dsDNA antibodies bind intrinsic glomerular structures like entactin, laminin or collagen (line 2, see also data in **Table 2**). Since these antibodies may bind ligands shared by mesangial matrix and GBM, the antibodies are expected to bind simultaneously in the mesangial matrix and in the GBM (line 2). Therefore, the cross-reactive antibodies might well-initiate a glomerular inflammation more similar to the renal inflammation in Goodpasture syndrome (line 3) than to the stepwise progression of lupus nephritis as illustrated in **(A)**, lines 2 and 3. This consequence has not been considered in the relevant literature. One possible exception for this Goodpasture-like inflammation would be an early production of antibodies specific for a ligand unique for the matrix (suggested in line 4) and that the mesangial

(Continued)

⁵Jenny Buckland. Therapeutic targeting of chromatin in lupus nephritis? Research Highlights *Nat Rev Rheum.* (2011) 7:132.

FIGURE 4 | nephritis promoted by this particular antibody incites an inflammation that down-regulates renal DNase 1 and a subsequent exposure of undigested chromatin fragments in complex with IgG anti-chromatin antibodies in GBM. This would promote the evolution of progressive lupus nephritis. In contrast to this hypothetical model, lines 5 and 6 summarize progressive lupus nephritis according to the chromatin model. These principally conflicting models are summarized in **(A)**, lines 2 and 3 for the chromatin model, and in **(B)**, line 2 for the cross-reactive model [This figure is a revised and extended version of Figure 4 in Rekviig et al. (182) with permission from Elsevier (License number 4832930988362)].



collagens with relatively high affinity (180), and are released locally in the glomerulus, these observations may explain the canonical progression of lupus nephritis from mesangial nephritis into end-stage disease as described by Weening et al.

(177). This process may have specific therapeutic consequences, since chromatin prone to be deposited in GBM may be removed by flushing kidneys with heparin or other analogous chaperone molecules **[Figure 4A** Line 4, (181)], and theoretically, the

process will assumedly be interrupted upon upregulation of renal expression of DNase 1 [(181), as indicated in **Figure 4A**, line 5]. Such experimental approaches are awaited.

In **Figure 4B**, the architecture of the glomerulus is repeated, and the transition of the mesangial matrix into the GBM is indicated (**Figure 4B**, line 1). Cross-reacting anti-dsDNA antibodies bind non-dsDNA, intrinsic mesangial matrix and GBM structures like entactin, laminin or collagen (**Figure 4B**, line 2); thus, these antibodies may from theoretical arguments simultaneously bind ligands shared by the mesangial matrix and GBM. If this model is correct, the cross-reactive antibodies might well initiate a glomerular inflammation similar to the renal inflammation in Goodpasture syndrome (**Figure 4B**, Line 3). The model may indicate that mesangial nephritis does not precede progressive lupus nephritis, but appear simultaneously. This consequence of a cross-reaction has not been considered in the literature.

One possible exception for this would be an early production of antibodies specific for a ligand unique for the matrix (suggested in **Figure 4B**, line 4) or that the mesangial nephritis promoted by this particular antibody incites an inflammation that down-regulates renal DNase 1 and subsequent exposure of large chromatin fragments in GBM and thereby the evolution of progressive lupus nephritis. In contrast to this hypothetical model, **Figure 4B** lines 5 and 6 summarize progressive lupus nephritis according to the chromatin model. These principally conflicting models are summarized in **Figure 4A**, lines 2–3 for the chromatin model, and **Figure 4B**, line 2 for the cross-reacting model.

The cross-reactive model also inherits another provoking problem that is not regarded in the literature. Since e.g., laminins, entactin, collagens, and other ligands are obligate constituents in all basement membranes, this is relevant also for basement membranes in glomeruli [discussed in (11)], alveoli (187) and skin (188). Accordingly, one would expect affection of glomeruli (**Figure 5A**), alveoli (**Figure 5B**) and also skin (**Figure 5C**) in analogy to Goodpasture syndrome [glomerulonephritis and alveolitis (189, 190)], and to autoimmune skin diseases (191–193, 197). Surprisingly, in context of studies on the impact of cross-reactive anti-dsDNA antibodies as central in the pathogenesis of lupus nephritis, the involvement in other organs has not been considered in relevant studies.

Concluding Remarks and Four Concise Hypotheses

SLE is a complex serious disease considered to rely on an autoimmune pathogenesis. One central question arises from the discussions above: Is SLE with nephritis another syndrome than SLE without nephritis? And are the same clusters of classification criteria, and the same sets of biomarkers linked to SLE with and without nephritis or with or without anti-dsDNA antibodies informing about the same fundamentals of the disease? We do not need more classification criteria

in the aftermath of those tentatively identified till now. For now, we first have to develop an understanding why they appear in clusters and thereby why they define the syndrome SLE. Today, these problems are hidden from our perspective on SLE in our search for new classification systems for SLE. We need a penetrating and better theoretical model for SLE, to generate a basis for new and stringent cohort studies. The discussions given above on classification systems for SLE, anti-dsDNA antibodies and phenotypes of lupus nephritis must be imperative to develop new concise and testable hypotheses.

The following hypotheses may be considered.

- Analyzing cohorts of SLE patients selected by ACR or SLICC criteria will identify a larger spectrum of deviating clinical and biological parameters than homogenous cohorts of patients selected based on e.g., proteinuria and anti-dsDNA antibodies.
- The “Anti-dsDNA antibody” has lower impact as a classification criterium than anti-dsDNA antibodies specific for certain unique dsDNA structures.
- Different assay systems detect antibodies with different structural dsDNA specificities and not different avidities of the antibodies detected in the individual assay systems, thus this may result in different phenotypic presentations of SLE.
- If crossreacting anti-dsDNA antibodies bind renal basement membrane structures like laminin, entactin and collagen, the probability that they bind basement membranes in other organs is high. If not, one may question whether cross-reaction is of clinical significance.

In sum, SLE remains an enigmatic disease despite (or because) implementing new classification criteria; anti-dsDNA antibodies in clinical medicine are still poorly defined; lupus nephritis pathogenesis needs to be defined with respect to specificity of nephritogenic anti-dsDNA antibodies: Specificity for dsDNA or cross-reacting renal antigens.

DATA AVAILABILITY STATEMENT

The present article is a review on hypotheses and theories related to murine and human SLE and lupus nephritis. All data are taken from original published studies approved by relevant ethical committees.

ETHICS STATEMENT

The present manuscript is a review on hypotheses and theories related to murine and human SLE and lupus nephritis. All data are taken from original published studies approved by relevant ethical committees. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

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

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Th17-Immune Response in Patients With Membranous Nephropathy Is Associated With Thrombosis and Relapses

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Membranous nephropathy (MN) is a rare autoimmune kidney disease. Most autoimmune diseases are associated with a pro-inflammatory Th17-immune response, but little is known about immune dysregulation in MN. In China, MN was associated with exposure to fine air particulate matter (PM_{2.5}) that could act as a danger signal and redirect immune response toward the Th2 or Th17 pathway. We aimed to analyze the cytokine profile of MN patients and to study the possible environmental factors involved in this immune reorientation, as well as the consequences on the prognosis of the disease. In this prospective study, 59 MN patients filled a comprehensive lifestyle questionnaire. Peripheral blood cells from MN patients were stimulated *in vitro* to measure the cytokines produced in supernatant. Cytokine profiles of MN patients were compared to 28 healthy donors and analyzed regarding individual PM_{2.5} exposure. Compared to healthy donors, MN patients had higher serum levels of Th17 and Th2 cytokines IL-17A (62 pg/ml [IQR, 16–160] versus 31 [IQR, 13–51], $P=0.035$), IL-6 (66767 pg/ml [IQR, 36860–120978] versus 27979 [IQR, 18672–51499], $P=0.001$), and IL-4 (12 pg/ml [IQR, 0–33] versus 0 pg/ml [IQR, 0–0], $P=0.0003$), respectively, as well as a deficiency of Th1 and regulatory T cell cytokines IFN- γ (5320 pg/ml [IQR, 501–14325] versus 18037 [IQR, 4889–31329], $P=0.0005$) and IL-10 (778 pg/ml [IQR, 340–1247] versus 1102 [IQR, 737–1652], $P=0.04$), respectively. MN patients with high IL-17A levels lived in areas highly exposed to PM_{2.5}: 51 $\mu\text{g}/\text{m}^3$ versus 31 $\mu\text{g}/\text{m}^3$ for patients with low IL-17A levels ($P=0.002$) while the World Health Organization recommends an exposition below 10 $\mu\text{g}/\text{m}^3$. MN patients with Th17-mediated inflammation had more venous thromboembolic events ($P=0.03$) and relapsed more often ($P=0.0006$). Rituximab treatment induced Th1 and regulatory T cell cytokines but did not impact Th17 cytokines. MN patients with Th17-mediated inflammation which appears to be related to an urban environment have worse

prognosis. Alternative strategies targeting dysregulated cytokine balance could be considered for these patients at high risk of relapse.

Keywords: membranous nephropathy, inflammation, Th17-profile, fine air particulate matter, thrombosis, relapse, non-invasive biomarker, prognosis

INTRODUCTION

For several decades, Western countries have been facing an increasing incidence of allergies and autoimmune diseases. Immune-mediated conditions are thought to result from a complex interplay between genetic predisposition, immune dysregulation, and environmental factors (1). Membranous nephropathy (MN) is a rare autoimmune disease (incidence 1.3 cases per 100 000 inhabitants affecting more men than women) (2) with an increasing prevalence (3), characterized by subepithelial immune deposits containing IgG and complement fractions with alteration of the glomerular basement membrane structure (4, 5) related to autoantibodies against podocyte proteins: M-type phospholipase A2 receptor 1 (PLA2R1) or thrombospondin type-1 domain-containing 7A (THSD7A) in 70% and 3% of patients, respectively (6–9). Anti-PLA2R1 and anti-THSD7A antibodies titers correlate with disease activity and predict disease outcome (10, 11). Disease evolution is highly variable with spontaneous remission, persistent proteinuria or end-stage kidney diseases (ESKD) (12). Rituximab is a first line anti-CD20 immunosuppressive drug often used to treat MN patients which induces remission in about 35% to 67% of cases (13–15), while MN recurs after remission in about 20% of cases (14). Nephrotic syndrome has been correlated with an exceptionally high risk of venous thromboembolic events (16, 17).

In spite of these advances in diagnosis, prognosis and treatment, little is known about the risk factors leading to the onset of MN. Several genome-wide association studies identified alleles at two genomic loci associated with idiopathic MN: HLA-DQA1 and single-nucleotide polymorphisms (SNPs) on PLA2R1 (18–26). More recently, a novel genome-wide significant (GWAS) risk locus for MN with large effects encoding two transcriptional master regulators of inflammation, NFKB1 and IRF4, has been discovered suggesting MN is an inflammatory disease (27).

Little is known about immune dysregulation in MN. While Th1 is usually associated with intracellular pathogens (Th1 cells secrete IFN- γ), Th2 is activated by parasites or allergens and secrete IL-4 and IL-5 (28). Th17-immune response is a pro-inflammatory immune pathway associated with autoimmune diseases (29, 30). Th17 cells require specific cytokines, such as transforming growth factor- β (TGF- β) combined with IL-6 or IL-21 for their differentiation (31). Th17 cells secrete a characteristic profile of cytokines including IL-17A, IL-17F, IL-21, and IL-22, which recruit and activate neutrophils and macrophages to fight against extracellular pathogens or mediate the development of autoimmune diseases (32). Th17 and regulatory T cells (Treg cells) are two subsets with opposite actions (33). They play an important role in the prevention of

autoimmunity and in the regulation of immune responses against infections and cancer in a cell-contact-dependent manner or by secreting inhibitory cytokines like IL-10 or TGF- β (34).

In MN, several studies led to different conclusions regarding the predominant type of immune response. Kuroki et al. showed 15 years ago that MN is associated with a Th2-type immune response with increased IL-4 levels (35–37) using real time PCR. Recently, Rosenzweig et al. and Roccatello et al. described a low level of Treg cells in two cohorts of MN (n=25 and n=17, respectively) at diagnosis and an increasing level of these cells after remission induced by rituximab (38, 39), associated with low levels of a Treg cytokine IL-35 that also increases with remission (38). Moreover, Rosenzweig et al. did not identify cytokine profiles in non-stimulated cells with a decrease of IL-17A in MN patients compared to healthy donors (39), which could be explained by a cytokine discharge in urine due to high proteinuria. Very recently, Li et al. showed an increased number of Th17 cells in primary MN patients after a stimulation with leukocyte activation cocktail (BD GolgiPlug™) using a flow cytometry-based intracellular cytokine detection method, and an increased plasma level of Th17 cytokines by ELISA (40).

Environmental factors may play a role in the pathophysiology of MN. The incidence of post-infectious MN is declining in industrialized countries, in part due to vaccination campaigns against hepatitis B, while its autoimmune form is stable or even increasing (41–44), which could be explained by hygiene hypothesis: low exposure to infectious agents in childhood induces a Th1/Th2 or Th17 imbalance causing allergic and autoimmune diseases. Environmental factors (pollution, vitamin D deficiency, smoking) could act as danger signals and redirect immune response toward the Th2 or Th17 pathway (1, 45). In China, Xu and al. showed a geographical correlation between the occurrence of MN (399 MN diagnosed in 2014) and the satellite analysis of exposure to fine particles (PM_{2.5}) detected over the same period (3).

We aimed to clarify which immune response plays a major role in MN. We analyzed the cytokine profile in the supernatant of peripheral blood cells from MN patients after a non-specific stimulation of immune cells and sought to establish whether this profile could be related to the environmental data collected.

MATERIALS AND METHODS

Study Design and Population

We performed a prospective cohort study in the Department of Nephrology at Nice University Hospital. The inclusion criteria were: 1) patients with biopsy proven MN or with either anti-PLA2R1- or anti-THSD7A-associated MN, at diagnosis or at

relapse; 2) urine protein/creatinine ratio (UPCR) >1 g/g and immunological activity (for PLA2R1- or THSD7A-related MN); 3) not having received immunosuppressive therapy in the 6 months before inclusion; 4) followed in the Department of Nephrology at Nice University Hospital between January 2016 and January 2018; 5) ability to sign an informed consent. Fifty-nine MN patients were recruited, and for 26 of them with active disease at inclusion (UPCR >3.5g/g) an additional follow-up sample at complete or partial remission was collected. Complete remission was defined as proteinuria <0.3g per 24 h and partial remission as proteinuria >0.3 but <3.5g per 24 h or a decrease in proteinuria by at least 50% from the initial value and <3.5g per 24 h, according to international Clinical Practice Guidelines (KDIGO) (46). Healthy donors of the same age and living in the same environment were also recruited. Patients and healthy donors with infection or breastfeeding at the time of sampling were excluded.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the appropriate institutional review committee (NCT02199145). Written informed consent was obtained from participants prior to inclusion in the study.

Blood Collection and Cytokine Assay

One milliliter of whole blood was collected and stimulated with immune ligands (anti-CD3 as T-cells stimulant, R848 as TLR 7/8 agonist, or both) on single lyophilized spheres (LyoSphere™, Qiagen) within 8 h from blood collection. Stimulated blood samples were incubated for 16 to 24 h at 37°C and then centrifuged at 2,000 to 3,000 × g for 15 min to harvest the stimulated serum. Stimulated serum was stored at -20°C until the analysis and freeze-thaw cycles were minimized to preserve the quality of the samples. Serum levels of 11 cytokines (IL-17A, IL-6, IL-1β, IFN-γ, IL-12p70, TNF-α, IL-10, IL-5, IL-4, IL-13, and GM-CSF) were measured using the ProcartaPlex™ Immunoassay Kit (Luminex™, ThermoFisher) or the custom-designed cartridges Ella (ProteinSimple™), following the manufacturers' instructions. ProcartaPlex™ Immunoassays incorporate magnetic microsphere technology to enable simultaneous detection and quantification of multiple cytokines in serum, and Ella measures cytokines in a microfluidic Simple Plex cartridge. All samples were measured both as pure and diluted 1:100, as the detection threshold differs among cytokines.

Data Collection

Clinical and environmental data were collected during the medical consultation, using the patient's medical record and *via* a questionnaire which focused on lifestyle, usual area of residence and medical history before the first symptoms of MN.

Exposure level to air pollutants (PM_{2.5}, PM₁₀, NO_x, CO, SO₂, and C₆H₆) was publicly available (Atmosud (a French public institution)). We used cumulative exposition during the year before the diagnosis or the relapse measured by Atmosud using sensors present at different points in the region studied (Supplemental Figure 1).

Statistics

For descriptive statistics, data are presented as mean and standard deviation for quantitative variables with Gaussian distribution, as median and range for quantitative variables with non-Gaussian distribution, or as numbers and percentages for qualitative variables. The Shapiro-Wilk test was used to determine if a variable had a Gaussian distribution or not. Quantitative variables were compared by the unpaired *t*-test or one-way ANOVA if the values were normally distributed and by the Mann-Whitney test if they were not. Qualitative variables were compared using Chi-square test or Fisher's exact test as appropriate. Receiver Operating Characteristic (ROC) curve was used to define an IL-17A threshold above which patients would be considered positive. A Wilcoxon matched pairs signed rank test was used to compare two measurements of a quantitative variable performed on the same subjects. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) or SAS 9.0. Differences were considered significant when *P* value < 0.05.

RESULTS

Serum Cytokine Levels in MN Patients Compared to Healthy Subjects After *In Vitro* Stimulation of Immune Cells

Serum Cytokine Levels in MN Patients

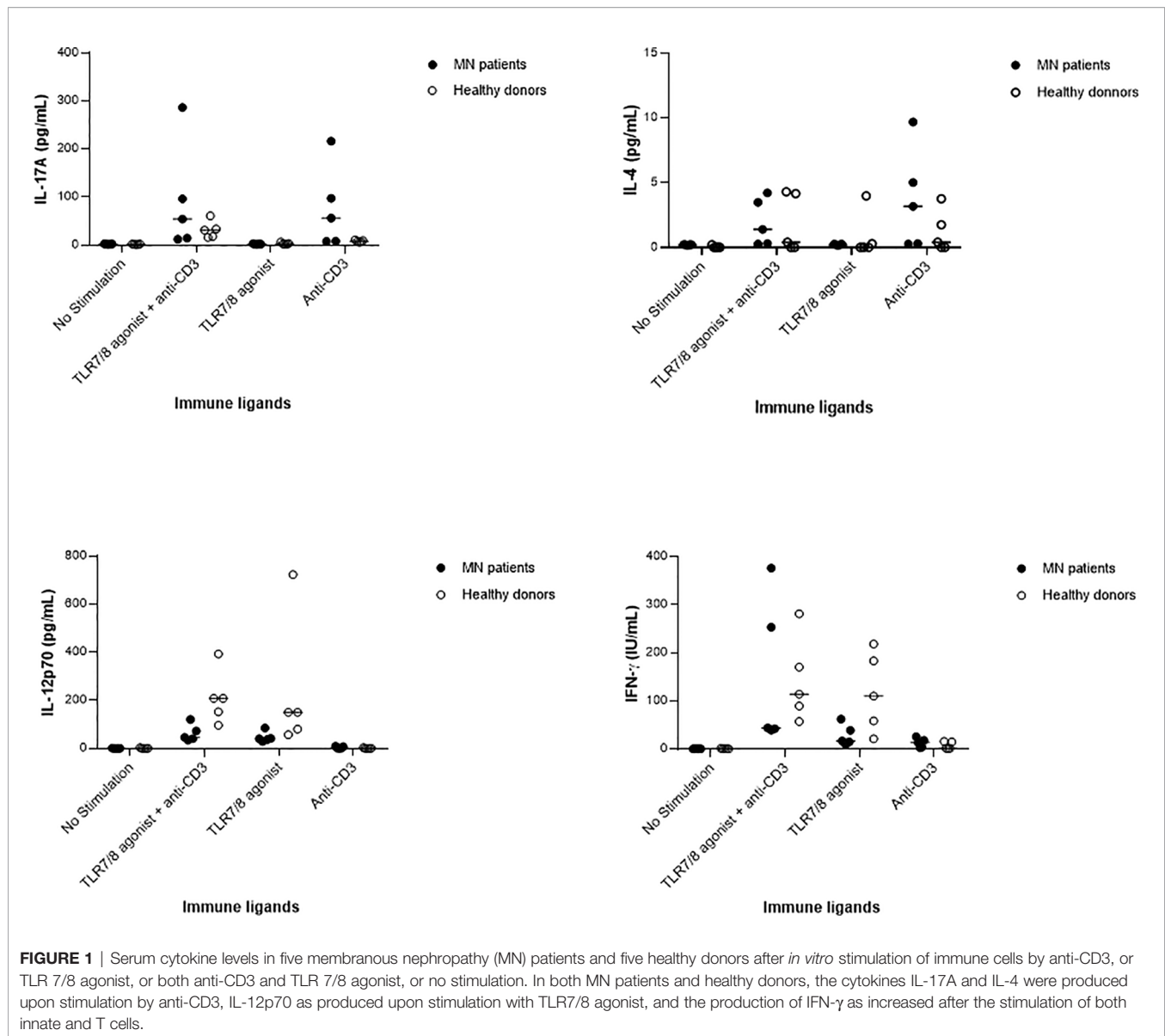
Without *in vitro* stimulation, a very low level of all cytokines was detected in serum (for example, IL-17A: 40.94 pg/ml [IQR, 0–96] after stimulation by anti-CD3 and R848 *vs* 0 pg/ml [IQR, 0–0] without stimulation, *P*=0.0005; IFN-γ: 4418 pg/ml [IQR, 1248–10350] after stimulation by anti-CD3 and R848 *vs* 0 pg/ml [IQR, 0–0] without stimulation, *P*<0.0001) (Supplemental Figure 2). This experiment was carried out on 14 MN patients before the start of the study to confirm the relevance of stimulating immune cells.

In Vitro Stimulation of Innate Cells Versus T Cells

Immune cells of 5 MN patients and 5 healthy donors paired for age (MN: mean age 45 ± 24 years *vs* HD: mean age 39 ± 16 years, *P*=0.63) and gender were stimulated with immune ligands: anti-CD3 as T-cells stimulant, TLR 7/8 agonist as a stimulant of macrophages and dendritic cells, or with both anti-CD3 and TLR 7/8 agonist. As shown in Figure 1, MN patients appeared to have increased levels of anti-CD3-induced IL-17A and IL-4, and impaired levels of IL-12p70 and IFN-γ after stimulation with the TLR7/8 agonist. This preliminary descriptive result suggests an enhanced Th17 and Th2 response and an impairment of the Th1 pathway in MN patients, as recently proposed by Li et al. (40).

Cytokine Levels in MN Patients After *In Vitro* Stimulation of Both Innate and T Cells

To approximate the pathophysiological conditions of a viral stimulation, described as a frequent immune trigger in MN, we induced *in vitro* stimulation of innate response by TLR7/8



agonist (47), and T lymphocytes by anti-CD3, in MN patients and controls, mimicking an activation of both innate and specific immune pathways. Fifty-nine patients with active MN (39 men and 20 women; mean age 53 ± 17 years) were enrolled, as well as 28 healthy donors (mean age 48 ± 14 years). For 26 MN patients who entered into remission follow-up samples were available. Patient characteristics are summarized in **Table 1**. Forty-two patients (71%) had anti-PLA2R1 antibodies, two (3%) had anti-THSD7A antibodies and 15 patients (26%) were negative for both anti-PLA2R1 and anti-THSD7A.

Supernatant IL-17A, IL-6, IL-1 β , IFN- γ , IL-12p70, TNF- α , IL-10, IL-5, IL-4, IL-13, and GM-CSF were measured after *in vitro* stimulation. The concentration of inflammatory cytokines IL-1 β and IL-6 implicated in innate immune response was significantly higher in MN patients than in the healthy control

group (IL-1 β : 8405 pg/ml [IQR, 5224–12065] versus 4522 pg/ml [IQR, 3183–6418], $P=0.0002$; IL-6: 66767 pg/ml [IQR, 36860–120978] versus 27979 pg/ml [IQR, 18672–51499], $P=0.001$). Th17 and Th2 cytokines IL-17A and IL-4, respectively, were also significantly increased in MN patients in comparison to healthy subjects (IL-17A: 62 pg/ml [IQR, 16–160] versus 31 pg/ml [IQR, 13–51], $P=0.035$; IL-4: 12 pg/ml [IQR, 0–33] versus 0 pg/ml [IQR, 0–0], $P=0.0003$) (**Figure 2**).

On the other hand, the concentration of Th1 cytokines IFN- γ and IL-12p70, as well as of a regulatory T cells cytokine IL-10 was lower in MN patients in comparison to healthy subjects (IFN- γ : 5320 pg/ml [IQR, 501–14325] versus 18037 pg/ml [IQR, 4889–31329], $P=0.0005$; IL-12p70: 23 pg/ml [IQR, 5–48] versus 83 pg/ml [IQR, 24–189], $P<0.0001$; IL-10: 778 pg/ml [IQR, 340–1247] versus 1102 pg/ml [IQR, 737–1652], $P=0.04$) (**Figure 2**).

TABLE 1 | Baseline characteristics of MN patients (n=59).

Characteristics	Value
Age (years)	53 ± 17
Sex	
Male	39 (66%)
Female	20 (34%)
Etiology	
Anti-PLA2R1-associated MN	42 (71%)
Anti-THSD7A-associated MN	2 (3%)
Double negative patients	15 (26%)
Laboratory evaluations	
UPCR (g/g)	4.29 [2.42 – 7.80]
PLA2R1-Ab titer (RU/ml)	43.5 [16.0 – 199.3]
Serum creatinine (μmol/L)	118.5 [86.0 – 230.0]
Albuminemia (g/L)	31.85 [21.88 – 36.75]
Urea (mmol/L)	8.65 [5.90 – 14.10]
Lymphocyte count (G/L)	1.7 [1.4 – 2.3]

Ab, antibody; MN, membranous nephropathy; PLA2R1, phospholipase A2 receptor 1; THSD7A, thrombospondin type-1 domain-containing protein 7A; UPCR, urine protein to creatinine ratio.

There was no difference in TNF-α, IL-5, IL-13 and GM-CSF concentrations between MN patients and healthy subjects (*data not shown*).

These data suggest an activation of the Th17 and Th2 pathways and a deficit of the Th1 and Treg pathways in MN patients as observed in many autoimmune diseases (29, 30, 39).

Th17 Profile and Air Pollutants

A threshold of 58 pg/ml was defined to distinguish the patients with positive IL-17A activity, determined using ROC analysis with a sensitivity of 86% and a specificity of 52% (AUC=0.64, $P=0.035$) (**Supplemental Figure 3**): 31 patients (53%) had a high level of IL-17A.

Forty-one patients (69%) lived in urban areas and 18 (31%) in rural areas. As previous studies demonstrated that exposure to PM_{2.5} induces production of pro-inflammatory cytokines (IL-1β and IL-6) (48) and is correlated with a higher risk of MN (3), we aimed to analyze the levels of air pollution according to the Th17 profile of MN patients.

IL-17A-positive patients were exposed to significantly higher levels of PM_{2.5} than IL-17A-negative patients at their usual area of residence: 51 μg/m³ [IQR, 46–51] versus 31 μg/m³ [IQR, 21–36], respectively ($P=0.002$) (**Figure 3**). The mean French exposition is evaluated at 12 μg/m³ and the World Health Organization recommends an exposition below 10 μg/m³ (49).

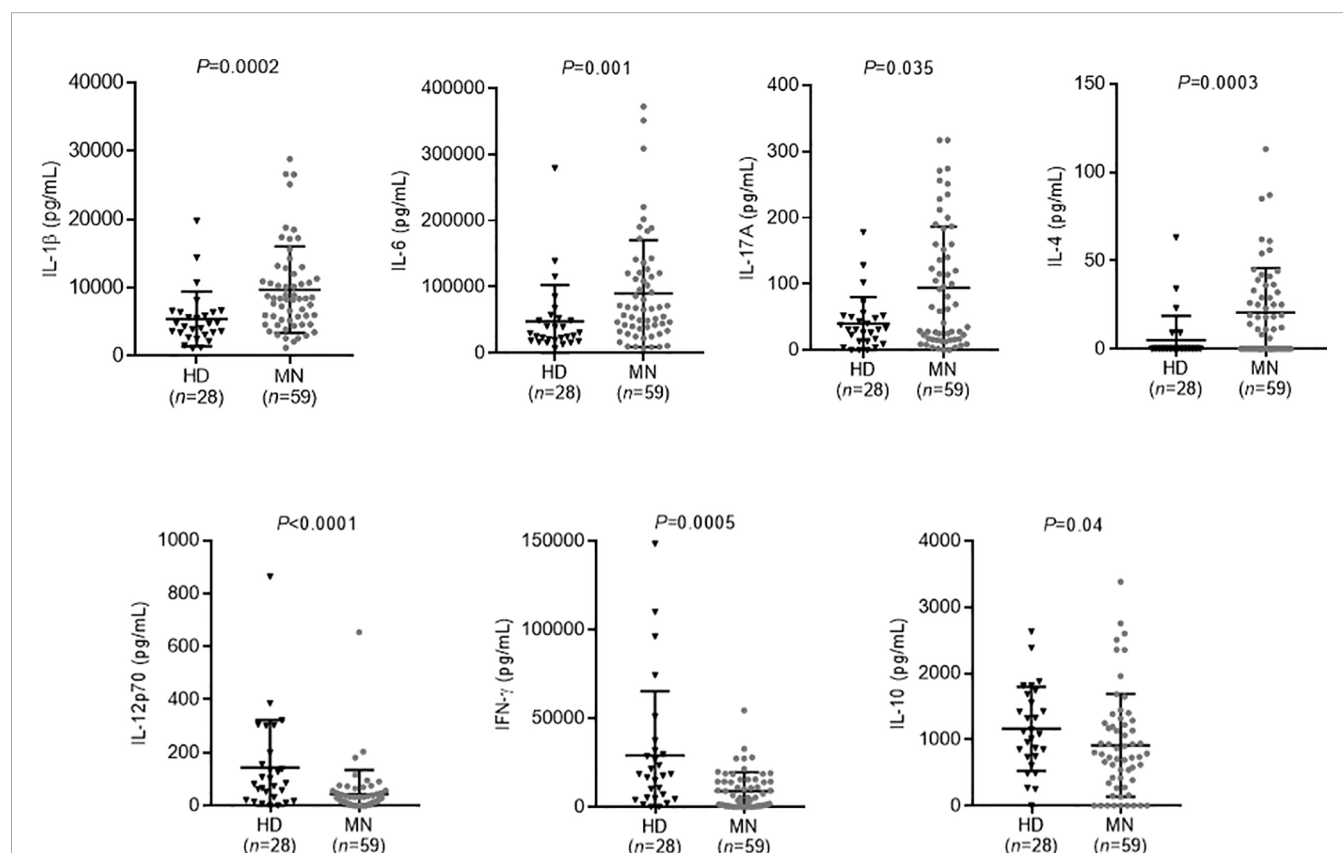


FIGURE 2 | Serum cytokine levels in membranous nephropathy (MN) patients and healthy donors after *in vitro* stimulation of immune cells by both anti-CD3 and TLR 7/8 agonist. Compared to healthy donors, MN patients had increased levels of IL-1β, IL-6, IL-17A, and IL-4, and decreased levels of IL-12p70, IFN-γ, and IL-10. A non-parametric two-tailed test (Mann-Whitney) was used to compare the level of cytokines in patients with that in healthy donors. MN, membranous nephropathy; HD, healthy donors.

Cytokine Profiles and Prognosis

Th17 Profile and Prognosis of MN

We then analyzed the impact of this inflammatory profile on MN prognosis. We found no difference in proteinuria, albuminemia, serum creatinine, urea, lymphocyte count, anti-PLA2R1 activity and remission between IL-17A-positive and IL-17A-negative patients but IL-17A positive patients presented more thromboembolic complications (phlebitis, pulmonary embolism, renal vein thrombosis) and relapsed more often ($P=0.03$ and $P=0.0006$, respectively, **Table 2**).

We then focused on patients who had thromboembolic complications or who relapsed within one year after rituximab treatment. These patients had significantly higher IL-17A levels than the patients without thromboembolic complications ($P=0.004$) and the patients who did not relapse ($P=0.0005$) (**Figures 4A, B**). An IL-17A threshold at 73 pg/ml was defined to identify patients at risk of relapse, using ROC curve with a sensitivity of 78% and a specificity of 75% ($AUC=0.77$, $P<0.001$, *data not shown*). Patients with more than 73 pg/ml of IL-17A were 10.50-times more likely to relapse (odds ratio=10.50 [IQR, 3.13–35.20]) and renal survival without relapse was significantly lower for these patients ($P=0.0085$) (**Figure 4C**). It is important to note that in our patient population with active MN but often non-nephrotic proteinuria, albuminemia and serum creatinine levels do not correlate with the occurrence of venous

TABLE 2 | Characteristics of MN patients according to interleukin-17A level.

	IL-17A-positive patients (n=31)	IL-17A-negative patients (n=28)	P
Demography			
Age (years)	54 ± 16	52 ± 19	0.66
Sex ratio (F/M)	9/21	11/17	0.58
Laboratory evaluations			
PLA2R1-Ab titer (RU/ml)	29 [8 – 271]	53 [19 – 95]	0.66
UPCR (g/g)	3.95 [1.89 – 6.59]	4.61 [3.53 – 8.15]	0.15
Serum creatinine (μmol/L)	123 [88 – 159]	117 [76 – 255]	0.86
Albuminemia (g/L)	34.5 [24.7 – 37.2]	30.3 [17.8 – 35.8]	0.32
Urea (mmol/L)	9.6 [5.9 – 13.0]	8.6 [6.2 – 15.4]	0.60
Lymphocyte count (G/L)	1.6 [1.4 – 2.5]	1.7 [1.2 – 2.1]	0.47
Environmental data			
PM2.5 level (μg/m ³)	51 [46–51] ^a	31 [21–36] ^b	0.002
Venous thromboembolic events			
Remission at 6 months after rituximab treatment			
	Remission: 12/22 No remission: 10/22	Remission: 8/16 No remission: 8/16	0.0006
Relapses			
	21	6	0.0006

Ab, antibody; MN, membranous nephropathy; PLA2R1, phospholipase A2 receptor 1; THSD7A, thrombospondin type-1 domain-containing protein 7A; UPCR, urine protein to creatinine ratio.

^aData was missing for two patients.

Bold values are p value statistically significant.

^bData was missing for six patients.

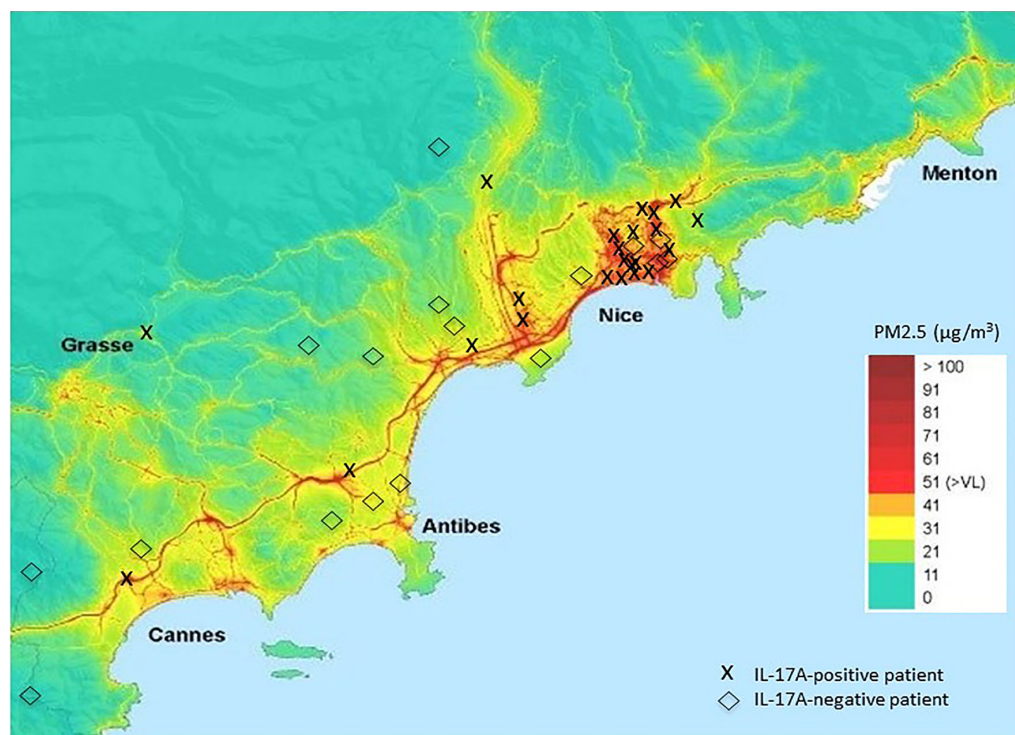


FIGURE 3 | Superposition of the usual area of residence of membranous nephropathy (MN) patients and air pollution. Cumulative PM_{2.5} exposition in 2016 in the French region Provence-Alpes-Côte d'Azur is presented as heat map. The usual area of residence of each MN patient living in the French region Provence-Alpes-Côte d'Azur is represented by a cross (IL-17A-positive MN patients) or a rhombus (IL-17A-negative MN patients). IL, interleukin; PM, particulate matter.

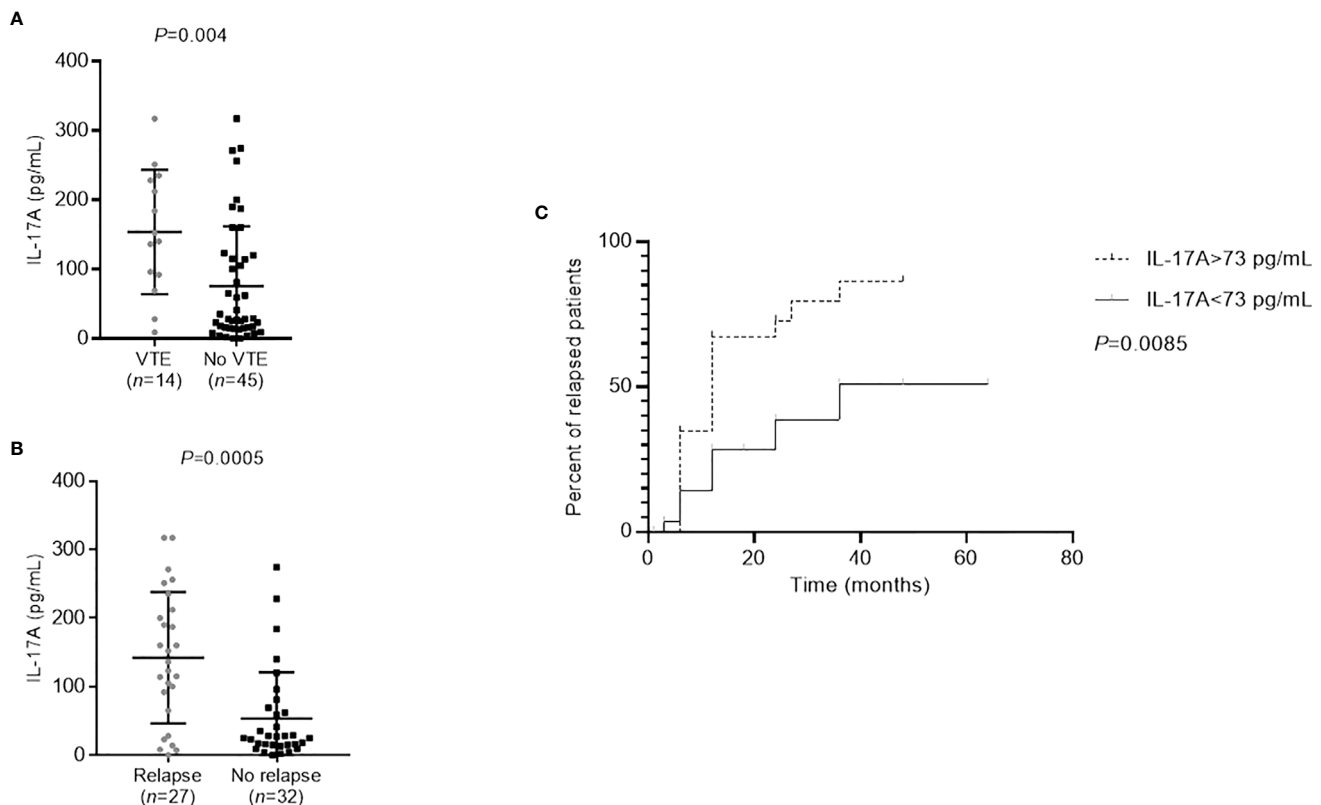


FIGURE 4 | Prognosis of membranous nephropathy (MN) patients according to the level of cytokines. **(A)** Patients with thromboembolic complications (VTE) had significantly higher IL-17A levels than those without ($P=0.004$). Statistical significance was determined by a Mann-Whitney test. **(B)** Patients who relapsed within one year after rituximab treatment had significantly higher IL-17A levels than those who did not relapse ($P=0.0005$). Statistical significance was determined by a Mann-Whitney test. **(C)** Relapse-free survival was lower in patients with higher IL-17A levels at diagnosis. The threshold of 73 pg/ml, as determined by ROC curve (sensitivity of 81% and specificity of 76%), was used to distinguish the patients with low or high levels of IL-17A. Kaplan-Meier analysis was used to estimate the relapse-free survival of MN patients ($n=59$) based on their IL-17A level. IL, interleukin; MN, membranous nephropathy; VTE, venous thromboembolic event.

thromboembolic events (VTE) (albuminemia: 26.1 mg/L [18.7–35.3] in patients with VTE vs 33.0 mg/L [22.9–36.8] without VTE, $P=0.21$; serum creatinine: 111 $\mu\text{mol/L}$ [88–143] in patients with VTE vs 124 $\mu\text{mol/L}$ [81–253] without VTE, $P=0.56$) or relapses (albuminemia: 34.2 mg/L [27.0–36.8] in relapsing patients vs 26.2 mg/L [19.2–36.3] in non-relapsing patients, $P=0.11$; serum creatinine: 133 $\mu\text{mol/L}$ [97–247] in relapsing patients vs 96 $\mu\text{mol/L}$ [80–192] in non-relapsing patients, $P=0.23$).

Cytokine Profile of MN Patients in Remission

We measured cytokine profile in the serum of 26 patients both in active phase of the disease and in remission induced by rituximab (1g at two-weeks interval). IL-10 and IL-12p70 levels increased at remission (IL-10: 467 pg/ml [IQR, 0–1005] in the active phase versus 1382 pg/ml [IQR, 798–1676] at remission, $P=0.0005$; and IL-12p70: 15 pg/ml [IQR, 0–48] versus 45 pg/ml [IQR, 17–73], respectively, $P=0.004$) (Figures 5A, B). No changes were observed for IL-17A (Figure 5C) suggesting that rituximab did not impact on Th17 profile.

Evolution of IL-17A Levels After Rituximab Treatment

During follow-up, IL-17A levels were stable during active disease and at remission for most patients (Figure 5C). One IL-17A-negative patient relapsed after rituximab treatment and developed anti-rituximab antibodies at the moment of the cytokine profile switch toward the Th17 pathway (IL-17A: 27 versus 168 pg/ml) (Figure 5D) (50). In this patient the onset of the Th17-mediated inflammation was thus associated with the immunization against rituximab, reappearance of anti-PLA2R1 antibodies and MN relapse (Figure 5D).

DISCUSSION

Membranous nephropathy is a rare autoimmune renal disease with an increasing prevalence. Previous studies showed a polarization toward Th2 immune response in idiopathic MN patients (35–37) (using RT-PCR) with little or no information on Th17 pathway cytokines, which are involved in the development of autoimmune diseases (29, 30). Rosenzwaig et al. observed a

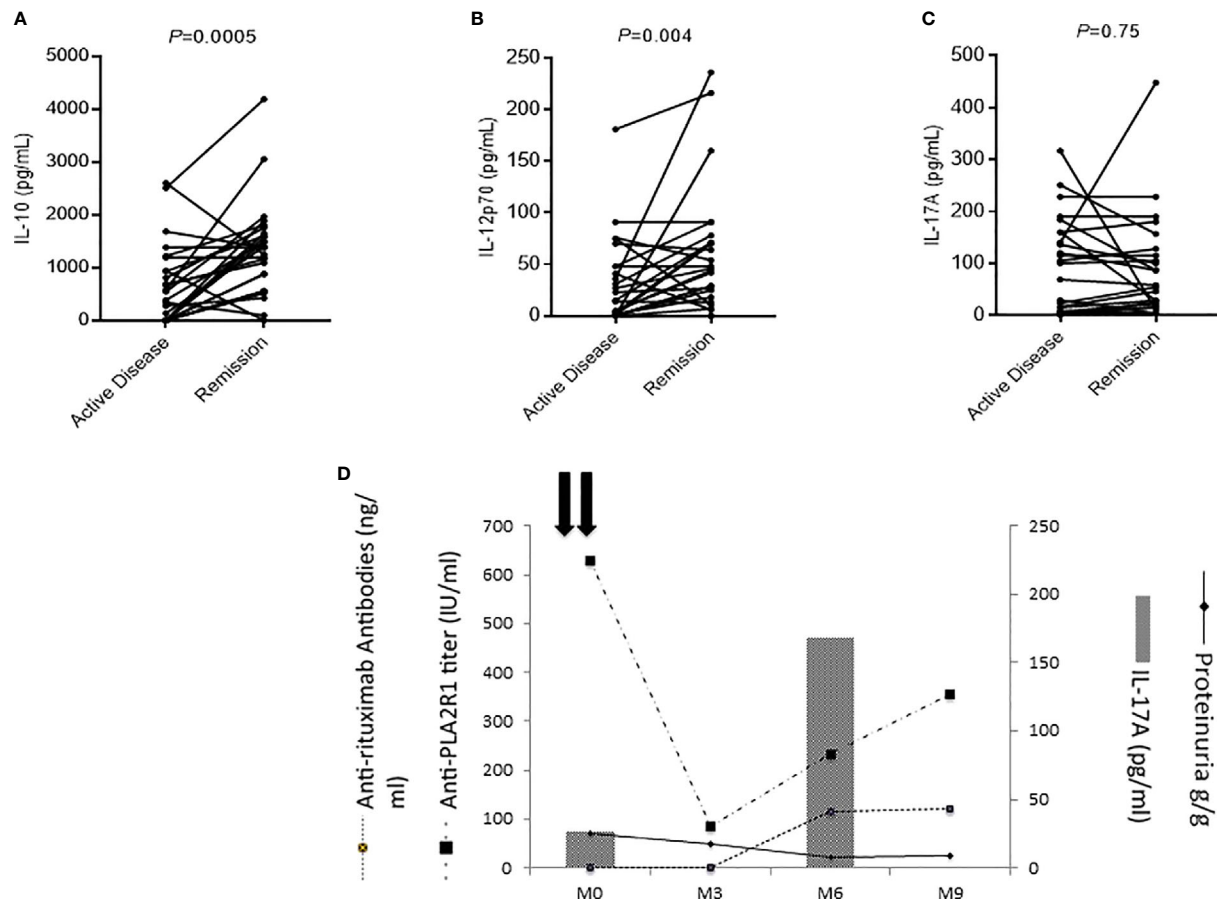


FIGURE 5 | Evolution of membranous nephropathy (MN) patients according to the level of cytokines. **(A–C)** Evolution of serum cytokine levels in MN patients who entered into remission ($n=26$). Remission was associated with a significant increase of IL-10 **(A)** and IL-12p70 **(B)** levels, while the level of IL-17A remained unchanged **(C)**. A Wilcoxon matched pairs signed rank test was used to compare cytokine levels performed on the same MN patients during active disease and in remission. **(D)** Individual evolution of interleukin-17A levels of a MN patient according to his clinical evolution. This patient had low IL-17A levels before treatment with rituximab but developed antibodies against rituximab at month-6 associated with an increase of anti-PLA2R1 antibodies and an increase of IL-17A demonstrating a shift toward a Th17 profile.

decrease of IL-17A in MN patients that could be related to urinary leakage of proteins in nephrotic patients (39) whereas Li and al. suggested an increased Th17 immune response detected by intracellular cytokine assay following stimulation with a leukocyte activation cocktail. (40). *In vitro* non-specific stimulation of lymphocytes and innate immunity cells prior to measuring cytokines allowed us to detect several cytokines in the serum of MN patients despite nephrotic range proteinuria. Our study showed a significant increase of pro-inflammatory cytokines (IL-1 β and IL-6) and of Th2-related cytokines (IL-4) in MN patients compared to healthy subjects, as well as a decrease in Treg (IL-10) and Th1-related cytokines (IL-12p70 and IFN- γ), as previously shown (35–39). We have also shown an activation of Th17 pathway in MN patients, confirming GWAS data published by Xie et al. (27).

This polarization toward Th17 immune response in MN patients appears to be associated with an urban environment:

MN patients with Th17-positive profile were more exposed to PM_{2.5}. In accordance with our results, a Chinese team recently showed in a double-blind, randomized crossover study that higher PM_{2.5} exposure is positively associated with a higher level of cytokines IL-1 β and IL-6 (48). Since a geographical correlation between the occurrence of MN and the satellite analysis of exposure to fine particles (PM_{2.5}) has already been demonstrated (3), our results reinforce the hypothesis of an environmental origin of the onset of MN in a subset of patients exposed to air pollution with a Th17-profile. In addition, exposure to PM_{2.5} could also be considered as a danger signal which induces activation of autoreactive T lymphocytes, which may be a trigger required to induce an autoimmune response (51).

Clinically, serum IL-17A concentrations were associated with poor prognosis of MN defined by more thromboembolic complications and more relapses. The remission was heralded by a recovery of the Treg and Th1 pathways as previously

described (38, 39). In contrast to Berti et al. who showed that IL-6 was reduced in response to rituximab during anti-neutrophil cytoplasmic antibody-associated vasculitis (52), in our study MN patients in complete remission maintained a Th17 profile and treatment with rituximab did not allow a reorientation of the Th17-mediated inflammatory response. The stability of the immune response toward the Th17 pathway despite remission and immunosuppressive therapy could be explained by the persistence of pro-inflammatory environmental triggers. These data suggest that the detection of Th17-mediated inflammation at diagnosis could raise the question of a treatment with anti-IL-6 or anti-IL-17A associated with rituximab.

This study has several limitations. First, it is limited to a single territory with a poor contrast between polluted and unpolluted areas. A large-scale study on a larger territory should be initiated to confirm the association between air pollution and the Th17 profile of MN patients. Second, we did not identify the environmental factor(s) associated with non-Th17 related MN in patients from rural areas. It would be interesting to study the role of certain more specific exposures of this population such as pesticides, wood combustion, the use of diesel engines etc. Third, this is an association study that cannot confirm the causal link between the urban environment, the modification of the cytokine profile of patients and the development of MN. *In vitro* studies on PBMC and podocytes, and *in vivo* studies on animal models are necessary to assess the impact of exposure to particulate matter on the cytokine profile. These three points are the objectives of a French hospital clinical research program called Immunopathological Analysis in a French National Cohort of Membranous Nephropathy (NCT04326218) which started including MN patients in July 2020.

In conclusion, 53% of MN patients have Th17-mediated inflammation appearing to be related to an urban environment. Clinically they have a poor prognosis defined by more thromboembolic events and relapses, and their Th17 cytokine profile remains unchanged after treatment with rituximab. Treatment inducing re-orientation of the immune system could be beneficial for these patients.

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.

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ETHICS STATEMENT

The studies involving human participants were reviewed and approved by French local etc committee NCT02199145. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BS-P designed the study. VB and MC carried out experiments. MC, BS-P, VB, SP, and FD analyzed and interpreted the data. BS-P, VE, MC, SB-S, MA, and AG provided medical oversight. MC, BS-P, and VB drafted and revised 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.2020.574997/full#supplementary-material>

SUPPLEMENTARY FIGURE 1 | Location of pollutants measuring stations in the French region Provence-Alpes-Côte d'Azur (PACA) (Atmosud data).

SUPPLEMENTARY FIGURE 2 | Serum cytokine levels in MN patients with and without *in vitro* stimulation of immune cells by anti-CD3 and TLR 7/8 agonist (R848). No cytokines were detected in serum without *in vitro* stimulation of immune cells by anti-CD3 and TLR 7/8 agonist. A non-parametric two-tailed test (Mann-Whitney) was used to compare the level of cytokines. IL, interleukin; MN, membranous nephropathy.

SUPPLEMENTARY FIGURE 3 | Receiver operating characteristic curve to distinguish IL-17A-positive patients and IL-17A negative patients. An IL-17A level greater than 58 pg/mL was associated with an IL-17A positivity (sensitivity of 86% and specificity of 52%). AUC, area under the curve; ROC, receiver operating characteristic curve.

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The remaining 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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Vasculature-Associated Lymphoid Tissue: A Unique Tertiary Lymphoid Tissue Correlates With Renal Lesions in Lupus Nephritis Mouse Model

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Lupus nephritis (LN) is a common complication in young patients and the most predominant cause of glomerulonephritis. Infiltrating immune cells and presence of immunocomplexes in the kidney are hallmarks of LN, which is closely associated with renal lesions (RLs). However, their regulatory mechanism in the kidney remains unclear, which is valuable for prevention of RL development. Here, we show the development of vasculature-associated lymphoid tissue (VALT) in LN, which is related to renal inflammatory cytokines, indicating that VALT is a unique tertiary lymphoid tissue. Transcriptomic analysis revealed different chemokines and costimulatory molecules for VALT induction and organization. Vascular and perivascular structures showed lymphoid tissue organization through lymphorganogenic chemokine production. Transcriptional profile and intracellular interaction also demonstrated antigen presentation, lymphocyte activity, clonal expansion, follicular, and germinal center activity in VALT. Importantly, VALT size was correlated with infiltrating immune cells in kidney and RLs, indicating its direct correlation with the development of RLs. In addition, dexamethasone administration reduced VALT size. Therefore, inhibition of VALT formation would be a novel therapeutic strategy against LN.

Keywords: tertiary lymphoid tissue, lupus nephritis, chemokine, kidney, renal lesions, dexamethasone

INTRODUCTION

The vital functions of the mammalian kidney include filtration of blood to excrete metabolic wastes and toxins through urine, maintenance of body fluid balance, pH, and absorption of minerals to sustain life (1). The prevalence of patients suffering from chronic kidney disease (CKD) is 8–13%, overall (2). Moreover, it has been estimated that more than 19 million people in the USA and about 22% of adult humans in Japan suffer from complications from CKD (3, 4). Therefore, CKD is a major public health concern as is associated with end-stage renal disease and cardiovascular

complications (5). Since kidney is a non-regenerative organ, understanding the pathogenesis of CKD and identification of therapeutic targets is valuable for developing remedial strategies. Different studies using human biopsy samples and experimental animals showed that infiltration of inflammatory cells in the kidney is a hallmark of CKD, including lupus nephritis (LN) (1, 6). Our previous study showed that the number of infiltrating immune cells and deposition of immune complexes in the kidney were closely related to the severity of the renal pathological lesions in the LN model mice (1, 7, 8). However, the origin and local regulation of immune cells and autoantibodies in the LN remain unclear.

Effective and prompt immune response to infection or damage is mediated by a well-established immune system. The secondary lymphoid organ (SLO) in adults provides a critical microenvironment for the interaction between immune cells and antigens to propagate an effective adaptive immune response (9). However, during chronic inflammation, ectopic lymphoid tissue can form in inflamed non-lymphoid tissues and demonstrates the most common features of SLO, including stromal chemokine production to attract immune cells, lymphatic vascularization, germinal center (GC) formation, and antibody production. These ectopic lymphoid tissues are called tertiary lymphoid tissues (TLT) and are found in many diseases, including atherosclerosis, persistent infection, cancer, and autoimmune diseases (10–12). TLTs are considered to function as local sites for antigen presentation, perpetuation of antibodies against self-antigens, clonal expansion, and lymphocyte activation (10, 12, 13). The role of TLTs is either beneficial or detrimental depending on the context. Formation of TLTs is beneficial when they clear antigens or pathogens in diseases like viral diseases, cancer, and atherosclerosis (11, 14), while in other diseases such as autoimmune diseases, TLTs are detrimental due to vigorous and sustained response to self-antigen and destruction of normal tissue (11). In the latter context, TLTs may serve as therapeutic targets.

At the beginning of TLT formation in non-lymphoid tissue, resident stromal cells or fibroblasts secrete lymphorganogenic chemokines (LC) (*Cxcl13*) to attract leukocytes for homing (15). Arterial smooth muscle cells (SMCs) also act as lymphoid tissue organizers (LTo) and secrete LC, in atherosclerosis (16). In the skin, the perivascular area is rich in adventitial fibroblasts, and alteration of these fibroblasts leads to the formation of perivascular TLT (17). In addition, aged mice (12 months) subjected to ischemia-reperfusion injury also showed perivascular TLT (15). This study showed that the development of TLT is fully dependent on aging and the presence of acute injury, implying that perivascular fibroblasts only act as LTo. However, there are no reports regarding the role of both vascular and perivascular structures as LTo in TLT development and its correlation with renal histopathology in comparatively younger individuals.

LN is a common complication in young females and the predominant cause of glomerulonephritis (18). Our previous studies used MRL/MpJ-Fas^{lpr/lpr} (LPR) and BXSB/MpJ-Yaa (Yaa) mice as a systemic lupus erythematosus model to investigate LN and other lupus-related disease pathogenesis

(1, 19, 20). Our previous study also showed that female LPR mice developed a well-organized mediastinal fat-associated lymphoid cluster that had more detrimental effects on the lungs than in male LPR mice (20). In the present study, we show a unique perivascular TLT development in the kidney using two LN model mice at different ages and named vasculature-associated lymphoid tissue (VALT). We showed that LPR mice developed inflammation and VALT at an earlier stage (3 months), but VALT became larger and directly correlated with glomerular and tubulointerstitial lesions (GL and TIL) at a later stage (6 months). We also identified the molecular cues responsible for VALT formation, antigen presentation, and GC activity of VALT in the lupus model mice, by using routine histopathological techniques and transcriptomic analysis (TA). Moreover, we identified a therapeutic target to ameliorate LN lesions through ablation of VALT.

MATERIALS AND METHODS

Experimental Mice and Ethical Statement

The authors adhered to ethical guidelines (Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University, approval No. 16-0124) throughout the experiments using experimental mice. LPR mice with their respective control MRL/MpJ (MRL), Yaa, and normal control C57BL/6 (B6) mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Mice were maintained in special-pathogen-free housing at 1:1 dark and light conditions with *ad libitum* food and water supply.

Sample Preparation

Mice at 1, 3, and 6 months of age were deeply anesthetized with a mixture of an aesthetic agent as previously described (1), and the kidney slices were fixed with 10% neutral buffer formalin (NBF), 4% paraformaldehyde (PFA), and 2.5% glutaraldehyde (GTA) for histopathological, immunohistochemical, and electron microscopy studies, respectively.

Dexamethasone (Dex) Administration

Twelve-week-old LPR female mice were divided into two groups: Dex (n = 6) and control group (n = 6). Mice in the Dex group received Dex daily with drinking water and weekly intraperitoneal injections of 0.4 mg/kg body weight, whereas the control group received normal drinking water daily and saline once intraperitoneally every week. Both groups of mice were sacrificed at 22 weeks of age.

Immunohistochemistry and Immunofluorescence

NBF-fixed paraffin blocks were cut and stained with hematoxylin and eosin (HE), and periodic acid Schiff-hematoxylin (PAS-H) to examine the renal histopathology. Immunodetection of cell markers was performed as previously described (1) for B cells (B220), T cells (CD3), macrophages (Iba1), lymphatic vessels (LYVE-1), high endothelial venule (PNAd), Vimentin, LCs

(CCL8, CXCL9 and CXCL13), smooth muscle actin (SMA), follicular dendritic cell (CD21), MHC II, plasma cell (CD138), immunoglobulin (IgG and IGM), and damaged tubules (interleukin 1, family member 6, IL-1F6). The staining conditions are listed in **Supplementary Table 1**.

Combination of Immunohistochemistry and Aniline Blue Staining

Immunohistochemistry against CXCL13 was performed as previously described. The same tissue sections were stained with aniline blue to colocalize CXCL13 and collagen fibers.

Histoplanimetry

HE, PAS-H, and immunohistochemistry stained slides from three different planes of the kidney were converted to virtual slides using Nano Zoomer 2.0 RS (Hamamatsu Photonics Co., Ltd.; Hamamatsu, Japan), and the stained area and positive cells were counted using NDP.view2 software (Hamamatsu Photonics Co., Ltd.).

Reverse Transcription and Real-Time PCR

Total RNA, complementary DNA synthesis, and real-time PCR with Brilliant III SYBR Green QPCR master mix and Mx3000P (Agilent Technologies, La Jolla, CA, USA) was performed as described in our previous study (8). The primers used are listed in **Supplementary Table 2**.

In Situ Hybridization

In situ hybridization was performed for *Ccl8*, *cxcl9*, and *cxcl13* as described in our previous study (8). The probes used are listed in **Supplementary Table 2**.

Microarray Analysis

The excised kidney was treated with RNAlater (Thermo Fisher Scientific) overnight at 4°C. Then, the RNAlater was replaced with TRIzol reagent and stored at -80°C for further analysis (Life Technologies, California, USA). Total RNA was isolated using Trizol reagent following the manufacturer's instructions. RNA integrity was validated using an Agilent 2100 Bioanalyzer II (Agilent Technologies, Santa Clara, CA, USA), and complementary RNA was synthesized using a Low Input Quick Amp Labeling Kit (Agilent Technologies). Gene expression was analyzed using an Agilent Technologies Microarray Scanner and SurePrint G3 Mouse 8x60K v2.0 (Agilent Technologies), and the raw data normalized by 75Percentile shift (GeneSpring; Agilent Technologies). Toppgene Suite (<https://toppgene.cchmc.org/>) and Heatmapper (<http://www.heatmapper.ca/>) were used for gene ontology (GO) analysis and heatmap preparation, respectively, for genes that showed ≥ 10 folds compared to the control. Microarray data were deposited in a public repository (GSE160488, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160488>).

Scanning Electron Microscopy (SEM)

We modified the sample preparation for SEM for the examination of VALT. Briefly, the mice were perfused with 2.5% GTA through the ventricle. The excised kidney was sliced and fixed with GTA and PFA for 4 and 12 h, respectively, to

prepare the paraffin block. The paraffin blocks were cut at 5 mm thickness and mounted on a glass slide. The sections were deparaffinized, fixed, and post-fixed with GTA and osmium tetroxide. The specimen was coated with ion sputter (Hitachi; Tokyo, Japan) for 1 min and examined with an S-4100 SEM (SU 8000, Hitachi) with an accelerated voltage of 10 kV.

Statistical Analysis

Control and diseased mice were compared using the nonparametric Mann-Whitney *U* test ($P < 0.05$). The Kruskal-Wallis test was used to compare three or more populations, and multiple comparisons were performed using Scheffe's method when significant differences were observed ($P < 0.05$). The genes that showed expression ≥ 10 folds compared to the control were selected for statistical analysis, and significant differences between control and disease were calculated by analysis with a 2-tailed Student's *t* test. Spearman's rank correlation coefficient ($P < 0.05$) was used to examine the correlation among different parameters.

RESULTS

Prevalence of Perivascular Cellular Cluster (PCC) in the Kidney at Different Ages

We examined the prevalence of PCC in lupus prone LPR mice and its corresponding control MRL mice kidney at different ages (**Figure 1**). There was no PCC in any of the mice at 1 month of age (**Figures 1A, B**). At 3 months, PCC was absent in MRL mice but was observed in LPR mice (**Figures 1C, D**). At the later stage (6 months), PCC was observed in both MRL and LPR mouse kidneys, but it was larger in LPR mice (**Figures 1E, F**). We also examined PCC in another lupus-prone mice (Yaa) and normal control B6 mouse kidneys at different ages (**Supplementary Figure 1**). There was no PCC in 1 and 3-month-old B6 and Yaa mice kidneys (**Supplementary Figures 1A–D**). At 6 months of age, PCC was observed in both B6 and Yaa mice kidneys, but it was comparatively larger in Yaa mice (**Supplementary Figures 1E, F**).

The percentage of PCC and its relative size were greater in LPR mice than in MRL mice, at 3 and 6 months of age (**Figures 1G, H**). The percentage of appearance and size of PCC was also higher in LPR mice at 6 months of age compared to the respective controls at the same age and all mice at other ages.

Cellular Characterization of PCC

We characterized the PCC cluster cells by immunohistochemistry using different cell markers (**Figure 2**). We observed that PCCs were composed of B-, T-cells, and macrophages (**Figures 2A–L**). Next, we examined the presence of high endothelial venules (HEV) and lymphatic vessels in or around the clusters. We did not observe any high HEV either in or around the PCC in either MRL or LPR mice (data not shown). However, LYVE-1⁺ lymphatic vessels accompanied by arteries were found in MRL mice at 3 months of age, though no cellular cluster was observed (**Figure 2M**), whereas lymphatic vessels were observed in and around PCC in 6 month old MRL, and 3 and 6 month old LPR mice (**Figures 2N–P**). The

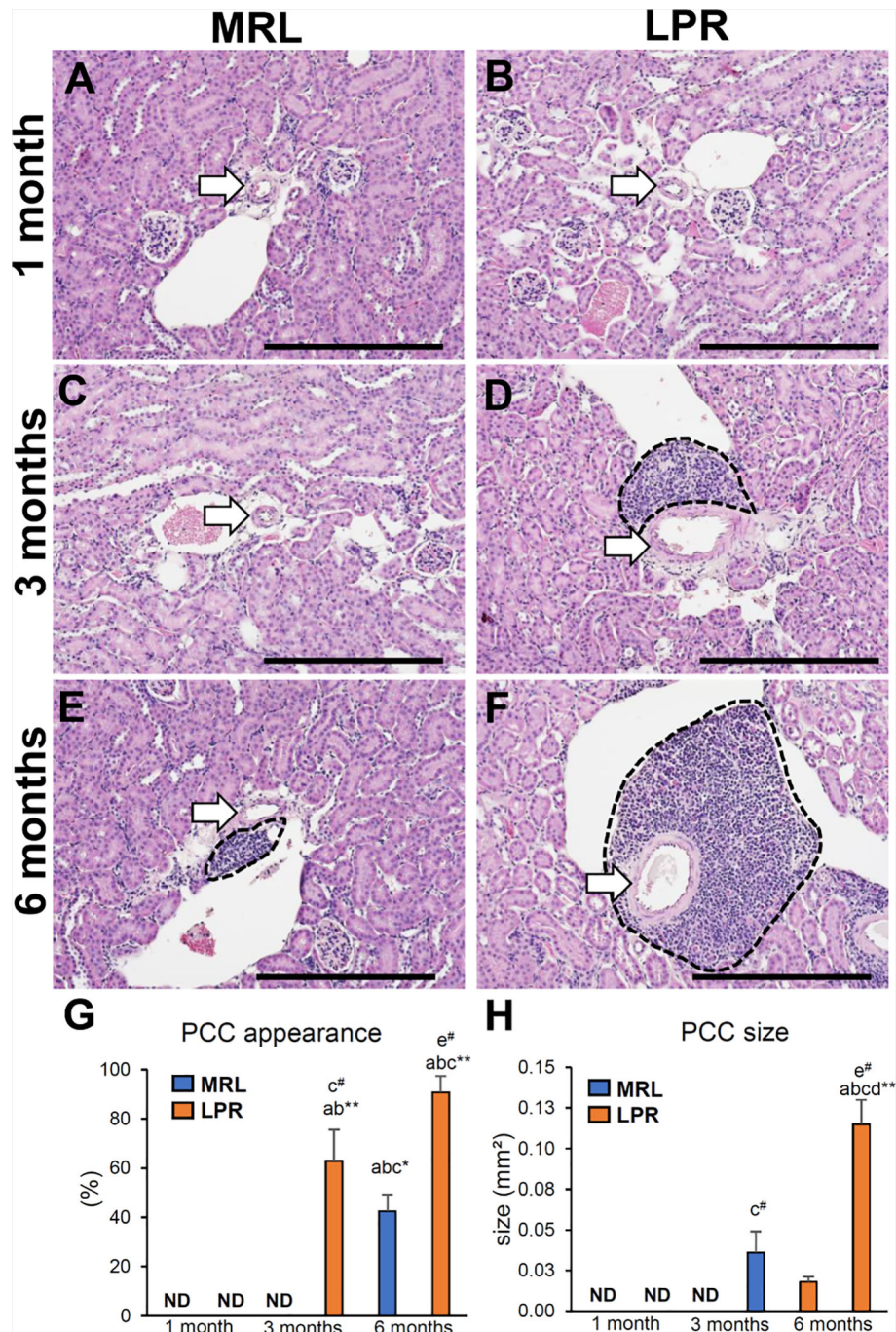


FIGURE 1 | Appearance of PCC at different ages of lupus nephritis model mice kidney. **(A, B)** Absence of PCC near the blood vessel (arrow) in MRL and LPR mice kidney at 1 month of age. **(C)** Absence of PCC near the blood vessel (arrow) in MRL mice kidney at 3 months of age. **(D)** Presence of PCC (dashed area) near the blood vessel (arrow) in LPR mice kidney at 3 months of age. **(E, F)** Presence of PCC (dashed area) near the blood vessel (arrow) in MRL and LPR mice kidney at 6 months of age (HE stain). **(G)** Percentage of PCC appearance in MRL and LPR mice at different ages. **(H)** Size of PCC in MRL and LPR mice at different ages. The values are the mean \pm standard error (s.e.). Significant difference from the control in the same age group is indicated by # ($P < 0.05$, Mann-Whitney U -test). Significant differences from the other groups are indicated by * ($P < 0.05$, Kruskal-Wallis test followed by Scheffe's method). $n = 4$. A, B, C, D, E, and F denote 1 month old MRL, 1 month old LPR, 3 month old MRL, 3 month old LPR, 6 month old MRL, and 6 month old LPR mice, respectively. Bars = 100 μ m. PCC, perivascular cellular cluster; HE, hematoxylin and eosin; ND, not detectable.

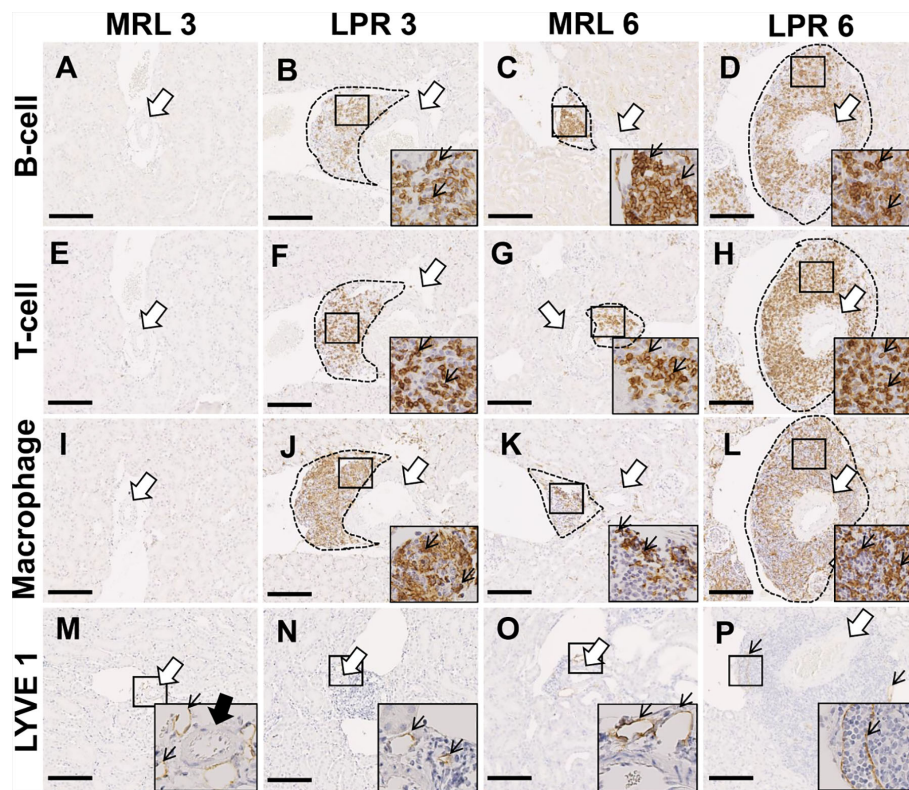


FIGURE 2 | Cellular characterization of PCC at 3 and 6 months of age. **(A–D)** B-cell infiltration (thin arrow) near the blood vessel (arrow) in PCC (dashed area) of MRL and LPR mice kidney at 3 and 6 months of age (IHC). **(E–H)** T-cell infiltration (thin arrow) near the blood vessel (arrow) in PCC (dashed area) of MRL and LPR mice kidney at 3 and 6 months of age (IHC). **(I–L)** Macrophage infiltration (thin arrow) near the blood vessel (arrow) in PCC (dashed area) of MRL and LPR mice kidney at 3 and 6 months of age (IHC). **(M–P)** LYVE1⁺ lymphatic vessels (thin arrow) near the blood vessel (arrow) in PCC (dashed area) of MRL and LPR mice kidney at 3 and 6 months of age (IHC). Bars = 100 μ m. PCC, perivascular cellular cluster; IHC, immunohistochemistry.

number of B-, T-cells, and macrophages in the cluster was higher in LPR mice than in control mice at all ages, but highest in LPR mice at 6 months of age (**Figures 3A–C**). We also examined PCCs by SEM (3D). No abnormality of the vascular wall was observed, and cluster cells were arranged in chambers made by stromal anastomoses (**Figures 3D–A', B'**).

Evaluation of LC and Molecules Responsible for Lymphoid Tissue Formation

As the PCC consists of B-, T-cells, and macrophages and has lymphatic vessels, we examined the principle LC (*Cxcl13*) and its receptor (*Cxcr5*) responsible for lymphoid tissue formation in 3 and 6-month-old mice to distinguish PCC from the perivascular cuffing (**Figure 4**). The expression of both *Cxcl13* and *Cxcr5* was upregulated in LPR mice compared to their respective controls (**Figures 4A, B**). Higher levels of serum anti-dsDNA antibody were found in the LPR mice compared to the control (**Figure 4C**). Examination of the inflammatory cytokines in the whole kidney revealed upregulation of *Ifng* compared to their respective controls, only in LPR mice (**Figure 4D**). Importantly, serum anti-dsDNA antibody was correlated with *Cxcl13* expression,

PCC appearance, and *Ifng* expression level (**Supplementary Figures 2A–C**). Moreover, *Ifng* expression was correlated with the expression level of *Cxcl13* and the appearance of PCC (**Supplementary Figures 2D, E**). Since we observed a higher prevalence of large PCC, upregulation of LCs, serum anti-dsDNA antibody, and inflammatory cytokines in LPR mice at 6 months of age, we performed TA on kidneys from MRL and LPR mice at this age and selected genes that showed >10-fold upregulation, for further analysis, to correlate with our histologic findings. GO analysis using upregulated transcripts revealed an LPR kidney expression signature associated with lymphoid tissue formation (GO:0048534, $P = 4.14e^{-25}$) (**Figure 4E**). GO also identified an expression signature associated with leukocyte migration (GO:0050900, $P = 9.24e^{-18}$) (**Figure 5A**). Moreover, TA analysis revealed upregulation of HEV forming molecules (*Glycam* and *St8sia4*) and adhesive molecules (*Icam* and *Vcam*) (**Figure 5B**).

Localization of Structures Producing LCs for Lymphoid Tissue Formation in PCC

We aimed to characterize the 3 most abundantly expressed chemokines (*Ccl8*, *Cxcl9*, and *Cxcl13*) in TA analysis related to

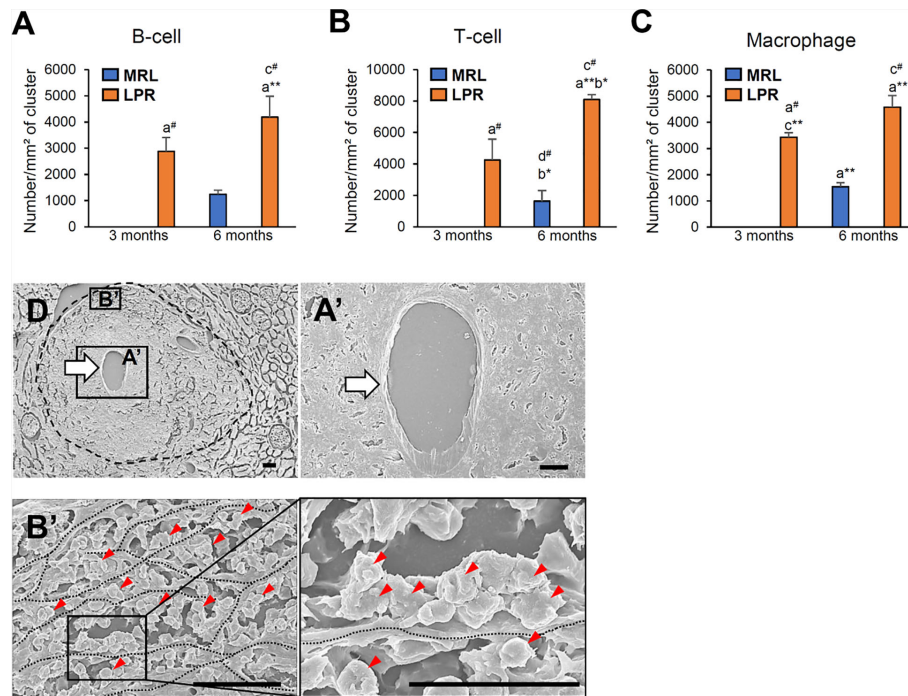


FIGURE 3 | Number of different cellular infiltrates and their cellular organization in PCC. (A–C) Number of infiltrating B cells (A), T-cells (B), and macrophages (C) in PCC of MRL and LPR mice kidney at 3 and 6 months of age. The values are the mean \pm s.e. Significant difference from the control in the same age group is indicated by [#] ($P < 0.05$, Mann–Whitney *U*-test). Significant differences from the other groups are indicated by * ($P < 0.05$, ** $P < 0.01$, Kruskal–Wallis test followed by Scheffe's method). $n = 4$. A, B, C, and D denote 3 months-old MRL, 3 months-old LPR, 6 months-old MRL, and 6 months-old LPR mice, respectively. (D) A PCC (encircled dashed area) surrounds the artery (thick arrow), which has no abnormality in wall (A', thick arrow). Immune cells (red arrowheads) are arranged in chambers made by stromal anastomoses (B') (dotted line). Immune cells (red arrowheads) are attached to the stromal wall (inset) (dotted line). SEM. Bars = 10 μ m. PCC, perivascular cellular cluster; SEM, scanning electron microscopy.

leukocyte chemotaxis and migration (**Supplementary Data Sheet 1**). Therefore, we examined the protein localization of chemokine CCL8, CXCL9, and -13 in PCC. CCL8 localization was not observed in MRL mice kidney at 3 months of age, but its localization was observed with vimentin in the PCC of LPR mice at 3 months of age, and in MRL and LPR mice at 6 months of age (**Figures 6A–D**). CXCL9 localization was not observed in MRL mice kidney at 3 months of age, but its localization was observed with vimentin in the PCC of LPR mice at 3 months of age, and in MRL and LPR mice at 6 months of age (**Figures 6E–H**). Similarly, CXCL13 localization was not observed in MRL mice kidney at 3 months of age, but its localization was observed with vimentin in the PCC of LPR mice at 3 months of age, and in MRL and LPR mice at 6 months of age (**Figures 6I–L**). *Ccl8*, *Cxcl9*, and *Cxcl13* expression was not observed in MRL mice kidney at 3 months of age, but its expression was observed in PCC of LPR mice at 3 months of age, and in MRL and LPR mice at 6 months of age (**Figures 6M–X**).

Vascular and Perivascular Structures Produced LCs

We also examined the role of vascular and perivascular structures in the production of LCs for lymphoid tissue

formation. Protein localization and expression for *Ccl8* and *Cxcl9* in vascular structures was not observed in mice at any stage (data not shown). CXCL13 protein was not observed in MRL mice vascular structures at 6 months of age, but it was co-localized with SMC in the tunica media of the arteries in LPR mice at the same age (**Figures 7A–F**). This result was also confirmed by *in situ* hybridization, which revealed that SMC of the vascular tunica media expressed *Cxcl13* in LPR mice at 6 months of age (**Figures 7G, H**). TA showed upregulation of different collagen fibers in the kidney of LPR mice, compared to that in MRL mice (**Figure 7I**). The combination of immunohistochemistry for CXCL13 and aniline blue staining revealed colocalization of collagen fiber and CXCL13 (**Figures 7J–A', B'**) in LPR mice at 6 months of age. PCC was termed as vasculature-associated lymphoid tissue (VALT) hereafter, since vascular and perivascular structures produce LCs for PPC formation.

Antigen Presentation in VALT

GO analysis revealed several transcripts for genes related to immunological synapses (GO:0001772, $P = 9.03e^{-09}$), major histocompatibility complex (MHC) class II protein binding (GO:0042289, $P = 2.38e^{-04}$), MHC class II protein complex

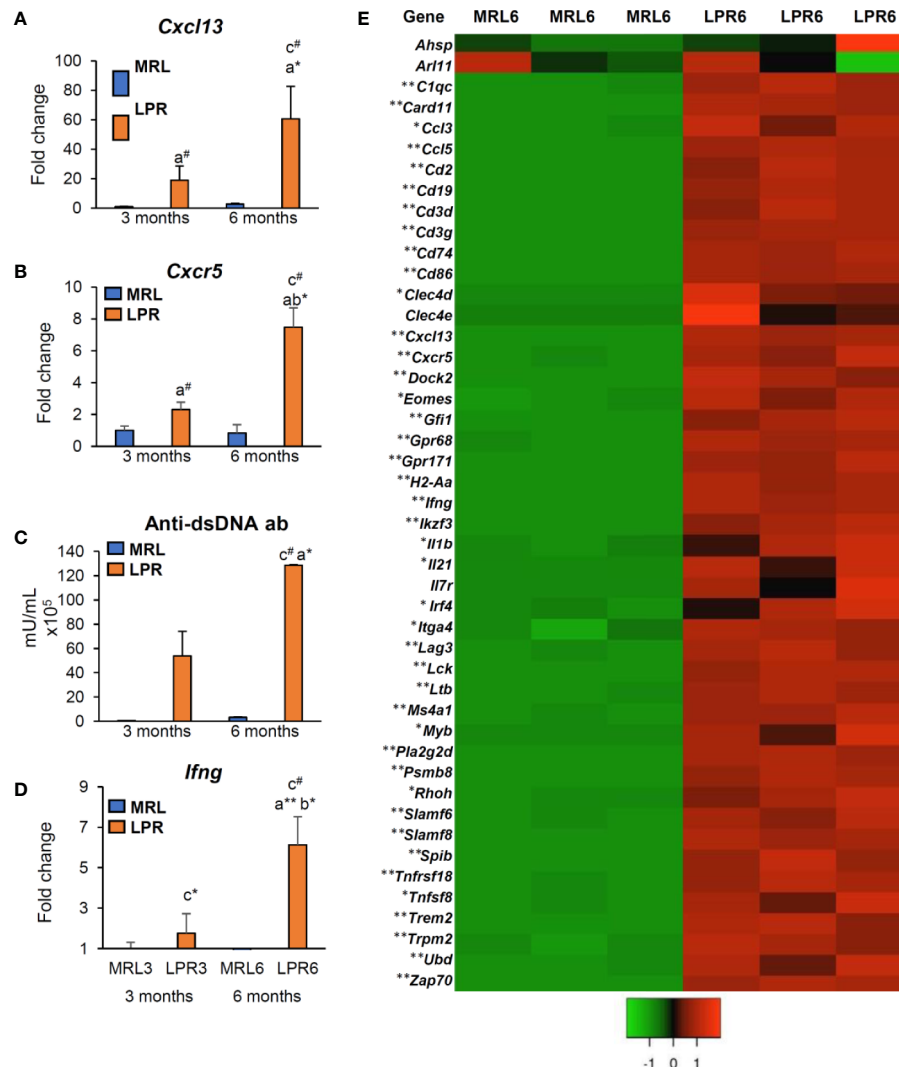


FIGURE 4 | Expression of lymphorganogenic chemokines, cytokines, and costimulatory molecules. **(A, B)** Relative mRNA expression of *Cxcl13* **(A)** and *Cxcr5* **(B)** in MRL and LPR mice at 3 and 6 months of age (qPCR). **(C)** Serum anti-dsDNA antibody in MRL and LPR mice at 3 and 6 months of age (ELISA). **(D)** Relative mRNA expression of *Ifng* in MRL and LPR mice at 3 and 6 months of age (qPCR). The expression levels were normalized to the levels of *actb*. The values are the mean \pm s.e. Significant difference from the control in the same age group is indicated by # ($P < 0.05$, Mann-Whitney U -test). Significant differences from the other groups are indicated by * ($P < 0.05$, ** $P < 0.01$, Kruskal-Wallis test followed by Scheffé's method). $n = 4$. A, B, C, and D denote 3 months old MRL, 3 months old LPR, 6 months old MRL, and 6 months old LPR mice, respectively. **(E)** Expression of transcripts for lymphorganogenic chemokines, cytokines, co-stimulatory molecules, and their receptors in MRL and LPR mice kidney at 6 months of age. Microarray analysis, significant difference from the control is indicated by * ($P < 0.05$, ** $P < 0.01$, 2-tailed Student's t test). $n = 3$. qPCR, Quantitative PCR; dsDNA ab, double strand DNA antibody; ELISA, enzyme-linked immunosorbent assay; *Ifng*, interferon gamma.

binding (GO:0023026, $P = 4.56 \times 10^{-6}$), antigen binding (GO:0003823, $P = 258 \times 10^{-4}$), and positive regulation of T cell activation (GO:0050870, $P = 1.32 \times 10^{-17}$) (**Figure 8A**). We also evaluated VALT infiltrates for evidence of antigen presentation and found colocalization of CD3⁺ lymphocytes and Iba1⁺ macrophages as well as a combination of CD3⁺ lymphocytes and MHC II molecules on antigen-presenting cells, consistent with antigen presentation to T lymphocytes in VALT (**Figures 8B, C**). Moreover, SEM revealed juxtaposition of lymphocytes with antigen-presenting cells (**Figure 8D**).

VALT in Lupus-Prone Mice Mimics the Lymphoid Follicle With GC Activity

Following determination of the nature and organization of cellular infiltrates in VALT, we characterized the individual cells and transcription factors to determine whether VALT in lupus-prone mice mimics lymphoid follicle with GC activity (**Figure 9**). Higher expression of transcripts for T-cell plasticity and acquisition of T-follicular helper cells (T_{FH}), including *Batf*, *Cd40*, *Cxcr5*, *Foxp3*, *Gpr183*, *Icos*, *Il21*, and *Il27ra* were observed in LPR kidney (**Figure 9A**). Higher expression of transcription

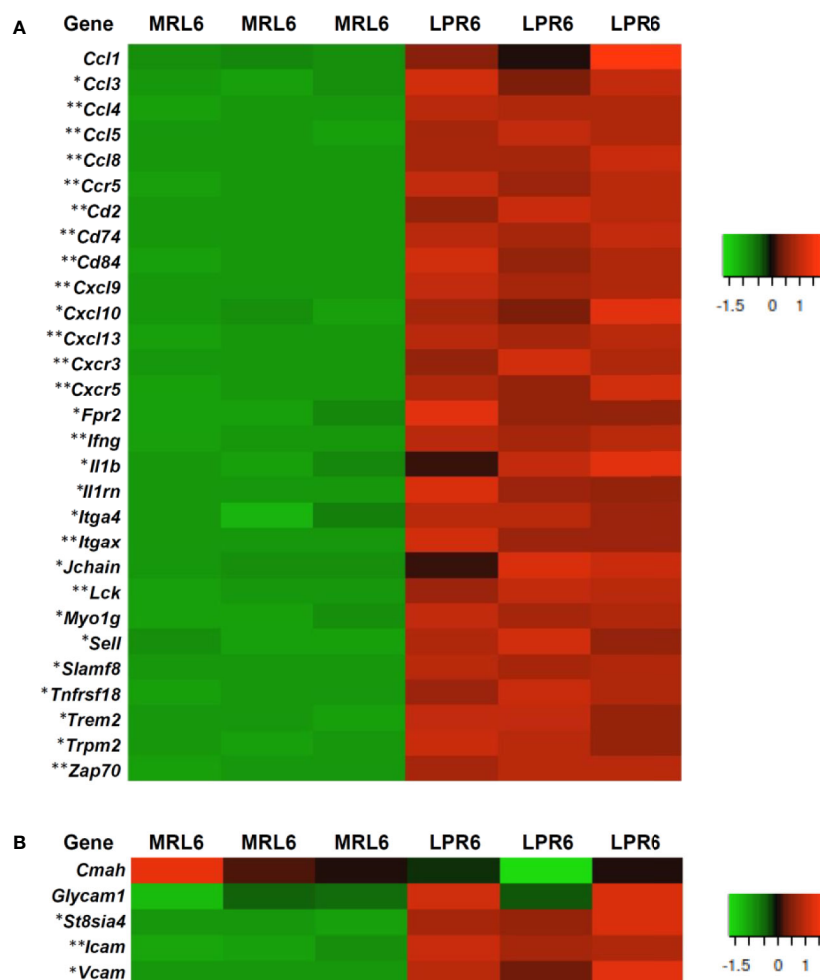


FIGURE 5 | Expression of different transcripts related to leukocyte migration, high endothelial venules forming molecules, and adhesion molecules. **(A)** Gene transcripts related to leukocyte migration in MRL and LPR mice at 6 months of age. Microarray analysis, significant difference from the control is indicated by * (* $P < 0.05$, ** $P < 0.01$, 2-tailed Student's t test). $n=3$. **(B)** Gene transcripts related to high endothelial venules forming molecules and adhesion molecules in MRL and LPR mice at 6 months of age. Microarray analysis, significant difference from the control is indicated by * (* $P < 0.05$, ** $P < 0.01$, 2-tailed Student's t test). $n=3$.

factors for B-cell receptor signaling, B-cell survival, maturation, proliferation, GC activity, and antibody class switching (*Ada*, *Cmtm3*, *Cmtm7*, *Ccr6*, *Ebi3*, *Gpr183*, *Klhl6*, *Lyn*, *Tnfrsf13b*, and *Tnfrsf13c*) was observed in the kidney of LPR mice (**Figure 9A**). CD21⁺ follicular dendritic cells (FDC), CD138⁺ plasma cells, IgM, and IgG⁺ cells were observed in VALT of LPR mice kidney at 6 months of age (**Figures 9B–E**). Moreover, upregulation of transcripts for different autoantigens and BrdU⁺ proliferating cells was also observed (**Supplementary Figures 3A, B**).

VALT in Lupus-Prone Mice Correlated With Renal Lesions

Infiltrating B-, T-cells, and macrophages in the glomerulus were higher in 6-month-old LPR mice (Data not shown), who also developed glomerulosclerosis. Semiquantitative glomerular damage score was higher in LPR mice than in MRL mice at the same age (**Figures 10A–C**). The size of VALT was correlated

with infiltrating B-, T-cells, and macrophages in the glomerulus and glomerular damage score (**Figure 10D**). Infiltrating B-, T-cells, and macrophages in the tubulointerstitium was higher in LPR mice at 6 months of age (Data not shown). IL-1F6⁺ damaged tubules were more abundant in LPR mice at 6 months of age than in MRL mice at the same age (**Figures 10E–G**). The size of VALT was also correlated with infiltrating B-, T-cells, and macrophages in tubulointerstitium and IL-1F6⁺ damaged tubules (**Figure 10H**).

Ablation of VALT Using Immunosuppressive Drugs

We administered Dex as an immunosuppressive drug to ablate VALT. The Dex group showed lower serum anti-dsDNA antibody and spleen to body weight ratio compared to the saline control (**Figures 11A, B**). The Dex group had almost no or reduced size VALT compared to the control group (**Figures 11C, D**).

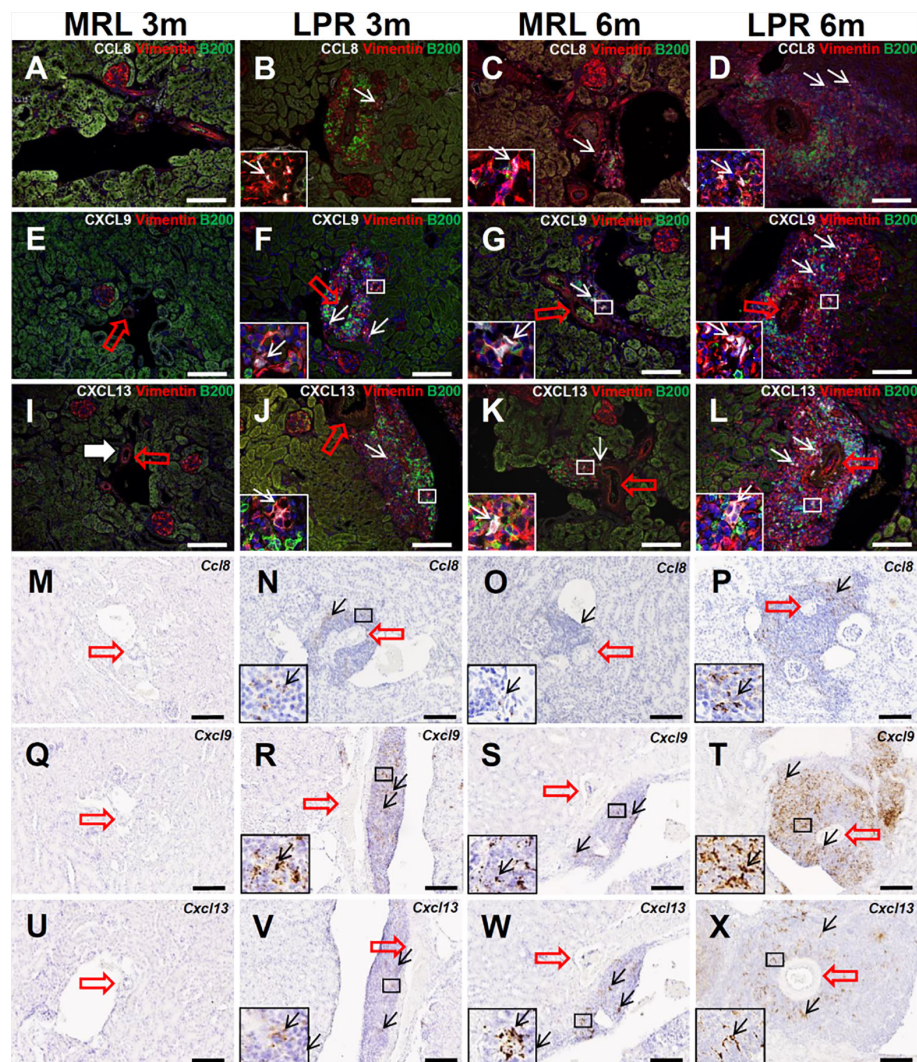


FIGURE 6 | Localization and expression of lymphorganogenic chemokines in PCC. (A–D) Colocalization (arrows) of CCL8 with vimentin and B200⁺ B cells in PCC near the blood vessel (thick arrow) of MRL and LPR mice at 3 and 6 months of age (IF stain). (E–H) Colocalization (arrows) of CXCL9 with vimentin and B200⁺ B cells in PCC near the blood vessel (thick arrow) of MRL and LPR mice at 3 and 6 months of age (IF stain). (I–L) Colocalization (arrows) of CXCL13 with vimentin and B200⁺ B cells in PCC near the blood vessel (thick arrow) of MRL and LPR mice at 3 and 6 months of age (IF stain). (M–P) Expression (arrows) of *Ccl8* in PCC near the blood vessel (thick arrow) of MRL and LPR mice at 3 and 6 months of age (ISH). (Q–T) Expression (arrows) of *Cxcl9* in PCC near the blood vessel (thick arrow) of MRL and LPR mice at 3 and 6 months of age (ISH). (U–X). Expression of *Cxcl13* in PCC near the blood vessel (thick arrow) of MRL and LPR mice at 3 and 6 months of age (ISH). Bars = 100 μm. PCC, perivascular cellular cluster; IF, immunofluorescence; ISH, *in situ* hybridization.

Moreover, *Cxcl9* and *Cxcl13* expression was significantly reduced in the Dex group compared to that in the control (Figures 11E, F).

DISCUSSION

Our present study clarified the development of VALT in LN using two widely used lupus-prone model mice (LPR and Yaa) at different ages. This study shows that VALT develops at an earlier stage (3 months) in lupus-prone mice only. VALT development in LN is associated with chemokines, cytokines, and

costimulatory molecules related to typical lymphoid tissue development and organization. Interestingly, lupus-prone mice showed large VALT, which was associated with renal histopathology at 6 months of age, while the respective control mice developed small VALT in the absence of renal pathology.

We first determined the age at which VALT first appears and measured its average size at different ages. At the earlier stage, VALT was found only in lupus-prone mice (Figure 1). While lupus model mice developed large VALT at 6 months of age, the control MRL mice developed small VALT (Figure 1). At these stages, cellular characterization of VALT allowed distinction from perivascular cuffing. VALT was found to compose of B-,

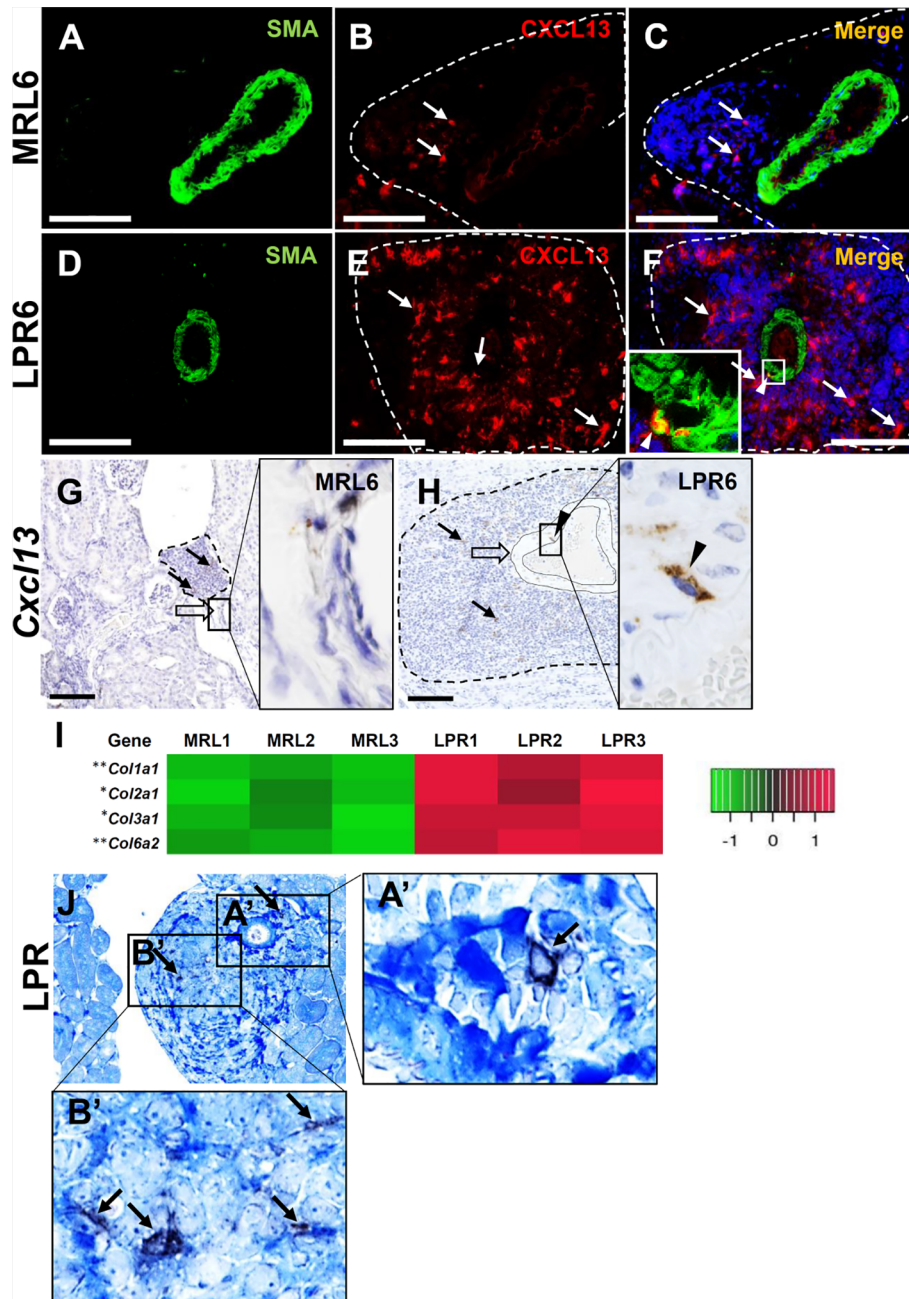


FIGURE 7 | Lymphorganogenic chemokine localization and expression in vascular and perivascular structure. **(A–C)** CXCL13 (arrow) did not colocalize with SMA⁺ smooth muscle cells of the arteries in MRL mice at 6 months of age (IF stain). **(D–F)** CXCL13 (arrow) colocalized (arrowhead) with SMA⁺ smooth muscle cell of the artery in LPR mice at 6 months of age (IF stain). **(G, H)** *Cxcl13* was not expressed at the arterial wall of MRL mice **(G)** at 6 months of age but was expressed at the arterial wall of LPR mice at the same age **(H)** (ISH). **(I)** Transcripts for different genes of collagen in MRL and LPR mice at 6 months of age. Microarray analysis, significant difference from the control is indicated by * (**P* < 0.05, ***P* < 0.01, 2-tailed Student's *t* test). *n* = 3. **(J)** Colocalization of CXCL13 (arrow) with collagen fiber in PCC of LPR mice at 6 months of age (IHC and aniline blue stain). Bars = 100 μm. PCC, perivascular cellular cluster; IF, immunofluorescence; ISH, *in situ* hybridization; IHC, immunohistochemistry.

T-cells, and macrophages, and was accompanied by lymphatic vessels (**Figure 2**). Moreover, SEM revealed that this did not result from arterial damage but rather from the presence of different types of cells in the stromal chambers, as observed in

typical lymphoid organs (**Figure 3**). These results suggest that VALT appearance and development might be associated with inflammatory conditions, aging, and developing the features of TLT. Importantly, previous studies showed that TLT formation

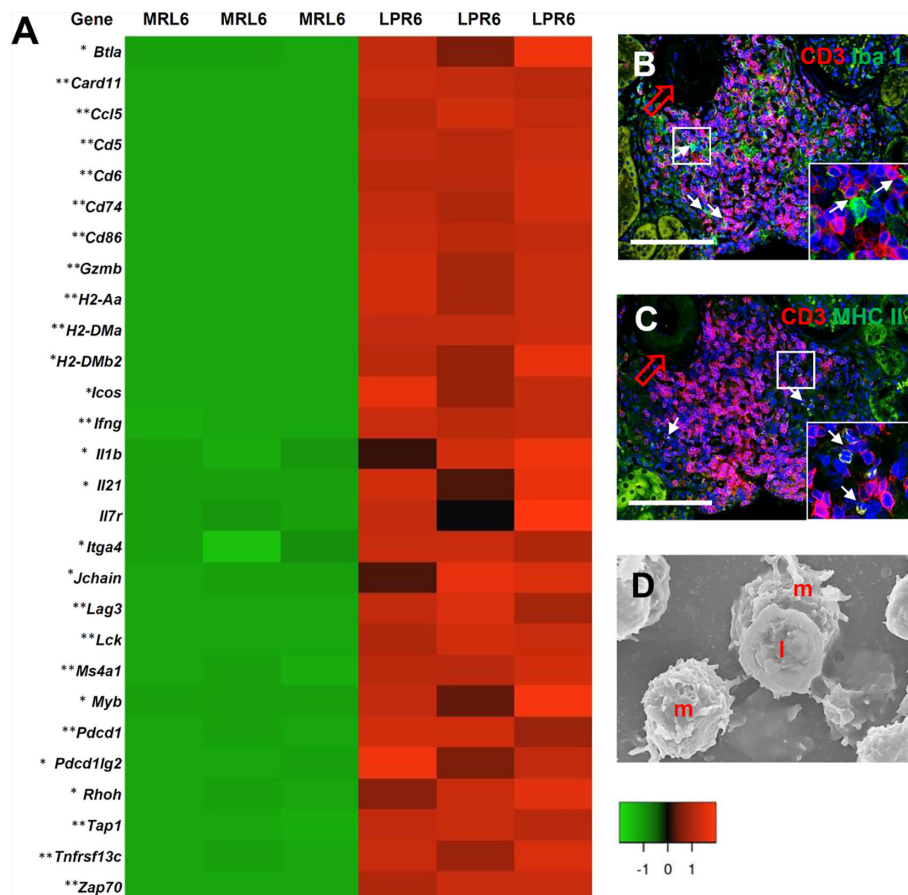


FIGURE 8 | Antigen presentation and lymphocyte activity in VALT. **(A)** Expression levels of different chemokines, receptors, and costimulatory molecules for antigen presentation and cellular activity in VALT of MRL and LPR mice at 6 months of age. Microarray analysis, significant difference from the control is indicated by * (* $P < 0.05$, ** $P < 0.01$, 2-tailed Student's t test). $n = 3$. **(B)** CD3⁺ lymphocytes are localized adjacent to Iba1⁺ macrophages (arrows) in VALT of LPR mice at 6 months of age (IF stain). **(C)** CD3⁺ lymphocytes are localized near MHC II molecules on antigen-presenting cells (arrows) in VALT of LPR mice at 6 months of age (IF stain). **(D)** Lymphocytes (l) are juxtaposed on macrophages (m) in VALT of LPR mice at 6 months of age (SEM). Bars = 100 μ m **(B, C)** and 5 μ m **(D)**. VALT, vasculature-associated lymphoid tissue; IF, immunofluorescence; SEM, scanning electron microscopy.

was triggered by chronic inflammation and autoimmunity (9, 21). Therefore, we examined the expression level of major LC (*Cxcl13*) (22) and its receptor (*Cxcr5*), serum anti-dsDNA antibody levels, and inflammatory cytokines in kidneys from MRL and LPR mice at both stages and found that their expression was higher in LPR mice kidney compared to MRL at both stages (**Figure 4**). Moreover, serum anti-dsDNA antibody and *Ifng* expression correlated with *Cxcl13* expression and percentage of VALT appearance (**Supplementary Figure 2**). These results proved that these VALTs are TLT and result from autoimmune condition.

We further examined how VALT was formed around the blood vessels. RNA sequencing has proven to be a powerful technique for identifying the multifaceted and complex transcriptional activity that is responsible for TLT formation (23). Therefore, we performed TA using kidney tissues from LPR and MRL mice at this age to reveal the chemokines, cytokines, and costimulatory molecules responsible for VALT formation. GO analysis revealed highly upregulated genes, consistent with

TLT formation (**Figure 4**). Typical lymphoid tissue is usually developed by the interaction of hematopoietic lymphoid tissue inducer (LTi) and mesenchymal origin LTo (22). LTi cells expressing CXCR5 and IL7R chemokines accumulate in newly formed lymphoid tissues in response to local production of CXCL13 and IL7 by LTo cells (24). Therefore, our TA clearly revealed that hematopoietic LTi cells are responsible for VALT formation.

We examined the localization and *in situ* expression of highly expressed chemokines in VALT to identify their local sources. We observed that CCL8, CXCL9, and CXCL13 proteins were colocalized with vimentin (**Figure 6**). Moreover, *in situ* expression of *Ccl8*, *Cxcl9*, and *Cxcl13* was observed in the interstitial space among infiltrating cells in VALT. These results indicate that mesenchymal-originated cells such as stromal cells are responsible for the expression of LCs and act as LTo for VALT. Moreover, our TA also showed higher expression of adhesion molecules (*Icam* and *Vcam*), which facilitates the retention of cells in VALT (**Figure 5**).

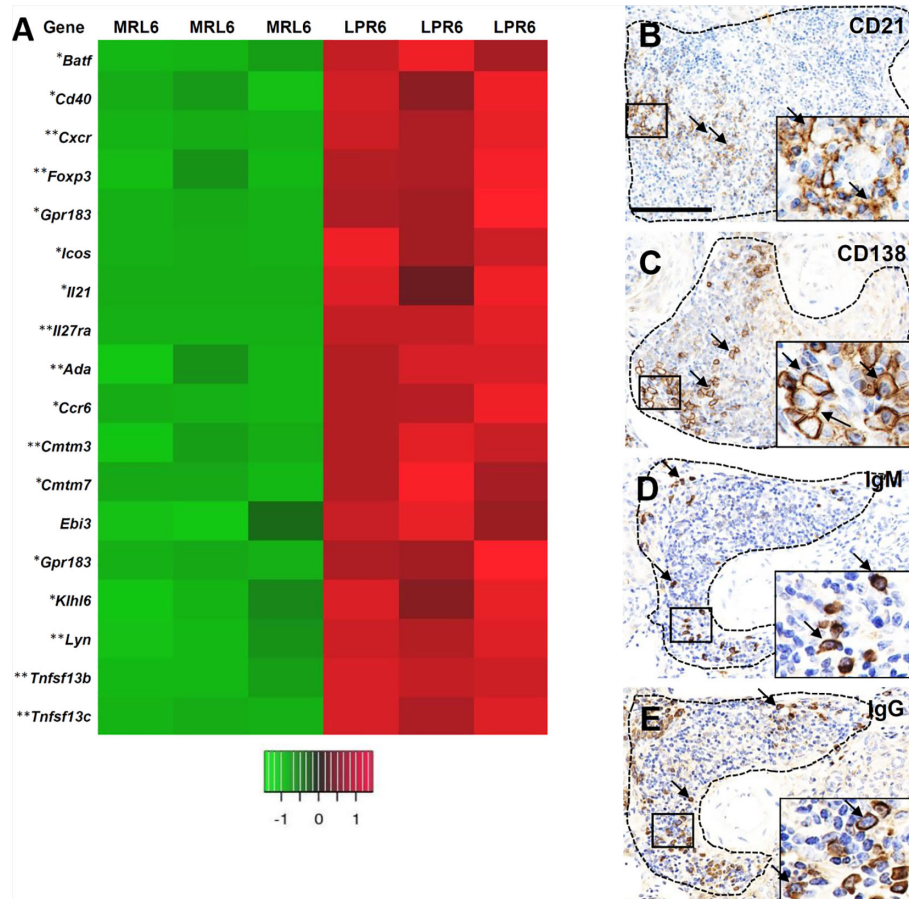


FIGURE 9 | VALT shows lymphoid follicular and germinal center activity. **(A)** Different gene transcripts related to lymphoid follicular and germinal center activity in MRL and LPR mice at 6 months of age. Microarray analysis, significant difference from the control is indicated by * ($P < 0.05$, 2-tailed Student's t test). $n = 3$. **(B)** CD21⁺ follicular dendritic cells (arrows) in VALT of LPR mice at 6 months of age (IHC). **(C)** CD138⁺ plasma cells (arrows) in VALT of LPR mice at 6 months of age (IHC). **(D)** IgM⁺ cells (arrows) in VALT of LPR mice at 6 months of age (IHC). **(E)** IgG⁺ cells (arrows) in VALT of LPR mice at 6 months of age (IHC). Bars = 100 μ m. VALT, vasculature-associated lymphoid tissue; IHC, immunohistochemistry.

Cellular clusters were confined only to the perivascular area. It is not surprising that the space between the artery and the vein harbored clusters of infiltrating cells, as it provides space in this solid organ. However, the formation of these clusters was determined by examining the role of vascular and perivascular structures as LTo (**Figure 7**). We observed the colocalization of CXCL13 with SMC of arterial tunica media, by immunohistochemistry and *in situ* hybridization. In addition, TA also showed higher transcription for many collagen-related genes, including *col1*, -2, -3, and -6. Therefore, we examined the role of these perivascular collagen fibers as LTo. Surprisingly, we found colocalization of CXCL13 with perivascular collagen fibers. Taken together, SMC and perivascular collagen fibers also produced LCs to attract leukocytes. Therefore, vascular SMCs and collagen fibers act as LTo in addition to perivascular stromal cells in the formation of VALT. This VALT is different from the recently characterized lymph node-like structures on the renal pelvic wall in lupus nephritis model mice kidney because VALT developed separately at an early stage around

the large blood vessels and had different lymphatic vasculatures and lymphoid tissue organizers (25). Moreover, serial sectioning examination confirmed that VALT is connected to the lymphoid cluster on the renal pelvic wall only at the late stage.

Usually, TLTs are vascularized with high endothelial venules and lymphatic vessels for lymphocyte trafficking (26). However, we observed only LYVE1⁺ lymphatic vessels in the VALT. Although PNAd expression in VALT could not be detected, a higher expression for transcripts of *Glycam 1* and *St8sia4* (**Figure 5**) were detected, indicating that PNAd constituting proteins undergo local posttranslational modification, suggesting that leukocyte trafficking into VALT may also be directed by elements, in addition to vasculatures. Moreover, our previous study also showed peritubular capillary injury and extravasation of inflammatory cells in the tubulointerstitial space in LN (27). This indicates that extravasated inflammatory cells also have the potential to be attracted by LCs and contribute to the VALT population in LN.

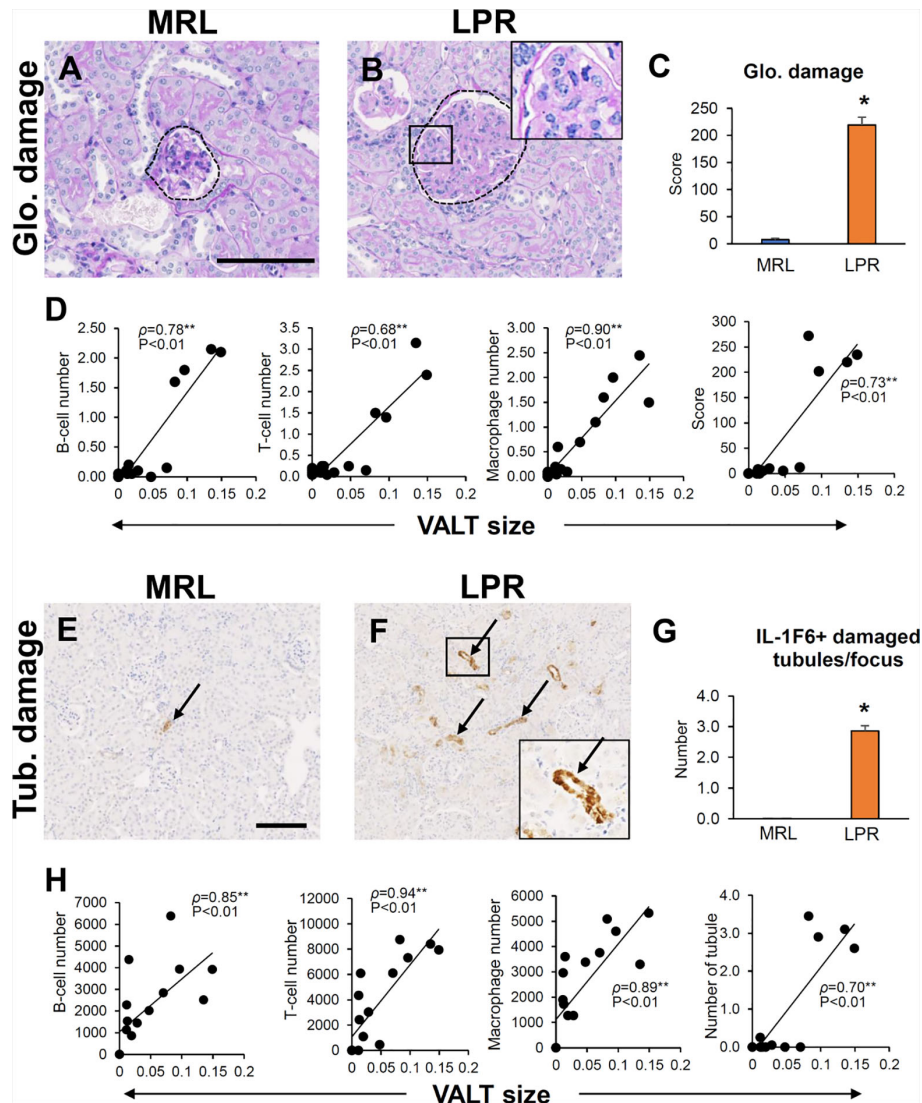


FIGURE 10 | Association of VALT and renal pathology. (A, B) Glomerular damage in MRL and LPR mice at 6 months of age (PAS-H stain). (C) Glomerular damage score in MRL and LPR mice at 6 months of age. (D) Correlation between VALT size and GL parameters. (E, F) IL-1F6⁺ damaged tubules in MRL and LPR mice at 6 months of age. (G) Number of IL-1F6⁺ damaged tubules in MRL and LPR mice at 6 months of age. (H) Correlation between VALT size and TIL parameters. The values are the mean \pm s.e. *significantly different from control mice (Mann-Whitney *U*-test, $P < 0.05$; $n = 4$). ** $P < 0.01$, Spearman's rank correlation coefficient, $n = 16$. Bars = 100 μ m. VALT, vasculature-associated lymphoid tissue; PAS-H, periodic acid *Schiff*-hematoxylin; IHC, immunohistochemistry; GL, glomerular lesion; TIL, tubulointerstitial lesion; glo, glomerular; Tub, tubular.

TLTs function as local sites for the generation of antibodies as a result of local antigen presentation, lymphocyte activation, and maturation (28). In this study, we evaluated the antigen presentation, GC activity, and antibody production in VALT to clarify its functional activity. We evaluated the infiltrates in VALT to examine antigen presentation and found colocalization of CD3 and Iba1⁺ cells as well as CD3 and MHC-II, indicating local antigen presentation to T cells. Similarly, SEM observation also revealed colocalization of lymphocytes and macrophages (Figure 8). Moreover, GO demonstrated the enrichment of genes related to antigen presentation, lymphocyte activation, and immunological synapse (Figure 8). *Il2rb2* stimulates T cell

proliferation towards Th1 by enhancing *Ifng* through *Il27*. T_{FH} cells are characterized by their surface markers *Cxcr5* and *Icos* and by the expression of *Il21* upon stimulation (29, 30). TA revealed higher transcription for *Cxcr5*, *Icos*, *Il12rb2*, *Il27*, *Il21*, and *Cd40* (Figure 9). Therefore, we concluded that helper T cells become T_{FH} cells in VALT and promote formation of GC, where B cells rapidly differentiate into antibody-producing plasma cells with greater affinity. TA also revealed several genes which support the GC activity, including *klhl6* (B cell receptor transduction signals), *Ada* (GC B-cell survival), *Tnfrsf13b* (BAFF), and *Tnfrsf13c* (BAFF receptor). Moreover, FDC plays a crucial role in B-cell activation and affinity maturation through

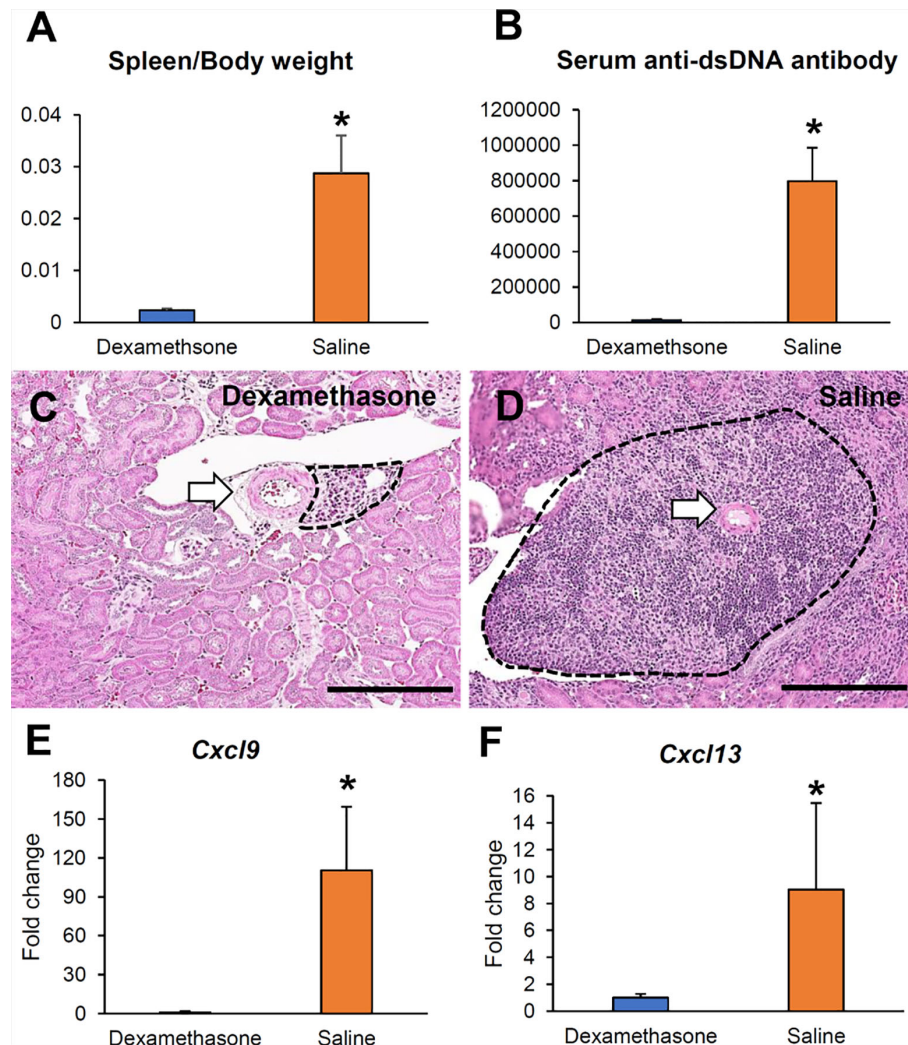


FIGURE 11 | Reduction of VALT size by dexamethasone. **(A, B)** Serum anti-dsDNA antibody level and spleen to body weight ratio in the Dex and saline groups. **(C, D)** Smaller VALT in the Dex **(C)** group compared to the control group **(D)**. HE stain. Bars = 100 μ m. **(E, F)** *Cxcl9* and *-13* expression levels in the Dex and control groups. qPCR. The expression levels were normalized to the levels of *Actb*. The values are the mean \pm s.e. *, significantly different from control mice (Mann-Whitney *U*-test, $P < 0.05$); $n = 4$. Dex, dexamethasone; HE, hematoxylin and eosin; qPCR, quantitative PCR.

the continuous presentation of antigens to B cells (31). We detected CD21⁺ FDC in VALT (**Figure 9**) and identified several autoantigens in the kidney of LPR mice (**Supplementary Figure 3**). Therefore, we concluded that FDCs continuously present autoantigens to B-cells for the perpetuation of local antibodies. Importantly, we observed CD138⁺ plasma cells as well as IgM and IgG antibodies at the periphery of VALT. This result is consistent with previous results that showed that TLT was associated with autoantibody production in autoimmune disease (21, 32, 33). In addition, BrdU⁺ cells were observed in VALT, indicating that local cells undergo proliferation and VALT activation.

Next, we clarified the role of VALT in renal lesion development, as TLT has detrimental effects on the residing organ (21). We found larger VALT as well as more infiltrating immune cells in the kidney, and VALT size was correlated with GL and TIL (**Figure 10**). In

addition, there were many immune cells as well as IgM and IgG antibodies at the periphery of VALT invading adjacent tubules and glomerulus. These results indicate that VALT functions as a TLT for the perpetuation of adaptive immune responses by providing a local source of antibody that is generated as a result of local antigen presentation, lymphocyte activation, and maturation in the newly formed structure.

As VALT formation is associated with renal lesions, therapeutics targeting VALT formation could ameliorate renal lesions. This study clearly showed the molecular bases of VALT development, which could be considered as therapeutic targets, including major LCs, mechanical removal of VALT, and administration of immunosuppressive drugs. The first two approaches are not feasible as the administration of antibodies targeting the reduction of LCs might affect lymphoid tissue

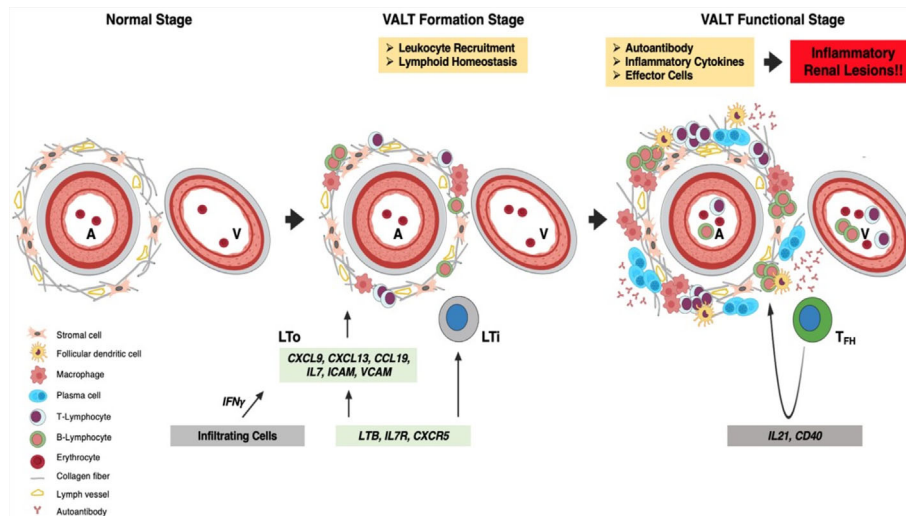


FIGURE 12 | Summary of VALT formation and its effects. The perivascular space contains stromal cells and collagen fibers in the normal kidney. At the VALT formation stage, *Ifng* secreted by infiltrating cells stimulates LTo to secrete LC (*Cxcl9*, *Cxcl13*, *Ccl19*, *Il7*, *ICAM*, *VCAM*) secreted from LTo. Interaction between LTI and LTo also increases LC production. The retention of infiltrated cells is maintained by costimulatory molecules (*VCAM* and *ICAM*) secreted from LTo. At the functional stage, VALT becomes larger where helper T-cells become follicular T_{FH} cells with progression of inflammation and helps in germinal center formation and activity. VALT functions in the perpetuation of autoantibodies against autoantigens and effector cells, which aggravates inflammatory renal lesions. A, artery; V, vein; LTI, lymphoid tissue inducer; LTo, lymphoid tissue organizer; LC, lymphorganogenic chemokinesand; T_{FH} , T follicular helper cell.

necessary for body defense. Moreover, VALT surrounds the large vessels, and mechanical removal of VALT affects the vessel. Therefore, mechanical removal of VALT from mouse kidneys as well as human patients suffering from lupus nephritis is not realistic. Therefore, we chose the latter target since VALT developed due to renal inflammatory conditions. Moreover, prophylactic treatment with Dex effectively reduces the incidence of CKD after cardiac surgery (34). Surprisingly, we observed that administration of Dex reduced serum anti-dsDNA antibody and spleen to body weight ratio and almost ablated the VALT with the least expression of LCs (Figure 11). Dex has multifaceted effects on different immune cells and can reduce the development of pathogenic lesions directly or indirectly, although this remedy is also accompanied by side effects (15). Therefore, refining the Dex dose, starting time, and duration of administration should be considered before using it as a therapeutic to reduce LN lesions through ablation of VALT.

In conclusion (Figure 12), the perivascular space contains stromal cells and fibers in the normal kidney. At the beginning of LN, *Ifng* from infiltrating cells stimulates stromal cells and fibers to secrete LCs. Recruited LTI cells also stimulate LTo to secrete more LCs to attract leukocytes for homing around the artery to form VALT. With the progression of LN, VALT becomes larger and helper T cells acquire the phenotype of T_{FH} cells, which stimulates B cells for proliferation, affinity maturation, and GC formation. Autoantibodies and effector cells are formed in VALT upon stimulation with autoantigens, which aggravates renal lesions. Moreover, administration of Dex ablates VALT in LN. Therefore, this study revealed the cell types and molecules that

governed the formation of VALT in LN, and thereby opening a new window for therapeutic intervention of LN through ablation of VALT.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University, approval No. 16-0124.

AUTHOR CONTRIBUTIONS

MM: conceptualization, formal analysis, funding acquisition, investigation, methodology, and roles/writing—original draft. OI: conceptualization, funding acquisition, investigation, and roles/writing—original draft. YE: conceptualization, investigation, roles/writing—original draft. YO: formal analysis, investigation, and methodology. TN: formal analysis, investigation, and methodology. YK: conceptualization, funding acquisition,

investigation, supervision, and roles/writing—original draft. All authors contributed to the article and approved the submitted version.

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design, execution of experiments, data collection, manuscript preparation, and decision to publish.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.595672/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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Highly Sensitive Flow Cytometric Detection of Residual B-Cells After Rituximab in Anti-Neutrophil Cytoplasmic Antibodies-Associated Vasculitis Patients

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Background: B-cell depletion with rituximab (RTX) is an effective treatment for anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients. Nevertheless, relapses are frequent after RTX, often preceded by B-cell repopulation suggesting that residual autoreactive B-cells persist despite therapy. Therefore, this study aimed to identify minimal residual autoimmunity (MRA) in the B-cell compartment of AAV patients treated with RTX.

Methods: EuroFlow-based highly-sensitive flow cytometry (HSFC) was employed to study B-cell and plasma cell (PC) subsets in-depth in AAV patients before and after RTX treatment. Additionally, peripheral blood mononuclear cells (PBMCs) of these RTX-treated AAV patients were cultured and *in vitro* stimulated with CpG, IL-2, and IL-21 to induce antibody-secreting cells (ASC). (ANCA)-IgG was measured in these supernatants by ELISA.

Results: By employing EuroFlow-based HSFC, we detected circulating CD19⁺ B-cells at all timepoints after RTX treatment, in contrast to conventional low-sensitive flow cytometry. Pre-germinal center (Pre-GC) B-cells, memory B-cells and CD20⁺CD138[−] plasmablasts (PBs) were rapidly and strongly reduced, while CD20[−]CD138[−] PrePC and CD20[−]CD138⁺ mature (m)PCs were reduced slower and remained detectable. Both memory B-cells and CD20[−]PCs remained detectable after RTX. Serum ANCA-IgG decreased significantly upon RTX. Changes in ANCA levels strongly correlated with changes in naive, switched CD27⁺ and CD27[−] (double-negative) memory B-cells, but not with plasma cells. Lastly, we demonstrated *in vitro* ANCA production by AAV PBMCs, 24 and 48 weeks after RTX treatment reflecting MRA in the memory compartment of AAV patients.

Conclusion: We demonstrated that RTX induced strong reductions in circulating B-cells, but never resulted in complete B-cell depletion. Despite strongly reduced B-cell numbers after RTX, ANCA-specific memory B-cells were still detectable in AAV patients. Thus, MRA is identifiable in AAV and can provide a potential novel approach in personalizing RTX treatment in AAV patients.

Keywords: ANCA-associated vasculitis, rituximab, B-cells, immunomonitoring, glomerulonephritis, ANCA antibodies, minimal residual autoimmunity, highly sensitive flow cytometry

INTRODUCTION

B-cell depletion with rituximab (RTX) is an effective treatment strategy for patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) (1, 2) and is increasingly prescribed as induction and/or maintenance treatment (3–5). The rationale for B-cell depletion in AAV patients is the reduction of autoreactive, ANCA-producing B-cells (6). RTX, a chimeric anti-CD20 antibody, results typically in a rapid CD20⁺ B-cell depletion, while bone marrow (BM) precursors and long-lived plasma cells (PCs), which lack CD20 expression, remain unaffected (7).

Despite the success with RTX as remission-induction therapy in AAV patients (1, 2), in the RAVE-trial, one-third of the patients experienced a relapse within 18 months after RTX (8). In this trial, increases in ANCA levels did not predict relapses in either the RTX or the cyclophosphamide treatment group. Additionally, relapses were preceded by B-cell repopulation in 88% of the patients, but B-cells also returned in the two-thirds of patients who did not experience a relapse. Importantly, relapses were rare in the absence of both B-cells and ANCA (8). Nevertheless, serum ANCA-levels and B-cell numbers have been proposed as potential biomarkers that can predict these relapses and guide RTX retreatment, based on the premise that return of ANCA or B-cells are a hallmark of disease relapse (8–14). Already before the use of RTX, a meta-analysis demonstrated that rising ANCAs and persistent presence of ANCAs were associated with future relapses (positive likelihood ratio (LR+): 2.84 [1.65–4.9] and 1.97 [1.43–2.7], respectively) (15). Also, we recently demonstrated that PR3-positivity predicted future relapses in AAV patients after remission-induction therapy with RTX (16), which was also supported by another study (13). Absence of B-cell repopulation strongly predicted a relapse-free status in both PR3- and MPO-ANCA positive patients. Altogether, ANCA was found to associate with relapses in several studies, whereas the potential of circulating B-cells was not always evident. However, studies that investigated the B-cell compartment of AAV patients after RTX more in-depth found several phenotypes that were associated with relapses, e.g. incomplete B-cell depletion (17); or B-cell repopulation with relatively high number of plasmablasts (PBs) (18); switched memory B-cells (19); relatively low number of naive B-cells (17) or decreased CD5⁺ regulatory B-cells (20, 21), whereas the latter also inversely correlated with ANCA levels (22). Altogether, strongly indicating that specific subsets of (autoreactive) B-cells are involved in the pathogenesis of relapses.

Importantly, the method of analyzing B-cells, especially after RTX, and specifically the sensitivity of the method, determines the detection level of B-cell depletion and reconstitution (23). Conventional “low sensitive” flow cytometry (LSFC), applied in standard clinical care for AAV patients, is only able to detect CD19⁺ B-cells starting from 1 cell/ μ l. As a consequence, <1 cell/ μ l is then defined as “complete B-cell depletion”, without defining different B-cell subsets. In contrast, highly-sensitive flow cytometry (HSFC), originally developed to detect minimal residual disease (MRD) in hematologic malignancies (24), is able to pick up B-cells with up to 100 times more sensitivity.

In the present study, we performed an in-depth phenotypic and functional analysis of B- and plasma cells after RTX treatment in AAV patients. We employed EuroFlow-based HSFC (25, 26) after RTX and studied ANCA-specific memory B-cells *in vitro* to identify minimal residual autoimmunity (MRA).

MATERIALS AND METHODS

Study Population

This observational prospective single cohort study was conducted at the expert center for Lupus-, Vasculitis-, and Complement-mediated systemic autoimmune diseases (LuVaCs) of the Leiden University Medical Center (LUMC) in the Netherlands. In this study, AAV patients treated with RTX were eligible and informed consent was required for study participation. The study was approved by the local medical ethics committee of the LUMC. Eleven unique AAV patients that received RTX were included in this study (**Table 1**). Seven patients received RTX as remission-induction treatment for active disease, of which 6 were included for flow cytometry studies which are shown in **Figures 2–4**. Additionally, four other patients and three patients from the previous group received up to 4 times maintenance treatment with 500 mg RTX every 6 months (**Supplementary Tables 1 and 2**), which were allowed to re-enter the study (**Supplementary Figure 1**). There was a total of 17 RTX maintenance treatments, of which 8 were included for flow cytometry studies (**Supplementary Table 1**). The flow cytometry data of these RTX maintenance patients were shown in the **Supplementary Figures 5 and 7**. Regarding the PBMC culture experiments, all available PBMC samples at week 0, 24, and 48 weeks after all RTX treatments in all patients were included, except one ANCA-negative patient (n = 23).

TABLE 1 | Patient characteristics.

	AAV patients (n = 11)	Flow cytometry studies (n = 6)
Demographics		
Age	59 (33–77)	42 (33–77)
Male	6 (55%)	3 (50%)
Caucasian	11 (100%)	6 (100%)
ANCA associated vasculitis		
GPA	4 (36%)	2 (33%)
MPA	6 (55%)	3 (50%)
eGPA	1 (9%)	1 (17%)
Immunology		
ANCA immunofluorescence		
c-ANCA	3 (27%)	1 (17%)
p-ANCA	7 (64%)	4 (67%)
negative	1 (9%)	1 (17%)
ELISA		
PR3	4 (36%)	2 (50%)
MPO	6 (55%)	4 (67%)
negative	1 (9%)	1 (17%)
Low sensitive flow cytometry		
CD19+ B-cells (10 ⁶ /L)	27 (0–311)	183.5 (1–311)
Organ involvement		
Constitutional symptoms	6 (55%)	3 (50%)
Mucocutaneous	3 (27%)	1 (17%)
Musculoskeletal	4 (36%)	2 (33%)
ENT	6 (55%)	2 (33%)
Renal	6 (55%)	3 (50%)
Respiratory	7 (64%)	3 (50%)
Cardiovascular	1 (9%)	1 (17%)
Central nervous system	1 (9%)	0 (0%)
Peripheral nervous system	0 (0%)	0 (0%)
Ophthalmology	3 (27%)	0 (0%)
Abdominal	3 (27%)	2 (33%)
Disease parameters		
BVAS	6 (0–30)	11.5 (6–30)
VDI	3 (0–12)	0.5 (0–7)
Reason for treatment		
New diagnosis	4 (36%)	4 (67%)
Relapse	1 (9%)	0 (0%)
Persistent disease	2 (18%)	2 (33%)
Maintenance treatment	4 (36%)	0 (0%)
Treatment		
Rituximab		
2× 1,000 mg	6 (55%)	6 (100%)
1× 500 mg	5 (45%)	0 (0%)
Methylprednisolone 3× 1,000 mg	4 (36%)	4 (67%)
Plasmapheresis	1 (9%)	1 (17%)
Cyclophosphamide (2× 500 mg)	1 (9%)	1 (17%)
High-dose corticosteroids	5 (45%)	5 (83%)
Previous remission-induction treatment		
Rituximab	5 (45%)	0 (0%)
Cyclophosphamide oral	3 (27%)	1 (17%)
Cyclophosphamide IV	2 (18%)	0 (0%)

For numerical values the median with (range) and for categorical values the frequency with percentages is shown.

Rituximab Treatment Schedules

Treatment with RTX as remission-induction therapy was given as two times 1,000 mg RTX i.v. with a 2 week interval or as 500 mg RTX every 6 months as maintenance therapy. Directly before every RTX infusion, all patients received oral acetaminophen, clemastine i.v. and 100 mg methylprednisolone i.v. Additionally, patients were allowed to receive other immunosuppressive medication according to standardized local protocol including

up to three times 1,000 mg methylprednisolone i.v. daily, followed by high dose oral corticosteroids (1 mg/kg/day, maximum 60 mg) with tapering over 3 months. Additionally, patients with severe renal and/or lung involvement were allowed to receive 500 mg cyclophosphamide i.v. and/or plasmapheresis. All patients also received prophylactic treatment with co-trimoxazole 480mg/day, proton-pump inhibition, vitamin D, calcium supplementation and, if indicated, bisphosphonates.

Clinical and Laboratory Measurements

Clinical and laboratory data were collected at study visits just before RTX infusion and 2, 4, 12, 24, and 48 weeks after RTX treatment. Serum and PBMCs were stored for experimental studies at each study visit. Birmingham Vasculitis Activity Score (BVAS-3) was used to score disease activity during the study (27). The clinical diagnostics lab measured total immunoglobulin (Ig) levels in the sera of the patients. Standardized measurements of circulating CD19⁺ B-cells by routine flow cytometry protocols were performed at the laboratory of Hematology, with a detection limit of 1×10^6 cells/L for CD19⁺ B-cells, further referred to as low-sensitive flow cytometry (LSFC).

Euroflow-Based Highly-Sensitive Flow Cytometry

Peripheral blood was collected in EDTA-coated tubes at each visit. Samples were processed within 4 h after collection and analyzed by flow cytometry after bulk-lyse standard operating procedure (www.EuroFlow.org), as described before (25, 26). According to these protocols, the membranes of 20×10^6 nucleated cells per patient sample were stained with the EuroFlow 13-color IgH-isotype B-cell tube. Per sample, 10 – 20×10^6 leucocytes were measured in LSR Fortessa X-20 flow cytometer (Becton Dickinson Biosciences, San Jose, Calif). Instrument set-up and calibration were performed according to EuroFlow standard operating procedures www.EuroFlow.org (28). For data analysis, Infinicyt software version 2.0.1 (Cytognos S.L., Salamanca, Spain). was used.

Gating strategy for the identification of different major B-cell and plasma cell subsets was shown in previous publications (25, 26). B-cells were identified based on their positive CD19 expression and low forward (FSC) and sideward scatter (SSC) properties, which is typical for lymphocytes. PCs were identified by high expression for CD38 and CD27, with low expression of CD24 and CD21 in conjunction with low-to-intermediate forward light scatter and sideward light scatter. Both switched memory and PCs were subsequently subclassified based on their maturation stage and expression of distinct Ig isotypes and Ig subclasses, as previously described (25, 26). This strategy resulted in the following definitions of B-cell subsets:

Pre-germinal center B-cells, including:

CD27[−]CD38^{hi}CD24^{hi}CD5⁺smIgM⁺⁺IgD⁺: immature/transitional B-cells;

CD27[−]CD38^{lo}CD24^{het}CD5⁺smIgM⁺IgD⁺⁺: CD5⁺ naive B-cells;

CD27[−]CD38^{lo}CD24^{het}CD5[−]smIgM⁺IgD⁺⁺: CD5[−] naive B-cells;

Memory B-cells, including:

CD27⁺CD38^{lo}CD24^{het}smIgM⁺⁺IgD⁺: unswitched memory B-cells;

CD27⁺CD38^{lo}CD24^{het}smIgM[−]IgD[−]: switched memory B-cells;

CD27[−]CD38^{lo}CD24^{het}smIgM[−]IgD[−]: switched “double negative” memory B-cells;

Plasma cells (PCs)

CD20⁺CD138[−]CD27^{hi}CD38^{hi}CD21[−]CD24[−]: plasmablasts;

CD20[−]CD138[−]CD27^{hi}CD38^{hi}CD21[−]CD24[−]: prePCs;

CD20[−]CD138⁺CD27^{hi}CD38^{hi}CD21[−]CD24[−]: mature PCs;

Switched memory B-cells were divided in further subsets based on CD27 expression (CD27⁺ and CD27[−]), the latter also referred to as “double negatives”. PCs were further subdivided in plasmablasts (PBs), prePCs and mPCs based on CD20 and CD138. Additionally, switched memory B-cells and PCs were further subclassified according to their Ig isotypes and Ig subclasses smIgA1⁺, smIgA2⁺, smIgG1⁺, smIgG2⁺, smIgG3⁺, and smIgG4⁺ memory B-cells and smIgM⁺-only, smIgD⁺-only, smIgA1⁺, smIgA2⁺, smIgG1⁺, smIgG2⁺, smIgG3⁺, smIgG4⁺, and smIg[−] PCs, respectively. Absolute counts were calculated by using total leucocyte cell counts, which was measured by the clinical diagnostics lab of the LUMC. For HSFC, reliable interpretation was set at a minimum of 20 acquired events, according to the EuroFlow guidelines. Counts below 20 acquired events were considered less robust interpretation and therefore indicated in the graphs with gray areas. The absolute detection limit of HSFC (<1 acquired event was 1×10^3 cells/ul (average of all patients and timepoints), which was indicated by the dotted lines in each graph.

PBMC Cultures

Isolation

EDTA-anticoagulated peripheral blood from patients was drawn, followed by PBMC isolation with the use of Ficoll-amidotriozate (Poli Apotheek LUMC, Leiden, The Netherlands). After washing with PBS (B. Brain Medical N.V., Oss, The Netherlands), peripheral blood mononuclear cells (PBMCs) were stored in Gibco[®] RPMI Medium 1640 (1×) containing L-Glutamate and 25 nM HEPES (Thermo Fisher Scientific, Waltham, MA, USA), 10% Fetal Calf Serum (Bodinco BV, Alkmaar, The Netherlands), 200 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen until further use. Buffy coats from healthy donors, who gave written informed consent for scientific use of the buffy coats, were purchased from Sanquin Blood Bank, Amsterdam, The Netherlands. PBMCs from these buffy coats were isolated following the same protocol as described above.

Culture

After thawing, PBMCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing L-Glutamate and 25 nM HEPES (Thermo Fisher Scientific, Waltham, MA, USA), 10% Fetal Calf Serum, 200 U/ml Penicillin-Streptomycin, and 4 nM 2-Mercaptoethanol (Sigma-Aldrich, Darmstadt, Germany). One million PBMCs were cultured in 1,000 µl IMDM per well in a sterile Costar[®] 48-Well Flat Bottom Plate (Corning Inc, Corning, NY, USA). Up to five wells per patient or HC sample were cultured per experiment. PBMCs were polyclonally stimulated with 3.2 µg/ml class B CpG ODN (InvivoGen, Toulouse, France), 1,000 IU/ml IL-2, and 100 ng/ml IL-21 (PeproTech EC Ltd., London, United Kingdom) to induce

antibody secreting cells (ASCs), adjusted from protocols that were previously published (29–31). Culture plates were incubated at 37°C, 5.0% CO₂, and 92% RH for 10 days. After 7 days, the PBMCs from one well per sample were analyzed with flow cytometry to assess the viability of the cells and proliferation of plasma cells. After 10 days supernatants of all wells were harvested and stored at –20°C.

Identification of Antibody-Secreting Cells

PBMCs were analyzed with a simple standard flow cytometry panel at start and after 7 days of culturing to identify antibody-secreting cells (**Supplementary Figure 2**). Cells were stained in the dark for 30 min at 4°C with the following antibody panel: anti-CD45-PerCP, anti-CD3-FITC, anti-CD14-APC-H7 (BD Biosciences, Franklin Lakes, NJ, USA), anti-IgD-PE, anti-CD19-eFluor450, anti-CD27-PeCy7 (Thermo Fisher Scientific, Waltham, MA, USA), and anti-CD38-APC (BioLegend, San Diego, CA, USA). All washing steps were performed using PBS containing 1% BSA and 0.01% azide (FACS buffer). Stained cells were analyzed in a LSR-II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with FACSDiva software version 8.0.2 (BD Biosciences, Franklin Lakes, NJ, USA). FlowJo™ version 10.4.2 (FlowJo, LLC, Ashland, OR, USA) was used for gating of the different cell populations (**Supplementary Figure 2**). After live cell and single cell selection, all CD45⁺CD3[–]CD14[–]CD19⁺ cells were selected and considered B-cells. Of these B-cells, all CD27^{hi}CD38^{hi} cells were considered PCs. Absolute numbers of B-cells and plasma cells were calculated based upon the absolute counts of surviving PBMCs per well in combination with CD45⁺ population in the flow cytometry.

ELISA

In-house ELISA protocols were used to determine total IgG and IgM concentrations in the supernatants of the cultures and anti-MPO IgG and anti-PR3-IgG in sera of the patients and supernatants of the cultures. Nunc MaxiSorp™ flat-bottom ELISA plates (ThermoFisher Scientific, Waltham, MA, USA) were coated overnight at room temperature in coating buffer (0.1M Carbonate, pH 9.6) with goat anti human IgG Fc (Bethyl Laboratories, Inc, Montgomery, TX, USA), rabbit anti human IgM (Thermo Fisher Scientific, Waltham, MA, USA), human sputum myeloperoxidase (Elastin Products Company, Inc, Owensville, MO, USA) or PR3 (isolated from neutrophils according to (32)). Subsequently the plates were blocked with PBS/2% Casein for 1 h at room temperature. After washing, the plates were incubated for 1 h with sera, supernatants, and antibodies were diluted in PBS/0.05% Tween/2% Casein (Tween® 20, Sigma-Aldrich, Darmstadt, Germany). All washing steps were performed with PBS/0.05% Tween. Goat-anti-Human IgG-HRP (Bethyl Laboratories, Inc, Montgomery, TX, USA) was used for the total IgG ELISA. Monoclonal anti human IgM biotin antibody (Sigma-Aldrich, Darmstadt, Germany) and poly streptavidin-HRP (Sanquin, Amsterdam, The Netherlands) was used for the IgM ELISA. ABTS containing 2 µl hydrogen peroxide 30% (Sigma-Aldrich,

Darmstadt, Germany) was used as a substrate for the horseradish peroxidases (HRPs) used in the IgG and IgM ELISAs. Anti-human IgG-AP antibody (Sigma-Aldrich, Darmstadt, Germany) was used for anti-MPO and anti-PR3 IgG followed by substrate: 5 mg phosphatase substrate tablet (Sigma-Aldrich, Darmstadt, Germany) in 10 ml pNPP buffer (97 ml diethanolamine, 0.1g MgCl₂, and 0.2 NaN₃ in 1-L water, pH 9.8). Because MPO and PR3 are active enzymes HRP was not suitable to be used in this ELISA. ELISAs using ABTS were measured at 415 nm, while those using phosphatase substrate were measured at 405 nm.

Statistics

All descriptive clinical data were expressed as median with [range] for numerical data or given as percentage for nominal data. All flow cytometry data were expressed as median with [range] for absolute numbers or ratio as compared to baseline. Statistical difference between two groups was determined with Mann-Whitney U test. Correlations were tested with Pearson's correlation test (flow cytometry methods) or spearman's correlation statistical test (changes of ANCA/B-cell subsets). All data were analyzed using SPSS statistics (IBM, New York, USA), version 23 or Prism version 8.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

AAV Study Population

The characteristics of AAV patients treated with RTX were summarized in **Table 1**. Median [range] BVAS scores in patients with active disease (n = 6) that received remission-induction treatment with RTX decreased from 11.5 [6–30] to 0 [0–7] after 24 weeks and remained 0 [0–10] after 48 weeks (**Supplementary Figure 3A**). Patients that received maintenance therapy (n = 8) had a median [range] BVAS score at baseline of 4 [0–7] which decreased to 0 [0–4] after 24 weeks and to 0 [0–0] after 48 weeks (**Supplementary Figure 3B**).

CD19+ B-Cells Were Always Detectable With HSFC After RTX in Contrast to LSFC

Both standard clinical “low-sensitive” flow cytometry (LSFC) and EuroFlow-based HSFC (25, 26) were applied to detect B-cells in 14 AAV cases before and 2, 4, 12, 24, and 48 weeks after RTX, resulting in 68 samples. LSFC was unable to detect CD19⁺ B-cells in 43/54 samples after RTX, in contrast to HSFC which was able to detect CD19⁺ B-cells in all patients at all timepoints after RTX. **Figure 1A** illustrates the increased sensitivity of HSFC to detect B-cells up to 0.001*10⁶/L, while there is a strong positive and linear correlation between the methods (Pearson's r = 0.99, CI [0.98–0.99]; p < 0.0001). Additionally, the number of CD19⁺ B-cells over time was shown for each individual patient after treatment with RTX as measured with LSFC (**Figure 1B**) and with HSFC (**Figure 1C**). These figures clearly emphasized the increased capability of HSFC to detect B-cells at all timepoints in contrast to LSFC.

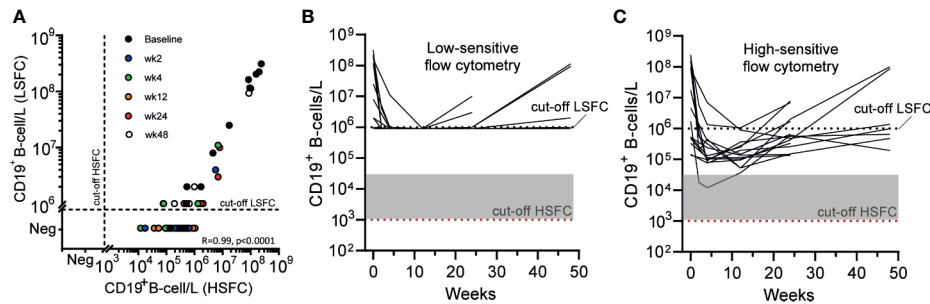


FIGURE 1 | Comparing the detection of circulating CD19⁺ B-cells with LSFC versus HSFC (A). Absolute numbers of circulating CD19⁺ B-cells were analyzed with HSFC (x-axis) and correlated to LSFC (y-axis) with, respectively, a detection limit of 1×10^3 B-cells/L and 1×10^6 B-cells/L. Each dot represents one timepoint for one patient before and 2, 4, 12, 24, or 48 weeks after RTX treatment measured with LSFC (C). Each line represents the absolute CD19⁺ B-cell count for an individual patient during RTX treatment measured with HSFC. In (B, C), the dotted line indicated the detection limit for LSFC (10^6 cells/L). Gray area indicates 1–20 analyzed events. Red dotted line indicated the detection limit for HSFC (10^3 cells/L).

Residual Memory and Plasma Cells After RTX

With the ability to reliably detect low numbers of circulating B-cells with HSFC, kinetics of different B-cell and plasma cell subsets could now be assessed in AAV patients. At baseline, AAV patients with active disease ($n = 6$), had a median [range] of $125 [1.70–238] \times 10^6$ CD19⁺ B-cells/L while AAV patients in remission on maintenance RTX therapy ($n = 8$) had significantly less CD19⁺ B-cells/L with a median of $0.50 [0.14–17.1] \times 10^6$ ($p = 0.003$). Given the reduced B-cell numbers due to previous RTX maintenance treatments (Supplementary Figure 4A), the kinetics of B-cell and plasma cell subsets were studied separately in patients with active disease that received remission induction treatment with $2 \times 1,000$ mg RTX ($n = 6$) (Figure 2). Of note, the effects of RTX as maintenance therapy ($n = 8$) are depicted in Supplementary Figure 5.

After remission-induction therapy with RTX, the nadir of circulating CD19⁺ B-cells was reached after 12 [4–24] weeks. At the nadir, concentration of CD19⁺ B-cells was $0.07 [0.01–1.05] \times 10^6$ B-cells/L (Figure 2A), corresponding to a significant decrease of -99.7% ($p = 0.03$) (Figure 2E). After the nadir, total circulating B-cells increased up to $0.58 [0.15–6.90] \times 10^6$ B-cells/L at 24 weeks and up to $0.65 [0.19–10.0] \times 10^6$ B-cells/L at 48 weeks after RTX, but remained strongly reduced as compared to baseline. As expected, RTX remission-induction treatment ($2 \times 1,000$ mg) decreased total B-cells significantly more than RTX maintenance treatment (500 mg) at all timepoints (Supplementary Figure 4B).

At baseline, the predominant B-cell population was pre-GC B-cells while plasma cells represented only a small subset of total B-cells (Figure 2F). The pre-germinal center (Pre-GC) B-cells, including immature, CD5⁺ and CD5[−] naïve B-cell populations

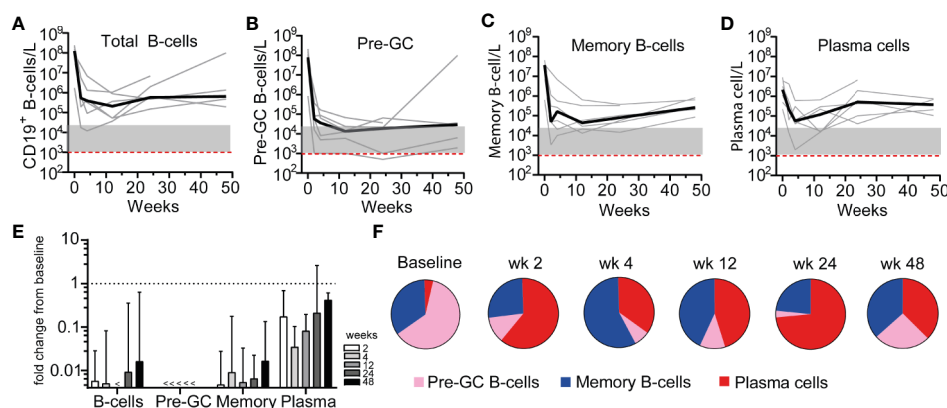


FIGURE 2 | Residual memory and plasma cells after RTX. Absolute counts of (A) total CD19⁺ B-cells (B), Pre-GC B-cells (C), memory B-cells, and (D) plasma cells are shown for each individual patient that received remission-induction therapy with RTX ($n = 6$). The median is indicated by the thick black line. Red dashed line indicates the mean detection limit for HSFC. Gray area indicates 1–20 analyzed events (E). Median \pm IQR fold change as compared to baseline for each B-cell subset per timepoint is shown (F). Mean distribution of B-cell subsets per timepoint during RTX treatment is shown.

(**Supplementary Figure 6**) were rapidly reduced after RTX and 67% of the patients reached their nadir between 12 and 24 weeks, which was 0.01 (0.001 – 0.04) $\times 10^6$ cells/L, corresponding to a significant decrease of 99.98% (**Figures 2B, E**). Additionally, the memory B-cell compartment was rapidly reduced (**Figures 2C, E**) and surprisingly also the plasma cell compartment (**Figures 2D, E**). However, the reduction of plasma cells was less profound than the memory B-cells, and the time to nadir was 12 [2–24] weeks for memory B-cells and 4 [4–12] weeks for plasma cells. The increasing numbers of total CD19⁺ B-cells 24 weeks after RTX was mainly due to an increased number of circulating plasma cells (**Figure 2F**). Because plasma cells were the least targeted population by RTX, the predominant B-cell population during RTX maintenance therapy was plasma cells (**Supplementary Figure 5F**). Of note, the absolute counts of Pre-GB B-cell subsets after maintenance therapy with RTX are shown in **Supplementary Figure 7**.

Phenotyping Residual Memory and Plasma Cells After RTX

To further study the residual memory B-cells and PCs after RTX treatment, we subsequently investigated their phenotype by HSFC. All memory subsets, including “unswitched memory B-cells” (CD27⁺ with IgM⁺ and/or IgD⁺), “switched memory B-cells” (CD27⁺ with IgA⁺ or IgG⁺) and “double negative” (DN) B-cells (CD27⁺ with IgA⁺ or IgG⁺) were rapidly and strongly reduced after RTX (**Figures 3A, B**). No significant different responses among these subgroups nor among IgG⁺ or IgA⁺ memory B-cells were detected after RTX (**Figure 3B**). Noteworthy, increasing numbers of unswitched and switched

memory B-cells could be reliably detected at 48 weeks which was not the case for DN B-cells (**Figure 3A**).

Subsequently we investigated the residual plasma cell subsets based on their maturation stage and their expression of Ig isotypes after RTX (**Figure 3C**). Not unexpectedly, CD20⁺CD138[−] plasmablasts (PBs) were rapidly undetectable after RTX, while CD20[−]CD138[−] “Pre-PCs” and CD20[−]CD138⁺ mature PCs (mPCs) were reduced but remained detectable at all timepoints (**Figures 3C, D**). The distribution of IgA⁺, IgG⁺, and IgM⁺ plasma cell subsets was shown in **Supplementary Figure 8**. At baseline and after RTX, the most prevalent Ig subtype on PBs, PrePCs and mPCs was IgA.

Changes of Circulating ANCAs Associated With Changes of Naive and Memory B-Cells But Not With Plasma Cells After RTX

Because HSFC provided measurable, longitudinal changes of B- and plasma cell subsets in AAV patients after RTX, we investigated whether these changes reflected simultaneous changes of circulating Ig and ANCA levels. The absolute serum levels of IgM, IgG and IgA after RTX in patients that received RTX as remission-induction treatment are shown in **Supplementary Figure 9**. IgM levels decreased gradually without recovery after RTX in most patients to a median decrease of −54% [−61; +37] ($p = 0.31$) after 48 weeks (**Figure 4A**). Total serum IgG levels reached their nadir after 4 weeks, corresponding to a median decrease of −21% [−53; −13] ($p = 0.03$) (**Figure 4A**). Subsequently, IgG levels gradually recovered back to baseline levels at 48 weeks. Total serum IgA levels remained largely unaffected by RTX treatment, but

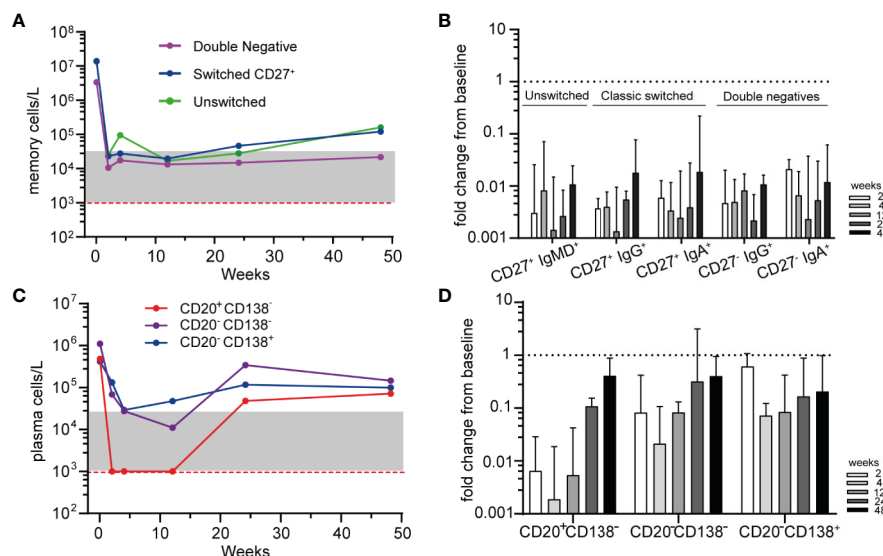


FIGURE 3 | Phenotyping residual memory and plasma cells after RTX (**A**). Median absolute counts of unswitched memory (CD27⁺IgM⁺ and/or IgD⁺), switched memory (CD27⁺IgG⁺ or IgA⁺) and double negative (DN, CD27⁺, IgG⁺, or IgA⁺) B-cells are shown for AAV patients ($n = 6$) after remission-induction treatment with RTX (**B**). Median \pm IQR fold change from baseline for different memory B-cell subsets are shown for each timepoint after RTX (**C**). Median absolute counts of PBs (CD20⁺CD138[−], red), PrePCs (CD20[−]CD138[−], purple) and mPCs (CD20[−]CD138⁺, blue) are shown for AAV patients ($n = 6$) after RTX as remission-induction therapy (**D**). Median [IQR] fold change from baseline for the subsets in A are shown for each timepoint after RTX. Red dashed line indicated the detection limit for HSFC. Gray area indicates 1–20 analyzed events.

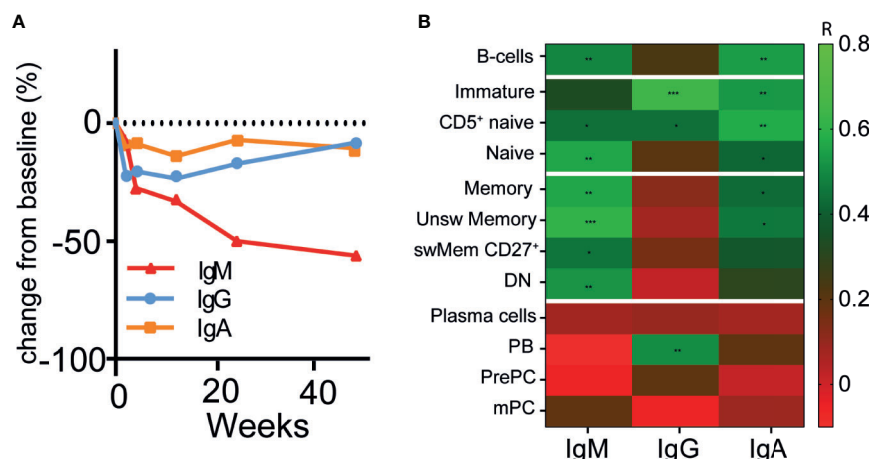


FIGURE 4 | Changes of circulating immunoglobulins were not reflected by plasma cell kinetics after RTX (A). Median percentage change in total immunoglobulin -M (red line), G (blue line) and -A (orange line) levels after RTX as remission-induction treatment over time (n = 6) (B). Heatmap of spearman correlations of the change in IgG, IgM, and IgA with the change in different B-cell subsets as compared to baseline for all timepoints (n = 6). Gradients indicates Spearman's R. *p < 0.05, **p < 0.01. ***p < 0.001.

decreased to a median change of -11% [-44; +40] as compared to baseline (Figure 4A). In AAV patients that received RTX maintenance treatment circulating Ig levels did not change much (Supplementary Figure 10).

Surprisingly, the changes in serum IgM, -G, and -A levels were not associated with the changes in the plasma cell compartment ($r < 0.2$) (Figure 4B), which was also the case for patients that received maintenance treatment with RTX (Supplementary Figure 10E). Moreover, changes in IgM levels were strongly associated with changes in unswitched memory B-cells ($r = 0.61$, $p = 0.001$) and naive B-cells ($r = 0.56$, $p = 0.002$) (Figure 4B). The changes in IgG levels were strongly associated with changes of the immature B-cell compartment ($r = 0.64$, $p = 0.0001$), CD5⁺ naive B-cells ($r = 0.44$, $p = 0.02$), and PBs ($r = 0.51$, $p = 0.006$). Changes in IgA levels were associated with changes of CD5⁺ naive B-cells ($r = 0.57$, $p = 0.002$) and unswitched memory B-cells ($r = 0.46$, $p = 0.01$).

Figure 5 illustrates the relative changes from baseline of serum anti-PR3-IgG and anti-MPO-IgG in patients treated with RTX as remission-induction and as maintenance treatment. Absolute values are shown in Supplementary Figure 11. All ANCA IgG, against PR3 or MPO significantly decreased for all patients that received remission-induction treatment with RTX (Figure 5A). ANCA IgG was less affected during maintenance treatment with RTX (Figure 5B). Altogether, changes in both anti-PR3 and anti-MPO IgG autoantibodies were strongly associated with changes of naive B-cells (resp. $r = 0.76$, $p = 0.0001$ and $r = 0.73$, $p = 0.0001$) and DN memory B-cells (resp. $r = 0.72$, $p = 0.0001$ and $r = 0.58$, $p = 0.008$) (Figure 5C). Surprisingly, but in line with the total Ig data, changes in ANCA did not associate with changes of plasma cell subsets except a weak association of changes in anti-PR3 IgG and mPCs ($r = 0.43$, $p = 0.02$) (Figure 5C). Of interest, changes in MPO-ANCA IgM and IgA autoantibody levels demonstrated

similar dynamics and correlations as MPO-ANCA IgG, which were significant correlations with the changes in naive B-cells (resp. $r = 0.76$, $p = 0.0001$ and $r = 0.75$, $p = 0.0001$) and DN memory B-cells (resp. $r = 0.58$, $p = 0.008$ and $r = 0.55$, $p = 0.01$), and no association with any of the plasma cell subsets (data not shown).

Minimal Residual Autoimmunity: Presence of ANCA-Specific Memory B-Cells After RTX

To ultimately demonstrate whether the observed residual memory B-cells with HSFC harbored ANCA-specific B-cells, we investigated *in vitro* ANCA production before and after RTX treatment. To do so, total PBMCs from AAV patients were polyclonally stimulated to induce antibody-secreting cells (ASCs) and subsequently (ANCA) IgG was measured in their supernatants as a reflection of ANCA-specific memory B-cells (Figure 6A). At baseline of the PBMC culture, the number of CD19⁺ B-cells for HCs was $61[56-74] \times 10^3$ /well out of 1×10^6 PBMCs/well, corresponding to normal range references values of HCs (~6%) (33) (Figure 6B). Both PR3- and MPO-ANCA AAV patient samples had significantly lower starting numbers of B-cells in the culture as compared to HCs, possibly due to previous immunosuppressive treatment (Figure 6B).

Polyclonal stimulation of PBMCs from HCs resulted in a median [range] of $70 [45-170] \times 10^3$ CD27⁺⁺CD38⁺⁺ ASCs per well after 7 days (Figure 6C). Polyclonal stimulation of PBMCs from PR3-ANCA and MPO-ANCA AAV patient samples before RTX treatment resulted in $0.17 [0.001-100] \times 10^3$ and $2.6 [1-61] \times 10^3$ ASCs (Figure 6C). After 10 days of culturing, PBMCs from HCs produced a median of $8.3 [2.7-20.7] \mu\text{g/ml}$ IgG, while $0.03 [0-6.1] \mu\text{g/ml}$ IgG by PR3-ANCA patient samples and $0.7 [0-7.3] \mu\text{g/ml}$ IgG by MPO-ANCA patient samples was detected

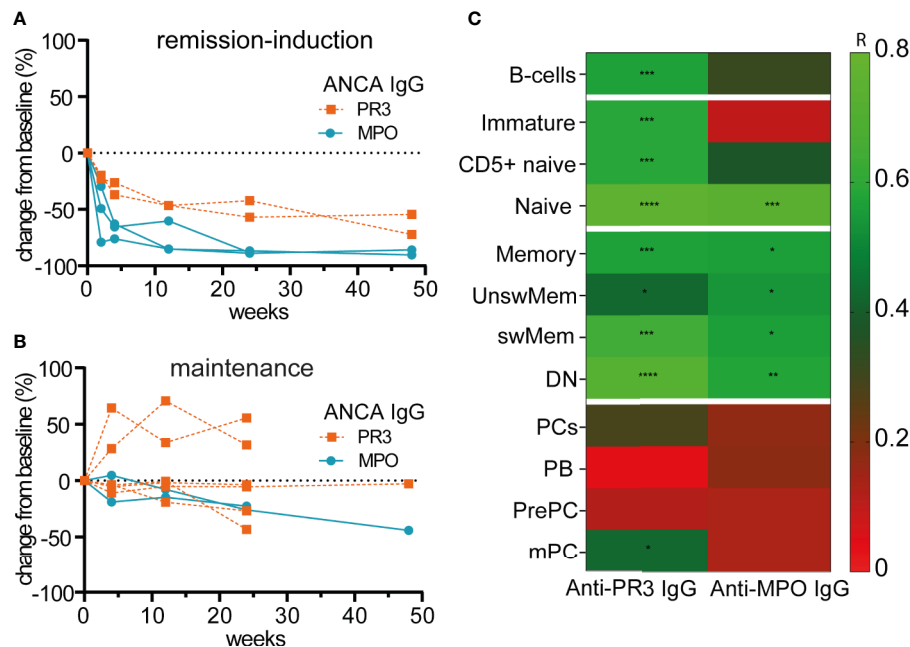


FIGURE 5 | Changes of circulating ANCA-associated changes of naive and memory B-cells but not with plasma cells after RTX (A). Individual percentage change as compared to baseline of anti-PR3 and anti-MPO IgG serum levels after remission-induction with RTX treatment ($n = 5$) (B). Individual percentage change as compared to baseline of anti-PR3 and anti-MPO IgG serum levels is shown for each patient that received RTX as maintenance treatment ($n = 8$) (C). Heatmap of the Spearman correlations of changes in anti-MPO IgG ($n = 6$) and anti-PR3 IgG ($n = 8$) with changes in B-cell subsets as compared to baseline for all timepoints ($n = 14$). Gradients indicates Spearman's R. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(Figure 6D). Importantly, relevant IgG levels could also be detected at 24 and 48 weeks after RTX. Most importantly, ANCA IgG production was detectable in 3/12 PR3-ANCA and 9/11 MPO-ANCA patient samples at baseline. At 24 weeks after RTX, ANCA IgG production was detectable in 4/12 PR3-ANCA and 7/9 MPO-ANCA patient samples and at 48 weeks after RTX in 0/2 PR3-ANCA and 3/4 MPO-ANCA patient samples (Figure 6E). Altogether, these PBMC cultures demonstrated that both PR3-ANCA and MPO-ANCA patients had residual ANCA-specific memory B-cells after RTX.

DISCUSSION

This study aimed to investigate MRA in the B-cell compartment of AAV patients after RTX. We demonstrated that despite significant reductions in circulating B-cell numbers after RTX, B-cells always remained detectable when employing Euroflow-based HSFC. Residual B-cells after RTX were predominantly memory B-cells and CD20⁺ plasma cells. Longitudinal changes in the plasma cell compartment were not associated with changes in serum ANCA levels. Within residual B-cells after RTX, we demonstrated the presence of ANCA-specific memory indicative of MRA in AAV patients. RTX is an effective treatment for AAV patients which is increasingly used both for remission-induction and maintenance treatment. Nevertheless, relapses are common after RTX, which are not always reliably

predicted through immunomonitoring of serum ANCAs and/or total circulating CD19⁺ B-cell numbers. Still, much evidence points toward the role of B-cells in the pathogenesis of relapses in AAV patients.

Euroflow-based HSFC always detected circulating CD19⁺ B-cells after RTX in any patient, demonstrating that B-cell depletion after RTX is never absolute and that it depends on the sensitivity of the flow cytometry method, which is at best 1×10^6 B-cells/L in routine clinical practice and large clinical studies (5, 34). However, it was previously demonstrated that after RTX, AAV patients with residual B-cells, either defined as $\geq 1 \times 10^6$ B-cells/L (16) or $\geq 0.1 \times 10^6$ B-cells/L (17) had worse clinical responses. It is also well described that patients can relapse with B-cells below the conventional threshold of flow cytometry (5, 35, 36). Additionally, the return of B-cells after RTX has also been recognized as a risk factor for relapse (16, 37), and successfully used as a biomarker to reduce RTX infusions (5). Moreover, different studies have shown that specific B-cell populations have a distinct role in AAV disease. The repopulation of naive B-cells after RTX at 6 months was associated with a reduced risk of relapse (17). Also regulatory B-cells (Breg) have been described as a key B-cell subgroup responsible for maintaining self-tolerance (38). Indeed, these Bregs, present among CD5⁺ B-cells inversely correlated with disease activity in AAV patients after RTX (20, 21). Recently, CD27⁺CD38⁺⁺ plasma cells were increased in patients at baseline that relapsed in the future (18). Altogether, these studies support

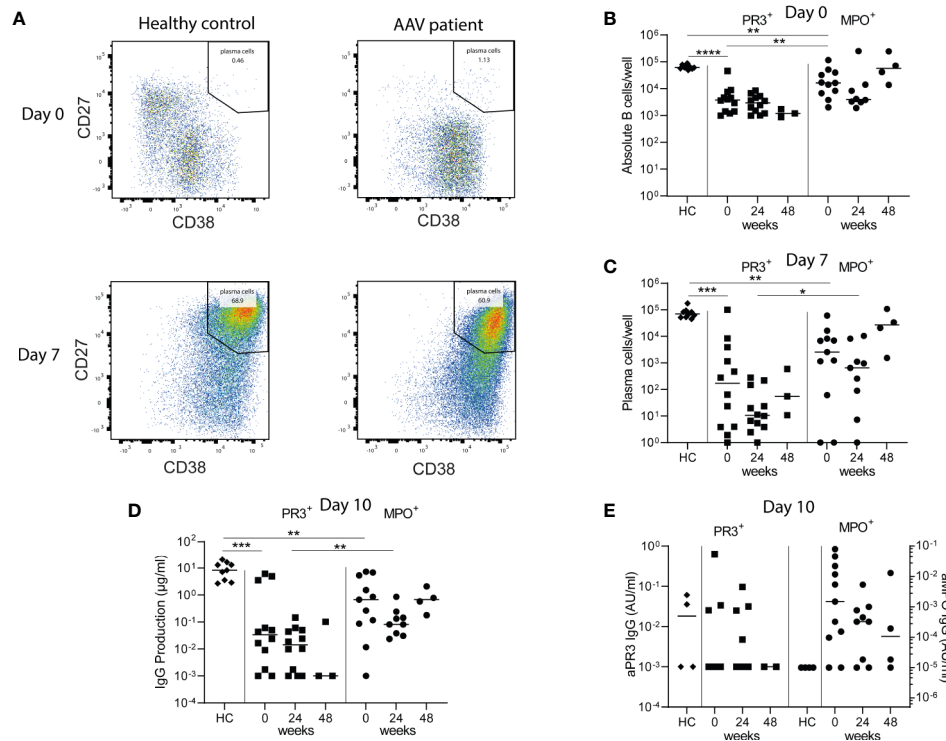


FIGURE 6 | Minimal residual autoimmunity after RTX: presence of ANCA-specific memory B-cells. 1×10^6 PBMCs/well from healthy controls (HCs) and AAV patients before, 24 and 48 weeks after RTX treatment, were stimulated for 10 days with CpG ODN class B, IL-2, and IL-21 to induce antibody-secreting cells (ASCs) in a 48-well plate (A). Representative bivariate dot plots of ASCs at day 0 and day 7 of PBMC cultures demonstrated the induction of CD27⁺CD38⁺ ASCs 7 days after polyclonal stimulation of PBMCs from a HC and an AAV patient (MPO-ANCA) (B). Absolute counts of total CD19⁺ B-cells were shown for each individual at baseline of the cultures (day 0) (C). Absolute counts of induced ASCs per well were shown for each individual after 7 days of culturing (D). Total IgG production was measured in the supernatants of each well after 10 days of culturing. Here the median of 5 wells is shown per individual (E). Total ANCA-IgG production was measured in the supernatants of each well after 10 days of culturing. Anti-PR3 IgG and anti-MPO IgG are, respectively, shown on the left and right y-axis. Each dot represents the median of 5 wells for each individual sample. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

the implementation of HSFC as an immunomonitoring tool in AAV patients. With HSFC, potential new biomarkers for RTX can be identified which are more closely associated to its B-cell depleting mechanism of action than, for example, serum ANCA levels. Therefore, further studies are needed to investigate the added value of HSFC to predict treatment efficacy and to guide personalized treatment strategies in AAV patients.

An interesting finding in our study was that the changes in ANCA levels closely related to reductions in naive, switched memory and DN B cells, but not plasma cells. There are reports that naive B-cells can contain antigen-experienced cells, including B-cells recognizing RhD and tetanus (39). Also, PR3⁺ B-cells were detected in the naive B-cell compartment in both HCs and PR3⁺ AAV patients previously (40). In this study, the feasibility of detecting PR3-specific B-cells by HSFC was demonstrated (40). There was no correlation between PR3-ANCA titer and % PR3⁺ B-cells, while PR3⁺ B-cells were phenotypically enriched in the switched memory B-cell and PB subsets. Whether PR3-specific B-cells can be detected within low B-cell levels after RTX remains to be demonstrated. We demonstrated here the proof-of-concept that indeed ANCA-specific memory B-cells persist after RTX.

Moreover the correlation of unswitched memory B-cells and serum IgM levels were consistent with a previous study (41). In this study marginal zone (MZ)-like B-cells (CD19⁺CD27⁺IgD⁺IgM^{high}, comparable with unswitched memory B-cells in our study), negatively correlated with MPO- and PR3-ANCA levels, whereas we found a positive significant correlation of the changes in MPO- and PR3-ANCA levels with the changes in unswitched memory B-cells. Possibly this is due by the setting of the study: we studied the changes in ANCAs and B-cells after RTX treatment in our study, whereas Appelgren et al. studied the correlations of absolute ANCA levels and MZ-like B-cells in active treatment naïve patients.

Another interesting finding in this study employing HSFC was the observation that RTX not only reduced CD20⁺ B cell subsets but was also associated with significant reductions in CD20⁺ prePCs and mPCs. This observation strongly suggested that these reductions were an indirect effect of RTX most likely explained by the strong decrease of their precursors and/or migration of CD20⁺ PCs out of the circulation while entry of their precursors lacked.

The present study had some limitations. First, the study was designed as a proof-of-concept study to investigate the potential

use of HSFC after RTX treatment. As such, the number of patients included in this study was too low to assess the value of HSFC as biomarker in relation to disease activity or relapses. To achieve this, the study population should also have been treated in a homogenous, standardized RTX-based treatment strategy. Secondly, it needs to be acknowledged that RTX is known to mask the CD20 epitope in competition with diagnostic antibodies used for flow cytometry (42). As such, CD20 expression up to 12 weeks after RTX needs to be interpreted with caution, notably the CD20⁺CD138⁻ PB and CD20⁻CD138⁻ PrePCs in our study. Thirdly, when investigating *in vitro* cultures of PBMC, the detection of ANCA in supernatants depends upon the frequency of ANCA-specific B-cells. Therefore, because B-cell numbers are low after RTX, we cannot firmly conclude that *in vitro* undetectable ANCA levels in the supernatant actually relate to the successful eradication of ANCA-specific B-cells.

In conclusion, this study demonstrates the presence of residual ANCA-specific memory B-cells in AAV patients after RTX. Moreover, EuroFlow-based HSFC demonstrated that RTX strongly reduced circulating B-cells but never fully depleted them. Residual B-cells consisted predominantly of memory B-cells and plasma cells. Changes in ANCA levels associated predominantly with changes in naive, switched or DN memory B-cells but not plasma cells. Altogether, we provide evidence for MRA in the memory B-cell compartment after RTX in AAV. Further studies are warranted to better assess MRA and its association with disease activity and relapses in AAV patients.

DATA AVAILABILITY STATEMENT

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

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ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CME LUMC LEIDEN. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LD, JO, SK, and EJA carried out the experiments, LD, MB, and JO analyzed the data. LD made the figures. LD, OWB, MB, JD, CK, and YT designed the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.566732/full#supplementary-material>

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Rituximab Induces Complete Remission of Proteinuria in a Patient With Minimal Change Disease and No Detectable B Cells

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Minimal change disease (MCD) is a common cause of nephrotic syndrome. Treatment with steroids is usually effective, but frequent relapses are therapeutic challenges. The anti-CD20 antibody rituximab has shown promising results for treatment of steroid-sensitive nephrotic syndrome. Since predictive biomarkers for treatment efficacy and the accurate rituximab dosage for effective induction of remission are unknown, measurement of CD19⁺ B cells in blood is often used as marker of successful B cell depletion and treatment efficacy. A male patient with relapsing MCD was successfully treated with rituximab, but developed relapse of proteinuria 1 year later, although no B cells were detectable in his blood. B and T cell populations in the patient's blood were analyzed before and after treatment with rituximab using FACS analysis. Rituximab binding to B and T cells were measured using Alexa Fluor 647 conjugated rituximab. We identified a population of CD20⁺ CD19⁻ cells in the patient's blood, which consisted mostly of CD20⁺ CD3⁺ T cells. Despite the absence of B cells in the blood, the patient was again treated with rituximab. He developed complete remission of proteinuria and depletion of CD20⁺ T cells. In a control patient with relapsing MCD initial treatment with rituximab led to depletion of both CD20⁺ B and T cells. Rituximab induces remission of proteinuria in patients with MCD even if circulating B cells are absent. CD20⁺ T cells may play a role in the pathogenesis of MCD and might be a promising treatment target in patients with MCD.

Keywords: minimal change disease, rituximab, CD20⁺ T cells, B cell depletion therapy, nephrotic syndrome

INTRODUCTION

Minimal change disease (MCD) is responsible for 10–25% of all cases of a nephrotic syndrome in adults (1). The exact pathomechanisms of MCD remain elusive. However, a circulating factor, most probably secreted by immune cells, is assumed to lead to effacement of podocyte foot processes, leakage of the glomerular filtration barrier, and development of a nephrotic syndrome (2). T cells have been suggested to contribute to the development of MCD (1). The role of the adaptive immune

system in MCD is also supported by the presence of functionally impaired regulatory T cells in these patients (3, 4). In addition, the association of reduced regulatory T cells in patients with relapse of the disease (5–7) and the altered transcription regulators reported in B and T cells of MCD patients (8) suggest a role of the adaptive immune system in this disease. This assumed immune-mediated pathogenesis of MCD is the rationale why patients with this disease are treated with immunosuppressive drugs (1, 9).

Steroids lead to remission of proteinuria in 75–80% of adult MCD patients, but relapses occur in up to 56–76% of all cases (1, 10–12). Frequent relapses, steroid-dependence, or steroid-resistance require repeated courses of treatment (9). Thereby, high doses and long-term steroid treatments are needed, frequently leading to adverse effects and toxicity (1, 13). Therefore, alternative immunosuppressive treatments are applied in these patients, including alkylating agents, calcineurin inhibitors, mycophenolate mofetil, and rituximab (1, 10). Rituximab is a mouse-human chimeric anti-CD20 antibody, which induces direct cell death, complement dependent cytotoxicity, and antibody-dependent cell-mediated cytotoxicity in CD20 expressing cells (14, 15). The membrane protein CD20 is a B cell marker and is expressed in human B cells at different stages of their development (16, 17). On the other side, CD20 is not expressed on human podocytes (18). Depletion of CD20 expressing cells using rituximab has shown promising results in the treatment of MCD (10, 13, 19, 20), leading to the hypothesis that B cells have a pathogenetic role in MCD. Nonetheless, the precise mechanisms of action of rituximab in the treatment of MCD are unknown (9, 21). Measurement of CD19⁺ B cells in the blood is used to assess successful B cell depletion, but stable remission has been observed in some patients despite reconstitution of CD19⁺ B cells (22). Recently, the reconstitution of memory B cells but not total CD19⁺ B cells has been shown to correlate with a shorter time to MCD relapse (21). Deciphering the role of both B and T cells in MCD is an ongoing challenge in the understanding of the pathomechanisms of MCD.

Here, we present the case of a patient with MCD, who developed relapses of proteinuria and was successfully treated with rituximab, despite having no detectable CD19⁺ B cells in the peripheral blood. CD20⁺ T cells, which were present in the blood of this patient prior to rituximab treatment and were depleted afterwards, might account for the therapeutic impact of rituximab in this patient.

MATERIAL AND METHODS

Diagnostic Laboratory Results

Proteinuria was quantified by photometric measurement of urinary excreted protein in 24 h urine samples and the urinary protein excretion rate (PER; g/24 h) was calculated. Nephrotic-range proteinuria was defined as PER >3.5 g/24 h according to the Kidney Disease: Improving Global Outcomes (KDIGO) nomenclature (23).

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

EDTA blood was obtained from the index MCD patient and a control MCD patient before and after rituximab treatment. Blood from a healthy donor was used as a control. PBMC were freshly isolated by Ficoll's protocol and stored in liquid nitrogen until further use. Briefly, EDTA blood was diluted 1:3 in PBS and layered upon Biocoll separating solution 10 mM HEPES (Biochrom, Berlin, Germany). After gradient centrifugation at 1,000 g for 25 min at 20°C, the PBMC layer was carefully removed and washed twice with cold PBS. Lysis of erythrocytes was performed by hypotonic shock. Afterwards PBMC were stored until further use in 10% DMSO (Sigma Aldrich, St. Louis, MO, USA), 30% FCS (Thermo Fisher Scientific, Waltham, MA, USA), and 60% RPMI1640 (Thermo Fisher Scientific) in liquid nitrogen. The study was approved by the local ethics committee of the chamber of physicians in Hamburg (PV4806) and conducted in accordance with the ethical principles stated by the Declaration of Helsinki. An informed consent was obtained from patients and the healthy donor prior to study inclusion.

Conjugation of Rituximab

Rituximab was conjugated to the fluorophore Alexa Fluor 647 using the Alexa Fluor 647 Protein Labeling Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 450 µl of rituximab (1.6 mg/ml) were incubated with 50 µl of AF647 reaction dye and 50 µl sodium bicarbonate (1 M) at room temperature for 70 min. Afterwards, matrix column purification was performed to separate excess fluorochrome from labelled rituximab molecules (RTX-AF647). RTX-AF647 was stored at 4°C until use. For all FACS experiments, RTX-AF647 was used at a final dilution of 1:100 in PBS.

FACS Analysis

All FACS measurements were conducted using a FACS Celesta (BD Biosciences, Franklin Lakes, NJ, USA). PBMC were thawed rapidly at 37°C. After washing with cold PBS, cells were counted using a Neubauer counting chamber and diluted to desired concentrations. For FACS analysis, PBMC were blocked with human serum for 30 min at 4°C and incubated with a pre-titrated antibody cocktail including RTX-AF647 as well as AF750 (Thermo Fisher Scientific), for live-dead staining. For FACS analysis, in each sample at least 200,000 cells were regularly acquired. If not otherwise indicated, all antibodies were obtained from BioLegend (San Diego, CA, USA). The following antibodies and fluorophores were used: V450 anti-CD27 (clone: M-T271), V500 anti-IgD (clone: IA6-2), BV650 anti-CD3 (clone: OKT3), BV785 anti-CD45 (clone: HI30), FITC anti-CD38 (clone: HIT2; BD Biosciences), PE anti-IgM (clone: MHM-88), PerCP anti-CD4 (clone: L200), PE-Cy7 anti-CD19 (clone: HIB19), AF700 anti-CD8a (clone: HIT8a). PBMC were washed twice and measured using a FACS Celesta (BD Biosciences). Graphical analysis was performed using FlowJo version 10.6.1 (FlowJo, Ashland, OR, USA). Of note, in contour plots not all cells are depicted as single dots. For gating strategy see supplemental information (**Figure S1**).

Histology and Immunohistochemistry

PAS staining and electron microscopy were performed following standard protocols. For immunohistochemical staining for IgG and CD20, 1–2 μ m thin slices from formalin-fixed, paraffin-embedded renal biopsies were deparaffinized and pretreated for 15 min at pH6 and 117°C in the autoclave (for CD20) or with proteinase (protease P-8038, Sigma-Aldrich, St. Louis, MO, USA) at 40°C for 15 min (for IgG) followed by incubation with normal serum (Vector S2000, CA, USA) for 10 min. Primary antibodies for IgG (1:7,500) (mouse monoclonal antibody, 209-005-088, Dianova, Hamburg, Germany) and CD20 (1:2,000) (DAKO M0755, CA, USA) were added for 30 min at 40°C. Bound antibodies were then visualized manually using a standard APAAP protocol.

RESULTS

Clinical Case

A 70-year-old male patient presented at our outpatient clinic with nephrotic syndrome. A kidney biopsy revealed the diagnosis MCD (**Figure 1**). Treatment with steroids resulted in complete remission of proteinuria. However, the patient developed frequent relapses. The first relapse appeared a few months after

the initial treatment, while steroids were being withdrawn. Because the patient also had diabetes mellitus type 2 and severe osteoporosis, re-treatment with steroids had to be avoided. He was alternatively treated with rituximab and developed complete remission of proteinuria. In the next 8 years the patient had eight relapses of the nephrotic syndrome, which were all successfully treated with rituximab and the patient went again into complete remission. One year after the last treatment with rituximab, the patient presented again in our outpatient clinic with a relapse of nephrotic syndrome and proteinuria of 8.3 g/24 h. Peripheral blood CD19⁺ B cells were still depleted with a cell count of 1 B cell/ μ l (normal range 80–500 B cells/ μ l). Serum creatinine and total leukocyte count in the peripheral blood were within the normal range (1.1 mg/dl and 4,500 cells/ μ l, respectively). The patient was treated with 1 g rituximab, combined with 60 mg/day prednisolone for 3 days, which was tapered over a time period of 4 weeks, leading to a complete remission of proteinuria within 21 days (proteinuria 0.3 g/24 h).

Characterization of Peripheral Blood Cells Bound by Rituximab

Because of the effect of rituximab treatment on proteinuria in the absence of CD19⁺ B cells in the blood, we aimed to better understand the potential underlying mechanism how rituximab unfolds its effect in this patient. We hypothesized

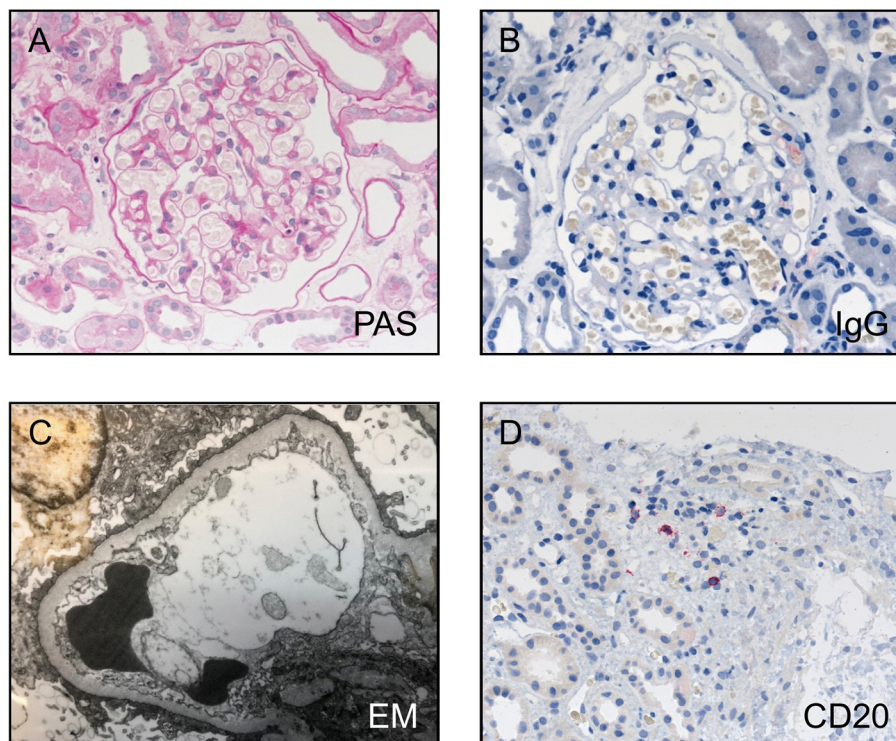


FIGURE 1 | Histological findings in the kidney biopsy of the patient. (A) PAS staining, (B) IgG staining, and (C) electron microscopy confirm the diagnosis of minimal change disease with no IgG positivity, no electron dense deposits, and diffuse loss of podocyte foot processes. (D) Only very few CD20 positive cells were detectable, mostly in the area of tubular atrophy and interstitial fibrosis.

that rituximab targets and depletes CD19⁺ circulating blood cells. Therefore, we first assessed rituximab binding to CD19⁺ blood cells. For this purpose, rituximab was conjugated to the fluorophore AF647 (RTX-AF647) and used to stain circulating CD20⁺ cells. Staining of peripheral CD45⁺ lymphocytes from a 27-year-old male healthy donor with RTX-AF647 led to the identification of CD19⁺ B cells (blue) and a population of CD19⁺ CD20⁺ cells (**Figure 2A**, left panel). A majority (59.6%) of CD19⁺ CD20⁺ cells expressed the T cell receptor associated marker CD3 on the surface. These cells were considered a T cell population (red; **Figure 2A**, right panel) and showed a similar CD3 expression as RTX-AF647⁺ T cells (green), but were enriched for CD8⁺ T cells (**Figure 2B**). RTX-AF647⁺ T cells did not express B cell markers, such as CD19 and surface IgD (**Figure 2B**). CD27 expression was present in 76.1% of RTX-AF647⁺ T cells and 78.5% of RTX-AF647⁺ CD3⁺ T cells, respectively (**Figure 2B**).

Rituximab Treatment Depletes RTX-AF647⁺ T Cells

Next, we analyzed the B and T cell populations in PBMC from the index MCD patient before and after rituximab treatment by flow cytometry. Interestingly, only CD19⁺ RTX-AF647⁺ cells and no CD19⁺ B cells were detected in the blood of the index MCD patient prior to rituximab treatment (**Figure 3A**, left, **Figures S1**

and **S2A**). These CD19⁺ RTX-AF647⁺ cells consisted mostly of CD3⁺ T cells (64.9% of RTX-AF647⁺ CD19⁺ cells; **Figure 3A**, right panel). The RTX-AF647⁺ T cell population in the blood of the patient consisted of 55.1% CD4⁺ T cells and 30.6% CD8⁺ T cells (**Figure 3B**), and was therefore enriched for CD8⁺ T cells compared to the total circulating RTX-AF647⁺ T cell population in the same sample, which consisted of 68.4% CD4⁺ T cells and 11.4% CD8⁺ T cells (**Figure 3C**). Noteworthy, in healthy controls, CD4⁺ T cells and CD8⁺ T cells account for 23–52% and 13–40% of the circulating lymphocytes respectively (24). Double negative T cells were found in a lower frequency of 8.16% of RTX-AF647⁺ T cells (**Figure 3B**), while they comprised 15.4% of total RTX-AF647⁺ T cells (**Figure 3C**).

Before rituximab treatment, 0.12% of CD3⁺ T cells of the index MCD patient were bound by RTX-AF647 (**Figure 4A**, left panel). After rituximab treatment, the frequency of RTX-AF647⁺ T cells was reduced by 91.7 to 0.01% (**Figure 4A**, right panel).

Next, we aimed to confirm that rituximab treatment leads to depletion of a subpopulation of CD3⁺ T cells in a control patient with relapsing MCD. We performed an identical FACS analysis in a second patient with MCD (control MCD patient). This patient was a 43-year-old female, who was treated for the first time with rituximab. She had both circulating B and T cells prior to rituximab treatment (**Figure S2B**). In the blood of the control MCD patient, we detected RTX-AF647⁺ T cells at a frequency of

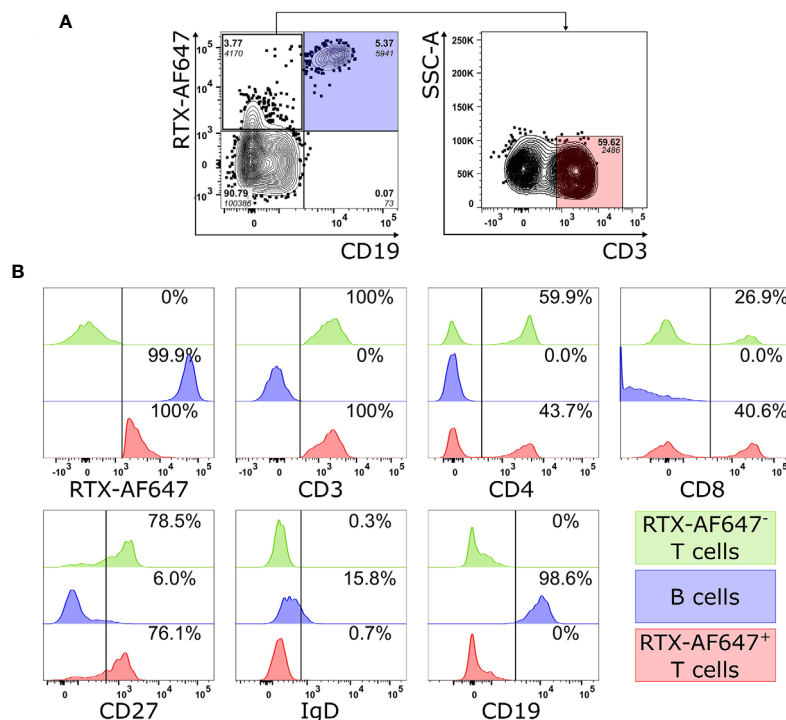
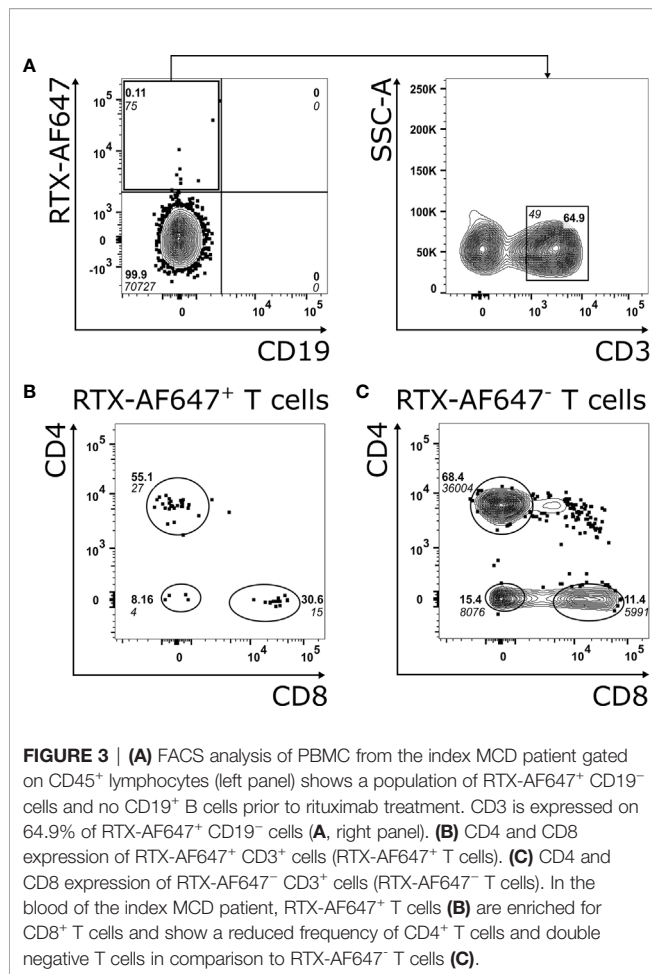


FIGURE 2 | (A) FACS analysis of PBMC from a healthy donor gated on CD45⁺ lymphocytes (left panel) shows a population of CD19⁺ RTX-AF647⁺ cells, 59.6% of which expresses the T cell marker CD3 (right panel). **(B)** The expression patterns of RTX-AF647, CD3, CD4, CD8, CD27, CD19, and IgD were assessed in equal counts of CD19⁺ B cells (blue), RTX-AF647⁺ CD3⁺ cells (RTX-AF647⁺ T cells, red), and RTX-AF647⁻ CD3⁺ cells (RTX-AF647⁻ T cells, green). RTX-AF647⁺ T cells (red) express CD3, CD4, CD8, and CD27 at a similar fluorescence intensity as RTX-AF647⁻ T cells (green) and lack the expression of the B cell markers CD19 and surface IgD. For gating strategy see supplemental data (**Figure S1**). Event counts for each gate are indicated in italic below the frequency (bold).



1.67% of CD3⁺ T cells prior to rituximab treatment (**Figure 4B**, left panel). After rituximab treatment of the control MCD patient, the frequency of RTX-AF647⁺ T cells was reduced by 70.7 to 0.49% (**Figure 4B**, right panel). Similar findings were made when RTX-AF647⁺ CD19⁻ cells were analyzed. Their frequencies decreased after rituximab from 0.11 to 0.01% in the index MCD patient (**Figure S2A**) and from 1.31 to 0.21% in the control MCD patient (**Figure S2B**), respectively. In the control MCD patient, CD19⁺ B cells were also depleted to a non-detectable level following rituximab treatment, while as shown before, in the index MCD patient CD19⁺ B cells were non-detectable even prior to rituximab treatment (**Figure S2**).

The frequency of CD3⁺ T cells did not significantly change in both the index MCD patient and the control MCD patient after rituximab treatment (**Figure 4C**). The frequency of RTX-AF647⁺ T cells was reduced by 91.7 and 70.7% in the index MCD and control MCD patient, respectively (**Figure 4D**). We further characterized the RTX-AF647⁺ T cell subsets before and after rituximab treatment in both patients. In the index MCD patient CD4⁺ RTX-AF647⁺ T cells were reduced by 91.2% (**Figure 4E**) and CD8⁺ RTX-AF647⁺ T cells were completely depleted (**Figure 4F**). In comparison, in the control MCD patient CD4⁺ RTX-

AF647⁺ T cells were reduced by 63.9% and CD8⁺ RTX-AF647⁺ T cells by 88.5% after rituximab treatment.

DISCUSSION

Frequent relapsing and steroid-dependent MCD patients represent a therapeutic challenge. This is due to the toxicity of long-term steroid treatment. Rituximab is a treatment option for these patients, however, biomarkers to guide therapy are lacking. The B cell marker CD19 is commonly used to predict whether a B cell depleting therapy is a rationale in treating patients with autoimmune kidney diseases. Despite showing no CD19⁺ B cells circulating in the blood, the presented index MCD patient developed a relapse of nephrotic syndrome. Moreover, he developed a complete remission of proteinuria within 3 weeks of treatment with rituximab and steroids. Remission of proteinuria lasted almost 1 year, similar to the remission phases during the last 8 years of treatment with rituximab. As CD19 and not CD20 is commonly used as a B cell marker to follow the depletion of B cells after rituximab, we investigated whether CD19⁻ CD20⁺ cells were present in the patient's blood, which could be targeted by rituximab and might play a role for disease induction. We were able to characterize a population of CD19⁻ RTX-AF647⁺ cells in the blood of the patient, which consisted mostly of CD3⁺ T cells.

After rituximab treatment, proteinuria resolved and CD20⁺ T cells were depleted. There are several potential explanations for the relapse of the nephrotic syndrome in the absence of B cells and the successful induction of remission following rituximab treatment. Firstly, pathogenic B cells could have reconstituted without being detectable in the patient's circulation. Such CD20 positive cells may be resident in the tissue and thus are not detected in the blood. The depletion of these potentially pathogenic B cells may have contributed to the remission of proteinuria. Relapses of nephrotic syndrome have been shown to be associated with reconstitution of memory B cells, which reappear only after naïve B cells are detectable in the circulation (21). In our patient, however, neither naïve nor memory B cells were detected in the blood at the time of relapse. Therefore, cryptic reconstitution of memory B cells seems unlikely. Secondly, B cells could have reconstituted but escaped detection due to technical limitations. However, CD19⁺ B cells were detected successfully in the blood of the control MCD patient and a healthy control using our method of detection. Thirdly, RTX-AF647⁺ T cells could play a role in the relapse of a nephrotic syndrome and the depletion of these CD20⁺ T cells following rituximab treatment may be a relevant therapeutic mechanism.

Contrary to B cells, circulating CD20⁺ T cells were detected at the time of relapse in a low frequency (0.12% of T cells) in the blood of the index MCD patient. In the blood of healthy controls, the mean frequency of CD20⁺ T cells usually varies between 1.6 and 3.8% of CD3⁺ T cells (25, 26). This shows that in our index MCD patient CD20⁺ T cells had already reconstituted after the

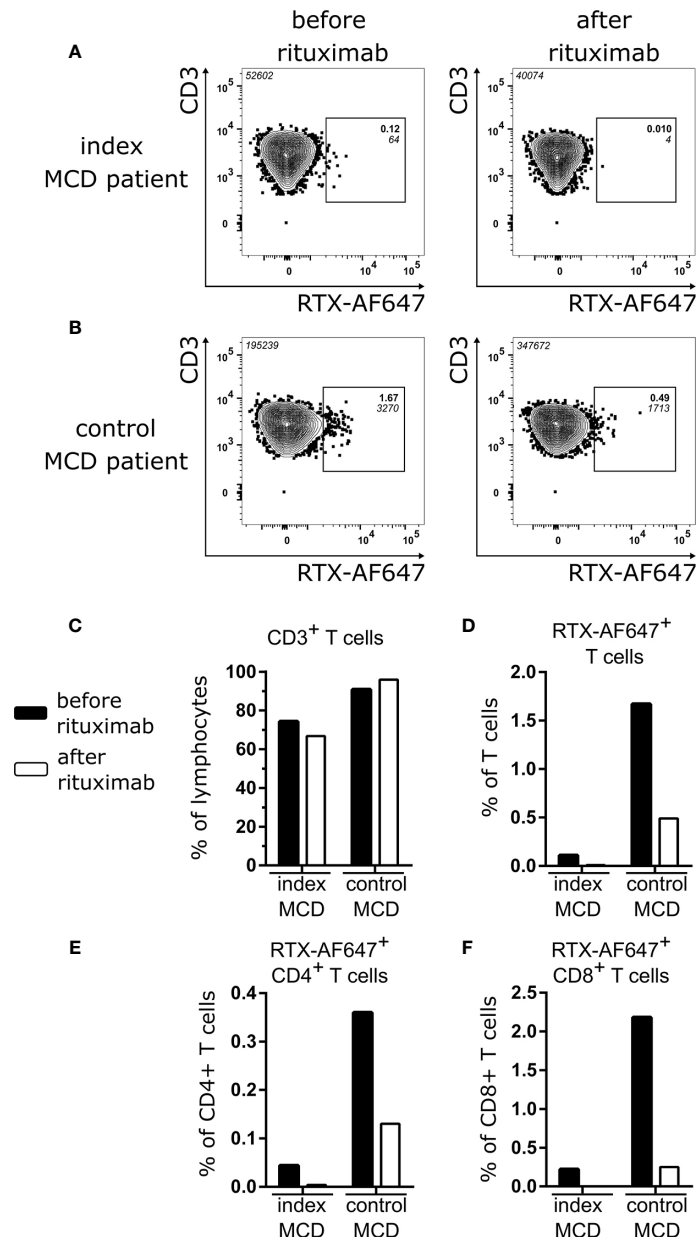


FIGURE 4 | RTX-AF647⁺ CD3⁺ T cells of the index MCD patient (A) and the control MCD patient (B) before (left panel) and after (right panel) rituximab treatment. (C) Frequency of CD3⁺ T cells as percentage of lymphocytes and (D) frequency of RTX-AF647⁺ cells among CD3⁺ T cells, (E) CD4⁺ T cells, and (F) CD8⁺ T cells before (black) and after (white) rituximab treatment in the index MCD patient and the control MCD patient.

previous rituximab treatment, in contrast to B cells. As RTX-AF647⁺ T cells were depleted after rituximab treatment in both the control MCD patient as well as the index MCD patient, we conclude that rituximab binds specifically to CD20⁺ T cells and depletes them *in vivo*.

MCD has been considered a T cell mediated disease (27, 28), while the role of B cells remains unclear (21). Nonetheless, CD20 targeted antibody therapies have shown promising results in the

treatment of relapsing MCD (7, 10, 19, 29, 30). The removal of autoreactive T cells has been proposed as a therapeutic mechanism of rituximab (31) and treatment of MCD patients with rituximab leads to the depletion of CD4⁺ CD45RO⁺ CXCR5⁺ T cells, invariant natural killer T cells and CD4[−] CD8[−] T cells (7, 32). The depletion of CD20⁺ T cells may represent a link connecting the successful application of anti-CD20 antibody treatment and the role of T cells in the pathogenesis of MCD.

Whether T cells are capable of expressing CD20 has been controversial since the first description of CD3⁺ T cells co-expressing CD20 (33). Several studies were able to show the expression of CD20 in T cells on a single cell level by imaging flow cytometry (34, 35), confocal microscopy (36), and immunohistochemistry (37). Together with the endogenous transcription of CD20 mRNA in CD20⁺ T cells (25, 26, 34, 35), this supports the view of endogenous synthesis of CD20 in a subset of T cells. Accordingly, CD20 antibody treatment depletes CD20⁺ T cells in the blood along with B cells (25, 26, 34–36, 38–42). CD20 expressing T cells have been described in several autoimmune diseases, e.g. multiple sclerosis (25, 35, 39, 41, 42), rheumatoid arthritis (26, 36, 43), primary Sjögren's syndrome (38), and psoriasis (40), as well as HIV infection (34). Our patient is the first case where these cells are reported in an immune-mediated nephrotic glomerular disease.

Clinical data describing the role of CD20⁺ T cells in autoimmune disease are scarce. In patients with psoriasis, frequency of circulating CD20⁺ T cells producing IL-17, IL-21, and TNF α correlates with disease activity (40). Recently, it has been suggested, that CD20⁺ T cells contribute to the pathogenesis of multiple sclerosis and depletion of CD20⁺ T cells may play a role in the therapeutic mechanism of anti-CD20 antibody treatment (42, 44, 45). In patients with relapse of multiple sclerosis, it was shown that after anti-CD20 antibody treatment B cells were hardly detectable in the blood at a time when CD20⁺ T cells were already replenished (25). This finding has similarities to our index MCD patient, who showed reconstitution of CD20⁺ T cells while B cells were still depleted at the time when proteinuria relapsed. Other studies have also shown that CD20⁺ T cells reconstitute months before B cells reappear in the circulation after rituximab treatment (25, 35).

This is the first report describing CD20⁺ T cells in the context of a frequent relapsing nephrotic syndrome in a patient with MCD. Rituximab combined with a short steroid treatment induced remission of proteinuria despite undetectable B cells. We found CD20⁺ T cells in the blood of the patient at the time of relapse, which were depleted after re-treatment with rituximab. Rituximab successfully induced complete remission of proteinuria despite of the absence of CD19⁺ B cells. Patients with relapse of nephrotic syndrome may benefit from rituximab treatment irrespective of the frequency of CD19⁺ B cells. Therefore, low or absent CD19⁺ B cells in the blood might not be used as disconfirming evidence for the effectivity of rituximab for relapse of nephrotic syndrome. More studies are needed to decipher the role of CD20⁺ T cells in autoimmune kidney diseases. This may help to guide clinical decisions for the use of CD20 antibody treatment in kidney autoimmune diseases and may lead to a better understanding of the pathogenesis of MCD.

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DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Written informed consent was obtained from the individuals for the publication of images and data included in this article.

AUTHOR CONTRIBUTIONS

EH, SH, and RS were responsible for the conception and design of the study. MW and LR performed the experiments. H-WM and MW developed the FACS strategy. EH, H-WM, and MW interpreted the FACS data. EH and SH analyzed and interpreted the clinical data. TW analyzed and interpreted the morphological and immunohistochemical findings. Drafting and revising of the article were performed by MW, EH, and SH. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Pathogenicity of Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody in Granulomatosis With Polyangiitis: Implications as Biomarker and Future Therapies

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Granulomatosis with polyangiitis (GPA) is a rare but serious necrotizing auto-immune vasculitis. GPA is mostly associated with the presence of Anti-Neutrophil Cytoplasmic Antibody (ANCA) targeting proteinase 3 (PR3-ANCA), a serine protease contained in neutrophil granules but also exposed at the membrane. PR3-ANCAs have a proven fundamental role in GPA: they bind neutrophils allowing their auto-immune activation responsible for vasculitis lesions. PR3-ANCAs bind neutrophil surface on the one hand by their Fab binding PR3 and on the other by their Fc binding Fc gamma receptors. Despite current therapies, GPA is still a serious disease with an important mortality and a high risk of relapse. Furthermore, although PR3-ANCAs are a consistent biomarker for GPA diagnosis, relapse management currently based on their level is inconsistent. Indeed, PR3-ANCA level is not correlated with disease activity in 25% of patients suggesting that not all PR3-ANCAs are pathogenic. Therefore, the development of new biomarkers to evaluate disease activity and predict relapse and new therapies is necessary. Understanding factors influencing PR3-ANCA pathogenicity, *i.e.* their potential to induce auto-immune activation of neutrophils, offers interesting perspectives in order to improve GPA management. Most relevant factors influencing PR3-ANCA pathogenicity are involved in their interaction with neutrophils: level of PR3 autoantigen at neutrophil surface, epitope of PR3 recognized by PR3-ANCA, isotype and glycosylation of PR3-ANCA. We detailed in this review the advances in understanding these factors influencing PR3-ANCA pathogenicity in order to use them as biomarkers and develop new therapies in GPA as part of a personalized approach.

Keywords: anti-neutrophil cytoplasmic antibodies, proteinase 3, granulomatosis with polyangiitis, pathogenicity, human neutrophils, biomarkers, new therapies

HIGHLIGHTS

- Despite recent advances, GPA is still a serious disease with a high risk of relapse which is inconsistently predicted based on PR3-ANCA level alone.
- PR3-ANCAs have a pathogenic role in GPA: their binding with neutrophils by their Fab (on membrane-bound PR3) and Fc fragments (on FcγR) leads to auto-immune activation of neutrophils.
- Correlation between disease activity and circulating PR3-ANCA level is inconsistent suggesting that not all PR3-ANCAs are pathogenic.
- Different factors (paratope and glycosylation) influencing PR3-ANCA pathogenicity need to be taken into account to develop new biomarkers and therapies.

INTRODUCTION

Granulomatosis with polyangiitis (GPA), formerly known as Wegener's disease, is a form of necrotizing auto-immune vasculitis affecting predominantly small to medium vessels with histological inflammatory lesions and granulomas (1). GPA is relatively rare with an incidence rate of 10 to 20 new cases per million inhabitants per year and a prevalence between 120 and 140 cases per million inhabitants in Europe and the United States (2). This prevalence continues to increase (3). Its manifestations vary but mainly develop in the ear–nose–throat region (ENT), in the lungs and kidneys resulting in a necrotizing sinusitis, pulmonary capillaritis sometimes with alveolar hemorrhage and glomerulonephritis, all of which can be severe (2).

In vasculitis nomenclature, GPA is part of a group of anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV), along with eosinophilic granulomatosis with polyangiitis (EGPA) and microscopic polyangiitis (MPA) (1). AAVs are classified by the antigen recognized by ANCA: either proteinase 3 (PR3-ANCA) or myeloperoxidase (MPO-ANCA) (4). PR3 is a neutrophilic serine protease contained in neutrophil granules but also exposed at the membrane (5). The high-quality immunoassays are now used as the primary screening method for patients suspected of AAV, and immunofluorescence has been abandoned (4). Concerning GPA, PR3-ANCAs are found in about three quarters of patients and appear to be very specific (specificity > 90%) (6–8). In contrast, only 10% of GPA patients have MPO-ANCA, and less than 10% have no detectable ANCA (6). PR3-ANCA can be found in other conditions than AAV (9). Furthermore, PR3-ANCA can be found in healthy people (10, 11). But these natural antibodies to PR3 are only detected when samples are prepared (10). However, PR3-ANCAs have a direct pathogenic role in the disease. Indeed, PR3-ANCAs bind neutrophils allowing their auto-immune activation which is responsible for vasculitis lesions in GPA (12, 13).

According to recommendations from the European League Against Rheumatism (EULAR), the current initial management of patients with GPA involves the use of long-term immunosuppressive

therapy, such as glucocorticoids, cyclophosphamide and, more recently, monoclonal antibodies as anti-CD20 (14). The total duration of these treatments after obtaining remission is at least two years (14). Despite treatment, GPA is a serious disease with an important mortality rate of 21.5% at five years when renal involvement is present (15), a significant morbidity related to the disease and its management (16) and a high risk of relapse of 30 to 50% within five years (17). Furthermore, the correlation between PR3-ANCA level, currently used for relapse management, and disease activity is inconsistent in the literature (18–23) except to predict relapse in patients with renal involvement (24, 25) or following treatment with rituximab (26, 27). Moreover, PR3-ANCA can persist in GPA patients during remission without predicting relapse (19, 22). Therefore, the development of new biomarkers to evaluate disease activity and predict relapse and new therapies is necessary.

PR3-ANCAs have a fundamental role in GPA by inducing auto-immune activation of neutrophils (12, 13). Therefore, understanding factors influencing PR3-ANCA pathogenicity, *i.e.* their potential to induce auto-immune activation of neutrophils, is necessary to develop new biomarkers to improve prediction of relapse and to develop new more specific therapies.

The aim of this review is to provide an overview of the advances in the understanding of the pathogenicity of PR3-ANCA in order to exploit them to develop new biomarkers and therapies in GPA. First, the importance of PR3-ANCA and neutrophils in the pathophysiology of the disease is discussed, then factors involved in the mechanism of auto-immune activation of neutrophils by PR3-ANCA are detailed and finally the understanding of these factors is examined to highlight avenues for the development of new biomarkers and therapies.

GRANULOMATOSIS WITH POLYANGIITIS PATHOPHYSIOLOGY: ARGUMENTS FOR A PATHOGENIC ROLE OF PROTEINASE 3-ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY

The role of PR3-ANCA in the pathophysiology of GPA has been studied widely since their discovery. In 1982, it was demonstrated that autoantibodies in sera of patients with segmental necrotizing glomerulonephritis stained the cytoplasm of neutrophils, later to be named ANCA (28). ANCAs have been then described for the first time in GPA patients in 1985 (29). Five years later, the proteinase 3 was identified as the ANCA antigen in GPA patients (30). The following section details clinical, *in vitro* and *in vivo* arguments supporting PR3-ANCA pathogenicity in GPA.

Clinical Arguments of Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody Pathogenicity

The first argument supporting the importance of PR3-ANCA in the pathogenicity of AAV is that classification of patients according to their ANCA specificity seems more relevant than the clinical presentation. Indeed, the presence of PR3-ANCA or

MPO-ANCA correlates better with genetic factors, prognosis, and response to treatment than the clinical phenotype of GPA or MPA (31–36). This underlines the importance of the antigen recognized by ANCA (PR3 or MPO) in the disease and highlights the concept of PR3-AAV and MPO-AAV (31). A second argument, developed below, is the frequent and regular, but not strictly correlated fluctuations of PR3-ANCA levels with the activity of the disease (18–27). Furthermore, the efficacy of current therapies seeking to eliminate these auto-antibodies, such as plasma exchange, although recently challenged, and anti-CD20 therapy, supports the importance of PR3-ANCA in GPA (14, 26, 37, 38). Finally, a key indirect argument of PR3-ANCA pathogenicity is the fundamental role in the disease of neutrophils, their target cells: activated neutrophils are found in inflammatory tissue, vessel samples, and in the circulation of GPA patients (13, 39, 40).

In Vitro Arguments of Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody Pathogenicity

The most widely accepted pathophysiological hypothesis in GPA, although contested by some authors (41), assumes the central role of PR3-ANCA and neutrophils.

PR3-ANCAs are initially produced by B lymphocytes pointing to a loss of tolerance to PR3. Several hypotheses have been put forward to explain this acquired loss of tolerance. The first is that PR3 contained in neutrophil extracellular traps (NETs) during inflammatory responses is exposed to the immune system (39). This hypothesis is supported by the finding that neutrophils from GPA patients are more likely to produce NETs and tend to have a lower DNase I activity leading to a lack of clearance of NETs. Consequently, PR3 is exposed to antigen presenting cells on extracellular immune enhancing components of neutrophils, thereby breaking self-tolerance (39, 42, 43). The second hypothesis postulates that a deficiency in clearance of apoptotic neutrophils overexpressing membrane-bound PR3 (mbPR3) maintains a prolonged state of inflammation favoring the generation of anti-PR3 antibodies (44, 45). Indeed, the overexpression of mbPR3 inhibits efferocytosis, a mechanism involved in the elimination of apoptotic cells by M2 macrophages during the resolution phase of inflammation (44). The third hypothesis is that the production of autoantibodies is triggered during the course of an immune response against *Staphylococcus aureus* which is associated with GPA (46) or another pathogen (47). Indeed, it has been shown that some patients with PR3-ANCA also had antibodies directed against a peptide translated from the antisense DNA strand of PR3 (complementary PR3, cPR3) which included sequences from *Staphylococcus aureus*. In this study, immunization of mice with the middle region of cPR3 induced antibodies against cPR3 but also PR3, showing that auto-immunity can be initiated through an immune response against a peptide that is antisense or complementary to the autoantigen, which subsequently induces anti-idiotypic antibodies (48). Another argument for the triggering of auto-immunity by *Staphylococcus aureus* is its strong capacity to induce NET production by neutrophils (42). Hyper-reaction to influenza vaccine was also

hypothesized in a case report to contribute to the development of AAV (49).

Once generated, PR3-ANCA binds to partially activated neutrophils primed *e.g.* by TNF alpha (TNF α) (50) and causes their excessive auto-immune activation responsible for vasculitis lesions (12, 13, 51). The widely accepted hypothesis of auto-immune activation of neutrophils by IgG ANCA was recently challenged by Popat and Robson (52). In their study, purified IgG from AAV patient sera, even in active disease, did not induce neutrophil activation. However, all experiments were performed on neutrophils obtained from only two healthy donors (52). Interestingly, in another study performed by the same group, the same IgG preparations obtained from MPO-ANCA positive patients promoted inflammation through monocyte stimulation but IgG preparations obtained from PR3-ANCA positive patients were not tested (53). Furthermore, excessive NET formation in AAV has been shown to be independent of the presence of ANCA while correlating with disease activity (54). Whatever, most of results found in literature are in favor of an auto-immune activation of neutrophils by PR3-ANCA.

There are two types of interaction between PR3-ANCA, mainly involving IgG, and neutrophils: one includes a link between the PR3-ANCA Fab (fragment antigen binding) and mbPR3 exposed on the surface of neutrophils and the other involves a link between the Fc (fragment crystallizable) of PR3-ANCA and Fc gamma receptors (Fc γ R) (55, 56) (**Figure 1**). *In vitro* auto-immune activation of neutrophils by PR3-ANCA has been studied with human purified polyclonal PR3-ANCA or murine/chimeric anti-PR3 mAbs *in vitro* but never, to our knowledge, with human anti-PR3 mAb. Nevertheless, *in vitro* auto-immune activation of neutrophils by PR3-ANCA results in an adherent phenotype (57), induction of NETosis (39, 43), production of intra- and extra-cellular reactive oxygen species (ROS) (58–60), degranulation with protease release (PR3, elastase, cathepsin G) (58), actin polymerization in a calcium-dependent manner (55), and production of pro-inflammatory cytokines, in particular IL-8 (61, 62). The excessive release of all these mediators leads to a damage of the vascular endothelium and, therefore, to vasculitis lesions (63). This release of these mediators moreover triggers a vicious circle: NETosis contributes to tolerance failure, leads to PR3 exposure to extracellular DNA and activates the alternative pathway of the complement system (39, 42). In addition, IL-8 production and vascular damage enhance the recruitment of other inflammatory cells towards the inflammatory site (62).

In Vivo Arguments of Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody Pathogenicity From Murine Models

In vivo ANCA pathogenicity was demonstrated more consistently with MPO-ANCA than PR3-ANCA (64). The study of PR3-ANCA *in vivo* in a murine model is difficult and complex. Indeed, the hydrophobic patch allowing human PR3 exposure on the neutrophil surface is lacking in murine PR3, and therefore murine PR3 is not expressed on the neutrophil surface (65). Furthermore, murine and human PR3 are only 63% homologous (65). To overcome these differences, a research team generated a

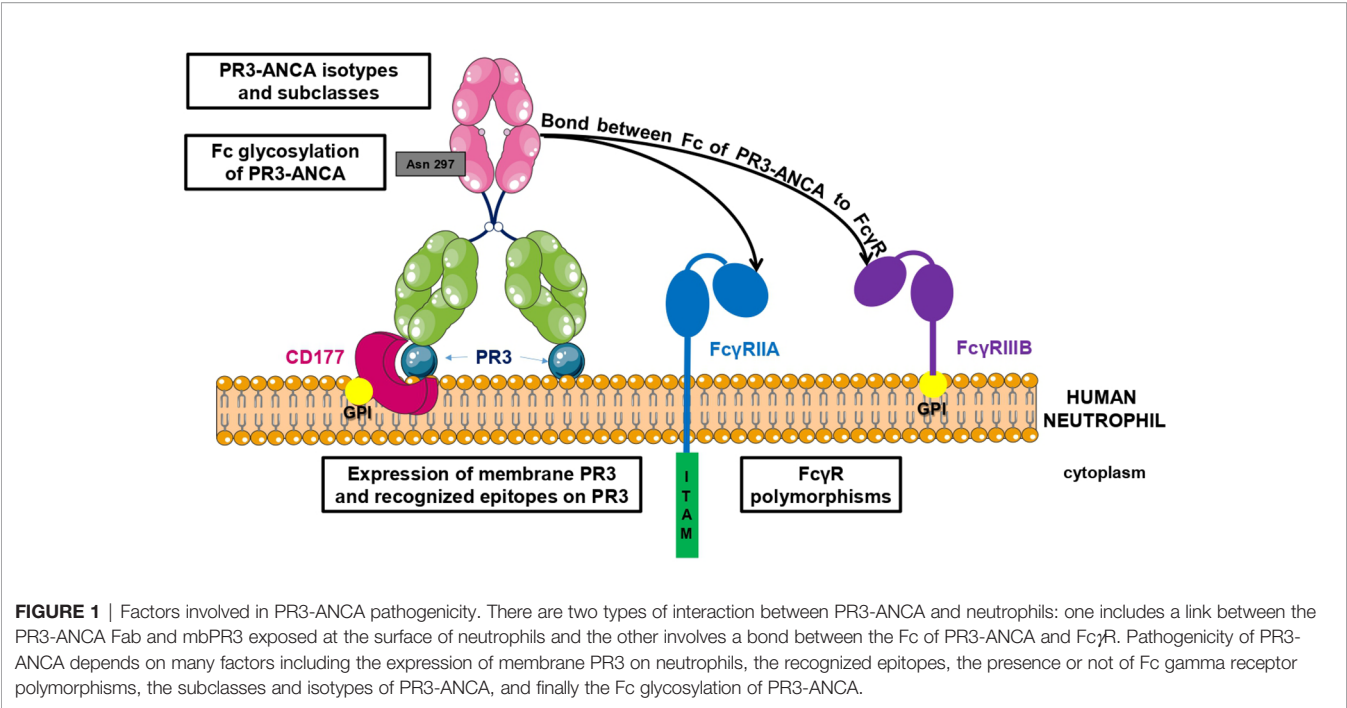


FIGURE 1 | Factors involved in PR3-ANCA pathogenicity. There are two types of interaction between PR3-ANCA and neutrophils: one includes a link between the PR3-ANCA Fab and mbPR3 exposed at the surface of neutrophils and the other involves a bond between the Fc of PR3-ANCA and FcγR. Pathogenicity of PR3-ANCA depends on many factors including the expression of membrane PR3 on neutrophils, the recognized epitopes, the presence or not of Fc gamma receptor polymorphisms, the subclasses and isotypes of PR3-ANCA, and finally the Fc glycosylation of PR3-ANCA.

transgenic mouse model in which murine neutrophils expressed human PR3. Despite this, passive transfer of anti-PR3 antibodies failed to induce glomerulonephritis, partially because mice did not process human pro-PR3 into mature PR3 properly (66). Nevertheless, some studies have shown interesting results. In a model of acute lung injury, co-perfusion of human TNFα-primed neutrophils and monoclonal anti-PR3 antibodies induced pulmonary edema dependent on ROS production on isolated rat lungs (67). Moreover, in a humanized mouse model, administration of purified human IgG PR3-ANCA was able to partially reproduce pulmonary and glomerular lesions (68).

To conclude on GPA pathophysiology, despite the implication of other factors, such as the alternative pathway of the complement system, the regulatory cytokine network, monocytes, and T lymphocytes (69–72), PR3-ANCAs appear to play a fundamental role in GPA by inducing auto-immune activation of neutrophils. Therefore, understanding factors involved in the mechanism of auto-immune activation of neutrophils by PR3-ANCA is a necessary prerequisite to consider in the development of new biomarkers and therapies.

UNDERSTANDING AUTO-IMMUNE
ACTIVATION OF NEUTROPHILS BY
PROTEINASE 3-ANTI-NEUTROPHIL
CYTOPLASMIC ANTIBODY IN
GRANULOMATOSIS WITH POLYANGIITIS

Mechanisms of auto-immune activation of neutrophils by PR3-ANCA are influenced by several factors summarized in **Table 1** and illustrated in **Figure 1**. They have been divided into two groups depending on whether PR3-ANCA binds with the neutrophil on PR3 or FcγR.

Factors Involved in mbPR3 and Proteinase
3-Anti-Neutrophil Cytoplasmic
Antibody Interaction
Characteristics of mbPR3

PR3 is a 29-kDa serine protease which can be found in neutrophil granules and also exposed on the outer leaflet of the plasma membrane. This mbPR3 on the outer surface of neutrophils is the

TABLE 1 | Factors influencing auto-immune activation of neutrophils by PR3-ANCA.

	Fab/PR3 interaction	Fc/FcγR interaction
PR3-ANCA characteristics	<ul style="list-style-type: none">• Epitope recognized on PR3• Cross-linking of PR3• Avidity for PR3• Affinity for PR3?	<ul style="list-style-type: none">• Isotype of immunoglobulin• IgG subclasses• Glycosylation on asparagine 297
Neutrophil characteristics	<ul style="list-style-type: none">• % of mbPR3⁺ neutrophils• Amount of mbPR3• Glycosylated isoforms of mbPR3	<ul style="list-style-type: none">• Affinity for FcγRIIA and FcγRIIIB?• Polymorphism of FcγR• Activation independent of FcγR engagement

Main consistent factors described in the literature are shown in bold.

fraction recognized by Fab of PR3-ANCA. Two mbPR3 forms have been described: constitutive mbPR3 and induced-mbPR3 (5). Expression of the constitutive mbPR3 follows a bimodal distribution with mbPR3⁺ positive and negative neutrophils. The ratio between negative and positive neutrophil subpopulations varies extremely between individuals (ranging from 0 to 100%), but remains extremely stable throughout life (56, 73, 74). This bimodal distribution seems to be correlated with the distribution of neutrophil antigen B1 (NB1) also called CD177. NB1 is a glycosylphosphatidylinositol (GPI)-anchored neutrophil-specific membrane receptor which would serve as a co-receptor to allow PR3 expression on the neutrophil membrane (75, 76). Induced mbPR3 is due to a signal-dependent translocation of PR3 from granules to the membrane *e.g.* in response to TNF α stimulation. This TNF α priming is necessary for neutrophil activation by PR3-ANCA (60, 77). Nevertheless, constitutive mbPR3, devoid of enzymatic activity, is also recognized by PR3-ANCA (73). Furthermore, unlike induced-mbPR3, constitutive mbPR3 is not solubilized by alpha1-antitrypsin (A1AT), a natural protease inhibitor, and is therefore a permanent target for PR3-ANCA (78).

It was also suggested that PR3-ANCA could directly activate neutrophils by binding circulating soluble PR3 and forming a PR3-antibody immune complex (55), signifying that PR3-ANCA could bind to the neutrophil and activate them only by their Fc fragment. Another hypothesis concerning involvement of immune complex in AAV was reported by van Paassen et al. (79): ANCA antigens, after their release, will bind to the endothelial cell surface and tissue matrix and will then be bound by ANCA resulting in the formation of immune complexes. These immune complex deposits will enhance further recruitment and activation of neutrophils sustaining an innate inflammatory vicious circle (79). However, in a recent study, no immune complex deposits were found in the majority of renal biopsies of AAV patients whereas C3d, C4d, and C5b-9 were found in a majority of analyzed renal biopsies suggesting the implication of the alternative pathway of the complement system (80). The implication of the complement system in AAV, particularly the alternative pathway, has also been suggested by others (72).

Epitopes on PR3

Implication of epitopes recognized by PR3-ANCA on PR3 in GPA has been studied extensively. Different epitopes and epitope regions have been identified with polyclonal immunoglobulins from patients or with murine/chimeric anti-PR3 mAb but never with human anti-PR3 mAb. Most ANCAs clearly recognize conformational epitopes (81–83), but many more studies have been performed using linear peptides. Concerning epitope regions described with murine anti-human PR3 monoclonal antibodies, five epitopes have been described. Epitopes 1, 2, and 4 are located near the active site while epitope 3 is located very remotely on its posterior face and epitope 5 is found on the hydrophobic patch, allowing PR3 exposure at the neutrophil surface and thus rendering it inaccessible on the mbPR3 (84, 85). Results from studies describing the epitopes recognized during the active phase of the disease support this. With these studies, we can conclude that pathogenic PR3-ANCAs found in active

disease have common characteristics: 1/they mainly bind PR3 close to its active site and close to the binding site of A1AT (84, 86, 87); 2/they have the capacity to modulate the enzymatic activity of PR3 *in vitro* (88–90) although the possible mechanisms of remote or direct interference remain to be clarified (91), and 3/they have the capacity to interfere with the complexation of PR3 with A1AT (87, 92). This interference with the complexation of PR3 with A1AT suggests that PR3-ANCA could have a direct pathogenic role through their Fab fragment (91). They could reduce the clearance of PR3 by A1AT allowing the prolongation of its inflammatory effects. They could also prolong its exposure to the immune system and perpetuate the vicious circle of auto-immunity. Decreased A1AT activity (93) and A1AT deficiency (94), observed in GPA, could act the same way in the disease process. The impact of PR3 glycosylation on its recognition by PR3-ANCA remains poorly studied. But it does not seem to be required (95, 96).

Factors Involved in Fc Gamma Receptors and Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody Interaction Neutrophil Activation Mediated by Fc γ R

Neutrophil activation by PR3-ANCA mainly occurs after Fc domain binding with Fc γ R on the neutrophil surface. This neutrophil activation primarily leads to degranulation (58), ROS production (58–60), NETosis (39, 43), adhesion to endothelial cells (57), and secretion of pro-inflammatory cytokines, especially IL-8 (62). Neutrophils constitutively express Fc γ RIIa (CD32a) and Fc γ RIIb (CD16b), which are both low-affinity and activating Fc γ Rs. These two receptors are not found in other species which also explains the difficulty to study GPA in animal models. Fc γ RIIa is a “classical” transmembrane Fc γ R with a cytoplasmic domain containing an immunoreceptor tyrosine-based activation motif (ITAM) domain. In comparison, Fc γ RIIb, mainly expressed on neutrophils and on a minor subset of basophils, is an atypical GPI-linked receptor without the intra-cytoplasmic part, and thus it is not capable of intracellular signaling. The main hypothesis explaining the mode of action of Fc γ RIIb is that this receptor colocalizes with other transmembrane receptors such as CD18 and Fc γ RIIa. Fc γ RI expression is induced after interferon gamma (IFN γ) stimulation and has been little studied in GPA (97–99).

Several characteristics of these Fc γ R and PR3-ANCA must be taken into account when trying to explain the modalities and consequences of the interaction between the Fc fragment of PR3-ANCA and Fc γ R of the neutrophil: the differential binding of immunoglobulin subtypes to the different Fc γ R leads to different neutrophil functions at varying intensities. Fc γ RIIa engagement induces increased L-selectin expression and is the predominant Fc γ R involved in phagocytosis, but this mechanism has not been identified as being involved in GPA (99–101). Fc γ RIIb engagement induces actin polymerization in a calcium-dependent way, activation of β 1-integrines, IL-8 secretion, and NET formation (98, 99). NETosis is an important phenomenon in the pathogenesis of auto-immune vasculitis and is induced particularly by ROS that are produced intracellularly after Fc γ RIIb signaling (102–104). Nevertheless, it is still unclear

which of these two Fc γ R is predominantly involved in neutrophil activation by PR3-ANCA and what role they play in ROS production. Some studies have shown a greater importance of Fc γ RIIa (105–107) while others state Fc γ RIIIb to be mainly involved (108). The hypothesis of a cooperation between these two receptors or with other surface neutrophil components such as complement receptor 3 (CR3) or β_2 integrins also seems relevant (97, 99, 109, 110). In the majority of studies, selective blocking of Fc γ RIIa or Fc γ RIIIb never abolished neutrophil activation completely suggesting that neither of these two receptors are exclusively responsible for neutrophil activation by PR3-ANCA (105, 111, 112). Therefore, the existence of a mechanism has been suggested by which PR3-ANCAs activate neutrophils in a manner distinct from conventional Fc γ R engagement. Direct stimulation of neutrophils with the Fab fragment of a PR3-ANCA was shown not to activate neutrophils whereas the Fab'₂ fragment of PR3-ANCA, lacking the Fc domain, was able to induce moderate activation of neutrophils and triggered distinct signaling pathways (111, 113, 114). This indicates that cross-linking of the PR3 antigen, by Fab'₂ PR3-ANCA could lead to neutrophil activation independent of signaling through Fc γ R.

Despite the importance of Fc γ R signaling in neutrophil activation, the association of Fc γ R polymorphisms with the disease remains unclear (115–117). In one study, the NA1 polymorphism of Fc γ RIIIb was associated with a higher NET production by neutrophils after *in vitro* PR3-ANCA stimulation and with the development of severe renal disease *in vivo* (118). The implication of NA1 polymorphism in MPO-AAV is an additional argument of its implication in AAV (115). On the other hand, a low FCGR3B copy number is associated with autoimmune diseases such as systemic lupus erythematosus (SLE), MPA, and GPA (119). Furthermore, the role of Fc γ R polymorphisms in GPA is supported by studies showing that patients homozygous for the Fc γ RIIA131H or Fc γ RIIIA158V alleles respond faster to immunosuppressive treatment with Rituximab and their disease progresses significantly faster than in other patients (120, 121).

Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody Isotypes and Subclasses Involved in Neutrophil Activation

Among the human immunoglobulin (Ig) isotypes, IgA and IgM PR3-ANCAs have been found in GPA patients but remain little studied, and their implication remains controversial. IgA is found in a quarter of patients and in a small number of patients with severe renal impairment (118). IgM is found in 15 to 40% of cases, most often transiently (122). The isotype IgG, however, is the most frequent and most studied immunoglobulin isotype. IgG1 and IgG4 have been reported to be the most abundant IgG PR3-ANCA subclasses in sera from GPA patients (123–125). In general, IgG1 and IgG3, which can bind to Fc γ RIIa, are the major PR3-ANCA subclasses able to activate neutrophils. IgG3 seems to be the most pathogenic IgG PR3-ANCA subclass in GPA as they seem to play the greatest role in ROS production and IL-8 response, which subsequently leads to

recruitment of other inflammatory cells and amplifies inflammation (62, 126). It should also be noted that IgG3 binds Fc γ RIIa and Fc γ RIIIb with a higher affinity than the other IgG subclasses (98). IgG2 PR3-ANCAs, which bind poorly or not at all Fc γ RIIIb (99), do not appear to have an important role in neutrophil activation in GPA. IgG4 has long been considered to weakly activate neutrophils because of its low affinity to Fc γ R. Furthermore, it is rather supposed to have an anti-inflammatory role partly due to a dynamic Fab arm exchange (127). Nevertheless, it was first shown that human polyclonal IgG4 PR3-ANCAs purified from patient sera were able to induce neutrophil activation (128). The same group then confirmed this result using a monoclonal chimeric IgG4 anti-PR3 antibody which was able to induce the release of superoxide, degranulation, and adhesion but not IL-8 secretion (101). Furthermore, they demonstrated, in this last study, that activation of neutrophil was dependent of Fc γ R engagement (101).

Particular Glycosylation of Proteinase 3-Anti-Neutrophil Cytoplasmic Antibody

The conserved glycosylation of asparagine 297 in the Fc domain of IgG is important for the interaction between IgG and Fc γ R. A modification of this glycosylation leads to a change of the Fc affinity towards Fc γ R and thus to an altered role in inflammatory processes (129–131). The presence and composition of this glycosylation seem to be fundamental in AAV. Indeed, enzymatic modification of IgG PR3-ANCA and MPO-ANCA glycans attenuates neutrophil activation (ROS production and degranulation) (132). Moreover, in the same study in a murine model of renal disease, complete deglycosylation of IgG MPO-ANCA induced by injection of the bacterial enzyme endoglycosidase S, led to a major decrease in the renal symptoms in these mice (132). This last result, only experimented for MPO-ANCA, should be tested for PR3-ANCA. Furthermore, modifications of this glycosylation are implicated in several autoimmune diseases, such as in GPA, as highlighted by Goulabchand et al. (133). IgG from GPA patients shows low levels of bisection, sialylation, and galactosylation in the active phase of the disease (134–138).

Therefore, the knowledge of these factors influencing PR3-ANCA pathogenicity, *i.e.*, their potential to induce auto-immune activation of neutrophils, could be exploited in view of improving GPA management (biomarkers and therapies) focused on the pathogenicity of PR3-ANCA.

APPLYING KNOWLEDGE ON PROTEINASE 3-ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY PATHOGENICITY

Perspectives to Develop New Biomarkers

PR3-ANCA level combined with clinical manifestations provide insufficient results in predicting relapse (18–21, 23) except in

patients with renal involvement, in whom this PR3-ANCA level correlates with disease activity (24, 25), and in patients after treatment with rituximab (26, 27). Furthermore, PR3-ANCA can persist in GPA patients during remission without predicting relapse (19). In a recent study of 126 patients, the utility of PR3-ANCA as a biomarker was examined. No strict clinical-immunological correlation was observed in 25% of the patients. PR3-ANCA remained positive in 21.7% of patients after the induction of treatment. Among patients with persistent PR3-ANCA, 27.4% did not relapse within 36–38 months, and 50% of them were in complete remission. Finally, 15% of patients in complete remission had persistently positive PR3-ANCA for more than 12 months (22). Therefore, GPA management cannot be based on PR3-ANCA level exclusively. It is essential to develop new biomarkers which could be used in combination as suggested by Osman et al. (139). Here, we have described several factors of PR3-ANCA that might influence their pathogenicity and which could be used as biomarkers. These factors are relevant either because of their difference between active disease and remission or because of their association with relapse. Although most of them are debated or insufficiently known, some of them are promising biomarkers: especially epitope specificities and the glycosylation pattern of PR3-ANCA.

The critical antigenic target of PR3-ANCA, *i.e.* mbPR3 expression, is important but has not been sufficiently investigated *in vivo* to be used as a biomarker of disease activity. Even though the percentage of mbPR3⁺ neutrophils was found to be higher in GPA patients than in healthy subjects and was correlated with the risk of relapse in some studies, other studies did not confirm this correlation (140, 141). The level of mbPR3 at the neutrophil surface correlates with ROS production and *in vitro* degranulation after stimulation of neutrophils with PR3-ANCA (142, 143). Nevertheless, only one study has highlighted that this level of mbPR3 changed during different stages of the disease and correlated with disease activity (144).

Contrary to MPO-ANCA associated vasculitis, in which a linear epitope (aa447–459) is exclusively associated with active disease (83), there is no epitope specifically pointing to disease activity in GPA. Nevertheless, these results obtained with MPO-ANCA allow the hope that epitopes associated with disease activity could be found in PR3-AAV. Several results, however, underline the importance of epitope-specificities of PR3-ANCA to determine their pathogenicity. Concerning epitopes targeted by PR3-ANCA in the active phase of the disease, they have common consistent characteristics which could be useful biomarkers. Indeed, pathogenic PR3-ANCAs: 1/bind PR3 close to the active site of PR3 (84, 86, 87), 2/inhibit the enzymatic activity of PR3 *in vitro* (88–90), and 3/have the capacity to interfere with the complexation of PR3 with its natural inhibitor A1AT (87, 92). Therefore, these common characteristics could be helpful in the diagnosis of the active form of the disease. Furthermore, they could enable a GPA patient and a healthy person with a positive PR3-ANCA result to be differentiated: PR3-ANCAs found in healthy donors target different epitopes than those found in GPA patients (86). Furthermore, it has been

demonstrated that PR3-ANCAs target epitopes on PR3 with different proportions between patients and with a different evolution in the same patient according to the state of disease activity (145, 146). Therefore, studying epitope shift during patient follow-up seems promising in predicting relapse. To date, epitope shift has been associated with relapse in one prospective study: among 12 patients with relapse, an epitope shift was observed in 11 cases from epitopes located in the C-terminal towards epitopes in the N-terminal part of PR3. Furthermore, in the same study, the relapse rate was significantly higher in the group of patients with predominantly C-terminal reactivity at diagnosis compared to the group with N-terminal reactivity (147).

Only one study has investigated the influence of interaction strength between PR3 and PR3-ANCA and found a correlation between the avidity of this interaction and relapses in patients with renal impairment (148). The affinity of PR3-ANCA to PR3 could also influence their pathogenicity but has never been investigated to our knowledge.

As described above, most isotypes of Ig, at different levels, appear to be involved in GPA and are able to activate neutrophils. IgG is generally studied *in vitro*; IgG1 and mostly IgG3 are the two main IgG subclasses able to induce neutrophil activation (62). In one study, respiratory burst induced by IgG fractions from patients correlated with the disease activity and was related to changes in the relative amount of the IgG3 subclass of PR3-ANCA (126). Nevertheless, measurement of IgG3 subclass of PR3-ANCA did not improve the predictive value of a rise in ANCA in another study (18). Clinical implications of different Ig isotypes in disease activity, however, are studied separately and provide few or contradictory results, offering no consistent data for use as biomarkers. IgG4:IgG RNA ratio, representing the number of IgG4-producing B-lineage cells, seems to significantly differentiate active disease from remission (149). One study postulated that IgA PR3-ANCA had a protective role (118), whereas in another, their level was related to disease activity (150). IgM has been associated with the severity of the disease, particularly in severe pulmonary impairment (122). In contrast, other authors proposed a protective role of IgM as it can be found in healthy donors and patients in remission (151). Furthermore, a reduced frequency of marginal zone-like B cells, which are the main producers of IgM, has been observed in the circulation of patients with auto-immune vasculitis (152). Therefore, the role of isotypes and subclasses of Ig in GPA pathophysiology requires further investigation. They could be relevant biomarkers as they can be studied easily in the laboratory.

Glycosylation of total IgG and IgG PR3-ANCA could also be used as relevant biomarker. Indeed, IgG1 from GPA patients in the active phase of the disease shows low levels of bisection, sialylation, and galactosylation (134–138) and clinical remission was associated with glycan normalization in one study (138). Espy et al. highlighted that hyposialylation of IgG PR3-ANCA was correlated with disease activity and with the *in vivo* production of ROS by neutrophils (135). Therefore, studying ANCA glycosylation, particularly sialylation, of total IgG and IgG PR3-ANCA could be helpful to evaluate disease activity and probably to affirm remission.

An interesting biomarker could be the PR3-ANCA producer cell itself. Cornec et al. found a higher proportion of PR3-specific B cells among patients with active disease (2.91%) than among patients in remission (0.99%), whereas there was no difference in serum PR3-ANCA levels between the two groups (153). Studying the global B-cell population after a remission induction treatment with rituximab also seems attractive in GPA patients. Indeed, B-cell repopulation was associated with relapses whereas their absence predicted a relapse-free status (27).

To further investigate PR3-ANCA pathogenicity, we developed in our laboratory human anti-PR3 mAbs obtained after immortalization of memory B cells from GPA patients at different stages of the disease. We obtained an original anti-PR3 mAb (called 4C3) from a GPA patient in remission having a persistently high PR3-ANCA level. Neutrophil stimulation with 4C3 did not induce auto-immune activation of neutrophils *in vitro*, which demonstrates for the first time that non-pathogenic PR3-ANCAs exist. The existence of non-pathogenic PR3-ANCA, as 4C3, must be confirmed by further studies. However, their existence could explain why PR3-ANCA can persist in some GPA patients in remission without predicting relapse (22) and also why they can be found in healthy people (10, 11). Furthermore, due to the absence of any functional defect in its Fc fragment, we hypothesize that its non-pathogenic character is related to the epitope recognized on PR3. Indeed, mAb 4C3 binds mbPR3 on a newly described epitope close to the hydrophobic patch (154). Epitopes associated with non-pathogenicity of PR3-ANCA could be a relevant biomarker.

Perspectives to Develop New Therapies

Despite current therapies, GPA is still a serious disease with an important mortality rate (15, 155), a significant morbidity related to the disease and its management (16) and a high risk of relapse (17). Furthermore, none of the treatments used or studied in research (51) are specific to the fundamental auto-immune activation of neutrophils by PR3-ANCA. The most recently used treatment is the application of rituximab, a chimeric monoclonal anti-CD20 antibody that depletes circulating B lymphocytes without specifically targeting those producing ANCA. This depletion is not immediate, with a decrease in ANCA of just 50% after one month (156), and includes several side effects such as significant risk of hypogammaglobulinemia, infection, lymphopenia, and neutropenia (156–159). Therefore, it seems necessary to look for more specific treatments for GPA which specifically block auto-immune activation of neutrophils by PR3-ANCA. Blocking this interaction could directly neutralize the effector cells more targeted and potentially suppress the inflammatory cascade faster. This new therapeutic approach could reduce the morbidity related to side effects of non-specific treatments currently used and also the morbidity related to complications of inflammation due to the disease.

Different treatment options could be considered to achieve this purpose. Elimination of PR3 autoantigen has already been

proposed by Korkmaz et al. with promising results for a cathepsin C inhibitor (160, 161). Cathepsin C is a central biosynthetic switch in the activation of many serine proteases in immune cells and is responsible for the maturation of pro-PR3 to active PR3 (161). Consequently, using a cathepsin C inhibitor could lead to a significant decrease in the level of both PR3 autoantigen and PR3-ANCA (162, 163). Moreover, inhibition of neutrophil activation by PR3-ANCA has already been demonstrated *in vitro* by neutrophils pre-incubated with A1AT (78, 164, 165). Nevertheless, A1AT has the disadvantage of not being very sensitive for PR3 (85) and not eliminating constitutively expressed mbPR3 making its clinical use difficult (78). In our laboratory, we chose to develop full human mAbs targeting PR3 by immortalizing B cells from patients with GPA. Compared to a small chemical molecule, an antibody has several advantages: a larger target binding area for better specificity, a greater ability to hide the target, and a longer half-life (166).

Considering all these aforementioned factors influencing the auto-immune activation of neutrophils by PR3-ANCA, it would be interesting to find an anti-PR3 mAb to neutralize this interaction on condition that it has the following characteristics: it should bind to mbPR3 with a high affinity and target the major epitopes of PR3, without binding to the “hydrophobic patch” which is not accessible on mbPR3, in order to prevent the fixation of the majority of pathogenic PR3-ANCA (84, 85). The antigenic moiety, essentially of mbPR3, should also be taken into account in the dose used because of its variation during the course of the disease (55). It should obviously not induce neutrophil activation.

Several strategies could be used to create an anti-PR3 mAb that does not activate neutrophils. The majority of these strategies aim to block FcγR engagement. A first avenue would be to keep the mAb in its complete form which would give it the advantage of having a longer half-life. Modification of its glycosylation could be a solution as IgG glycosylation is important in FcγR engagement (129, 138). Interestingly, in a murine model it has been shown that when MPO-ANCA is deglycosylated, *in vitro* neutrophil activation and *in vivo* vasculitis symptoms decrease significantly (132). Whatever, PR3-ANCA deglycosylation has never been studied *in vivo*. Changing the subclass, for example using an anti-PR3 IgG2 or IgG4 recombinant antibody seems to be risky. These two isotypes have weak affinities for FcγR expressed by neutrophils (98, 99), but still seem to be involved in the pathophysiology of GPA as anti-PR3 IgG4 has already been shown to be able to activate neutrophils *in vitro* (101, 128). Another strategy would be to use antibody fragments. Fab fragments of PR3-ANCA seem to be interesting because they were able to bind mbPR3 without causing neutrophil activation in most studies, while Fab′₂ fragments of PR3-ANCA did, probably through cross-linking the PR3 antigen (111, 113, 114). Despite this, it has the disadvantage of being rapidly eliminated because of its low molecular weight. To overcome this, a multi-specific recombinant Fab with an extended half-life could be a solution. Moreover, a combination of several different Fab fragments targeting different epitopes on the mbPR3 could be

used in order to prevent the majority of circulating pathogenic PR3-ANCA from binding with neutrophils.

Finally, we hypothesize that a human anti-PR3 mAb could directly prevent neutrophils from activating. As described above, we have produced the mAb 4C3, a non-pathogenic human anti-PR3 mAb which was shown to be unable to activate neutrophils *in vitro*. Moreover, this mAb is able to neutralize auto-immune activation of neutrophils induced by pathogenic PR3-ANCA from GPA patients at diagnosis (154). This promising result offers perspectives to develop new therapies in GPA but must be confirmed by further studies.

CONCLUDING REMARKS

GPA is a rare but serious auto-immune vasculitis in which auto-immune activation of neutrophils, enabled by their interaction with PR3-ANCA, plays a central role. GPA management (monitoring and treatment) could be improved based on a better knowledge of factors influencing PR3-ANCA pathogenicity. Therefore, a better understanding of these factors and the confirmation of the existence of non-pathogenic PR3-ANCA could lead to developing new potential biomarkers, such as paratope and glycosylation of PR3-ANCA. Indeed, pathogenic characteristics and total level of PR3-ANCA could be useful biomarkers to evaluate disease activity, to predict relapse in GPA patients, and to differentiate a GPA patient and a healthy person with positive PR3-ANCA. Furthermore, targeting PR3-ANCA interaction with neutrophils, especially with monoclonal antibodies or antibody fragments, seems to offer an attractive perspective and could represent a more focused therapeutic, thereby

avoiding overtreatments and achieving higher efficacy with fewer side effects than current therapies.

AUTHOR CONTRIBUTIONS

JG, RL, DN, SW, DJ, BK, and CH prepared and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Autoantibodies Targeting Intracellular and Extracellular Proteins in Autoimmunity

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Detecting autoantibodies provides foundational information for the diagnosis of most autoimmune diseases. An important pathophysiological distinction is whether autoantibodies are directed against extracellular or intracellular proteins. Autoantibodies targeting extracellular domains of proteins, such as membrane receptors, channels or secreted molecules are often directly pathogenic, whereby autoantibody binding to the autoantigen disrupts the normal function of a critical protein or pathway, and/or triggers antibody-dependent cell surface complement killing. By comparison, autoantibodies directed against intracellular proteins are recognized as useful diagnostic biomarkers of abnormal autoimmune activity, but the link between antigenicity and pathogenicity is less straightforward. Because intracellular autoantigens are generally inaccessible to autoantibody binding, for the most part, they do not directly contribute to pathogenesis. In a few diseases, autoantibodies to intracellular targets cause damage indirectly by immune complex formation, immune activation, and other processes. In this review, the general features of and differences between autoimmune diseases segregated on the basis of intracellular or extracellular autoantigens are explored using over twenty examples. Expression profiles of autoantigens in relation to the tissues targeted by autoimmune disease and the temporal appearance of autoantibodies before clinical diagnosis often correlate with whether the respective autoantibodies mostly recognize either intracellular or extracellular autoantigens. In addition, current therapeutic strategies are discussed from this vantage point. One drug, rituximab, depletes CD20+ B-cells and is highly effective for autoimmune disorders associated with autoantibodies against extracellular autoantigens. In contrast, diseases associated with autoantibodies directed predominately against intracellular autoantigens show much more complex immune cell involvement, such as T-cell mediated tissue damage, and require different strategies for optimal therapeutic benefit. Understanding the clinical ramifications of autoimmunity derived by autoantibodies against either intracellular or extracellular autoantigens, or a spectrum of both, has practical implications for guiding drug

development, generating monitoring tools, stratification of patient interventions, and designing trials based on predictive autoantibody profiles for autoimmune diseases.

Keywords: autoantibodies, autoimmune, treatment, onset, autoantigen

INTRODUCTION

The production of autoantibodies against self-proteins, called autoantigens, is an abnormal process characteristic of most autoimmune diseases. Autoantibody immunoreactivity in patient blood or CSF provides key diagnostic information when autoimmune disease is suspected. The spectrum of autoantibodies is often clinically informative for a given autoimmune disease. Some autoimmune diseases harbor autoantibodies against only one or a few target autoantigens, but in other conditions autoantibodies against multiple targets may co-exist. Among the seventy most common autoimmune diseases, approximately 100 out of the estimated 20,000 human proteins encoded by the genome comprise the most common antigenic targets (1). However, an increasing number of autoantibodies have been discovered in rare disorders suggesting additional diseases are likely to exhibit autoantibody-associated autoimmunity.

The treatment of many autoimmune diseases remains sub-optimal due to varying degrees of efficacy and the side-effects of available interventions (2). Advances in autoimmune disease therapeutics will require disease-specific information including identification of immune cells and underlying signaling pathways involved, the mechanisms governing loss of tolerance, and how autoantibodies participate in pathogenesis. To aid in this process, we have focused on conceptually defining autoimmune diseases based on whether the autoantibodies in a particular disorder target extracellular or intracellular proteins or a more complex mixture of both. Intracellular autoantigens are generally inaccessible to binding by autoantibodies and instead represent autoimmune biomarkers of abnormal immune cell activity. Conversely, extracellular antigenic proteins are readily accessible to autoantibodies whose targets include secreted proteins, cell surface channels, and receptors. Antibodies against extracellular proteins can directly cause disease by altering protein function or abundance and/or by recruiting complement-mediated cell killing. In this review, we explore the potential for classification of autoimmune diseases based on whether they have autoantibodies predominantly against intracellular or extracellular targets. These two distinct sites of autoantigen localization are described in the context of tissue expression, temporal appearance of autoantibodies in relation to disease diagnosis, and how this information provides a rational basis for treatment choices.

Autoimmune Diseases Enriched in Autoantibodies Against Intracellular Proteins

Some of the most common autoimmune diseases demonstrate a preponderance of autoantibodies directed against intracellular

targets including structural proteins, enzymes, splicing machinery, RNA-binding proteins, and RNA polymerases (3). Although it is well-recognized that T-cells play a central role in the destruction of the corresponding cells and tissue in autoimmune diseases showing autoantibodies against intracellular proteins additional mechanisms have been proposed to explain why these proteins become targets of B-cell responses including release from dying cells, ineffective clearance of apoptotic debris, protein modification during inflammatory responses, and molecular mimicry (4). In this section, we discuss several major autoimmune diseases characterized mainly by autoantibodies to intracellular proteins and describe the tissue expression patterns of their target autoantigens in relationship to the autoimmune process and its pathological manifestations.

Autoantibodies against intracellular proteins are important disease biomarkers in a number of rheumatological diseases including Sjögren's syndrome, systemic lupus erythematosus, systemic sclerosis, and myositis. In Sjögren's syndrome, an autoimmune disease defined by sicca symptoms of oral and ocular dryness, the major autoantibodies are against SSA and SSB. SSA is comprised of two different autoantigenic proteins, Ro52 (*TRIM21*) and Ro60 (*Trova2*), and SSB autoantibodies recognize a single protein, La (SSB) (**Figure 1A**). Despite the importance of those three autoantigens for diagnosing an underlying autoimmune basis for Sjögren's syndrome, they are ubiquitously expressed, rather than confined only to salivary glands, thus making a causal relationship to sicca symptoms difficult to establish (3). Both Ro60 and La are intracellular RNA-binding proteins, but Ro52 acts differently, as an important immunoglobulin receptor inside cells that mediates ubiquitylation-dependent destruction and neutralization of internalized immunoglobulin-pathogen complexes (4). Based on the biological function of Ro52 in pathogen clearance, one possible abnormal mechanism explaining its antigenicity in subjects with Sjögren's syndrome and other autoimmune diseases is that the entire protein complex of Ro52, immunoglobulins, and infectious agents such as virus may be recognized as "foreign" (5).

Systemic lupus erythematosus (SLE) is characterized by immune activation and widespread tissue destruction (6). A common feature of SLE is the high prevalence of autoantibodies to several intracellular proteins, as well as against cellular DNA. In addition to autoantibodies against Ro52, Ro60 and La proteins, RNA-binding proteins including RNP-A (*SNRPA1*), U1-70K (*SNRNP70*), and Sm-D3 (*SNRPD3*), are also important diagnostic autoantigens. It is important to point out that these autoantibodies against intracellular targets are not directly pathogenic *via* their autoantigen binding, but they can still contribute to disease by participating in immune complex

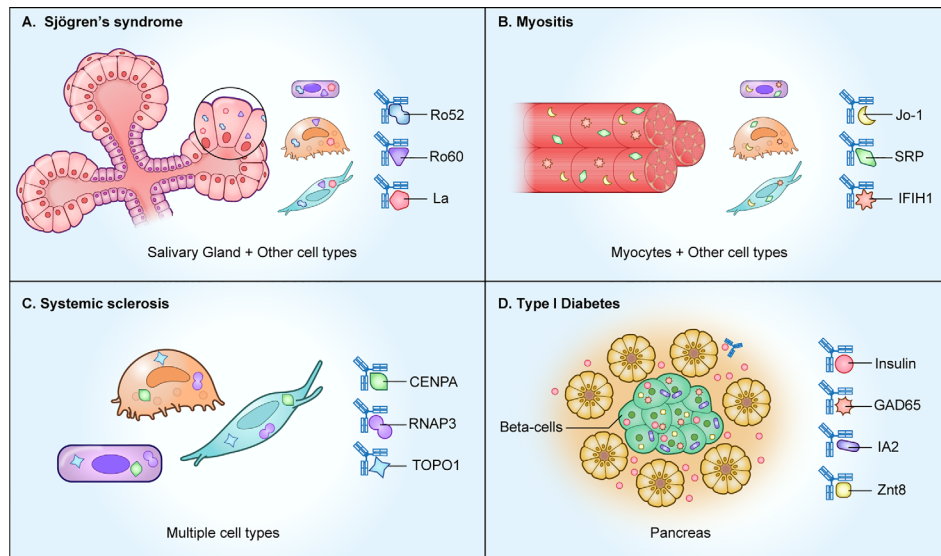


FIGURE 1 | Autoimmune diseases with autoantibodies directed against intracellular proteins. As shown, several autoimmune diseases including **(A)** Sjögren's syndrome, **(B)** myositis, and **(C)** systemic sclerosis, harbor autoantibodies against ubiquitously expressed intracellular proteins. However, in **(D)** type I diabetes, the intracellular autoantigens represent beta cell-specific proteins derived from the pancreas.

formation, complement activation, and immune activation (7, 8). Exactly why these various RNA-binding proteins are autoantigenic or what causes loss of tolerance to them is unknown. Autoantibody profiling reveals that most SLE patients can be segregated into one of two autoantibody clusters: those with Ro52, Ro60, and La as targets, or those who are enriched for Sm-D3, U1-70k, and RNP-A autoantibodies (9). Numerous clinical phenotyping efforts have found that autoantibodies to certain intracellular autoantigens correlate with specific SLE symptoms (10–12). For example, the presence of RNP-A autoantibodies is associated with patients having Raynaud's skin symptoms. Since none of these intracellular RNA-binding proteins are accessible to autoantibody binding, one possible explanation for their association with certain symptoms is that these autoantibodies may converge on common protein synthesis pathways involved in disease pathogenesis, and upon upregulation and release, these RNA-binding proteins become autoantibody-associated biomarkers.

Myositis represents a diverse spectrum of disease subtypes involving autoimmune-mediated muscle inflammation and subsequent muscle tissue destruction (13). All known myositis autoantigens that are targets of autoantibodies are intracellular proteins (**Figure 1B**). By profiling the autoantibody response against a panel of myositis-associated autoantigens, it is possible to segregate the disease into four subtypes: anti-synthetase syndrome, dermatomyositis, inclusion body myositis, or immune-mediated necrotizing myopathy (14). In anti-synthetase syndrome, the major autoantigens are involved in tRNA synthesis and include Jo1, an enzyme responsible for histidyl-tRNA synthesis (*HARS*), PL7, a threonyl tRNA synthetase (*TARS*), and PL-12 alanyl-tRNA synthetase (*ARS*). In dermatomyositis, autoantibodies against Mi2 histone acetylase, the tRNA synthetase proteins and anti-TIF- γ are often found.

Dermatomyositis patients with interstitial lung disease often harbor autoantibodies against an intracellular RNA sensor protein, MDA-5 (*IFIH1*), but the mechanism behind the association of autoantibodies with lung disease is not known. Lastly, in necrotizing myositis, autoantibodies directed against the intracellular signal recognition protein (SRP) complex and in some rare cases against 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*) are present. The association and pathways involved in generating autoantibodies against these diverse intracellular autoantigens in myositis remains unresolved.

The autoimmune manifestations in systemic sclerosis (scleroderma) are characterized by vascular dysfunction, inflammation, and fibrotic structural changes in the skin and internal organs (15). As shown in **Figure 1C**, patients exhibit autoantibodies against a variety of intracellular proteins that include Ro52, Ro60, topoisomerase 1 (top1), centromere proteins (*CENPA* and *CENPB*), PM/SCL (*EXOSC9* and *EXOSC107*), and RNA polymerase 3 complex (*POLR3A* and *POLR3K*) (16). Autoantibody-based diagnosis in systemic sclerosis patients requires a large panel of fifteen autoantigens for high diagnostic sensitivity to classify most systemic sclerosis patients into one of five clusters (17). Autoantibody-mediated pathways targeting extracellular proteins have been explored as possible drivers of fibroblast activation, but to date none have been discovered.

Type I diabetes (T1D) is an autoimmune disease commonly found in children involving T-cell mediated immune destruction of the insulin-producing beta cells in the pancreas (18). Autoantibodies against one extracellular and several intracellular proteins are typically found in T1D and represent important biomarkers for the disease (**Figure 1D**). Autoantibodies against the secreted peptide hormone insulin

(INS), an extracellular target, are one of the early indicators of prediabetic islet cell autoimmunity in T1D (19). Despite the accessibility of circulating insulin to serum autoantibodies, anti-insulin autoantibodies are not pathogenic because they do not cause the destruction of the insulin-producing beta-cells. The intracellular autoantigen IA-2 (*PTRN*) is a receptor type tyrosine-protein phosphatase localized to the membrane of dense core vesicles and highly expressed in the brain and pancreas. The region of IA2 directed toward the vesicle lumen is immunodominant, and this intracellular tail region is used to measure autoantibodies in most studies (19). Two more intracellular proteins, glutamate decarboxylase/GAD65 (*GAD2*) and Znt8 (*SLC30A8*), are also targets of autoantibodies in T1D. GAD65 is an enzyme responsible for synthesis of the neurotransmitter gamma-aminobutyric acid (GABA). Autoantibodies against GAD65 are not specific to T1D and can be found in several central nervous system autoimmune diseases, including Stiff-person syndrome and autoimmune-mediated encephalitis (20). Znt8 is an abundantly expressed zinc transporter protein found on insulin secretory granules of pancreatic beta cells. Besides beta cell-specific proteins, autoantibodies against the ubiquitously-expressed proteins tetraspanin-7 are found in T1D (*TSPAN7*) (21). Protein array technologies have also identified the intracellular peptidylprolyl isomerase like 2 (*PPIL2*) and DNA mismatch repair protein Mlh1 (*MSH1*), albeit the presence of these autoantibodies only occurs in a small subset (<8%) of T1D subjects (22, 23). Understanding the loss of B-cell tolerance to these rarer autoantigens may provide insight into patient subsets, autoantibody spreading, and/or mechanisms involved in T1D autoimmunity.

In addition to proteins that are strictly intracellular, there are some autoantigens that are transiently expressed on the cell surface, thereby becoming accessible to autoantibody binding. In systemic vasculitis, the intracellular target autoantigens are proteinase-3 (PR3) and myeloperoxidase (MPO), which have signal peptides that allow their association with and storage in secretory vesicles. In patients with vasculitis activated neutrophils, PR3 and MPO proteins traffic to the plasma membrane surface (24, 25), making these normally intracellular primary granule enzymes accessible to autoantibody binding. PR3 and MPO autoantibody binding can activate neutrophils by engaging Fcγ receptors (26). Passive transfer of MPO autoantibodies into recipient mice cause glomerulonephritis and vasculitis and provides further proof that these autoantibodies are pathogenic (27).

With the exception of T1D, where autoantigens are generated against highly expressed proteins of the insulin-producing beta cells of the pancreas, the relationships between autoantigenic proteins and target tissues in the other autoimmune disease examples remains poorly understood. For example, it is unclear how autoantibodies against Ro52 and Ro60 proteins in Sjögren's syndrome act as biomarkers for salivary gland dysfunction and why the ubiquitous t-RNA synthetases are targets of autoantibodies in myositis. Understanding the mechanisms involved in the loss of tolerance against these and other autoantigens would shed light on

how autoimmunity develops and what are the etiological triggers for these autoimmune diseases.

Autoantibody Diseases Harboring Autoantibodies to Extracellular Targets

Autoantibodies against extracellular proteins can directly cause a variety of autoimmune diseases. This direct pathogenicity is caused by binding of the autoantibody to the extracellular protein thereby disrupting normal function of a critical protein or pathway, and/or by triggering antibody-dependent cell surface complement killing. In order to classify an autoantibody against an extracellular target as pathogenic, it needs to fulfill several criteria: 1) the specific autoantibody is strongly associated with the relevant clinical presentation of the disease and absent in healthy individuals or other diseases, 2) the autoantigen is specifically localized to the diseased tissue, and 3) the autoantibody levels correlate with disease activity. Validating the pathogenicity of autoantibodies often involves animal models, whereby passive transfer of patient autoantibodies or antigen-induced immunization can recapitulate clinical features of the disease. An in-depth discussion of mechanisms underlying autoantibody-induced pathology can be found in a recent review (28). Here, we provide examples of several major autoimmune diseases harboring pathogenic autoantibodies to extracellular autoantigenic targets and describe how many of these autoantigen targets are cell/tissue-specific and fulfill the criteria for pathogenicity (**Figure 2**).

Myasthenia gravis is an autoimmune disease of muscle harboring pathogenic autoantibodies that interfere with cholinergic receptors and other proteins at the neuromuscular junction. The autoantibodies found in myasthenia gravis cause progressive skeletal muscle weakness (29). The major autoantibody target, found in approximately 85% of myasthenia gravis patients, is the extracellular N-terminal region of the alpha 1 subunit of the nicotinic acetylcholine receptor/nAChR (*CHRNA1*), which is highly enriched in skeletal muscle (**Figure 2A**). Autoantibodies against two additional targets, the muscle-associated cell surface tyrosine kinase (*MUSK*) and the low-density lipoprotein receptor-related protein (*LRP4*), are less common and found in about 1–10% and 1–3% of cases, respectively (29) (**Figure 2A**). Patients seropositive for either nAChR or LRP4 autoantibodies show classic myasthenic symptoms, yet interestingly patients with MuSK autoantibodies show more bulbar and cranial involvement, less muscle extremity involvement, and a high occurrence of respiratory problems. As illustrated in myasthenia gravis patients, and in most patients with autoantibodies to an extracellular target, the humoral response is directed against only one target protein, which typically drives pathogenesis.

Autoimmune diseases of the central nervous system also involve autoantibodies to extracellular proteins found in neurons or glia (30). For example, autoantibodies targeting the aquaporin-4 (*AQP4*) channel, which is enriched on the surface of astroglial cells and involved in maintaining integrity of the blood brain barrier, cause neuromyelitis optica (NMO/Devic's disease).

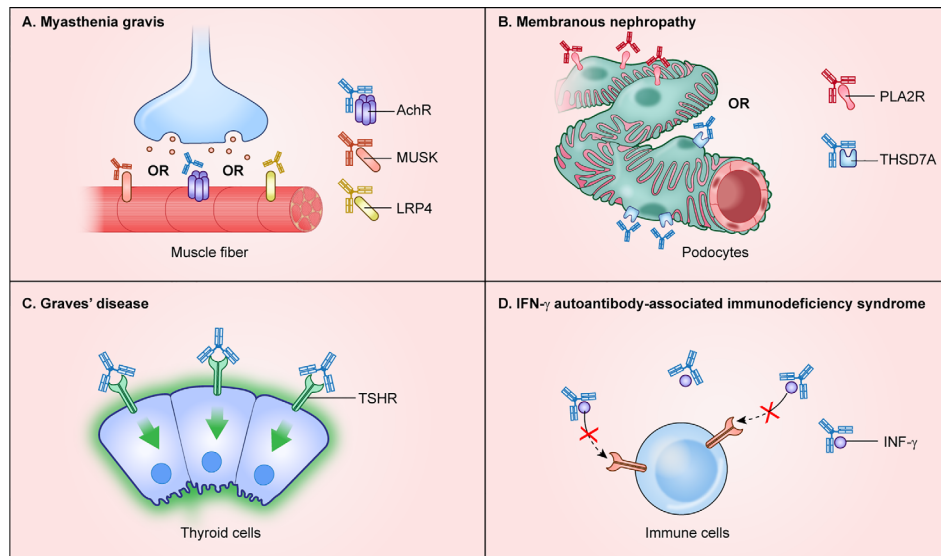


FIGURE 2 | Autoimmune diseases with autoantibodies directed against extracellular protein targets. Autoantibodies targeting extracellular proteins directly cause disease pathogenesis and are found in autoimmune disease including **(A)** myasthenia gravis, **(B)** membranous nephropathy, **(C)** Graves' disease, and **(D)** interferon- γ autoantibody immunodeficiency syndrome.

Similarly, autoantibodies against myelin oligodendrocyte glycoprotein (MOG) cause autoimmune demyelination disease in NMO. Autoantibodies targeting subunits of the N-methyl-D-aspartate receptor/NMDAR (e.g., *GRIN1*) on the surface of neurons cause encephalitis and other neurological problems (31). Additional autoimmune neurological diseases targeted by pathogenic autoantibodies involve alpha-amino-3-hydroxy-5methyl-4-isoxazolepropionic receptor/AMPA (*GRIA1* and *GRIA2*) and channel scaffold proteins such as the secreted leucine rich glioma inactivated 1 protein (*LG1*) and the neuroligin family protein contactin associated protein 2 (*CNTNAP2*) (30). Deleterious effects in the central nervous system often involve interference with the normal function of critical ion channels.

Several autoimmune diseases of the kidney are driven by autoantibodies to extracellular proteins. Anti-glomerular basement membrane disease (Goodpasture's syndrome), designated anti-GBM disease, is caused by autoantibodies against the collagen IV- α 3 chain (*COL4A3*) whose expression is enriched in lung and kidney (32). In anti-GBM disease, autoantibodies against collagen IV cause complement activation and leukocyte infiltration that damages the basement membrane lining the capillaries in the glomeruli of the kidney. A different autoimmune condition, membranous nephropathy, exhibits focal autoantibody deposits in the kidney sub-epithelial layer of the glomerular basement membrane adjacent to podocyte foot processes (33). Autoantibodies in membranous nephropathy are directed against at least two podocyte-specific membrane proteins with extracellularly exposed regions including phospholipase A2 receptor (*PLA2R*) (34) and thrombospondin type-1 domain-containing 7A (*THSD7A*) (35) (**Figure 2B**). The mRNAs and proteins for *PLA2R* and *THSD7A*

show some of the highest expression levels in the kidney. *PLA2R* autoantibodies are the most common cause of membranous nephropathy and can be used diagnostically or for monitoring responses to therapy, as well as for detecting relapse (36).

Autoantibodies to extracellular target proteins are typically assumed to cause a corresponding loss of function. However, in Graves' thyroiditis, binding of autoantibodies to the thyroid hormone stimulating receptor (*THSR*) found on follicular thyroid cells has an agonist-like activity that over-activates downstream signaling and results in high levels of circulating thyroid hormones (37) (**Figure 2C**). Clinical symptoms include hyperthyroidism, ophthalmopathy, and dermopathy (37). Besides the thyroid-stimulating hormone receptor, autoantibodies are also directed to intracellular autoantigens including thyroid peroxidase (*TPO*), which is involved in thyroxine biosynthesis, and to the secreted thyroid hormone binding protein, thyroglobulin (*TG*). Although thyroid-stimulating hormone receptor autoantibodies are well-established as the cause of hyperthyroidism in Graves' disease, less is known about the mechanisms underlying other features such as ophthalmopathy or dermopathy.

Autoantibodies against circulating hormones, growth factors, and cytokines cause a variety of autoimmune-mediated diseases. Cytokines are particularly important because they function as key regulators of the immune system by playing critical roles in the maturation of immune cells and orchestrating responses to pathogens. Several acquired autoimmune immunodeficiencies are caused by anti-cytokine autoantibodies (38). One anti-cytokine autoimmune disease is pulmonary alveolar proteinosis caused by autoantibodies against GM-CSF (*CSF2*) (39). Autoantibodies sequester GM-CSF and block its signaling, thereby preventing downstream production and maturation of

macrophages in the lung, thus leading to excessive accumulation of surfactant and other lipoproteins in the lower respiratory tract. While the lung is the most vulnerable organ, a second clinical phenotype found in patients with anti-GMCSF autoantibodies are opportunistic infections by microbes such as *Cryptococcus*, *Nocardia*, and *Histoplasma*, which are caused by defective phagocyte function (40, 41). Another acquired anti-cytokine immunodeficiency syndrome is caused by autoantibodies against interferon- γ (*IFNG*), in which patients develop severe mycobacterial infection (42). The IFN- γ autoantibodies detected in these patients are mainly of the IgG4 isotype and bind circulating IFN- γ , interfering with its normal signaling activity (Figure 2D). Consistent with this sequestration mechanism, serum autoantibodies harvested from patients were capable of neutralizing *in vitro* signaling activity downstream of the IFN- γ receptor as demonstrated by blockade of STAT1 phosphorylation (42). These and other examples of anti-cytokine autoimmune diseases highlight how vulnerabilities to specific infectious agents is driven by loss of function of specific cytokines responsible for proper immune cell signaling.

It is important to point out that some individuals with autoantibodies against extracellular proteins also occasionally have additional autoantibodies directed against intracellular proteins, but that the defining pathology is caused by autoantibodies against the extracellular autoantigen. Recognizing that autoantibodies against a specific target protein drive the clinical features of a disease is an important aspect to consider with regard to treatment. One benefit to monitoring serum levels of pathogenic autoantibodies is the ability to directly track responses to therapy, where the reduction or disappearance of circulating autoantibodies coincides with cure or remission.

Autoimmune Diseases Harboring Autoantibodies Against Extracellular Proteins Can Mimic Genetic Diseases for the Same Target Protein or Pathway

One interesting feature of pathogenic autoantibody diseases associated with extracellular autoantigens is that they frequently share clinical phenotypes with genetic mutations in the corresponding protein target or pathway (Table 1). This

relationship between autoimmune-mediated and a corresponding inherited genetic disease in the same protein is consistent with the loss-of-function phenotype induced by most acquired pathogenic autoantibodies. For example, in congenital forms of myasthenia gravis, patients possess mutations either in the $\alpha 1$ subunit of the acetylcholine receptor (*CHRNA1*), *LRP4*, or *MUSK* genes, all known targets of autoantibodies causing autoimmune forms of myasthenia gravis (43). In anti-GBM autoimmune disease, there are autoantibodies to the collagen IV- α 3 chain and in the genetic disease Alport syndrome, glomerulonephritis, and end-stage kidney disease are caused by mutations in the collagen IV- α 3 chain (44). Mutations in *GPIHBP1*, encoding a protein involved in blood lipid transport, causes hyperlipidemia (46) with clinical features mimicking autoimmune hyperlipidemia caused by inactivating autoantibodies against GPIHBP1 (46). In some cases, the genetic defect occurs in the receptor rather than the ligand, resulting in the same phenotype. For example, pulmonary alveolar proteinosis (PAP) patients have autoantibodies against the soluble GMCSF cytokine preventing the normal development of macrophages in the lung, but the genetic forms of PAP have mutations in the membrane-bound GMCSF receptor (*CSF2RA*) (51). Similarly, patients with mutations in the interferon-gamma receptor (*IFNGR*) (52) exhibit clinical features resembling patients with neutralizing autoantibodies against the cognate ligand, interferon-gamma (42), resulting in unusual opportunistic non-mycobacterial infections. While diseases caused by gene mutations are inherited as life-long conditions and are difficult to treat, the analogous autoimmune diseases are acquired and often highly treatable with immune therapies.

In contrast, there is little or no evidence that mutations in genes encoding intracellular autoantigens cause similar diseases. For example, autoantibodies against the intracellular autoantigen MDA5, encoded by the *IFIH1* gene, are found in myositis-associated lung disease, but mutations in the *IFIH1* gene cause an unrelated disease characterized by a spectrum of neuro-immunological features (53). Similarly, mutations in HARS, encoding the intracellular Jo-1 myositis autoantigen, do not affect muscle tissue but cause a genetic form of inherited Charcot-Marie-Tooth type 2 peripheral neuropathy (54).

Based on the observation that a variety of genetic diseases involve mutations in extracellular receptors and secreted molecules, we speculate that there are likely more unrecognized

TABLE 1 | Pathogenic autoantibody-mediated diseases mimic genetic diseases.

Gene or protein	Genetic mutation phenotype	Autoimmune phenotype
CHRNA1 (AChR1)	Congenital myasthenia gravis (43)	Myasthenia gravis (29)
MUSK	Congenital myasthenia gravis (43)	Myasthenia gravis (29)
COL4A3	Alport syndrome (44)	Anti-GBM disease (32)
LRP4	Congenital myasthenia gravis (43)	Myasthenia gravis (29)
GPIHBP1	Hyperlipidemia (45)	Hyperlipidemia (46)
GluR1 (NMDA)	Epilepsy (47)	Epilepsy and encephalitis (31)
ADAMTS13	Congenital thrombotic thrombocytopenic purpura (48)	Autoimmune thrombotic thrombocytopenic purpura (48)
FGF23	Familial hyperphosphatemic tumoral calcinosis (49)	Autoimmune hyperphosphatemic tumoral calcinosis (50)
CSF2RA (receptor) or GMCSF (ligand)	<i>CSFR2</i> mutations cause hereditary alveolar proteinosis (51)	GMSF autoantibodies cause alveolar proteinosis (39)
IFNGR (receptor) or IFN-γ (ligand)	<i>IFNRG</i> mutations cause opportunistic mycobacterial infections (52)	IFN- γ autoantibodies cause opportunistic mycobacterial infections (42)

autoimmune conditions involving autoantibodies to extracellular targets. This may be particularly applicable to patients with unknown disease etiology where whole exome sequencing has not identified coding mutations, thereby implicating alternative mechanisms. One recent example was the identification of autoimmune hyperphosphatemic tumoral calcinosis in a child exhibiting paradoxically high, unexplained levels of FGF23 cytokine, but showing resistance to FGF23 signaling (50). Targeted genetic analysis and whole exome sequencing of the affected child did not reveal mutations in any known or candidate genes. However, serological testing revealed that the child was robustly seropositive for autoantibodies against FGF23, and these anti-FGF23 autoantibodies were able to interfere with FGF23 signaling. This case study highlights how pathogenic autoantibodies directed against extracellular proteins associated with known genetic diseases can cause a similar clinical phenotype. These findings provide the rationale for evaluating autoantibodies against their corresponding extracellular targets as alternate mechanisms of disease pathogenesis.

Autoantibody Appearance Before Autoimmune Disease Diagnosis

The temporal appearance of autoantibodies before diagnosis is another parameter that distinguishes autoimmune diseases harboring autoantibodies against intracellular and extracellular autoantigens. These autoantibody studies are based on retrospective, longitudinal serum samples stored in biobank repositories. The first autoimmune disease to be interrogated for prediagnostic autoantibodies was T1D, in which autoantibodies against autoantigens such as insulin, GAD65, and IA2 are present approximately 4–10 years before patients require insulin replacement therapy (55). This lengthy interval of autoantibody seropositivity preceding T1D diagnosis corresponds to chronic subclinical autoimmune attack on the pancreatic beta cells that produce insulin (**Figure 3A**).

T1D is not unique in this respect. Studies of several other autoimmune diseases having mainly intracellular autoantigens also demonstrate a long subclinical phase. In systemic lupus erythematosus, seropositive autoantibodies against Ro52, Ro60,

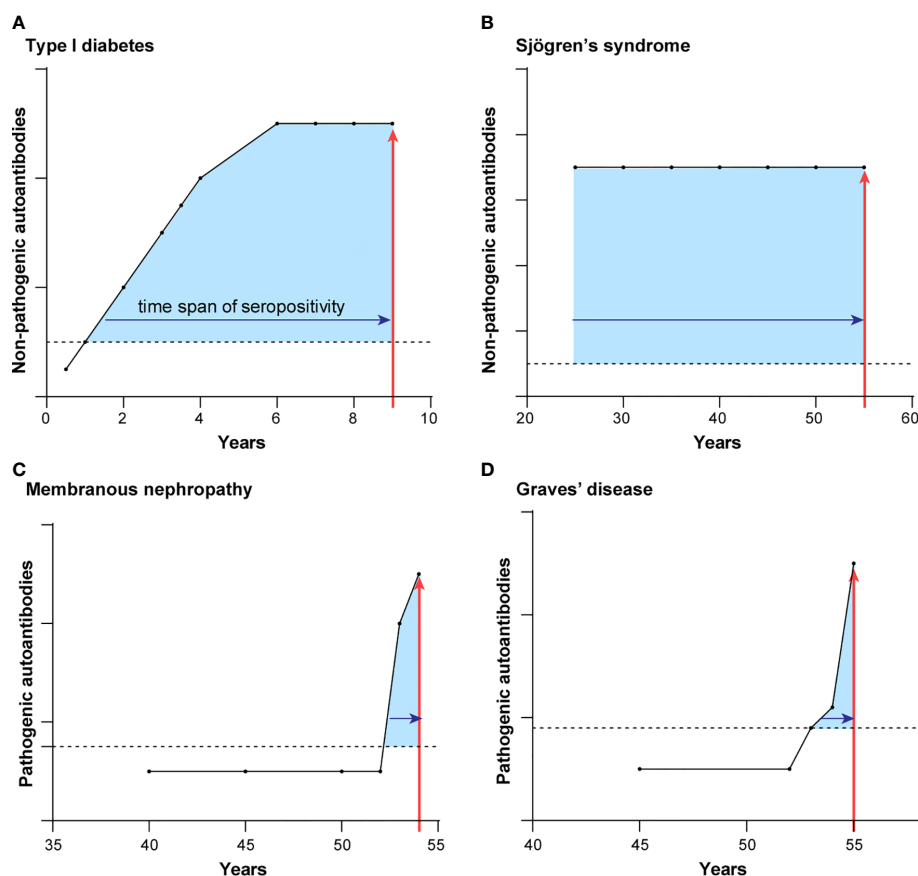


FIGURE 3 | Autoantibody appearance before autoimmune diagnosis differ for the two types of autoimmune diseases. Representative illustrations for the typical time course of prediagnostic autoantibodies before the diagnosis of diseases harboring autoantibodies to intracellular proteins (**A, B**) and extracellular proteins (**C, D**). Shown are the longitudinal appearance of autoantibodies in (**A**) T1D, (**B**) Sjögren's syndrome, (**C**) membranous nephropathy, and (**D**) Graves' disease. Time of autoimmune disease diagnosis is shown by the vertical red line arrow. The length of time of autoantibody seropositivity before the time of diagnosis is denoted by the shaded blue area under the curves.

and DNA were detectable on average 10 or more years before diagnosis (56). In rheumatoid arthritis (57, 58), Sjögren's syndrome (59), and systemic sclerosis (60), autoantibodies to a number of intracellular autoantigens were discovered that antedate the clinical diagnosis. In the case of Sjögren's syndrome (**Figure 3B**), autoantibodies against Ro52 and Ro60 are often detected in the earliest retrospective serum sample available and can appear up to 18 years before diagnosis (59). Detection of seropositive autoantibodies to intracellular proteins in advance of overt symptoms suggests that an underlying subclinical immune dysfunction may be present long before recognition of the clinical symptoms. It is important to note that for many of these diseases, the presence or extent of early tissue damage is simply unknown because longitudinal tissue biopsies are unavailable.

In contrast to the autoimmune diseases with autoantibodies against intracellular targets, the first detection of seropositive autoantibodies against extracellular autoantigen-driven diseases generally coincides with clinical diagnosis. This is consistent with the principle that these autoantibodies directly cause illness. In anti-GBM autoimmune disease, elevated autoantibodies against the collagen IV- α 3 autoantigen are only detectable approximately ≤ 1 year before diagnosis and not at earlier presymptomatic time points (61). However, in this same study autoantibodies against the intracellular MPO and PR3 were found years before autoimmune kidney disease onset at a average time of 3.25 years consistent with the possibility that they might reflect subclinical immune dysfunction. In membranous nephropathy, another autoimmune kidney disease, longitudinal analysis of future clinical cases showed that 56% of the seropositive patients became seropositive for PLA2R autoantibodies ≤ 1 year before diagnosis (**Figure 3C**) (62). Another 44% of membranous nephropathy cases showed PLA2R autoantibodies several years before diagnosis, reflecting the relapsing and remitting nature of this autoimmune disease (62). Graves' disease has provided particularly insightful information because this autoimmune disease shows autoantibodies to both intracellular and extracellular proteins (63). Pre-diagnostic samples from Graves' disease patients revealed that seropositive autoantibodies against the intracellular thyroid peroxidase were present at a frequency of 31, 49, and 57% at -7 years, -1 year and at the time of diagnosis, respectively (63). In contrast, autoantibodies against the extracellular thyroid-stimulating hormone receptor rose dramatically near the time of clinical presentation and diagnosis. Thyroid-stimulating hormone receptor autoantibody seropositivity was 2% at -7 years, 20% at -1 year, and 55% at the time of diagnosis (**Figure 3D**). These findings in Graves' disease highlight how autoantibodies against the intracellular protein can circulate for a long time, likely reflecting persistent, low-level autoimmune damage to the thyroid gland. However, the key drivers of productive, symptomatic presentation are the pathogenic autoantibodies that bind to extracellular thyroid-stimulating hormone receptor and thereby activate its signaling.

In summary, autoimmune diseases with autoantibodies against intracellular or extracellular proteins show markedly

different patterns of seropositivity during the course of the disease. Autoantibodies to extracellular targets appear close in time to diagnosis because they often cause the autoimmune disease. In contrast, autoimmune diseases harboring autoantibodies to intracellular proteins show detectable humoral responses several years, to even decades, before diagnosis, implying that autoimmune mechanisms are both active and persistent for a sustained period of time. It is possible that some subjects with such a prolonged subclinical phase could experience irreversible tissue damage, and this may in turn impede or prevent effective treatment. Nevertheless, the early warning sign indicated by autoantibody responses against intracellular autoantigens can potentially provide a window of opportunity to thwart the onset of frank autoimmune disease through interventional therapy.

Mechanistic Differences Imply Different Treatment Modalities for Autoimmune Diseases

Traditionally, autoimmune diseases have been treated with broadly immunosuppressive drugs including steroids, azathioprine, methotrexate, and cyclosporin, which inhibit many different types of immune cells. More recently, targeted therapies such as depletion of specific immune cell subpopulations, anti-cytokine blockade, or inhibition of immune cell signaling pathways are being used to treat autoimmune diseases. In this section, we describe various pathological mechanisms in different autoimmune diseases harboring autoantibodies against intracellular and extracellular proteins and focus on several diseases where this information guides specific treatment strategies.

Autoimmune diseases exhibiting pathogenic autoantibodies against extracellular target proteins often represent ideal cases to employ B lymphocyte-depleting therapies to reduce levels of deleterious autoantibodies. One targeted treatment approach that works well for many autoimmune diseases harboring pathogenic autoantibodies against extracellular targets is rituximab. This anti-CD20 monoclonal antibody therapy eliminates CD20-expressing B lymphocytes, but not plasma cells, stem cells or pro-B-cells. Rituximab acts by causing antibody-dependent complement cytotoxicity and antibody-dependent cell-mediated cytotoxicity, hence causing the death of the CD20-expressing B lymphocyte subpopulation (64, 65). As illustrated in **Figure 4**, rituximab shows efficacy for decreasing autoantibodies to extracellularly located autoantigens in a number of autoimmune diseases including anti-GBM disease (66), myasthenia gravis (67), neuromyelitis optica (68), pemphigus (69), and interferon-gamma autoantibody disease (70). Importantly, in those autoimmune diseases, a significant reduction in pathogenic autoantibodies typically coincides with clinical improvement.

In SLE, rituximab has proven therapeutic benefit and is able to decrease the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and significantly lower proteinuria (71). Anti-DNA autoantibodies are a key SLE biomarker, in which anti-DNA autoantibodies enhance cytokine production and can

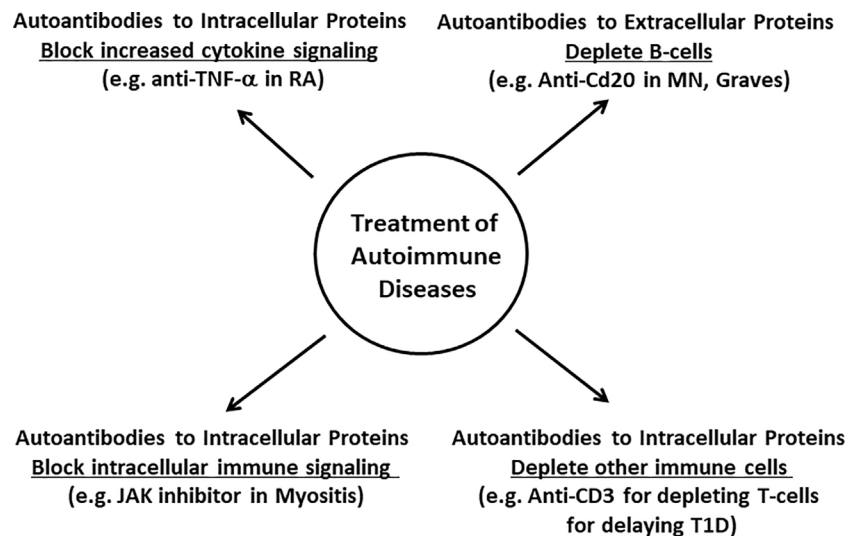


FIGURE 4 | Conceptual foundation for treating autoantibody diseases harboring either autoantibodies to intracellular or extracellular proteins. As shown above, autoimmune diseases with pathogenic autoantibodies to extracellular targets proteins can often be successfully treated with anti-CD20 therapy. In contrast, autoimmune diseases harboring autoantibodies to intracellular proteins require a more tailored approach involving drugs that deplete other immune cell types, block cytokines, or immune signaling pathways.

deposit in the kidney to cause pathogenesis (72). The clinical efficacy of rituximab in SLE may be due to its ability to decrease immune complex formation by anti-DNA and other autoantibodies and prevent complement activation, thereby limiting kidney and other tissue damage. In contrast, rituximab and other drugs targeting B-cells often have more variable outcomes when treating other diseases such as Sjögren's syndrome, or myositis. In Sjögren's syndrome, B-cell depletion with rituximab provides limited objective improvement of sicca symptoms (73–75). Rituximab shows variable efficacy for dermatomyositis or polymyositis (76). Another B-cell targeting biologic, belimumab, blocks B-cell activating factor, but it does not improve salivary flow or eye inflammation in Sjögren's syndrome (77). One limitation of using B-cell targeting drugs in diseases with intracellular autoantibodies such as Sjögren's syndrome is that patients typically seek therapy at late stages, by which time autoantibodies, immune activation, and tissue damage may have persisted for many years.

Many autoimmune disorders with autoantibodies to intracellular autoantigens often involve T-cell-mediated tissue destruction rather than B lymphocyte antibody-mediated damage. In type I diabetes, a large body of evidence suggests that T-cells play a major role in the destruction of pancreatic beta cells, consistent with the finding that T-cell targeted drugs can attenuate beta cell destruction (18). In accordance with these findings is the observation that anti-CD3 antibody therapy with the drug teplizumab, which kills T-cells, delays the progression of type I diabetes in high-risk patients by suppressing CD8+ lymphocytes and thereby blunting the T-cell mediated attack (Figure 4) (78).

Another strategy for treating autoimmune diseases is to target T-cell signaling pathways involved in immune activation. One

important molecule is the interferon- γ activated Janus kinase signal transducer (JAK) that acts downstream of cytokine signaling to activate STAT transcriptional targets (79). Presently, there are several JAK inhibitors in various phases of development (e.g., tofacitinib, baricitinib, and upadacitinib, figlotinib). The most well-studied among the FDA-approved JAK inhibitors is the orally-active small molecule, tofacitinib, which is therapeutically beneficial for rheumatoid arthritis and psoriatic arthritis (Figure 4) (80, 81). In patients with myositis-associated pulmonary disease harboring anti-MDA5 autoantibodies, tofacitinib showed promise by decreasing lung inflammation and improving pulmonary function (82). Many clinical trials with JAK inhibitors are ongoing and this class of drug shows significant promise for treating multiple autoimmune diseases.

Autoimmune diseases with autoantibodies against intracellular target proteins can also involve upregulated cytokine production as a driver of pathology. In several autoimmune diseases, one important treatment strategy is to counteract cytokine-mediated immune activation (Figure 4). In rheumatoid arthritis and psoriatic arthritis, elevated levels of the cytokine TNF- α mediates the inflammation that destroys joints and tissues (83, 84). For treatment of rheumatoid arthritis, several different TNF- α inhibitors, such as monoclonal antibody-based therapy (e.g., infliximab, adalimumab, golimumab), or a fusion protein that sequesters TNF- α and consists of the extracellular domain of TNF receptor 3 and IgG1-Fc (etanercept) are employed (85). In addition to elevated levels of TNF- α , gene expression profiling of SLE, myositis, and systemic sclerosis have identified a type I interferon activation signature (86). In SLE, levels of interferon- α cytokine are elevated and correlate with disease flare ups (87).

Based on these and other findings, sifalimumab, a monoclonal antibody that binds and blocks interferon- α activity, is efficacious for treating SLE (88), further supporting the key role of interferon-alpha signaling in the pathogenesis of this disorder (**Figure 4**). However, interferon alpha is only one of multiple but related cytokine proteins, and an alternative approach dampens signaling by targeting the common interferon alpha receptor (IFNAR) using the monoclonal antibody drug anifrolumab, which has been shown to reduce symptoms in moderate to severe SLE (89). A recent phase III trial of monthly anifrolumab met its primary endpoint and demonstrated a higher percentage of patients with a beneficial response compared to placebo; additionally, secondary analyses demonstrated decreased glucocorticoid use and reduced severity of skin disease (90).

In summary, autoimmune disease with pathogenic autoantibodies against extracellular targets will often respond to rituximab, if treated early. In contrast, autoimmune diseases characterized by autoantibodies mainly against intracellular proteins are typically driven by T-cells and other immune cells rather than B lymphocytes often do not, or only partially respond to rituximab. Tailored treatments for many of these diseases are less well-developed and involve therapies targeting several different types of immune cells, cytokines, and signaling pathways.

CONCLUSIONS

In this review, we describe how many autoimmune diseases can be segregated based on whether they have autoantibodies mainly against either extracellular or intracellular target proteins. This classification provides insight into mechanisms of autoimmunity, temporal appearance of the autoantibodies and rational foundations for treatment. While most patients with an autoimmune disease generally have one or the other type of autoantibodies exclusively, some patients show more complicated patterns. Some patients who initially feature autoantibodies against intracellular proteins may later acquire pathogenic autoantibodies to extracellular proteins as the disease progresses. This is best documented in Graves' disease, where autoantibodies against intracellular proteins appear early in the course of disease and autoantibodies against the extracellular thyroid stimulating hormone receptor develop later, coincident with clinical symptoms. As has been described for vasculitis, predominantly intracellular autoantigens such as PR3 and MPO are transiently expressed on the cell surface, and thereby they become accessible to autoantibody binding and are directly involved in disease pathogenesis. There likely are other, yet to be discovered, intracellular proteins that may be recognized by autoantibodies when presented transiently on the cell surface and thus can directly participate in the autoimmune process.

The segregation of autoimmune disease based on intracellular and extracellular autoantigens may also have limitations due to an incomplete assessment of autoantibodies. For example, several studies have shown that rituximab is beneficial in

systemic sclerosis (91, 92), a disease classified in this review as having autoantibodies only against intracellular autoantigens. Based on the positive clinical results following rituximab treatment, it is possible that unidentified pathogenic autoantibodies directed against extracellular targets are mediating autoimmunity and abnormal fibrotic matrix accumulation. Alternatively, the positive effects observed with rituximab may involve other functions of B-cells besides antibody production such as antigen presentation or interactions with immune cells.

There remain many unanswered questions about autoantibody production in the context of autoimmunity. For example, little is known about the source of B-cells producing the autoantibodies, whether there are B-cells located in ectopic lymphoid-like structures outside of the spleen, or whether lymph nodes are involved. In the case of Sjögren's syndrome, within the salivary glands of patients, germinal center-like structures have been found that produce the Ro52, Ro60, and La autoantibodies (93), although less is known about whether ectopic lymphoid-like structures contribute to autoantibody production in other conditions. In addition, new technologies can also be used to analyze autoantibodies, such as mass spectroscopy revealing that in certain autoimmune conditions the presence of public clonotypes of autoantibodies; common antibodies present in different patients (94, 95). This approach could be complemented by the cloning and sequencing of the B-cells producing autoantibodies. One recent study found that rheumatoid factor autoantibodies produced from the B-cells of Sjögren's syndrome patients had mutations in known B-cell lymphoma driver genes, potentially explaining the clonal expansion of autoantibody producing cells (95). Moreover, sequencing of the immunoglobulins from these cells revealed unique amino acid residues in the rheumatoid factor antibodies that cause insoluble aggregates of immunoglobulins to form, which likely explains why they precipitate from patients' sera at lower temperatures. Lastly, other technologies such as single-cell RNA sequencing and spatially resolved RNA sequencing could be exploited to characterize and provide new granular insight into the spatio-temporal alterations in immune cell populations and signaling present in the affected tissues of patients in the different autoimmune diseases.

In conclusion, a characterization of autoimmune disorders as we have presented here provides a framework for their mechanistic study and for developing appropriate therapeutic strategies. The observation that autoimmune disease with humoral responses against intracellular proteins have a prolonged seropositive incubation period suggests preemptive screening might identify patients who would benefit from early intervention to reverse or delay the onset of disease. Retrospective studies monitoring the exact temporal appearance of seropositive autoantibodies in parallel with other biomarkers may yield additional insight into potential environmental triggers and other factors that cause or drive disease progression. With regard to disorders caused by pathogenic autoantibodies against extracellular proteins, understanding mechanistic information about why some

autoimmune patients show spontaneous remission with the natural disappearance of their autoantibodies may yield new treatment approaches for these diseases. Future strategies will also be developed for treating pathogenic autoantibody diseases by targeting and ablating specific autoantibody-producing B-cells (96), which could provide substantial clinical benefit with fewer off-target side effects.

AUTHOR CONTRIBUTIONS

PB initially drafted the review. MI, JK, and BW contributed intellectually through multiple edits, revisions, and refinement of

the manuscript. PB and MI drafted the figures. All authors contributed to the article and approved the submitted version.

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Autoantibodies in the Diagnosis, Monitoring, and Treatment of Membranous Nephropathy

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The discovery of anti-podocyte antibodies in primary membranous nephropathy (MN) has revolutionized our approach toward the diagnosis and treatment of this disease. Evaluation of serum levels of anti-podocyte antibodies paved the way for non-invasive diagnosis and helped distinguish between primary and secondary MN although the relationship between anti-podocyte antibodies and cancer remains to be elucidated. Serum levels of anti-PLA2R antibodies directed against the major podocyte autoantigen are related to MN activity and the decrease in serum levels of anti-PLA2R antibodies in response to treatment (immunologic remission) also serves as an early indicator of the later putative proteinuric remission, enabling personalization of the treatment. The serum levels of anti-podocyte antibodies also enable the prediction of renal outcomes in terms of both remission and the risk of progression to end-stage renal disease. The positivity of anti-PLA2R antibodies before renal transplantation is associated with the risk of recurrence of MN. It remains to be established if all these relations observed in patients with anti-PLA2R antibodies are also valid for expanding spectrum of antibodies directed against recently discovered minor antigens (e.g., THSD7A, NELL-1, semaphorin 3B).

Keywords: membranous nephropathy, anti-PLA2R antibodies, anti-THSD7A antibody, remission, outcome

MEMBRANOUS NEPHROPATHY—A MAJOR CAUSE OF NEPHROTIC SYNDROME

Membranous nephropathy (MN) is defined by the typical histopathological patterns of thickening of the glomerular capillary wall with subepithelial immune deposits gradually embedded in newly formed glomerular basement membranes with positive granular immunofluorescence of IgG and C3 (1, 2). Although kidney biopsy is an invasive procedure, it remains the gold standard for the diagnosis of MN.

More than 80% of patients with MN present with nephrotic syndrome (3). MN is the cause of nephrotic syndrome in approximately 25% of adults (more frequently in males) and is the most common cause of nephrotic syndrome among older adults (4). The glomerular filtration rate at presentation is usually normal or only slightly impaired.

MN may be secondary, most often as one of the histologic classes (class V) of lupus nephritis, or pathogenetically related to cancer, hepatitis B, sarcoidosis, or some drugs (1, 2). However, in about 75% of patients with MN, the primary cause is not apparent, with the disease traditionally classified as idiopathic (until the discovery of anti-podocyte antibodies) or primary MN. Light microscopy does not reliably distinguish between primary and secondary MN. Immunofluorescent deposition

of IgG4 is typical for primary and IgG1, IgG2, and IgG3 for secondary MN with C1q often present in membranous (class V) lupus nephritis (1, 2).

Proteinuria and serum creatinine are still used to stratify the renal risk in patients with primary MN (5, 6). Patients with sub-nephrotic proteinuria have good long-term renal outcomes and should not be treated with immunosuppression. On the other hand, predicting renal outcomes in patients with nephrotic proteinuria is much more difficult as it ranges from spontaneous remission [in as much as 50% of patients during longer follow-up (7)] to the progression to end-stage kidney disease [in about 30% of patients within 10 years of follow-up (8)].

Due to the high propensity to spontaneous remission and toxicity of traditional treatment according to KDIGO guidelines (9) immunosuppressive treatment with alkylating agents is initiated only in patients with nephrotic syndrome persisting for at least 6 months, severe complications of nephrotic syndrome, or an increase in serum creatinine by 30% within 6–12 months.

EXPANDING SPECTRUM OF ANTI-PODOCYTE ANTIBODIES IN MN

The experimental model of MN, Heymann nephritis (10), was induced in its active form by the immunization of the rats with the material derived from the proximal tubule and in its passive form by injecting the rats with heterologous IgG to a crude rat tubular extract (11).

Heymann nephritis and human MN were always suspected to be autoimmune diseases putatively caused by anti-podocyte antibodies. As a proof of the concept in 1995, megalin, expressed by rat podocytes and on the luminal membrane of the cells of the proximal tubules (where it is involved in the uptake of a variety of proteins) was identified as an autoantigen of Heymann nephritis (12).

In humans, megalin is believed to be expressed only by the brush border of the proximal tubule, but not the human podocytes. Therefore, it could not be the target antigen in human MN. Recently, however, primary renal interstitial disease with anti-brush border antibodies and IgG-positive immune deposits along the tubular basement membrane, segmental subepithelial glomerular deposits, subnephrotic proteinuria, and circulating antibodies against megalin (LDL receptor-related protein 2—LRP2) was described (13, 14). Segmental MN in humans with anti-megalin antibodies is apparently due to some (although not as dense as in rats) expression of megalin in human podocytes (14). However, megalin is not the podocyte autoantigen in patients with typical MN.

The role of podocyte proteins in eliciting an immune response in MN was first demonstrated with the finding of neonatal MN in a child of a mother with a mutated MME gene for neutral endopeptidase (NEP), normally expressed by podocytes. During pregnancy, the mother raised antibodies against NEP of the fetus, which crossed the placenta and caused transient MN in the newborn (15).

However, the major podocyte autoantigen in MN was only identified in 2009 as the phospholipase A2 receptor [PLA2R

(16)]. Anti-PLA2R antibodies occur with disease presentation in about 70–80% of patients (more often men). The presence of anti-PLA2R antibodies is highly specific for primary MN. However, they may also be present in some patients with secondary MN (sarcoidosis or HBV-associated MN).

Since 2009, several minor podocyte autoantigens have been identified in patients with MN: antibodies against thrombospondin type-1 domain-containing 7A [THSD7A (17)] are present in 3–5% of patients (more often women) (6), antibodies against neural epidermal growth factor-like 1 protein [NELL-1 (18)] are present in 5–10% of patients, and antibodies against semaphorin 3B (19) are present namely in pediatric patients. Anti-NELL-1 antibodies were shown to be much more common in segmental MN (20). Very recently, antibodies against protocadherin 7 (PCDH7) and high temperature recombinant protein A1 /HTRA1, both of IgG4 subclass, were described in several patients with anti-PLA2R-negative primary membranous nephropathy (21, 22).

Additionally, antibodies directed against exostosin-1 and exostosin-2 (EXT-1 and EXT-2) were found to be specific for lupus MN (23) and antibodies against neural cell adhesion molecule-1 (NCAM-1) occur in about 6% of patients with lupus MN, but may also occur in 2% of patients with primary MN (24). In membranous lupus nephritis EXT1/EXT2-positive patients have compared to EXT1/EXT2-negative patients more often nephrotic proteinuria, but less glomerulosclerosis and interstitial fibrosis. EXT1/EXT2-negative patients progressed to end-stage kidney disease more quickly and more frequently (25).

Antibodies to EXT1/EXT2, NELL-1 and Sema3B are of IgG1 subtype and are complement-activating, antibodies to PCDH7 are, similarly as anti-PLA2R antibodies predominantly of IgG4 subtype and do not activate the complement.

Different autoantibodies may thus be associated with different pathogenesis, pattern of histologic damage and different clinical phenotype including response to treatment and outcome (26).

All as-yet identified autoantigens are responsible for up to 90% of cases of idiopathic (primary) MN, so the future discovery of further minor antigens cannot be excluded (27). Approximately 1% of patients with MN may have double positivity for both anti-PLA2R and anti-THSD7A antibodies (28, 29). In a large cohort of 1,012 patients with biopsy-proven MN, anti-THSD7A antibodies were identified in 2.8% of patients, eight of which (0.8%) were double positive for anti-THSD7A and anti-PLA2R antibodies (30).

Double positivity of MPO-ANCA and anti-PLA2R was described in two patients with ANCA-associated glomerulonephritis combined with MN-like lesions (31). In other studies necrotizing/crescentic glomerulonephritis was described in a small proportion (about 0, 3%) of patients with membranous nephropathy (32, 33). Although most of these patients have concomitant ANCA or anti-GBM antibodies this histologic pattern was observed also in several patients with anti-PLA2R antibodies only. It remains unclear if anti-GBM damage caused by ANCA or anti-GBM antibodies may expose podocyte antigens, or, on the other hand, subepithelial deposits in membranous nephropathy may enhance damage to GBM by ANCA or anti-GBM antibodies.

GLOMERULAR BINDING OF ANTI-PLA2R ANTIBODIES

The binding of anti-PLA2R IgG4 antibodies to podocytes can usually be detected in patients with MN using immunofluorescence or immunohistochemistry. However, it is not always parallel to circulating anti-PLA2R antibodies, if measured at the time of biopsy (34, 35). Although glomerular binding of anti-PLA2R antibodies is highly specific for primary MN, it also occurs in some patients with HBV, sarcoidosis, or cancer-associated secondary membranous nephropathies.

Glomerular binding of anti-PLA2R antibodies may persist for months even after the systemic autoimmune response is suppressed. Alternatively, the absence of circulating anti-PLA2R antibodies is observed in some patients with active MN and glomerular anti-PLA2R binding. It is speculated that the lack of circulating antibody detection could be due to the high avidity of anti-PLA2R antibodies to the podocytes (35).

In some patients with MN positive for glomerular PLA2R staining, circulating antibodies may not be detectable at presentation, but may occur later during follow-up (and treatment) of the disease (36). This seroconversion could be explained by the buffering capacity of the kidney, that is, circulating antibodies occur only after the binding capacity of the kidney is exceeded, emphasizing the importance of the evaluation of glomerular PLA2R staining or repeated evaluation of anti-PLA2R in anti-PLA2R negative patients, especially during the first 6-months of follow-up.

In some patients with positive glomerular staining, circulating anti-PLA2R antibodies may only become detectable at 12 months, or even during late relapse at 28 months (37). There can be even an early absence of glomerular IgG4-anti-PLA2R positive staining, as some patients may have initially IgG1 dominant immune deposits (potentially with another podocyte target antigen), that are PLA2R negative. This suggests putative later histologic conversion (to IgG4-PLA2R staining) which could be demonstrated only through repeat kidney biopsies (38). Positive glomerular staining of anti-THSD7A (39) and anti-NELL-1 antibodies (18) was also described in patients with MN with autoantibodies directed to these minor autoantigens, respectively.

ANTI-PODOCYTE ANTIBODIES IN THE PATHOGENESIS OF MN

The role of complement in the pathogenesis of MN was demonstrated by the deposition of C3 and C5b-9 in both Heymann nephritis and human MN. Moreover, glomerular injury in Heymann nephritis can be prevented by blocking the formation of the C5b-9 complex on the podocyte membrane (40).

Although the direct pathogenic role of anti-podocyte autoantibodies in MN has been supported by the observation in passive Heymann nephritis induced in rats by injection of IgG against tubular extract (11), in humans, it was difficult to reconcile the preponderance of non-complement activating IgG4 anti-PLA2R antibodies (at least at a later

course of anti-PLA2R antibody-positive MN) with the well-documented complement activation. One putative explanation could be the activation of the alternative complement pathway in MN recently demonstrated using proteomic analysis of microdissected glomeruli (41), which may be preceded by earlier glomerular deposition of other IgG subclasses that activated the classical complement pathway (35). The recently described anti-NELL-1 antibody is predominantly of the IgG1 class and maybe the cause of prominent C3 deposits (40). The role of complement regulatory proteins in the pathogenesis of MN also remains to be elucidated (42).

THSD7A (unlike PLA2R1) is expressed in human and murine podocytes. As a result, it was possible to show that administration of human anti-THSD7A antibodies to mice results in their binding to murine podocytes with subsequent foot process effacement, histologic changes typical of MN and proteinuria (43). However, in this model, proteinuria preceding complement deposition was only small and transient (44).

The pathogenic role of anti-PLA2R antibodies was recently demonstrated in a mouse model in which the major target antigen, human PLA2R, was expressed in mouse podocytes *in vivo* (45). In this model the administration of heterologous rabbit anti-PLA2R antibodies (with no data on IgG subclass) increased dose-dependently proteinuria (to the nephrotic range) with limited C3 deposition, leaving the proportional role of IgG4 predominant anti-PLA2R antibodies and membrane attack complex (C5b-9) unclear. It is conceivable that the early rise of proteinuria in experimental models of MN may be complement-independent as both anti-THSD7A and anti-PLA2R antibodies were shown to directly mediate podocyte damage in MN. However, their interaction with complement activation (if any) remains to be elucidated.

There is currently ongoing trial in anti-PLA2R antibody-positive primary membranous nephropathy with an oral inhibitor of factor B iptacopan (LNP023) directed of the alternative complement pathway (NCT04154787) and one small phase 2 safety trial with anti-MASP2 monoclonal antibody narsoplimab (NCT02682407) inhibiting complement lectin pathway.

Epitope spreading may play an important role in the severity of podocyte injury. Commonly in autoimmune diseases, primary response to immunodominant antigens may expand to another epitope on the same protein or to dominant epitopes on neighboring molecules (6, 46). It has been shown (47) that anti-PLA2R antibodies may be restricted only to the cysteine-rich (CysR) domain, or the anti-PLA2R response may also spread the C-type lectin domain 1 (CTLD1) and C-type lectin domain 7 (CTLD7) of PLA2R. Epitope spreading was shown to be associated with a more severe course of the disease.

GENETICS OF MN AND ANTI-PODOCYTE ANTIBODIES

An early GWAS in 556 European patients (48) identified two loci strongly associated with MN: the PLA2R1 gene on chromosome 2q24 and HLA-DQA1 on chromosome 6p21 with homozygosity

for both alleles, increasing the risk of MN more than 78 times. A similar kind of association (allele in the HLA system likely facilitating the response against the target antigen and the risk allele of the target antigen itself) has also been described for PR3-positive ANCA-associated vasculitis (49). The strong association of three single nucleotide polymorphisms (SNPs) within PLA2R1 and one within HLA-DQA1 was confirmed in 1,112 Chinese patients with MN (50). Risk alleles were associated with the presence of anti-PLA2R antibodies and their glomerular expression. Retrospective analysis of anti-PLA2R antibodies and genotyping of DQ alleles and analysis of PLA2R1 SNPs performed in 90 prevalent patients with idiopathic MN with a 90-month follow-up demonstrated that levels of anti-PLA2R antibodies were significantly linked to DQA1*05:01 and DQB1*02:01 (51).

A recent large GWAS in 3 782 patients of European and East Asian ancestry (52) identified two other risk loci (NFKB1 and IRF4) and provided a fine mapping of PLA2R1 (rs178312151). Interestingly, this GWAS demonstrated the ancestry-specific effect of HLA alleles: DQA1*0501 in Europeans, DRB1*1501 in East Asians, and DRB1*0301 in both ethnicities with identified loci explaining the 32% disease risk in East Asians and 25% in Europeans. All these studies clearly showed that MN and more specifically an autoimmune response to PLA2R are strongly genetically mediated. The genetic contribution of the identified risk loci is much stronger in MN compared with IgA nephropathy, where it explains no more than 5% of the overall disease risk (53).

ANTI-PODOCYTE ANTIBODIES AND NON-INVASIVE DIAGNOSIS OF MN

As anti-podocyte antibodies are highly specific for primary MN [well-documented for anti-PLA2R antibodies, where the specificity is 98–100% (54)] it has been suggested that the diagnosis of primary MN may be assessed in patients with nephrotic syndrome, normal renal function and positive anti-PLA2R antibodies even without renal biopsy (54). However, there are several drawbacks. Anti-PLA2R antibodies should be measured with ELISA, and the reference ranges should be better established (55). Limited data are available concerning anti-PLA2R antibody specificity in non-white populations. Larger studies are required to help to define the positive predictive value of anti-PLA2R positivity as this testing is usually performed in selected patient populations with and without nephrotic syndrome and, anti-PLA2R positivity was shown to precede clinical diagnosis of MN by months to years (56). In these patients, however, anti-PLA2R positivity could predict the future development of nephrotic syndrome. As expected, increasing sensitivity can be achieved only at the expense of decreasing specificity [$<75\%$ with a low cutoff level (57)].

The specificity of anti-PLA2R and anti-THSD7A antibodies is very high in terms of comparing patients with MN and other glomerular diseases. Both autoantibodies are also more frequent in primary MN (58); however, specificity with which anti-PLA2R positivity excludes secondary MN, especially cancer-associated

MN is lower. Similarly, anti-THSD7A antibodies are highly specific for MN; however, they also do not reliably distinguish between primary and secondary MN (39).

Of note, anti-PLA2R positivity cannot be used as a non-invasive diagnostic test in patients with impaired renal function and also, in patients with suspected concomitant diabetic kidney disease or other glomerular diseases. Renal biopsies also provide an estimate of the degree of tubulointerstitial damage, strongly associated with renal outcomes and may also have a substantial impact on the response to immunosuppressive treatment. Systemic disease, although rare, namely sarcoidosis or antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, should also be considered in patients with anti-PLA2R positive antibodies. Rare necrotizing/crescentic glomerulonephritis in MN cannot be also recognized without renal biopsy (32, 33).

ANTI-PLA2R AND ANTI-THSD7A ANTIBODIES AND CANCER

According to a recent meta-analysis (59), the prevalence of cancer in MN is 10% (most commonly lung cancer in 26% of patients, followed by prostate cancer in 15% of patients) with a diagnosis of cancer preceding the diagnosis of MN in 20% of patients.

THSD7A is expressed by some cancers and it has been suggested that anti-THSD7A antibodies are associated with an increased risk of cancer-associated MN (60), and screening for cancer was recommended in patients with anti-THSD7A positive MN (61).

In a large Chinese study, anti-THSD7A antibodies were positive in only 2% of patients with cancer-associated MN (29). Surprisingly, 41% of patients with cancer-associated MN had anti-PLA2R1 antibodies. In another Chinese study (62), 33% of patients with cancer-associated MN were positive for both circulating anti-PLA2R antibodies and glomerular (IgG4-dominant) PLA2R staining. Although 56% of patients had THSD7A positive staining in cancer tissues, circulating antibodies were not detected. These studies suggest that anti-PLA2R antibodies may be quite commonly (and anti-THSD7A uncommonly) positive in (at least Chinese) patients with cancer-associated MN. However, it is unclear whether these findings are purely coincident, or if PLA2R antibodies participate in the pathogenesis of cancer-associated MN.

In a recent study in Caucasian patients (63), anti-PLA2R antibodies were positive in 75% of patients with idiopathic MN and in 9 out of 32 (28%, in seven patients with cancer) with secondary MN. The specificity of anti-PLA2R antibodies decreased from 100 and 94.6% compared to normal and pathological (other glomerulonephritides) controls to only 71.9% vs. secondary MN.

Antigen-specific IgG subclasses and their association with cancer were studied in patients with anti-PLA2R antibodies or anti-THSD7A antibodies (64). All patients had the most frequent IgG4 antibodies. In patients with idiopathic MN, IgG subclass distribution levels of IgG4 antibodies were similar to those of patients with malignancy-associated MN. This suggested that the

pathogenesis of primary and malignancy-associated MN with either anti-PLA2R or anti-THSD7A antibodies may be similar to those in primary MN.

In the consecutive series of anti-PLA2R and anti-THSD7A negative patients 3.8% had **NELL-1** as a target antigen with segmental or incomplete IgG staining dominantly of IgG1 subclass. Out of them **33% had concurrent malignancy** which is higher proportion than in anti-PLA2R and anti-THSD7A positive patients (65).

Although cancer is more common in anti-PLA2R antibody-negative patients, diagnostic workup for cancer cannot be completely avoided, even in patients with anti-PLA2R positive antibodies (66). Elucidation of mechanisms underlying anti-podocyte autoimmunity induced by cancer warrants further research.

ANTI-PODOCYTE ANTIBODIES AND THE OUTCOME OF MN

Up to a third of patients with MN may develop spontaneous remission and can be spared immunosuppressive treatments, sometimes associated with serious adverse events. This is reflected by the recommendation that immunosuppressive treatment should be postponed by as many as 6 months in patients without poor prognostic signs, such as impaired glomerular filtration rate and high-grade proteinuria (9).

The chance of developing spontaneous remission was shown to be related to the titer of anti-PLA2R antibodies (67–70) and is comparatively low in patients with high titers of anti-PLA2R antibodies who could be treated with immunosuppression without unnecessary delay.

Spontaneous remission occurred significantly less often in patients with high antibody titers [38 vs. 4% in the lowest and highest tertiles (68)]. Patients with low anti-PLA2R levels had 2.72 times higher rates of spontaneous remission after a median follow-up of 2.9 years. Alternatively, high anti-PLA2R levels were associated with persistent proteinuria and a need for immunosuppressive therapy (70). Complete spontaneous remission was also more common in patients with lower anti-PLA2R levels at presentation (71).

The titer of anti-PLA2R antibodies was also closely linked to the outcome in terms of a higher risk of declining renal function during a follow-up period of 90 months (51). In another prospective study in patients with MN and positivity for anti-PLA2R antibodies with a median follow-up of 27 months, clinical endpoint (defined as an increase in serum creatinine by $\geq 25\%$ and serum creatinine reaching ≥ 1.3 mg/dL) was reached in 69% of patients in the highest tertile of the titer of anti-PLA2R antibodies compared to only 25% of patients in the lowest tertile at inclusion into study. The study endpoints were also significantly shorter in patients with the highest compared to the lowest tertile of anti-PLA2R antibodies [17.7 vs. 30.9 months (72)]. In another study, however, long-term renal survival (at 5, 10, and 15 years)

was high in all tertiles of anti-PLA2R antibodies (97, 93, and 89%, respectively), and was only related to the severity of proteinuria (70).

Poor renal outcomes in patients with anti-PLA2R antibodies may also be associated with epitope spreading of anti-PLA2R autoimmune response with less frequent nephrotic syndrome at presentation, a higher rate of spontaneous remission, and lower risk of progression to renal failure in patients with anti-PLA2R antibodies restricted to cysteine-rich domain of the PLA2R, compared with patients with concomitant positivity of antibodies to C-type lectin domain 1 and C-type lectin domain 7 (47). Epitope spreading is strongly correlated with titers of anti-PLA2R antibodies and a lower rate of remission at 6 months (73).

Epitope spreading is thus an important independent predictor of poor outcomes, and patients with epitope spreading should be treated without delay with higher doses of rituximab. As epitope-specific assays for anti-PLA2R are not available, epitope spreading cannot be evaluated in clinical practice and we must rely on the correlation of epitope spreading with anti-PLA2R titers (73). Similar to patients with high titers of anti-PLA2R antibodies, patients with high titer of anti-THSD7A antibodies also have poor clinical outcomes (30).

In summary, low baseline and decreasing anti-PLA2R antibody levels strongly predict spontaneous remission, thus favoring conservative therapy. Conversely, high baseline or increasing anti-PLA2R antibody levels are associated with nephrotic syndrome and progressive loss of kidney function, thereby encouraging prompt initiation of immunosuppressive therapy (58).

ANTIBODIES AND TRANSPLANTATION

Up to 40–50% of patients with primary MN (if untreated) may finally develop end-stage renal disease. MN may recur in transplant in 30–45% of patients with recurrence being more frequently reported by centers performing regular protocol biopsies (74). Anti-PLA2R antibodies are detected in 70–80% of patients with recurrence of MN in the renal allograft (75). The risk of recurrence is higher in patients with positive anti-PLA2R antibodies before transplantation (76, 77) and with anti-PLA2R antibody staining in renal allografts (77).

Using cutoff levels of circulating anti-PLA2R antibodies of 45 U/mL, the recurrence of MN was predicted with a sensitivity and specificity of 85% and a negative predictive value of 92%. Anti-PLA2R levels and recurrence of MN in the allograft were shown to be genetically mediated [associated with HLA DQA1* 05:01/05 and DQB1* 02:01 (77)]. In another study (78), anti-PLA2R antibodies were positive prior to transplantation in 83% of patients who experienced recurrence.

In the patient with anti-THSD7A-associated MN, who reached end-stage renal failure and was subsequently transplanted with early recurrence of MN in the graft, the anti-THSD7A antibodies were detectable both before and after transplantation. The staining for THSD7A was also documented

in the allograft, suggesting the pathogenic role of anti-THSD7A antibodies (43).

ANTI-PODOCYTE ANTIBODIES AND ACTIVITY OF MN

Titers of anti-PLA2R antibodies are strongly correlated with the clinical activity of the disease and proteinuria (67, 68, 79). This is demonstrated by the antibody titers in patients with active disease compared with patients with partial or complete remission (51). The reemergence of circulating antibodies predicts disease relapse (58, 80).

Titers of anti-THSD7A also decrease (or the antibody completely disappears) with the development of remission and once again reappear (increase) with the relapse of the disease (29). High titers of anti-THSD7A are also correlated with the activity of the disease. However, due to the disease sparsity, the evidence is less convincing than that of the PLA2R antibodies (30). Regular measurement of the levels of anti-PLA2R and anti-THSD7A antibodies may thus be used for monitoring the activity of the disease.

ANTI-PODOCYTE ANTIBODIES AND RESPONSE TO TREATMENT

Patients with primary MN are currently treated with either alkylating cytotoxic drugs, calcineurin inhibitors, B-cell depleting monoclonal antibody, or rituximab. Alkylating cytotoxic drugs are effective, but their use is associated with significant adverse events. The use of calcineurin inhibitors is associated with a high relapse rate on their tapering and withdrawal.

Reduction in the levels of circulating anti-PLA2R antibodies preceded by several months and predicted a decrease in the proteinuria in rituximab-treated patients with MN (68, 81–83).

In 133 adult patients with anti-PLA2R positive MN, the antibody levels decreased after 3 months of immunosuppressive treatment by 81% and proteinuria by 39% (72). Patients who developed remission 12 months later had significantly lower levels of anti-PLA2R at baseline compared to patients with no remission. Moreover, patients with high levels of anti-PLA2R antibodies achieved remission later than patients with low levels. The antibody levels remained elevated in patients who did not achieve remission of proteinuria.

In another study, 84 out of 132 rituximab-treated patients with idiopathic MN achieved complete or partial remission during a median follow-up of 30.8 months and 25 relapsed after remission (80). In 81 patients with positive anti-PLA2R antibodies, remission was strongly predicted by a lower antibody titer prior to treatment. These patients had complete antibody depletion 6 months after the initiation of rituximab treatment. In all patients, depletion of anti-PLA2R antibodies preceded complete remission. Early reduction of the titer of anti-PLA2R antibody by 50% was associated with a decrease in proteinuria by 50% by 10 months. In this study, response to rituximab treatment was not related to the polymorphisms of both PLA2R1 and HLA-DQA1.

The role of immunologic remission (depletion of anti-PLA2R antibodies) in MN was recently demonstrated in three randomized controlled trials, GEMRITUX (84), MENTOR (85) and STARMEN (86).

In the GEMRITUX trial (84), 75 patients with biopsy-proven MN and nephrotic syndrome were randomized either to rituximab or non-immunosuppressive antiproteinuric treatment. Anti-PLA2R depletion was achieved at 6 months in 50% of patients treated with rituximab and only in 12% of patients treated with non-immunosuppressive antiproteinuric treatment. Although at 6 months there was no significant difference between both limbs in combined complete and partial remission (35.1 vs. 21.1%), at the end of observational phase (after median follow-up of 17 months) remission rates were significantly higher in patients treated with rituximab compared with patients treated with non-immunosuppressive antiproteinuric treatment (64.9 vs. 34.2%). Early responses in terms of anti-PLA2R levels predicted the later development of clinical remission.

In the MENTOR trial (85), 130 patients were randomized to rituximab or cyclosporine. Early response (at 12 months) to either rituximab or cyclosporine was similar (complete and partial remission induced in 60% vs. 52% of patients, respectively). However, at 24 months, complete or partial remission was much more frequent in rituximab- than in cyclosporine-treated patients (60 vs. 20% of patients, respectively). It should be, however, stressed, that cyclosporin was withdrawn already at 12 months, so these data reflect namely the risk of early relapse after cyclosporine withdrawal. Importantly, the decline of anti-PLA2R antibodies was faster and of greater magnitude and duration in rituximab vs. cyclosporine. This again demonstrated that immunological remission (depletion of anti-PLA2R antibodies) was indispensable for clinical remission.

In a recently published STARMEN trial (86) which randomized 86 pts with membranous nephropathy at high risk or progression to either cyclical alternating treatment with corticosteroids and cyclophosphamide or tacrolimus followed by single dose of rituximab complete or partial remission of nephrotic syndrome at 24 months was reached in 83.7% of patients treated with corticosteroid-cyclophosphamide and in only 58.1% of patients treated with tacrolimus and rituximab. Immunological remission (depletion of anti-PLA2R antibodies) was achieved at 3 and 6 months in 77 and 92%, respectively, of pts in corticosteroid-cyclophosphamide limb compared to 45 and 70% of patients in the tacrolimus-rituximab limb—in fact, it reflects the efficacy of tacrolimus only as rituximab was given only at 6 months). Corticosteroid-cyclophosphamide treatment thus induced compared to tacrolimus-rituximab more often both complete and partial remission and induced immunological remission more quickly.

In a small open-label study belimumab (monoclonal antibody against B lymphocyte stimulating factor BAFF-BLyS) decrease of proteinuria was preceded by the decrease of the titer of anti-PLA2R antibodies (87). There is currently ongoing clinical trial (REBOOT, NCT03949855) in anti-PLA2R positive membranous nephropathy comparing the combination of belimumab with rituximab with rituximab only.

Rituximab was also shown to reverse epitope spreading in almost 60% of treated patients (88). In the GEMRITUX trial (84), epitope spreading was strongly correlated with the titer of circulating anti-PLA2R antibodies, and reversal of epitope spreading was observed in 10 out of 17 patients (58%) with epitope spreading at baseline treated with rituximab.

Available data suggest that the early decrease in levels of anti-PLA2R antibodies is a predictor of later clinical remission, which may develop slowly within more than 1 year. As the chance to develop clinical remission is very high in patients with treatment-induced depletion of anti-PLA2R antibodies, no further immunosuppression (e.g., maintenance treatment with rituximab) may be indicated. Instead, monitoring and waiting may be a safer and more reasonable approach (2, 58, 80).

Alternatively, a decrease in proteinuria without an immunological response (as in some patients treated with cyclosporine) should not be interpreted as real remission. There is a high risk of relapse when treatment (most frequently cyclosporine) is withdrawn. Persistence of high titer of anti-PLA2R antibody is a sign of ongoing immunologic activity (and possibly ongoing podocyte damage) despite lower proteinuria. Thus, the efficacy of any immunosuppressive treatment should be assessed based on the induction of immunologic remission. The reappearance of anti-PLA2R positivity and/or an increase in the previously decreased titer of anti-PLA2R antibody is a clear sign of the impending relapse of MN, sometimes preceding the rise of proteinuria. Serial measurements of anti-PLA2R antibodies (monthly, or at least bi-monthly) during and at the end of the cycle of immunosuppressive treatment may help personalize the treatment of MN (2).

Response to treatment in anti-PLA2R positive patients is characterized by a rapid decrease in the titer of anti-PLA2R antibodies. The response to treatment is worse in patients with high levels of anti-PLA2R antibodies before the start of treatment. Levels of anti-PLA2R antibodies are independent

predictors of response to treatment (in terms of achieving remission). Levels of anti-THSD7A antibodies are also related to the response to immunosuppressive treatment. In a subgroup of patients with serial titers, persistently elevated anti-THSD7A autoantibodies were observed in patients who wither did not respond to treatment or achieve remission (30). Hopefully, our experience will be soon extended to patients with anti-NELL-1 antibodies (18).

CONCLUSIONS

Identification of anti-podocyte antibodies elucidated the pathogenesis of primary MN and enabled its non-invasive diagnosis. Titers of anti-podocyte antibodies predict the renal outcome and response to treatment and help personalize the immunosuppressive therapy. Persistent remission and prevention of progression to end-stage renal disease cannot be achieved without suppression of the podocyte-directed autoimmune response. With the identification of several further minor podocyte antigens classification of primary membranous nephropathy will be based on the type of autoantibody, its IgG subclass and potential to activate complement resulting possibly in the future preference of either B cell- or complement-targeted therapeutic approaches.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the review of the available literature and preparing the manuscript.

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Case Report: Expanding Clinical, Immunological and Genetic Findings in Sideroblastic Anemia With Immunodeficiency, Fevers and Development Delay (SIFD) Syndrome

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Since the first description of the syndrome of sideroblastic anemia with immunodeficiency, fevers and development delay (SIFD), clinical pictures lacking both neurological and hematological manifestations have been reported. Moreover, prominent skin involvement, such as with relapsing erythema nodosum, is not a common finding. Up to this moment, no genotype and phenotype correlation could be done, but mild phenotypes seem to be located in the N or C part. B-cell deficiency is a hallmark of SIFD syndrome, and multiple others immunological defects have been reported, but not high levels of double negative T cells. Here we report a Brazilian patient with a novel phenotype of SIFD syndrome, carrying multiple immune defects and harboring a novel mutation on TRNT1 gene.

Keywords: SIFD, recurrent fever, erythema nodosum, B-cell deficiency, TRNT1

INTRODUCTION

A syndrome characterized by sideroblastic anemia, with associated B-cell immunodeficiency, periodic fevers and development delay (SIFD), was first described as an isolated entity in 2013, followed by the molecular identification of the causative gene, TRNT1, by the same group in 2014 (1, 2). Initially, diagnostic criteria specified the presence of clinical signs referred to in the acronym associated with the syndrome, yet the first report also highlighted several other observable clinical signs. Over time, pleiotropic clinical manifestations were observed, notably in the absence of sideroblastic anemia, developmental delay and a broad spectrum of immunological defects. Moreover, skin manifestations were not commonly observed in this disorder (3, 4).

The gene encoding transfer RNA (tRNA) nucleotidyl transferase 1 (TRNT1) is responsible for the formation of an enzyme essential to the synthesis of the 3'-terminal CCA sequence in tRNA molecules in the nucleus and mitochondria. Mutations in gene TRNT1 result in partial loss-of-function defects, leading to metabolic abnormalities in both the mitochondria and cytosol that

account for the multiple phenotypes thus far reported (5). At the time of this publication, no genotype and phenotype correlations had been reported.

Here we report a novel SIFD phenotype characterized by multiple immunological defects in a Brazilian patient harboring a novel bi-allelic mutation in gene *TRNT1*.

PATIENT AND METHOD

Clinical Data and Genomic Sequencing

Clinical data were retrieved from the patient's records after her parents provided written consent for the publication of any potentially identifiable images or data included herein. Genomic DNA was extracted from blood samples using a QIAamp® DNA Blood Maxi Kit (Qiagen®, Valencia, CA, USA). PBMCs were obtained by density gradient centrifugation ($d=1.077$ g/ml). Primers directly targeting exon 4 of the *TRNT1* gene were designed. Sanger sequencing was performed for genetic confirmation and familial segregation in accordance with standard procedures.

Quantification of B and T Lymphocyte Phenotypes by Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were stained with titrated mouse anti-human monoclonal antibodies (mAbs) (all from BD Biosciences). Fluorescence minus one (FMO) control was set up for CD45RA marker.

Flow cytometry was performed in FACSCanto II (BD Biosciences) and the analyses were made in FlowJo 9.9.5 software (TreeStar Inc, San Carlos, CA, USA). After exclusion of cell doublets, sequential gating of PBMC was performed in the

lymphocyte region. For T lymphocytes, after gate of CD3+ T cells, followed by discrimination of CD8+ and CD4+ markers, we analyzed the T lymphocyte naïve/memory subpopulations through Boolean gates: T naïve (CD45RA⁺CCR7⁺CD27⁺), T central memory (TCM) (CD45RA⁻CCR7⁺CD27⁺), T effector memory (TEM) (CD45RA⁻CCR7⁻CD27⁺) and T effector memory with RA re-expression (TEMRA) (CD45RA⁺CCR7⁻CD27⁻). To analyze the phenotypes CD3⁺ TCRαβ CD4⁺ CD8⁺, CD3⁺ HLA-DR⁺ and CD3⁺ B220⁺, the cells were gated on CD3⁺ region, after exclusion of doublets and death cells. The phenotype CD27⁺ was analyzed in B lymphocyte region (CD20⁺ cells).

Lymphocyte subsets absolute counts were calculated using the percentages obtained in flow cytometry. The subset percentages analyzed were referred to total lymphocyte counts for T and B cells.

RESULTS

Clinical Case

The patient, a 3-year-old Brazilian female, was born to non-consanguineous healthy parents and experienced recurrent episodes of fever since the first month of life. Initially, febrile episodes were characterized by high fever lasting five days and associated with arthritis and dactylitis. In her ninth month of life, the appearance of a diffuse painful skin eruption was noted, marked by nodules and plaques, erythematodematous rash and infiltrate, resembling erythema nodosum (**Figure 1**). The child was admitted on an almost monthly basis; on some occasions her symptoms were attributed to defined infections (mono-like), yet in other instances infections were undefined—these were somewhat responsive to systemic antibiotics. In her eleventh month of life,

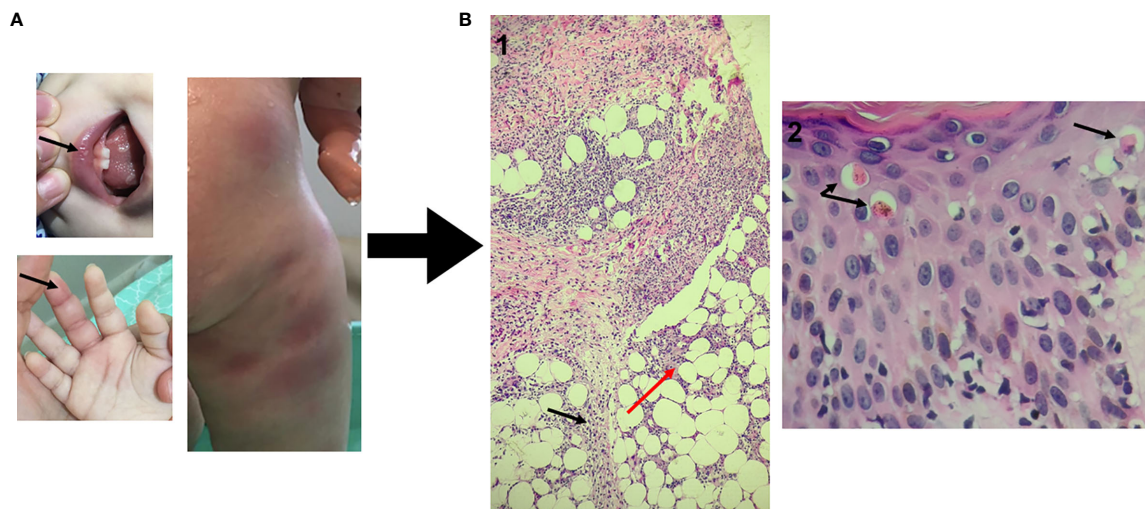


FIGURE 1 | Dermatological and histopathological findings. **(A)** - Oral ulceration and dactylitis (black arrows) and skin rash observed during fever flares resembling erythema nodosum. **(B)** Hematoxylin-eosin staining of skin biopsy (punch) previously fixed in 10% neutral buffered formalin. 1 - (100x magnification): Septal panniculitis (black arrow) with foci of inflammatory cells extending into adjacent fat lobule (red arrow). 2 - (400x magnification): epidermal spongiosis and lymphocyte exocytosis with dyskeratotic cells (arrow) and interface dermatitis with vacuolar changes in the basal layer.

during another episode of fever, low levels of immunoglobulin G prompted the initiation of intravenous immunoglobulin (IVIG) replacement. IVIG had an effect on infectious episodes and consequently reduced the frequency of hospital admissions, but skin rash and arthritis remained uncontrolled. A biopsied skin specimen from her left leg revealed septal panniculitis, thus confirming the clinical suspicion of erythema nodosum (**Figure 1**). At the age of two years, while high doses of steroids improved skin and osteoarticular symptoms, the patient failed to respond to several steroid sparing agents. Due to severe side effects, steroid administration was suspended. Monthly doses of IVIG (500 mg/kg) associated with anti-TNF alfa (etanercept) at the dose of 25 mg subcutaneously every week led to the control of fever, skin and osteoarticular symptoms. While anemia was a constant finding, sideroblastic changes were not evidenced in peripheral smears. In addition, no clinical signs of neurological development impairment were seen. All relevant laboratory analyses are summarized in **Figure 2**.

Genetic Analysis

Upon suspicion of an inborn error of immunity (IEI), commercially available whole exome sequencing was solicited, revealing two novel mutations in exon 4 of TRTN1 (c.361 G>A; p.Glu121Lys and c.407 C>G; p.Ala136Gly). Neither of these mutations had been previously reported in SIFD patients. Subsequently, both variants were confirmed and segregated by Sanger sequencing, confirming inherited transposition of the variants (**Figure 3**).

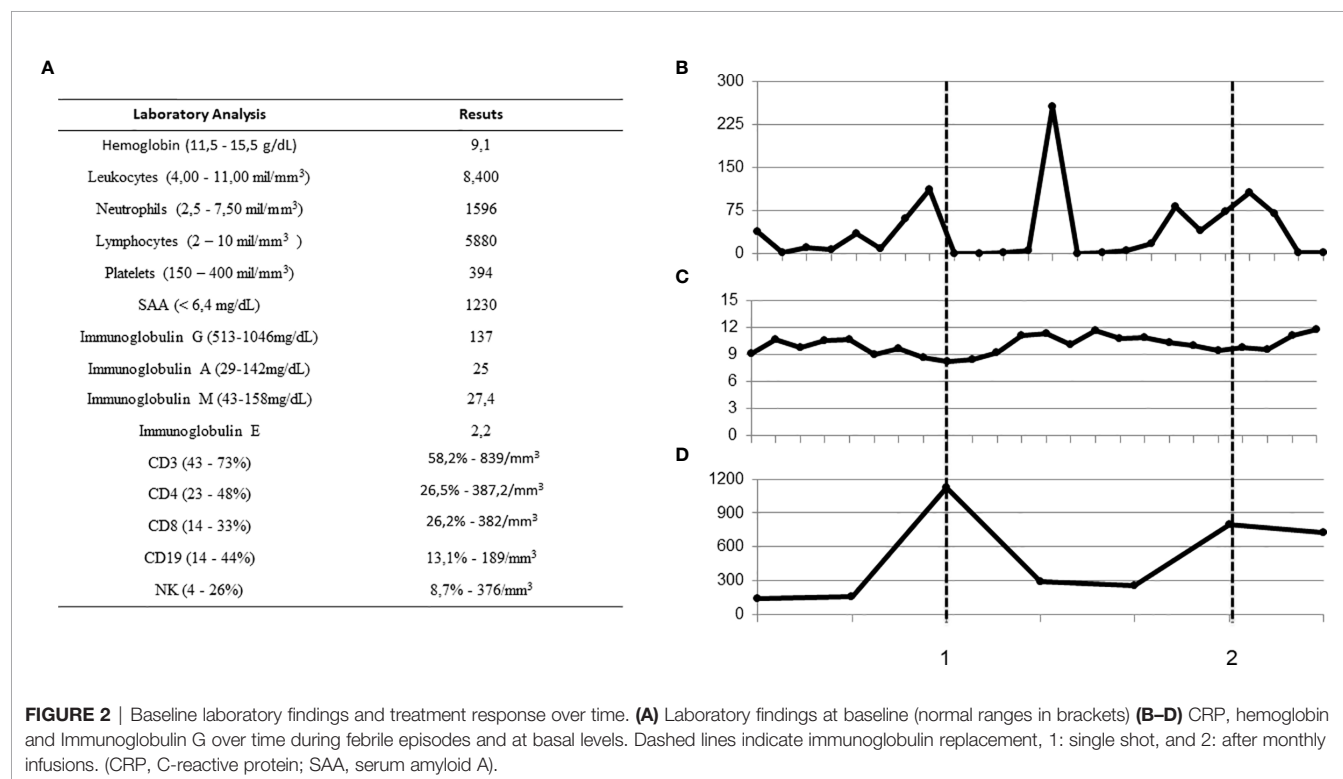
Immunological Analysis

Consistently with expected findings in SIFD patients, very low frequencies of CD19 (B lymphocytes), low B naïve cells, normal B memory cells and low levels of immunoglobulins G, M and A were found. The patient also presented positive IgG antibodies for herpes simplex virus and anti-VCA. Levels of dense fine speckled nuclear ANA fluctuated, with titers ranging from 1:160 to zero. Anti-ENA, as well as C3 and C4 complement titers, were within normal ranges (**Table 1** and **Figure 2A**).

The peripheral T lymphocyte repertoire evidenced multiple other immune defects in T naïve and memory cells, in addition to high frequencies of total T lymphocytes, as well as CD4 and CD8. T CD4 naïve and CD4 TEMRA cell expression were normal, but low frequencies of TCD4 CM and high TCD4 EM were noted. Expression levels of TCD8 naïve, TCM and TCD8 TEMRA were low, while high amounts of TCD8 EM cells were quantified. We additionally detected excessive peripheral expression of double-negative T cells (**Table 1**).

DISCUSSION

Here we report a case of a SIFD patient lacking signs of sideroblastic anemia and neurodevelopment delay and presenting with a new autoinflammatory phenotype characterized by recurrent episodes of fever and erythema nodosum. Skin manifestations in SIFD syndrome are rare; just one case with ichthyotic skin changes was initially reported (1). A later review of the 17 cases previously reported in the



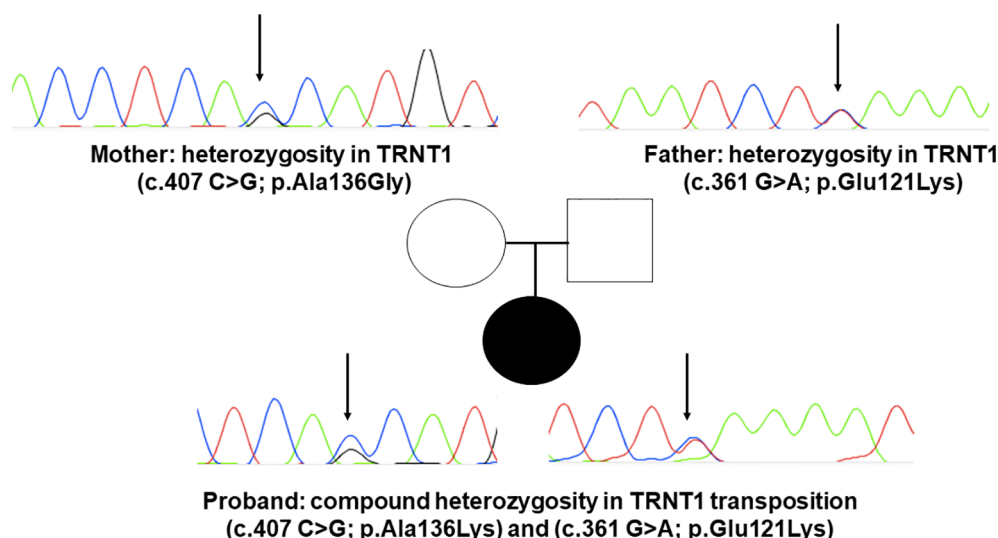


FIGURE 3 | DNA sequence electropherograms demonstrating mutations p.Ala136Lys and p.Glu121Lys in *TRNT1*. Sanger sequencing results for *TRNT1* mutations in SIFD in compound heterozygous status in the proband. The father carries p.Glu121Lys and the mother p.Ala136Lys, both in heterozygous fashion. Electropherograms detail each of the two newly reported mutations, indicated by black narrows.

TABLE 1 | Peripheral lymphocyte repertoire evidencing multiple immune defects.

Lymphocyte	Total number	%	Age Reference (3y)
CD3+	14936	76	66.2 (57.1-72.7)
CD4+	7493	50,2	37.7 (27.7-46.3)
CD8+	5068	33,9	21.9 (15.7-33.8)
CD19+	139	6,12	19.3 (13.3-26.7)
CD4+ naïve	1476	70,8	70.30 (46.14-84.40)
CD4+ TCM	247	11,8	26.40 (13.88-48.12)
CD4+ TEM	65	3,12	2.8 (0.94-6.46)
CD4+ TEMRA	4	0,192	0.2 (0.00-1.36)
CD8+ naïve	503	59,6	63.5 (36.80-83.16)
CD8+ TCM	78	9,24	15.8 (5.18-31.66)
CD8+ TEM	52	6,16	3.40 (0.70-11.22)
CD8+ TEMRA	18	2,13	15.5 (0.84-33.02)
CD20+ CD27+ (LB mem)	289	7,24	7.70 (3.60-18.55)
CD20+ CD27- (LB naïve)	461	11,4	76.20 (59.59-85.28)
CD3+ TCRab CD4- CD8-	390	6,3	<1,5%
CD3+ B220+	2266	36,7	-
CD3+ TCRab	4318	92	-
CD3+ TCRgd	610	13	-

In bold are the aberrant expressions when compared to the reference range. As expected in SIFD disease, marked low levels of B (CD19 cells) can be observed and an unusual observation of very high levels of double negative T cells (TCR α/β).

literature did not evidence consistent mucocutaneous manifestations in SIFD. However, one adult patient presented lichen sclerosus et atrophicus and morphea (6). Panniculitis, but not relapsing erythema nodosum, was previously reported in one SIFD patient (7).

Since the first publication on SIFD, several studies have described other patients harboring disease-causing mutations in *TRNT1*, notably bearing phenotypes incompatible with the original description, such as the present case. Since *TRNT1* is encoded in the nucleus, as required in both cytoplasm and in mitochondria, specific mutations may impact the ability of *TRNT1* to refold properly once transported into mitochondria,

thus giving rise to the spectrum of phenotypes observed. Indeed, studies mapping mutations along the *TRNT1* gene have suggested that mild phenotypes are located around the N or C terminal domains of this gene, as was seen in our patient (8, 9). We report two novel mutations in the *TRNT1* gene and even if functional analysis of the protein expression was not performed, taking into account the clinical and immunological phenotype altogether with the *in-silico analysis* of the mutations found we strongly believe that both are causative of SIFD syndrome.

Multiple immunological defects or phenomena have been described in patients with SIFD. While a significant reduction in B cells is noted, other lymphocyte classes seem to initially remain preserved, but then progressively decline, resulting in profound B, T and NK lymphopenia. One study that performed extensive immunophenotyping revealed increased TCD8 cells, which was also observed in our patient. Curiously, a low TCD8 frequency was found prior to IVIG replacement, which may reflect positive immune modulation by IVIG. Contrary to previous descriptions, we detected normal levels of TCD4 terminally differentiated effector memory helper T lymphocytes (CD4 TEMRA), as well as increased numbers of CD4 effector memory lymphocytes (CD4 EM). Similarly to other studies, we also found a lower percentage of switched memory B cells (1–3). We call attention to a relevant consideration, as no reference range for these cells exists in the Brazilian population; therefore, the range reported in a Chinese study (9) was employed, which must be taken into account when interpreting the present results.

A recent report on a patient carrying a mutation in the N terminal region of *TRNT1* identified high levels of interferon- α (IFN- α) and elevated expression of interferon-stimulated genes, and thusly hypothesized that this signature could be relevant in some clinical phenotypes; notably, no skin involvement was described (10). The IFN- α signature is a

hallmark of proteasome-associated autoinflammatory syndromes (PRAAS) in which skin eruptions, such as panniculitis, as was observed in our patient, are a common finding (11). The peripheral expression of double-negative (DN) T cells, a marker of apoptotic cell death, is a hallmark of Autoimmune Lymphoproliferative Syndrome (ALPS) and other ALPS-related syndromes, including some autoinflammatory disorders (12, 13). The present observation of considerably high numbers of DN T cells has not been previously reported in SIFD patients to date, thus expanding the immunological features of SIFD.

CONCLUSION

This present case constitutes the first report of SIFD in Brazil, and serves to enhance the range of clinical, immunological and genetic findings associated with this syndrome. IVIG replacement has appeared to have a positive immunomodulatory effect in affected patients, and *TRNT1* mutations should be considered in patients with ALPS-like syndromes. Autoinflammatory signs, such as recurrent fever and erythema nodosum, should also prompt consideration for *TRNT1* genetic screening. As aberrant *TRNT1* functioning seems to stress proteasome activity, further study may shed light on the therapeutical relevance of these cell machinery interactions in both SIFD and PRAAS patients.

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DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a article that does not adhere to our open data policies.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of São Paulo—School of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LM, AP, SB, and KK: article draft, sanger sequencing, performance of flow cytometry, and data analysis. IC, MB, and RD: derma-pathological analysis and data interpretation. LF, JK, FC, and MT-B: article review. All authors contributed to the article and approved the submitted version.

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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