AUTOIMMUNE VASCULITIS: ADVANCES IN PATHOGENESIS AND THERAPIES

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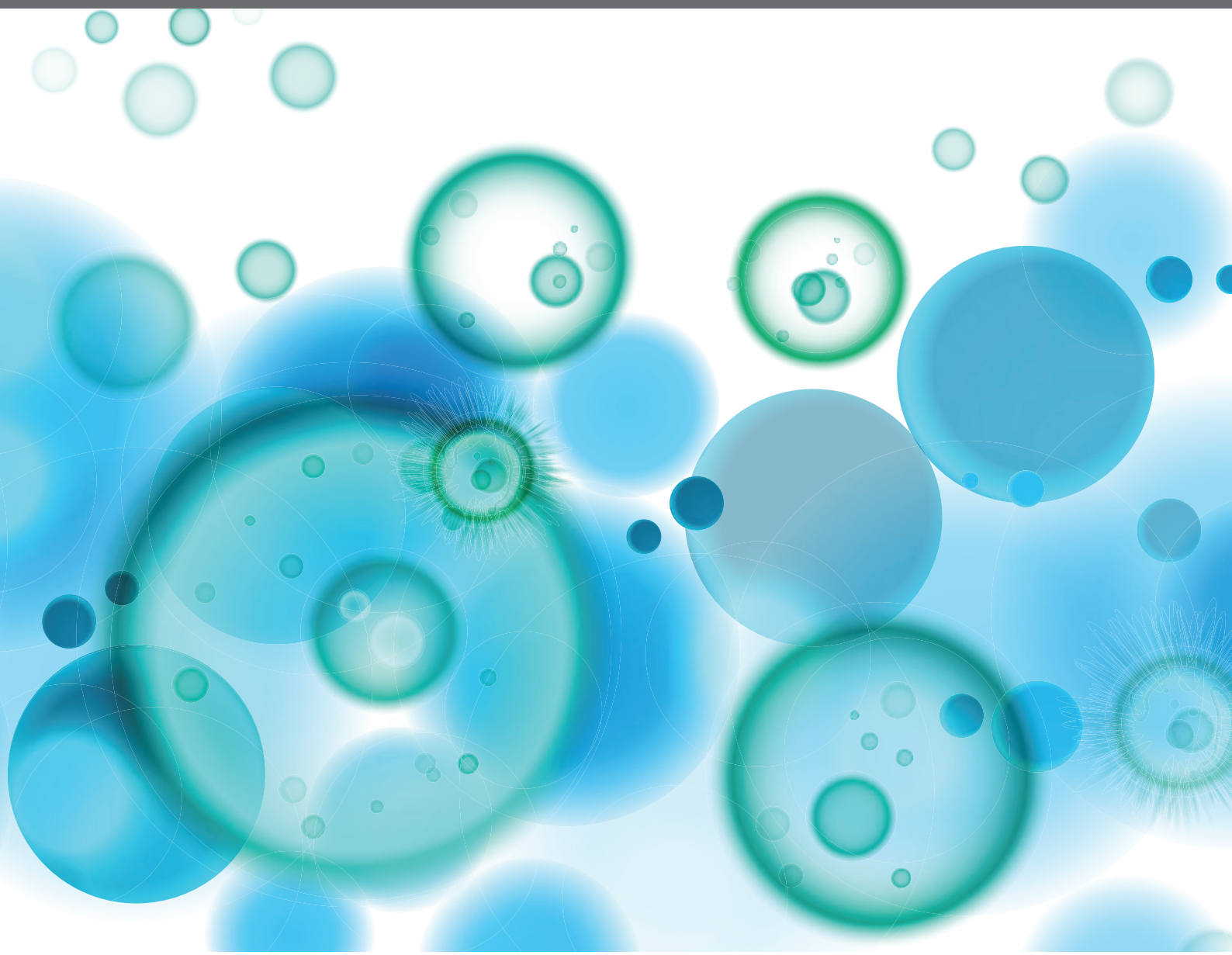

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AUTOIMMUNE VASCULITIS: ADVANCES IN PATHOGENESIS AND THERAPIES

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EDITORIAL
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Editorial: Autoimmune Vasculitis - Advances in Pathogenesis and Therapies

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Keywords: systemic vasculitis, autoimmune disease, autoimmunity, autoantibodies, T cell targeting, autoepitopes

Editorial on the Research Topic

Autoimmune Vasculitis - Advances in Pathogenesis and Therapies

Systemic vasculitis consists of a collection of heterogenous and rare autoimmune diseases, often with severe life-threatening manifestations. In this Research Topic, we sought to demonstrate the breadth of autoimmune vasculitis, the cutting-edge science being performed to better understand these diseases, and the latest developments in therapies. We were not disappointed. Here, we present 28 articles from across the globe, covering 16 different forms of systemic vasculitis. These include in-depth reviews, novel clinical observations, experimental mouse model studies, as well as the effect of COVID-19 on patients with autoimmune vasculitis. Collectively, the articles in this Topic highlight the complexities of these diseases, their similarities, and the strides that are currently being taken to develop better treatments.

In the ANCA-associated vasculitides (AAV), Li et al. review the genetic associations including both the MHC and non-MHC regions associated with AAV. They demonstrate that the MHC associations provide an additional basis for further dividing the AAV into three subtypes, while specific mutations in the immunoregulatory pathways provide a link to disease pathogenesis. Nozaki provides an up-to-date review on the latest in AAV treatment with biologicals, while O'Sullivan and Holdsworth expound on the relatively new phenomena of NETosis and how understanding its mechanism of injury can lead to the identification of new therapeutic targets. In original AAV research papers, Lin et al. correlate clinical observations of glomerular immune deposition in this traditionally pauci-immune disease with poorer renal survival, and Zeisbrich et al. identify PD-L1 on monocytes as a potential therapeutic target. In the study performed by Zeisbrich et al., the surface expression of the immune checkpoint (PL-L1) on peripheral blood monocytes was assessed in patients with AAV. Monocytes from AAV patients were found to have a lower expression of PD-L1 compared to healthy controls caused by reduced expression of CMTM6, which prevents PD-L1 from degradation. The lower expression of PD-L1 monocytes from AAV patients led to an increased capacity to induce T cells activation and proliferation while inhibiting lysosomal activity increased PD-L1 expression and reduced T cells stimulation by monocytes. This study reveals a potential novel strategy for the treatment of AAV by increasing the expression PD-L1 on monocytes.

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In anti-GBM disease, Shen et al. present an impressive study of 60 ultra rare cases of atypical anti-GBM disease, i.e. these patients have linear IgG deposits along their GBM but do not have circulating anti-GBM antibodies in their blood. They found that these atypical anti-GBM patients overall had a less severe renal and pulmonary injury.

In Behcet's disease, Perazzio et al. present an in-depth and timely review of disease pathogenesis with a focus on the role of innate immunity. The French Behcet's Network conducted the largest multicenter observational cohort study of apremilast, a phosphodiesterase 4 (PDE4) inhibitor, to treat Behcet's patients with joint and mucocutaneous symptoms refractory to colchicine and immunosuppressants. Overall, the network found that apremilast was effective in refractory patients.

In Cogan's syndrome, Venhoff et al. report that Certolizumab pegol, a TNF-α-inhibitor, is effective and well tolerated in two patients with Cogan's syndrome during pregnancy. This first of its kind study reveals the safety and efficacy of Certolizumab pegol in pregnant patients with inflammatory diseases.

In cutaneous vasculitis, Wang et al. show that HMGB1 blockade in a cutaneous reverse passive Arthus reaction mouse model of disease vastly reduces disease severity. This finding suggests that pursuing an anti-HMGB1 biological treatment in patients would be a promising strategy.

In Giant Cell Arteritis (GCA), we have in this Research Topic three distinct reviews. Akiyama et al. review the latest innate and adaptive immune pathways implicated in GCA; and point to currently available drugs that would be efficacious at inhibiting those pathogenic pathways. Robinette et al. provide a very informative comparison of the similarities and differences in immunopathology between GCA, polymyalgia rheumatica, Takayasu Arteritis and clinically isolated aortitis. Reitsema et al. focus their review on the recent developments in the role of CD8+ T cells in GCA as well as granulomatosis and polyangiitis (GPA).

In IgA nephropathy, Tang et al. apply single-cell RNA sequencing to kidney biopsies from patients to understand the pathways that lead to kidney injury. They show that tubule cells, in particular, were enriched for the TNF signalling, IL-17 signalling and NOD-like receptor signalling inflammatory pathways.

In Kawasaki Disease, Porritt et al. tested the role of anti-IL-1 treatment (anakinra) in suppressing disease using *Lactobacillus casei* cell wall extract (LCWE) injection mouse model. Interestingly, they found that although anakinra administration suppressed *Il6* and *Stat3* gene expression, disease was not attenuated.

Currently, systemic vasculitis is divided into different diseases based on clinical phenotype (1). The PedVas Initiative Investigators performed RNAseq on blood to enable classification of the autoimmune vasculitides based on disease aetiology. This is important because classifying disease based on endotype can inform treatment strategy. Based on whole blood gene expression profiling, the investigators identified two distinct endotypes, neutrophil degranulation and T cell receptor signalling. This promising work can lead the development of targeted therapies that treat the pathogenic mechanisms.

Lastly, in this COVID-19 era, there are two topical papers that look at autoimmune vasculitis and COVID-19. Schramm et al. present a case study of a patient with severe eosinophilic granulomatosis with polyangiitis (EGPA) who contracted COVID-19. They found no major complications despite the patient being highly immunosuppressed. Chen et al. perform a systematic review on cases of COVID-19 associated multisystem inflammatory syndrome in children (MIS-C), which manifests as a Kawasaki disease-like symptoms. Importantly, the authors discuss the immunopathogenesis of COVID-19 associated MIS-C and suggests potential life-saving treatments.

Overall, the papers in this Research Topic highlight the global effort taken to better understand the autoimmune mechanism of systemic vasculitides. As editors, each based in a different continent, we believe that the rarity of these diseases mean that international collaboration is necessary for ultimately developing cures for these severe diseases. Thus, we hope that this Topic serves as an impetus that shows autoimmune vasculitis research is active across the world and encourages vasculitis researchers to initiate collaborations with each other.

AUTHOR CONTRIBUTIONS

All authors contributed equally. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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COVID-19 in a Severely Immunosuppressed Patient With Life-Threatening Eosinophilic Granulomatosis With Polyangiitis

Markus A. Schramm[1], Nils Venhoff[1], Dirk Wagner[2], Jens Thiel[1], Daniela Huzly[3], Nils Craig-Mueller[1], Marcus Panning[3], Hartmut Hengel[3], Winfried V. Kern[2] and Reinhard E. Voll[1]*

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Immunosuppressive therapies increase the susceptibility of patients to infections. The current pandemic with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) compels clinicians to develop recommendations for successful clinical management and surveillance of immunocompromised patients at high risk for severe disease progression. With only few case studies published on SARS-CoV-2 infection in patients with rheumatic diseases, we report a 25-year-old male who developed moderate coronavirus disease 2019 (COVID-19) with fever, mild dyspnea, and no major complications despite having received high-dose prednisolone, cyclophosphamide, and rituximab for the treatment of highly active, life-threatening eosinophilic granulomatosis with polyangiitis (EGPA).

Keywords: COVID-19, SARS-CoV-2, vasculitis, eosinophilic granulomatosis with polyangiitis, EGPA, immunosuppression, rituximab, cyclophosphamide

INTRODUCTION

With a wide range of clinical outcomes in coronavirus disease 2019 (COVID-19), from being asymptomatic to fatal acute respiratory distress syndrome, questions have been raised about the safety of immunosuppressive therapies (1). Individuals with anti-neutrophil cytoplasm autoantibody (ANCA)-associated vasculitides require particular care, especially considering the life-threatening course of disease with multi-organ manifestations. Pulmonary disease manifestations and immunosuppression with glucocorticoids combined with cyclophosphamide and/or rituximab are associated with infectious complications. Thus, inadequate immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in such patients may predispose to severe COVID-19.

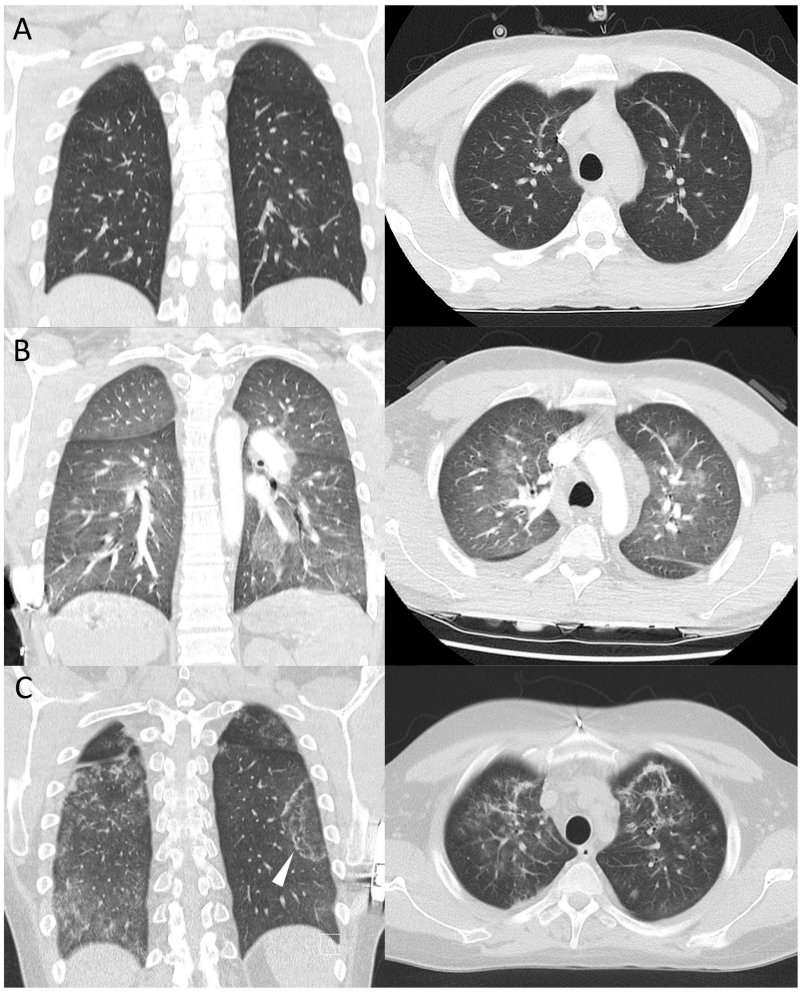

FIGURE 1 | CT scans (coronal, axial) of the chest **(A)** after diagnosis of EGPA, unremarkable for pulmonary disease manifestation, **(B)** showing ground glass opacities and interlobular septal thickening after diagnosis of alveolar hemorrhage by bronchoalveolar lavage, **(C)** demonstrating bipulmonary ground glass opacities and consolidations with minor reticulation. Presence of reversed halo sign (arrow) as previously described in COVID-19 pneumonia.

CASE PRESENTATION

We report the case of a 25-year-old male with nosocomial COVID-19 while receiving immunosuppressive treatment for eosinophilic granulomatosis with polyangiitis (EGPA). EGPA was newly diagnosed in early January 2020 when the patient presented at the emergency room with sinusitis, asthma, and a life-threatening myocardial infarction, resulting in a decreased ejection fraction of 30%. Blood eosinophils and serum concentrations of Immunoglobulin E (IgE) and C-reactive protein (CRP) were increased, ANCA-testing was negative, and a pulmonary CT scan unremarkable (**Figure 1A**). Immediately initiated immunosuppression with intravenous high-dose prednisolone and cyclophosphamide showed adequate therapeutic response. With conversion to oral glucocorticoid treatment at the end of January 2020, the patient unexpectedly developed a serious relapse of disease with peripheral neuropathy, pulmonary hemorrhage (**Figure 1B**) and a second myocardial infarction. Thus, due to severity and refractory disease the previously healthy patient was continuously hospitalized from January to March 2020, receiving intravenous cyclophosphamide (CYCLOPS-protocol, cumulative dose 4.76 g), rituximab (4×375 mg/m^2), and a long-term, slowly tapered high-dose prednisolone treatment (up to 1 g/day).

On presumed day 0 of COVID-19 (ongoing oral treatment with 60 mg prednisolone only, 9 days after last of five cyclophosphamide infusions and 19 days after the last of four rituximab infusions), he reported catarrh and a mild cough. A SARS-CoV-2 real-time reverse transcription PCR (rt-PCR) from oropharyngeal swab was positive (**Figure 2A**). On day 3, treatment with hydroxychloroquine (for 6 days) and lopinavir/ritonavir (for 8 days) was initiated while daily prednisolone was reduced from 60 to 15 mg. He developed a sore throat, hyposmia, headaches, myalgias, and diarrhea. Despite rhonchi/crackles on auscultation and a CT scan consistent with bilateral viral pneumonia (**Figure 1C**), the patient only

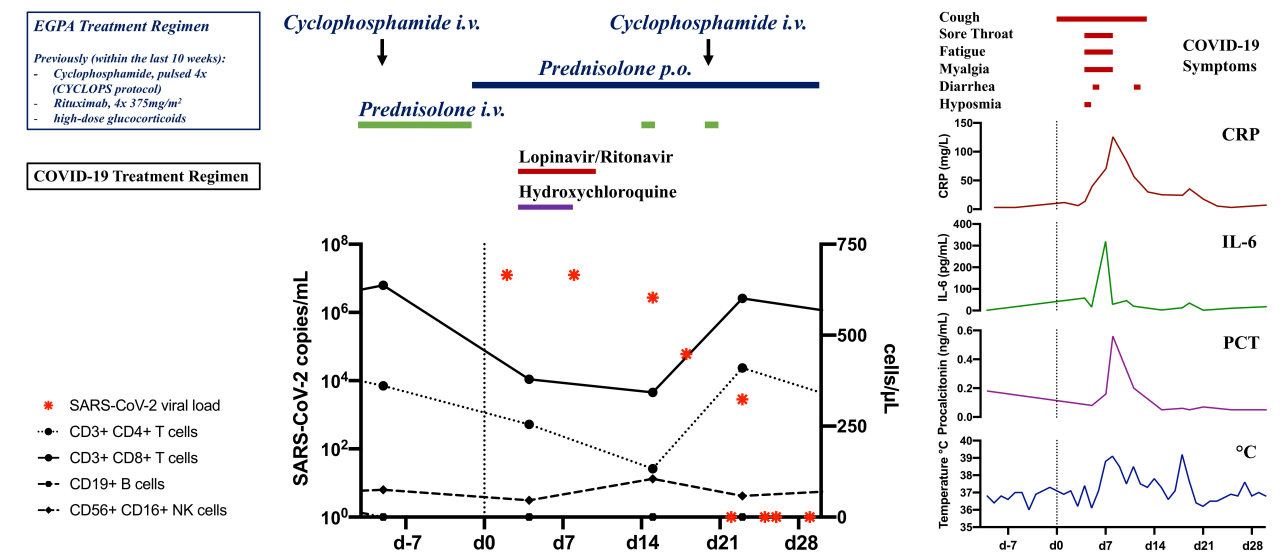

FIGURE 2 | (A) Timeline of SARS-CoV-2 real-time reverse transcription PCR results (copy numbers) and numbers of immune cells during COVID-19. Colored bars indicate treatment regimen employed pre and post SARS-CoV-2 infection. **(B)** Course of COVID-19 induced inflammation markers quantified in patient sera including CRP, IL-6, procalcitonin (PCT), and body temperature (°C). Colored lines indicate patient symptoms related to SARS-CoV-2 infection. Vertical dashed line indicates onset of COVID-19 related symptoms.

reported mild dyspnea. Short-term decrease of oxygen saturation (minimal SaO_2 85%) required oxygen supplementation for 3 days (low flow 2 L/min). With spiking serum concentrations of CRP (125.9 mg/L, reference range <5 mg/L), procalcitonin (0.56 ng/mL, reference range <0.05 ng/mL), and interleukin-6 (IL-6, 320 pg/mL, reference range <7 pg/mL), concomitant with decreasing $CD4^+$ and $CD8^+$ T-cell counts, the patient developed fever (max. 39.1°C) on day 7 (**Figure 2B**). Anti-IL6-receptor treatment was considered, however the patient steadily recovered and was free of COVID-19 symptoms 2 weeks after onset. Nevertheless, subsequent oropharyngeal swabs confirmed active SARS-CoV-2 infection with gradual decrease of viral RNA. Relapsing neurological symptoms of EGPA urged us to re-administer high-dose glucocorticoids and cyclophosphamide on days 14 and 20, respectively, without causing recurrence of COVID-19-related symptoms. Despite severe immunosuppression and complete peripheral B-cell depletion, SARS-CoV-2 RNA copy numbers in oropharyngeal swabs were below the threshold for reliable detection on days 25, 26, and 29. By day 46, there were no antibodies to SARS-CoV-2 spike protein detectable by ELISA (EUROIMMUN). Remarkably, interferon-gamma release upon polyclonal T-cell stimulation was normal on day 36.

DISCUSSION

Given the high-risk profile with sustained cardiac dysfunction, previous pulmonary hemorrhage, continued high-dose glucocorticoids, B-cell depletion, decreased T-cell counts, and secondary hypogammaglobulinemia (minimal IgG 4.47 g/L, reference range >7 g/L), it is remarkable that our patient overcame COVID-19 in a rather timely manner without complications. The effects of potential anti-viral agents hydroxychloroquine and lopinavir/ritonavir on the disease course remain unclear. Despite initially higher than average copies per oropharyngeal swab, which could be explained by the effect of immunosuppression during virus contraction, our patient showed a temporal pattern of viral load peaking within the first week after onset of symptoms and gradually declining over the following three weeks as previously described in COVID-19 patient cohorts (2, 3). Our observations might therefore suggest that a functional adaptive immune system with an effective B-cell response is not required to survive COVID-19. Moreover, our data points to an important role of innate immune mechanisms and perhaps T cells for SARS-CoV-2 control based on the coinciding increase of $CD8^+$ and $CD4^+$ T-cell numbers with declining viral RNA load. Notably, antibodies may often be insufficient for viral clearance (4). There is even evidence that antibodies against the SARS-CoV-2 spike protein can exacerbate pulmonary inflammation due to immunocomplex-mediated complement and Fcγ receptor activation with consecutive immune cell infiltration (5, 6).

While our knowledge of COVID-19 pathogenesis continues to evolve, strategies to avoid unfavorable outcomes of SARS-CoV-2 infection should continue to be mindful of potentially greater adverse outcome caused by tempering existing immunosuppressive or immunomodulatory treatment in autoimmune diseases.

DATA AVAILABILITY STATEMENT

The original data generated and analyzed for this study are included in the published article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

MS and RV wrote the manuscript. All authors were involved in direct patient care or acquisition of clinical data, made important intellectual contributions, revised and approved the final version of the manuscript.

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Clinical-Pathological Features and Outcome of Atypical Anti-glomerular Basement Membrane Disease in a Large Single Cohort

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Background: Atypical cases of anti-glomerular basement membrane (GBM) disease had absent circulating antibodies but linear IgG deposits along GBM in the kidneys. Herein, we reported the clinical-pathological features and outcome of these rare cases.

Methods: Linear IgG deposit along GBM were examined by immunofluorescence on renal specimens, with exclusion of diabetic kidney disease. Circulating anti-GBM antibodies were tested by commercial ELISA assay. Clinical, pathological and follow-up data were retrospectively analyzed.

Results: From 2013 to 2018, a total of 60 patients were diagnosed as atypical anti-GBM disease. They had a male predominance, with an average age of 51.7 ± 15.6 years. Three (5.0%) patients had alveolar hemorrhage. Forty five percent of them presented with acute kidney disease. All patients had linear IgG deposit along GBM, some in addition on tubular basement membrane and/or Bowmans' capsules. C3 deposition was found in 65.0% of the patients. 41.7% (25/60) of the patients showed crescent formation and the percentage of crescent was (34.7 ± 23.5)% in those patients. They had higher prevalence of hematuria and C3 deposit, higher levels of serum creatinine, worse renal and patient survival than those without crescent ($P < 0.05$). During the follow-up of 35.7 ± 21.4 months, 14 (23.3%) patients progressed to ESRD. The serum creatinine on diagnosis [per 200 μmol/L increase, HR (95% CI): 2.663 (1.372, 5.172), $P = 0.004$], serum C3 [per 0.1 g/L increase, HR (95% CI): 0.689(0.483, 0.984), $P = 0.040$] and the intensity of kidney C3 staining [per 1+ increase, HR (95% CI): 2.770 (1.115, 6.877), $P = 0.028$] were independent predictive factors for kidney outcome. Nine (15.0%) patients died of all causes.

Conclusions: Atypical anti-GBM disease manifested milder kidney injury and scarce pulmonary hemorrhage compared to the classical cases. Though heterogeneous, a substantial number of the patients had complement activation and crescent formation.

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Patients having crescents presented with more severe clinical course and worse outcomes. The poor kidney and patient prognosis emphasize prompt interventions from physicians. The immunosuppressive intervention was not associated with kidney or patient outcome. Further studies are needed to address the optimal therapeutic regimen.

Keywords: anti-GBM disease, crescentic glomerulonephritis, rapidly progressive glomerulonephritis, renal outcome, renal pathology

INTRODUCTION

Anti-glomerular basement membrane (GBM) disease is a rare *in situ* immune-complex vessel vasculitis that involves glomerular capillaries or pulmonary capillaries, or both (eponymously termed as Goodpasture syndrome) (1, 2). It is considered to be a prototypical autoimmune disease characterized by the burst of antibodies against the non-collagen domain one of α3 chain of type IV collagen [α3(IV)NC1] located in both GBM and alveolar basement membrane (3). The disease is documented as the most severe glomerulonephritis due to the rapidly progressive renal impairments with large amount of crescent in glomeruli and ∼40∼60% concurrence of lung hemorrhage including lethal massive hemoptysis (4). To improve kidney and patient outcomes, the combination regimen of plasmapheresis, steroids, and cyclophosphamide is recommended to start up immediately on diagnosis (5).

At present, the diagnosis of anti-GBM disease depends on the detection of circulating anti-GBM antibodies and/or linear IgG deposition along GBM on kidney biopsy (6). Clinical routine assay to detect circulating antibodies is enzyme-linked immunosorbent assay which utilizes recombinant human α3(IV)NC1 or purified bovine GBM as solid-phase antigen (7). The positive result is necessary for an early diagnosis and quick start of intensive treatments including plasma exchange and immunosuppressive therapy. However, in decades, atypical presentations of anti-GBM disease have been reported in case reports and case series (8–19), in which the circulating anti-GBM antibodies were often undetectable by commercial ELISA and the diagnosis was based on the linear deposit of immunoglobulins along GBM on renal specimens. The atypical condition brought challenges to the diagnosis and treatment of this aggressive disease. Whether these atypical cases are a homogeneous subtype of anti-GBM disease or a group of heterogeneous conditions is still not clear, nor are the causes and roles of the deposited antibodies in disease development. Therefore, it is of importance to explore their clinical and pathological characteristics and especially their outcomes from a large cohort.

In the present study, data from 60 consecutive "atypical" patients diagnosed from 2013 to 2018 were retrospectively screened, who presented with substantial linear deposits of IgG along GBM on immunofluorescence and without detectable circulating anti-GBM antibodies. We investigated the clinical-pathological characteristics and attempted to identify the predictive factors for kidney and patient survival in order to provide some clues for the pathogenesis and treatment of this rare entity.

MATERIALS AND METHODS

Patients

Sixty patients with atypical anti-GBM disease identified at Peking University First Hospital were retrospectively analyzed from January 2013 to December 2018. The diagnostic criteria of "atypical anti-GBM disease" were defined as follows: 1. Immunofluorescence of renal specimens exhibited substantial linear deposit of IgG along GBM (staining intensity ≥1+); 2. Detection of circulating anti-GBM antibodies were negative examined by commercial ELISA kits (Euroimmun, Luebeck, Germany); 3. Patients with diabetic kidney disease were excluded. A study flow diagram is drawn to summarize the study procedure (**Figure 1**).

Demographic, clinical, and laboratory data were collected at the time of kidney biopsy and during follow-up. Renal insufficiency was defined as the serum creatinine >133 μmol/L on diagnosis. All patients were followed up until they met the endpoints. The primary endpoint (renal survival) was set as end-stage renal disease (ESRD) defined as dialysis dependence for >3 months. Patients who had not progressed to ESRD before death were treated as censored data when analyzing renal survival.

This study complied with the Declaration of Helsinki and was approved by the Ethics Committee of Peking University First Hospital.

Kidney Pathology

Kidney biopsy was performed in all the 60 patients. The staining of IgG, IgA, IgM, C3, C1q, fibrinogen-fibrin related antigen (FRA), albumin, IgG subclasses and light chains were performed on frozen renal sections using fluorescein-conjugated rabbit/mouse anti-human IgG, IgA, IgM, C3c, C1q, FRA, albumin, light chain, IgG subclasses antibodies (Dako, Santa Clara, CA), and were evaluated under a fluorescence microscope (Nikon, Tokyo, Japan). The grades of staining intensity were ranged from 0+ to 4+. Light microscopy and electron microscopy examinations were performed as previously showed (20). All the pathological evaluations were performed by two renal pathologists blinded to each other.

Statistical Analysis

SPSS statistical software (version22.0, IBM) was applied for statistical analysis. Quantitative data were presented as mean ± SD when complying with normal distribution, or as median (1/4, 3/4) when disobeying normal distribution. Qualitative data were presented as number (%). Comparison between continuous variables was conducted by *t*-test for normally distributing

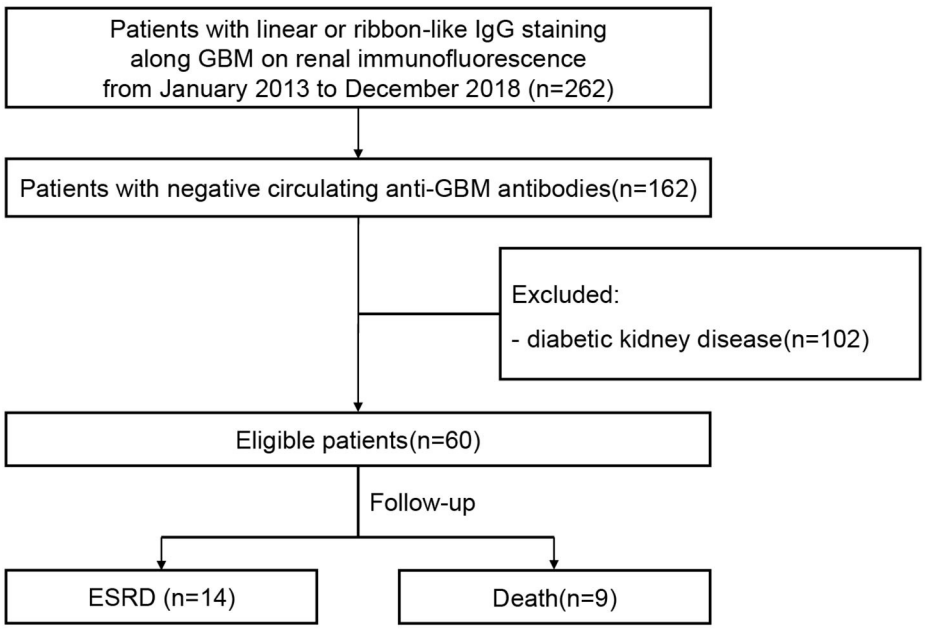

FIGURE 1 | Flowchart of patient recruitment and follow-up.

data or non-parametric test for non-normally distributing data. Differences between qualitative data were analyzed using χ^2 or Fisher exact test. Univariate survival analysis was operated using both Kaplan-Meier analysis (log-rank test) and univariate COX regression analysis to explore potential prognostic predictors. Candidate variables were then enrolled together in a COX regression models to undergo multivariate survival analysis. Output results were exhibited as hazard ratios (HRs) along with 95% confidence intervals (95% CIs). The difference was considered statistically significant as P-value < 0.05.

RESULTS

The Demographic and Clinical Features of Atypical Anti-GBM Patients

A total of 60 consecutive patients were retrospectively analyzed in this study, fitting the criteria of "atypical anti-GBM disease" from 2013 to 2018 (**Table 1**). They had a male predominance, and the ratio of male to female was 2.3:1. The ages of patients ranged from 19 to 87 years old, with an average age of 51.7 ± 15.6 years. 53.3% of patients were current or remote smokers. 13.3% of patients displayed prodromal infections before disease onset. 5.0% of patients manifested hemoptysis.

Thirty eight (63.3%) patients exhibited hematuria and 4 of them had macroscopic hematuria. Proteinuria existed in 56 (93.3%) patients and 26 of them reached nephrotic level. The median level of proteinuria was 2.7 (0.8, 6.3) g/24 h. Nineteen (31.7%) patients presented with nephrotic syndrome. 45.0% (27/60) of patients presented with acute kidney disease(AKD), among them 18.5% (5/27) underwent oliguria or anuria. The median level of serum creatinine at diagnosis was 142.5 (87.8, 257.5) μmol/L, and over half of the patients (32/60, 53.3%) showed renal insufficiency at presentation. The serum C3 level, available in 52 patients, was normal in 49 and low in three. The serum C4 level, available in 52 patients, was normal in 51 and low in one. Anti-neutrophil cytoplasmic antibodies(ANCA) were detectable in serum of 14.0% (7/50) of the patients, among whom six were MPO-ANCA positive and one was PR3-ANCA positive.

Kidney Pathology

All patients exhibited visible linear deposit of IgG along GBM with the intensity grade ranging from 1+ to 4+. Linear deposit of IgG could be observed at GBM (60/60, 100.0%), tubular basement membrane (37/60, 61.7%) and/or Bowmans' capsule (5/60, 8.3%). In 41 patients who had IgG light chains examined, all have both kappa and lambda chains deposit. IgG1 was the predominant subclass (27/59, 45.8%), followed by IgG2 (21/59, 35.6%), IgG4 (11/59, 18.6%), and IgG3 (7/59, 11.9%). Coexistence of IgA and IgM were shown in 27/60 (45.0%) and 33/60 (55.0%) patients. Complement deposits including C3 and C1q were found in 39 (65.0%) and 10 (16.7%) patients, respectively (**Table 2**).

41.7% (25/60) of all cases were observed of crescent formation and the percentage of crescent was (34.7 ± 23.5)% in those patients. Five of them had crescentic glomerulonephritis (defined by diffuse crescents occupying >50% of the glomeruli). In patients with crescents, the average proportion of cellular, cellulofibrous, and fibrous crescents was 25.8, 52.8, and 21.4%, respectively. 56.0% (14/25) of those patients showed crescents in synchrony, the remaining showed a mixture of acute and chronic lesions. There was a positive correlation between the percentage of crescents and the serum creatinine at diagnosis ($r = 0.427$, $P = 0.001$). Almost all patients showed tubular atrophy

TABLE 1 | Demographic and clinical characteristics of patients with atypical anti-GBM disease.

Characteristic	Total patients (*N* = 60)
Demography	
Male/female	42/18 (2.3/1)
Age, year	51.7 ± 15.6
Clinical feature	
Interval from onset to diagnosis, month	2.1 (1.1, 6.9)
Smoking, *n* (%)	32 (53.3)
Prodromal infection, *n* (%)	8 (13.3)
Hemoptysis, *n* (%)	3 (5.0)
[a]AKD and [b]AKI, *n* (%)	27 (45.0)
Oliguria/anuria, *n* (%)	5 (8.3)
Hematuria, *n* (%)	38 (63.3)
Macroscopic hematuria, *n* (%)	4 (6.7)
Proteinuria, *n* (%)	56 (93.3)
24 h Proteinuria, g/24 h	2.7 (0.8, 6.3)
Nephrotic level proteinuria, *n* (%)	26 (43.3)
Nephrotic syndrome, *n* (%)	19 (31.7)
Serum albumin, g/L	34.2 (23.8, 41.2)
Serum creatinine on diagnosis, μmol/L	142.5 (87.8, 257.5)
Renal insufficiency, *n* (%)	32 (53.3)
Hemoglobin, g/L	118.1 ± 26.3
Serum C3, g/L*	0.94 ± 0.26 (*n* = 52)
Serum C4, g/L*	0.25 ± 0.07 (*n* = 52)
[c]ESR, mm/h	36.5 (17.0, 71.3) (*n* = 52)
[d]ANCA, *n* (%)	7 (14.0) (*n* = 50)
[e]MPO-ANCA/[f]PR3-ANCA/both	6/1/0
Treatment	
[g]ACEIs/[h]ARBs, *n* (%)	26 (43.3)
Immunosuppressive therapy, *n* (%)	34 (56.7)
steroids, *n* (%)	32 (53.3)
cytotoxic drugs, *n* (%)	18 (30.0)
Plasmapheresis, *n* (%)	4 (6.7)
Outcome	
Follow-up duration, month	35.7 ± 21.4
Progression to ESRD, *n* (%)	14 (23.3)
Death, *n* (%)	9 (15.0)
1-year renal survival, *n* (%)	50 (83.3)
1-year patient survival, *n* (%)	57 (95.0)

*[a]AKD, acute kidney disease; [b]AKI, acute kidney injury; [c]ESR, erythrocyte sedimentation rate; [d]ANCA, anti-neutrophil cytoplasmic antibodies; [e]MPO, Myeloperoxidase; [f]PR3, proteinase 3; [g]ACEIs, angiotensin converting enzyme inhibitors; [h]ARBs, angiotensin receptor blocker. *Normal range of serum C3: 0.6–1.5 g/L, normal range of serum C4: 0.12–0.36 g/L.*

TABLE 2 | Pathological characteristics of patients with atypical anti-GBM disease.

Characteristic	Total patients (*N* = 60)
Immunofluorescence	
IgG linear deposition, *n* (%)	60 (100.0)
Intensity (scale 0~4+)	1.0 (1.0, 1.5)
Location (GBM/[a]TBM/Bowman's capsules), *n* (%)	60/37/5 (100.0/61.7/8.3)
IgG subclass (*n* = 59)	
IgG1/IgG2/IgG3/IgG4, *n* (%)	27/21/7/11 (45.8/35.6/11.9/18.6)
IgA deposit, *n* (%)	27 (45.0)
IgM deposit, *n* (%)	33 (55.0)
C3 deposit, *n* (%)	39 (65.0)
C1q deposit, *n* (%)	10 (16.7)
[b]FRA deposit, *n* (%)	20 (33.3)
Albumin deposit, *n* (%)	38 (63.3)
Light microscopy	
Number of glomeruli	25.0 (19.3, 36.0)
Crescent formation, *n* (%)	25 (41.7)
Percentage of crescents, %	27.3 (0.0, 49.7)
[c]TA/IF, *n* (%)	58 (96.7)
Electron microscopy	
Electric dense deposit, *n* (%)	33 (55.9) (*n* = 59)
Combined [d]GN, *n* (%)	35 (58.3)
[e]IgAN (including [f]HSP-GN), *n* (%)	12 (20.0)
[g]MN, *n* (%)	8 (13.3)
[h]MPGN, *n* (%)	6 (10.0)
[i]AAV, *n* (%)	4 (6.7)
[j]FSGS, *n* (%)	3 (5.0)
[k]TBMN *n* (%)	1 (1.7)
[l]TMA, *n* (%)	1 (1.7)

[a]TBM, tubular basement membrane; [b]FRA, fibrinogen-fibrin related antigens; [c]TA/IF, tubular atrophy and interstitial fibrosis; [d]GN, glomerulonephritis; [e]IgAN, IgA nephropathy; [f]HSP-GN, Henoch–Schönlein purpura glomerulonephritis; [g]MN, membranous nephropathy; [h]MPGN, membranoproliferative glomerulonephritis; [i]AAV, ANCA-associated vasculitis; [j]FSGS, focal segmental glomerulosclerosis; [k]TBMN, thin basement membrane nephropathy; [l]TMA, thrombotic microangiopathy.

and interstitial fibrosis (58/60, 96.7%), interstitial inflammatory cells infiltration (55/60, 91.7%) and arteriole injury (59/60, 98.3%). Electric dense deposit was observed in 33/59 (55.9%) patients. Foot process effacement of podocyte appeared in most of the patients (55/59, 93.2%). 58.3% (35/60) of all patients combined with other glomerulonephritis, including IgA nephropathy (12/60, 20.0%), membranous nephropathy (8/60, 13.3%), membranoproliferative glomerulonephritis (6/60, 10.0%), ANCA-associated vasculitis (4/60, 6.7%), focal segmental glomerulosclerosis (3/60, 5.0%), thin basement membrane nephropathy (1/60, 1.7%), thrombotic microangiopathy (1/60, 1.7%) (**Table 2**).

Treatment and Outcome

26/60 (43.3%) patients received only angiotensin converting enzyme inhibitors (ACEIs) or angiotensin receptor blocker (ARBs). Immunosuppressive therapies that were defined as administration of steroids and/or cytotoxic drugs, were applied in 34/60 (56.7%) patients. Among them, 16 patients received administration of steroids combined with cytotoxic drugs, 16 patients received steroids alone and two patients received cytotoxic drugs alone. In 18 patients who received cytotoxic drugs, 14 of them were treated with cyclophosphamide (CTX), the remaining with cyclosporine (two patients), tacrolimus (one patient) and mycophenolate mofetil (one patient), respectively.

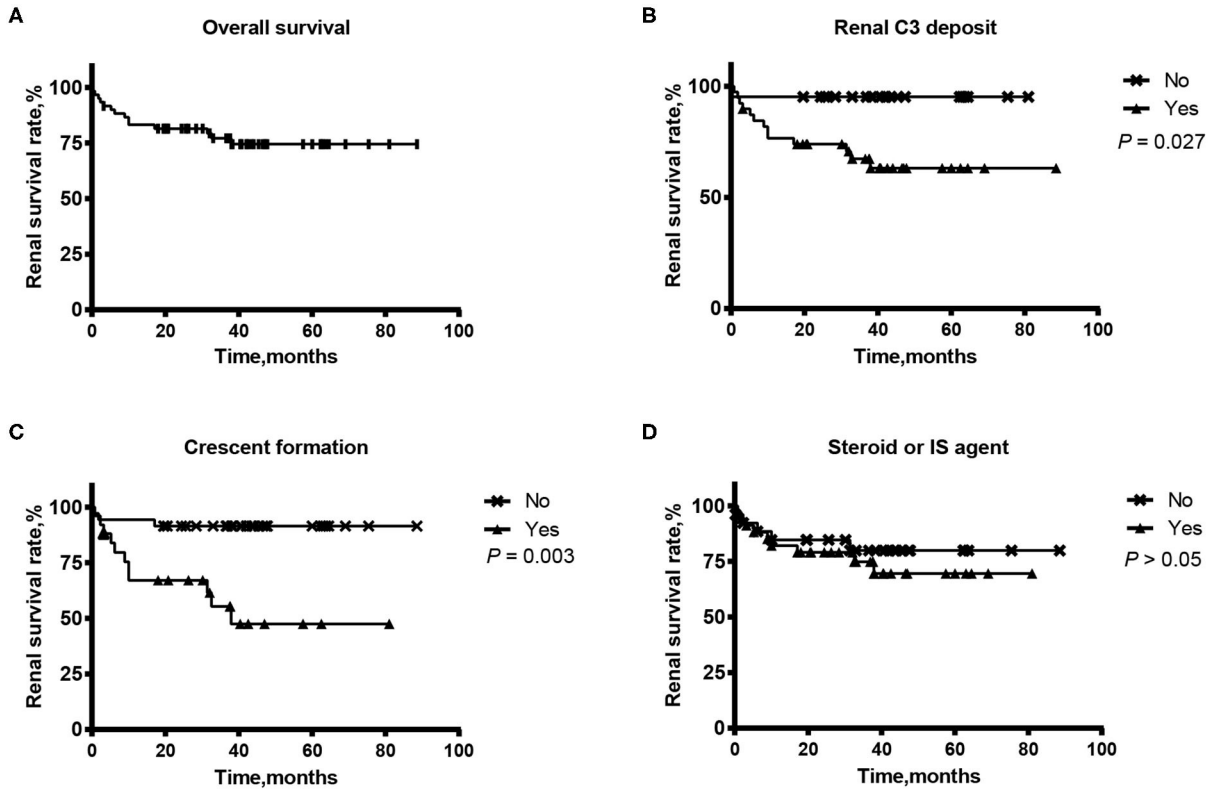

FIGURE 2 | Renal survival of a Chinese cohort of 60 patients with atypical anti-GBM disease: Overall renal survival **(A)** and according to the renal deposit of C3 **(B)**, the crescent formation in glomeruli **(C)** and the administration of steroids or immunosuppressant (IS) agents **(D)**.

Plasma exchange was performed in 4/60 (6.7%) patients (**Table 1**).

The follow-up ranged from 3 months to 89 months with an average of 35.7 ± 21.4 months. During follow-up, 14/60 (23.3%) patients progressed to ESRD. The 1-year renal survival rate was 83.3% (50/60). The prognostic values of clinical-pathological parameters and therapeutic strategies for kidney outcome were evaluated using Kaplan-Meier analysis (log-rank test) and Cox regression analysis, shown in **Figure 2**, **Table 3**. After univariate survival analysis, we found that the level of serum creatinine on diagnosis, level of serum C3, intensity of kidney C3 staining, kidney C1q positive staining, percentage of crescents and plasmapheresis were potential risk factors for ESRD. Multivariate analysis showed that serum creatinine on diagnosis [per 200 μmol/L increase, HR (95% CI): 2.663 (1.372, 5.172), $P = 0.004$], serum C3 (per 0.1 g/L increase, HR (95% CI): 0.689 (0.483, 0.984), $P = 0.040$) and the intensity of kidney C3 staining (per 1+ increase, HR (95% CI): 2.770 (1.115, 6.877), $P = 0.028$) were independent predictive factors for kidney outcome. Immunosuppressant therapies had no significant association with kidney outcome.

Nine (15.0%) patients died during follow-up. The 1-year patient survival rate was 95.0% (57/60). Four patients died of severe pneumonia and respiratory failure. One died of acute myocardial infarction. Four died of unknown reasons. In the nine died patients, four were dialysis dependence lasting for more than 3 months before death and were regarded as meeting the primary endpoint. The remaining five patients who did not progressed to ESRD before death were treated as censored data when analyzing renal survival. The predictive indicators for death were evaluated using Kaplan-Meier analysis (log-rank test) and Cox regression analysis, shown in **Table 4**. After univariate survival analysis, we found that age, the intensity of kidney C3 staining, and the percentage of crescents were potential risk factors for death. However, multivariate analysis did not come out with any independent predictive factors for death.

Comparison Between Atypical Anti-GBM Patients With and Without Crescent Formation

41.7% patients of the whole cohort presented with crescent formation in renal histological examinations. The clinical and pathological features of patients with and without crescent formation were compared (**Table 5**). The patients with crescents presented with more significant male predominance (84.0 vs. 60.0%, $P = 0.046$), higher levels of SCr at diagnosis [206.8 (123.9, 372.7) μmol/L vs. 109.9 (82.7, 161.5) μmol/L, $P = 0.003$], higher frequency of kidney C3/IgA/IgM deposit (92.0 vs. 45.7%, $P < 0.001$; 64.0 vs. 31.4%, $P = 0.012$; 72.0 vs. 42.9%, $P = 0.025$), worse

TABLE 3 | Potential prognostic factors for kidney outcome by univariate and multivariate COX regression analysis.

Variable	Univariable analysis ($N = 60$)		[a]Multivariable analysis ($N = 52$)	
	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**
Gender (female)	0.528	0.663 (0.185, 2.379)	-	-
Age	0.151	1.026 (0.991, 1.063)	-	-
Hematuria (0 = none; 1 = microscopic; 2 = macroscopic)	0.090	2.227 (0.883, 5.622)	-	-
Proteinuria (0 = none; 1 = non-nephrotic; 2 = nephrotic)	0.327	1.566 (0.639, 3.839)	-	-
SCr on diagnosis (increased by 200 μmol/L)	***<0.001***	2.355 (1.598, 3.471)	***0.004***	2.663 (1.372, 5.172)
Serum C3 (increased by 0.1g/L) ($n = 52$)	***0.002***	0.608 (0.445, 0.830)	***0.040***	0.689 (0.483, 0.984)
Kidney IgG staining >1+	0.271	1.803 (0.631, 5.154)	-	-
IgG deposit on TBM and/or Bowman's capsule	0.272	0.555 (0.195, 1.585)	-	-
Kidney C3 staining intensity (increased by 1+)	***0.006***	2.170 (1.252, 3.762)	***0.028***	2.770 (1.115, 6.877)
Kidney C1q positive staining	***0.041***	3.126 (1.045, 9.353)	0.780	0.805 (0.175, 3.699)
Percentage of crescents (increased by 10%)	***0.009***	1.258 (1.059, 1.494)	0.775	0.940 (0.616, 1.435)
Combined with other GN	0.364	1.711 (0.536, 5.459)	-	-
ACEIs/ARBs	0.484	0.677 (0.227, 2.021)	-	-
Steroids	0.340	1.703 (0.570, 5.087)	-	-
Cytotoxic drugs	0.597	1.343 (0.450, 4.010)	-	-
Plasmapheresis	***0.019***	4.692 (1.295, 17.005)	0.849	0.749 (0.038, 14.796)

[a]*Multivariable analysis was performed in a subgroup of 52 patients, of which the values of serum C3 were available at the presentation. Bold values represent P < 0.05.*

TABLE 4 | Potential prognostic factors for patient outcome by univariate and multivariate COX regression analysis.

Variable	Univariable analysis ($N = 60$)		Multivariable analysis ($N = 60$)	
	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**
Gender (female)	0.461	0.552 (0.113, 2.684)	-	-
Age	***0.028***	1.054 (1.006, 1.105)	0.080	1.046 (0.995, 1.099)
Hematuria (0 = none; 1 = microscopic; 2 = macroscopic)	0.634	1.291 (0.451, 3.695)	-	-
Proteinuria (0 = none; 1 = non-nephrotic; 2 = nephrotic)	0.773	0.850 (0.281, 2.568)	-	-
SCr on diagnosis (increased by 200 μmol/L)	0.457	1.207 (0.735, 1.981)	-	-
Serum C3 (increased by 0.1 g/L) ($n = 52$)	0.057	2.010 (0.980, 4.120)	-	-
Kidney IgG staining >1+	0.894	1.101 (0.267, 4.533)	-	-
IgG deposit on TBM and/or Bowman's capsule	0.377	2.032 (0.422, 9.797)	-	-
Kidney C3 staining intensity (increased by 1+)	***0.012***	1.937 (1.155, 3.248)	0.263	1.664 (0.682, 4.060)
Kidney C1q positive staining	0.710	1.347 (0.279, 6.500)	-	-
Percentage of crescents (increased by 10%)	***0.016***	1.272 (1.045, 1.547)	0.173	1.156 (0.938, 1.425)
Combined with other GN	0.755	0.811 (0.217, 3.030)	-	-
ACEIs/ARBs	0.191	0.351 (0.073, 1.688)	-	-
Steroids	0.140	3.269 (0.679, 15.741)	-	-
Cytotoxic drugs	0.812	1.183 (0.296, 4.735)	-	-
Plasmapheresis	0.628	1.673 (0.209, 13.396)	-	-

Bold values represent P < 0.05.

kidney survival (ESRD, 44.0 vs. 8.6%, $P = 0.001$) and higher proportion of death (28.0 vs. 5.7%, $P = 0.044$). More patients received immunosuppressive therapy in the group with crescent formation (76.0 vs. 42.9%, $P = 0.011$). Besides, hemoptysis (three cases) was only found in the patients with crescents.

DISCUSSION

To our best knowledge, the present study comprised the largest cohort of atypical anti-GBM disease. Atypical anti-GBM disease manifested milder clinical features and better kidney outcomes compared to classical anti-GBM disease. Though rather heterogeneous, a substantial number of the patients had complement activation and crescent formation. Patients having crescents presented with more severe clinical course and worse renal and patient outcomes than those without crescents. It is of note that nearly a quarter of these patients progressed to ESRD and 9/60 patients died with a median follow up of 36 months. The poor kidney and patient prognosis, not favorable as expected, emphasizes the attention to atypical anti-GBM disease

TABLE 5 | Comparison of clinical and pathological features between patients with and without crescents.

Characteristic	With CGN[a] (*n* = 25)	Without CGN (*n* = 35)	*P*-value
Clinical feature			
Male/female	21/4	21/14	***0.046***
Age, year	52.4 ± 16.8	51.2 ± 14.9	0.784
Smoking, *n* (%)	18 (72.0)	14 (40.0)	***0.014***
Prodromal infection, *n* (%)	7 (28.0)	1 (2.9)	***0.015***
Hemoptysis, *n* (%)	3 (12.0)	0 (0.0)	0.133
AKD or AKI, *n* (%)	14 (56.0)	13 (37.1)	0.148
Oliguria/anuria, *n* (%)	2 (8.0)	3 (8.6)	1.000
Hematuria, *n* (%)	21 (84.0)	17 (48.6)	***0.005***
24 h Proteinuria, g/24 h	3.8 (2.0, 6.9)	1.5 (0.5, 6.3)	0.089
Serum albumin, g/L	31.4 (23.9, 36.5)	36.2 (23.2, 42.8)	0.195
SCr on diagnosis, μmol/L	206.8 (123.9, 372.7)	109.9 (82.7, 161.5)	***0.003***
ANCA, n/N (%)	6/24 (25.0)	1/26 (3.8)	0.081
Pathology			
IgG deposit intensity (scale 0∼4+)	1.0 (1.0, 2.3)	1.0 (1.0, 1.5)	0.188
IgA deposit, *n* (%)	16 (64.0)	11 (31.4)	***0.012***
IgM deposit, *n* (%)	18 (72.0)	15 (42.9)	***0.025***
C3 deposit, *n* (%)	23 (92.0)	16 (45.7)	***<0.001***
C1q deposit, *n* (%)	6 (24.0)	4 (11.4)	0.349
FRA deposit, *n* (%)	12 (48.0)	8 (22.9)	***0.042***
Electric dense deposit, n/N (%)	19/24 (79.2)	14/35 (40.0)	***0.003***
Treatment			
ACEIs/ARBs, *n* (%)	6 (24.0%)	20 (57.1)	***0.011***
Immunosuppressive therapy, *n* (%)	19 (76.0)	15 (42.9)	***0.011***
Steroids, *n* (%)	19 (76.0)	13 (37.1)	***0.003***
Cytotoxic drugs, *n* (%)	11 (44.0)	7 (20.0)	***0.046***
Plasmapheresis, *n* (%)	4 (16.0)	0 (0.0)	0.054
Outcome			
Follow-up duration, month	27.4 ± 21.1	41.9 ± 19.7	***0.009***
Progression to ESRD, *n* (%)	11 (44.0)	3 (8.6)	***0.001***
Death, *n* (%)	7 (28.0)	2 (5.7)	***0.044***
1-year renal survival, *n* (%)	17 (68.0)	33 (94.3)	***0.012***
1-year patient survival, *n* (%)	22 (88.0)	35 (100.0)	0.067

[a]*CGN: crescentic glomerulonephritis. Bold values represent P < 0.05.*

from physicians. Our study showed that the immunosuppressive intervention was not associated with kidney or patient outcome. In future, prospective and controlled studies might be needed to address the optimal therapeutic regimen.

Our retrospective study unearthed that the clinical and pathological features of patients with atypical anti-GBM disease were rather heterogeneous, and milder than classical anti-GBM patients. Less than half of the patients underwent a course of AKD or AKI. Kidney injuries were much slighter than that in classical anti-GBM disease, manifested as less crescent formation and lower levels of SCr at presentation (21, 22). However, the degree of kidney impairment varied as 1/5 of patients exhibited SCr levels >300 μmol/L, while 1/2 of patients presented normal kidney function. Although half of the patients were current or former smoker, the manifestation of hemoptysis was rather rare in these patients, in contrast to ∼40–60% of classical anti-GBM patients presenting pulmonary involvement (11). Distinguished from mild to moderate proteinuria in classical anti-GBM disease (7), the degree of proteinuria was much more severe in atypical patients. Nearly half of the patients showed nephrotic-range proteinuria and 1/3 of them suffered from nephrotic syndrome. Almost all patients with atypical anti-GBM disease showed tubular-interstitial and arteriole injury, which was less common in typical anti-GBM disease. These histopathological features implied a more chronic course in atypical anti-GBM disease.

In our cohort of atypical patients, around half of all cases had crescent formation. Though less than classical patients (21), the percentage of crescents were associated with serum creatinine on diagnosis. A further comparison analysis showed that the kidney outcomes of patients with crescents were worse than those without crescents. Univariate survival analysis showed that the percentage of crescents was associated with renal survival. These results were similar to previous reports in classical anti-GBM disease that the proportion of crescents was an independent predictor for ESRD (23). It is of notice that nearly all patients with crescents had positive C3 staining, in contrast to merely half in patients without crescents. Moreover, higher level of serum C was an independent protective factor and the intensity of kidney C3 staining was an independent risk factor for kidney outcome in this cohort. Renal C3 deposit generally implies the activation of complement system in the kidneys, which promotes the formation of membrane attack complex to damage the tissues (24). As previously reported, almost all patients with anti-GBM disease have C3 deposit in glomeruli (21). Therefore, we speculated that the deposited linear IgG in a substantial atypical anti-GBM patients might also act as "classical pathogenic antibodies," which causes the activation of complement resulting in kidney injuries and crescent formation. However, the positive rate of C3 staining is lower in patients with atypical anti-GBM disease, which again reflects the heterogeneity in these patients and summons further investigations on the renal complement activation and its association with kidney outcome.

Previous studies have proven that the combination of plasmapheresis, steroids and CTX could improve renal and patient outcomes in classic anti-GBM disease (25–27). However, there were no unified recommendations for the treatment of atypical anti-GBM disease at present, given the heterogeneity of these patients (15, 18, 28). Treatments varied in different patients, which were highly dependent on the clinical judgments by physicians. In our cohort, only half of the patients received immunosuppressive treatments and 1/10 received plasmapheresis. Patients received steroids or immunosuppressant agents were usually those presented with more severe renal damage. A higher proportion of patients receiving steroids or immunosuppressant agents presented with crescent formation and renal dysfunction. The heterogeneity of treatments made it difficult to investigate on the association of immunosuppressive therapy and renal outcome in the current study. Considering a relatively high incidence of

complement activation and poor renal outcome, the role of immunosuppressive treatment in atypical anti-GBM disease may need to be further explored in future studies.

Concurrent AAV and MN in patients with anti-GBM disease had been well-documented in previous articles (2), and sporadically IgAN (29, 30), HSP-GN (31), TMBN (8), TMA (32) et al. In our present study, a high proportion (58.3%) of patients with atypical anti-GBM disease coexisted with other glomerular diseases including IgAN, MN, MPGN, FSGS, TMBN, AAV, and TMA. Typically, those diseases presented no linear IgG deposit alongside GBM, therefore, we speculated that they might have underlying relations with the occurrence of anti-GBM disease in this rare entity. The local glomerular damage caused by the already existing glomerular diseases exposed the sequestered autoantigens in GBM and then elicited autoimmune responses toward the GBM. Another explanation might be that the anti-GBM antibodies elicited immuno-inflammatory reactions in glomeruli, caused local tissue injury and facilitated other glomerular diseases.

There were several underlying explanations for the absence of circulating anti-GBM antibodies: (1) Instead of α3(IV)NC1, antibodies of some patients recognized unconventional antigens located on GBM which were beyond routine assays. Serum anti-GBM antibodies could be detected by indirect immunofluorescence using normal kidney tissues in a few patients of our cohort, which collaborated this hypothesis (data not shown). (2) Antibodies with low affinity could only be discovered by higher sensitive assays such as western blot and biosensor experiments rather than routine methods (33). (3) Similar like other autoimmune diseases, during the reconstruction of immune homeostasis in disease retrieval, the production of antibodies paused and circulating antibodies were obliterated by liver, but the tissue antibodies were hard to eliminate and presented a longer half life time (34).

There are several limitations of this study. First of all, the follow-up duration is short for survival analysis for this rare disease. Secondly, the treatments of patients had a high heterogeneity in our cohort, thus the role of immunosuppressive therapy in patient and kidney survival might be underestimated due to data bias. Lastly, the current study comprised of patients from Chinese population which might be lack of generalizability to other races.

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 the Ethics Committee of Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CS participated in the research design, performance of the research, data analysis, and article writing. XJ participated in the research design, data analysis, and article writing. ZC participated in the research design and data analysis. XY participated in the performance of the research and data analysis. MZ participated in the research design and article modification. All authors were involved in revising the manuscript and approved the final version.

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The Anti-inflammatory Effects of HMGB1 Blockades in a Mouse Model of Cutaneous Vasculitis

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In our previous study, we have found increased serum levels of HMGB1 in patients with Henoch– Schonlein purpura (HSP), allergic vasculitis (AV), and urticarial vasculitis (UV) and altered HMGB1 distribution in lesional skin in patients with HSP. HMGB1 plays a pro-inflammatory role in the pathogenesis of HSP. To further investigate the role of HMGB1 in the pathogenic mechanism of vasculitis, we investigated the anti-inflammatory effects of HMGB1 blockades (including anti-HMGB1 mAb and glycyrrhizin) in a mouse model of a cutaneous reverse passive Arthus (RPA) reaction. A total of 36 balb/c mice were randomly divided into four groups: the control group, IC model group, HMGB1 monoclonal antibody (anti-HMGB1-mAb) group and the glycyrrhizin group, with nine mice in each group. A cutaneous RPA reaction mouse model was established by injections of the OVA antibody and the OVA antigen. Mice of the anti-HMGB1-mAb group and glycyrrhizin group were pre-treated with anti-HMGB1 mAb or glycyrrhizin, respectively, before the RPA reaction. Our results indicated that HMGB1 blockades (anti-HMGB1 mAb and glycyrrhizin) obviously extenuated the severity of vasculitis skin damage and improved the histological evolvement of inflammatory cells infiltration, vascular fibroid necrosis, and vasodilation in a cutaneous RPA reaction mouse model. In addition, HMGB1 blockades reduced the infiltration of neutrophils, DCs, and T cells and decreased the mRNA expression of IL-6 and CCL5 in skin lesions in the cutaneous RPA reaction mouse model. We suggest that HMGB1 blockades may represent a new direction for the treatment of cutaneous vasculitis.

Keywords: high mobility group box-1, glycyrrhizin, reverse passive arthus reaction, vasculitis, inflammation

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INTRODUCTION

Vasculitis, that can occur in all sizes and types of blood vessels in almost all organs, is a procedure of clinical pathology, characterized by an infiltration of inflammatory cytokines around the blood vessel wall and blood vessels. Cutaneous vasculitis (CV) may be the most representative symptom of the varying degrees of vasculitis or may be the part most associated with other primary systemic diseases. It has been found in histology that CV is intimately related to immunopathological mechanisms and may be caused by immune cells (such as neutrophils, lymphocytes, or eosinophils) that mediate inflammation (1–3). It is well-established that the production and release of proinflammatory cytokines such as TNF- α and IL-6 play crucial roles in IC-induced inflammation (4–6).

High mobility group box 1 (HMGB1), which is described as a highly conserved non-histone DNA-binding protein, was discovered to be a crucial cytokine that mediates the response to infection, injury, and inflammation (7, 8). Antigen presenting cells (APC) can activate immune responses against pathogens. With the absence of pathogens, endogenous molecules (e.g., HMGB1 is passively released from necrotic cells or secreted by stressed cells to respond to cellular injury) activate APCs, resulting in autoimmune diseases and transplant rejections (9, 10). It has been proven in our previous study that HMGB1 is involved in the pathogenesis of inflammatory and autoimmune disorders, such as, Henoch– Schonlein purpura (HSP), allergic vasculitis (AV), and urticarial vasculitis (UV). It also altered HMGB1 distribution in lesional skin in patients with HSP (11–13). However, the function and mechanism of HMGB1 in vasculitis has not been clearly stated.

Glycyrrhizin is an active ingredient of licorice, and can be extracted or chemically synthesized (14). Glycyrrhizin has been confirmed to have both anti-inflammatory and anti-viral influences by combining directly to HMGB1, and inhibits its chemoattractant and mitogenic activities (15). Our previous study found that glycyrrhizin suppresses TNF- α induced chemokine production in HMEC-1 cells (16). We also found that the serum HMGB1 level of 16 Henoch– Schonlein purpura patients was significantly lower after treatment with glycyrrhizin (11).

Cutaneous reverse passive Arthus (RPA) reaction is a classical animal model of CV, in which immune-complex-induced endothelial inflammatory responses play essential roles (11). In our research, the anti-inflammatory effects of HMGB1 blockades stems from an understanding of the biological basis of the HMGB1 inflammation, and we further investigate the role of HMGB1 in the pathogenic mechanism of vasculitis in a mouse model of cutaneous reverse passive Arthus (RPA) reaction.

MATERIALS AND METHODS

Mice

Thirty-six balb/c mice aged from 6 to 8 weeks old were chosen in this study from the Sichuan University Animal Center (Sichuan, China) with free access to drinking water and food. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chengdu Second People's Hospital and carried out in accordance with guidelines for the Care and Use of Laboratory Animals of National Institute of Health.

Animal Model

A total of 36 balb/c mice were randomly divided into four groups: the control group, IC model group, HMGB1 monoclonal antibody (anti-HMGB1-mAb) group and glycyrrhizin group, with nine mice in each group. At the beginning of the experiment, the mice of the anti-HMGB1-mAb group were injected i.p. with anti-HMGB1 mAb (Sino Biological, Beijing, China) 2 mg/kg once every other day three times. For the glycyrrhizin group, mice were injected i.p. with glycyrrhizin (20 mg/kg; Nippon Kayaku, Tokyo, Japan) once every day for 6 days. The mice of the control group and IC model group, an equal volume of phosphate-buffered saline (PBS) was injected i.p. once every day for 6 days.

A mouse model was established on the last day of treatment mentioned above in each group. Mice were subjected to intradermal injection of the OVA antibody. Tail vein injections were given with the OVA antigen, some mice were injected by tail vein with the OVA antigen and 1% Evan's blue solution. Mice in the control group were given an intradermal injection with PBS solution on the back. The lesional skin on the back of the mice and the extent of exudation of 1% Evan's blue solution were observed. After that, mice were euthanized. Tissue samples in different groups were obtained.

Histological Examination and Immunohistochemical Staining

According to standard techniques, hematoxylin–eosin (HE) staining was performed on tissue sections of histological observation and immunohistochemistry with CD3 (Abcam, Cambridge, UK), CD11c (Beijing Biosynthesis, Beijing, China) and myeloperoxidase (MPO; Beijing Biosynthesis, Beijing, China). Morphological changes of skin lesions were observed by light microscopy (BX60; Olympus, Tokyo, Japan).

Real-Time Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (qPCR) tests were taken to detect the mRNA expression of IL-6 and CCL5 in the skin of every group. Total RNA in every groups was extracted from the Trizol reagent (Invitrogen Corp, Carlsbad, CA, USA) in conformity with the manufacturer's instructions. In the established model system, qPCR primers and the SYBR Green Master Mix were used to carry out the experiments. The following primers of IL-6 (MQP036632; GeneCopoeia, Rockville, MD, USA) and CCL5 (MQP030981; GeneCopoeia, Rockville, MD, USA) were used. In our established model, the reference gene and glyceraldehyde 3-phosphate dehydrogenase were analyzed by way of $2^{-\Delta\Delta CT}$ and the expression levels and fold changes of these cytokine controls were analyzed. GAPDH was used as the internal reference gene. The reaction conditions in this experiment were: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s for a total of 40 cycles.

Statistical Analysis

Mean ± SD were used for all results; one-way analysis of variance was used for statistical differences between groups. Data were analyzed with the Graphpad Prism software (GraphPad Sotware, La Jolla, CA, USA). Statistical significance was accepted at the level of $P < 0.05$. All experiments were carried out at least three times.

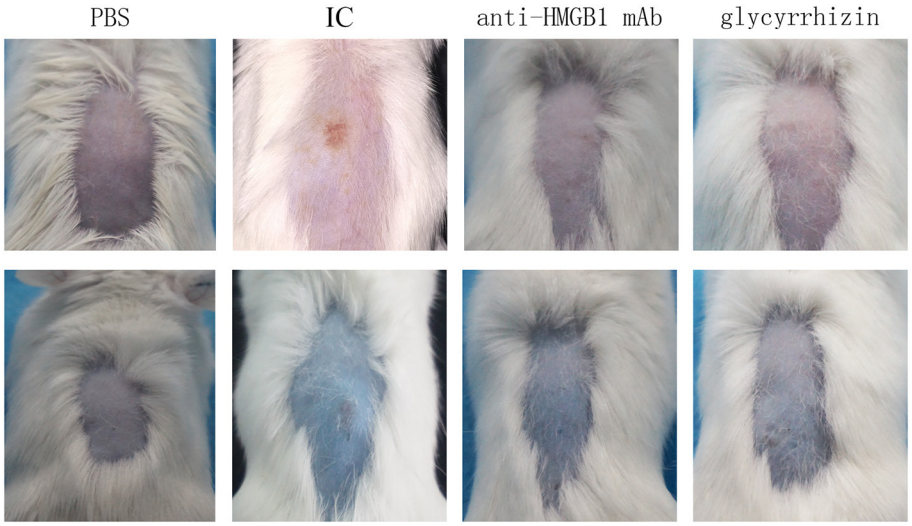

FIGURE 1 | Mice were injected i.p. with PBS, anti-HMGB1 monoclonal antibody, or glycyrrhizin, respectively. Mice were subjected to intradermal injection of the OVA antibody. Tail vein injections were given with the OVA antigen and 1% Evan's blue solution. Mice in the control group were given an injection with the PBS solution on the back. The macroscopic presentation of mice back skin was shown. IC showed obvious vasculitic lesions and Evan's blue exudation on the back contrasting with the other groups. IC, model control group; anti-HMGB1 mAb, anti-HMGB1 monoclonal antibody. $n = 9$ per group.

RESULTS

HMGB1 mAb and Glycyrrhizin Obviously Extenuated the Severity of Vasculitis Skin Damage

We observed skin lesions in all groups on the back of the mice and the extent of exudation of 1% Evan's blue solution. As presented in **Figure 1**, compared with the PBS control group, the model group showed obvious vasculitic lesions and Evan's blue exudation on the back. While treatment with the HMGB1 monoclonal antibody and glycyrrhizin, the skin on the back of the mice was significantly reduced in the local inflammatory response compared with the model group.

Significant Improvement in Inflammatory Cells Infiltration, Vascular Fibroid Necrosis, and Vasodilation Treated With the HMGB1 Blockade

Morphological changes of skin lesions were observed by light microscopy. During the histological examination, in contrast with the PBS group, we found that there was more inflammatory cell infiltration, vascular fibroid necrosis, and vasodilation in the model group, nevertheless, there was a significant improvement in the tissue treated with the HMGB1 monoclonal antibody and glycyrrhizin as seen in **Figure 2**.

HMGB1 Blockade Reduces Inflammatory Cell Infiltration in Vasculitis Mice Model

Immunohistochemistry was adopted to analysis the infiltration of the inflammatory cells. In flagrant contrast with PBS group, there was a marked reduction of lymphocytes, neutrophils, and dendritic cells infiltrating the model group. However, compared with the model group, the above inflammatory cells were significantly reduced after treatment with the HMGB1 antibody and glycyrrhizin as shown in **Figure 3**.

The mRNA Expression of IL-6 and CCL5 Were Reduced Evidently in the HMGB1 Monoclonal Antibody Group and Glycyrrhizin Group

As displayed in **Figure 4**, compared with the PBS group, the results of qPCR demonstrated that the mRNA expression of IL-6 and CCL5 had reduced evidently in the scathing tissue of the HMGB1 monoclonal antibody group and glycyrrhizin group ($P < 0.05$), yet there was a marked increase in the control group.

DISCUSSION

The high mobility group box 1 protein (HMGB1) is universal in all the cells of higher eukaryotes, is secreted by inflammatory cells (such as: activated monocytes, macrophages, mature dendritic cells, and natural killer cells), and may act as a potent mediator of inflammation (17–20). Our previous work found that the expression of HMGB1 was elevated in a variety of vasculitis diseases, and it was decreased in patients with HSP after treatment (13). HMGB1 may play an important role in the pathogenic mechanism of vasculitis. In this study, a HMGB1 blockade was used to treat vasculitis in a mouse model of cutaneous vasculitis. Then we detected that local skin lesions and tissue inflammatory cell infiltration were both significantly reduced. It meant that a HMGB1 blockade can effectively improve the clinical and pathological manifestations of vasculitis in mice.

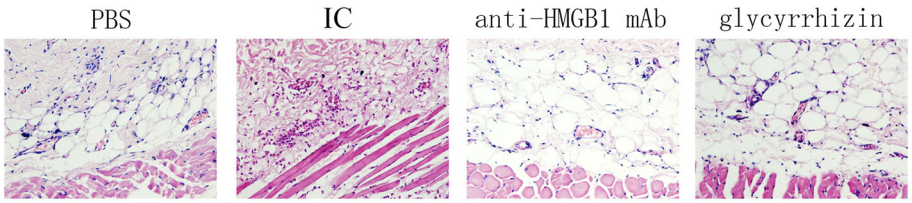

FIGURE 2 | Shown as a histological examination, Phenotypical presentation of mouse back skin which was injected with PBS, anti-HMGB1 monoclonal antibody, or glycyrrhizin, respectively, were applied and hematoxylin–eosin (HE) staining of tissue sections treated as described. There was more inflammatory cell infiltration, vascular fibroid necrosis, and vasodilation in the model control group, compared with the other groups. $n = 6$ per group.

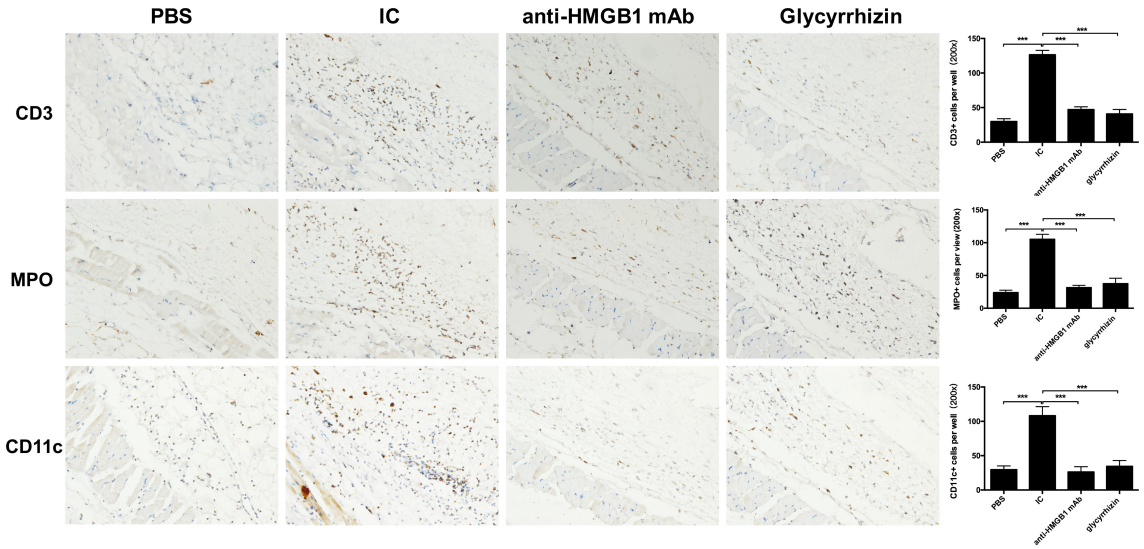

FIGURE 3 | Results of immunohistochemical staining of four groups in CD3, MPO, and CD11c. Image shows high power (200x). More inflammatory cell infiltration, vascular fibroid necrosis, and vasodilation in the IC when compared with the other groups. $n = 6$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$.

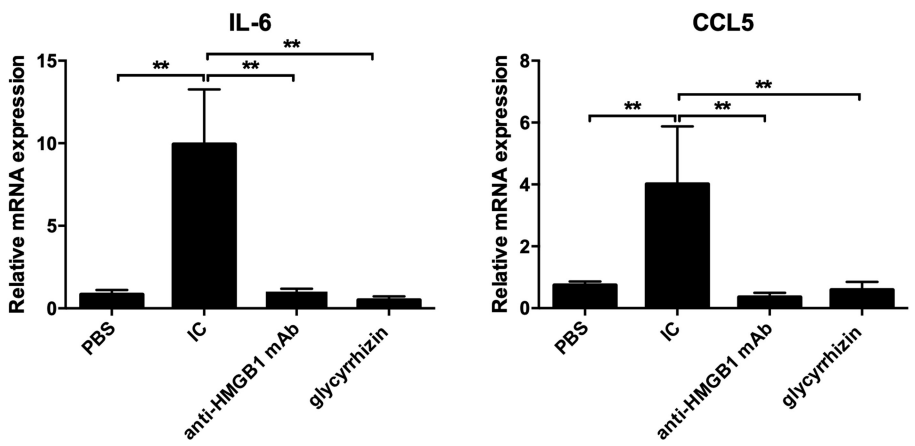

FIGURE 4 | PCR was taken to detect the mRNA expression of IL-6 and CCL5. Total RNA were extracted from the Trizol reagent. In contrast with PBS, the mRNA level of IL-6 and CCL5 was markedly higher in IC, yet there were reduced in other groups. $n = 6$ per group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

It is well-established that the production and release of proinflammatory cytokines such as TNF- α and IL-6 play crucial roles in IC-induced inflammation (4–6). In this study, we found that the expression of related inflammatory cytokines IL-6 and CCL5 were markedly increased in the skin samples of mice from the IC group compared with the control group.

The HMGB1 protein is not only a nuclear factor but also a secreted protein (8). It can stimulate activated monocytes, neutrophils, and the production of IL-7, IL-8, TNFα, MMP, and other pro-inflammatory cytokines, and can also promote the development of various chemokines. HMGB1 can also promote the local production of the tumor-necrosis factor (TNF), interleukin-6 (IL-6), and interferon-γ (7, 21). HMGB1 can induce phosphorylation of the inhibitor of κB-a (IκBa) and the nuclear translocation of nuclear factor-κB (NF-κB) p65 in HMEC-1 cells. The signaling pathway leads to the activation of NF-κB, which leads to an increased expression of CCL5, which in turn triggers a subsequent inflammatory response, resulting in more inflammatory cells infiltrating the damaged tissue, further aggravating pathological damage (18, 22). Maeda et al. (23) found that anti-HMGB1 antibodies inhibited the production of TNF-α and IL-6 by blocking extracellular HMGB1. In order to find out whether it reduced the inflammatory response by inhibiting HMGB1, we used anti-HMGB1 mAb and the HMGB1 blocker compound glycyrrhizin to treat the mice model. Then, we found the expression of related inflammatory cytokines IL-6 and CCL5 were significantly decreased after treatment, it may further reduce the damage of vasculitis. That means that a HMGB1 blockade can effectively inhibit the infiltration of inflammatory cells and the expression of inflammatory cytokines in the skin lesions of vasculitis mice (18, 19).

Previous studies suggest that glycyrrhizin has anti-inflammatory, antiviral, antimicrobial, antioxidative, anticancer activities, and immunomodulatory effects (24, 25). In Japan, a glycyrrhizin preparation called Stronger Neo-Minophagen C (SNMC) has been used as an anti-allergic and anti-hepatitis agent in clinical treatment for 60 years (26, 27). It has been reported that glycyrrhizin can effectively inhibit the cytoplasmic transduction of HMGB1. Our previous studies also found that glycyrrhizin can target the inhibition of cytoplasmic transduction in HMGB1 and thus inhibit the expression of inflammatory cytokines, and it has been proven that glycyrrhizin is a direct inhibitor of HMGB1 (13). This study used glycyrrhizin to effectively treat vasculitis mice and reduce inflammation. It was once again demonstrated that glycyrrhizin acts as an inhibitor of HMGB1 and also has an inhibitory effect on vasculitis.

In general, we have experimentally demonstrated that HMGB1 is closely related to the pathogenesis of vasculitis, and that the HMGB1 blockade can significantly improve the inflammatory response of vasculitis, resulting in a more effective, safe, and potentially new treatment for vasculitis.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Chengdu Second People's Hospital.

AUTHOR CONTRIBUTIONS

LF and TC: concept, design, and definition of intellectual content. JW and LF: literature search. LF, JW, and KC: experiment studies. QS and HY: data acquisition and data analysis. JW and LF: manuscript preparation. JW, LF, and TC: manuscript editing. TC: manuscript review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Understanding Behçet's Disease in the Context of Innate Immunity Activation

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Behçet´s disease (BD) is a heterogeneous condition consisting of idiopathic systemic vasculitis affecting large and small blood vessels of different types (i.e., arteries, veins, or capillaries). The disease frequently occurs in young adults without gender predilection, differently from several other autoimmune conditions. This challenging illness has recently been proposed by some authors as an example of complex autoinflammatory syndrome. Although much remains unanswered about BD pathogenesis, recent understanding of some aspects of innate immunity have clarified a few issues (and raised others). *HLA-B*51* represents the strongest genetic risk factor for BD to date, albeit several other HLA-independent loci have also been associated with the disease. The consistent hyper-reactivity against *Streptococcus sanguinis* antigens and alterations in oral and gut microbioma suggests that infectious agents may play an important role. Moreover, functional abnormalities of pattern recognition receptors, especially Toll-like receptors in monocytes, have been demonstrated in patients with BD and can be associated with the development of the disease. Neutrophil hyperactivity is one of the most consistent findings in BD pathogenesis, as demonstrated by exacerbated constitutive oxidative burst, chemotaxis and NET formation. However, some studies suggest that the phagocyte-activated status in BD is not primary to the disease itself, but rather restricted to a fraction of patients with severe disease activity, and probably secondary to activating soluble factors carried by serum/plasma from BD patients. Herein we review the state of the art on BD etiopathogenesis with special emphasis on the participation of the innate immune system

Keywords: Behçet's disease, innate immunity, phagocytes, neutrophil hyperactivity, alarmin, *Streptococcus sanguinis*

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INTRODUCTION

Behçet's disease (BD) is an idiopathic systemic vasculitis affecting large and small blood vessels. It was initially described as recurrent oral and genital ulcers associated with anterior uveitis with hypopyon by Hulusi Behçet (1) and Benediktos Adamantiades (2) in the 1930s. Progressively, however, a variety of musculoskeletal, neurological, gastrointestinal and vascular manifestations was associated to the syndrome.

The epidemiology of BD exhibits an interesting geographic distribution throughout the ancient "Silk route", with higher prevalence in Turkey, Iran and Japan. The disease frequently occurs in

young adults (mean age: 25–30 years old) (3). The lack of gender predominance is one of the arguments favoring the non-autoimmune nature of BD pathogenesis. This challenging illness received special attention in the last years, culminating with novel insights on the possible autoinflammatory etiopathogenesis of the disease, which encouraged some authors to consider BD as an example of complex autoinflammatory syndrome (4). Much remains unanswered in BD pathogenesis, as recent progress in the understanding of some aspects of the innate immunity has raised unforeseen questions. Herein we review the state of the art on BD etiopathogenesis with special emphasis on the participation of innate immunity activation.

THE IMMUNOGENETIC BASIS OF BEHÇET'S DISEASE

Histocompatibility Leucocyte Antigen

Histocompatibility leucocyte antigen (HLA)-B*51 represents the strongest genetic risk factor for BD to date. It was initially reported in the Japanese (5) and reproduced among several other ethnic groups (6). A meta-analysis reported an overall odds ratio of 5.78 (95% CI=5.00–6.67) for *HLA-B*51* carriers to develop BD, independently of the ethnicity (6). Similar results were confirmed in two different genome-wide association studies (GWAS) in Japanese (7) and Turkish BD patients (8). However, recently other loci have been demonstrated to increase the risk of BD as well. Kuranov et al. (9) studied HLA-B*51-negative patients and showed a significant association of BD and HLA-Bw4-80I, an epitope present on B locus-derived proteins, characterized by the presence of an isoleucine at amino-acid position 80 in the α1 helix of the HLA-B*04. Additionally, these authors found an association with HLA-A*26 independent from HLA-B*51, which was confirmed in other studies (8, 10). In addition, Hughes et al. (10) demonstrated that HLA-A*03, B*15, B*27, B*49 and B*57 also contribute to BD risk independently, although this has not been replicated to the moment. Another study identified additional independent risk factors for BD located at HLAB/MICA and at the region between HLA-F and HLA-A (11).

Other Genetic Risk Factors

GWAS in BD patients identified extra-HLA genetic risk factors. By analyzing 311,459 SNPs in 1,215 BD patients and 1,278 controls, Remmers et al. (8) identified two novel susceptible loci for BD: *IL23R-IL12RB2* and *IL10* (allele rs1518111 A, associated with low mRNA and protein expression). Recently, association between *IL10* polymorphisms and BD was also demonstrated in Chinese patients (12). These data emphasize the possible role of IL-10 in BD pathogenesis and raise the question of possible participation of adaptive immunity, especially Th17 and Treg cells, in BD (13).

Copy number variation (CNV) of Complement component *C4* genes was investigated in BD. In contrast to systemic lupus erythematosus (14), there is an increased frequency of more than 2 copies of the *C4A* gene in BD patients and this represents a risk factor independent from *HLA-B*51* (15). Moreover, the authors also demonstrated that BD patients with high *C4A* copy number had increased production of IL-6, an important mediator of the innate immunity acting as an acute phase reactant.

Another interesting observation is the presence of a specific chromosomal abnormality in a number of patients with BD: trisomy of chromosome 8 (16, 17). Considered a risk factor for myeloid leukemia (18) and myelodysplastic syndrome (19), appearing in 5-10% of patients, trisomy 8 also seems to play a role in BD. As shown in a recent study, its frequency was reported as high as 86% in patients with concurrent BD and myelodysplastic syndrome (20). Apparently, these cases present frequently with prominent gastrointestinal involvement and no geographical preference (21). Interestingly, chromosome 8 harbors some pivotal genes related to innate immunity modulation and NF-κB pathway activation, such as *IKBKB*.

ALARMINS AND MICROORGANISMS

Alarmins are a group of proteins with the ability of initiating the innate immune response after quick release following cell necrosis. Alarmins activate pattern recognition receptors (PRR), such as Toll-like receptors (TLR), and are essential to restore homeostasis after tissue damage. In fact, alarmins are considered a subtype of DAMP (damage-associated molecular patterns), which consist of stereotyped molecular patterns shared by molecules originated after exposure to physical or chemical agents capable of inducing tissue damage (e.g., radiation, heat and cold, among others).

The High Mobility Group Box 1 (HMGB1) is probably the most studied alarmin in systemic autoimmune rheumatic diseases (22–26). Ahn et al. (27) demonstrated that HMGB1 serum levels are increased in BD patients, especially those with gastrointestinal involvement. Conversely, our group found higher HMGB1 levels in BD patients compared to controls, regardless of disease activity, disease manifestations or therapy with prednisone and azathioprine (28). Han et al. (29) reported increased serum levels of alarmin S100A12 in BD, independently of disease activity, although at higher magnitude in active phase. Accordingly, S100A12 serum levels decreased after the treatment and the protein expression was increased in skin biopsies of active erythema nodosum lesions from BD patients.

There is also some evidence regarding the role of microorganisms on BD. The hyper-reactivity against *Streptococcus sanguinis* antigens and the homology and potential cross-reactivity of some of its proteins with human heat-shock proteins (HSP) (30), as exemplified by the activation of T $\gamma\delta^+$ cells by the pathogen and HSP 60/65 kDa (31), suggest this infectious agent might play an important role in BD pathogenesis (32). Herpes simplex virus 1 (HSV-1), *Staphylococcus aureus*, *Mycobacterium tuberculosis* and some *Prevotella* species have also been identified as potential candidates (33). HSV-1 RNA and DNA were found in increased frequency in cells from BD patients (34, 35). Moreover, mycobacterial HSP peptides stimulate $\gamma\delta^+$ T cells from BD

patients, which, in turn, are increased in peripheral blood and mucosal lesions (36). Despite these intriguing set of data, a direct causal relationship between infectious agents and BD, as well as the precise role of $\gamma\delta^+$ T cells on the disease pathogenesis, remain unclear.

TOLL-LIKE RECEPTORS AND OTHER PRR

Evidence of increased serum levels of alarmins and hyperactivity against some microorganisms turns plausible the hypothesis that PRR participate in BD pathogenesis. Indeed, functional abnormalities of PRR and their activation cascades have been identified in previous studies and can be associated with BD development. Yavuz et al. (37) demonstrated that TLR6 expression is significantly increased in granulocytes from BD patients after stimulus with *Streptococcus sanguinis* or HSP-60 compared to rheumatoid arthritis patients and healthy controls. Interestingly, monocytes from BD patients presented lower TLR2 expression after the same stimuli. Of interest, Neves et al. (38) and Do et al. (39) showed that TLR2 and TLR4 expression in monocytes from BD patients was constitutively increased; however, this finding was not observed in neutrophils from these patients.

A recent GWAS with 2,461 BD cases and 2,458 healthy controls showed protective *TLR4* and *NOD2* polymorphisms, respectively associated with decreased response to lipopolysaccharide and muramyl dipeptide (40). Furthermore, a multicenter study with Chinese and Dutch patients has provided evidence that polymorphisms in TLR2 are involved in ocular BD susceptibility (41). A SNP of *TIRAP*, a MyD88-adapter-like molecule with a regulatory role in TLR2 and TLR4 signaling, has been associated with BD in a British cohort (42), but results were not replicated in Middle-Eastern, Turkish or Italian patients (43).

Taken together, these findings implicate innate immunity and bacterial sensing mechanisms as important players in BD pathogenesis, with participation of diverse gene polymorphisms according to different ethnicities, and represent a promising investigational area in BD.

THE NF-κB PATHWAY

Downstream signaling leading to internalization of the two nuclear factor κB subunits (p50 and p65) represents the canonical signal transduction pathway after activation of several PRR. Despite the inflammatory characteristics of BD suggesting NF-κB hyperactivation in BD patients (44), there are few studies in the area, but this has progressively been changing lately.

Polymorphisms in *NFKB1* promoter (–94 insertion⁄deletion ATTG) (45) and *NFKBIA* (rs696) (46) were demonstrated to enhance the risk for BD in the Turkish population. It seems that NF-κB plays a pivotal role in controlling T cells apoptosis in BD. Although CD95 is highly expressed on T cells from BD patients, Todaro et al. (47) demonstrated decreased sensitivity to CD95-induced apoptosis, possibly attributed to the inhibitory action of anti-apoptotic genes (*CFLAR*, *BCL2L1*, *BCL2*, *CASP3*, *CASP8*) and up-regulated expression of Iκκ, IκB, and NF-κB. Interestingly, thalidomide, a therapeutic agent used in severe mucocutaneous manifestations of BD, and NF-κB small interfering RNA down-regulated cFLIP and Bcl-xL expression levels, ultimately increasing activated T cells sensitivity to CD95-induced apoptosis in BD.

Constitutive NF-κB canonical pathway hyperactivation in BD phagocytes was previously reported by our group, as indicated by the over-expression of phosphorylated p65 subunit (44). Similarly, a monogenic form of an autoinflammatory disorder resembling BD was described in five families carrying heterozygous germline mutations of *TNFAIP3*, a potent inhibitor of the NF-κB canonical pathway (48). The mutant *TNFAIP3*-derived transcript A20 is not capable of modulating intracellular signaling, ultimately culminating in phagocyte hyperactivation and increased NF-κB-mediated proinflammatory cytokines secretion. Moreover, carriers of *NFKB1* variants have been reported to present a monogenic BD-like disease, characterized by pathergy-like lesions and striking macrophage inflammasome activation. Finally, an autosomal-dominant mucocutaneous ulceration disorder was recently associated with *RELA* mutations, encoding the NF-κB subunit p65 (49).

Previous studies further support the clinical overlap between BD and other autoinflammatory diseases with shared etiopathogenesis, such as familial Mediterranean fever (4, 50). *MEFV* M694V mutation frequency is increased in Turkish BD patients (40). Rare genetic variants of undetermined significance in inflammasome components upstream of NF-κB have also been found in BD patients, especially *NOD2* and *NLRP3* (51–53). Some of these variants may contribute to the disease onset, but others could be only single nucleotide polymorphisms without any effect. Anyhow, inflammasome-activated NF-κβ pathway dysregulation seems to be a common finding in disorders with BD-like phenotypes (54).

ENDOTHELIAL CELL DYSFUNCTION

As a pro-thrombotic condition, one would expect the existence of some sort of endothelial dysfunction in BD. A study in Turkish patients showed that patients with active disease presented lower nitric oxide serum levels than those in remission (55). Since endothelial cells are major producers of nitric oxide, the authors suggested a putative dysfunction in these cells. This would be probably mediated by increase in oxidative stress due to augmented malondialdehyde (a metabolite of polyunsaturated lipids oxidation by reactive oxygen species – ROS) serum levels in active BD patients.

Fadini et al. (56) originally demonstrated a progressive decrease of circulating endothelial progenitor cells in BD patients, which might represent a vascular damage mechanism, since these cells are involved in vascular homeostasis and repair.

The authors also showed a positive correlation between the number of endothelial progenitor cells and both BD activity score and C-reactive protein.

A systematic review aimed to evaluate subclinical atherosclerosis in BD by endothelial-mediated dilatation and by measurement of intima media thickness (IMT) of carotid arteries (57). Among nine studies, endothelial-mediated dilation was demonstrated to be impaired in BD even in inactive state. IMT was greater in BD patients, despite considerable variation that reflects the clinical heterogeneity of the disease.

NEUTROPHIL HYPERACTIVITY

Clinical and pathological data strongly suggest that neutrophil hyperactivity is a prominent feature in BD pathogenesis. Exacerbated neutrophil activity can be determined by evaluating oxidative burst, phagocytic and microbicide activities, activation of intracellular signaling pathway, among others. Takeno et al. (58) showed that ROS production is increased not only in BD patients but also in asymptomatic *HLA-B*51* carriers and even in transgenic mice expressing *HLA-B*51*. These observations suggest a mechanistic connection between the already known immunogenetic background of BD and its pathogenesis.

Carletto et al. (59) described that peripheral blood and skin (obtained by cutaneous abrasion) neutrophils from patients with active BD present higher migration capacity than those from healthy controls and BD patients with inactive disease. The increased migration capability was normalized when patients attained remission, suggesting that this mechanism is involved in the inflammatory state of the disease. In contrast, no significant abnormalities were observed in other neutrophil functions, such as adhesion or superoxide production after zymosan, phorbol-myristate-acetate (PMA) or N-formylmethionine-leucyl-phenylalanine (fMLP) stimuli. However, Yoshida et al. (60), using a chemoluminescence method to determine the superoxide production in 20 BD patients and healthy controls, demonstrated a significantly higher superoxide production by neutrophils from BD patients after the same stimuli used by Carletto et al. (59).

Eksioglu-Demiralp et al. (61) studied the oxidative burst after stimuli with PMA or fMLP and the phagocytic activity against *E. coli* in neutrophils from healthy controls, BD patients (*HLA-B*51* carriers or not), septic patients and patients with inflammatory arthropathy (namely, rheumatoid arthritis and ankylosing spondylitis). Oxidative burst was decreased in stimulated neutrophils from BD and septic patients, suggesting that phagocytes were exhausted and hypo-responsive *in vitro* due to previous *in vivo* hyper-activation. Interestingly, phagocytic activity was significantly increased in septic and inflammatory arthropathy groups, but did not differ between BD patients and healthy controls.

The same Turkish group published in 2002 another study reassessing phagocytic activity and oxidative burst profile after PMA stimulus of neutrophils from healthy controls, BD patients and "inflammatory patients" (septic, primary vasculitis, systemic lupus erythematous, osteomyelitis and pneumonia) (62). Exclusively BD patients presented a decreased oxidative burst after stimulus, which was inhibited by nitric oxide synthase inhibitors, although significantly less than the healthy controls. There was no difference in phagocytic activity among the groups. Once again, the authors attributed the results to a possible *in vivo* pre-activated/exhaustion state of BD neutrophils.

Altogether, these data support the concept that neutrophils play a pivotal role in BD pathogenesis. However, there might be other factors contributing to BD development, many of them still unknown. It is unclear, for example, if the striking neutrophil hyperactivation occurs constitutively or if it is secondary to a yet unknown stimulus, such as bacterial (e.g.: *Streptococcus*) or viral infections. Therefore, in an attempt to clarify this doubtful issue, our group designed a study aiming to assess the classical phagocyte functions (i.e., oxidative burst, *in vitro* cytokine production, phagocytic and microbicide activities) before and after stimulus with pathogens and several microbial components in 30 healthy controls, 25 septic patients, 31 inactive, and 30 active BD patients (63). We observed that phagocytes from BD patients with severe manifestations exhibit significantly higher oxidative burst activity, both before and after PMA stimulation, compared to cells from patients with mild BD manifestations. Furthermore, we found significant positive correlations between BD patients' scores on the simplified *Behçet´s Disease Current Activity Form* (BR-BDCAF), a validated tool to measure disease activity, and *Streptococcus sanguinis*-stimulated production of IL-23 by peripheral blood mononuclear cells (PBMC) and IL-8 by neutrophils. In addition, significant positive correlations were also found between BR-BDCAF score and constitutive production of TNFα, IFNγ, IL-6, and IL-23 by PBMC. Thus, our study corroborates the participation of phagocyte in BD pathogenesis by the evidence that patients with severe BD exhibit phagocytic dysfunction and some extent of constitutive activation.

In contrast, an important aspect of neutrophil biology has largely been ignored despite the striking body of evidence of involvement of these cells in BD: neutrophil extracellular traps (NET) release. Our group originally showed an increased constitutive NET release in BD patients (64). Interestingly, NETosis was markedly stimulated by soluble CD40L, especially from plasma of active BD patients. Similarly, Le Joncour et al. (65) recently demonstrated that circulating NET components are elevated in active BD patients, mainly in those with vascular involvement, suggesting that NET may represent a potential therapeutic target for BD-associated thrombotic risk.

Despite some controversy in the literature regarding neutrophil dysfunction in BD (summarized in **Table 1**), the bulk of evidence suggests that the activated status of phagocytes in BD is not a constitutive feature, but rather restricted to a fraction of severely active patients, and probably secondary to an unknown soluble factor (**Figure 1**).

ROLE OF MONOCYTES IN BD

Like neutrophils, monocytes are important agents of the innate immune system by means of their phagocytic activity, oxidative

TABLE 1 | Controversies regarding phagocyte activity in Behçet's disease (BD).

Author	Year	Brief description	Reference
Takeno et al	1995	Increased oxidative burst in BD patients and HLA-B51 healthy controls.	(58)
Sahin S et al	1996	Increased adhesion.	(66)
Carletto A et al	1997	Increased migration (active BD only). No difference regarding oxidative burst.	(59)
Yoshida et al	1998	Constitutively increased oxidative burst in BD neutrophils. Increased oxidative burst in neutrophils from healthy controls after pretreatment with serum from BD patients.	(60)
Eksioglu-Demiralp E et al	2001	Decreased oxidative burst. No difference regarding phagocytic activity compared to healthy controls.	(61)
Atalay G et al	2002	Decreased oxidative burst (active BD only). No difference regarding phagocytic activity.	(62)
Neves et al	2009	Both normal and BD neutrophils increased chemotactic capacity after incubation with BD plasma. No difference regarding chemotaxis.	(38)
Perazzio et al	2015	Increased oxidative burst (severe BD only). Positive correlation between activity score and constitutive or *Streptococcus sanguinis*-stimulated production of cytokines *in vitro*. No differences regarding phagocytic and microbicide activities.	(44)
Perazzio et al	2017	Plasma from BD patients exerted a stimulus on neutrophil extracellular traps release and oxidative burst, probably induced by sCD40L	(44)
Le Joncour et al	2019	Circulating neutrophil extracellular traps markers are elevated in BD and contribute to the procoagulant state	(65)

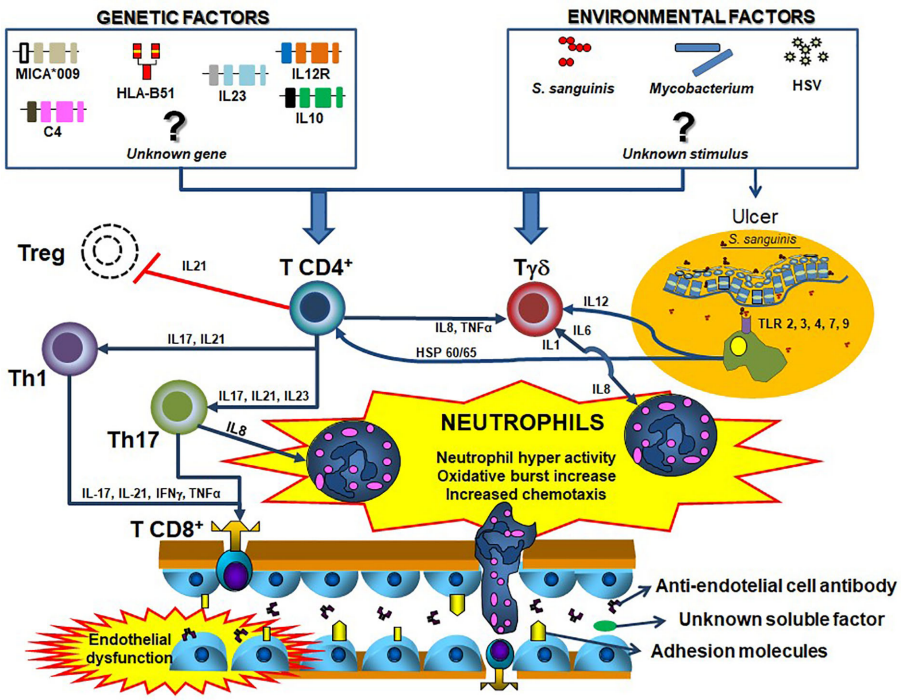

FIGURE 1 | Summary of the possible Behçet´s disease pathogenesis. Distinct T helper cells mainly Th1 and Th17 have the ability of stimulating T effectors and T regulatory cells especially by the action of cytokines. Although some remain unknown, several genetic (e.g.: *HLA-B*51*, MICA, C4 copy number variation, among others),,, and environmental factors (e.g., *Streptococcus sanguinis*, *Herpes-simplex virus*, mycobacteria, among others) are involved in the process, by facilitating the activation of T cells. Similarly, the antigen presenting cells, especially macrophages from mucosa, stimulate immune cells by Toll-like receptors activation. All these innate and adaptive immune pathways culminate with the sequential neutrophil activation, considered the most important element in BD pathogenesis. blue closed arrows represent stimulation of a cell subtype mediated by cytokines, while red open lists represent inhibition.

burst and cytokine production. Thus, it is reasonable to suppose that their function and response to stimuli may bear some similarity to those of neutrophils, especially in diseases with neutrophil hyperactivity.

Indeed, Gogus et al. (67) showed that monocytes from BD and familial Mediterranean fever patients present higher oxidative burst activity than those from rheumatoid arthritis patients and healthy controls, especially when stimulated by sodium monourate crystals. Moreover, interactions between neutrophils and monocytes have received particular attention. For example, peptides released from activated human neutrophils stimulate monocyte adhesion and transmigration as well as macrophage oxidative burst (68).

Interestingly, monocytes from BD patients present higher expression of TLR2. Moreover, bacterial-derived lipoteichoic acid activated TLR2 increases neutrophil chemotaxis and

adhesion to endothelial cells (38). Furthermore, active DB is associated with higher expressions of TLR2 and TLR4 in monocytes, as well as with a higher frequency of pro-inflammatory $CD14^+CD16^+$ monocytes in the peripheral blood compared to healthy controls (39). *In vitro* lipopolysaccharide-stimulated monocytes from BD patients produced similar amounts of TNFα compared to healthy controls cells; however, a higher *in vitro* production of TNFα was observed in monocytes from clinically active BD patients in comparison to those from quiescent BD patients (69). It would be intriguing to further explore this aspect in BD since monocytes seem to play a role in its pathogenesis as these cells may contribute to neutrophil activation after bacterial triggers in BD patients.

SOLUBLE FACTORS AND AUTOANTIBODIES

Cytokines and soluble receptors are major effectors of innate immunity and some of them have already been associated with BD pathogenesis. A previous study showed an increased expression of Th1 cytokines (IL-12, IL-18, and IFNγ) in skin and oral ulcers from active BD patients (70). In fact, Yanaginori et al. (71) demonstrated that stimulation with Streptococcal antigens specifically increased gene and protein expression of IL12p40, in conjunction with IL12p70 induction, in PBMC from BD patients. This finding provides evidence for Th1-skewed anti-bacterial host response mediated by IL-12 in BD patients. Two studies showed that serum and cerebrospinal fluid (CSF) levels of IL-15 and IL-18, two crucial Th1 cytokines, were increased in neuro-Behçet patients (72, 73).

Moreover, in the strict context of innate immunity, some studies showed that serum levels of IL-8, a potent neutrophil activator and chemotactic factor, are increased in BD patients (66, 74), especially in active disease (75). IL-8 levels were also elevated in synovial fluid from BD compared to osteoarthritis patients, which suggests an important role for this cytokine in BD pathogenesis (76). Inflammasome or NF-κB-derived IL-1β, TNFα, and IL-6 are also representatives of strictly innate immunity soluble mediators and act individually or altogether in systemic inflammation, inducing acute phase reactants and phagocyte activation. These cytokines may play pivotal role on BD etiopathogenesis, as therapeutical blockade is indicated by current clinical guidelines (77). Among immunobiological therapies for BD, certainly anti-TNF are the most frequently prescribed and are indicated for refractory mucocutaneous lesions, peripheral vascular symptoms (deep venous thrombosis and arterial aneurisms) and as alternative for parenchymal central nervous system and ocular manifestations. Anti-IL-6 therapy has been indicated on refractory central nervous system manifestations (78–80) and anti-IL-1β showed promising results for severe ocular (81, 82) and mucocutaneous clinical phenotype (83, 84).

Interestingly, Alpsoy et al. (85) demonstrated that *IL8* gene expression was increased in macrophages from BD patients and healthy controls after incubation with serum from active BD patients. Similarly, (85) several other studies demonstrated the capacity of serum or plasma from BD patients to stimulate the innate immune system. Yoshida et al. primed neutrophils from healthy controls with BD serum and observed an increase in the production of superoxide similar to that observed after stimuli with zymosan, PMA or fMLP. (60). However, the absence of objective BD disease activity determination was a caveat of that study. Sahin et al. (66) demonstrated an increased adhesion ability of normal neutrophils to human umbilical vascular endothelial cells and increased expression of adhesion molecules (CD11a, CD18, and ICAM-1) when exposed to BD serum, compared to the stimulus of normal serum. However, the authors could not find any difference between serum from patients with active and inactive disease, possibly due to similarly high IL-8 serum levels in both groups. Another study from the same group showed that BD patients presented higher monocyte expression of CD14, a monocyte-activating marker, as well as higher soluble CD14 serum levels than healthy controls (86). Furthermore, the supernatant of BD monocyte culture significantly increased the adhesion ability of normal neutrophils to endothelial cells *in vitro*. These results indicate that BD monocytes are active and produce a milieu of pro inflammatory cytokines, which may play a role in the chronic inflammation of BD.

Neves et al. showed that chemotaxis was similar in neutrophils from BD and normal controls after stimulation with lipoteichoic acid (38). Interestingly, both healthy and BD neutrophils presented increased chemotactic capacity when incubated in the presence of BD plasma or stimulated with C5a, B4 leukotriene or fMLP. Similarly to Sahin et al. (86), CD14 expression in monocytes and soluble CD14 serum levels were increased in BD patients. Additionally, the authors showed a positive correlation between BDCAF and soluble CD14 serum levels, suggesting that the soluble proinflammatory factors produced in BD correlate with disease activity.

Our group also demonstrated that NET release and oxidative burst were stimulated with plasma from BD patients (64). In addition, markedly elevated sCD40L serum levels in conjunction with CD40L overexpression on $CD4^+$ T cells from BD patients were observed. Interestingly, we originally described that both NET release and oxidative burst were exacerbated by recombinant sCD40L and decreased after sCD40L blockade, suggesting a possible role of this mediator on BD pathogenesis.

Serum and plasma seem not to be the only carriers of soluble factors related to phagocyte activation in BD. Chemokine levels in aqueous humor are apparently also increased in BD patients with ocular manifestations. El-Asrar et al. (87) demonstrated that CXCL1 and CXCL10 were significantly higher in aqueous humor of patients with BD compared to patients with Vogt-Koyanagi-Harada disease and *HLA-B*27*-associated uveitis. Additionally, CCR5 and CXCR3 had increased expression in biopsy specimens of oral ulcers from BD patients compared to healthy controls (88), and MIP-1β (macrophage inflammatory protein 1β) had increased serum levels in BD (70), indicating a Th1-skewed immune response on BD immunopathology. Interestingly, another study showed increased expression of transmembrane

CXCL16 on circulating plasmacytoid dendritic cells from BD patients, which might contribute to the high serum IFN-α levels seen in patients with BD (89).

Although BD is not a typical autoantibody-associated condition, anti-α-enolase antibodies have been associated with the disease. One study demonstrated that *Streptococcus sanguinis* or BD serum stimuli increase α-enolase expression on human microvascular endothelial dermal cells, a target for autoantibodies observed in a fraction of BD patients (90). Thus, hyper-expressed α-enolase could react to anti-α-enolase antibodies present in BD serum, eliciting immune response (91). Additionally, the same authors previously identified that heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 is a cross-reactive target of anti-α-enolase antibodies and that *Streptococcus sanguinis* or serum from active BD patients are capable of inducing *in vitro* expression of hnRNP A2/B1 in human microvascular endothelial dermal cells (92). Orem et al. (93) also described that plasma from BD patients impaired nitric oxide production by human umbilical vascular endothelial cells, suggesting an inhibitory effect over endothelial NO synthase.

Despite several pieces of evidence, the literature is still controversial regarding the potential role played by a possible soluble phagocyte- or endothelial-activating factor carried by serum or plasma (**Figure 1**). As discussed above, it is unclear whether phagocyte activation is constitutive in BD or secondary to a soluble factor stimulation. Additionally, even considering the existence of such a factor, the identity and the cells responsible for its production remain unknown. Therefore, this is an area of great interest and further research is warranted to clarify these questions.

INNATE VERSUS ADAPTIVE IMMUNITY: A PARADIGM FROM THE PAST

Although BD is considered an example of strong innate immunity activation, it is important to highlight that not all immune cells can be assigned strictly to either the innate or the adaptive arm of the immune system. The existence of bridge populations between the two classic arms of the immune system expands the paradigm of innate versus adaptive immunity and, thus, sheds doubt on the concept of "innate" versus "adaptive" immune-mediated diseases. The modern understanding of the immune system consists of an organizational continuum, rather than a dichotomic system, especially due to the acknowledgment of the bridge population subsets, which present functions comprehending the innate and adaptive poles (94). Macrophages, for example, can phagocyte and destroy microorganisms by the induction of oxidative burst, but bridges the gap between innate and adaptive immunity by processing and presenting antigens to lymphocytes. Other elements involved in the integration of innate and adaptive immunity include NKT cells, γδ T cells, CD8α T cells, and B1 cells. In addition, innate lymphoid cells also produce large amounts of cytokines attributed to adaptive immunity, such as IFNγ, IL-4 and IL-17A.

This review refers mainly to the innate arm of the immune system in BD. However, there are several pieces of evidence supporting the participation of the adaptive immunity in BD pathogenesis. Keller et al. (95) described a prominent $CD4^+CXCL8^+CCR6^+$ T cell infiltrate in three different "neutrophilic" diseases: BD, pustular psoriasis and generalized exanthematous pustulosis. Interestingly, these cells produced predominantly CXCL8 and GM-CSF, but not IL-5 and IFNγ. Therefore, it is possible that these cells constitute a different subset of T cells, since their phenotype and functions differ from those of other classical $CD4^+$ cells, such as Th1, Th2, Th17, and are associated to a unique inflammation cascade that promotes neutrophil hyperactivation.

Additionally, Th1 cells producing TNFα, IFNγ, IL-8, IL-12, CCR5, CXCR3, and MCP-1 (macrophage chemoattractant protein 1) were reported in several BD lesions, including oral and genital ulceration, pseudofolliculitis, pathergy pustules and bowel ulcers (88, 96). Data regarding regulatory T (Treg) cells are scarce and conflicting. Some studies demonstrated a high number of Treg cells ($CD4^+CD25^{high}Foxp3^+$) in peripheral blood and cerebrospinal fluid (CSF) from BD patients (97–99). On the other hand, one study reported a decreased frequency of Treg in peripheral blood (100) and another one showed no difference in Treg frequency between BD and healthy controls. However, BD patients presented a decreased frequency of activated Treg cells ($CD45RA^+CD25^{+++}$) (101).

Th17 cells are also apparently important in BD pathogenesis, especially by recruiting neutrophils *via* G-CSF (102). Indeed, the percentage of peripheral Th17 cells and IL-17 production are increased in active BD (103). Noteworthy, T γδ and NKT cells are also capable of IL-17 production and apparently are associated with BD pathogenesis as well (104, 105). Geri et al. (101) demonstrated an increase in the number of Th17 cells and a reduction of Treg cells in the peripheral blood from BD patients, as well as increased serum levels of IL-21 compared to controls. In addition, healthy control $CD4^+$ T cells stimulated *in vitro* with sera from active BD patients showed high IFNγ and IL-17A and decreased T reg cells differentiation compared to stimulus with sera from BD patients in remission. Moreover, Bassyouni et al. (106) showed that Th17 polarization in BD patients is induced by high levels of the inflammatory mediator serum amyloid-A. Thus, IL-17 axis seems to coordinate interactions between lymphocytes and neutrophils in BD and may represent a potential therapeutic target. In fact, the adaptive immune system apparently can stimulate neutrophil functions, contributing to the hyper-activated status of these cells. **Figure 1** summarizes a proposition for integrated pathogenesis of Behçet`s disease.

CONCLUSION

Understanding Behçet's disease pathogenesis is a pivotal step for the development of novel and efficacious therapies. Nevertheless, polygenic inheritance with the participation of several unknown environmental factors contributes to heterogeneity among patients and extra challenge for elucidating its pathogenesis.

Evidence indicates that innate immunity is prominently involved in BD, which is illustrated by the striking neutrophil hyperactivity and its interaction with monocytes. However, adaptive immunity also seems to be important in BD, with particular emphasis on Th1 and Th17 responses.

Key messages

1. Phagocyte hyperactivity, with increased oxidative burst and chemotaxis, is a hallmark of Behçet's disease.
2. Soluble factors carried in the plasma contribute to phagocyte dysfunction in BD
3. Innate and adaptive immunity play an important role in BD pathogenesis and the IL-17 axis seems to play a pivotal role in the integration of the two arms of the immune system in this disease.

AUTHOR CONTRIBUTIONS

SP conceived, reviewed the literature, designed, wrote, and reviewed the manuscript. LA reviewed and improved the manuscript. AS reviewed and improved the manuscript. All authors contributed to the article and approved the submitted version.

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Vasculitis as a Major Morbidity Factor in Patients With Partial RAG Deficiency

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Vasculitis can be a life-threatening complication associated with high mortality and morbidity among patients with primary immunodeficiencies (PIDs), including variants of severe and combined immunodeficiencies ((S)CID). Our understanding of vasculitis in partial defects in recombination activating gene (RAG) deficiency, a prototype of (S)CIDs, is limited with no published systematic evaluation of diagnostic and therapeutic modalities. In this report, we sought to establish the clinical, laboratory features, and treatment outcome of patients with vasculitis due to partial RAG deficiency. Vasculitis was a major complication in eight (13%) of 62 patients in our cohort with partial RAG deficiency with features of infections and immune dysregulation. Vasculitis occurred early in life, often as first sign of disease (50%) and was complicated by significant end organ damage. Viral infections often preceded the onset of predominately non-granulomatous-small vessel vasculitis. Autoantibodies against cytokines (IFN-α, -ω, and IL-12) were detected in a large fraction of the cases tested (80%), whereas the majority of patients were anti-neutrophil cytoplasmic antibodies (ANCA) negative (>80%).

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Genetic diagnosis of RAG deficiency was delayed up to 2 years from the onset of vasculitis. Clinical cases with sole skin manifestation responded well to first-line steroid treatment, whereas systemic vasculitis with severe end-organ complications required second-line immunosuppression and/or hematopoietic stem cell transplantation (HSCT) for definitive management. In conclusion, our data suggest that vasculitis in partial RAG deficiency is prevalent among patients with partial RAG deficiency and is associated with high morbidity. Therefore, partial RAG deficiency should be included in the differential diagnosis of patients with early-onset systemic vasculitis. Diagnostic serology may be misleading with ANCA negative findings, and search for conventional autoantibodies should be extended to include those targeting cytokines.

Keywords: vasculitis, primary immumunodeficiencies, rag deficiency, severe combined immunodeficiencies (SCID), autoimmunity, combined immunodeficiency with granuloma and/or autoimmunity, atypical SCID

INTRODUCTION

The recombination-activating gene 1 (*RAG1*) and *RAG2* encode lymphoid-specific proteins that are essential for V(D)J recombination, promoting diversification the T and B cell repertoire (TCR, BCR), and receptor editing (1, 2). First described in 1996 by Schwarz et al. null mutations in the *RAG1/RAG2* genes result in T- and B-cell-negative SCID (3). The spectrum of the disease was soon extended to include patients with Omenn syndrome and leaky SCID (LS), with relative recombinase activity lower than 5% resulting in the generation of restricted, oligoclonal lymphocytes that are enriched for self-reactive specificities (3, 4). Hypomorphic variants with more preserved relative recombinase activity (in average 5–30%), compared to OS and LS, result in a broader spectrum of distinct phenotypes, including, combined immunodeficiency with granuloma and/or autoimmunity (CID-G/A) (5, 6), primary antibody deficiencies (7–9), idiopathic $CD4^+$ T cell lymphopenia (10), hyper-IgM syndrome (11), and sterile chronic multifocal osteomyelitis (12). This highly vulnerable patient population presents with a variety of autoimmune and hyperinflammatory complications including refractory cytopenias (84.1%), granulomas (23.8%), and inflammatory skin disorders (19.0%) (13).

Vasculitis is observed in various chronic diseases; it is characterized by inflammation of blood vessels, and is classified into large, medium, and small vessel vasculitis, based on the diameter of the affected vessels. While the inflammatory process may be confined to a single organ or site, it may also involve several organ systems, resulting in a vast variety of clinical presentations. Although the specific pathogenesis has yet to be identified, most vasculitides have complex etiology, and both genetic and environmental factors appear to contribute to the pathogenesis (14). In recent years, vasculitis has been described as a feature of various forms of PID, including those with pathogenic STAT1 gain-of-function variants, adenosine deaminase 2 (ADA2) deficiency, X-linked lymphoproliferative syndrome (XLP) type 1, Wiskott-Aldrich-syndrome (WAS), TAP1/2 deficiency, complement deficiency, and NOD2 deficiency (15–21).

Systemic vasculitis has been described as severe complication with significant end-organ damage in patients with partial RAG deficiency (pRD) (13). However, our understanding of vasculitis in RAG deficiency is limited, with no published systematic evaluation of clinical evolution, diagnostic, and therapeutic modalities. Herein we sought to describe the clinical, laboratory features, and treatment outcome of patients with vasculitis due to pRD.

MATERIALS AND METHODS

We maintain a curated patient database (IRB protocol #Pro00025693) of 62 cases of RAG deficiency with autoimmune/hyperinflammatory complications from which we collected the following information: sex, age (current as of March 2020, at clinical diagnosis of immunodeficiency and/or autoimmunity, at molecular diagnosis of RAG deficiency, and at death or HSCT), genotype (specific *RAG1 or RAG2* variants), immune phenotype (lymphocyte counts and function, immunoglobulin levels, and autoantibodies), vasculitis (type, age at onset, preceding infections if available, length, and severity), other autoimmune/hyperinflammatory complications, and therapies trialed (including response and complications) (13). This database is continuously updated with relevant cases following literature search and/or personal communication. Patients with vasculitis were identified from our curated database and physicians were individually contacted for additional details. All patients remained deidentified and were previously consented locally. A structured datasheet was utilized to collect clinical information from the treating physician. All patients were assigned as CID-G/A based on published criteria by Delmonte et al. (22). Although we do acknowledge, that currently CID-G/A has not been fully defined by either the Primary Immunodeficiency Consortium (PIDTC) or the Expert Committee of International Union of Immunodeficiency Societies (IUIS) (23). In **Table 1** we provide detailed clinical information on patients with vasculitis and pRD. Predicted relative V(D)J recombination activity was recorded as previously described (24, 25). Lymphocyte panel and immunoglobulin

TABLE 1 | Detailed clinical information on patients with vasculitis due to RAG deficiency.

ID	Age	Gene	Mutation	RAG activity	RAG phenotype	Type of vasculitis	Severity of vasculitis	Age at onset of vasculitis	Diagnosis	Autoantibodies	Other Autoimmunity	Treatment			Respose to therapy	Overall outcome (cause of death)	Reference
												First-Line	Second-Line	Third-Line			
Patient 1	6 yrs	*RAG1*	a.W522C b.H994R	a. 41.6% b. n.a.	CID-G/AI	Henoch schonlein purpura	Severe/ multiorgan (CNS vasculitis, stroke)	2.5 yrs	Serology Imaging	Anti-IFN-α/ω, anti-IL12, Coombs+ p-ANCA	AIHA	Steroids, IVIG	–	HSCT (MUD)	Good	Deceased, 6 yrs (stroke)	Unpublished
Patient 2	2.7 yrs	*RAG1*	a.R396C b.M435V	a. 0.6% b. 23.6%	CID-G/AI	Non-granulomatous-small vessel vasculitis	Severe/ multiorgan (digital necrosis)	1.5 yrs	Serology Imaging Biopsy	Anti-IFN-α/ω, anti-IL12, Coombs+	AIHA	Steroids	Cyclophosphamide. rituximab	HSCT (MUD)	Poor	Deceased, 2.7 yrs (idiopathic pneumonia syndrome)	Unpublished
Patient 3	48 yrs	*RAG1*	a.M1V b.R737H	a. n.a. b. 0.2%	CID-G/AI	Leukocytoclastic vasculitis	Mild/skin only	8 yrs	Serology Imaging Biopsy	Anti-IFN-α, ANA, anti-dsDNA, RF, anti-TG/TPO/TSHR	None	Steroids, IVIG	–	–	Good	Deceased, 48 yrs (COPD)	(1)
Patient 4	2 yrs	*RAG1*	a.R841Q b.F974L	a. 0% b. 56.5%	CID-G/AI	Non-granulomatous-small vessel vasculitis	Severe/ multiorgan (digital necrosis)	0.5 yrs	Serology Imaging	APLA, Coombs+, anti-platelet, anti-TPO	AIHA, ITP, AN, inflamatory myopathy, AIH	Steroids, IVIG	Rituximab	–	Good	Deceased, 2 yrs (enterobacter sepsis)	(6)
Patient 5	3.4 yrs	*RAG2*	a.G35A b.A456D	a.22.1% b. n.a.	CID-G/AI	n.a.	Severe/ multiorgan (n.a.)	0.5 yrs	n.a.	–	ITP	Steroids, IVIG	Alemtuzumab	HSCT (n.a.)	Good	Alive	Unpublished
Patient 6	15 yrs	*RAG1*	a.fs188X b.A444V	a. 2.7% b.1.4%	CID-G/AI	Non-granulomatous-small vessel vasculitis	Mild/ skin only	12.5 yrs	Serology Imaging Biopsy	–	Cutaneous granulomatosis	Steroids, IVIG	–	–	Good	Deceased, 15 yrs (pulmonary fibrosis)	(26)
Patient 7	5 yrs	*RAG1*	a.b.A444V	a.b. 1.4%	CID-G/AI	Kawasaki disease	Mild/ skin only	1.5 yrs	Serology	Anti-IFN-α, ANA	Macrophage activation syndrome (MAS), SLE	Steroids	–	–	Good	Alive	(28)
Patient 8	7.5 yrs	*RAG1*	a.b.R699W	a.b. 19.3%	CID-G/AI	Polyarteritis nodosa	Severe/ multiorgan (digital necrosis)	2.5 yrs	Serology Biopsy	–	AIHA	Steroids	Cyclophosphamide. azathioprine	–	Partial	Alive	(27)

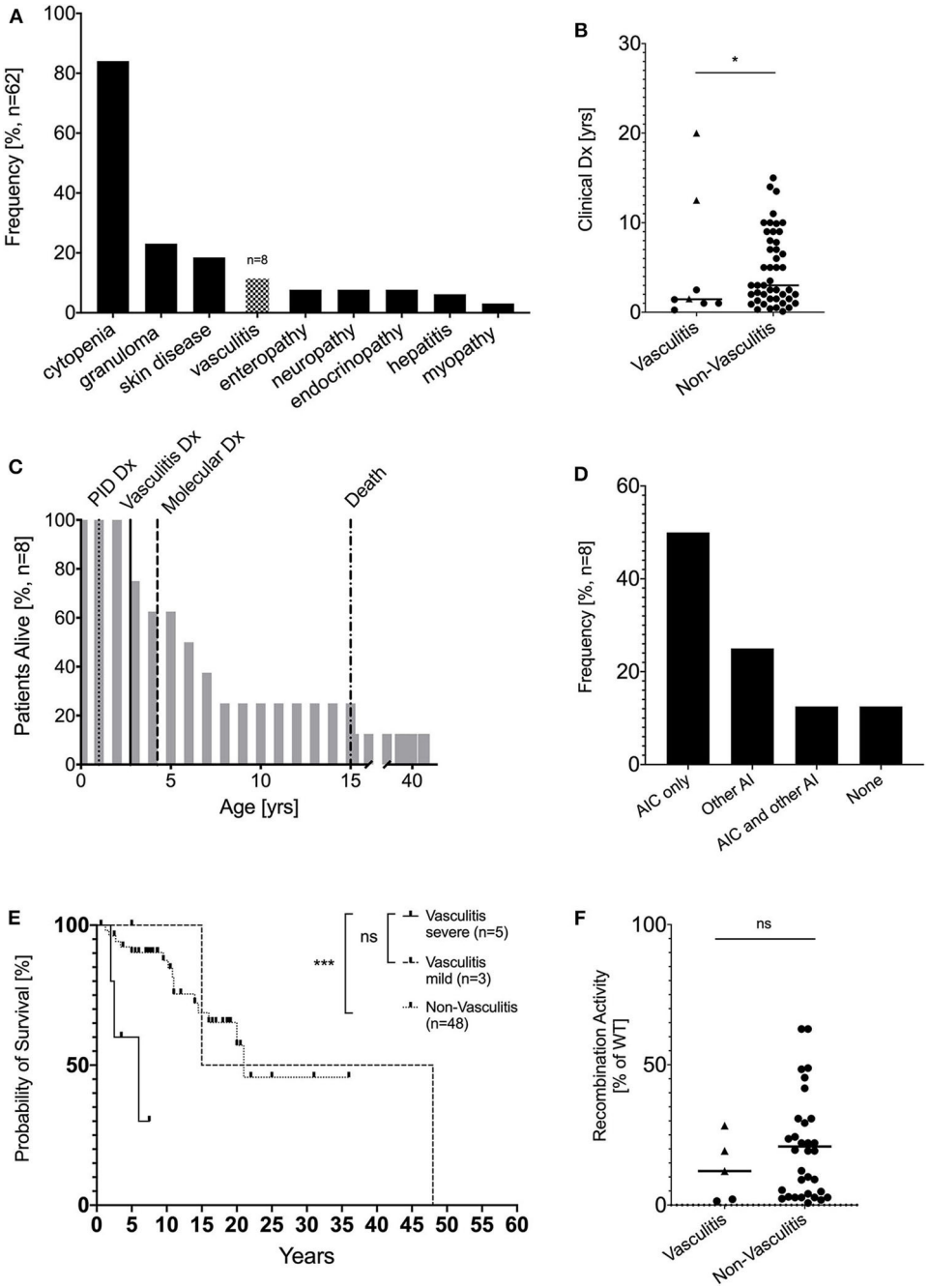

FIGURE 1 | Demographic and clinical characterization patients with vasculitis due to RAG deficiency. **(A)** Vasculitis is the fourth most common complication of pRD with immune dysregulation in a cohort of 62 patients (modified from 13) **(B)** Clinical diagnosis of PID in years compared between patients with pRD ($n = 8$, circles = severe/multiorgan, rectangle = mild/skin-only) or without ($n = 54$) vasculitis and RAG deficiency (*$p < 0.05$) **(C)** Percent of patients alive by age and annotate clinical milestones **(D)** Kaplan-Meier curves comparing survival of RAG-deficient patients with ($n = 8$, dotted line) and without ($n = 54$, straight line) vasculitis **(E)** Overall frequency of autoimmune complications besides vasculitis in adult patients with RAG deficiency, AIC… autoimmune cytopenia, AI… autoimmunity **(F)** Recombination activity from all available RAG1/2 alleles (average of % wild-type protein). For the non-vasculitis control group only patients with CID-G/AI and AS phenotype were considered. (circles = severe/multiorgan, rectangle = mild/skin-only) (ns statistically not significant, *$p \leq 0.05$, ***$p \leq 0.001$).

levels were determined by clinical laboratory testing at the patient's home institution. Similarly, ANCA and antinuclear antibodies (ANA) were detected by indirect immunofluorescence assay, other autoantibodies were detected by Enzyme-Linked Immunosorbent Assay (ELISA) as part of the routine medical care (6, 9, 26–28). Anti-cytokine antibodies were detected by

ELISA as previously described (29). For phenotypic description, healthy age matched blood donors ($n = 25$), and RAG deficient patients with similar clinical phenotypes (CID-G/A and atypical SCID, $n = 48$) served as healthy and disease controls. Statistical comparisons were performed by calculating the Mann Whitney *U*-test using Prism Graphpad 8.4 software. Statistically significant differences obtained in intergroup comparisons were confirmed by Kruskal–Wallis one-way analysis of variance using Prism Graphpad 8.4 software. Kaplan-Meier curves were compared using a log-rank (Mantel-Cox) test. Values of $p < 0,05$ were considered as significant (ns statistically not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$).

RESULTS

Demographics and Clinical Characterization of Patients With Vasculitis due to RAG Deficiency

In our cohort of 62 patients with hypomorphic RAG variants and autoimmune and/or hyperinflammatory complications, we identified 8 patients (12.9%) with episodes of vasculitis (**Figure 1A**). There was equal distribution of female ($n = 4$) and male patients ($n = 4$). The designated clinical phenotype was combined immunodeficiency with granuloma and/or autoimmunity (CID-G/A) in all 8 RAG deficient patients with vasculitis based on criteria described above (22). Patients with severe/multiorgan vasculitis were diagnosed with PID early in life ($n = 5$, median age of 1 years; age range of 0.25–2.5 years), in contrast patients with mild/ skin manifestation ($n = 3$, median age 12.5 years; age range 5–20 years, $p =$ ns) and those without vasculitis ($n = 48$, median age 3 years, age range 0–15 years, $p = 0.0171$) were diagnosed later in life (**Figure 1B**). In 4 of 8 patients, vasculitis was the first clinical signs of immune dysregulation. The median duration of vasculitis episodes was 1.25 years, with no significant difference between severe/multiorgan and mild/skin manifestations (data not shown). Genetic diagnosis of underlying RAG deficiency was obtained at the median age of 4.25 years (range: 1.5–46 years) (**Figure 1C**). Besides development of vasculitis, the majority ($n = 6$) of the patients developed autoimmune complications. Cytopenia was the most common autoimmune complication, being present in 50% of the patients in our cohort, similar to other recently reported cohorts (21–77%) (13). Systemic autoimmunity/inflammatory conditions were observed in three patients, including inflammatory myopathy, cutaneous granulomatosis, and macrophage activation syndrome (MAS), and systemic lupus erythematodes (SLE). Only one patient developed no additional autoimmune complications besides vasculitis (**Figure 1D**).

The course of the disease was complicated by significant end organ damage, which was associated with a high mortality rate of 62.5% (5 of 8 patients) and a significantly reduced ($p = 0.0436$) median survival of 15 years compared to non-vasculitis pRD patients with immune dysregulation who had a median survival of 21.1 years. Although not significant, patients with severe/multiorgan vasculitis had overall reduced median survival of 6 years compared to patients with mild/skin limited vasculitis with a median survival of 15 years (**Figure 1E**). Leading causes of death in RAG patients with vasculitis included respiratory failure (idiopathic pneumonia syndrome post HSCT, pulmonary fibrosis, chronic obstructive pulmonary disease ($n = 3$), followed by sepsis with multi-organ failure (enterobacter sepsis, $n = 1$) and stroke due to central nervous system (CNS) vasculitis ($n = 1$) (**Table 1**).

The majority ($n = 7$) of the patients carried pathogenic *RAG1* variants, while one patient was compound heterozygous for pathogenic *RAG2* variants. To our knowledge, this is the first reported case of RAG2 deficiency and vasculitis. There was no significant difference in the relative recombinase activity level between RAG variants presenting with or without vasculitis and between severe and mild vasculitis manifestation (**Figure 1F**).

Detailed Clinical Description of Vasculitis and Treatment Outcome in Patients With RAG Deficiency

Childhood vasculitis is classified based on vessel size, including large, medium, and small vessel vasculitis (30). Detailed clinical information of vasculitis was available in 7 patients with RAG deficiency. In our cohort, we observed predominately non-granulomatous-small vessel vasculitis ($n = 5$), including one case of Henoch-Schönlein purpura (IgA vasculitis), one case of cutaneous leukocytoclastic vasculitis and 3 cases of unspecified non-granulomatous-small vessel vasculitis. Two patients displayed medium vessel vasculitis, one case of childhood polyarteritis nodosa and one case of Kawasaki disease. There were no cases of large vessel nor granulomatous-small vessel vasculitis identified (**Figure 2A**). Vasculitis was diagnosed based on clinical history, serology, imaging, and/or biopsy (**Table 1**). The disease was complicated by severe end organ complications. In particular, skin involvement was seen in all seven patients, and digital necrosis in four CNS vasculitis and cardiovascular complications were seen in one patient each (**Figure 2B**).

Vasculitis may develop as a result of infectious or non-infectious triggers (31). We therefore tried to correlate potential infectious triggers with the onset of vasculitis. Five patients developed vasculitis following viral infection (varicella zoster virus, adenovirus, or respiratory syncytial virus) or administration of attenuated vaccine (measles, mumps, and rubella), four patients had bacterial infections (*E. faecalis*, *C. difficile*, *Enterobacteria*, *Streptococcus spp.*), two patients had fungal infection (Candida). No correlation of a potential infectious trigger and development of vasculitis could be identified in two patients (**Figure 2C**). The median duration of time elapsed from viral infections or vaccination to the development of vasculitis was 5 months (range 0–9 months, $n = 5$) (data not shown).

In addition, we analyzed if autoantibodies associated with systemic vasculitis can be used as a diagnostic biomarker in RAG deficient patients. The majority of the patients were anti-neutrophil cytoplasmic antibodies (ANCA) and antinuclear

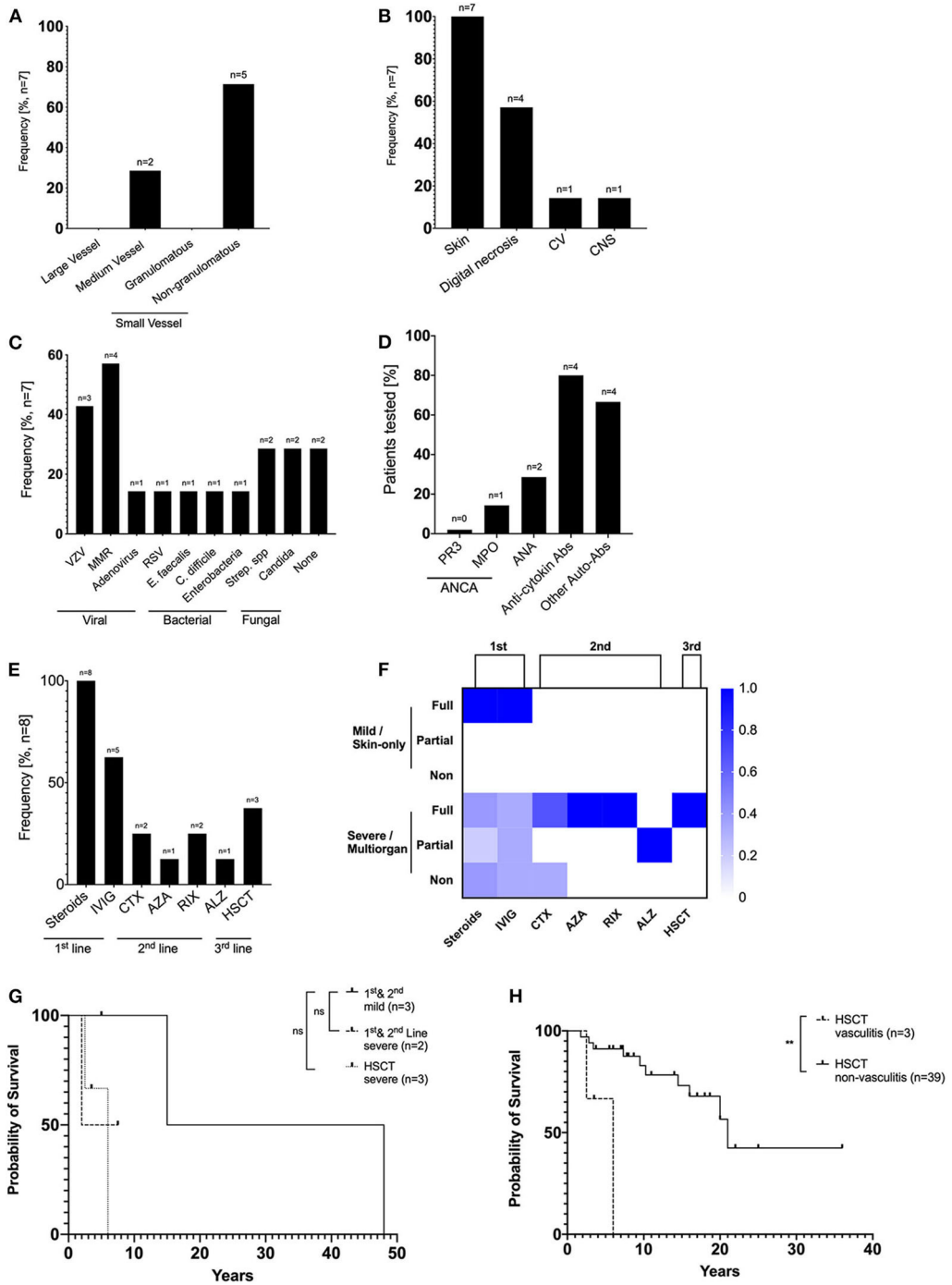

FIGURE 2 | Detailed description of vasculitis and treatment outcome in patients with RAG deficiency. **(A)** Relative frequency of large, medium and small vasculitis among RAG patients **(B)** Relative frequency of target organs affected by vasculitis **(C)** Relative frequency of potential infectious trigger preceding episodes of vasculitis. **(D)** Prevalence of autoantibodies among RAG patients. Anti-cytokine included antibodies against IFN α, ω, and IL-12. Other autoantibodies included anti-erythrocyte, platelet, dsDNA, TPO, TG, ALPA antibodies. **(E)** Treatment strategies used in patients with vasculitis due to RAG deficiency **(F)** Treatment response scored for first line (steroids ± IVIG), second line (biologicals or immunosuppressives), or third line (Haematopoietic stem cell transplantation) was scored using the following criteria: "non," no clinical response or side effects were limiting; "partial," clinical improvement but therapeutic escalation was required; or "full," clinical improvement with no escalation. **(G)** Kaplan-Meier curves comparing survival of RAG-deficient patients with severe, systemic multiorgan vasculitis that underwent HSCT ($n = 3$, dashed line), patients with severe, systemic multiorgan vasculitis that received first/second line therapy ($n = 2$, dotted line) and patients with mild, skin limited vasculitis ($n = 3$, straight line). **(H)** Kaplan-Meier curves comparing survival of RAG-deficient patients with vasculitis ($n = 3$, dashed line) and RAG-deficient patients without vasculitis ($n = 39$, straight line) that underwent HSCT (ns statistically not significant, $^{**}p \leq 0.01$).

antibodies (ANA) negative (ANCA-negative: 5/6; ANA-negative: 5/7). However, 4 out of 5 patients were positive for anti-cytokine antibodies (targeting IFN-α, -ω, and IL-12), confirming what has been described in previous reports (29) (**Figure 2D**).

Topical and systemic steroids [± immunoglobulin replacement therapy (IgRT)] were used in all patients as first line therapy and were sufficient to induce remission of vasculitis limited to skin manifestations in three patients. First (steroids, IVIG) and second-line treatment (cyclophosphamide, azathioprine rituximab, and alemtuzumab) had limited effectiveness in four patients with severe, systemic multiorgan complications. Three patients were referred for HSCT as third line therapeutic approach, leading to remission of vasculitis in all of them (**Figures 2E,F**). Comparisons of overall survival between first/second line treatment and HSCT revealed no statistically significant difference. Patients with severe/multiorgan complications that underwent HSCT ($n = 3$) had a median survival of 6 years, whereas patients with severe complications that received first/second line treatment ($n = 2$) had a median survival of 7 years. Patients with mild, skin-limited vasculitis ($n = 3$) had a median survival of 15 years (**Figure 2G**). RAG deficient patients with vasculitis (median survival 6 years) that were treated with HSCT had an overall worse outcome than patients without vasculitis that underwent HSCT (median survival 21 years, $p = 0{,}0018$) (**Figure 2H**).

Phenotypic Description of RAG Deficient Patients With Vasculitis

Next, we compared the immunologic phenotype of RAG deficient patients with vasculitis to those without vasculitis and healthy pediatric and adult controls. The dominant laboratory feature among patients with vasculitis associated with pRD was a severe T cell lymphopenia [mean T cell count: 220 cells/μl (range 65–727), $p = 0.0073$]. In comparison, T cell lymphopenia was less pronounced in patients with pRD and immune dysregulation but without vasculitis (mean T cell count: 635 cells/μl; range 106–2,678). We observed a trend toward lower counts of $CD4^+$ T cells (mean: 104 cells/μl; range: 30–611, $p = 0.0577$) and of naïve $CD4^+$ T cells (mean: 5 cells/μl; range 1.69–8.5, $p = 0.0991$) in vasculitis patients than in the non-vasculitis group ($CD4^+$ T cells, mean: 257 cells/μl; range 66–958; naïve $CD4^+$ T cells, mean: 7 cells/μl; range 0.04–47), although this did not reach significance. Interestingly, all patients with vasculitis were severely $CD8^+$ T cell lymphopenic with a mean of 81 cells/μl (range 7–194, $p = 0.0116$) compared to the non-vasculitis group (mean $CD8^+$ cells count: 304 cells/μl; range 11–1,731). B cell counts were variably low compared to controls with a mean of 81 cells/μl (range 6–359). There was no significant difference in NK cell numbers between different groups (**Figure 3A**). T cell proliferation to phytohemagglutinin (PHA) was comparable between patients with vasculitis (in average 25,601 cpm) and patients without vasculitis (in average 21,000 cpm) (data not shown). IgG levels could not be assessed because the majority of the patients were on IgRT, and no native IgG levels were recorded. IgA (85.2 mg/dl, 0–200) and IgM (112.6 mg/dl, 16–230) serum levels were highly variable, and no significant difference to non-vasculitis pRD patients could be observed. Elevated IgE was detected in 4/6 of cases (**Figure 3B**).

DISCUSSION

In the last decade, the spectrum of PIDs has extended from being defined by a susceptibility to infections alone to include features of immune dysregulation (29). A recent study of the French national PID registry observed a wide range of autoimmune and autoinflammatory complication (26.2%) among PID patients (32). All types of PIDs were associated with a risk to develop immune dysregulation, although T-cell PIDs and CVID appear to have the greatest risk (32). Among PIDs with CID with immune dysregulation, partial RAG deficiency is one of the most common entities (33, 34). Initially found to account for T- and B-cell-negative SCID, pathogenic RAG *gene* variants have been subsequently associated with a broader spectrum of phenotypes, including autoimmunity and immune dysregulation (13).

Herein, we have presented vasculitis as key component of morbidity among patients with hypomorphic *RAG* variants. Vasculitis was associated with a high mortality rate of 62.5% and a reduced median survival of 15 years. Although RAG-mutated patients with vasculitis were recognized earlier than those without vasculitis, their overall survival and life expectancy were severely reduced, confirming that autoimmunity worsens the prognosis in patients with PIDs. Treatment strategies need to be carefully examined to balance the efficacy and toxicity of biologic and non-biologic immunosuppressive drugs in RAG deficient patients.

Abnormalities of central and peripheral T and B cell tolerance play key mechanisms in immune dysregulation in patients with hypomorphic RAG variants. Central B cell tolerance is affected by a failure to reexpress the RAG complex during receptor editing of immature B cells in the bone marrow (35). Peripheral B cell tolerance is disturbed by an inability to deplete anergic self-reactive B cells due to survival in a milieu with increased BAFF levels (36, 37). Impaired B cell tolerance in RAG deficiency is highlighted by a wide spectrum of serum autoantibodies, including neutralizing antibodies against interferon-α, interferon-ω, and IL12 observed in our cohort. The majority of RAG deficient patients with vasculitis were positive for anti-cytokine antibodies, which were demonstrated to aggravate immune dysregulation, hyperinflammation with increased type-1 interferon signature and increased susceptibility to prolonged viral infection (29). As an example, for hyperinflammation in the setting of infections, it has been described for vaccine-derived rubella in cutaneous granuloma formation in RAG deficient patients (38). While we tried to correlate the development of vasculitis with potential infectious trigger, further research needs to be done to identify a causative trigger.

Recent studies have identified vasculitis as an uncommon complication of PIDs, having been observed in 1–1.6% of the patients reported in the French national PID registry and in the USIDNET registry (32, 39). In contrast, we identified

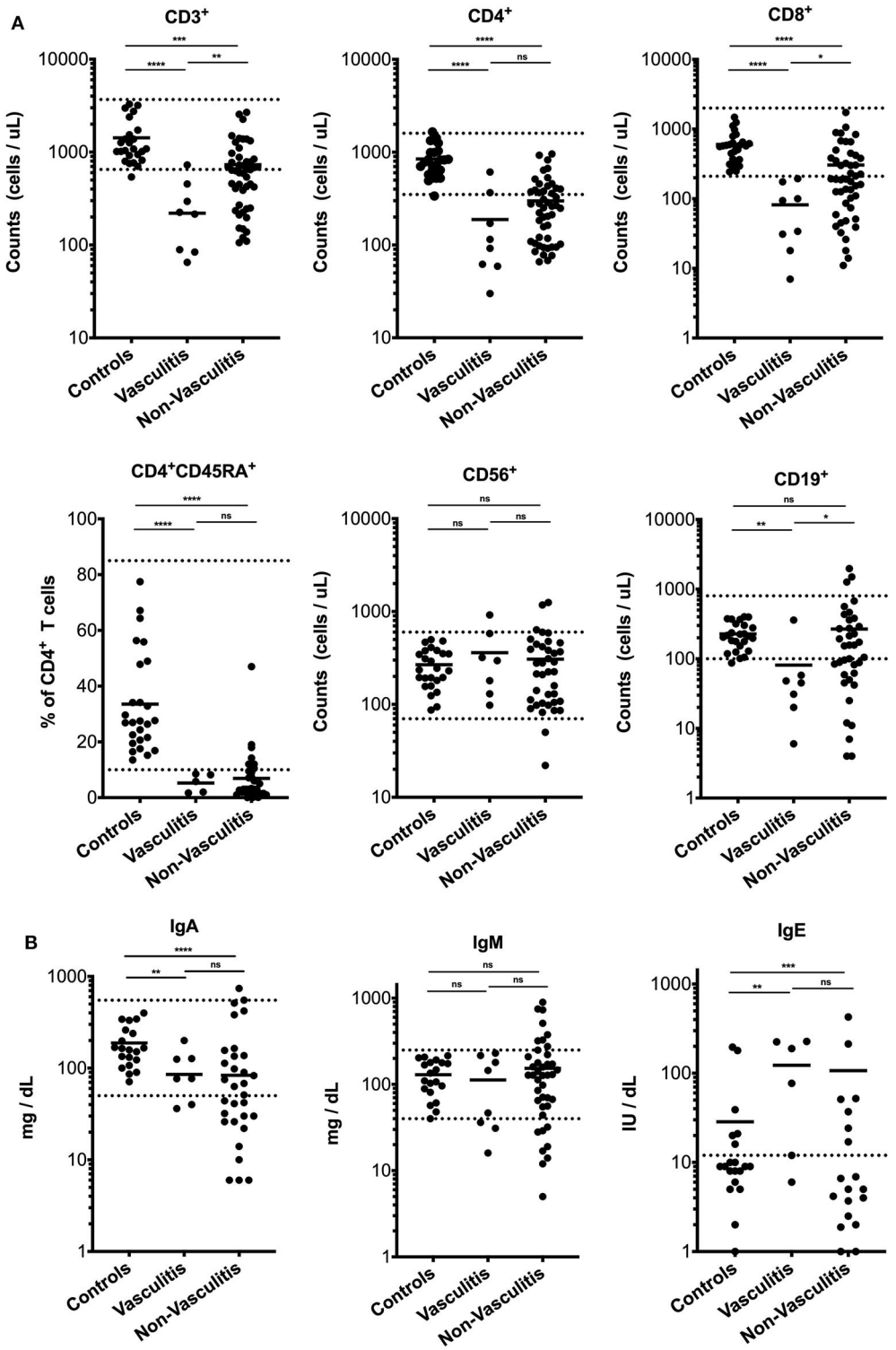

FIGURE 3 | Lymphocyte cell counts and serum immunoglobulins in patients with vasculitis due to RAG deficiency. **(A)** $CD3^+$ T cells, $CD4^+$ T cells, naïve $CD4^+CD45RA^+$ T cells, $CD8^+$ T cells, $CD56^+$ NK cells, and $CD19^+$ B cells of age-matched healthy controls ($n = 25$), RAG deficient patients with ($n = 8$) without ($n = 46$) vasculitis **(B)** Immunoglobulin titers of age-matched healthy controls ($n = 25$) RAG deficient patients with ($n = 8$) without ($n = 46$) vasculitis. Dashed line represents normal values. Statistically significant differences obtained in intergroup comparisons were confirmed by Mann Whitney *U*-test. Values of $p < 0.05$ were considered as significant (ns statistically not significant, $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$).

vasculitis to be a prevalent complication among patients with hypomorphic *RAG* variants and immune dysregulation (12.9%). Similar to RAG deficiency, there are other PIDs specifically associated with vasculitis. The differential diagnosis should include ADA2 deficiency (40), CVID (38%) Wiskott-Aldrich syndrome (WAS) (26%) (39). Unlike in ADA2 deficiency, where stroke is predominant, in our cohort of 8 patients, only one patient had a stroke.

We have recently reported that pathogenic variants in the *RAG* genes can result in significant phenotypic variability, and may occur in 1 in 500 patients with antibody deficiency, including CVID (41). We therefore recommend that partial RAG deficiency should be considered for patients with antibody deficiency and vasculitis, especially when associated with other autoimmune manifestations, and/or progressive T cell lymphopenia. Autoantibodies that are frequently associated with typical forms of vasculitis may be lacking in patients with hypomorphic *RAG* variants, as indicated by the fact that the majority of patients presented with ANCA negative small vessel vasculitis. Therefore, conventional vasculitis autoantibody panel should be extended to test for antibodies targeting cytokines, and in particular IFN-α, -ω, and IL-12 (29).

Given the importance of providing optimal care for patients with PIDs, further prospective studies are needed to identify potential pathogenic mechanisms and help guide in the development of optimal treatment of vasculitis in patients with RAG deficiency.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Clinical data were collected, and research laboratory studies were performed on de-identified samples under IRB approved protocols at University of South Florida (IRB protocol #Pro00025693 at University of South Florida for JW). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CG conceived the presented idea, interpreted and analyzed the results, and wrote the manuscript. JF, ZF, BU, and JS curated the patient database, assisted with data interpretation performed functional assays. JWS, SP, MD, S-YP, LH, MH, BN, DM, SS, SM, PY, EN, SÖ, KB, JF, and HW provided clinical data. ME, LN, and LC reviewed the clinical information presented. JW encouraged to describe this cohort and clinical course in the context of internationally accepted guidelines and supervised the findings of this work. All authors discussed the results and contributed and agreed to the final manuscript.

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REVIEW
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IFN-I Mediates Dysfunction of Endothelial Progenitor Cells in Atherosclerosis of Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease including the cardiovascular system. Atherosclerosis is the most common cardiovascular complication of SLE and a significant risk factor for morbidity and mortality. Vascular damage/protection mechanism in SLE patients is out of balance, caused by the cascade reaction among oxidative stress, proinflammatory cytokines, Neutrophil Extracellular Traps, activation of B cells and autoantibodies and abnormal T cells. As a precursor cell repairing vascular endothelium, endothelial progenitor cells (EPCs) belong to the protective mechanism and show the reduced number and impaired function in SLE. However, the pathological mechanism of EPCs dysfunction in SLE remains ill-defined. This paper reviews the latest SLE epidemiology and pathogenesis, discusses the changes in the number and function of EPCs in SLE, expounds the role of EPCs in SLE atherosclerosis, and provides new guidance and theoretical basis for exploring novel targets for SLE treatment.

Keywords: atherosclerosis, endothelial cell, endothelial progenitor cell, pathogenesis, systemic lupus erythematosus, IFN-I

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INTRODUCTION

SLE is an immune complex-mediated autoimmune disease involving multiple systems. Its prevalence and incidence rate can be as high as 241/100,000 per year and 23.2/100,000 per year, and the rate of premature death is 2–3 times that of healthy people (1). Since 2000, the prevalence rate of adult SLE in women has been 30–150/100,000, and the incidence rate is 2.2–23.1/100,000 per year (2). SLE is also an autoimmune disease characterized by cardiovascular disease (CVD). A multicenter study found that a quarter of the nearly 10,000 deaths from SLE were caused by CVD (3). Current studies have demonstrated that the inherent factors of SLE are independent risk factors for the premature occurrence of atherosclerosis in SLE patients (4). With the improvement of the diagnosis and treatment, the early mortality of SLE patients has been dramatically reduced. However, atherosclerosis is still one of the leading causes of death of late SLE patients. It is of considerable significance to explore the natural course and mechanism of SLE combined with atherosclerosis, find useful therapeutic targets, provide evidence for clinical intervention, and delay the death of SLE.

Vascular endothelial dysfunction is the starting point in SLE atherosclerosis. Endothelial progenitor cells (EPCs) are closely related to vascular endothelial function. Therefore, the relationship between atherosclerosis and EPCs in SLE is a research direction worth exploring. However, in recent decades, there are few studies on the relationship between atherosclerosis and EPCs in SLE, and the results are controversial. This paper analyzes the changes in the number and function of EPCs in SLE and reviews the potential role of EPCs in SLE atherosclerosis.

MECHANISM OF ATHEROSCLEROSIS IN SLE

Arteriosclerosis is a series of aggregation events of leukocytes and vascular smooth muscle cells (VSMCs) in intima triggered by endothelial dysfunction and lipoprotein retention, resulting in fibrous plaques. Then fibrous plaques rupture, followed by thrombosis. This process requires the immune response's help (5, 6). The abnormal immune response driven by SLE enhances vascular injury mechanism and weaken repair mechanism, breaking vascular dynamic balance which determines the occurrence of CVD (**Figure 1**).

Oxidative Stress

Mitochondrial dysfunctions, abnormal bioenergetics/immunometabolism and telomere/telomerase disequilibrium endowed SLE patients with intense oxidative stress (7). Among the three main targets of oxidative stress, oxidized lipids—oxLDL and proinflammatory HDL (piHDL)—play a prominent role in accelerating SLE atherosclerosis (8). OxLDL participates in many stages of atherosclerosis, from endothelial dysfunction to plaque rupture (6, 9). Normal HDL plays a role in protecting atherosclerosis by promoting cholesterol outflow, inhibiting vascular inflammation and scavenging oxidizing substances. However, lupus-altered HDL shifts from a normal anti-inflammatory state to a proinflammatory state, causing atherosclerosis (10). Increased piHDL weakens the ability to prevent LDL oxidation (8).

Cytokines

Cytokines, the primary regulators of immune responses, regulate and coordinate multiple stages of atherosclerosis. There is a cascade reaction between these proinflammatory cytokines in accelerating SLE atherosclerosis (**Figure 2**).

IFNs are divided into three classes: IFN-I (IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, IFN-ω), IFN-II (IFN-γ), IFN-III (IFN-λ1, IFN-λ2, IFN-λ3). IFNs participated in the whole process of atherosclerosis, especially IFN-I (15, 43–45). For example, IFN-α and IFN-γ promote lipoproteins' oxidation (15, 16). IFN-α promotes endothelial dysfunction by accelerating endothelial cells (ECs) apoptosis and damaging EPCs, one of the vascular repair mechanisms (15, 46–53). IFN-α enhances the expression of chemokine and adhesion molecules without leukocytes adhesion (53); while IFN-γ can regulate the attraction and adhesion of leukocytes (54). IFN-α induces the up-regulation of SR-A expression in monocytes/macrophages, then promoting the lipid uptake and the formation of macrophage-derived foam cells (55); IFN-γ not only up-regulates SR-A, but also up-regulate ACAT1 (56) and inhibit specific anti-atherosclerotic MSRN proteins (APOE and C3) in macrophages (57) to reduce cholesterol efflux. IFN-α prevents smooth muscle progenitor cell (SMPC) from maturation which

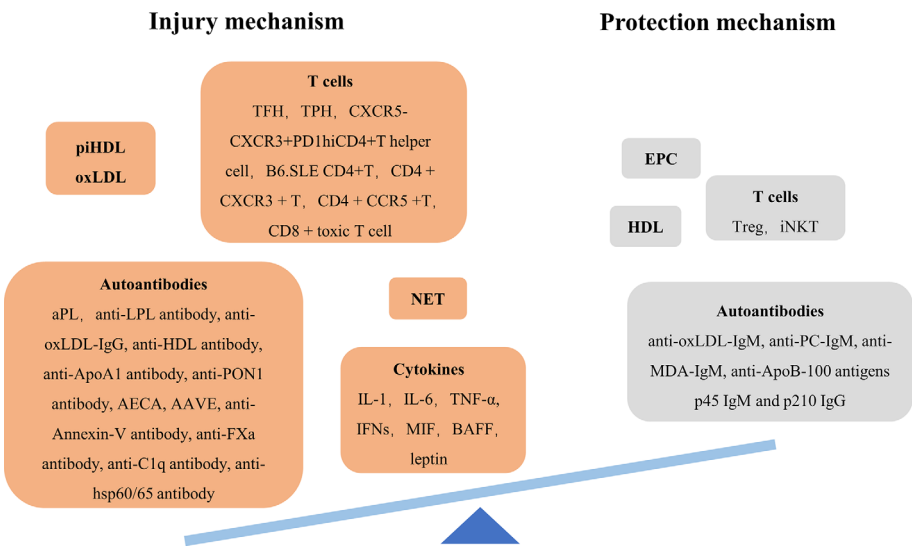

FIGURE 1 | Imbalance of injury/protection mechanism of SLE arteriosclerosis. AAVE anti-vascular endothelial-cadherin antibody AECA anti-endothelial cell antibody aPL antiphospholipid antibody BAFF B cell-activating factor EPC endothelial progenitor cell iNKT invariant natural killer T cell LPL lipoprotein lipase MDA malondialdehyde; MIF, macrophage migration inhibitory factor; NET, neutrophil extracellular trap; PC, choline phosphate; TFH, follicular helper T cell; TPF, peripheral helper T cell.

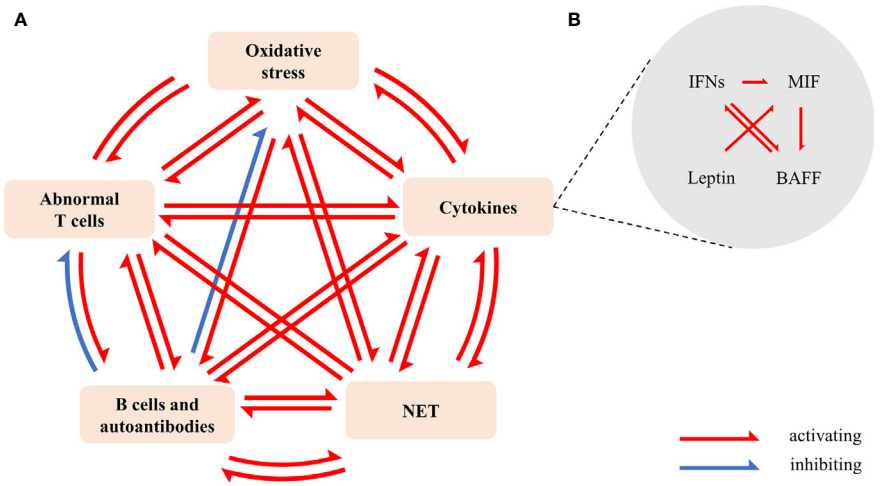

FIGURE 2 | Cross-talk between oxidative stress, cytokines, NETs, activation of B cells and autoantibodies, and abnormal T cells in SLE. **(A)** Oxidative stress promotes the production of IFN-I (11), NETs (12), autoantibodies (13), and the imbalance of Th17/Treg (14). IFN-α and IFN-γ promote lipids oxidative modification (15, 16); BAFF promotes the production of autoantibodies (17), the release of NETs (18) and the activation of T cells (19); leptin promotes the production of autoantibodies, the release of NET and the imbalance of Th17/Treg (20, 21). NET encourages oxidation HDL (22), the expression of IFN-α (23) and IL-1β (24), and activates NET-specific memory B cells to proliferate and secrete polyclonal IgG (25). Overactive T cells increase ROS (26) and cytokines, especially IFN-γ; TFH (27, 28), CXCR5-CXCR3^{+}PD1hiCD4^{+}T helper cell (29), and peripheral helper T cell (TPH) (30) promote the differentiation of B cells and the production of antibodies. SLE-related autoantibodies and immune complexes induce the release of NET (31); anti-ApoA1-IgG guides the expression of cytokines (32). Anti-PC-IgM increases Tregs (33); anti-PC-IgM and anti-MDA-IgM reduce oxidative stress (34). **(B)** IFN-I (35, 36) and IFN-II (37) induce the expression and mobilization of BAFF. BAFF promotes the activation of B cells by IFN (38). Moreover, IFN-I encourages the production of MIF (39). MIF/CD74 signal regulates BAFF (40, 41). Leptin enhances MIF-induced inflammation (42). Besides, IFNs, MIF and leptin strengthen the expression of chemokine, adhesion molecule, TNF- α and ILs. BAFF, B cell-activating factor; MDA, malondialdehyde; MIF, macrophage migration inhibitory factor; NET, neutrophil extracellular trap; PC, choline phosphate; ROS, reactive oxygen species; TFH, follicular helper T cell; TPF, peripheral helper T cell.

could give rise to macrophages and eventually foam cells (58); IFN-γ enhances VSMCs' proliferation and migration (56). IFN-α and IFN-γ induce VSMC and macrophages apoptosis in atherosclerotic plaques, contributing to plaque instability (59–61). Moreover, IFN-α inhibits the expression of type I collagen gene COL1A1 in VSMCs (62) and induces the synthesis of TNF-α, IL-12 and MMP-9 (63); while IFN-γ inhibits the expression of type I collagen gene COL1A2 in VSMCs (64) and induces the synthesis of MMP-1, MMP-2 and MMP-9 (56). Besides, IFN-α forms an IFN-α-platelet-CD154-CD40 forward feedback loop to promote thrombosis (65, 66).

Macrophage migration inhibitory factor (MIF) is an inflammatory and chemokine-like cytokine and an upstream regulator of innate immunity. MIF enhances LDL uptake (67), recruits monocytes and T cells (68–70), migrates VSMCs (71), resulting in plaques. MIF also increases the expression of MMP-1 and MMP-9, inducing plaques rupture (72, 73).

B-Cell Activating Factor (BAFF) is a critical factor in B cell maturation, survival and function, and an independent factor in accelerating SLE atherosclerosis (17). BAFF/BAFF-R axis supports pathogenic B cells producing pathogenic anti-IgG-oxLDL antibodies (74, 75), which is over-activated in SLE (76). The co-expression of BAFF/TNFSF13B and APRIL/TNFSF13 in the plaque lymphocytes and macrophages up-regulate FURIN, the primary Proprotein convertase subtilisin/Kexin (PCSK), which inactivates lipases and regulates inflammation in atherosclerosis (19). And BAFF weakens EPCs' function and promotes EPCs' apoptosis (77).

As an immunopotentiator (78), leptin significantly increases the risk of atherosclerosis in SLE patients (79). And the serum leptin level ≥ 34ng/dL was significantly correlated with carotid plaque (79). Leptin induces oxidative stress, increases MCP-1, TNF-α, IL-6 and endothelin-1, accompanied by the expression of other EC adhesion molecules, MMPs and VEGF, which damages VSMCs and ECs (80). And leptin promotes the secretion of atherosclerotic factor (42, 81). Besides, leptin promotes the production of autoantibodies, increases the release of NET and imbalance of Th17/Treg in SLE (20).

Neutrophil Extracellular Traps (NETs)

NET is a unique form of neutrophils death, characterized by the extrusion of chromatin and a driver of SLE atherosclerosis (82–87). NETs damages ECs. They promote vascular leakage and endothelial-to-mesenchymal transition through the degradation of VE-cadherin and the activation of β-catenin signaling (87); they induce EC death through the activation of endothelial MMP-2 (88). NETs also kill VSMCs (89). Moreover, NETs mediate HDL's oxidation, interfering with cholesterol outflow (22). NETs induce the secretion of IFN-α (23) and IL-1β (24). Serine proteases from NETs degrade tissue factor pathway inhibitor (TFPI) (90) and promote FXII (91) that activate coagulation cascade and thrombosis.

The Activation of B Cells and Autoantibodies

B cells mainly affect atherosclerosis by producing autoantibodies: B1 cells secrete protective natural IgM and IgA antibodies, whereas B2 cells produce pathogenic IgG antibodies. And the tendency of overactive B cells to produce pathogenic IgG antibodies is a potential risk factor for lupus-associated atherosclerosis (17). In particular, antiphospholipid antibodies (aPL) have been identified as independent predictors of atherosclerotic plaque progression in SLE (92, 93).

Anti-HDL-IgG induces LDL to enter the ECs, which is a major contributor to atherosclerosis in SLE. Recently, Kurien BT et al. found that SLE RNP and anti-Ro/LaRNP antibodies probably increase the level of anti-oxLDL antibodies (94). Anti-HDL antibody, anti-ApoA1 antibody and anti-PON1 antibody probably have a common atherogenic pathway—they unbalance PON-1/MPO, which enhances lipids oxidative modification and interferes with HDL's anti-inflammation (95–97). Besides, anti-ApoA1-IgG has two pathways that induce atherosclerosis in a TLR2/TLR4/CD14-dependent manner: it activates transcriptional nuclear factor NF-kB to guide the expression of inflammatory factors; it provides an alternative (or a concomitant) signal to PI3K in an Src-dependent pathway, activates L-type $Ca2^+$ channels and potassium/calcium exchangers, resulting in the depolarization of myocardial plasma membrane (32). Anti-FXa-IgG unbalances hemostasis and thrombosis by inhibiting the FXa enzyme (98) and promotes endothelial dysfunction by enhancing FXa-PAR-mediated $Ca2^+$ signal transduction (99). Recent studies have found that IgA-AECA is involved in SLE endothelial damage by recognizing the membrane proteins of ECs (100). Anti-C1q antibody plays a role in atherosclerosis by reducing C1q's level and protective effects (101, 102), which polarizes macrophages towards an M2-like anti-inflammatory phenotype (103) and improves macrophages' survival and excretion (104).

There are potential protective autoantibodies in SLE patients, such as anti-oxLDL-IgM, anti-ApoB100 antibodies, anti-choline phosphate (PC) antibodies and anti-malondialdehyde (MDA) antibodies. The first three have a synergistic effect: they reduce the level of oxLDL, the uptake of oxLDL, and the formation of foam cells (105–107). And Anti-PC-IgM increases Tregs in SLE and atherosclerosis, reduces IL-17 and TNF-α, and makes dendritic cells (DCs) immature (33). The combined application of anti-PC-IgM and anti-MDA-IgM has a doubly preventive impact on atherosclerosis (34). However, SLE patients showed a low level of protective autoantibodies (34, 107). Some dietary and metabolic factors may be responsible for the low levels of anti-PC-IgM and anti-MDA-IgM (108).

SLE increases the risk of CVD by promoting pathogenic autoantibodies and inhibiting potential protective autoantibodies.

The Abnormal T Cells

Abnormal T cell subsets are considered to be an essential factor leading to endothelial dysfunction and CVD in SLE patients. Tregs are protective T cells in atherogenesis, inhibiting atherogenic T cell subsets and inflammation. And Treg/Th17 imbalance is common in SLE, becoming a risk factor for atherosclerosis (109). In human circulation, atherosclerosis's severity is not directly related to the number of Tregs (110) but is closely related to the dysfunction of Tregs (111). During atherosclerosis, most Treg lost Foxp3 expression and its immunosuppressive function, then transform into follicular helper T cell (TFH) (112), which is used to stimulate the formation of germinal center (GC) and the selection of high-affinity B cells in GC (27). TFH has also been shown to accelerate atherosclerosis, although not necessarily by inducing the production of pathogenic IgG (112, 113). Besides, $CD4^+$T cells in peripheral blood of SLE patients highly express CCR5 and CXCR3 promoting the migration of inflammatory T cells to the arterial wall in a chemokine-dependent way (114, 115). In particular, CCR5 is the critical factor for CD4+T cells homing to atherosclerotic plaques (116).

A recent study has shown that Invariant natural killer T (iNKT) in SLE patients has an anti-atherosclerotic phenotype which induces macrophages to polarize into anti-inflammatory and anti-atherosclerotic M2 phenotype (117). The protection is triggered in early atherosclerosis but is lost or submerged in the development of clinical atherosclerosis (117).

Oxidative stress, cytokines, NETs, activation of B cells and autoantibodies, and abnormal T cells in SLE interact with each other, amplifying their pro-atherogenic effects (**Figure 2**). As a result, the dynamic vascular homeostasis is broken in SLE patients, characterized by enhanced injury mechanism and weakened protection mechanism. Subclinical atherosclerosis in SLE accelerates, even in environments with low disease activity (92).

THE ROLE OF EPCS IN ARTERIOSCLEROSIS

Atherosclerosis is a manifestation of the imbalance between vascular injury and protection mechanisms, especially in endothelial dysfunction. EPCs are the primary protection mechanism for endothelial dysfunction, which promote angiogenesis and maintains endothelial integrity with a series of reactions. But the situation of this protection mechanism in SLE is not optimistic.

Classification, Immunophenotype, and Physiology of EPCs

Scientists have reached a consensus that EPCs isolated by cell culture are distinguished into two different groups: myeloid angiogenic cells (MACs), used to identify early EPCs (118); endothelial colony forming cells (ECFCs), used to identify late EPCs (119). They promote vascular repair through different mechanisms (120). ECFCs, considered to be real EPCs, can differentiate into ECs promoting vascular repair and neovascularization (121), with the immunophenotype positive for CD31, CD105, CD146, and negative for CD45, CD14 (120). MACs can't become ECs but secretes angiogenic cytokines to promote angiogenesis through a paracrine mechanism (122),

with the immunophenotype positive for CD45, CD14, CD31, and negative for CD146, CD133, and Tie2 (120) (**Table 1**).

The Role of EPCs in Vascular Repair

After the injury, vascular repair occurs by accelerating the replacement of ECs. Re-endothelialization is a self-repairing process that maintains vascular endothelial protection after injury, including the proliferation and migration of adjacent intact ECs, resident EPCs and recruited EPCs. EPCs provide an endogenous repair mechanism to counteract persistent cell damage induced by risk factors. Scientists suggested EPCs are a useful tool for the treatment of endothelial injury in regenerative cardiovascular medicine (123–126). Thus, EPCs have been studied as biomarkers for the diagnosis and prognosis of CVD (127–129).

ECs

Healthy ECs protect atherosclerosis by promoting vasodilation, antioxidant and anti-inflammatory and inhibiting leukocyte adhesion and migration, and smooth muscle cell proliferation and migration. Remarkably, ECs can repair themselves. VEGF activates Cdc-42 and Rac1, mediates the formation of filamentous pseudopodia and plate pseudopodia, leading to EC migration (130). SDF-1 activates GPCR-dependent p110γPI3K, increases the expression of FoxM1 in ECs, participates in the transcriptional regulation of cell cycle progression genes, promoting the proliferation of ECs (131). Also, FoxM1 promotes re-adhesion between ECs through transcriptional control of β-catenin (132). When cells exfoliate after injury, surrounding ECs proliferate and migrate to coverage the basement membrane. However, mature ECs have limited ability to replace damaged ECs. Compared to ECs, EPCs show a higher proliferation potential, thus can serve as an additional source of ECs.

TABLE 1 | Classification, immunophenotype and physiology of EPCs.

Classification	Physiology	Immunophenotype
MACs	Secreting angiogenic cytokines	Positive : VEGFR2,CD133,CD45, CD115,CD14,CD31 Negative : CD146, CD34,Tie2
ECFCs	Differentiating into ECs	Positive : VEGFR2,CD34,C D31, CD105,CD146 Negative : CD133,CD45, CD115,CD14

EPCs

EPCs could differentiation into ECs. EPCs locate at the site of vascular injury, restore endothelial integrity and participate in neovascularization. The process of re-endothelialization includes mobilization, chemotaxis, homing, proliferation and differentiation (**Figure 3**). Early EPCs release growth factors, adhesion molecules and chemokines to promote the proliferation, survival and migration of late EPCs; late EPCs directly participate in the formation of endothelium (133). EPCs also release exocrine bodies to respond to injured ECs (134, 135).

Mobilization

The mobilization is the first step and is strictly regulated. EPCs are mainly seen in the bone marrow and in an inactive state which bind to bone marrow stromal cells (BMSCs) through the interaction of integrins (α4β1 and β3) and VCAM-1 (136). Under hypoxia, hypoxia-inducible factor 1 (HIF-1) rapidly increases, then weakens the interaction between EPCs and

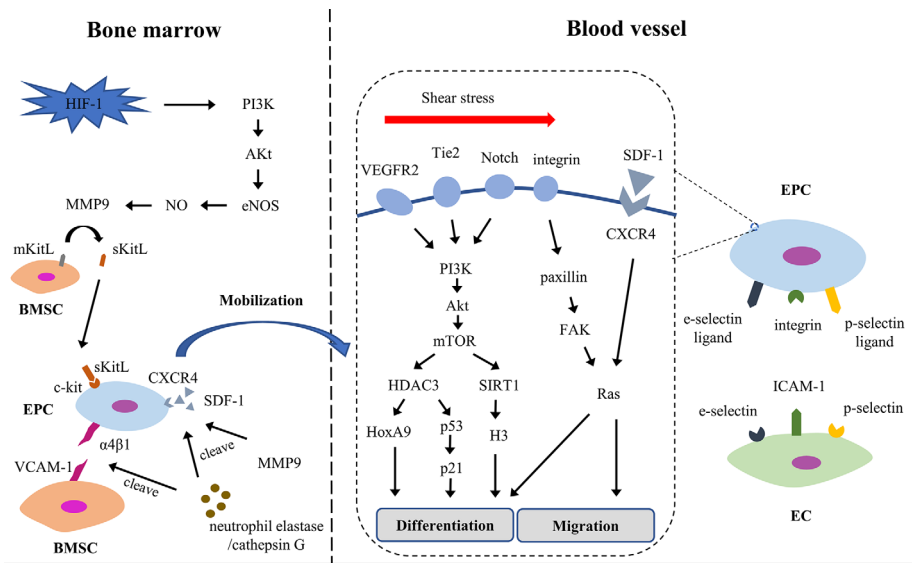

FIGURE 3 | The process of EPC re-endothelialization. Akt, Protein kinase B; BMSC, bone marrow stromal cell; CXCR4, chemokine (C-X-C motif) receptor 4; EC, endothelial cell; EPC, endothelial progenitor cell; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; HDAC3, histone deacetylase 3; HIF-1, hypoxia-inducible factor 1; HoxA9, Homeobox A9; ICAM-1, intercellular adhesion molecule-1; mKitL, membrane-bound form of Kit ligand; MMP9, matrix metalloproteinase-9; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3-kinase; SDF-1, stromal cell-derived factor-1; sKitL, soluble Kit-ligand; VCAM-1, vascular cell adhesion molecule-1; VEGFR2, vascular endothelial growth factor receptor-2.

BMSC through NO and MMP-9 in PI3K/AKt/eNOS-dependent manner. Moreover, neutrophil elastase and cathepsin G prevent EPCs from combining with BMSCs by cutting integrin and VCAM-1; and they cooperated with MMP9 to degrade SDF-1 in peripheral blood matrix niches forming a high SDF-1 concentration gradient. Under the synergistic action of elastase, cathepsin G and MMPs, EPCs are driven into the peripheral circulation (137).

Homing

After entering the peripheral circulation from the bone marrow, EPCs are summoned and stay at the site of endothelial injury in the tissue. This process involves multi-step cascade adhesion and signaling events, including chemotaxis, involvement, adhesion and migration (138). The SDF-1/CXCR4 axis regulates the downstream signal Rac, changes the polarity and cytoskeleton of the cells, maintains the motor state of the transitional cells, and navigates the EPCs to the target organ (139). Meanwhile, integrin, p-selectin ligand and e-selectin ligand expressed on EPCs interact with p-selectin, e-selectin and ICAM-1 expressed on activated ECs, supporting EPCs adhesion and migration to ECs (140, 141). Some studies have shown that SDF-1 increases the expression of e-selectin in microvascular ECs and then increases the adhesion of EC-EPC (142).

Differentiation

On the way to the target organ, EPCs begin to differentiate into ECs. During differentiation, cytokines and shear stress trigger a series of events, which cause EPCs to acquire some phenotypic characteristics of ECs. Shear stress supports the differentiation and proliferation of EPCs *via* VEGFR2, Tie2, Notch, and β1/3 integrin signaling (143). It stabilizes and activates histone deacetylase 3 (HDAC3) through the VEGFR2/Tie2/Notch/PI3K/Akt/mTOR pathway, which in turn deacetylated p53, leading to increased cell cycle arrest protein p21 and endothelial markers (144). The homeobox transcription factor HoxA9 contributes to HDAC-mediated differentiation (145). Histone deacetylase SIRT1, another downstream factor of shear stress/PI3K/Akt pathway, is overexpressed in EPCs and decreases histone H3 acetylation, upregulating endothelial markers (146). Beside, integrins β1 and β3, also overexpressed, enhance the expression of endothelial markers *via* paxillin/FAK/RAS/ERK pathway (147–149).

Mobilized EPCs enter into the peripheral blood and build a cell pool, repairing the endothelium by forming a patch at the site of intimal injury. EPCs represent negative feedback in intravascular homeostasis. The number and function of EPCs are regulated by the same molecular pathway, so the decrease of EPCs number is related to weakened function, and the increase of EPCs number is related to enhanced function.

Changes in the Number and Function of EPCs in SLE

There are 15 research articles about the number and function of SLE EPCs by searching "(Endothelial Progenitor Cells) AND (Lupus Erythematosus, Systemic)" in PubMed, which have shown inconsistent results (**Table 2**). Most of the results on the quantitative studies of SLE EPCs have shown a low level. Four studies have shown different results. The difference in the detection, quantification and identification of EPCs and the active phase of SLE might explain the quantitative differences. Studies on the qualitative of SLE EPCs also showed different results. Ablin JN et al. shown enhanced adhesion of SLE EPCs (156), while the others shown weakened proliferation/migration/adhesion/differentiation (46–49, 77, 150, 153, 154, 157–159). The different adhesion test and quantification seems to be the reason.

Causes of Reduced Number and Impaired Function of EPCs in SLE

Although the results are controversial, we believe that SLE EPCs show a trend of reduced number and impaired function. The risk factors (IFN-I, BAFF, OPG, IL-10, IL-18) and protective factors (Tang) both exist in SLE. The reduced number and impaired function of SLE EPCs seem to be the result of the game between the two sides.

There is no doubt that IFN-I accelerates SLE atherosclerosis, whether in the initiation or development of the disease (15, 52). The adult and mouse models' researches conclude that IFN-I accelerating SLE atherosclerosis by interfering with EPCs (15, 46–49, 51, 52, 160). Like adult-onset SLE, childhood-onset SLE also shown reduced number and impaired function of EPCs (150). But there was no significant correlation between IFN-I activity and childhood-onset SLE subclinical atherosclerosis and endothelial function (150). We need a longitudinal assessment in the future to assess whether vascular damage in childhood-onset SLE is related to IFN-I. Inflammatory body activation is a key downstream pathway leading to vascular abnormalities. The interaction between IFN-I and inflammatory factors mediates reduced number and impaired function of SLE EPCs. IFN-α down-regulates IL-1β and VEGF (52) and up-regulates IL-18 and its activator caspase-1 (51)— IL-1β promotes the differentiation of EPCs (52); IL-18 inhibits the differentiation of EPCs (51). IL10 inhibits EC differentiation and enhances IFN-α-mediated EPC dysfunction (50). OPG plays a pathogenic role in atherosclerosis. OPG binds to syndecan 4, the receptor of OPG on EPC, then induces oxidative stress, causing apoptosis of EPC (151). Spinelli FR et al. has observed that BAFF receptors are expressed in both EPC and EC, and mediated the apoptosis of EPC (77). The addition of BAFF inhibitor—belimumab—restored the quantity and quality of EPCs *in vivo* and *in vitro*, which further proved this point (77).

Tang, a specific T cell group expressing CD3, CD31 and CXCR4, promotes early EPCs differentiation and activates locally resident ECs (161). And the percentage of circulating Tang increased in SLE patients (162–164). However, the chronic inflammatory environment of SLE accelerates autoimmune aging. Aging Tang (CD28null-Tang) is not protective but cytotoxic, secreting inflammatory mediators and releasing cytolytic molecules from intracellular particles to induce EC damage and accelerates atherosclerosis in most SLE patients (165). And the frequency of CD28null-Tang increased in SLE patients with traditional CVD risk factors and active diseases (165).

TABLE 2 | Quantitative analysis of circulating EPCs between SLE and healthy control.

Results	Research objects	Surface labelings for the determination of EPCs	Detection methods	Quantization methods	References
Low level of EPCs in the SLE group	18 patients with SLE	$CD34^+$ $VEGFR2^+$	Flow cytometry Cell colony	Relative to the number of lymphocytes	(77)
	132 children with SLE	$CD34^+$ $CD133^+$	Flow cytometry	Absolute count per unit of blood	(150)
	90 patients with SLE	$CD34^+$ $VEGFR2^+$	Flow cytometry	Absolute count per unit of blood	(151)
	17 patients with SLE	$CD34^+$ $CD133^+$ $VEGFR2^+$ /$CD34^+$ $VEGFR2^+$/$CD133^+$ $VEGFR2^+$	Flow cytometry	Absolute count per unit of blood	(152)
	70 patients with SLE	$CD34^+$ $VEGFR2^+$	Flow cytometry Cell colony	Absolute count per unit of blood	(47)
	135 patients with SLE	$CD34^+CD133^+$	Flow cytometry Cell colony	Absolute count per unit of blood	(48)
	44 patients with SLE	$CD34^+CD133^+$	Flow cytometry	Absolute count per unit of blood	(153)
	15 patients with SLE	$CD34^+VEGFR2^+$	Flow cytometry	Absolute count per unit of blood	(154)
	gld.apoE-/- mice	$Sca\text{-}1^+$ $VEGFR2^+$	Flow cytometry	Relative to the number of lymphocytes	(155)
	gld.apoE-/- mice	$Sca\text{-}1^+$ $VEGFR2^+$	Flow cytometry	Relative to the number of lymphocytes	(46)
	NZB/W mice	$CD34^+$ $VEGFR2^+$	Flow cytometry	Relative to the number of lymphocytes	(49)
No significant difference	31 patients with SLE	$Tie\text{-}1^+$ $VEGFR2^+$ $CD31^+$	Cell colony	The number of colony	(156)
	35 patients with SLE	$CD34^+$ $VEGFR2^+$	Flow cytometry Cell colony	Relative to the number of lymphocytes	(157)
	31 patients with SLE	$CD34^+$ $VEGFR2^+$ $CD\ 133^+$	Flow cytometry Cell colony	Relative to the number of lymphocytes	(158)
Low level of CD34 +VEGFR2+ cells and high level of CD133+VEGFR2+ cells in the SLE group	19 patients with SLE	$CD133^+VEGFR2^+$ cells represent early EPCs, and $CD34^+VEGFR2^+$ cells represent late EPCs	Flow cytometry	Absolute count per unit of blood	(159)

Therefore, we speculate that Tang activates the vascular endothelial protective mechanism in the early SLE. With the progress of the disease, the chronic inflammatory environment of SLE not only accelerates the aging of Tang but also enriches a variety of risk factors for EPCs, which leads to the dysfunction of EPC in SLE patients.

THE ROLE OF IFN-I IN THE INJURY OF EPCS IN SLE

The Immune Mechanism of IFN-I Production in SLE

The IFN-I system in SLE is chronically active. pDCs (plasmacytoid pre-dendritic cells) are the primary source, which have high levels of interferon regulatory factor (IRF) 7, facilitating rapid and large-scale IFN-α generation (166). Up-regulated interferon-induced genes such as MX1, ISG54, and ISG56 and transcription factors of interferon pathway such as IRF5, IRF7, IRAK1, TREX1, STAT4, and PTPN22 mediate abnormal immune responses and the production of ICs, resulting in abnormal activation of pDCs (167). And other immune cells such as neutrophils, NK cells, T cells, B cells and platelets enhance IFN-I production by IC-stimulated pDCs; IFN-I, in turn, stimulates the activation of these immune cells, forming a self-magnifying pathogenic loop (65, 66, 168–173).

During exploring the signaling pathway, the increased exposure of nuclear contents to corresponding nucleic acid biosensors is the critical risk factors. Under normal physiological conditions, self DNA/RNA exists in different cell compartments and is isolated from the nucleic acid biosensor in the cytoplasm. Due to the insufficient clearance of apoptotic/necrotic cells, SLE patients are rich in endogenous free DNA/RNA, which form ICs with anti-DNA/RNA antibodies (174). Exogenous microbial DNA/RNA also induce autoimmune response (175–177). Exposed RNA and DNA stimulate the relevant nucleic acid biosensor in the form of ICs. DNA biosensors are divided into two types: endosomal membrane receptors and intracellular receptors (178). TLR9 is the only known DNA biosensor based on endosomes, which is mainly expressed in pDCs. The DNA ICs are absorbed and transported into the endosome through the Fcγ RIIa in pDCs, activating TLR9-MyD88-IRF7 pathway (166). Moreover, TLR9 can bind to

the curli-DNA complex, composed of bacterial DNA and amyloid protein curli—a component of bacterial biofilms (175, 176). Compared with TLR9, cytoplasmic DNA biosensors are widely expressed in mammalian cells. Thirteen cytoplasmic DNA biosensors have been found so far and cGAS is the most important cytoplasmic DNA biosensor (178). cGAS binds to cytoplasmic DNA to produce cGAMP, which then activates ER-resident STING protein. The activated STING is transported from the endoplasmic reticulum to the ER-resident Golgi apparatus and recruits TBK1 to enter the endosome. TBK1 activates IRF3 and IRF7, leading to the expression of IFN-I (179). Major RNA biosensors include TLR7 and RIG-I/MDA5. TLR7 also belongs to the endosomal membrane receptor, activated by single-stranded RNA. The U1snRNA induces PDCs to produce IFN-α through Fcγ RIIa-TLR7-MyD88-IRF7 pathway in SLE patients (180, 181). RIG-I/MDA5 signal is mainly used to deal with viral infections. After recognizing viral double-stranded RNA, intracellular RNA helicases (RIG-I and MDA5) undergo conformational changes to induce MAVS, and activates IRF3/7 through TRAF6/3, resulting in the secretion of IFN-I (182). Recent studies have shown that RIG-I/MDA5 signal may reduce the degradation capacity of insoluble virus-like aggregates, inducing a continuous increase of IFN-I (177).

The Pathways of IFN-I Damaging EPCs

IFN-I is one of the causes of impaired EPCs, but the specific mechanism remains to be elucidated. IFN-I damages EPCs in two ways: direct toxicity and indirect toxicity (**Figure 4**).

IFN-I actively induces the production of ELR-negative CXC chemokines CXCL9, CXCL10 and CXCL11, which mediate angiostasis through the receptor CXCR3 (183). CXCR3 exists in three different splice variants, CXCR3A, CXCR3B, and CXCR3-alt (184). CXCR3A recruits leukocytes, especially in Th1 lymphocytes (185). CXCR3-alt has a higher affinity for CXCL11, but its role in angiogenesis remains to be determined (186). Conversely, CXCR3B, expressed in ECs, is the main angiostatic variant of CXCR3 and is the primary angiostatic receptor for CXCL9, CXCL10, and CXCL11, inducing anti-proliferation and anti-migration (187–189). CXCR3A and CXCR3B differ for 52 amino acids at the NH2 end and couple different types of G proteins, triggering different signal transduction pathways, CXCR3A-Gi-PI3K-MAPK and CXCR3B-Gs-AC-cAMP-PKA (187, 190). The coupling of CXCR3B with Gs results in the selective activation of adenylyl cyclase (AC) and a consequent increase of intracellular cAMP levels (187). Up-regulation of cAMP in ECs directly activates PKA, inducing apoptosis (191).

Moreover, IFN-I enhances the toxicity of ILs and BAFF, which are EPC risk factor as well. IFN-I interacts with inflammatory factor ILs to damage EPC synergistically. IL-10 enhances the effect of IFN-α on SLE EPC (50). IFN-I down-regulates angiogenic molecules IL-1β and VEGF (52) and up-regulates IL-18 and its activator caspase-1 (51), enhancing the anti-angiogenic effect. There was a positive correlation between the levels of IFN-I and BAFF in SLE (192). IFN-I induces the expression and mobilization of BAFF in SLE monocytes and neutrophils (35, 36). The expression of BAFF is directly induced by IFN-I through IRF1 and IRF2 (36). IFN-α stimulates the secretion of IL-17, then IL-17 and BAFF promote the survival and differentiation of B cells and production of autoantibodies, which enhances IFN by pDCs, forming a closed vicious circle (192).

Therefore, IFN-I has direct and indirect toxic effects on EPC, resulting in endothelial dysfunction, which starts atherosclerosis in SLE. It is proved once again that IFN-I plays a central pathogenic role in SLE CVD.

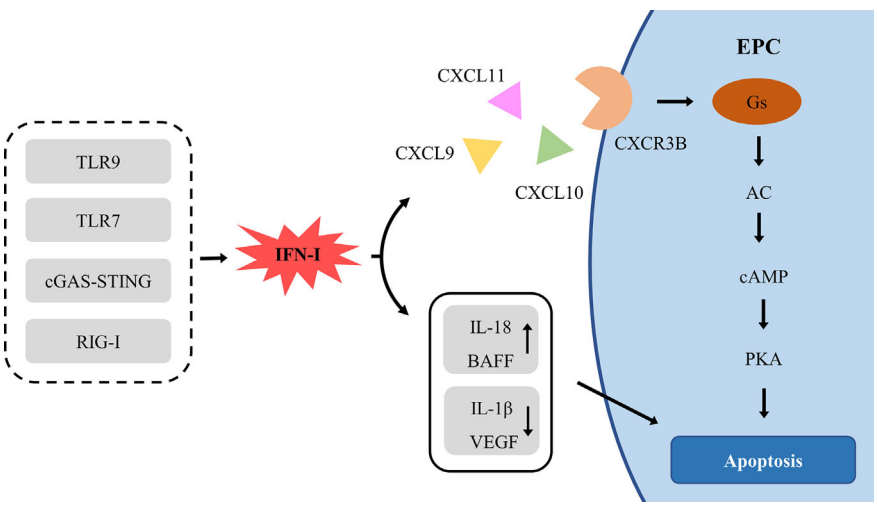

FIGURE 4 | The signal pathway of IFN-Idamaging EPC. AC, adenylyl cyclase; BAFF, B cell-activating factor; cAMP, cyclic adenosine monophosphate; cGAS, cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase; CXCL, chemokine (C-X-C Motif) ligand; CXCR, chemokine (C-X-C motif) receptor; EPC, endothelial progenitor cell; PKA, Protein kinase A; RIG-I, retinoic acid-inducible gene I; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

CONCLUSION

Long-term activation of IFN-I system in SLE induces the expression of CXCL9/10/11, activating CXCR3B-Gs-AC-cAMP-PKA signal pathway to promote the dysfunction of ECs and EPCs; and CXCR3A-Gi-PI3K-MAPK signaling pathway to recruit leukocytes into the inflammatory site. Besides, IFN-I enhances the toxicity of other EPCs dysfunction factors, indirectly accelerating arteriosclerosis. Overexpression of IFN-I through the activation of TLR7/9 signal decreases the number and function of EPCs and increases atherosclerotic lesions in SLE patients (46), suggesting that targeted therapy of cGAS and RIG-I signal pathway may have a potential therapeutic effect on SLE atherosclerosis. Targeted therapy of the IFN-I system has a potential therapeutic effect on early atherosclerosis in SLE patients.

AUTHOR CONTRIBUTIONS

XD did the literature search and drafted the article. WX and XH gave insight. XH revised the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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A Neutrophil-Driven Inflammatory Signature Characterizes the Blood Transcriptome Fingerprint of Psoriasis

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Transcriptome profiling approaches have been widely used to investigate the mechanisms underlying psoriasis pathogenesis. Most researchers have measured changes in transcript abundance in skin biopsies; relatively few have examined transcriptome changes in the blood. Although less relevant to the study of psoriasis pathogenesis, blood transcriptome profiles can be readily compared across various diseases. Here, we used a pre-established set of 382 transcriptional modules as a common framework to compare changes in blood transcript abundance in two independent public psoriasis datasets. We then compared the resulting "transcriptional fingerprints" to those obtained for a reference set of 16 pathological or physiological states. The perturbations in blood transcript abundance in psoriasis were relatively subtle compared to the changes we observed in other autoimmune and auto-inflammatory diseases. However, we did observe a consistent pattern of changes for a set of modules associated with neutrophil activation and inflammation; interestingly, this pattern resembled that observed in patients with Kawasaki disease. This similarity between the blood-transcriptome signatures in psoriasis and Kawasaki disease suggests that the immune mechanisms driving their pathogenesis might be partially shared.

Keywords: psoriasis, transcriptomics, blood, Kawasaki disease, systems biology

INTRODUCTION

Inflammation has an important role to play as part of the host defense against infection. However, prolonged or excessive inflammation can cause notable pathology (1–3). One example of such a pathology is psoriasis, which affects ~100 million individuals worldwide (4). This common, immune-mediated disease results in a unique skin barrier abnormality caused by excessive epidermal proliferation and inflammation (5, 6). Psoriasis pathogenesis is likely driven by many factors, including environmental triggers, genetic susceptibility, and even microbiome composition

(1, 6, 7). At the cellular level, an interaction between innate and adaptive immune responses, and the activation of Th17 and Th1 cells are key to the immunopathogenesis (8). Plasmacytoid dendritic cells (DCs) found in psoriatic skin are activated by antigens and subsequently release IFN-α. Meanwhile, myeloid DCs secrete IL-23 and IL-12, which favors T-cell differentiation into Th17 and Th1 pathways, respectively (9, 10). In turn, Th17-derived cytokines including IL-17A and IL-22 play a dominant role in driving keratinocyte activation and proliferation. Finally, TNF-α secreted by DCs, Th17, and Th1 cells, and keratinocytes in psoriatic skin amplifies and perpetuates inflammation. Current treatment modalities include topical glucocorticoids, vitamin D analogues, phototherapy, conventional immunosuppressives (e.g., ciclosporin, methotrexate), and various biologics that target TNF-α (e.g., infliximab), the IL-17 pathway (e.g., secukinumab), and IL12/IL-23 (e.g., ustekinumab) (9–12). But despite such progress in understanding the molecular mechanisms driving psoriasis, we are still far from having a complete understanding of the immunopathogenesis and developing highly effective therapeutics.

To gain a better understanding of psoriasis pathophysiology, many researchers have compared the transcriptome profiles of diseased *vs.* healthy skin tissues isolated from affected patients (13–17). By contrast, only a few research groups have profiled genome-wide transcript abundance in blood samples from patients (18, 19). Measuring transcript abundance in the blood might seem less applicable to studies of skin diseases such as psoriasis; however, the blood presents the advantage of being highly accessible and amenable to serial sampling. Thus, blood transcriptional profiling could be harnessed to monitor dynamic treatment responses. Another advantage is the availability of numerous public blood transcriptome datasets, which allows us to make comparative analyses across various inflammatory diseases.

Here, we compared the blood transcriptome fingerprints of two publicly available psoriasis datasets (18, 19) with those derived from a collection of 16 reference patient cohort datasets (20). Our functional interpretations relied on extensive annotations and expression patterns observed in purified leukocyte populations.

METHODS

Collection of Public Datasets

The public datasets used in this re-analysis and interpretation were available in the NCBI GEO repository (21) (**Supplementary Table 1**). They include psoriasis blood transcriptome datasets as well as a collection of reference transcriptome datasets used for contextual interpretation. They are described in brief here:

Two psoriasis blood transcriptome datasets were identified for which data analysis was performed as detailed below. Both comprised control groups of subjects. Findings were reported in the literature by their original contributors:

- The GSE55201 dataset contributed by Wang et al. was generated using an Affymetrix U133 Plus (microarray) and consists of profiles for 81 samples. The study examined the role of IL-17 in ameliorating systemic inflammation and its impact on psoriasis complications, such as atherosclerosis and ischemic cardiovascular disease (19).
- The GSE123786 dataset contributed by Catapano et al. was generated using an Illumina HiSeq 2000 platform (RNA-Seq) and consists of profiles for 16 samples. This study examined the involvement of IL-36 in extracutaneous manifestations of psoriasis (18).

Other blood transcriptome datasets were used as reference:

- The GSE100150 dataset (20) contributed by our group was generated using an Illumina HumanWG-6 v3.0 BeadChip and consists of 16 reference patient cohorts encompassing the profiles of 985 subjects/samples.

Two other reference datasets were used to functionally interpret gene signatures:

- The GSE24759 dataset contributed by Novershtern et al. (22) was generated using Affymetrix U133A GeneChip and consists of 211 samples. The samples were collected from 4 to 7 donors and a wide range of hematopoietic cell populations from both adult and cord blood were profiled.
- The GSE60424 dataset (23) contributed by our group was generated using an Illumina RNA-Seq platform and consists of 134 subject/samples profiles. In this study, leukocyte populations were isolated from the blood of healthy individuals and patients with diabetes mellitus type 1, amyotrophic lateral sclerosis, multiple sclerosis (MS; pre- and post-interferon treatment) or sepsis.

Data Processing

The analysis workflow determines for each module the proportion of its constitutive transcripts that significantly differ in comparison with a given baseline (e.g., healthy controls). Thus, at the module level, changes are expressed as the proportion of transcripts constituting a given module being significantly increased (0 to 100%) or decreased (0 to −100%) compared with healthy controls. By design, changes in abundance among transcripts within a given module tend to be coordinated. However, when both significant increases and decreases are observed for the same module, the dominant trend is retained.

Data pre-processing steps for the two public psoriasis blood transcriptome datasets were performed as follows: The Wang *et al.* dataset (GSE55201) was generated using Affymetrix GeneChip and normalized with GCRMA (24). The Catapano *et al.* dataset (GSE123786) was generated *via* RNA-Seq data and the data are presented as RPKM values (reads per kilobase of transcript, per million mapped reads) after mapping with the HG38 genome build; the read counts were calculated using htseq-count (25).

Transcriptional Module Repertoire Analyses

Modular repertoire analyses were performed at the group level on both GSE55201 and GSE123786 psoriasis datasets using the BloodGen3Module R package: https://github.com/Drinchai/

BloodGen3Module (26). The pre-determined repertoire of 382 co-expressed blood transcriptional modules that served as a framework for this analysis was described by Altman et al. (20). Briefly, it was constituted based on co-expression observed across a collection of 16 reference datasets encompassing 985 unique blood transcriptome profiles and 14,168 transcripts. A wide range of immune states are represented in this collection of reference datasets, including several infectious diseases, autoimmune diseases, inflammatory disorders as well as cancer, pregnancy, and solid organ transplantation. Because this module repertoire is "fixed" and destined for reuse as a generic framework for blood transcriptome analyses, considerable efforts were dedicated to its annotation and functional characterization. This work included functional enrichment analyses (ontologies, pathways, literature terms), and the generation of heatmaps representing transcript abundance patterns for reference datasets. The latter included, for instance, profiling data from isolated leukocyte populations. Interactive presentations were established to provide access to the large compendium of analysis reports and heatmaps that were generated as part of these annotation efforts. The presentation for a subset of 21 modules associated with inflammation that will be discussed in more detail as part of this work can be accessed *via* this link: https://prezi.com/view/GkH4wHb0jhIbDGt7Ibwi/. A demonstration video can be accessed *via* this link: https://youtu.be/fTfqGhcCNdE. However, it should be noted that module annotation has an element of subjectivity and is still a work-in-progress. Additionally, some of the functional "labels" that have been assigned are still tentative and subject to change as data analyses and interpretations progress across several projects.

Blood Transcriptome Fingerprint Visualizations

The percent of increased or decreased transcripts computed per module were represented on a fingerprint grid plot. In brief, modules occupy a fixed position on the grid and changes for that module are indicated by a red spot (increased abundance compared to controls) or a blue spot (decreased abundance compared to controls). All fingerprints plots show changes in transcript abundance in cases compared to respective healthy controls (run concomitantly and matched for demographics). Modules arranged on the same row belong to one of 38 "module aggregates." These aggregates are formed based on similarities in the patterns of transcript abundance changes across the 16 reference datasets. Thus, a vertical reading of the grid across the rows gives an indication of the patterns of change in a given set of patients at the least granular level (aggregates). A second horizontal reading within each row and across the columns gives an indication of the changes occurring at a more granular level (modules). Functional interpretations are indicated by a color code that is overlaid on the grid plot (**Supplementary Figure 1**). Because the positions of the modules on the grid are fixed, different fingerprints generated for independent groups of patients can be compared. Fingerprint grid plots for all of the 16 reference cohorts can be generated dynamically using a previously developed app: https://drinchai.shinyapps.io/dc_gen3_module_analysis/#.

Screening of Drug Targets

Transcripts among the A35 modules were screened for the presence of drug targets using the "open targets platform" that is available *via* the open targets consortium at https://www.targetvalidation.org/ (27). The batch query functionality was used. The transcripts encoding targets of existing drugs (referred to in the results as "targets with clinical precedence") were retrieved (**Table 1**).

RESULTS

Blood Transcriptome Signatures of Independent Psoriasis Datasets Share a Similar Modular Component

We first aimed to determine whether we could measure robust changes in transcript abundance in the blood of psoriasis patients in comparison to heathy controls. We presumed that such signatures, if present, could then be "benchmarked" against that of other inflammatory or autoimmune diseases.

For this first step, we harnessed data from two psoriasis blood transcriptome datasets of a relatively modest size that have been published and made available *via* the NCBI GEO repository (18, 19). The technology platforms used to generate each dataset were quite dissimilar: Wang *et al.* used microarrays while Catapano *et al.* performed RNA sequencing. We previously showed that differences in transcript abundance summarized at the level of coordinately expressed gene sets (modules) are more amenable to cross-platform comparisons than when differences are expressed at the individual gene level (28). We therefore used a pre-determined repertoire of blood transcriptome modules that was recently developed and characterized by our group (20) (see *Methods*). Briefly, we formed this repertoire on the basis of co-expression measured across 16 reference patient cohorts, encompassing 985 unique blood transcriptome profiles. Two-dimensional reduction levels are built into the repertoire. The least reduced level has 382 variables, which are the modules that are constituted by sets of genes. The most reduced level has 38 variables, which are module aggregates that are constituted by sets of modules that altogether encompass the 382-module repertoire. Changes between cases and controls are expressed as a proportion of the transcripts constituting a given module found to be significantly increased (max +100%, all transcripts are increased) or decreased (−100%). We thus determined differences in transcript abundance for each of the 382 modules for the Wang *et al.* and Catapano *et al.* datasets. We represented these differences on a fingerprint grid plot, where the assignment of modules to a given position on the grid was fixed (**Figure 1**).

The fact that positions of modules on the grid are fixed ensures that the generated fingerprints are directly comparable. Here, we found a good level of concordance between the two datasets, with both predominantly showing changes for the 21 modules forming row A35 on the grid. At a high level, the module aggregate A35 is functionally associated inflammation

TABLE 1 | Transcripts comprised in aggregate A35 for which the gene products are targetable by existing drugs, and the drugs tested in psoriasis or Kawasaki disease (see *Methods*).

ID	Drug targets with clinical precedence	Drugs tested with psoriasis as an indication and their corresponding targets (underlined)	Drugs tested with KD as an indication	Open targets report
M15.84	MAPK14	MAPK14: BMS-582949 (phase II), doramapimod (phase II)	None	https://bit.ly/3iynGZF
M13.16	CASP4, CASP5, CSF2RB, CXCR2, KCNJ2, MGAM, NAMPT	CXCR2: navarixin (phase II)	None	https://bit.ly/38x3sey
M13.1	CASP1, FGR, FKBP1A, IMPDH1, MAPK1, MAPK3, S1PR4, NCSTN, NOTCH1, PRKCD, RARA, RXRA, SELL, SYK, TNFSF13B	RARA/RXRA: acitretin (phase IV), tazarotene (phase IV), etretinate (phase IV), alitretinoin (phase II); FKBP1A: tacrolimus (phase III); SELL: bimosiamose (phase II); S1PR4: amiselimod (phase II), PRKCD: sotrastaurin (phase II); IMPDH1: mycophenolate mofetil (phase II).	None	https://bit.ly/3iD3CW7 https://bit.ly/3e5UqGp https://bit.ly/3e3XQJF https://bit.ly/2W76iSz https://bit.ly/38uAT18 https://bit.ly/3e17rkn
M15.37	IL1B, NDUFB3,	NDUFB3: metformin (phase III)	None	https://bit.ly/3iBFByl
M15.113	BMX, IL1R1, MAPK14	None	None	
M12.10	ALOX5, IL13RA1, RAF1, TBXAS1, TNFRSF1A	None	None	
M13.12	CA4, F5, FCGR1A, HPSE, MMP9, TLR5	None	None	
M15.105	PSMB3	None	None	
M15.109	IL6R, NAMPT	None	None	
M13.22	C5AR1, FGR, HCK, HSPA1A, IFNAR1, IL8RA (CXCR1), LY96	IL8RA (CXCR1): Navarixin (Phase II)	None	https://bit.ly/2ZLoOAV
M14.28	None	None	None	
M15.26	EGLN1, FKBP1A, HPSE, MCL1, TLR4	FKBP1A: tacrolimus (phase III)	None	https://Bit.ly/3e5UqGp
M14.65	CD14, IFNGR2, ITGB2	IFNGR2: interferon gamma-1b (phase 0, terminated)—actimmune (early phase I)	None	https://bit.ly/3f77kVR
M16.79	CASP4, IL10RB	None	None	
M16.98	ADORA2B, CACNA1E, VDR	ADORA2B: caffeine (phase I completed) VDR: calcipotriene (phase IV), calcitriol (phase II), becocalcidiol (phase II), pefcalcitol (phase II), ergocalciferol (psoriasis vulgaris-phase 0), cholecalciferol (phase 0)	None	https://bit.ly/38wYAGh
M13.3	APH1B, CSF2RA, GBA, HDAC4, MAPK1, OPRL1, PDK3, PIM3	None	None	
M14.7	ECGF1, JAK2	JAK2: tofacitinib (phase III), baricitinib (phase II), ruxolitinib (phase II), lestaurtinib (phase II), peficitinib (phase II)	None	https://bit.ly/3f6RlHf
M14.74	None	None	None	
M15.43	COL18A1, MGC18216 (IGF1R), PTPRC, TNFSF14, TXNRD1	None	None	
M15.78	ANPEP, CSF3R, IL4R	None	None	
M15.81	PIK3CD	None	None	

(detailed below). In addition, the Catapano *et al.* dataset showed increases for modules forming row A28. The module aggregate A28 is functionally associated with interferon responses. Notably, an interferon signature was also reported by Catapano and colleagues (18), and seems to be associated with generalized pustular psoriasis, which is a severe form of the disease (1).

The fact that an increase in abundance of A35 modules was observed in both datasets suggests that this modular signature constitutes the main component of the blood transcriptome fingerprint associated with psoriasis overall. At a high level, seven of the 21 modules forming aggregate A35 were associated with inflammation, three with neutrophils, two with cytokines/chemokines, one with macrophages, and one with protein synthesis (**Figure 1**). The remaining seven modules were not associated with any given functional annotations due to lack of convergence between the functional profiling results obtained *via* different methodologies. The reports from gene ontology (GO), pathway and literature keyword enrichment analyses upon which these determinations were made (**Figure 2**), are available *via* an interactive presentation (https://prezi.com/view/7Q20FyW6Hrs5NjMaTUyW/) and all functional annotations of the A35 module are readily available (**Table 2** and **Supplementary File 1**).

In summary, this step identified that modules forming aggregate A35 are conserved between two independent psoriasis blood transcriptome datasets. Notably, this convergence was evident even though distinct technology platforms were used to generate the respective datasets. Altogether, these findings indicate that a blood transcriptional signature can consistently be observed in the blood of psoriasis patients.

The Psoriasis Blood Transcriptome Signature Is Associated With Neutrophils and Inflammation

We next aimed to determine the relevance of the increase in A35 transcripts in the context of psoriasis pathogenesis. To do so, we proceeded with the functional interpretations of this signature.

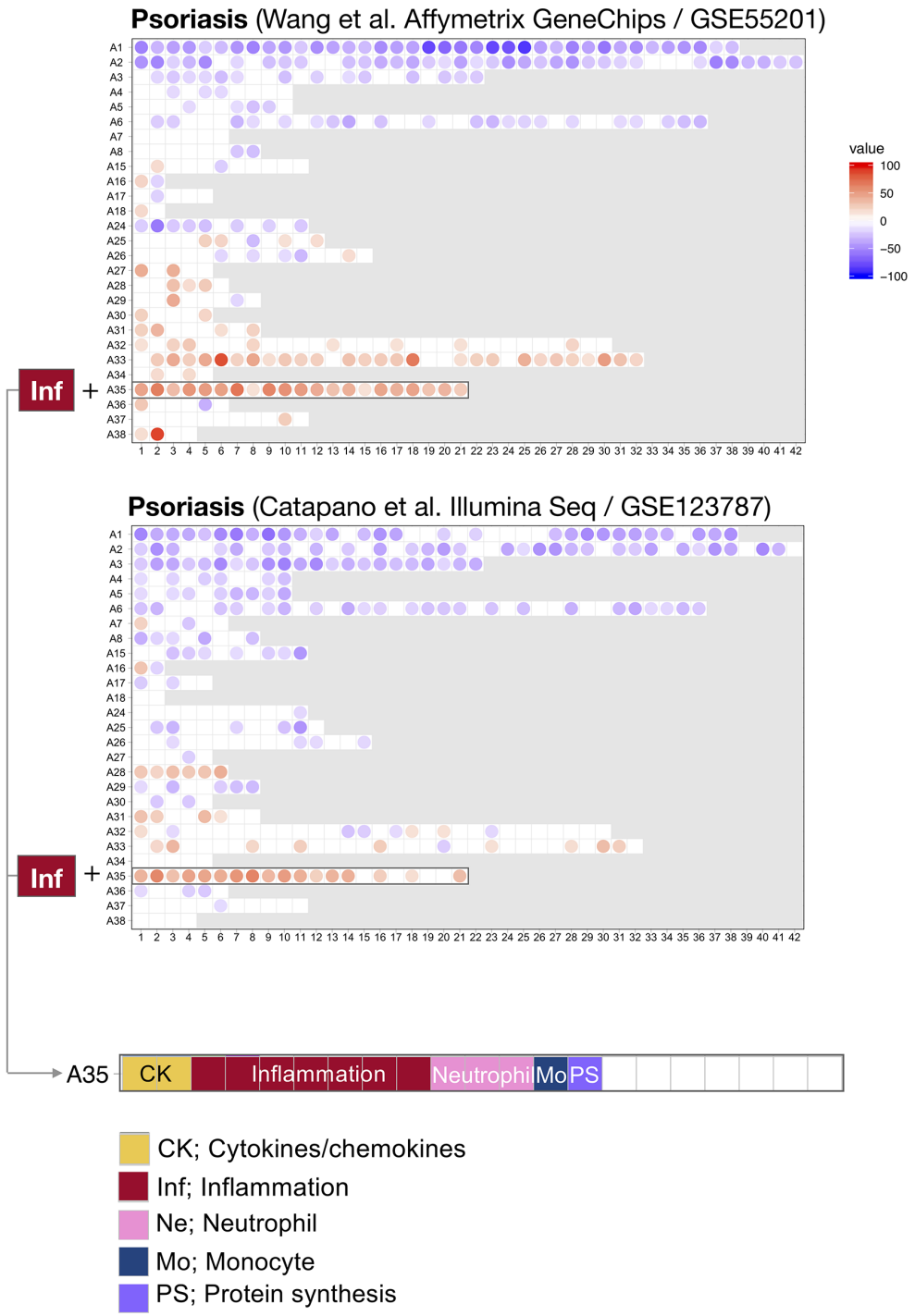

FIGURE 1 | Blood transcriptome fingerprints of psoriasis. Differences in the levels of blood transcript abundance in patients with psoriasis and controls were mapped on a grid for the two public datasets from Wang *et al.* (GSE55201: 30 controls and 51 subjects) and Catapano *et al.* (GSE123786: 7 controls and 9 subjects). Each position on the grid is occupied by a given module (pre-determined set of co-expressed genes). A blue spot indicates a module for which constitutive transcripts are predominantly present at lower levels in patients *vs.* controls. Conversely, a red spot indicates a module for which constitutive transcripts are predominantly present at higher levels in patients *vs.* controls. No spots on a white background indicate that there are no changes for the module in question. A gray background means that there are no changes in the module at this position. The modules are arranged by rows in "module aggregates" and ordered by their similarity in expression patterns across a set of 16 disease or physiological states (reference dataset collection). A consistent increase was observed for modules constituting aggregate A35. This aggregate is highlighted on the grid and functional annotations are provided (bottom panel). Functional annotations for the entire gird are provided in **Supplementary Figure 1**.

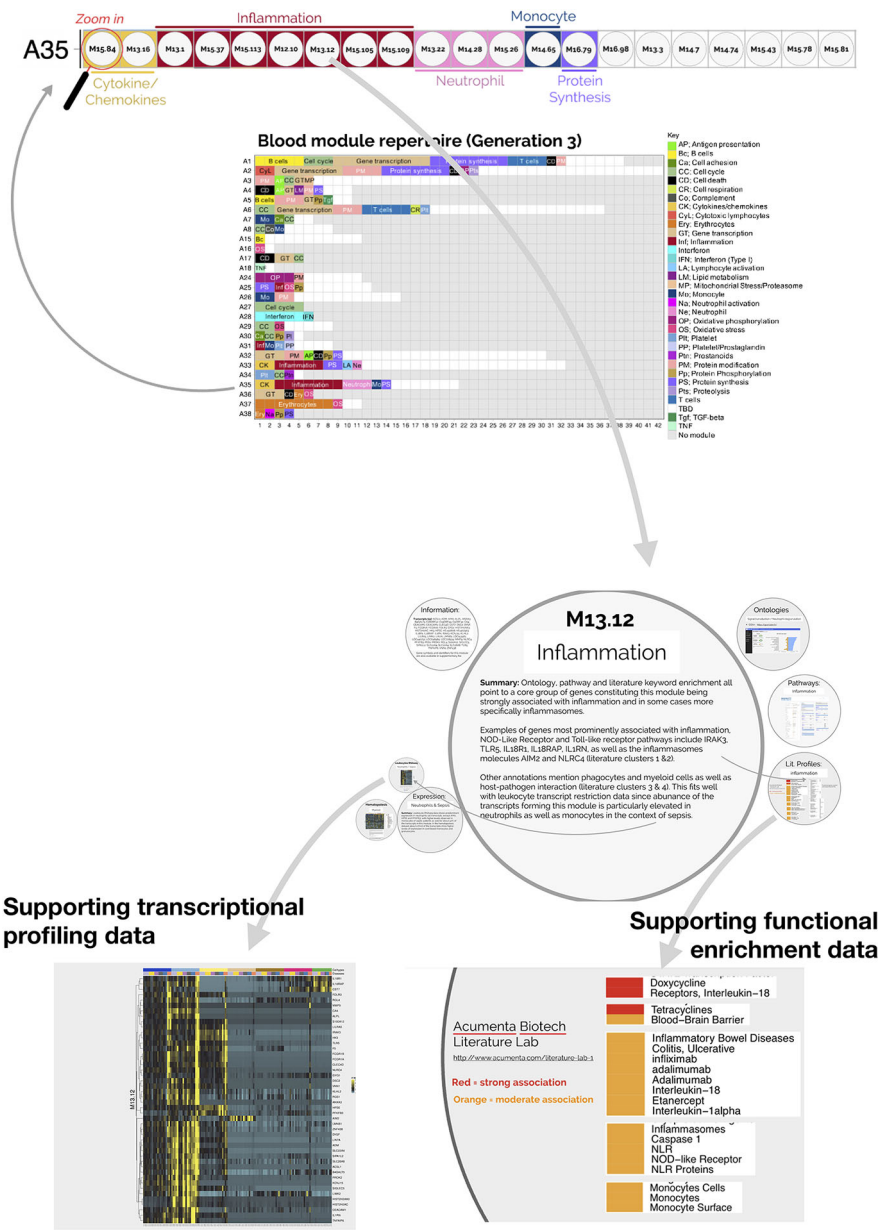

FIGURE 2 | Interactive presentation providing transcriptional profiling and functional enrichment data for modules constituting aggregate A35. An interactive presentation has been developed that allows for exploration of the modules constituting aggregate A35. A gene list is provided for each module, along with gene ontology, pathway or literature term enrichment results and transcriptional profiling data for the reference transcriptome datasets (circulating leukocyte populations, hematopoiesis). A summary of the findings is also given. The interactive presentation is available *via*: https://prezi.com/view/7Q20FyW6Hrs5NjMaTUyW/. The presentation provides zoom in/out functionalities for close-up examination of the text and figures embedded in the presentation.

The two converging themes that emerged through the extensive annotation work mentioned above were "neutrophil" and "inflammation." For instance, enriched literature terms included "neutrophil degranulation," "inflammation," and "inflammasome." Consistently, some of the genes in these modules are most recognizable as being involved in inflammatory processes, including those coding for inflammasome components. For example, NLR protein families were found across different modules within this aggregate, including NLRX1, NLRC4, NLRP12. Furthermore, "neutrophil activation involved in immune responses" (GO:0002283) was one of the most over-represented GO terms, with 121/784 transcripts forming modules belonging to aggregate A35. Thus, both gene composition and functional enrichment analyses suggest that this set of 21 modules constituting aggregate A35 is involved in inflammatory processes.

To complement our functional profiling analyses, we examined the expression patterns of the genes belonging to

TABLE 2 | The 21 modules constituting aggregate A35.

ID	Grid position	Number of transcripts	Functional annotation	Representative genes
M15.84	A35-1	20	Cytokines/chemokines	S100P, TLR2, MAPK14, FCAR
M13.16	A35-2	39	Cytokines/chemokines	BTNL8, CR1, FFAR2, FPR2, TLR6, ALPK1
M13.1	A35-3	137	Inflammation (innate immune response activation)	PYCARD, CLEC4A, SYK, CD300A, PRKCD, PELI1, LILRA2, MYD88, HSPA1B
M15.37	A35-4	33	Inflammation (leukocyte migration)	LAT2, SLC7A8, IL1B, FPR2, SLC16A3, GPSM3
M15.113	A35-5	16	Inflammation	SOCS3, RAB20, MAPK14, BMX, RASGRP4
M12.10	A35-6	53	Inflammation (neutrophil degranulation)	CRISPLD2, ALOX5, LAMP2, RAB24, ITGAX, TIMP2, SIRPA, RNASE3, LILRB3, IGF2R
M13.12	A35-7	55	Innate immunity, myeloid cells, inflammasomes	AIM2, TLR5, SIGLEC5, IL18RAP, IL18R1, S100A12, NLRC4, IRAK3, TNFAIP6, CLEC4D, LILRA5, FCGR1A, FCGR1B
M15.105	A35-8	16	Inflammation (myeloid cells, arginase pathway)	MAP3K3, TYROBP, PSMB3, LILRB2
M15.109	A35-9	17	Inflammation (defense response, leukocyte migration)	IL1RN, IL6R, TNFRSF10B, CR1, TLR8, FCGR2A
M13.22	A35-10	65	Neutrophils (response to LPS)	AKIRIN2, SLC11A1, C5AR1, LY96, TRIB1, LITAF, IFNAR1
M14.28	A35-11	20	Neutrophils (neutrophil degranulation)	BST1, MMP25, SERPINA1, FCER1G, ITGAM, SLC2A3, LILRA2, OSCAR
M15.26	A35-12	38	Neutrophils (activation, exocytosis)	PREX1, CEACAM3, ATP8B4, PLAUR, RAB27A, HPSE, SIRPB1
M14.65	A35-13	15	Monocyte (host defense)	ITGB2, CYBA, CD14, GNS, RAB7A, IFNGR2
M16.79	A35-14	27	Protein synthesis (secretion)	PYCARD, CNN2, FAM49B, RHOT1, DNAJC5, GAPDH, MCU, LILRA5
M16.98	A35-15	18	TBD	IL22, VDR, KREMEN1, LOXL3, ADORA2B, MAK, TIFA
M13.3	A35-16	100	TBD (response to stress)?	ERO1A, MAP3K2, G6PD, GADD45A, EDEM2, GBA, WIPI1
M14.7	A35-17	31	TBD	MFN2, JAK2, BATF, TFE3, CPEB3
M14.74	A35-18	14	TBD	MOSPD2, CD58, CKLF, CD53, TLE4, RNASEL
M15.43	A35-19	30	TBD (protein secretion)?	RCN3, COP1, CARD16, CLEC4E, CAMK2G
M15.78	A35-20	20	TBD (signal transduction)?	CSF3R, IL4R, SEMA4B, MKNK1, CREBRF, GPAT3, REM2
M15.81	A35-21	20	TBD (neutrophil degranulation)	PKM, GAA, ALDOA, AGPAT2

Detailed information can be found in ***Supplementary File 1****, and an interactive presentation that is accessible via this link: (https://prezi.com/view/GkH4wHb0jhIbDGt7Ibwi/; demonstration video (https://youtu.be/0j-kcE1tlXAc). (Acronym TBD, to be determined).*

module A35 in reference transcriptome datasets (see *Methods*). In particular, a dataset that we previously generated and deposited in the GEO showed that among circulating leukocyte populations, the expression of A35 transcripts was predominant in neutrophils [Linsley et al. (GSE60424) (23), http://sepsis.gxbsidra.org/dm3/miniURL/view/Q0)] (**Figure 3**). In another dataset, also contributed by our group, we found that the expression of A35 transcripts was upregulated in neutrophils exposed to plasma from septic patients *in vitro* (29); http://sepsis.gxbsidra.org/dm3/miniURL/view/Q2.

Altogether, functional and gene expression profiles observed in this reference dataset suggest that the A35 signature is associated with neutrophil-driven inflammation.

The Blood Transcriptome Fingerprint of Psoriasis Resembles That of Patients With Kawasaki Disease

As mentioned, a benefit of examining transcriptome signatures in blood rather than skin samples from patients with psoriasis is that it lends itself to making comparisons across a wide range of diseases. Carrying out such comparisons allows us to draw parallels or identify differences with diseases for which the pathogenesis might be better understood and managed clinically.

To achieve this, we compared the module repertoire fingerprints of psoriasis with those of the 16 other diseases comprising the reference collection of datasets used to construct our module repertoire (20). As was the case for psoriasis, we observed an increase in abundance of A35 modules in the blood repertoire fingerprints of systemic lupus erythematous (SLE), systemic onset juvenile idiopathic arthritis (SoJIA), and Kawasaki disease (**Figure 4**). In the case of SLE and SoJIA, the increase in abundance of A35 transcripts was one of many "perturbations" of the blood transcriptome repertoire, which is consistent with the systemic inflammation that characterizes these two diseases [for example: modules in aggregates/rows A27-A29 (SLE) or A30-A38 (SoJIA)]. The fingerprints of patients with acute infections (e.g., bacterial sepsis, tuberculosis, or influenza infection) also showed pronounced changes (such fingerprints can be generated dynamically *via* our web application accessible at: https://drinchai.shinyapps.io/dc_gen3_module_analysis/#). Notably, the fingerprints of patients with sepsis closely resembled those of patients with SoJIA. In both pathologies, other modules functionally associated with inflammation, such as A33, also showed a robust increase in abundance; such increases were not observed in the context of psoriasis or Kawasaki disease. Indeed, patterns of abundance of A33 and A35 modules across the 16 reference patient cohorts and two psoriasis datasets indicated that A35 modules tend to be more ubiquitously increased in comparison to A33 modules (**Figure 5**). The relative difference in intensity of A33 and A35 signatures between the two psoriasis datasets also suggests that those signatures might be non-synonymous and represent distinct inflammation pathways. Conversely, the Kawasaki disease blood transcriptome repertoire fingerprint was more subtle and, like that of psoriasis, was mostly restricted to an increase in abundance of

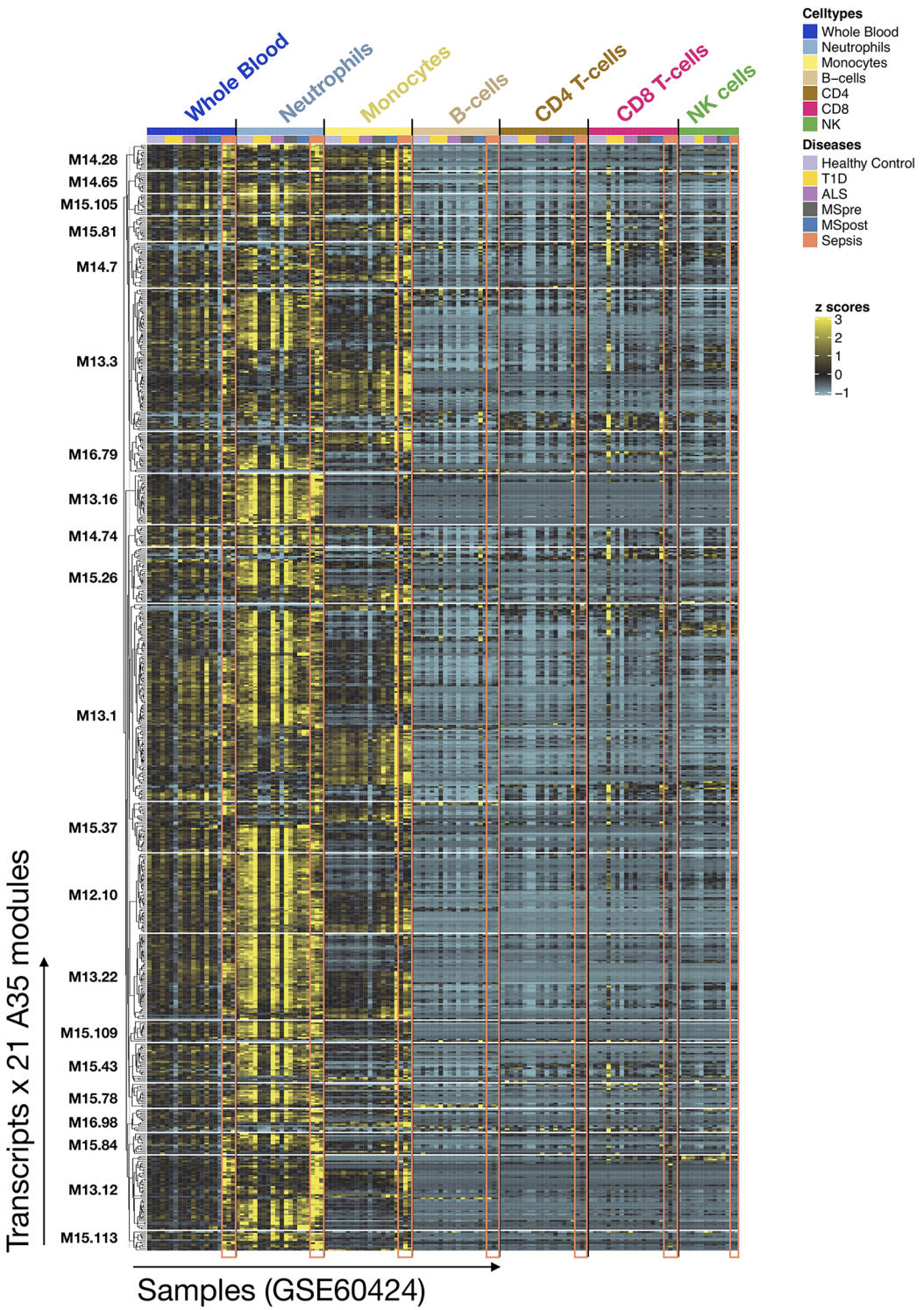

FIGURE 3 | Expression patterns of genes constituting A35 modules across whole blood and purified leukocyte populations. The expression levels for genes constituting A35 modules are shown on a heatmap for a reference dataset comprising the profiles of isolated leukocyte populations (GSE60424). The rows represent genes, with each cluster of rows representing a module. The columns represent samples. This study compared the whole transcriptome signatures of six immune-cell subsets and whole blood from patients with one of an array of immune-associated diseases. Fresh blood samples were collected from healthy subjects and those diagnosed with type 1 diabetes, amyotrophic lateral sclerosis, sepsis, or multiple sclerosis (before and 24 h after the first dose of IFN-beta). RNA was extracted from each of the indicated cell subsets and whole blood samples, and then processed for RNA sequencing (Illumina TruSeq; 20M reads).

FIGURE 4 | Blood transcriptional fingerprints of other autoimmune or autoinflammatory diseases. The differences in the levels of transcript abundance in the blood of patients with Kawasaki disease (top), systemic lupus erythematous (middle), or systemic onset juvenile idiopathic arthritis (bottom) are mapped on a grid, as described in **Figure 1**. The modules belonging to aggregate A35 are highlighted on this grid.

A35 module transcripts (**Figure 4**). This finding might reflect the fact that inflammation is typically localized at the onset of these diseases: to the skin for psoriasis and the vasculature endothelium for Kawasaki disease.

Taken together, these results suggest that the extent of changes in blood transcript abundance tends to correlate with the disease manifestation, from local (a low number of modules perturbed) to generalized inflammation (a high number of modules perturbed). Furthermore, the A35 transcriptional modules constituted the least common denominator across these inflammatory pathologies as it was the only set for which increases in transcript abundance were observed in all these immune-mediated diseases.

A35 Modules Comprise Transcripts Which Are Targetable by Existing Drugs

The identification of a robust transcriptional signature in the blood of psoriasis patients has several implications. For instance, it opens up the possibility of including blood transcript profiling assays in patient monitoring studies. For instance, such studies might be designed to predict the risk of flares or to monitor responses to therapy. We thus went on to examine the presence of transcripts among A35 modules that encode molecules that are targets for existing drugs and could be included in immune monitoring panels.

Among the 784 transcripts constituting the A35 modules, 81 are encoding targets for existing drugs (see *Methods*) (**Table 1**). Among these, we sought to identify targets for which drugs have been tested in clinical trials for psoriasis (14 targets) or Kawasaki disease (none). Notable examples for targets among A35 transcripts for which drugs have been considered for treatment of psoriasis include JAK2. This member of the Janus kinase family participates in signaling events downstream of a broad range of cytokine and hormone receptors. Drugs targeting this molecule that have been tested in the context of psoriasis include tofacitinib, which seems to be safe and to confer a clinical benefit (30, 31). Other immunosuppressive drugs have also been evaluated, such as the selective, pan-protein kinase C inhibitor sotrastaurin that inhibits the kinase PRKCD (32, 33).

Overall, we found that a sizeable number of transcripts comprised in A35 modules encode targets for existing drugs: a minority of these have already been tested in patients with psoriasis. We posit that other suitable candidates might be included in this list that have not yet been evaluated in the context of psoriasis. Furthermore, given the parallels in the blood transcriptome signatures of psoriasis and Kawasaki disease, there is good cause to consider investigating repurposing drugs showing clinical benefit in patients with psoriasis for the treatment of patients with Kawasaki disease.

DISCUSSION

Involved skin tissue is the ideal sample source to investigate psoriasis pathogenesis. However, despite being less relevant to this disease, blood presents the advantage of being amenable to repetitive sampling with minimal risk or discomfort. The blood can also harbor information regarding the immune status of affected patients. Such information can be obtained *via* blood transcriptome profiling, whereby all RNA species that are present in a given sample are measured simultaneously. Maybe more importantly, vast amounts of blood transcriptome profiling data are available in public repositories that can be used for contextual interpretation and "benchmarking" of blood transcriptional signatures.

Here, we compared the blood transcriptome fingerprints derived from several inflammatory diseases with those derived from patients with psoriasis. We found that blood transcriptome profiling may indeed serve to assess the extent of systemic involvement in these pathologies. Interestingly, we saw that the repertoire of changes characterizing the psoriasis blood transcriptome signature is much narrower than what is observed in other systemic inflammatory diseases. We also established that modules assigned to the aggregate A35 and associated with neutrophil-driven inflammation are a hallmark of the psoriasis blood transcriptome signature.

The role played by neutrophils in psoriasis pathogenesis has received particular attention over recent years (34, 35). Consistently, the data from our study suggest that blood transcriptome profiling studies might be of value for further patient-based investigations. While such an approach has been relatively under-utilized in this context, our findings suggest the possibility of employing blood transcriptional profiling as a means to assess the extent of systemic inflammation in psoriasis patients. Whether these measurements add value to those obtained using more traditional inflammatory markers (e.g., measurement of serum protein markers or neutrophil: lymphocyte ratios) remains to be investigated. It may be particularly relevant to assess utility of such blood transcriptional markers for the evaluation of cardiovascular diseases (CVD) risk in patients suffering from inflammatory disorders. Indeed, systemic inflammation associated with psoriasis was recently linked with development of CVD in this patient population (36, 37), while the risk of cardiovascular symptoms in Kawasaki disease is well established (38). And the question of the relative benefits of the available psoriasis treatment options with regards to addressing this risk remains to be fully addressed (36, 39).

Blood transcriptome profiling may also help stratify psoriasis patients according to molecular/immunological types. Such classification may be achieved through the delineation of distinct, biologically relevant modular A35 "sub-signatures." For instance, our previous work identified distinct modular interferon signatures that formed the basis of a stratification system for SLE patients (20). Psoriasis classification might also be informed by measuring the changes in the abundance of other aggregates/modules. For instance, we observed changes in the abundance of transcripts comprising the A33 and A28 modules, (also associated with inflammation and interferon responses, respectively) in either one of the two psoriasis datasets. This was the case for A28 (interferon) in the GSE123787 fingerprints (**Figure 1**), which is in line with the interpretation contributed earlier by Catapano et al. (18).

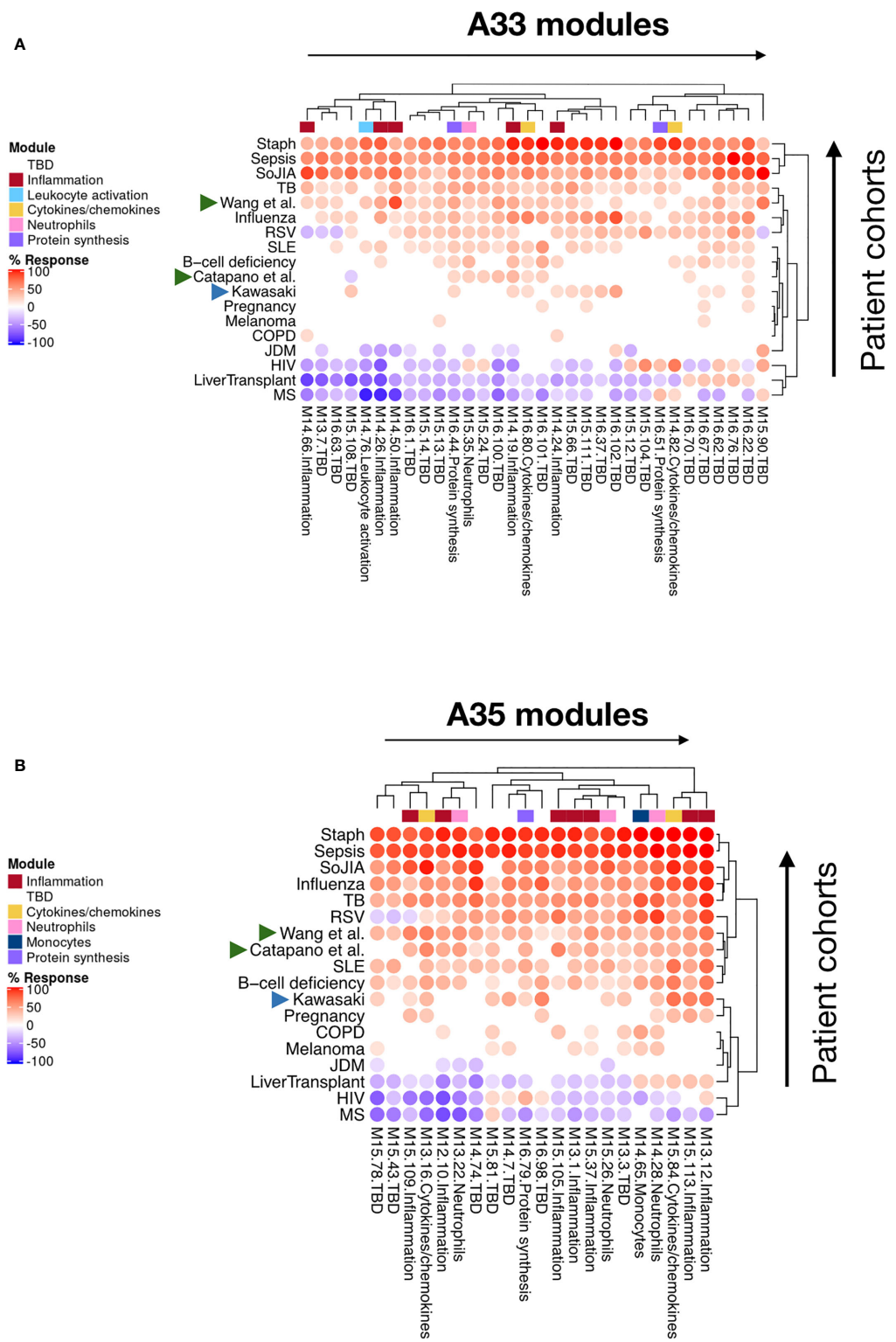

FIGURE 5 | Patterns of abundance for modules forming aggregates A33 **(A)** and A35 **(B)**. Heatmaps displaying the changes in transcript abundance for modules (columns) belonging to two aggregates associated with inflammation (A33 and A35), across 16 reference datasets and two psoriasis datasets (rows). The two psoriasis datasets are designated by the names of the researchers who contributed them and are indicated by green arrowheads. An increase or decrease in the abundance of transcripts constituting these modules is shown by a red or blue spot, respectively. The rows (datasets from each disease cohort) and columns (modules) were arranged by hierarchical clustering based on similarities in patterns of transcript abundance.

Some of our findings may also be relevant from a drug discovery/repurposing standpoint. For one, a number of the gene products comprising the A35 signature are targeted by drug candidates for psoriasis treatment (**Table 2**). This finding suggests that—according to the principle of "guilt by association"—other valuable targets might be identified among the genes constituting these modules. Secondly, similarities observed between the psoriasis and Kawasaki disease fingerprints suggest that the pathogenesis and/or pathophysiology of these diseases might be driven, at least in part, by similar immune mechanisms. Kawasaki disease, also known as mucocutaneous lymph node syndrome, is a rare childhood disease that mostly affects children <5 years old (38, 40). This disease presents as an acute, self-limiting vasculitis that sometimes targets coronary arteries and causes ischemic heart disease (41–43). The parallels we drew between psoriasis and Kawasaki disease blood transcriptional signatures are consistent with the growing body of evidence showing that patients with Kawasaki disease can develop psoriasiform eruptions (44–48). Among the treatments approved for psoriasis, drugs inhibiting IL17 might be considered good candidates for repurposing in Kawasaki disease (9, 49). Independent reports have also associated Th17 responses (defined by IL17 production) with Kawasaki disease (50–53), and IL17 is a known driver of neutrophil development, recruitment, and activation (54, 55). We therefore posit that IL17 might constitute one of the factors underpinning the A35 signature that we identified here in patients with psoriasis and Kawasaki disease.

Several aspects of the benchmarking exercise that we have conducted here across independent studies are inherently limiting and need to be addressed in follow-on investigations. First, while the use of respective healthy control groups as common denominators permits comparisons across independent studies, further investigations should comprise cohorts of patients with psoriasis, Kawasaki disease, and healthy controls. Samples should be collected and processed using harmonized protocols and the generated data should be analyzed concomitantly using the same platform (RNA-seq). This approach would permit direct comparisons of psoriasis and Kawasaki disease profiles while minimizing technical sources of variation. Inclusion of healthy controls would help with the interpretation of the data and would also permit future data re-use and meta-analyses across independent studies.

Second, the cohort size should be sufficient to allow for investigations into inter-individual variability. Such investigations would permit, for instance, the identification of "endotypes" or distinct molecular phenotypes within each patient population. The relatively low cost of recently introduced RNA-seq protocols might help realize such sample sizes (e.g., QuantSeq 3' mRNA-Seq by Lexogen: <$100/sample). Consolidating the results from multiple studies would remain feasible but any level of coordination or consultation between groups/centers could prove helpful.

Third, future studies are needed to clarify whether the A33/A35 signature observed in patients with psoriasis or Kawasaki disease is due to neutrophil priming secondary to inflammation or is a causal component of psoriasis pathophysiology. It can be difficult to ascertain in patient-based studies whether signatures are merely associated with or drive pathogenesis. Monitoring changes in transcript abundance at a high temporal frequency, either prior to a worsening of the clinical course of the disease or in response to therapy might provide useful indications in that sense. From a practical perspective, such studies could be implemented using protocols for at-home self-collection of low blood volumes and RNA stabilization (56, 57).

Finally, investigations into immune changes in the periphery and in bulk whole blood samples have inherent limitations. While systemic inflammation and interferon responses can be measured in whole blood, a dissection of the immune response at a more granular level (e.g., cellular subsets) might not be possible. Indeed, it is possible that we did not identify some responses associated with psoriasis pathogenesis (e.g., T-cell responses) for this reason. In addition, at least some immune responses may only be observed in affected skin tissues. Studies harnessing single-cell RNA-seq in a subset of patients are now warranted to further interrogate and interpret the psoriasis immune signatures measured in the peripheral blood.

Overall, our study provides a proof-of-principle for the use of fixed transcriptional module repertoires for blood transcriptome signature "benchmarking" and cross-study comparisons. It highlights the pertinence of using transcriptomic approaches for monitoring systemic inflammation. And it may also provide the necessary justification for further blood transcriptome studies in the context of psoriasis and Kawasaki disease.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/ (the NCBI Gene Expression Omnibus).

AUTHOR CONTRIBUTIONS

AR, and DC: conceptualization. AR, KS and DC: data curation and validation. AR and DR: visualization. AR, AM, TK, MK, and ZT-C: analysis and interpretation. AR, DR, MT, MG, BK, DB, and NK: methodology development. AR and DC: writing of the first draft. AR, DR, MT, AM, TK, MG, ZT-C, NK, DB, MK, KS, and DC: writing—review and editing. The contributor's roles listed above follow the Contributor Roles Taxonomy (CRediT) managed by The Consortia Advancing Standards in Research Administration Information (CASRAI) (https://casrai.org/credit/). 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.2020.587946/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Module annotation grid. Complete annotation for the grid used to map the differences in transcript abundance between the cases and controls shown in **Figure 1**. Each position on the grid corresponds to a different module. Each of the 382 modules are constituted by a set of transcripts found to be co-expressed across a range of disease and physiological states. In turn, the modules are arranged in rows based on similarities in gene expression. Gene ontology, pathway or literature keyword enrichment analyses provide the basis for attribution of biological functions to the modules; these are indicated by the color-coded abbreviations list below the grid. An interactive presentation is available that permits the exploration of functional enrichment results and expression patterns for the A35 modules: https://prezi.com/view/7Q20FyW6Hrs5NjMaTUyW/.

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CASE REPORT
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Case Report: Effective and Safe Treatment With Certolizumab Pegol in Pregnant Patients With Cogan's Syndrome: A Report of Three Pregnancies in Two Patients

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Cogan's syndrome is a rare autoimmune disease characterized by ocular inflammation and audiovestibular manifestations. Treatment consists of systemic glucocorticoids and other immunosuppressive agents including methotrexate, cyclophosphamide and TNF-α-inhibitors. Due to potential ovarian or fetal toxicity immunosuppressive treatment options are limited during pregnancies. Thus far there is a paucity of reports on pregnancies in Cogan's syndrome. With minimal transplacental transfer, Certolizumab pegol is considered to be safe for the use in pregnant patients with underlying inflammatory diseases. However, there is no literature on the use of this TNF-α-inhibitor in Cogan's syndrome in general and especially during gestation. Here we report three pregnancies in two Cogan's Syndrome-patients treated with Certolizumab pegol. Treatment with Certolizumab pegol was effective and well tolerated in patients with Cogan's syndrome and seems to be a safe treatment option during pregnancy.

Keywords: Cogan's syndrome, pregnancy, vasculitis, TNF-α inhibitor, Certolizumab pegol

INTRODUCTION

Cogan's syndrome (CS) is a very rare chronic autoimmune disease characterized by ocular inflammation and audiovestibular symptoms including tinnitus, vertigo and hearing loss (1). It mainly affects young adults without gender predominance (2). Standard treatment consists of systemic glucocorticoids that may be combined with glucocorticoid-sparing agents like methotrexate, azathioprine, cyclosporine, cyclophosphamide or tumor necrosis factor-α (TNF-α) inhibitors (2–4). Management of inflammatory diseases in general is particularly challenging in female patients of reproductive age. On the one hand, adequate disease control at conception and during pregnancy is crucial to ensure maternal and fetal health, on the other hand, available treatment options are limited because of potential ovarian or fetal toxicity. Therefore, treatment with cytotoxic or teratogenic substances like methotrexate or cyclophosphamide should be stopped months before conception. Furthermore, TNF-α inhibitors are often discontinued after the first trimester of pregnancy to limit placental transfer of the drug to the fetus (5). Monoclonal

anti-TNF-α-antibodies of IgG1 isotype are actively transported *via* the neonatal fragment crystallizable (Fc) receptor during the second and third trimester) (6). Certolizumab pegol (CZP) is a pegylated Fab fragment of a humanized anti-TNF-α-antibody, approved for the treatment of rheumatoid arthritis and other inflammatory autoimmune diseases. Because of the lacking Fc fragment CZP does not bind to the neonatal Fc receptor and is not actively transferred across the placenta (6). The CRIB study in pregnant women receiving CZP for approved indications showed no quantifiable CZP concentrations in the neonates at time of delivery and during follow-up, indicating zero to minimal placental transfer or fetal exposure during the third trimester (7). Accordingly, CZP is considered safe for the use in pregnant women (5). Cumulatively, only eight pregnancies in six female patients with CS have been described in literature, with no reports existing on the use of biological agents, e.g., CZP (8–13). Here we report the first three pregnancies in two Caucasian patients with CS who received CZP treatment in standard dose with subcutaneous injections of 200 mg every other week. Therapy in both patients was started after a risk-benefit analysis, shared decision making and written informed consent.

CASE DESCRIPTIONS

Patient A

At the age of 27, patient (A) experienced several episodes of steroid-sensitive hearing loss accompanied by vertigo and tinnitus, as well as recurrent bilateral conjunctivitis and keratitis (**Table 1**). The patient's right-sided deafness was treated with a cochlear implant. No further disease manifestations were detectable. The initial immunosuppressive treatment consisted of glucocorticoids in combination with azathioprine, which was later switched to methotrexate due to adverse drug reactions. Additionally, topical treatment with 5% dexpanthenol eye and nose ointment (Bepanthen®) and eye drops with hyaluronic acid for dry eyes were used during episodes of keratitis. Upon incomplete therapeutic response, the treatment regimen was

TABLE 1 | Patients' characteristics and treatment information.

		Patient A	Patient B
Age at time of diagnosis (y)		28	30
Origin		Caucasian / Europe	Caucasian/Europe
Medical history		Hypothyroidism	No other diseases or comorbidities
Family history		Inconspicuous	Inconspicuous
Previous pregnancies		None	One pregnancy with an uncomplicated course and a healthy child
Smoking status		Former smoker	Non-smoker
Body weight; Body mass index (BMI)		69 kg ; 26 kg/m²	55 kg; 20.7 kg/m²
Clinical manifestations			
Vestibulo-auditory manifestations		bilateral hearing impairment, unilateral hearing loss (right ear), bilateral tinnitus, vertigo	bilateral hearing impairment, unilateral hearing loss (left ear), bilateral tinnitus, vertigo
Eye manifestations		Bilateral interstitial keratitis Bilateral conjunctivitis	Bilateral conjunctivitis
General symptoms		fever, weight loss, arthralgia	subfebrile temperature, fatigue, myalgia
Other physical examination including neurological status		unremarkable	unremarkable
C-reactive protein serum concentration as biomarker for disease activity			
CRP before CZP		28.3 mg/L	6 mg/L
CRP within the first 3 months of CZP		Normalization to values <5 mg/L	Normalization to values <5 mg/L
CRP during follow-up under CZP		At normal dose <5 mg/L, After dose reduction <10 mg/L, After treatment cessation >10 mg/L	Continuously <5 mg/L
Treatment before start of CZP			
Topical treatment		5% Dexpanthenol eye and nose ointment (Bepanthen®), Eye drops with hyaluronic acid for dry eyes during episodes of keratitis	Intratympanic steroid injection, 5% Dexpanthenol eye and nose ointment (Bepanthen®), Eye drops with hyaluronic acid for dry eyes during episodes of conjunctivitis
Prednisolone treatment			
	intravenous bolus	PRED 3× 250 mg	PRED 3× 250 mg
	initial oral dose	PRED 60 mg/day	PRED 15 mg/day
	long term dose	PRED ≥ 10 mg/day	0
	treatment duration	32 months	11 months
Synthetic DMARDs		AZA 100 mg/day MTX 20 mg/week	AZA 100 mg/day
Biological DMARDs		ADA 40 mg EOW	ADA 40 mg EOW

ADA, adalimumab; AZA, azathioprine; BMI, body mass index; CRP, c-reactive protein; CZP, certolizumab pegol; DMARDs, disease modifying antirheumatic drugs; EOW, every other week; IGRA, interferon-gamma release assay; MTX, methotrexate; PRED, prednisone; y, years.

supplemented by adalimumab, a human recombinant IgG1 monoclonal antibody directed against TNF-α, resulting in rapid clinical response with good tolerability. Latent tuberculosis was ruled out by chest X-ray and interferon-gamma release assay (IGRA) prior to initiation of adalimumab. Due to a planned pregnancy methotrexate was paused and adalimumab was replaced by CZP in combination with low-dose prednisolone six months before conception. The slightly overweight patient had stopped smoking years before; a mild hypothyroidism was adequately treated. Prior to conception and throughout pregnancy CS was in clinical remission, and serum C-reactive protein (CRP) concentrations were normal or only slightly increased. Screening for antinuclear antibodies (ANA), antiphospholipid antibodies (APA), rheumatoid factor (RF), anti-neutrophil cytoplasmic antibodies (ANCA) was negative at time of initial diagnosis and during follow-ups. Serum concentrations of complement factors C3 and C4 as well as immunoglobulins IgG, IgA and IgM were within normal ranges. Results of tone audiometric monitoring remained stable during follow-up. The patient experienced no complications during pregnancy and delivered a healthy girl by spontaneous vaginal delivery at gestational week 38 after a percentile appropriate intrauterine development. Adherence to CZP was excellent during pregnancy and the prednisolone dose was continuously kept ≤5 mg/day. To prevent loss of bone mineral density 1000 IE of vitamin D3 were substituted daily. CZP-treatment was well tolerated without local reactions at the injection site or other clinically relevant adverse events. Neither the child nor the mother had any postpartum complications. After two years without relevant CS activity, under continued combination therapy of CZP and low dose prednisolone, the patient became pregnant again and gave birth to a healthy boy after 40 unremarkable gestational weeks (**Table 2**). Both children were formula-fed, reached all developmental milestones, and did not develop any serious infections or malignancies during the current follow-up time of five years.

In total, the patient was continuously treated with CZP for more than six years. Between her two pregnancies, the patient tried to lower the CZP dose at least twice by extending the application interval to three weeks. As these extensions of the treatment interval resulted in no signs of clinical activity but increased serum CRP concentrations, the CZP standard dosing regimen was implemented again. A complete cessation of CZP treatment more than a year after her second pregnancy led to rising CRP serum concentrations up to 20 mg/L, accompanied by fatigue and mild hearing impairments. Immediately after re-initiated administration of CZP 200 mg every other week, in combination with oral prednisolone 10mg/day for one week and consecutive tapering, CRP levels were normal again and clinical symptoms disappeared without any permanent damage.

Patient B

Three months after the delivery of her first child, the thirty-year-old patient B experienced several episodes of mild conjunctivitis of both eyes and bilateral hearing impairment with tinnitus, vertigo and subsequent unilateral hearing loss (**Table 1**). Myalgias were accompanied by an increase in serum concentrations of creatine kinase and CRP. No further disease manifestations were detectable by extensive physical examination including a detailed neurological assessment. Screening for autoantibodies (ANA, APA, RF, ANCA) was negative, and serum concentrations of C3, C4 and immunoglobulins were within normal range. The conjunctivitis with dry eyes was initially treated topically with 5% dexpanthenol eye and nose ointment (Bepanthen®) and eye drops with hyaluronic

TABLE 2 | Disease activity, course of pregnancy and nursing period.

	Patient A		Patient B
Pregnancies			
Prior pregnancies	None		One pregnancy
	1st pregnancy	**2nd pregnancy**	**3rd pregnancy**
CS activity at conception	complete remission	complete remission	complete remission
age at delivery (y)	32	35	33
pregnancy duration (gw)	38	40	41 + 3
Mode of delivery	spontaneous vaginal in labor	spontaneous vaginal in labor	spontaneous vaginal in labor
Child's condition at birth	healthy	healthy	healthy
APGAR score	10/10	9/10	10 / 10
Umbilical cord pH	7.25	7.29	7.22
Neonatal weight (g)	3270	3740	399
Neonatal length (cm)	54	54	57
Head circumference (cm)	35	33.5	36.5
Intrauterine development	percentile-appropriate	percentile-appropriate	percentile-appropriate
Postpartum complications	None	None	none
Treatment during pregnancy and nursing period			
1st trimester	CZP, PRED 5mg	CZP, PRED 4mg	CZP
2nd trimester	CZP, PRED 4mg	CZP, PRED 4mg	CZP
3rd trimester	CZP, PRED 4mg	CZP, PRED 4mg	CZP
Breast feeding	no breast feeding	no breast feeding	yes (CZP continued)
Follow up after birth (m)	59	29	28
Total duration of CZP treatment (months)	74		41

*APGAR, **A**ppearance, **P**ulse, **G**rimace, **A**ctivity, **R**espiration Score; cm, centimeters; CS, Cogan's syndrome; CZP, certolizumab pegol; g, gram; gw, gestational weeks; m, months; PRED, prednisone; y, years.*

acid. The ear manifestations required stronger immunosuppressive treatment with intratympanic steroid installation and systemic prednisolone treatment in combination with azathioprine, which subsequently had to be stopped because of hepatic side effects. Second-line treatment with TNF-α blocking agent adalimumab was effective and resulted in normalization of CRP levels, however, was switched to CZP because of the patient's desire to have children. After an unremarkable pregnancy with normal fetal development, she delivered a healthy boy in the 42nd gestational week (**Table 2**). Under continuation of CZP, her child was breastfed and displayed normal development within the first two years without any notable infections. Since initiation of treatment with TNF-α-inhibitors the patient was in complete, relapse-free remission. The patient continuously presented with normal CRP values and even showed an improvement in tone audiometry of the inner ear hearing threshold by 15–20 decibel hearing level compared to pre-treatment values. Currently, the patient is still under treatment with CZP to prevent relapse of disease.

DIAGNOSTIC ASSESSMENT

Before, during and after pregnancy the activity of CS was regularly monitored by CRP serum concentrations, along with clinical assessments including body weight, blood pressure and pulse rate, subjective hearing levels and tone audiometry, if necessary. In addition to the diagnostic parameters assessed above, blood smear with differential leukocyte count, and measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK) and creatinine, together with urine analyses were performed at least quarterly. The fetal development was monitored by the patients' gynecologists *via* ultrasound during every trimester of pregnancy. At gestational week 20 the fetus was screened for abnormalities and regular organ development. Additional Doppler imaging of placental blood flow was performed at specialized prenatal diagnostic centers twice during pregnancy. After childbirth the Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score was assessed, and umbilical cord pH, neonatal weight, length and head circumference were measured. Developmental abnormalities were ruled out by regular pediatric screening tests, which are provided after birth and within the first years of life on a regular basis by the German health system.

DISCUSSION

Cogan's syndrome is a systemic chronic inflammatory disease which can lead to severe functional visual and hearing impairments. It requires interdisciplinary clinical management and individualized treatment approaches by all specialists involved (2–4). Even more challenging is the adequate disease management in young female patients wishing conception or with ongoing pregnancy. Due to its systemic and inflammatory nature, pregnant patients with this complex vascular disease require unique attention and continuous clinical monitoring. The impact of a pregnancy itself on the course of CS is fairly unknown. Also, evidence-based treatment guidelines for CS are very limited with a paucity of randomized, double-blind trials comparing the efficacy of immunosuppressive agents. The current literature predominantly consists of retrospective chart analyses, small case series and single case reports. A recent review collected data on 87 adult CS patients and 17 pediatric patients (4) showing that methotrexate (MTX) was the most frequently used second-line therapy. Besides MTX there are reports on the successful use of azathioprine, cyclosporine, and cyclophosphamide as steroid-sparing immunosuppressive agents. However, immunosuppressive agents like MTX, cyclophosphamide and mycophenolate are contraindicated for their teratogenicity during pregnancy. More recently biological agents, especially TNF-α antagonists, and particularly infliximab, have shown favorable outcome (2–4).

Half of previously reported CS patients did not need any systemic immunosuppressive treatments throughout pregnancy, instead intermittent topical treatment with ophthalmic steroids was sufficient (10–13). Other pregnant patients in remission were successfully treated with hydroxychloroquine, or a combination therapy of either cyclosporine or intravenous immunoglobulin application (IVIG) with azathioprine and prednisolone (8, 9, 12). In contrast, our patients had the necessity of therapy escalation to anti-TNF therapy prior to conception because of disease activity and adverse effects. Accordingly, with its neglectable to minimal transplacental transfer in the third trimester of pregnancy (7), CZP was a particularly attractive second line therapy to avoid inflammatory flares and thereby protect mother and her unborn child. The European League Against Rheumatism (EULAR) recommends certolizumab as most favorable biological DMARD for the use throughout pregnancy and lactation in patients with inflammatory rheumatic diseases (5). Evidence for these recommendations is provided by two prospective pharmacokinetic studies, CRIB (NCT02019602) (7) and CRADLE (NCT02154425) (14). The CRIB study showed CZP plasma concentrations within the expected therapeutic range in 16 pregnant women at delivery, whereas no detectable or only minimal CZP serum concentrations were found in their newborns (7). The CRADLE study investigated CZP concentrations in human breast milk to estimate the daily dose of maternal CZP transferred to the infant by breast feeding (14). In most breast milk samples no measurable CZP was detectable, indicating no to minimal CZP transfer from maternal plasma to breast milk. Additionally, CZP absorption by the infants *via* breast milk is unlikely because of its low oral bioavailability. The mean age of all patients included in the CRIB and the CRADLE studies was approximately 30 years and therefore comparable to the CS patients reported here. Of relevance, none of the patients in these two studies had Cogan's syndrome or any other form of systemic vasculitis. The most frequent indications for CZP treatment in CRIB/CRADLE were rheumatoid arthritis, followed by Crohn's disease, psoriatic arthritis and axial spondyloarthritis (7, 14). Similar to our findings the gestational age and weight at birth of all 16 newborns in the CRIB study were within the expected range for healthy children (7). However, one limitation of our report is the low number of patients included, with CS being a very rare disease. Furthermore, we have no data

on CZP pharmacokinetics in the three infants and their mothers. Though, we have no indication to expect results that are relevantly different to previously reported data in the CRIB and CRADLE studies. The serological and clinical improvement in both mothers before, during and after pregnancy reflects sufficient CZP serum concentrations in our patients. The uncomplicated pregnancies and the normal development of the children afterwards, without any infectious or other complications, support our hypothesis that the infants were exposed to relevant CZP doses during pregnancy or lactation period.

Another important finding is the fact that decreasing the CZP dose in patient A resulted in an increase of CRP serum concentrations. Cessation of CZP treatment more than a year after the second pregnancy, with the patient being in complete remission, led to a relapse of CS with typical clinical symptoms and high CRP concentrations.

Re-start of CZP lead to complete serological and clinical remission without relevant side effects, which demonstrates the long-term efficacy and safety of CZP in this patient. Also, the chronic disease course in this patient illustrates that CS may need long-term immunosuppressive treatment, occasionally even requiring TNFα-inhibitor administration.

To our knowledge this is the very first report on both the treatment with the monoclonal anti-TNFα-inhibitor CZP in CS and the first report on biological treatment during pregnancies in CS. In both of our patients CZP was effective and well tolerated before, during and after pregnancies. CRP values normalized, and tone audiograms showed stable or improved results compared to pre-CZP-treatment findings. All three pregnancies were without complications. All children were born healthy, at term, and developed regularly during follow-up.

In summary, CZP was shown to be effective and safe in the treatment of Cogan's syndrome and should be considered as potential treatment option during pregnancy.

PATIENT PERSPECTIVE

Patient A: I agree to report my rare disease for a better medical understanding and improvement of treatment options in Cogan's syndrome. I hope that this report enables physicians in Germany and other countries around the world to treat women with Cogan's syndrome having a childbearing wish with a highly effective and, in my case, safe treatment. We are very happy to live a more or less normal life now as a family with two healthy and happy kids.

Patient B: I agree to report on my rare disease (Cogan's syndrome), the pregnancy including the data of my child. I hope that this report will help and support other physicians and especially young female patients with Cogan's syndrome.

DATA AVAILABILITY STATEMENT

The original data generated and analyzed for this study are included in the published article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Written informed consent was obtained from the individual for themselves and their child/children for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

NV and CG wrote the manuscript. All authors were involved in direct patient care or acquisition of clinical data and contributed to the article and approved the submitted version.

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REVIEW
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Abdominal Aortic Aneurysm: Roles of Inflammatory Cells

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Abdominal aortic aneurysms (AAAs) are local dilations of infrarenal segment of aortas. Molecular mechanisms underlying the pathogenesis of AAA remain not fully clear. However, inflammation has been considered as a central player in the development of AAA. In the past few decades, studies demonstrated a host of inflammatory cells, including T cells, macrophages, dendritic cells, neutrophils, B cells, and mast cells, etc. infiltrating into aortic walls, which implicated their crucial roles. In addition to direct cell contacts and cytokine or protease secretions, special structures like inflammasomes and neutrophil extracellular traps have been investigated to explore their functions in aneurysm formation. The above-mentioned inflammatory cells and associated structures may initiate and promote AAA expansion. Understanding their impacts and interaction networks formation is meaningful to develop new strategies of screening and pharmacological interventions for AAA. In this review, we aim to discuss the roles and mechanisms of these inflammatory cells in AAA pathogenesis.

Keywords: abdominal aortic aneurysm, inflammation, T cells, macrophages, inflammasome, neutrophil extracellular traps

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INTRODUCTION

Abdominal aortic aneurysm (AAA) is one of the most common types of true aneurysms in the world. AAA is defined when the maximal abdominal aortic diameter reaches 30 mm or 1.5 times of the normal ones. The estimated AAA prevalence in men aged over 60 years is about 4–8%, and the prevalence in women gets 0.5–1.5% or so (1). The major risk factors of AAA include cigarette smoking, aging, male gender and corresponding family history (2, 3). The most common cause of death for AAA patients is aneurysm rupture, which accounts for an approximately 60% of mortality (4).

In the past decades, AAA has been regarded as a result of long-term atherosclerotic lesions, which shares the same pathogenesis with other cardiovascular diseases (CVD), due to similar risk factors such as male sex, tobacco consumption, family history, hyperlipidemia and elder population (5, 6). However, diabetes mellitus (DM), a common comorbidity of atherosclerotic disease, is conversely related to AAA development. Patients with DM have a reduction of morbidity by nearly 30 percent (7). Besides, in contrary to the infrarenal segment of aorta, which is the most commonly involved part of AAA, the external iliac artery is often aneurysm-resistant, but it is strongly

vulnerable to atherosclerotic occlusive disease (8). Another phenomenon is that the lipid profiles of patients with AAA are not always abnormal like other CVD patients. These findings indicate that the atherosclerotic lesion may be independent of AAA formation.

Recent studies suggest the pathophysiology of AAA is a multifactorial process consisting of inflammation responses, matrix metalloproteinase (MMP) activation, oxidative stress, intraluminal thrombus, smooth muscle apoptosis and extracellular matrix (ECM) degeneration (9–11). The proteases secreted by inflammatory cells can induce degradation of ECM. In the meanwhile, due to destruction of ECM structure and loss of resistance of tunica media, soluble blood components like inflammatory cells are transferred and accumulated in tunica media through the highly vascularized adventitia, resulting in infiltration of inflammatory cells into the vascular media. These processes together with platelet accumulation and coagulation system activation promote intraluminal thrombosis, and subsequently causes aortic dilation and increased vulnerability to AAA rupture (12). Intraluminal thrombosis is capable to create an inflammatory microenvironment containing neutrophils, cytokines, proteases, and reactive oxygen species, and thereby decrease aortic wall strength. These phenomena indicate that inflammatory cells are in the central position of the whole process. This review is an update of recent advances of inflammatory cell-related mechanisms during AAA development.

INFLAMMATORY MICROENVIRONMENT

The aortic wall can be generally divided into three layers: tunica adventitia, tunica media and tunica intima, of which tunica adventitia is fully vascularized and permit leukocyte diapedesis. The aortic wall inflammation is characterized as a multicellular-participating process including mononuclear cell infiltration, immunoglobulin (Ig) secretion and cytokine production, suggesting that both innate and adaptive immune responses are involved (13). The histological specimen of human aortic aneurysm tissue reveals that there were a variety of inflammatory cells gathering in the aortic wall. Recent studies showed that perivascular adipose tissue (PVAT) played an essential role in the process of in leukocyte infiltration. When the vascular damage initiates, PVAT increases its volume and then upregulates the expression of inflammatory factors such as resistin, leptin, cytokines and chemokines (14), which induce infiltration of inflammatory cells, including neutrophils, macrophages, natural killer cells (NK cells), dendritic cells (DCs), T and B lymphocytes and mast cells. All these inflammatory cells are implicated in the formation of AAA (13), and the interactions among them formed the inflammatory microenvironment of aortic walls. For example, cytokines secreted by T cells are essential for macrophage activation, while DCs and macrophages can present antigens to T cells to stimulating primary T cell responses (15). Decreasing the activity of inflammatory cells may be a therapeutic strategy to treat non-ruptured AAAs. Daphnetin was recently proved to be eligible to suppress AAA generated with elastase by reducing the infiltration and accumulation of inflammatory cells such as macrophages, T cells and B cells (16). In addition, suppressing the infiltration of $CD11b^+$ macrophage and $CD4^+$ T cell with antagonism of toll-like receptor 2 significantly ameliorated $CaCl_2$-induced aneurysms (17). The fact that animals can benefit from inhibitors of inflammatory cells independent of models proved the central role of these cells in pathogenesis of AAA.

INNATE IMMUNE CELLS

Macrophages

There are generally two origins of macrophages involved in the pathogenesis of AAA: tissue-resident macrophages arising from embryonic precursors, and monocyte-differentiated macrophages from peripheral blood (18). Single-cell RNA sequencing has revealed markedly expansion and activation of aortic resident macrophages, blood-derived monocytes and inflammatory macrophages in the samples of elastase-induced AAA models (19). Tissue-resident macrophages are self-renewed independently of bone marrow activity and can continuously migrate to peripheral tissues. However, the circulating monocytes are the major origin of macrophages gathering in aortic walls (20).

Circulating monocytes originating from the bone marrow play a critical role in encoding antimicrobial and phagocytosis-related proteins (21). When the local environment undergoes inflammatory changes, blood monocytes can be recruited to the tissue and differentiated into macrophages. In response to different inflammatory stimuli, blood monocytes migrate to the tissue and differentiate into distinct macrophages subgroups, including classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (22). This process is termed as macrophage polarization. Interestingly, these two subgroups of macrophages serve almost opposite roles in the pathogenesis of AAA.

M1 macrophages are preferentially located in the tunica adventitia of the aortic wall (20). They can be activated by the stimuli like lipopolysaccharide (LPS) and IFN-γ (23). By upregulating massive inflammatory cytokines including TNF-α, IL6, IL12, IL1β, chemokine (C-C motif) ligand 2, and nitric oxide (NO) (24), M1 macrophages aggravate local inflammation and promote the aortic dilation as well as vascular remodeling. On the other hand, M2 macrophage polarization is typically induced by Th2 cytokines like IL-4 and IL-13 (23, 25). By mobilizing together with mast cells and NK cells, M2 macrophages can regulate angiogenesis, cell recruitment, and collagen deposition (26). With the progression of AAA, the aortic walls undergo a switch from M1 macrophage dominance to M2 macrophage dominance, which reflects a compensatory mechanism of the anti-inflammatory and tissue-repair effect of M2 macrophages (20). The counteracting effects of M1 and M2 macrophages in AAA make them eligible for therapeutic applications to control inflammation and destruction of aortic

walls. Cheng et al. introduced Notch receptor inhibitors which upregulated M2 macrophages and downregulated M1 macrophages to *Apoe*$^{-/-}$ mice with AAA, and identified this intervention remarkably ameliorated progression of AAA (27).

Neutrophils

Neutrophils are a kind of polymorphonuclear leukocytes, which are consistently generated in the bone marrow from myeloid precursors (28) Neutrophils are one of the most abundant immune effector cells of the human immune system, whose main functions include phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) (29, 30). Some studies suggest circulating neutrophils may be an important contributor to AAA formation in the early phase. Eliason et al. found AAA of wild-type animals (WTs) grew faster than mice with neutropenia 4 days after elastase perfusion to induce AAA, although there was not a significant difference in the 7th day (31). A cohort study showed that there were strong associations between elevated neutrophil counts and AAA (32). Li et al. that identified FAM3D, a novel chemokine, was strikingly upregulated in human AAA tissues, and *Fam3d*$^{-/-}$ mice had decreased levels of neutrophil infiltration than WTs. Besides, administration of FAM3D neutralizing antibody markedly suppressed AAA expansion (33).

The effective integrant of neutrophils is composed with granules and secretory vesicles consisting of various enzymes (28). There are three kinds of granules within neutrophils in total. The azurophilic granules contain myeloperoxidase (MPO), an enzyme essential for the oxidative burst, and other components including defensins, lysozyme and some proteases such as neutrophil elastase and proteinase 3 (34). The specific (secondary) granules are peroxidase-negative and storage lactoferrin, hCAP18, NGAL, lysozyme, and NRAMP-1 (35). The last type is called gelatinase (tertiary) granules. Although there are very few antimicrobials in gelatinase granules, they contain a host of MMPs (34).

NETs are net-like structures protruding from cell membranes of neutrophils or released from ruptured neutrophils (36). When neutrophils are activated, a process named NETosis (**Figure 1**) initiates. The first way of NETosis starts with nuclear delobulation and decondense of chromatin, followed by cellular depolarization and membrane rupture to release NETs. Another kind of NETosis, which is termed as non-lytic form of NETosis, proceeds with expulsion of chromatin and degranulation (37). NETs may have several impacts on aortic wall. To begin with, the proteases hanging on NETs like MMPs can cause direct damage to aortic walls after chromatin are cleaved by DNases (38). Besides, NETs can increase the transcription of IL-6 and pro-IL-1β in macrophages, induce Th17 cell differentiation and recruit more inflammatory cells (30). Another possible effect of NETs on AAA pathogenesis is promoting vascular occlusion. The net-like structure of NETs can render blood cell gathering within the aorta and finally cause thrombosis (36). NETs also help establish the bridge between neutrophils and other immune cells. Cathelicidin-related antimicrobial peptide exposed by NETs can bind to self-DNA

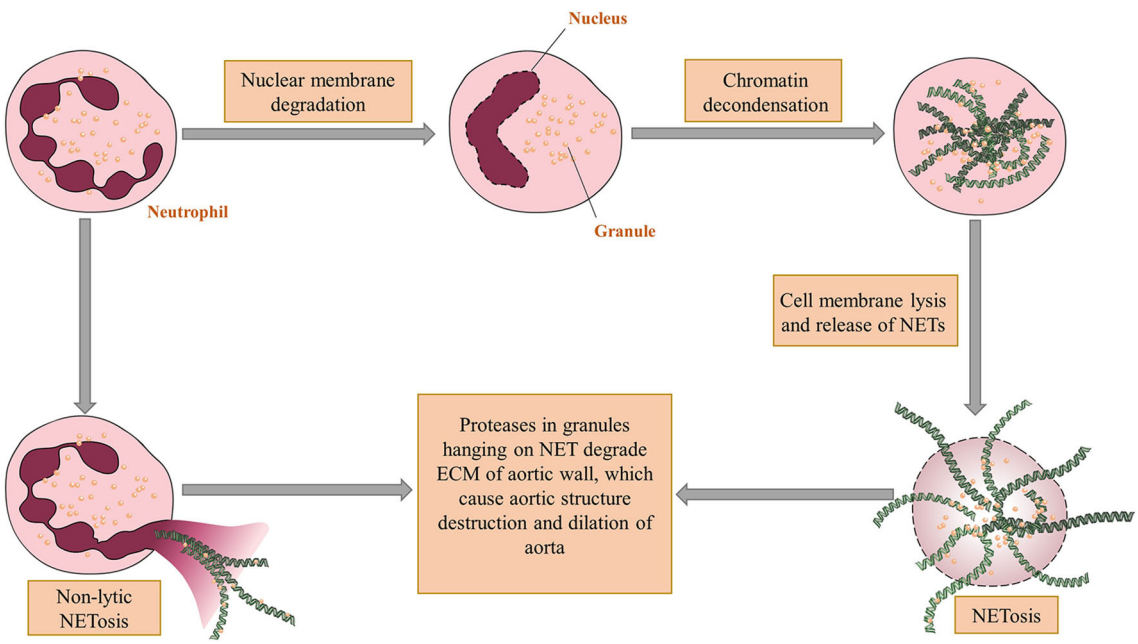

FIGURE 1 | The mechanism of NET formation and acting on aortic walls. There are two ways for neutrophil extracellular traps (NET) come into being. The first one is called NETosis in which nuclei of neutrophils undergo delobulation, chromatin decondensation and nuclear membrane lysis. After that neutrophil granules adhering to released chromatin enter extracellular spaces through ruptured cell membranes. The other way, which is a non-lytic form of NETosis, occurs after partial depolarization of nuclei and render granules hanging on chromatin out of plasma without cell deaths. The proteases within granules can thereby directly degrade the vascular structure and cause aortic dilation. Figures were produced using Servier Medical Art (www.servier.com).

and subsequently recruit plasmacytoid DCs (pDCs) that induce type I interferon synthesis (39).

Dendritic Cells

Dendritic cells (DCs) are a kind of antigen presenting cells (APC) which are able to process and expose antigen components to T lymphocytes, play a key role in the induction of innate immune responses and are implicated in the immune tolerance to self-antigens (40, 41). Krishna et al. indicated that depletion of $CD11c^+$ cells can significantly decrease the maximum diameter of AAAs 28 days after angiotensin II infusion (40), which suggests that DCs may also have important impact on the development of AAA.

DCs generally express CD11c and major histocompatibility (MHC) class II molecules. The four subsets of DCs are conventional DCs (cDCs), Langerhans cells, monocyte-derived DCs and pDCs (42). In that the main resident site of Langerhans cells are the epidermis and mucosa, the effective types of DCs on AAAs are cDCs, monocyte derived DCs and pDCs. All kinds of DCs derive from macrophage and DC precursors (MDP), which give rise to monocytes and the common DC precursors (CDP) (43). CDP could further differentiate into pDCs and pre-cDCs. pDCs are a special DC subset which can promote antiviral responses and are also involved in pathophysiology of autoimmune diseases (44). pDCs are able to produce type I interferons, such as IFN-α and IFN-β, to promote proinflammatory responses through activating effector T cell, cytotoxic T cells, and NK cells (39, 45). These inflammatory cells can further facilitate AAA development. cDC1s and cDC2s are two subsets differentiated from pre-cDCs. cDC1s are well known for their cross-presenting functions, and are involved in immune responses to bacterial and viral infections. cDC2s are specialized for sensing danger signals and producing high levels of IL-6 and IL-8 (46). These two phenotypes of cDCs are both characterized as regulatory mediators of immune responses. cDC1 can activate $CD8^+$ T cells, promote T helper type 1 (Th1) activation by MHC class I, and activate natural killer responses with by IL-12 (47, 48). cDC2 can cross-present antigens to induce the proliferation of Th1 cells though MHC class II molecules (49). Their effects enrich the communications in the inflammatory microenvironment of AAA tissues. The process that monocytes differentiate into DCs under the induction of GM-CSF plus IL-4 has been observed *in vitro* culture. Monocyte-derived DCs have the potential to transform into cDCs, and *in vivo* experiments showed they can induce Th1 and Th17 cell polarizations (50). However, the detailed roles of DC subsets in AAA need to be explored.

Mast Cells

Mast cells are widely distributed in the tunica adventitia and media of aortic wall. The mast cell count is positively correlated with the maximum of AAA diameter (51). The roles of mast cells in AAA have been intensively discussed in Shi et al.'s review, that elevated proteases of mast cells like chymase and tryptase in patients with AAA, and these proteases contribute to leukocyte adhesion and migration, vascular smooth muscle cells (VSMC) apoptosis, foam cell formation, and expression of MMP and cathepsins (52). Cathepsin is a kind of enzyme containing in mast cells. Cathepsin C (*Ctsc*) acts as an upstream activator of tryptases, chymases and other cathepsins by cleaving the N-terminal pro-peptide of the zymogen forms of these proteases (53). Cathepsin G has similar function with chymases, which can generate angiotensin II from angiotensin I. Mice deficient of *Ctsc* were resistant to elastase perfusion-induced AAA compared with WT mice, and suffered from less transmural inflammatory cell infiltration (54). However, controlling mast cells solely are not efficient enough as a medical treatment option for aortic aneurysms. A randomized clinical trial showed that pemirolast, a potential mast cell stabilizer, could not inhibit the development of AAA at several different doses, which may be due to the limited influences of pemirolast on plasma tryptase concentration (55, 56). In addition to directly suppress the activity of mast cells, diminishing their impact like inducing VSMC apoptosis might be an alternative way to treat AAAs. A master regulator of autophagy and lysosome biogenesis named transcription factor EB, for example, was shown to prevent VSMC apoptosis and attenuate AAA development (57).

Natural Killer Cells

NK cells are lymphocytes which have important effects on innate immune responses to tumors and infections (58). Although the fraction of NK cells is not that high as T cells in AAA tissues, they have an impact on aneurysm development both through causing aortic wall damage and through accelerating atherosclerotic changes (59–61). NKT cells, a special subtype of immune cells that express both T cell receptor and markers characteristic of NK cells, are amplified both *in vivo* and *in vitro* after injected with Ang II. NKT cells exacerbate aneurysm progression by increasing matrix degrading enzymes in VSMC and macrophages, and by secreting cytokine downregulating VSMC viability (62, 63). Forester et al. reveal peripheral level and cytotoxicity of NK cells are increased in AAA patients than control groups, and these NK cells retained amount and cytotoxicity to destruct VSMC even after aneurysm repair (64).

ADAPTIVE IMMUNE CELLS

$CD4^+$ T Cells

The most predominant infiltrated inflammatory cells in AAA specimens are T lymphocytes (65), and the majority are $CD4^+$ T cells (mainly helper T cells). The distinct phenotypes and functions of $CD4^+$ T cells are summarized in **Table 1**. Depending on surface markers and functions, $CD4^+$ T cells can be differentiated into diverse subsets in response to various microenvironment stimuli, including Th1 cells, Th2 cells, Th17 cells, regulatory T cells and follicular helper T (Tfh) cells (66). Specifically, these $CD4^+$ T cells express various immune molecules, including αβ T cell receptors, T cell activation markers, memory cell phenotypes ($CD45RO^+CD45R\ A^-$ $CD62L^-$), and distinct patterns of cell surface molecules

TABLE 1 | Differentiation, function, and role of various phenotypes of CD4+ T cells in AAA.

	Th1	Th2	Th17	Treg	Tfh
Activators	IFN-γ, IL-12	IL-2, IL-4	IL-1, IL-6, TGF-β	TGF-β, IL-2	IL-21, Bcl-6
Affiliated cell	Macrophage, $CD8^+$ T cell	B cell, eosinophil, mast cell	Neutrophil		B cell
Products	IFN-γ, IL-2 and TNF-β	IL-4, IL-5, IL-6 and IL-10, FasL	IL-17, IL-21, GM-CSF	TGF-β, IL-10, IL-35	CXCR5, IL-21
Role in AAA	Activate macrophage, inhibit collagen synthesis	↓Macrophage cytotoxicity and MMP secretion, ↑VSMC apoptosis	↑Macrophage and neutrophil recruitment	↓T cell proliferation and IFN-γ production, ↓Inflammatory cell chemotaxis, arterial wall remodeling, and angiogenesis	May upregulate autoantibody secretion through assisting B cell proliferation

(including CD54, CD31, CD11a, CD29, CD44, CD95, and CD27) (67).

Th1 and Th2 Cells

The most significant effect of $CD4^+$ T cells on AAAs rely on cytokine secretions, such as Th1 cytokines (IFN-γ, IL-2 and TNF-β) and Th2 cytokines (IL-4, IL-5, IL-6 and IL-10) (13, 67). Some of these cytokines are associated with macrophage activation, regulation of VSMC apoptosis and direct destruction of aortic walls (68). Deletion of *Il12b* can inhibit macrophage expansion, decrease production of cytokines like IL-6 and TNF-α in the early stage of AAA, and suppress aneurysm development (69). Another research determined a strikingly higher level of circulating IL-4 in patients with AAA than healthy individuals (70). Wanfen et al. showed that aneurysm dilation and MMP secretion were prevented in *Ifng* deficient mice (71).

Th1 cells, Th2 cells also have effects on aortic wall degradation. There are profound interactions between various types of helper T cells and vascular smooth muscle cells (VSMCs) through autoimmunity. Fas ligand (FasL) expressed by Th2 cells are indicated to promote VSMC death (72). Besides, TNF and IFN-γ released by Th1 cells can further inhibit collagen synthesis (73, 74). A study aiming to investigate the interactions among immune cells in AAAs reveals that $CD4^+$ T cells could promote VSMC proliferation through direct cell-to-cell contact (60). VSMC, the main cellular constituent of the aortic wall (75), subsequently induce NK cells aggregation and finally result in VSMC apoptosis. Extracellular matrix (ECM) enables artery wall to obtain the blood containing function, and the main component of ECM, especially collagen and elastin, are synthesized and processed by VSMC. Collagen defects can lead to aneurysm rupture, while elastin depletions are associated with continuous dilation (11). All these results demonstrate the essential position of Th1 and Th2 in aneurysmal diseases.

Th17 Cells

Th17 cells, the main origin of IL-17, are elevated in AAA tissues (76). IL-17 secreted by Th17 cells mediates a quantity of immune responses like neutrophil recruitments and plays a central part in vascular superoxide production (77). This can sharpen oxidative stress in aortic walls. Oxidative stress is one of the major pathogenic factors of AAA, and a study proved riboflavin (vitamin B2), a kind of antioxidant, could prevent aneurysm formation in rat models (78), which suggests inhibiting oxidative stress by controlling IL-17 synthesis and activity of Th17 cells may be a potential therapeutic target for AAA patients.

Owing to their various cytokines in addition to IL-17, such as IL-17F, IL-21 and granulocyte-macrophage colony-stimulating factor (GM-CSF), Th17 cells have been implicated in several autoimmune diseases, including inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis (79). Therefore, it is rational to anticipate that Th17 cells is also probably of great relevance to AAA. Ashish et al. showed that there is a evidently higher expression of IL-17 in AAAs. Besides, *Il17a*$^{-/-}$ mice are relatively resistant to AAA, and plasma concentration of inflammatory cytokines are also decreased, which proved the proinflammatory and atherosclerotic properties of IL-17 (76). Wei et al. introduced digoxin to antagonize retinoic acid-related orphan receptor gamma thymus, a master transcription of Th17 cell differentiation, and found out that this can attenuate aneurysm expansion in two different kinds of models with AAA (80). These findings indicate the role of Th17 cells in AAA development.

Tfh Cells

Tfh cells express CXCR5, a chemokine receptor that helps guide cells into B cell follicles (81). Tfh cells could provide assistant to B cells activation through autocrine or interactions with B cells, and are essential for formation and maintenance of germinal centers (82). Tfh cells have a role in atherosclerosis. Gaddis et al. found that deletion of *Bcl6*, a transcription factor of Tfh cells, prevented plaque formation in *Ldlr*$^{-/-}$ murine models (83). This finding suggests decreasing Tfh cells activity may slow down the exacerbation of aneurysms. However, the roles of Tfh cells in AAA need to be established.

Regulatory T Cells

Regulatory T (Treg) cells are a specific kind of $CD4^+$ T cells which express forkhead box protein 3 (FOXP3) and regulate the effects of other T cell subsets (84). Treg cells have an impact on suppressing local inflammation, and compromised Treg functions may promote AAA growth (85). The suppressive effect is determined by acetylation levels of FOXP3, which is lower in human aneurysm tissue. SIRT1 can specifically regulate the acetylation of FOXP3 (86). Studies have shown that EX-527, an inhibitor of SIRT1, can recover the acetylation levels of FOXP3, increase the number of active Treg cells and bring

back their suppressive functions on AAA (86). Zhou et al. found that Treg cells could release IL-10 and thereby suppress inflammatory cell chemotaxis, arterial wall remodeling, and angiogenesis (87). Another study showed that the proportion of Treg cells in peripheral mononuclear cells were markedly decreased in AAA patients than controls (88). The average aortic diameters of *Foxp3*$^{-/-}$ mice were larger than WTs after $CaCl_2$ induction, while infusion of normal Treg cells to *Foxp3*$^{-/-}$ mice can render their similar aortic size with WTs after $CaCl_2$ induction (88). Administration of IL-2 to expanse FOXP3$^+$ Treg cells also reduced the incidence and mortality of AAA in *Apoe*$^{-/-}$ mice with angiotensin II infusion (89). Besides, Treg cells are an essential source of TGF-β, which is a matrix-protecting and anti-inflammatory cytokine in human. Wang et al. concluded that systemic neutralization of TGF-β would increase the activity of MMP-12 and subsequently contributed to aneurysm progression and rupture (90). This growing body of evidence suggests an important role of Treg cells in enhancing inflammation and inducing AAA enlargement.

CD8$^+$ T Cells

CD8$^+$ T cells represent a considerable part of adaptive immunity. According to the immune state, CD8$^+$ T cells can be generally divided into effector cells and memory cells, which can provide both immediate clearance and long-term protective effect on killing tumor cells and virally infected cells (91). CD8$^+$ T cells are found to be elevated in AAA wall and perivascular tissues (92). Zhou et al. indicated that IFN-γ released by CD8$^+$ T cells could promote cellular apoptosis *in vivo* and MMP-producing macrophage recruitment (93). CD8$^+$ T cells exert versatile impacts on atherosclerosis. Chemokines like MCP-1 and CCL-2, which can induce monocytes infiltration in atherosclerotic lesions, were observed to be deceased in mice depleted of CD8$^+$ T cell (94). However, CD8$^+$ T cells can promote apoptosis of antigen presenting cells and suppress functions of CD4$^+$ T cells, which can resist progression of atherosclerosis (95). This discrepancy may result from production of inflammatory cytokines and lysis of endothelial cells by CD8$^+$ T cells. The pro-atherogenic and protective effects of CD8$^+$ T cells may also regulate the enlargement of AAA, but need to be further explored.

γδ T Cells

In contrast to αβ T cells, γδ T cells are independent of MHC class II or β2 microglobulin for development and activation (96), suggesting that they are eligible to generate rapid immune responses in blood. γδ T cells can produce various cytokines including TNF-α, IL−17, IL-22, and IFN-γ (97). Besides, γδ T cells also secrete chemokines, which influence recruitment of other immune cells at the site of inflammation and modulate the function of other innate and adaptive immune cells (97). These features establish distinct role of γδ T cells in sterile and non-sterile inflammation. γδ T cells were found to be present in samples of AAA patients (98), so the special immune properties of γδ T cells may play of role in early stage of aneurysm formation.

B Cells

B cells serve as essential functional parts in humoral immunity of the adaptive immune system through secreting antibodies. B cell can be divided into three subpopulations, including B1, B2 and regulatory B cells (99). Schaheen et al. discovered that depletion of B1 and B2 cells with anti-CD20 antibody significantly limit AAA growth in animals treated with elastase perfusion or angiotensin II-infusion (45). However, B2 cell refusion was exhibited to ameliorate AAA exacerbation in B cell-deficiency murine models (100). This anomalous phenomenon might be due to upregulation of Treg cells and TGF-β despite of the atherogenic effects of B2 cells (101), and also serves as another proof that AAA is an inflammation-driven disease rather than simple atherosclerotic lesions. The complex impact of B cells on AAA development may need more studies to verify, such as purely B1 cell deficiency murine models.

In addition to producing cytokines like TGF-β, the main function of B cells is to secrete immunoglobulins. After contacting with antigens, the activation-induced cytidine deaminase (AID)-driven somatic hypermutation (SHM) of the variable regions of immunoglobulin genes generate a number of mutated B cells that can differentiate into immunoglobulin-secreting plasma cells and memory B cells, which provide both immediate and persistent effects on the same antigens (102). Some of these B cells are overactive and produce autoantibodies after stimulated by autologous components of human tissues, and result in a variety autoimmune diseases including AAA (103, 104). Immunoglobulins were found widely deposited in mouse AAA tissues, and these autoantibodies can not only induce secretions of IL-6 and MMP-9 from T cells and macrophages, but directly cause local destruction of aortic walls (105). For example, B cell-derived anti-beta 2 glycoprotein I antibody was shown to exacerbate HHcy-aggravated vascular inflammation and AAA expansion (106). In addition, a study isolated antiphospholipid (aPL) antibody (a kind of autoantibody able to cause blood clots) from human AAA tissue, and found that more aPL-positive patients underwent AAA progression that aPL-negative patients (107). Another study purified antibodies against Chlamydia pneumoniae outer membrane proteins (OMPs) from serum of AAA patients, and used these antibodies to analyze the aortic walls of AAA patients with western blot and found positive reactions in all of the tested samples, which could be an evidence of the association between the *Chlamydia pneumoniae* OMP antigens and AAA (108). Besides, some of the immunoglobulin subtypes can interact with other immune cells. For instance, IgE can affect macrophage polarization and induce mast cell activated to synthesize various elastases (109, 110). These dramatically increasing evidences indicate that B cell may be an ideal target to treat AAA patients, and subsequent experiments confirmed this hypothesis. Zhang et al. reported that vinpocetine could alleviate AAA development by suppressing TNF-α-induced B cell activation and proinflammatory mediator expression in primary cultured macrophages both *in vitro*, and *in vivo* (111). The interactions of between B cells and other immune cells are illustrated in **Figure 2**.

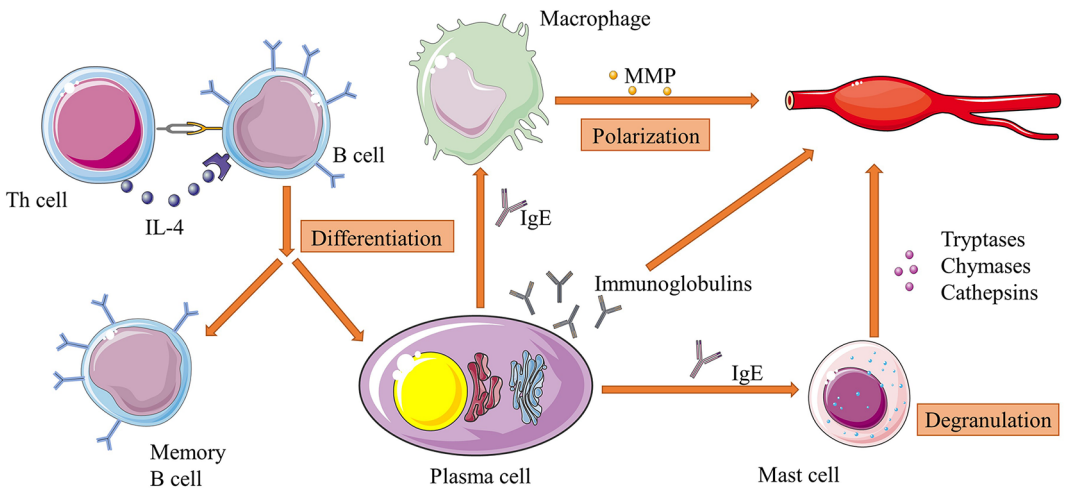

FIGURE 2 | Interactions of between B cells and other immune cells in AAA. B cells can differentiate into plasma cells and memory B cells under the stimulation of IL-4 from Th cells. Plasma cells continuously secrete immunoglobulins, which directly attack aortic walls. Specifically, IgE can activate macrophage polarization and mast cell degranulation and subsequently increase their productions of proteases such as MMPs and cathepsins. These factors work together in the pathogenesis of extracellular matrix degradation of aorta, and is an example of immune cell interactions in the whole process of AAA development. Figures were produced using Servier Medical Art (www.servier.com).

OTHER INFLAMMATORY-INVOLVED MECHANISMS

Matrix Metalloproteinases

MMPs have been implicated in the pathologic origin of AAAs. MMPs have significant destructive effects on elastin fiber integrity, and thereby cause elastin to lose its mechanical properties (112). Several types of MMPs can be secreted by AAA tissue, such as MMP-2, MMP-3, MMP-8, MMP-9, MMP-12 and MMP-13 (113, 114). MMP-9 is the most abundant elastolytic proteinase found in AAA tissue and is predominantly expressed by macrophages infiltrated in AAA (115). Several studies showed that *Mmp9* and *Mmp2* knockout mice are protected from $CaCl_2$ challenging, indicating the important role of MMPs in AAA developments (116). Besides, targeted delivery of MMP inhibitors with nanoparticles was shown to inhibit aneurysmal progression (113). Robert et al. found that the relative resistant to AAA formation in *Mmp9* deficient mice was related to the preservative structure of elastic lamellae despite the presence of infiltrating mononuclear phagocytes and neutrophils (115). It has also been found that MMP-9 can hardly cause local tissue injury without the presence of MMP-2, because MMP-2 can initiate cleavage of the triple-helix-structured collagen into one-quarter and three-quarter lengths, which complement the effects of MMP-9 (116). Netrin-1, a neuronal guidance signal that can specifically regulate the activity of MMP-3, was found to be elevated in murine and human AAA tissues, and targeted depletion of *Ntn1* in macrophages evidently decreased the risk of developing murine AAA (117).

All of above mechanisms give MMP the potential to be a target of screening and therapy for AAA patients. As a specific history hallmark of aneurysm formation, fragmentation of ECM by MMPs has been frequently studied to investigate particular biomarkers in AAA patients (118). A meta-analysis including eight case-control studies revealed strikingly increase of circulating MMP-9 levels in AAA patients (119). Hovsepian et al. found that the elevated MMP-9 had a sensitivity of 48% and a specificity of 95% to establish AAA diagnosis (120). Several other types, such as MMP-1, -2, -3, -7,-12 and -13 have been shown to have an increased level accompanied with reduction of their inhibitors by some researchers (121–123). Doxycycline is a kind of tetracycline antibiotic which is capable to suppress a cast of MMPs, and has been shown to be effective in reducing elastin degradation and aneurysm development in murine AAA models (1). Small randomized clinical trials showed doxycycline suppressed the expansion of AAA (124). A meta-analysis, however, concluded that patients with doxycycline prescription had no significant growth rate reduction of aneurysm diameter than control groups (125).

Inflammasomes

Inflammasomes are large multimolecular complexes that are able to induce inflammation reactions and control the activation of caspase-1, which regulates the proteolytic maturation of IL-1β and IL-18 (126, 127). These intracellular molecular protein scaffolds work through inducing pyroptosis (an inflammatory form of cell death) and necroptosis (a lytic form of inflammatory cell death) by cleaving the N-terminal of pro-IL-1β and pro-IL-18 with caspase-1 (128). Five kinds of receptor proteins have been identified so far to assemble inflammasomes, including nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing protein (NLR) family members NLRP1, NLRP3 and NLRC4, as well as the proteins absent in melanoma 2

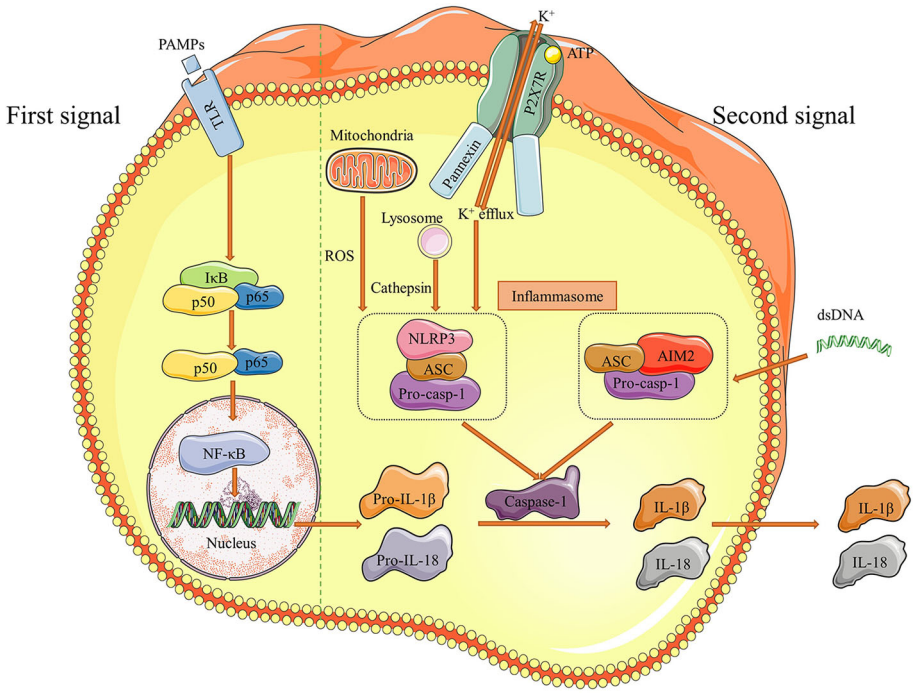

FIGURE 3 | Pathways of NLRP3 and AIM2 inflammasome activation. There are two distinct signals needed for inflammasome to be effective. Initially, pathogen-associated molecular patterns (PAMPs) as the first signal binds to Toll like receptors (TLRs) and stimulate NF-κB, which increases downstream pro-IL-1β and pro-IL-18 production. Then, efflux of K^+ and dsDNA are the second signals correspondingly to induce NLRP3 and AIM2 inflammasome formation. The pathway of NLRP3 inflammasome activation usually proceed under the assistant of cathepsin released by lysosome and ROS mtDNA from mitochondria. The final result of inflammasome activation is cleaving pro-casp-1 into caspase-1, which transforms pro-IL-1β and pro-IL-18 to IL-1β and IL-18. These two effective cytokines are secreted out and participate the inflammatory responses in aortic walls. Figures were produced using Servier Medical Art (www.servier.com).

(AIM2) and pyrin (126). It has been shown that inflammasomes are involved in a cast of inflammatory disorders (126). Recent works suggest that NLRP3 and AIM2 inflammasomes are implicated in the pathogenesis of AAA, and we summarized the process of these inflammasome activations in **Figure 3**.

A pilot study demonstrated an upregulation of the inflammasome core components ASC (apoptosis associated speck-like protein containing a caspase activation and recruitment domain), caspase-1 and IL-1β in AAA tissue compared to normal aortas and claimed AAA-associated lymphoid cells could carry on inflammasome signaling (129). Some subsets of inflammasomes like AIM2 were significantly increased in circulating granulocytes, monocytes, B lymphocytes of AAA patients, and IL-1β released by peripheral blood mononuclear cells of AAA patients was significantly higher than controls (130). Another study found expression of NLRP3 and AIM2 were notably lower in control samples than AAA. However, with the AAA lesion progression, inflammasome expressions decreased (131), which suggests the inflammasome-induced signaling plays a more important role in early AAA pathogenesis. Markus et al. found that necrotic cell debris from autologous cells promotes AIM2 and NLRP3 inflammasomes in VSMC of late stage AAA tissues, and thereby activates downstream inflammatory attacks (132). Ren, et al. found that NLRP3 inflammasomes directly activate MMP-9 by cleaving its N-terminal inhibitory domain, so blocking the inflammasome pathway with MCC950, a potent selective small-molecule NLRP3-inflammasome inhibitor, could prevent aortic aneurysm formation (133). Similarly, silencing of NLRP3 in macrophages remarkably ameliorated AAA formation (134). In the meanwhile, NLPR3, caspase 1, and IL-1β levels were elevated in hyperhomocysteinemia (HHcy) models compared with WTs, and administration of folic acid to reverse the HHcy-accelerated AAA could alleviate activation of inflammasomes in the tunica adventitia (134). These studies demonstrate inflammasomes may be a promising target for medical intervention of AAA.

PERSPECTIVES

AAA still remains to be a life-threatening disease. In the current review, we summarized the updated pathogenic roles of inflammatory cells in AAA development. The roles of T cells and macrophages in AAA have been predominantly studied, including inflammatory cytokines, MMPs, inflammasomes, etc. However, how the other types of inflammatory cells influence AAA are still not fully verified. Despite of the advances of endovascular aneurysm repair and open surgery for large or ruptured AAA, there is still lacking efficient medical therapy

choices for asymptomatic patients. This review lists a considerable number of pathways of inflammatory cell effects, and provides evidences from studies that suppressing corresponding pathways may influence the development of AAA in murine models or patient samples *in vitro*. These evidences not only prove the irreplaceable roles of inflammatory cells in AAA, but provide new methods to develop ideal drugs for researchers and physicians. Specific targets, such as inflammatory cytokines and MMPs, have been investigated for biomarker screening and possible medical therapies for asymptomatic AAA. These novel applications may serve as advanced strategies for early identification and therapeutic intervention for AAA.

It should be noted that most studies on detailed cellular mechanisms were conducted in animal models or *in vitro* experiments, which could not entirely mimic the pathogenesis of AAA in humans. Studies bridging pre-clinical mechanisms and clinical data are needed. Furthermore, most of the animal studies were only focused on the initiation of diseases, while how to prevent AAA rupture in real-world patients are more challenging. Further studies on different stages of AAA will be helpful.

AUTHOR CONTRIBUTIONS

YZ, YL, JW, JQW, JY, and ZX drafted, edited, and approved the manuscript and figures. All authors contributed to the article and approved the submitted version.

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Autoantibodies Against Lysosome Associated Membrane Protein-2 (LAMP-2) in Pediatric Chronic Primary Systemic Vasculitis

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Background: Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a small vessel vasculitis in adults and children that commonly affects the kidneys. Although the frequent antigenic, and presumed pathogenic, targets of ANCA in AAV are proteinase-3 (PR3) and myeloperoxidase (MPO), ANCA against lysosome associated membrane protein-2 (LAMP-2), a lesser known ANCA antigen that is expressed on the glomerular endothelium, are present in some adults with AAV-associated renal disease. LAMP-2-ANCA has not been assessed in children with chronic systemic vasculitis, and, if present, would be a potentially valuable biomarker given that treatment decisions for these pediatric patients at diagnosis are largely informed by kidney function.

Methods: A custom ELISA, using commercially available reagents, was designed to detect autoantibodies to human LAMP-2 in serum. Sera obtained from 51 pediatric patients at the time of diagnosis of chronic primary systemic vasculitis (predominantly AAV) were screened. LAMP-2-ANCA titers were evaluated for correlation with clinical metrics of disease activity (pediatric vasculitis activity score [pVAS], C-reactive protein [CRP] concentration, and erythrocyte sedimentation rate [ESR]), MPO- and PR3-ANCA titers, and renal function (glomerular filtration rate [GFR], renal-specific pVAS, and serum creatinine concentration).

Results: LAMP-2-ANCA (>1,000 ng/ml) were detected in 35% (n = 18) of pediatric systemic vasculitis patients, of which, 10 (20% of all patients) were found to have high positive titers (>1,500 ng/ml). Undetectable or negative titres (<500 ng/ml) were identified in 12% (n = 6) of patients, those with titers between 500 and 1,000 ng/ml were considered low with unknown clinical relevance (53%, n = 27). Although LAMP-2-ANCA titers did not significantly differ between patients with AAV versus ANCA-negative vasculitis, only AAV patients had high concentrations (>1,500 ng/ml) of LAMP-2-ANCA. LAMP-2-ANCA titers

did not correlate with measures of disease activity (pVAS, CRP, or ESR) at the time of diagnosis. In contrast, for patients with 12-month post diagnosis follow-up, a negative correlation was observed between the change in GFR (from diagnosis to 12-month follow-up) and LAMP-2-ANCA titer at diagnosis.

Conclusions: Moderate to high LAMP-2-ANCA titers were detected in 35% (18/51) of children with chronic systemic vasculitis affecting small-to-medium vessels. Although the highest concentrations of LAMP-2-ANCA in this population were observed in individuals positive for classic ANCA (MPO- or PR3-ANCA), similar to previous reports on adult patients, LAMP-2-ANCA titers do not correlate with classic ANCA titers or with overall disease activity at diagnosis. Renal disease is a common manifestation in systemic small-medium vessel vasculitis (both in adults and children, though more severe in children) and our preliminary data suggest LAMP-2-ANCA at diagnosis may be a risk factor for more severe renal disease.

Keywords: anti-neutrophil cytoplasmic antibody, ANCA-associated vasculitis, LAMP-2, lysosome-associated membrane protein-2, pediatric, systemic vasculitis

INTRODUCTION

Anti-neutrophil cytoplasmic antibodies (ANCA) are a family of autoantibodies that are reactive against multiple proteins that are predominantly contained within intracellular granules of neutrophils (1, 2). These autoantibodies were first observed in individuals with glomerulonephritis (3) and forms of systemic small vessel vasculitis (4, 5) that were subsequently named ANCA-associated vasculitis (AAV). In AAV, there are two major classes of ANCA that are defined by the antigenic target: PR3-ANCA directed against proteinase-3 (PR3) and MPO-ANCA directed against myeloperoxidase (MPO). PR3-ANCA and MPO-ANCA are predominantly, but not exclusively, associated with different AAV subtypes (respectively, granulomatosis with polyangiitis and microscopic polyangiitis), and are used clinically to aid phenotype classification. More recently, the presence and specificity (for PR3 or MPO) of ANCA have helped to define disease-associated risks in adult AAV subtypes that do not overlap with the phenotypic classification (6). For example, patients positive for PR3-ANCA often have a more relapsing disease course, increased risk of severe inflammatory lung disease, and systemic disease involving multiple organs at diagnosis (7, 8). In contrast, MPO-ANCA positive patients are more likely to have more severe renal-limited disease (9, 10). Some data on adult patients also supports the value of serially measuring ANCA titers as a marker of disease activity (7, 11), but whether ANCA are informative to organ-specific disease processes, which is a primary determinant in treatment decisions, remains to be shown. Although there is a high incidence of kidney disease in AAV, MPO and PR3 are not expressed by the glomerular endothelium, the primary site of injury in patients with renal involvement (10). Although MPO and PR3 released by neutrophils may associate with the endothelium and in this manner target the endothelium for ANCA-mediated damage, it is also possible that disease processes are the result of the indirect action of ANCA, or are independent of autoantibodies, as may be the case in patients with ANCA-negative vasculitis (2).

A search for autoantigenic targets expressed on the membrane of glomerular cells that may serve as a more direct target of autoimmune processes led to the discovery by Kain et al. in 1995 (12) of antibodies against lysosome associated membrane protein-2 (LAMP-2/CD107b). These LAMP-2-ANCA were detected in adults with active necrotising and crescentic glomerulonephritis (12) and whom were also frequently positive for PR3-ANCA or MPO-ANCA. It was further demonstrated that one of the most common ANCA recognition epitopes on LAMP-2 has 100% homology with the Type I fimbriated bacterial adhesion protein, FimH. Notably, FimH-immunized rats developed pauci-immune focal necrotizing glomerulonephritis and ANCA to both rat and human LAMP-2 (13). Despite this *in vivo* evidence of LAMP-2-ANCA pathogenicity and subsequent findings of LAMP-2-ANCA in cohorts of adults with small-to-medium sized vessel vasculitis (12–15), other studies demonstrate similar LAMP-2-ANCA titers in healthy individuals and patients (16). These contradictory findings may reflect the absence of a standardized assay for LAMP-2-ANCA, impact of immunosuppressive therapy on ANCA titers, and patient selection criteria (17, 18).

The prevalence of LAMP-2-ANCA has not been assessed in children with vasculitis due in large part to the rarity of the disease relative to adult-onset vasculitis. The aim of this study was to conduct a preliminary screen of a retrospective collection of sera from pediatric patients with small-to-medium vessel chronic primary systemic vasculitis for the presence of LAMP-2-ANCA. Without a commercially available assay for LAMP-2-ANCA, we designed a custom enzyme-linked immunosorbent assay (ELISA) and quantified the concentration of LAMP-2-ANCA in sera from 51 pediatric vasculitis patients at the time of

Abbreviations: AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; GN, glomerulonephritis; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; LAMP-2, lysosome associated membrane protein-2; MPO, myeloperoxidase; PAN, polyarteritis nodosa; PR3, proteinase 3; pVAS, pediatric vasculitis activity score; UCV, unclassified vasculitis; TOD, time of diagnosis.

diagnosis. Our findings demonstrate that LAMP-2-ANCA are present in children with systemic vasculitis and provide preliminary evidence that LAMP-2-ANCA titers at the time of diagnosis can indicate worse renal outcomes.

MATERIALS AND METHODS

Pediatric Patients, Clinical Data, and Samples

Patients described in this study were enrolled in the Pediatric Vasculitis Initiative (PedVas), an international study on chronic primary systemic vasculitis in children. Eligibility criteria for PedVas have been described previously (19). The study protocol was approved by the Children's and Women's Research Ethics Board of the University of British Columbia [H12-00894] and the respective ethical committees or IRBs at participating PedVas sites. At the time of diagnosis, participating centres collected sera and clinical data (including, but not limited to, positivity for PR3-ANCA and MPO-ANCA, and glomerular filtration rate) as described (20). Using entered information from participating sites, patients were formally classified into small-to-medium vasculitis subtypes using a pediatric modified algorithm of the European Medicines Agency (EMA) (21). Disease activity at the time of sample collection was calculated using the pediatric vasculitis activity score (pVAS) (22). Pediatric inflammatory disease controls included five patients diagnosed with an autoinflammatory disease/periodic fever syndrome and followed at the BC Children's Hospital, Vancouver, BC. All inflammatory controls were enrolled in a research study approved by Children's and Women's Research Ethics Board of the University of British Columbia [H15-00351]. All participants (pediatric vasculitis patients and autoinflammatory controls) contributed blood in K_2EDTA and/or serum separation tubes (both from BD Biosciences, NJ, USA) during a flare in disease. Blood was processed to serum and plasma according to standard protocols from the manufacturer and aliquots were stored at −80°C.

Enzyme-Linked Immunosorbent Assays

Concentrations of C-reactive protein (CRP) were measured in sera using a human CRP ELISA kit (ThermoFisher, MA, USA) according to manufacturer's instructions (23). Concentrations of PR3-ANCA (ORG518, Orgentec) and MPO-ANCA (425–2380, BioRad) were measured according to manufacturer's instructions and as described previously (23). Concentrations of LAMP-2-ANCA were measured by a custom indirect ELISA (**Supplementary Figure S1**) as follows: Nunc MaxiSorp™ flat-bottom 96-well plates (ThermoFisher, MA, USA) were coated with 50 µl of 5 µg/ml recombinant human (rh) LAMP-2 protein (R&D Systems, MN, USA) diluted in 0.2 M carbonate-bicarbonate, pH 9.4 coating buffer (ThermoFisher, MA, USA), and incubated overnight at 4°C. Wells were washed 3× with 250 µl/well wash buffer (WB; PBS containing 0.05% Tween®20 [FisherScientific, MA, USA]). Blocking buffer (BB; PBS containing 0.05% Tween®20 and 2% bovine serum albumin [MilliporeSigma, MA, USA]) was added (300 µl/well) and incubated at room temperature (RT) for 1 h. BB was discarded and standards/samples were added without washing the plate. Sample (serum or plasma) was diluted 1/10 in BB. Detection reagents were prepared in PBS containing 0.01% Tween®20 and 0.4% BSA. Standards were generated using anti-human LAMP-2 monoclonal antibody (H4B4) (Invitrogen, CA, USA) serially diluted in BB, with optimal dilution range of 50–1,000 ng/ml. Diluted standards and samples were plated (100 µl/well) in duplicate and incubated at RT for 1 h. Wells were washed 5× with 300 µl/well WB then incubated 1 h at RT with 100 µl/well of 1 µg/ml CaptureSelect™ biotin anti-IgG-Fc (multi-species) conjugate (ThermoFisher, MA, USA). Wells were washed 5× with 300 µl/well WB then incubated at RT 30 min with 100 µl/well of 0.5 µg/ml horseradish peroxidase (HRP)-conjugated streptavidin (ThermoFisher, MA, USA). Tetramethylbenzidine (TMB) substrate solution (ThermoFisher, MA, USA) was added (100 µl/well) and incubated for 30 min at RT. TMB stop solution (ThermoFisher, MA, USA) was added 50 µl/well, and absorbance read on the Tecan Infinite M200 spectrophotometer (Tecan, Switzerland) at 450 nm, with a reference read at 620 nm. By fitting the standard curve to a sigmoidal, 4 parameter logistic regression (4PL) equation, unknown values with an absorbance (Abs) at 450 nm (Abs_{450}) were interpolated between 0.402 AU (lower-limit) and 2.776 AU (upper-limit). Optimal sera dilution was found to be 1/10 (data not shown). The ELISA was validated with human sera from young-onset AAV patients, previously reported to be positive (n = 5) or negative (n = 1) for LAMP-2-ANCA at the Medical University of Vienna (13, 14).

Statistical Analysis

Statistical analyses were done using GraphPad Prism v8.0 Statistical Software (GraphPad Software, CA, USA). Group differences were analyzed by ANOVA and subsequent two-tailed t-tests. Correlations were assessed by Pearson correlation coefficient. For all analyses, a confidence interval of 95% was used; a p-value < 0.05 was considered significant.

RESULTS

LAMP-2-ANCA Are Present in Children With Chronic Systemic Small-Medium Vasculitis

A custom ELISA (described in methods and **Supplementary Figure S1**) was designed to determine if LAMP-2-ANCA are present in sera obtained from children with systemic vasculitis affecting small-to-medium sized vessels, the most common of which is ANCA-associated vasculitis (AAV). The ELISA was validated with human sera from individuals with early-onset AAV and known to be positive (n = 5, with high titers in two samples and moderate-low titers in three samples) and negative (n = 1) for LAMP-2-ANCA (13, 14). Concentrations of LAMP-2-ANCA in these samples as determined by the ELISA were as expected; the negative sample contained the lowest calculated concentration (229.8 ng/ml) of LAMP-2-ANCA, and titers in the low-moderate to high-positive samples ranged from 828.3–3,768.95 ng/ml (**Figure 1A**). Sera from five children with systemic inflammation due to an autoinflammatory disease were screened as controls; LAMP-2-ANCA concentration in 4/5 samples were on

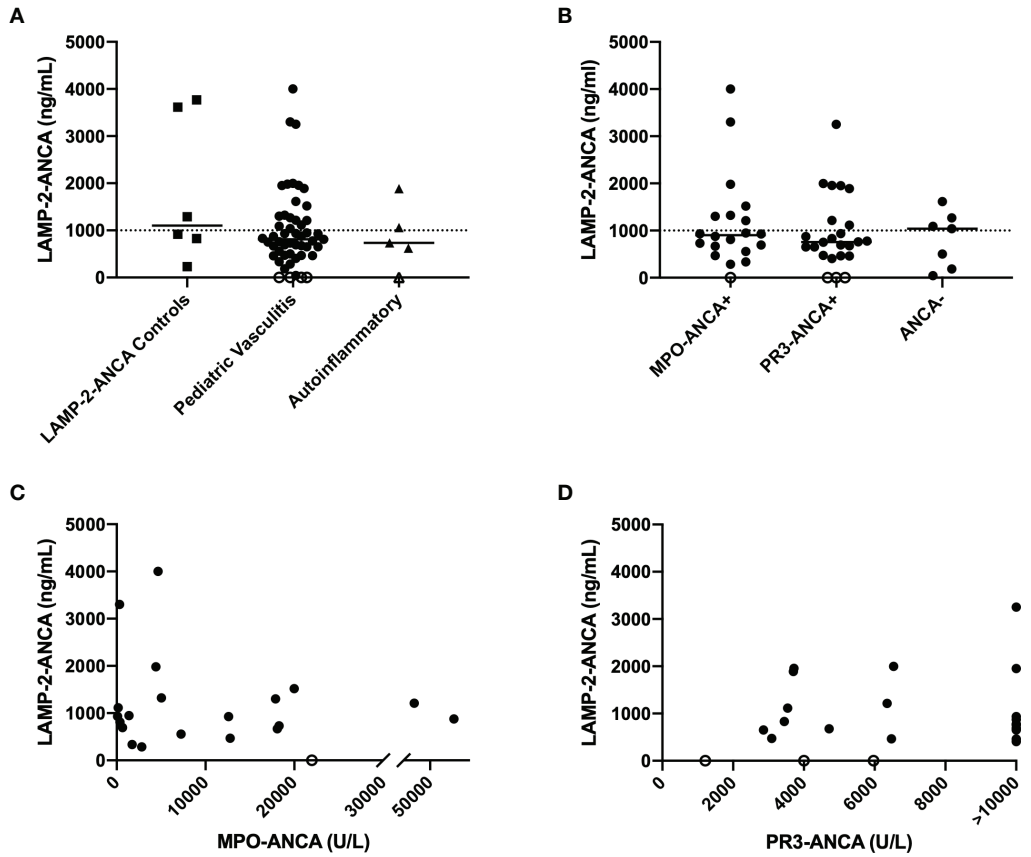

FIGURE 1 | Concentration of LAMP-2-ANCA in pediatric chronic small-to-medium vessel vasculitis patients. **(A)** LAMP-2-ANCA concentration (y-axis; ng/mL) in serum of individuals with early-onset vasculitis that are known to be positive (n = 5) and negative (n = 1) for LAMP-2-ANCA (squares) (14), children with vasculitis (n = 51, circles), and children with systemic autoinflammatory disease (n = 5, triangles). **(B)** LAMP-2-ANCA concentration (y-axis; ng/mL) in pediatric patients grouped (x-axis) based on positivity for MPO-ANCA (n = 19), PR3-ANCA (n = 23), or neither MPO- or PR3-ANCA (ANCA-, n = 7), and **(C, D)** LAMP-2-ANCA concentration (y-axis; ng/mL) plotted against **(C)** MPO-ANCA (x-axis; U/L) (n =19) and **(D)** PR3-ANCA (x-axis; U/L) (n = 23). Bars show median. Horizontal line divided low (<1,000 ng/mL) and moderate-high positive LAMP-2-ANCA (>1,000 ng/mL). Open symbols on the x-axis denote samples below the lower limit of detection of the assay (n = 4 patients with vasculitis, and n = 1 patient with autoinflammatory disease).

the lower end of the positive range (<1,062 ng/ml) (**Figure 1A**). Using these interpolated measures and for the purpose of this study, we reasoned that LAMP-2-ANCA concentrations <250 ng/ml would be considered negative and <1,000 ng/ml were low titers with unknown clinical relevance. Titers >1,000 ng/ml were considered positive with high titer measuring >1,500 ng/ml.

Using these established boundaries, 51 pediatric patients diagnosed with chronic primary systemic vasculitis affecting small-to-medium sized vessels (**Table 1A**) were screened for the presence of LAMP-2-ANCA. Of these, 19 patients were positive for MPO-ANCA, 23 were positive for PR3-ANCA, one patient had both MPO- and PR3-ANCA, and eight patients were ANCA-negative. The mean age of onset of disease was 12.6 years, and the ratio of males to females was equally distributed between groups. Similar to LAMP-2-ANCA-positive control sera, LAMP-2-ANCA concentrations in pediatric vasculitis samples ranged from undetectable (n = 4 patients) to levels over 3 μg/ml (n = 3 patients) (**Figure 1A**). Overall, 12% (n = 6) had undetectable or negative (< 250 ng/ml) LAMP-2-ANCA and 53% (n = 27) were found to have low titers (<1,000 ng/ml) of unknown clinical significance. The remaining 35% (n = 18) of pediatric vasculitis patients had a minimum of 1,000 ng/ml of LAMP-2-ANCA, with 56% of those individuals (and 20% of total patients) having high-positive titers (>1,500 ng/ml).

Although the highest concentrations of LAMP-2-ANCA were present in patients also positive for PR3-ANCA or MPO-ANCA (compared to ANCA-negative patients), LAMP-2-ANCA titers did not significantly differ between patients positive for the classic ANCA subsets (MPO-ANCA, PR3-ANCA) or ANCA-negative patients (n = 47, $p = 0.5715$) (**Figure 1B**). Moreover, within the subset of ANCA-positive patients, there was no correlation between LAMP-2-ANCA titers and titers (**Table 1B**) of either MPO-ANCA (n = 19, $p = 0.6054$, **Figure 1C**) or PR3-ANCA (n = 21, $p = 0.9897$, **Figure 1D**). No correlation was observed between LAMP-2-ANCA titer and age of onset or sex (data not shown).

TABLE 1A | Pediatric vasculitis cohort.

ID	Diagnosis[a]	Onset[b]	Sex	ANCA	Renal[c]	Treatment[d]
1	uAAV	16	Male	MPO	No	prednisone
2	MPA	15	Female	MPO	Yes	prednisone
3	GPA	12	Female	MPO	Yes	none
4	GPA	11	Female	MPO	Yes	prednisone
5	GPA	12	Female	MPO	No	prednisone, methotrexate
6	UCV	17	Female	MPO	Yes	prednisone, cyclophosphamide
7	GPA	15	Male	MPO	Yes	none
8	GPA	5	Female	MPO	Yes	prednisone, methotrexate, rituximab, azathioprine
9	GPA	16	Female	MPO	Yes	none
10	GPA	2	Male	MPO	Yes	none
11	GPA	13	Female	MPO	Yes	none
12	GPA	17	Female	MPO	Yes	prednisone, cyclophosphamide, rituximab
13	GPA	15	Male	MPO	Yes	prednisone
14	UCV	17	Female	MPO	Yes	none
15	MPA	16	Male	MPO	Yes	none
16	MPA	17	Female	MPO	Yes	cyclophosphamide
17	GPA	17	Male	MPO	Yes	prednisone, cyclophosphamide
18	GPA	1	Female	MPO	Yes	prednisone, rituximab
19	GPA	5	Female	MPO	Yes	prednisone, cyclophosphamide
20	MPA	5	Female	MPO	Yes	prednisone
21	GPA	10	Male	PR3	Yes	none
22	GPA	15	Female	PR3	Yes	none
23	GPA	13	Female	PR3	Yes	prednisone
24	GPA	15	Male	PR3	Yes	none
25	NA	17	Female	PR3	Yes	prednisone
26	GPA	12	Female	PR3	Yes	prednisone, rituximab
27	GPA	15	Male	PR3	Yes	prednisone
28	GPA	15	Female	PR3	Yes	prednisone, rituximab
29	uAAV	12	Female	PR3	No	prednisone, methotrexate
30	GPA	14	Male	PR3	No	prednisone, rituximab
31	GPA	14	Female	PR3	Yes	none
32	GPA	14	Female	PR3	Yes	prednisone, cyclophosphamide
33	uAAV	13	Male	PR3	Yes	prednisone
34	UCV	13	Female	PR3	Yes	prednisone, cyclophosphamide
35	GPA	12	Female	PR3	Yes	prednisone, cyclophosphamide
36	GPA	12	Female	PR3	Yes	prednisone, cyclophosphamide, rituximab
37	UCV	14	Female	PR3	Yes	prednisone
38	GPA	15	Female	PR3	Yes	prednisone, cyclophosphamide, rituximab
39	GPA	14	Female	PR3	Yes	prednisone, rituximab
40	GPA	15	Male	PR3	Yes	prednisone, rituximab
41	GPA	18	Male	PR3	Yes	prednisone, rituximab
42	GPA	16	Male	PR3	Yes	prednisone, rituximab
43	GPA	16	Male	PR3	yes	prednisone, rituximab
44	MPA	13	Male	Both	Yes	none
45	uAAV	10	Female	Negative	No	prednisone
46	uAAV	9	Male	Negative	No	prednisone
47	cPAN	2	Male	Negative	Yes	prednisone, infliximab
48	UCV	15	Female	Negative	Yes	prednisone
49	GPA	7	Female	Negative	Yes	none
50	UCV	4	Male	Negative	No	prednisone
51	PAN	16	Female	Negative	No	none

[a]According to the EMA classification algorithm (21). MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; PAN, polyarteritis nodosa; uAAV, unclassified ANCA-associated vasculitis, UCV, unclassified vasculitis. [b]Age in years when symptoms associated with vasculitis first presented [c]Renal involvement determined by renal component of the pVAS => 4 [d]Current treatments at diagnosis, coincident with sample and data collection.

LAMP-2-ANCA Titers Do Not Correlate With Clinical Disease Activity Measures

We next assessed whether concentrations of LAMP-2-ANCA correlated with standard clinical measures of disease activity, namely, C-reactive protein (CRP, mg/L), erythrocyte sedimentation rate (ESR, mm/hr), and pediatric vasculitis activity score (pVAS). ESR and pVAS were derived from clinical data entered at the participating site, and CRP was measured in house by commercial ELISA (see methods). Neither CRP (n = 47, $p = 0.3115$) nor ESR (n = 41, $p = 0.9707$) were found to correlate with LAMP-2-ANCA titers (**Figures 2A, B**). Likewise, LAMP-2-ANCA titers did not correlate with the pediatric vasculitis activity score (pVAS, **Figure 2C**) (n = 46,

TABLE 1B | Measures of disease activity, and LAMP-2-, PR3- and MPO-ANCA.

ID	pVAS[a,b]	CRP[b] (mg/L)	ESR[b] (mm/hr)	LAMP-2-ANCA[b] (ng/mL)	MPO-ANCA (U/mL)	PR3-ANCA (U/mL)
1	10	19.7	1	1,301.1	17,896.5	–
2	12	20.0	1	948.6	1,392.1	–
3	19	8.0	127	1,323.7	5,034.3	–
4	20	3.7	150	668.3	18,087.8	–
5	10	3.4	17	468.0	12,777.2	–
6	25	9.7	9	729.5	18,284.9	–
7	22	5.3	44	875.9	68,007.5	–
8	17	32.6	100	4,000.5	4,660.0	–
9	14	722.4	61	1,520.0	19,978.4	–
10	20	15.1	78	33,02.7	324.2	–
11	31	23.5	90	932.5	109.4	–
12	30	129.2	107	555.9	7,241.3	–
13	21	244.7	nd[c]	809.1	364.0	–
14	18	23.4	nd[c]	693.9	663.1	–
15	16	163.3	130	ND[d]	21,974.3	–
16	21	14.3	100	337.1	1,746.9	–
17	20	6,332.1	104	1,979.9	4,404.1	–
18	19	38.7	nd[c]	284.7	28,25.2	–
19	16	122.9	nd[c]	928.1	12,609.5	–
20	14	313.6	140	1,208.4	38,083.8	–
21	20	23.9	16	1,950.2	–	>10000
22	19	272.8	38	3,250.6	–	44,685.8
23	32	219.9	96	1,890.3	–	3,700.4
24	38	2,560.2	nd[c]	692.3	–	>10000
25	18	8.0	23	ND[d]	–	12,13.5
26	31	7.8	9	874.1	–	14,263.1
27	21	7.3	nd[c]	460.0	–	550,151.0
28	21	7.2	18	678.0	–	4,713.3
29	7	80.4	26	464.8	–	6,472.5
30	21	116.3	36	405.6	–	>10000
31	30	11.4	72	777.6	–	>10000
32	23	8.6	115	653.8	–	>10000
33	21	79.7	140	758.6	–	>10000
34	nd[b]	13.8	95	831.2	–	3,444.7
35	21	9.2	170	1,212.6	–	6,351.7
36	19	28.6	120	937.2	–	10,196.8
37	23	24.8	nd[c]	434.9	–	>10000
38	50	249.2	130	652.2	–	2,858.3
39	33	6.4	40	ND[d]	–	5,970.7
40	33	82.7	110	1,997.9	–	6,533.4
41	31	472.6	70	474.4	–	3,089.1
42	28	731.9	53	1,954.8	–	3,718.1
43	20	7.6	98	ND[d]	–	4,001.1
44	15	163.4	54	1,115.9	173.4	3,542.3
45	6	103.6	17	1,614.2	–	–
46	5	70.8	87	186.3	–	–
47	7	21.8	68	45.1	–	–
48	15	790.2	15	1,266.8	–	–
49	17	962.5	97	501.4	–	–
50	12	128.4	78	1,089.7	–	–
51	9	5.3	85	1,039.6	–	–

[a]*pVAS: pediatric vasculitis activity score.*
[b]*Measurement taken at time of diagnosis.*
[c]*nd, not done;* [d]*ND, not detected (Abs_{450} below the lower limit of detection).*

p = 0.9737), a pediatric adaption of the adult BVAS, which is a cumulative weighted score of disease activity of nine organ systems (mean pVAS = 20.4 +/- 8.8 at TOD, n = 46) (22). Consistent with these findings, LAMP-2-ANCA titers did not differ between samples collected prior to or shortly after immune suppressive induction therapy (n = 47, p = 0.2068) (**Figure 2D**).

LAMP-2-ANCA Titers Are Associated With Worsening Renal Disease

Both adult and pediatric ANCA-associated vasculitides are frequently associated with kidney disease. In our cohort, eighty-four percent (n = 43) of patients screened for LAMP-2-ANCA had renal involvement, as determined by the renal

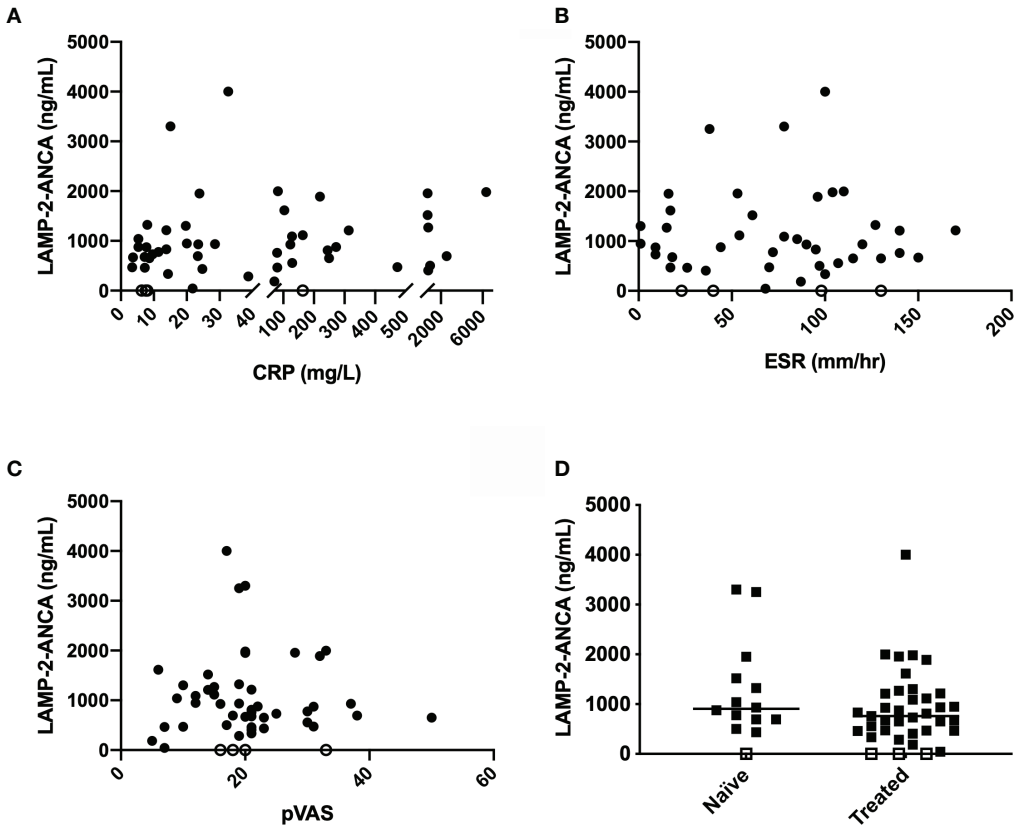

FIGURE 2 | Comparison of LAMP-2-ANCA titer with standard clinical measures of disease activity. Concentration of LAMP-2-ANCA (y-axis; ng/mL) in pediatric vasculitis patients plotted against **(A)** C-reactive protein (CRP) concentration (x-axis; mg/L) (n = 51), **(B)** erythrocyte sedimentation rate (ESR) (x-axis; mm/h) (n = 44), and **(C)** pediatric vasculitis activity score (pVAS) (x-axis) (n = 51) at the time of diagnosis, and **(D)** blood samples taken prior to (naïve, n = 14), or after (treated, n = 37), induction of immune suppressive therapy. Bars show median. Horizontal line divided low (<1,000 ng/mL) and moderate-high positive LAMP-2-ANCA (>1000 ng/mL). Open symbols on the x-axis denote samples below the lower limit of detection of the assay (n = 4).

component of the pVAS being ≥ 4 (**Tables 1A**, **C**). The renal component of the pVAS takes into account renal hypertension, glomerular filtration rate (GFR), and the presence of hematuria, RBC casts, and proteinuria. Mean renal pVAS at the TOD was 11.4 +/- 8.3 (n = 50). As observed for overall score of disease activity (total pVAS, **Figure 2C**), no correlation between LAMP-2-ANCA and renal pVAS was observed (n = 46, $p = 0.9734$; data not shown).

We next looked for correlations with individual indicators of renal function: proteinuria, GFR, and serum creatinine concentration. While no significant difference was observed, the presence of proteinuria was found in all patients with high LAMP-2-ANCA titers at the time of diagnosis (**Figure 3A**). Similarly, no significant correlation was observed between GFR at the time of diagnosis (**Table 1C**) and LAMP-2-ANCA titers ($r^2 = 0.0164$, $p = 0.4767$, n = 33; data not shown). In adults with ANCA-associated vasculitis, poor renal outcomes are associated with a negative change in GFR at 12 months (24), where negative values indicate a decrease in kidney function. For a subset of our pediatric patients (n = 27) that had follow-up clinical data, we also observed a negative correlation between the change in GFR (from time of diagnosis to 12-month follow-up) and LAMP-2-ANCA titers ($r^2 = -0.2111$, $p = 0.0314$) (**Figure 3B**). Similarly, there is a trending increase in LAMP-2-ANCA titers in patients with worsening renal disease at 12 months, as determined by a decrease in GFR > 10 ml/min/1.73m^2 (**Figure 3C**). As serum creatine concentration at disease onset has been shown to be a risk factor for end stage renal disease (10, 25), the correlation with LAMP-2-ANCA was assessed, however, no correlation was observed (n = 24, $r^2 = 0.0625$, $p = 0.2387$) (**Figure 3D**).

DISCUSSION

ANCA positivity and specificity for either PR3 or MPO aids phenotype classification in adult and pediatric AAV, and in adult-onset AAV is associated with general features of disease course (7–10). Their utility as prognostic markers for renal disease, which has a high prevalence among patients with AAV, may have limitations given that MPO and PR3 are not expressed on the glomerular endothelium. Unlike MPO or PR3, a lesser known ANCA antigen, LAMP-2, is expressed on the surface of the renal microvascular endothelium and LAMP-2-

TABLE 1C | Renal metrics.

ID	Renal pVAS[a]	GFR (mL/min/1.73m^2)	
		TOD[b]	12-month
1	0	119	97
2	28	9	85
3	26	20	34
4	20	56	96
5	0	nd[c]	nd[c]
6	20	nd[c]	nd[c]
7	14	nd[c]	nd[c]
8	10	nd[c]	nd[c]
9	4	102	93
10	4	99	nd[c]
11	28	2	nd[c]
12	22	nd[c]	nd[c]
13	14	53	64
14	28	nd[c]	nd[c]
15	10	54	62
16	12	8	nd[c]
17	12	4	nd[c]
18	12	3	nd[c]
19	12	6	27
20	12	13	58
21	6	94	nd[c]
22	16	91	10
23	22	25	7
24	26	26	6
25	6	nd[c]	nd[c]
26	6	182	148
27	6	146	130
28	10	112	90
29	0	nd[c]	nd[c]
30	0	nd[c]	nd[c]
31	10	nd[c]	nd[c]
32	10	nd[c]	nd[c]
33	10	121	67
34	24	0	62
35	10	147	113
36	24	5	22
37	10	97	94
38	12	80	nd[c]
39	10	133	93
40	12	142	108
41	12	10	nd[c]
42	12	49	nd[c]
43	12	7	8
44	14	nd[c]	nd[c]
45	0	136	111
46	0	nd[c]	nd[c]
47	4	nd[c]	nd[c]
48	4	nd[c]	115
49	6	148	nd[c]
50	0	129	111
51	0	127	nd[c]

[a]*Renal pVAS score at time of diagnosis.*
[b]*TOD, time of diagnosis.*
[c]*nd, not done.*

ANCA have been detected in adults with AAV-associated renal disease. The prospect of evaluating LAMP-2-ANCA for direct role(s) in the pathogenesis of renal disease associated with vasculitis or as a biomarker of glomerular damage (13) is inviting, particularly in children with AAV that, compared to adult-onset disease, present with more severe disease involving multiple organs (19, 26) and more than half of patients experience kidney damage early in disease course (27).

Herein, we conducted a preliminary screen of time of diagnosis sera from children (n = 51) with primary systemic small-to-medium sized vessel vasculitis (predominantly AAV) for the presence of LAMP-2-ANCA. Using a custom, in-house indirect ELISA, our data demonstrate that LAMP-2-ANCA are present in pediatric vasculitis patients. The majority of individuals were positive for low levels of LAMP-2-ANCA (53%), the clinical utility of which is unknown. An additional 35% of patients in the cohort had moderate-high titers of LAMP-2-ANCA (>1,000 ng/ml) and the remaining 12% of patients were negative for LAMP-2-ANCA. LAMP-2-ANCA titers did not correlate with positivity (or lack thereof) or titers of the classic PR3-ANCA and MPO-ANCA. LAMP-2-ANCA titers were also not correlated with elevated systemic disease activity as indicated by a validated pediatric vasculitis clinical scoring algorithm, pVAS, and general inflammatory markers, CRP and ESR. LAMP-2-ANCA titers may however be informative of renal function, which is affected in the majority of patients (84% in this cohort). Increasing LAMP-2-ANCA titers were observed in patients with declining glomerular filtration rate (GFR), indicative of worsening renal disease one-year post diagnosis.

Within the cohort, 88% of patients were positive for LAMP-2-ANCA with titers for the majority overlapping with concentrations detected in a control (autoinflammatory) cohort. Titers in the moderate to high range (>1,000 ng/ml), that, arguably, have a higher likelihood of disease association, were identified in 35% of patients in the cohort. The number of patients in our cohort with "moderate-high titer" positivity falls between conflicting rates of LAMP-2-ANCA positivity reported in two independent cohorts of adults with AAV, ranging from 21% (16) to >80% positivity for LAMP-2-ANCA (14). As summarized previously (18), these variable prevalence rates could be due to characteristics of the individual cohorts or assays used to assess LAMP-2-ANCA concentration. LAMP-2-ANCA titers are highly sensitive to immunosuppressive therapy, decreasing rapidly following treatment induction (14). As may be expected, higher prevalence rates of LAMP-2-ANCA were observed in patients with active disease and not on treatment. While the majority of the pediatric patients assayed in our study were not treatment naïve, samples were drawn early in disease course when disease activity was high. This may explain why LAMP-2-ANCA titers in our cohort were not significantly higher in the subset of treatment naïve patients.

In the highest reported prevalence rate of LAMP-2-ANCA in >80% of adults with AAV-associated renal disease, a recombinant, non-glycosylated human LAMP-2 protein was utilized in the immunoassays (14). While patient derived LAMP-2-ANCA have previously been shown to bind epitopes within non-glycosylated sites of the protein backbone (12, 13), non-human mammalian protein expression systems, such as the mouse myeloma line used to produce the rhLAMP-2 used in the described ELISA, may induce glycosylation patterns not found in humans (18). This potentially apparent glycosylation of LAMP-2 could block the endogenous LAMP-2-ANCA epitope — another possible

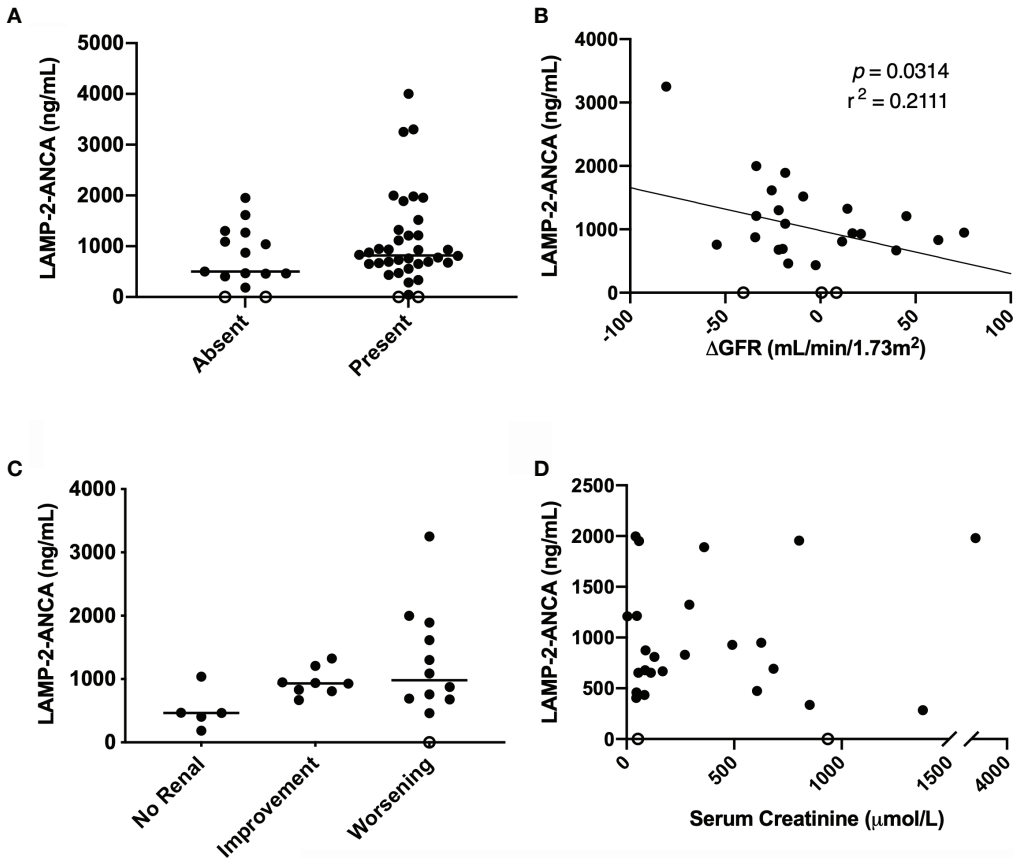

FIGURE 3 | Comparison of LAMP-2-ANCA titer with renal metrics. Concentration of LAMP-2-ANCA (y-axis: ng/ml) in pediatric vasculitis patients: **(A)** with the absence (n = 15) and presence (n = 36) of proteinuria (x-axis) at the time of diagnosis. **(B)** plotted against change in GFR from the time of diagnosis to 12-month follow-up (x-axis, ml/min/1.73m^2) (n = 25, p = 0.0314). **(C)** with no renal involvement (renal PVAS < 4; n = 5), and either renal improvement (increase in GFR at 12-month > 10 ml/min/1.73m^2, n = 8) or worsening (decrease in GFR at 12-month > 10 ml/min/1.73m^2, n = 12) from diagnosis to 12-month follow-up. **(D)** plotted against serum creatinine concentration (x-axis; μmol/L) (n = 26, p = 0.2149). Bars show median.

explanation to the varying prevalence rate of LAMP-2-ANCA observed in our pediatric cohort compared to other cohorts.

Reported prevalence rates are also dependent on where the positive and negative thresholds are drawn. While LAMP-2-ANCA were detected in 88% of our cohort of pediatric vasculitis samples, the majority were deemed low titers (<1,000 ng/ml). Low LAMP-2-ANCA titers were also observed in pediatric autoinflammatory controls, with one control having a high titer (>1,500 ng/ml). The observation of high LAMP-2-ANCA in a disease control cohort is similar to previous reports, where LAMP-2-ANCA were detected in 10 - 16% of disease controls (14, 16). These results are not unexpected, as it's not uncommon to detect autoantibodies in otherwise healthy individuals (28). In particular, given the molecular mimicry hypothesis (13), an individual with a previous Type I fimbriated bacterial infection could theoretically develop antibodies to LAMP-2.

The presence of LAMP-2-ANCA in some healthy individuals augments the importance of determining clinical utility of these autoantibodies. This can be difficult for rare populations, such as pediatric vasculitis, but our preliminary data suggest that, despite the lack of a correlation with markers of systemic disease activity (pVAS, CRP, ESR), LAMP-2-ANCA titers at diagnosis were negatively correlated with the change in GFR (from diagnosis to 12-months), a marker of renal function. As well, there was a trending increase in LAMP-2-ANCA at diagnosis in patients with worsening renal involvement at 12-month follow-up—patients with higher LAMP-2-ANCA at diagnosis, generally had worsening renal function after 12-months. Although sample numbers are a limitation in our study, these data suggest that LAMP-2-ANCA titers have potential utility as predictive marker of renal outcome.

In summary, a custom ELISA was designed to detect LAMP-2-ANCA in serum. This ELISA was used to screen a cohort of pediatric patients with AAV, to assess, for the first time, if LAMP-2-ANCA are prevalent in pediatric vasculitis. While LAMP-2-ANCA showed no correlation with MPO- or PR3-ANCA or markers of disease activity, evidence suggests a possible role for LAMP-2-ANCA as a predictive marker for renal outcome. As renal disease is a common manifestation in both children and adults with systemic small-medium vessel vasculitis, and often more severe in children, a prognostic biomarker could be invaluable to help guide

effective treatment. Screening of a larger pediatric cohort with detailed follow-up will be necessary to elucidate the role of LAMP-2-ANCA in renal outcomes in children with chronic systemic vasculitis.

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 Children's and Women's Research Ethics Board of the University of British Columbia. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KG, RK, RL, CR, DC, and KB contributed to conception and design of the study. RK provided sera controls for LAMP-2-ANCA. KG acquired data and performed the statistical analyses. 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.2020.624758/full#supplementary-material

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Apremilast in Refractory Behçet's Syndrome: A Multicenter Observational Study

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Objective: Mucocutaneous and joint disorders are the most common manifestations in Behçet's syndrome (BS) and are frequently clustered in the so-called minor forms of BS. There remains a need for safe and effective treatment for joint lesions in BS. We report the long-term safety and effectiveness of apremilast in refractory joint and mucocutaneous manifestations of BS.

Methods: French nationwide multicenter study including 50 BS patients with either active joint and/or mucocutaneous manifestations resistant to colchicine and/or DMARDs. Patients received apremilast 30 mg twice a day. Primary effectiveness endpoint was the proportion of patients with complete response (CR) of articular symptoms at month 6 (M6), defined as resolution of inflammatory arthralgia and arthritis, with joint count equal to zero.

Results: At inclusion, the median tender and swollen joint count was of 4 [2-6] and 2 [1-2], respectively. The proportion of CR in joint disease at M6 was 65% (n = 15/23), and 17% (n = 4/23) were partial responders. CR of oral and genital ulcers, and pseudofolliculitis at M6 was 73% (n = 24/33), 94% (n = 16/17) and 71% (n = 10/14), respectively. The overall response at M6 was 74% for the entire cohort and 70% for the mucocutaneous-articular

cluster (n = 27). The median Behçet's syndrome activity score significantly decreased during study period [50 (40–60) *vs.* 20 (0–40); p <0.0001]. After a median follow-up of 11 [6-13] months, 27 (54%) patients were still on apremilast. Reasons for apremilast withdrawal included adverse events (n = 15, 30%) and treatment failure (n = 8, 16%). Thirty-three (66%) patients experienced adverse events, mostly diarrhea (n = 19, 38%), nausea (n = 17, 34%) and headache (n = 16, 32%).

Conclusion: Apremilast seems effective in BS-related articular disease refractory to colchicine and DMARDs. Discontinuation rates were significantly higher than that reported in clinical trials.

Keywords: Behçet, apremilast, efficacy, safety, joint, skin, cohort

INTRODUCTION

Behçet's syndrome (BS) is a chronic, relapsing, inflammatory disease of unknown etiology, typically characterized by oral and genital ulcers with several potential systemic manifestations (1). Mucosa, skin, and joint involvement are among the most frequently reported manifestations. These symptoms frequently cluster in the so-called minor forms of BS (2, 3). Mucocutaneous manifestations constitute the hallmark of the syndrome, with the most common skin lesions being pseudofolliculitis and erythema nodosum. Joint involvement, mainly arthralgia, involve half of the patients, and may inaugurate BS (4). In contrast to major organ involvement, mucocutaneous and articular manifestations do not have a major impact on mortality (5, 6), but can be extremely disabling. The main therapeutic goal for these patients is to improve quality of life while minimizing side effects. Despite a wide number of topical and immunosuppressive drugs available in this context, their level of evidence remains limited (7), and the recommended therapeutic lines (i.e., colchicine and disease-modifying antirheumatic drugs - DMARDs) do not effectively control all patients (8). Moreover, following a phenotype-based treatment approach in BS, strategies effective against both mucocutaneous and articular manifestations are increasingly desirable (9).

Apremilast is an orally available small-molecule that selectively inhibits phosphodiesterase 4 (PDE4), and ultimately modulates both anti- and pro-inflammatory downstream mediators. By increasing intracellular levels of cyclic adenosine monophosphate (cAMP), apremilast upregulates interleukin-10 (IL-10) gene transcription, while inhibiting nuclear factor-κB (NF-κB)-driven genes, such as tumor necrosis factor (TNF) (10). Its efficacy has been proven in BS oral ulcers in phases II and III randomized placebo-controlled clinical trials (11, 12), leading to its approval by the FDA in 2019 (13). This effect was further confirmed in short-term small case series (14–16). Nevertheless, the efficacy of apremilast on other manifestations, and specifically on the joints, is still lacking. In addition, the prevalence and impact of its side effects in large real-life cohort with long-term follow-up period has not been assessed.

The present study aims to further investigate the effectiveness and safety of apremilast in a nationwide multicenter cohort of BS patients with refractory joint and mucocutaneous manifestations.

PATIENTS AND METHODS

Patients

We conducted a nationwide observational cohort study within the French Behçet's network. All patients were adults meeting the criteria of International Study Group for Behçet's Disease (1), and had either recurrent active joint and/or mucocutaneous manifestations that were refractory to colchicine, conventional synthetic (csDMARDs), and/or biological disease-modifying antirheumatic drugs (bDMARDs). The study was conducted in compliance with the Declaration of Helsinki, and no formal consent from participants was required according to local ethics committees. All data were collected from electronic medical records, including demographic features, BS characteristics at diagnosis, and previous treatments. Data on medications, safety, and disease activity, such as oral and genital ulcers, cutaneous, and articular disease or any other BS manifestations were collected at the time of apremilast initiation, at months 3 and 6 (M3, M6), and at last visit (end of follow-up).

Design

Apremilast was administered orally by increasing the doses gradually over 1 week up to a dose of 30 mg twice daily. Colchicine, prednisone and other immunosuppressive therapies were allowed if given at a stable dose over the month prior inclusion and during the study period. Patients who needed temporary increase in prednisone dose or any additional immunomodulatory therapy during the study period were considered as non-responders to apremilast.

Study Endpoints

The primary effectiveness endpoint was the proportion of patients with complete response of joint involvement at M6, defined as resolution of inflammatory arthralgia/arthritis and tender/swollen joint count (TJC, SJC) = 0. Secondary endpoints included (i) the proportion of patients with a complete response of ulcerations (defined as no oral and genital ulcers) (ii) the proportion of patients with a partial response (defined as patients who had a reduction of 50% or more in the number and frequency of oral and genital ulcers, inflammatory arthralgia, arthritis, and joint counts, and skin lesions); (iii) proportion of non-responders (defined as treatment failure and/or the needed for temporary increase in prednisone dose or any additional immunomodulatory therapy during the study period); (iv)

effectiveness on other BS manifestations (i.e., ocular, vascular, neurological or gastrointestinal tract involvement); (v) the overall response at M6 for the whole cohort and for the mucocutaneous-articular phenotype (those interrupting treatment before it, regardless of the reason, were considered as non-responders); (vi) BSAS score (17) between baseline and the end of follow up (EOF); (vii) relapse rate under apremilast; (viii) steroids sparing effect of apremilast between day 0 and EOF, and (ix) safety, as all adverse events were prospectively collected during the follow-up.

Statistics

Data are presented as the median and interquartile range [IQR] for continuous variables and as number (n) and percentage (%) for qualitative variables. Wilcoxon signed rank test with continuity correction was used to compare paired continuous variables. *P* values less than 0.05 were considered significant. Statistical analyses were performed using the software R version 3.6.3.

RESULTS

Characteristics of BS Patients

We included 50 patients [27 (54%) females, with median age of 42 (34–48) years]. Main baseline and treatment characteristics are summarized in **Tables 1** and **2**. All patients had active joint and/or mucocutaneous manifestations resistant to colchicine and/or DMARDs. The most common previous manifestations of BS were oral ulcers (98%), arthralgia (76%), genital ulcers and pseudofolliculitis (70%), vascular (22%), and ocular involvement (18%).

Ninety-eight percent of patients had already received colchicine, and 52% and 62% had been previously treated with steroids or DMARDs, respectively. Before apremilast treatment, BS patients had received a median number of previous treatment lines of 2 [1-3].

At inclusion, 30 patients (60%) had refractory joint manifestations with a median TJC and SJC of 4 [2-6] and 2 [1-2], respectively. Forty-seven (94%) and 23 (46%) patients had recurrent oral and genital ulcers, respectively. Pseudofolliculitis was present in 18 (36%) patients and erythema nodosum in 5 (10%). Median BSAS was 50 [40-60].

At the time of apremilast initiation, 18 (36%) and 14 (28%) patients continued to receive stable dose of colchicine, and prednisone (median dose = 6 [5-15] mg), respectively. Three (6%) patients pursued csDMARDs (i.e., methotrexate), and three

TABLE 1 | Clinical and demographic characteristics of 50 patients with Behçet's syndrome.

Demographic features	
Age, median [IQR] years	42 [34–48]
Female sex, n (%)	27 (54)
HLA-B51, n (%)†	9 (39)
Disease duration, median [IQR] years	5 [1–9]
Clinical features at diagnosis	
Oral ulcers, n (%)	49 (98)
Genital ulcers, n (%)	35 (70)
Pseudofolliculitis, n (%)	35 (70)
Erythema nodosum, n (%)	10 (20)
Positive pathergy test, n (%)	4 (8)
Arthralgia, n (%)	38 (76)
Arthritis, n (%)	13 (26)
Vascular involvement, n (%)	11 (22)
Ocular involvement, n (%)	9 (18)
Gastrointestinal involvement, n (%)	4 (8)
CNS involvement, n (%)	3 (6)
Disease status at the beginning of Apremilast	
Oral ulcers, n (%)	47 (94)
- Number of lesions, median [IQR]	2 [2–3]
Genital ulcers, n (%)	23 (46)
- Number of lesions, median [IQR]	1 [1-1]
Pseudo folliculitis, n (%)	18 (36)
Erythema nodosum, n (%)	5 (10)
Joint involvement, n (%)	30 (60)
- Arthritis, n (%)	7 (14)
Eye involvement, n (%)	1 (2)
BSAS, median [IQR]	50 [40-60]

†*HLA-B51 had been performed in 23 patients.*
BSAS, Behçet's syndrome Activity Score; IQR, interquartile range.

TABLE 2 | Treatments received as part of Behçet's syndrome (BS) before and during apremilast.

Previous treatments during disease course	
Number of treatment lines, median [IQR]	2 [1-3]
Colchicine, n (%)	49 (98)
Corticosteroids, n (%)	26 (52)
csDMARDs, n (%)†	22 (44)
- Number of csDMARDs, median [IQR]	1 [1-2]
bDMARDs, n (%)‡	9 (18)
- Number of bDMARDs, median [IQR]	1 [1-5]
Medications in use before apremilast start	
Colchicine, n (%)	29 (58)
- Median dose [IQR], mg	1.5 [1-2]
Prednisone, n (%)	15 (30)
- Median dose [IQR], mg	6 [5-15]
csDMARDs, n (%)	11 (22)
- Methotrexate, n (%)	4 (8)
- Azathioprine, n (%)	3 (6)
- Thalidomide, n (%)	3 (6)
- Dapsone, n (%)	1 (2)
bDMARDs, n (%)	5 (10)
- Ustekinumab, n (%)	2 (4)
- Adalimumab, n (%)	1 (2)
- Certolizumab, n (%)	1 (2)
- Secukinumab, n (%)	1 (2)
Combination treatment with Apremilast	
Colchicine, n (%)	18 (36)
Prednisone, n (%)	14 (28)
- Median dose [IQR], mg	6 [5–15]
csDMARDs, n (%)	3 (6)
- Methotrexate, n (%)	3 (6)
bDMARDs, n (%)	3 (6)
- Adalimumab, n (%)	1 (2)
- Certolizumab, n (%)	1 (2)
- Ustekinumab, n (%)	1 (2)

†*Previous csDMARDs included azathioprine, dapsone, hydroxychloroquine, methotrexate, and thalidomide.*
‡*Previous bDMARDs included anakinra, low-dose interleukin-2, secukinumab, tocilizumab, anti-tumor necrosis factor (adalimumab, certolizumab, etanercept, and infliximab), and ustekinumab.*
bDMARDs, biologic disease-modifying antirheumatic drugs; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; IQR, interquartile range.

(6%) continued bDMARDs (i.e., adalimumab, certolizumab, and ustekinumab).

Effectiveness

Six months after apremilast initiation, 65% of patients (n = 15/23) presented complete response (CR) of joint involvement and 17% (n = 4/23) had partial response (PR), while 17% (n = 4/23) were non-responders (**Table 3**). Median TJC and SJC remained zero from M3 until the EOF. Among 22 patients at the EOF with joint involvement, 12 (59%) were complete responders, two (9%) partial responders and eight (36%) had no response.

Mucocutaneous response is shown in **Table 4**. The proportion of complete responders for oral and genital ulcers at M6 was 73% (n = 24/33) and 94% (n = 16/17), respectively. At the EOF, no response was seen in 25% (n = 7/28) of oral ulcers and 20% (n = 3/15) of genital ulcers. As for pseudofolliculitis, 71% (n = 10/14) were complete responders at M6, and remarkably no patient had non-response during follow-up. For the two patients with erythema nodosum, one had CR and the other PR at M6. Noteworthy, the only patient presenting ocular involvement at baseline experienced a complete resolution of his refractory keratitis.

The overall response for the whole cohort at M6 was 74% (CR = 48%, PR = 26%). Regarding specifically the mucocutaneous-articular cluster (n = 27), the overall response at M6 was 70% (CR = 30%, PR = 40%). Median BSAS significantly decreased from baseline to EOF (50 [40-60] *vs.* 20 [0-40]; $p < 0.0001$). Among BS patients on steroids, median daily dose of prednisone significantly decreased from baseline to EOF (6 [5-15] *vs.* 5 [5-9] mg; $p = 0.021$). Two (14%) patients discontinued corticosteroids.

A total of 14 patients (28%) experienced BS relapses while on apremilast. Six of them had isolated mucocutaneous reactivations, five presented articular and mucocutaneous concomitant flares, and three experienced exclusively articular activity. Median time to relapse was 6 [4-11] months. A patient who had been presenting complete mucocutaneous response until then developed an unprecedented ileitis after the sixth month of treatment. No other major organ involvement was observed during the study period.

TABLE 3 | Effectiveness of apremilast on articular manifestations.

	Baseline	M3	M6	EOF
n	30	27	23	22
Complete response, n (%)	–	17 (63)	15 (65)	12 (55)
Partial response, n (%)	–	3 (11)	4 (17)	2 (9)
TJC, median [IQR]	4 [2–6]	0 [0–3]	0 [0–2]	0 [0–3]
SJC, median [IQR]	2 [1–2]	0 [0–0]	0 [0–0]	0 [0–0]

EOF, end of follow-up; IQR, interquartile range; SJC, swollen joint count; TJC, tender joint count.

TABLE 4 | Effectiveness of apremilast on mucocutaneous manifestations.

	M3	M6	EOF
Oral ulcers			
n	41	33	28
Complete response, n (%)	26 (63)	24 (73)	15 (54)
Partial response, n (%)	12 (29)	8 (24)	6 (21)
Genital ulcers			
n	20	17	15
Complete response, n (%)	15 (75)	16 (94)	11 (73)
Partial response, n (%)	3 (15)	1 (6)	1 (7)
Pseudofolliculitis			
n	16	14	11
Complete response, n (%)	11 (69)	10 (71)	7 (64)
Partial response, n (%)	4 (31)	4 (29)	4 (36)
Erythema nodosum			
n	4	2	2
Complete response, n (%)	3 (75)	1 (50)	1 (50)
Partial response, n (%)	0	1 (50)	0

EOF, end of follow-up.

Safety

Apremilast was discontinued in 23 patients (46%). Treatment interruption was mainly due to side effects (n=15, 30%), and treatment failure (n=8, 16%) (six relapses, one lack of response, and one disease progression). Six patients (12%) presented an early intolerance, with median time to treatment interruption of 7 [5-9] days. Among all AEs requiring discontinuation, gastrointestinal disorders, headache, and sleep disorder were the most frequent, reported in 10 (67%), nine (60%), and three (20%) patients, respectively.

Apremilast dose reduction was tried in seven (14%) patients presenting poor tolerance to conventional dosage despite good initial response. After a median follow-up of 11 [6-13] months for the entire cohort, 27 (54%) patients were still on apremilast.

Thirty-three (66%) patients experienced adverse events (AE), with median time to onset of 4 [1-4] weeks. Most common side effect included diarrhea (n = 19, 38%), followed by nausea (n = 17, 34%) and headache (n = 16, 32%). Adverse events frequency is detailed in **Table 5**. Two (4%) patients experienced suicidal ideation leading to treatment discontinuation, with one of them being hospitalized for its management. Moreover, four (8%) patients experienced infections, namely mycobacteria reactivation, cat scratch disease, herpes simplex, and an acute gastroenteritis. None of them were on concomitant DMARDs.

DISCUSSION

This multicentric study reports the largest real-life cohort of patients with BS treated with apremilast. The main conclusions drawn are: 1) 65% of BS patients with refractory joint manifestations at 6 months had a complete response; 2) Discontinuation rates were three times higher than that reported in clinical trials; and 3) BS patients with refractory skin disease respond to apremilast.

Management of mucocutaneous and articular symptoms in BS can be challenging. Current recommendations place colchicine as the first-line option, followed by several DMARDs, such as azathioprine, thalidomide, interferon-alpha and tumor necrosis factor inhibitors for refractory cases (8, 18). While some of these drugs have conflicting results in terms of efficacy, others have safety concerns, making management even

TABLE 5 | Adverse events during apremilast treatment.

≥ 1 Adverse events, n (%)	33 (66)
Number of adverse events, median [IQR]	2 [1–3]
Time to onset, median [IQR] weeks	4 [1–4]
Adverse events leading to discontinuation, n (%)	15 (30)
Adverse events leading to hospitalization, n (%)	1 (2)
Adverse events frequency	
Diarrhea, n (%)	19 (38)
Nausea, n (%)	17 (34)
Headache, n (%)	16 (32)
Abdominal pain, n (%)	10 (20)
Sleep disorder, n (%)	9 (18)
Fatigue, n (%)	5 (10)
Infection, n (%)	4 (8)
Suicidal ideation, n (%)	2 (4)
Depression, n (%)	2 (4)
Anorexia, n (%)	2 (4)

IQR, interquartile range.

more difficult (7). With the increasing availability of bDMARDs, new targets have been assessed in BS recently. Ustekinumab – a monoclonal antibody targeting interleukin-12 and -23 – was evaluated in a prospective, open-label study, showing promising results in mucocutaneous and articular manifestations resistant to colchicine in BS (19). In a retrospective study, the anti-interleukin-17 secukinumab was evaluated in the mucocutaneous-articular cluster refractory to initial treatment, revealing itself as a potential alternative in this subgroup (20). Herein, we report encouraging data on apremilast for BS refractory mucocutaneous-articular phenotype, notably regarding joint disease. The proportion of patients experiencing articular improvement at M6 was up to 82%, with 65% of complete responders. After the first 6 months of treatment, 64% of BS patients were still being improved. So far, only one small study reported articular outcomes in 14 BS being treated with apremilast. A complete response was obtained in 28% of cases over a 3-month period (16). In contrast, apremilast's efficacy has been better described in psoriatic arthritis (PsA). Similar to our results, a real-life PsA cohort showed that 61% of patients were responders at 6 months (21). Another real-life study with 131 PsA patients highlighted 40% of remission or low disease activity at 3 months and a drug-retention rate of 72% at 6 months (22). In a pooled analysis from clinical trials using the American College of Rheumatology (ACR) response criteria, 55% and 26% of PsA patients receiving apremilast maintained an ACR20 and ACR50 response at 1 year, respectively (23). In clinical trials, 72% of PsA patients were still on apremilast after a year. Despite its superiority against placebo, apremilast is reported to have low to moderate efficacy when compared to other bDMARDs in active PsA (24). The greater efficiency highlighted in our study may lie in the fact that BS presents with milder articular features (e.g., absence of bone erosions, arthralgia rather than arthritis).

In the absence of phase IV studies, the long-term safety of apremilast is unknown in BS. In BS clinical trials, most patients (71%–91%) experienced at least one adverse event (11, 12). Along this line, we found a similar frequency of AE (66%). Despite this high rate, AEs leading to discontinuation in BS controlled studies did not exceed 11% (11, 12). Strikingly, 30% of our patients interrupted apremilast owing to poor tolerance, of which 12% discontinuation as of one week. Another 16% ceased treatment due to failure, which is also higher than the 2%–7% seen in phase II/III placebo-controlled studies (11, 12). Indeed, a gap between clinical trials and real-life studies has been noted in other apremilast label indications. In PsA, pooled data from phase III trials reported withdrawal due to AE in 7.6% of patients over a 1-year period (25). Conversely, real-life studies have demonstrated higher rate of apremilast discontinuation ranging from 20% to 38% (21, 26). This contrast seems less pronounced in psoriasis, as 3-year pooled trial data showed 11% of AE resulting in discontinuation (27), whereas in real-life cohorts this rate varied between 16% and 19% (28, 29). Interestingly, a network meta-analysis evaluating safety among 12 different bDMARDs in PsA pointed out apremilast as the only medication with significantly higher chance of withdrawal due to AE (30).

Regarding the type of AE, a similar profile was reported in BS, PsA or psoriasis studies, with diarrhea, nausea, and headache accounting for the most common events (11, 12, 14, 15, 25, 27). Although gastrointestinal side effects represented the leading symptom motivating discontinuation in our cohort, two patients (4%) interrupted apremilast because of suicidal ideation. This serious AE has been consistently reported in post-marketing surveillance and continued pharmacovigilance is warranted (31). Upper respiratory tract infection has been the most reported infection in association to apremilast. Although we did not find any cases of it, 8% presented infections in our study, notably one mycobacterial reactivation. So far, no case of mycobacterial infection has been reported under apremilast in BS. In a large database cohort evaluating immunosuppressants infectious risk among psoriasis and PsA patients, only two tuberculosis codes were identified concomitantly to apremilast prescription over a follow-up of 12,842 person-years (32).

Our study highlighted apremilast's effectiveness in BS refractory skin disease. Patients with pseudofolliculitis achieved a sustained a complete response in nearly 70% over the study period. Phase III placebo-controlled study did not show efficacy of apremilast in BS skin disease (12), and two case series reported contrasting responses (100% vs. 0%) (15, 16). In psoriasis skin lesions, the long-term benefit of apremilast has been well established. Over a 2-year period, up to 52% of psoriasis patients maintained ≥ 75% reduction in Psoriasis Area and Severity Index (PASI) score from baseline (33). As BS share several common features with psoriasis (34), it is not surprising that apremilast could also work for BS manifestations other than oral ulcers. Finally, we confirm the efficacy of apremilast on BS oral ulcers consistently with phase III trial results (12). When compared to these, we found slightly higher complete response rates at 3 months for oral (63% vs. 53%) and genital ulcers (75% vs 71%). Real-life case series had further confirmed this significant impact on oral ulcers and demonstrated a positive trend on genital ulcers in smaller samples (14–16).

Our study has some limitations. The continuation of systemic therapy (i.e., colchicine, DMARDs) at steady-state doses was possible during apremilast treatment, and as there was no protocol limiting its concomitant use, this could represent a potential confounder in effectiveness evaluation. Nevertheless, colchicine and DMARDs were already at their optimized dosage, and only two patients needed additional combination therapy during the study, being considered as non-responders. Moreover, compared to the phase III trial where only 50% of patients had been previously received colchicine (12), all of our patients were refractory to colchicine, DMARDs, and/or prednisone.

In conclusion, this nationwide multicenter cohort study shed new lights on the effectiveness and tolerability of apremilast in BS patients with refractory joint and mucocutaneous manifestations. Besides oral ulcerations, apremilast seems to improve refractory joint and skin manifestations in those who manage to persist on treatment. However, the discontinuation rate was high mainly for safety issues. This raises the question of whether this treatment can be used for long-term management of BS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

MVi, MVi, CC, and DS conceptualized this work. MVa, SuB, MVa, AL, YJ, MG-V, LB, EL, SaB, LM, DG, TG, OF, KS, and PS were involved in data collection and analysis. MVi wrote the initial draft of the manuscript. Authors contributed to the article and approved the submitted version.

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Different Disease Endotypes in Phenotypically Similar Vasculitides Affecting Small-to-Medium Sized Blood Vessels

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Objectives: Chronic primary vasculitis describes a group of complex and rare diseases that are characterized by blood vessel inflammation. Classification of vasculitis subtypes is based predominantly on the size of the involved vessels and clinical phenotype. There is a recognized need to improve classification, especially for small-to-medium sized vessel vasculitides, that, ideally, is based on the underlying biology with a view to informing treatment.

Methods: We performed RNA-Seq on blood samples from children (n = 41) and from adults (n = 11) with small-to-medium sized vessel vasculitis, and used unsupervised hierarchical clustering of gene expression patterns in combination with clinical metadata to define disease subtypes.

Results: Differential gene expression at the time of diagnosis separated patients into two primary endotypes that differed in the expression of ~3,800 genes in children, and ~1,600 genes in adults. These endotypes were also present during disease flares, and both adult and pediatric endotypes could be discriminated based on the expression of just 20 differentially expressed genes. Endotypes were associated with distinct biological processes, namely neutrophil degranulation and T cell receptor signaling.

Conclusions: Phenotypically similar subsets of small-to-medium sized vessel vasculitis may have different mechanistic drivers involving innate vs. adaptive immune processes. Discovery of these differentiating immune features provides a mechanistic-based alternative for subclassification of vasculitis.

Keywords: vasculitis, neutrophils, transcriptome, inflammation, ANCA

INTRODUCTION

Vasculitis (1) is a group of complex rare diseases that are characterized by inflammation in the blood vessel walls. The disease can present in childhood and in adulthood, and can be life- and/or organ-threatening. The primary framework for classifying vasculitis syndromes is according to the predominant size of the involved vessels (small, medium, large), the clinical phenotype (pattern of organs affected), and histopathology of involved vessels (2–5). Recently, distinctive etiological/pathological processes have been incorporated in the classification framework; for example, an association with anti-neutrophil cytoplasmic antibodies (ANCA) against intracellular granule proteins proteinase-3 (PR3) and myeloperoxidase (MPO) enables classification of small-to-medium sized, ANCA-associated vasculitis (AAV) (3, 4, 6, 7).

AAV encompasses three specific diseases: microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA). An absence of specific classification criteria for MPA and the considerable phenotypic overlap with GPA, however, makes it challenging to distinguish GPA and MPA (6–8). In adult clinical trials, they are frequently analyzed collectively for convenience (9), despite important clinical and biological differences that argue for tailored treatment. Specifically, some studies show that GPA has a more refractory and relapsing disease course than does MPA (10, 11), although in more recent studies the association with relapse seems to be stronger with the presence of PR3-ANCA. ANCA specificity (for PR3 or MPO) has been suggested as an alternative to the clinical phenotype classification, or as a "biomarker" in updated classification criteria for AAV. However, PR3-ANCA, despite being predominantly associated with GPA, is also present in one-quarter of patients with MPA. Similarly, MPO-ANCA is differentially, but not exclusively, associated with MPA (7, 12), and a proportion of patients with AAV do not have ANCA to either PR3 or MPO. It is also noteworthy that GPA and MPA have overlapping clinical features with polyarteritis nodosa (PAN) (a medium-sized vessel vasculitis), and other small-to-medium sized vessel vasculitides that remain "unclassifiable" according to existing classification criteria.

Here, we considered if classification of small-to-medium sized vessel vasculitides could be improved by a deeper understanding of the molecular events underlying the disease and distinct disease subsets. To explore this hypothesis, and despite the disease being especially rare in children compared to adults, we focused on a cohort of children and adolescents with small-to-medium sized vessel vasculitis for mechanistic discovery. The study of pediatric patients can be advantageous (13, 14); children have limited confounding disease comorbidities and may have more predominant genetic factors that lead to early disease manifestations, compared to adults that have multiple environmental factors contributing to the onset of disease, which, for vasculitis, typically occurs after 50 years of age. Using RNA-Seq on blood obtained from pediatric patients with different clinically defined subtypes of small-to-medium sized vasculitis, we were able to cluster patients into two groups with distinct transcriptomic profiles and associated immune processes. Individuals with adult-onset disease could also be categorized in a similar manner, together suggesting that both adult- and pediatric-onset small-to-medium sized vessel vasculitides within the same disease "category" due to overlapping clinical features, might have different endotypes (15, 16).

Abbreviations: AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; ARChiVe, A registry for childhood vasculitis; BVAS, Birmingham vasculitis activity score; DCVAS, diagnostic and classification criteria for vasculitis; DE, differentially expressed; EMA, European medicines agency; FDR, false discovery rate; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; MPO, myeloperoxidase; PAN, polyarteritis nodosa; PR3, proteinase 3; PVAS, pediatric vasculitis activity score; UCV, unclassified vasculitis.

MATERIALS AND METHODS

Participants

Patients described in this study were enrolled in the Pediatric Vasculitis Initiative (PedVas) (17, 18) and included children (18 yrs of age and younger) and adults with small-to-medium sized blood vessel vasculitis. Two pediatric cohorts were used for this study: Cohort 1 contained a total of 30 patients that contributed samples at diagnosis ($n = 25$) or relapse ($n = 5$); at the time of sample and data collection, disease activity was high (indicated by PVAS; see clinical data description) and this cohort was used for initial transcriptomic discovery. Pediatric Cohort 2 consisted exclusively of patients (n = 11) at relapse and was used to validate gene expression patterns observed in Cohort 1. Major relapse was defined as a new or recurrent appearance of life- or organ-threatening disease activity that occurs more than 18 months post diagnosis and requires a change in treatment. Adult participants with chronic primary vasculiltis were first enrolled in DCVAS, the Diagnostic and Classification Criteria for Vasculitis study.

Clinical Data

Physicians collected data from pediatric participants (see **Table 1** for Cohort 1 and **Supplementary Table 1** for Cohort 2) (12, 17, 18) and entered it into A Registry of Childhood Vasculitis (ARChiVe), the RedCap data collection platform for PedVas. Generation of a pediatric vasculitis activity score (PVAS) (19) was a component of data entry; active and inactive disease was defined as a PVAS of > 2 and ≤ 2, respectively. The subtype of vasculitis was determined by a pediatric modified algorithm of the European Medicines Agency (EMA). ANCA status was reported by the participating site and validated in serum samples using a standard ELISA for anti-PR3 antibody (ORG518, Orgentec) and anti-MPO antibody (425-2380, BioRad). For adult patients, clinical data (**Table 2**) were collected through DCVAS. All DCVAS clinical data was reviewed independently by a panel of experts 6 months after the baseline assessment to provide a definitive, agreed diagnosis in accordance with the DCVAS protocol. For patients with GPA, MPA, and EGPA, there was ∼25% rejection of the submitting physicians original diagnosis following the review.

RNA Sequencing and Analysis

Blood (2.5 ml) was collected from study participants in Tempus Blood RNA tubes (Applied Biosystems^{TM}, CA, USA) at the time of diagnosis or flare (flare; ≥18 months post diagnosis with a major change in the PVAS and sustained escalation of treatment) when disease activity was high (pediatric vasculitis activity score [PVAS] range 5–33). Extracted RNA (Tempus Spin RNA Isolation Kit, Thermo Fisher) underwent PolyA enrichment (NEBNEXT Poly(A) mRNA magnetic isolation kit, New England BioSciences), RNA-Seq library preparation (75bp or 100bp single end, KAPA Stranded total RNA kit, Roche) and was sequenced on an Illumina Genome Analyzer IIx or an Illumina HiSeq 2500. Fastq files were checked for quality (FastQC v0.11.8 and MultiQC v0.8) and aligned to the human genome (Ensembl GRCh38.93) using STAR v2.6 (20). HTSeq-count (HTSeq 0.61p1) was used to generate read count tables (21). Read counts from globin genes were removed bioinformatically and batch correction for sequencing date [shown to contribute to variance by the R package eigenR2 (22)] was performed using the ComBat function of the SVA package (23). Raw RNA-Seq counts were normalized for library size and heteroskedasticity by variance stabilizing transformation (vst). Differential gene expression analysis was performed using DESeq2 package v1.14.1 (24, 25). Pathway over-representation analysis was conducted in the ReactomePA v1.26.0 package for R (26) and network visualization was conducted using NetworkAnalyst (27). Differentially expressed genes identified by Grayson et al. (28) in nasal brushings from adults with GPA (compared to controls) were obtained and analyzed with ReactomePA. Entrez IDs and Ensembl IDs were then used to compare microarray probeset labels (28) to RNA-Seq data.

Statistics

Differential gene expression from DESeq2 analysis of RNA-Seq data was defined as a ≥ ± 1.5-fold change (FC) and ≤0.05 false discovery rate. Pathway enrichment analysis in ReactomePA used hypergeometric overlap and *p*-values were adjusted for multiple testing using false discovery rate. Significant enrichment was defined as a false discovery rate ≤ 0.05. Rotation gene set testing (ROAST) (29) and competitive gene set testing (CAMERA) (30) were used to determine whether the 20 gene signature, identified in pediatric patients at diagnosis, was also significantly different in pediatric patients in relapse and in adult patients. Clinical metadata (see **Supplementary Table 2**) were analyzed using the Description of Categories and Multiple Factor Analysis (MFA) functions from the FactoMineR package for R (31).

Data Availability

RNA-Seq data have been submitted to the Gene Expression Omnibus data sharing repository, and are accessible through GEO Series accession number: GSE129752.

RESULTS

Whole Blood Gene Expression Patterns Delineated Distinct Endotypes of Pediatric Small-to-Medium Sized Vessel Vasculitis

To identify the underlying molecules and pathways associated with different pediatric small-to-medium vessel vasculitides, we sequenced the whole blood transcriptomes of the 30 children and adolescents in Cohort 1. Study samples were collected from the majority at first disease onset and included EMA-defined subtypes of GPA ($n = 16$), MPA ($n = 4$), PAN ($n = 2$), unclassified ANCA-associated vasculitis (unclassified AAV; $n = 7$), and unclassified (ANCA-negative) vasculitis (UCV; $n = 1$). Unsupervised hierarchical clustering of global gene expression (without consideration of EMA classification) placed the samples into two major and one minor cluster (**Figure 1** and **Table 1**).

Distinct patterns of whole blood gene expression and a total of 3,809 genes were differentially expressed (± 1.5 FC, FDR ≤ 0.05) between the two major clusters. One major cluster (A) contained samples ($n = 13$) from 4 male and 9 female patients: 9 with GPA, and 4 with unclassified AAV (uAAV). PR3-ANCA were present in 9 of the 13 ANCA-positive individuals in this group. The other major cluster (B) contained samples ($n = 14$) from 5 male and 9 female patients: 5 with GPA, 4 with MPA, 2 with PAN, 2 with uAAV, and 1 with UCV. All patients with MPA and PAN were in this cluster and MPO-ANCA were present in 7 of the 13 ANCA-positive individuals in this group.

Patients in cluster A had greater overall disease activity (**Supplementary Table 2**: mean PVAS = 21, p-value = 0.013 compared to cluster B mean PVAS = 14, p-value = 0.006), and specifically, higher disease activity in the respiratory domain (mean chest PVAS = 4, p-value = 0.022). These children (cluster A) were also diagnosed at significantly older ages (mean age = 14, p-value = 0.015) and showed significantly greater neutrophil counts than patients in cluster B (mean neutrophil count in Endotype A = 10.4, p-value = 0.018) (**Supplementary Table 2** and **Supplementary Figure 1**).

These clusters (A and B) were consistent with the predominant EMA subtype in each cluster, that is, GPA in A and MPA/PAN in B, although overlap was observed especially for patients with GPA (**Figure 1** and **Table 1**). These data together with the distinct patterns of whole blood gene expression associated with each major cluster suggests that the four EMA-defined (phenotypically classified) subtypes of small-to-medium sized vessel vasculitis in our cohort may fall under two major endotypes, A and B. The final, minor cluster contained three samples; 2 from patients with GPA and one from a patient with uAAV, and they could conceivably represent a rarer endotype.

Neutrophil Degranulation and T Lymphocyte Activation Were Associated With Pediatric Vasculitis Endotype A and B, Respectively

Among the 3,809 differentially expressed (DE) genes between Endotype (cluster) A and Endotype (cluster) B, a total of 2,217 genes were expressed higher in Endotype A (relative to

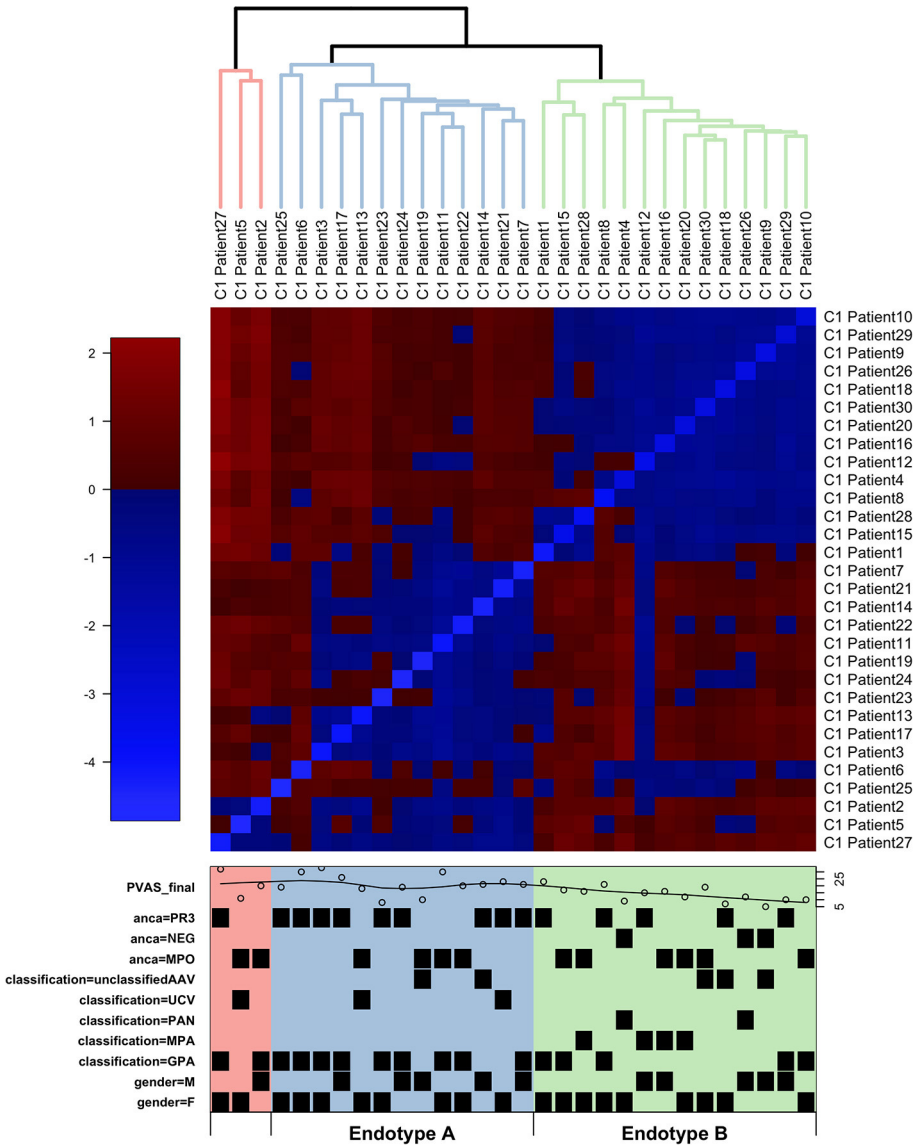

FIGURE 1 | Hierarchical clustering of RNA-Seq data from children with small-to-medium sized vessel vasculitis. Hierarchical clustering (blue lines, Endotype A; green lines, Endotype B; red lines, "other") and heatmap of normalized gene expression (variance stabilized counts) based on RNA sequencing of whole blood. Individual patient characteristics depicted in the lower box (also see **Table 1**): PVAS is the pediatric vasculitis activity score of disease activity at the time of sample collection; PR3 and MPO indicate positivity (or absence, NEG) for, respectively, anti-PR3 and anti-MPO antibodies; EMA classifications were uAAV, UCV, PAN, MPA, or GPA; M = male and F = female.

Endotype B) and 1,592 genes were expressed higher in Endotype B (relative to Endotype A). Genes with higher expression in Endotype A were involved in Toll-like receptor (TLR) signaling, interleukin signaling, Rho GTPase signaling, cellular senescence, and neutrophil degranulation (**Supplementary Figure 2** and **Supplementary Table 3A**). Within the latter pathway, 178 of the 479 known neutrophil degranulation genes (∼37%) were differentially expressed (FDR adjusted p-value for significance of association $= 1.1 \times 10^{-56}$). Functional interactions between the encoded proteins are shown in a protein:protein interaction network in **Figure 2**. Related, gene expression of biomolecules (including 16 histone genes, S100A8, S100A9, and PADI4) that are associated with neutrophil extracellular traps (NETs) (32) were higher (FDR < 0.05) in Endotype A compared to Endotype B.

In contrast, genes with elevated expression in Endotype B were associated with T cell receptor (TCR) signaling, RNA processing, interferon (IFN) signaling and the differentiation and regulation of T cells and NK cells (**Supplementary Figure 2** and **Supplementary Table 3B**). Dysregulated pathways in this cluster included: phosphorylation of CD3 and TCR zeta chains, PD-1 signaling, co-stimulation by the CD28 family, TCR signaling,

TABLE 1 | Characteristics and classification of Cohort 1 pediatric vasculitis patients.

ID	[a]Endotype	[b]EMA	[c]ANCA	Time	Sex	[d]Organ system involvement	[e]PVAS
C1 Patient 3	Endotype A	GPA	PR3	Diagnosis	Female	Skin, ENT, lung, CNS, MSK, renal	33
C1 Patient 23	Endotype A	GPA	PR3	Relapse	Female	ENT, lung, renal	8
C1 Patient 24	Endotype A	GPA	PR3	Relapse	Female	Skin, eye, ENT, lung, MSK, renal	19
C1 Patient 6	Endotype A	GPA	PR3	Diagnosis	Female	Skin, eye, ENT, lung, MSK, renal	30
C1 Patient 25	Endotype A	GPA	PR3	Diagnosis	Female	Lung, renal	19
C1 Patient 7	Endotype A	GPA	PR3	Diagnosis	Male	Skin, lung, MSK, renal	21
C1 Patient 17	Endotype A	GPA	PR3	Diagnosis	Male	ENT, lung, renal	26
C1 Patient 11	Endotype A	GPA	MPO	Diagnosis	Female	Skin, ENT, lung, renal	30
C1 Patient 22	Endotype A	GPA	MPO	Diagnosis	Female	Skin, lung, renal	20
C1 Patient 14	Endotype A	uAAV	PR3	Diagnosis	Male	Eye, ENT, renal	21
C1 Patient 21	Endotype A	uAAV	PR3	Diagnosis	Female	Lung, renal	23
C1 Patient 13	Endotype A	uAAV	MPO	Diagnosis	Female	Renal	18
C1 Patient 19	Endotype A	uAAV	MPO	Diagnosis	Male	Skin, ENT, lung	10
C1 Patient 10	Endotype B	GPA	MPO	Diagnosis	Female	Lung, MSK	10
C1 Patient 15	Endotype B	GPA	MPO	Diagnosis	Female	ENT, renal	17
C1 Patient 1	Endotype B	GPA	PR3	Diagnosis	Female	Skin, ENT, lung, renal	23
C1 Patient 8	Endotype B	GPA	PR3	Diagnosis	Female	Skin, lung, MSK, renal	21
C1 Patient 29	Endotype B	GPA	PR3	Relapse	Male	ENT, lung, renal	10
C1 Patient 20	Endotype B	MPA	MPO	Diagnosis	Female	Renal	12
C1 Patient 28	Endotype B	MPA	MPO	Diagnosis	Female	Skin, MSK, renal	16
C1 Patient 16	Endotype B	MPA	MPO	Diagnosis	Male	Skin, eye, renal	16
C1 Patient 12	Endotype B	MPA	PR3	Diagnosis	Male	Eye, renal	15
C1 Patient 4	Endotype B	PAN	NEG	Diagnosis	Female	Skin	9
C1 Patient 26	Endotype B	PAN	NEG	Diagnosis	Male	Skin, eye, ENT	12
C1 Patient 30	Endotype B	uAAV	MPO	Relapse	Female	Eye, renal	19
C1 Patient 9	Endotype B	UCV	NEG	Diagnosis	Male	Eye, MSK	5
C1 Patient 18	Endotype B	uAAV	PR3	Diagnosis	Female	ENT	7
C1 Patient 27	other	GPA	PR3	Diagnosis	Female	Skin, ENT, lung, MSK, renal	32
C1 Patient 2	other	GPA	MPO	Diagnosis	Male	Skin, lung, cardiac, renal	20
C1 Patient 5	other	uAAV	MPO	Relapse	Female	Lung	11

[a]Hierarchical cluster (Endotype A, light grey; Endotype B, dark grey) based on RNA-sequence analysis. [b]European Medicines Agency classification (GPA, blue; MPA, green; unclassified, white). [c]PR3 and MPO indicate positivity for, respectively, anti-PR3 (yellow) and anti-MPO (red) antibodies. NEG means that neither anti-PR3 nor anti-MPO antibodies were detected. [d]ENT = ear, nose, throat; MSK = musculoskeletal; CNS = central nervous system. [e]PVAS is the pediatric vasculitis activity score of disease activity at the time of sample collection.

and immunoregulatory interactions between lymphoid and non-lymphoid cells. Children in Endotype B had a moderate but significantly lower ratio of Th1:Th2 cells marker expression (TBX21:GATA3) (p-value = 0.038 by Wilcoxon test) compared to Endotype A (**Figure 3** and **Supplementary Figure 3**), and a much overall higher ratio of expression of the Th2 marker GATA3 (p-value = 3.0×10^{-6}). Individuals in Endotype B also had a higher expression ratio of the Treg marker FOXP3 (p-value = 0.014).

Endotypes Present at Diagnosis Also Defined Inflammatory Mechanisms at Relapse

To determine if the endotypes (and immunologic pathways) identified in Cohort 1 were associated with subsequent relapses in disease post diagnosis, we performed RNASeq on samples collected from a smaller cohort of pediatric patients (Cohort 2, n = 11) during a major disease relapse. Using unsupervised hierarchical clustering of RNASeq data as was done with Cohort 1, patients in Cohort 2 also sorted into two major clusters, 'Relapse cluster 1' and 'Relapse cluster 2'. Two outlier samples did not fall into either cluster and did not cluster with each other (**Supplementary Figure 4A**).

Relapse cluster 1 contained 4 patients: 2 with GPA, 1 with uAAV, and 1 with UCV. ANCA status was unknown for 1 patient and the remaining 3 patients were all positive for PR3-ANCA. Relapse cluster 2 contained 5 patients: 3 with GPA and 2 with MPA. This cluster contained 4 ANCA-positive patients: 3 with MPO-ANCA and 1 patient with both MPO- and PR3-ANCA. We noted that all patients (4/4) in relapse cluster 1 were female, and suggest that this is a reflection of Cohort 2 being small, and predominantly female (7/11 patients); a separation of sexes between Endotypes A and B was not observed in Cohort 1, which was larger and balanced between male and female patients.

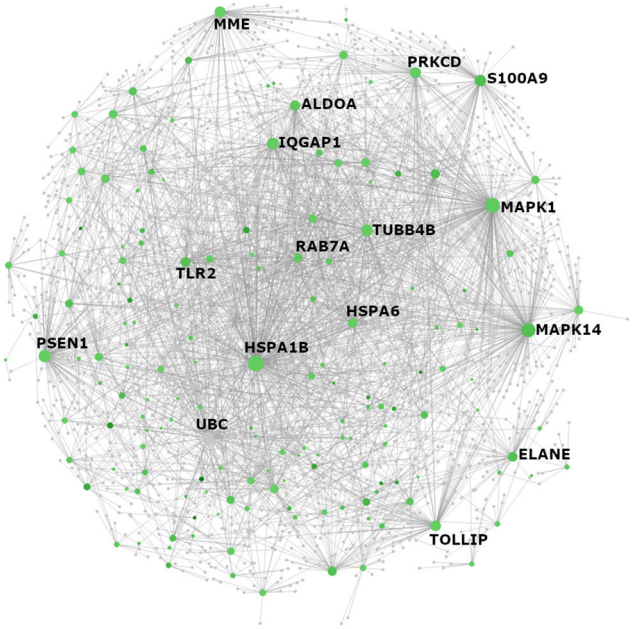

FIGURE 2 | NetworkAnalyst visualization of Reactome pathway "neutrophil degranulation" genes with elevated expression in individuals from Endotype A. All genes with significantly higher expression in Endotype A that appeared in the Reactome PA category "neutrophil degranulation" (R-HSA-6798695) were visualized as a protein:protein interaction network using NetworkAnalyst. The color of each node reflects fold change between Endotypes A and B where green indicates higher expression in Endotype A. Gray nodes are genes that are not themselves differentially expressed (DE) but interact with other DE neutrophil degranulation genes. The size of each node (gene) reflects the number of documented interactions with other DE genes in the network. Thus, the largest circles have the greatest number of DE interacting partners and are predicted to be the most important regulators ("hub proteins") of this network: inflammatory biomarker S100A8/A9, mitogen-activated protein kinases MAPK1 (ERK2) and MAPK14 (p38 MAPK), heat shock protein HSPA1B, the lysosome-associated glycoprotein LAMP2, tubulin TUBB4B, and Toll-like receptor protein TLR2 and its regulator/adaptor TOLLIP.

Using the more than 1,000 genes that were differentially expressed between relapse clusters 1 and 2, we performed Reactome analysis and compared pathway enrichment in the "relapse clusters", (**Supplementary Figure 4B** and **Supplementary Table 4**) to those in the "diagnosis clusters" (i.e., Endotype A and Endotype B from Cohort 1). Our results revealed an overlap in enriched pathways between relapse cluster 1 and Endotype A, and between relapse cluster 2 and Endotype B, suggesting that the same driving mechanisms (behind the distinct Endotypes) are present at diagnosis and disease relapse.

Pediatric Derived Molecular Pathways and Endotypes Defined Adult-Onset Small-to-Medium Sized Vessel Vasculitis

Unsupervised hierarchical clustering of RNASeq data from a small subset of adult vasculitis patients ($n = 11$, **Table 2**) also sorted into two distinct endotypes (Adult Endotype A and Adult Endotype B; **Supplementary Figure 5A**). Adult Endotype A contained 5 patients: 2 with MPA, 2 with GPA, and 1 with complex vasculitis (labeled COMP/GBM). Of these, 2 were positive for MPO-ANCA and 3 for PR3-ANCA. Adult Endotype B also included 5 patients: 1 with GPA, 1 with eosinophilic GPA (EGPA), 2 with MPA, and 1 with leucocytoclastic cutaneous vasculitis (labeled OSV). Three adult Endotype B patients were ANCA negative, 1 was positive for MPO-ANCA, and 1 was positive for PR3-ANCA. A total of 1,682 genes were differentially expressed between Adult Endotypes A and B. Similar to the pediatric endotypes, Adult Endotype A was enriched for neutrophil degranulation pathways, IL-4 and IL-13 signaling, and antimicrobial peptides, while Adult Endotype B was enriched for T cell receptor signaling, IL-2 signaling, CD28 dependent PI3K/AKT signaling, and translocation of ZAP-70 to the immunological synapse (**Supplementary Figure 5B**).

Due to the small cohort size, we also compared our results to a transcriptomic study by Grayson et al. (28) that identified 339 genes significantly DE in nasal brushings from adult GPA patients compared to healthy controls. Of these DE genes, 141 were highly expressed in pediatric Endotype A (Fisher's Exact test: p-value $= 2.2 \times 10^{-16}$, odds ratio $= 7.6$), and 62 were also highly expressed in Adult Endotype A (Fisher's Exact test: p-value $= 2.2 \times 10^{-16}$, odds ratio $= 5.2$). An overlap in Reactome pathways enriched in the nasal brushing dataset and Endotypes identified from our dataset were also observed (**Supplementary Figure 5C**).

Together, these findings indicate potentially common mechanisms between pediatric and adult vasculitis endotypes and suggest that gene signatures and disease processes in affected tissues may be evident in blood.

Differential 20-Gene Signature Defines Pediatric and Adult Vasculitis Subtypes

The differential gene expression patterns in Endotypes A and B, consistent in both the pediatric and adult cohorts, suggested fundamentally different disease mechanisms associated with each endotype. We therefore asked whether a small set of DE genes could reliably separate individual samples into the respective Endotypes (A and B). A 20 gene signature was identified from pediatric Cohort 1 (see **Figure 4** and Materials and Methods) that, using hierarchical clustering, placed all samples within the same pediatric Endotypes as the full RNA-Seq dataset (**Figure 4A** and **Supplementary Figure 6**).

This 20 gene signature, when applied to pediatric Cohort 2 (relapse samples; **Figure 4B**) and the adult cohort (**Figure 4C**), also separated samples into the same Endotypes A and B as the entire RNA-Seq dataset with the exception of only 2 samples from the relapse cohort. The application of gene set significance testing, using both ROAST and CAMERA, showed that this 20 gene signature was also significantly differentially expressed between Endotypes A and B at relapse (ROAST p-value $= 6.6 \times 10^{-03}$, CAMERA p-value $= 1.1 \times 10^{-03}$). Similarly, the gene sets were also highly significantly different between endotypes (ROAST p-value $= 4.10 \times 10^{-03}$, CAMERA p-value $= 7.72 \times 10^{-04}$) in the adult cohort. Among the 20 gene signature identified in both pediatric and adult RNA-Seq data, 25% (5/20) of these genes were differentially expressed (28) in nasal brushings from adults with GPA.

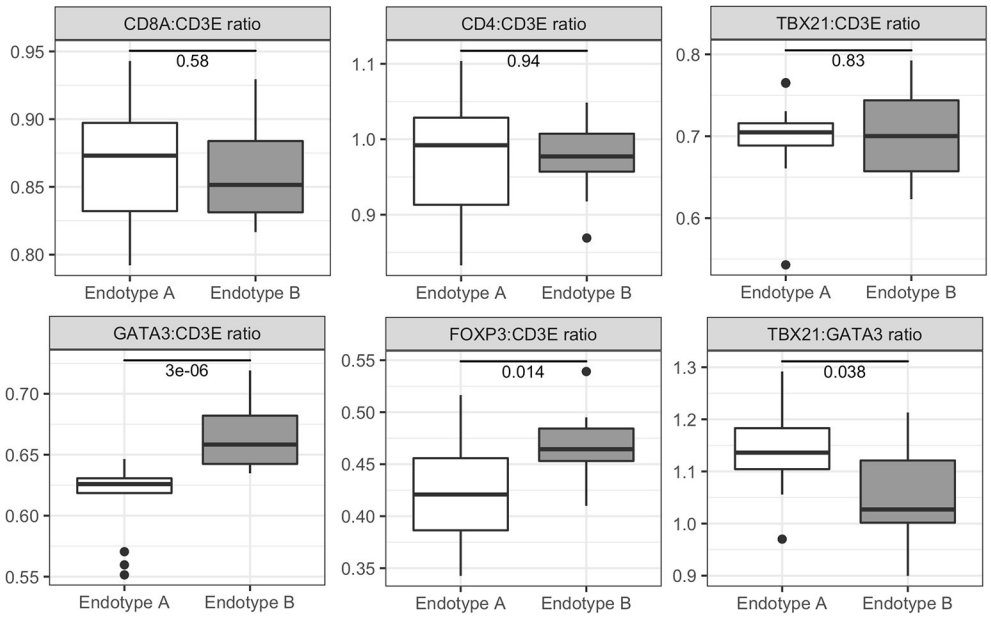

FIGURE 3 | Expression ratios of T cell related genes for pediatric vasculitis Endotypes A and B. Relative abundance and ratios (y-axis) of the expression of genes for T cell markers in Endotype A and Endotype B patients (x-axis). T cell marker genes included CD3E (present in all T cells), CD8A (CD8+ cells), CD4 (CD4+ cells), TBX21 (Th1 cells), GATA3 (Th2 cells), FOXP3 (Tregs), and RORC (Th17 cells). Gene expression ratios were calculated from variance stabilized counts. Significance of the ratio between the clusters is reported within each boxplot and was determined by the Wilcoxon Rank Sum test. Additional data shown in **Supplementary Figure 3**.

TABLE 2 | Characteristics and classification of adult vasculitis patients.

ID	[a]Cluster	[b]Physician diagnosis	[c]ANCA	Sex	[d]Organ systems involved	[e]BVAS
Adult Patient 2	Adult Endotype A	GPA	PR3	Female	Systemic, skin, eyes, ENT, chest, abdominal, renal	45
Adult Patient 5	Adult Endotype A	GPA	PR3	Male	ENT, chest, renal	18
Adult Patient 3	Adult Endotype A	COMP/GBM	PR3 and GBM	Male	Systemic, ENT, renal	19
Adult Patient 4	Adult Endotype A	MPA	MPO	Female	Systemic, skin, abdominal, renal	26
Adult Patient 6	Adult Endotype A	MPA	MPO	Male	Systemic, skin, renal	17
Adult Patient 8	Adult Endotype B	MPA	PR3	Male	Lungs, kidneys	16
Adult Patient 9	Adult Endotype B	MPA	MPO	Female	Systemic, skin, eyes, abdominal, chest, renal	31
Adult Patient 7	Adult Endotype B	GPA	NEG	Male	Lungs	5
Adult Patient 10	Adult Endotype B	OSV	NEG	Male	Systemic, skin	9
Adult Patient 11	Adult Endotype B	EGPA	NEG	Male	ENT, chest, abdominal, neurological	25
Adult Patient 1	other	MPA	MPO	Male	Systemic, renal, ENT	18

[a]Hierarchical cluster based on RNA-sequence analysis (Endotype A, light grey; Endotype B, dark grey). [b]Classification by expert consensus: (GPA, blue; MPA, green; EGPA, COMP and OSV are white) COMP/GBM = complex, anti-glomerular basement membrane disease, OSV = other small vessel vasculitis (leucocytoclastic cutaneous vasculitis). [c]PR3 and MPO indicate positivity for, respectively, anti-PR3 (yellow) and anti-MPO (red) antibodies. NEG means that neither anti-PR3 nor anti-MPO antibodies were detected. [d]ENT = ear, nose and throat. [e]BVAS is the Birmingham vasculitis activity score of disease activity.

DISCUSSION

Despite step-wise improvements in classification criteria for small-to-medium sized vessel vasculitis, the ability to use current classification systems to accurately diagnose, prognosticate and tailor treatment remains limited due to overlapping clinical features, unclassifiable patients and variable disease trajectories/outcomes of patients classified as having the same disease. For pediatric patients, classification is even more challenging. Even relatively recent pediatric adaptations of the American College of Rheumatology (ACR) criteria (originally based on adult data) (3, 4) fail to uniquely classify 25% of pediatric patients (12).

In this study, we investigated a biological basis to differentiate small-to-medium sized vessel vasculitis in children. Our results

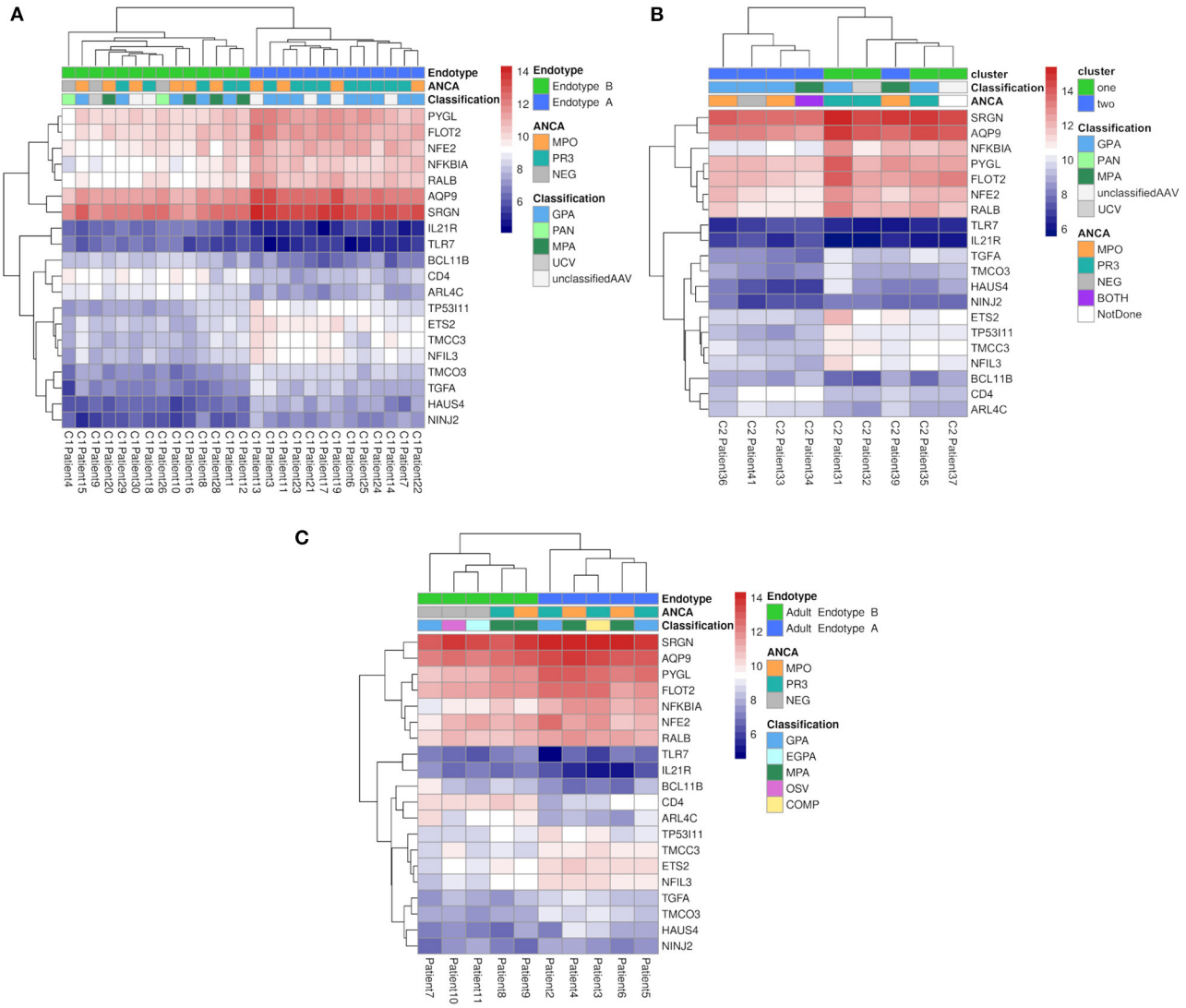

FIGURE 4 | Hierarchical clustering of vasculitis patients based on expression data from 20 genes. Hierarchical clustering (hclust function of R) was performed using the average linkage method with Euclidean distances calculated from vst counts. The variance for all genes (as batch corrected vst counts) was calculated separately for major clusters. Genes with the lowest within-group variance, an absolute FC ≥ 2 between the clusters, the 50% least variable genes in both clusters, and an overall average count of at least 100 were selected and assessed for their ability to separate samples into the same clusters as the entire gene dataset. The heatmap represents expression (variance stabilized counts) of each of 20 genes (y-axis, right) that were (a) maximally divergent between Endotypes A and B (i.e.: significantly DE with a FDR adjusted *p*-value <0.05 and fold change >1.5) and (b) had low variance within each cluster to minimize the margin of error for testing individual samples. **(A)** Pediatric Cohort 1 patients ($n = 27$, x-axis) in Endotypes A (blue) and B (green). **(B)** Pediatric Cohort 2 patients ($n = 9$, x-axis) in relapse Endotypes A (blue) and B (green). **(C)** Adult patients ($n = 10$, x-axis) in Adult Endotypes A (blue) and B (green).

demonstrate that the majority of patients fell into two primary groups with distinctive gene expression patterns and clinical phenotypes that were predominantly, but not exclusively, either GPA or MPA. The groups differed in the expression of approximately one-third of all expressed human genes and could be discriminated based on the differential expression of just 20 "biomarker" genes. These transcriptome-based signatures, which were elucidated from predominantly disease-onset (diagnosis) pediatric samples, were also found in a small cohort of pediatric patients experiencing a relapse in disease, and in a small cohort of adult patients at diagnosis of a variety of clinically-defined vasculitides affecting small-to-medium sized vessels.

The concept that mechanistic differences may enable separation of complex diseases into different "endotypes" has been demonstrated in other chronic inflammatory diseases, for example, asthma (15) and diabetes (16) that have a singular "disease category" yet intrinsic heterogeneity in symptoms and outcomes. In our cohort, the transcriptome-defined endotypes were not significantly associated with MPO/PR3 ANCA positivity or EMA classification for GPA, indicating

differentiating biological factors between endotypes that are not apparent based on current clinical classification or ANCA status. Of interest, unbiased cluster analysis of clinical metadata, including data used in the EMA classification algorithm, organized patients in our cohort into two "new" clinically-defined groups (**Supplementary Figure 7**) that had substantial overlap with RNASeq data-defined endotypes. In one clinical cluster, 10/13 patients were associated with Endotype A (correlation analysis p-value = 0.0018), and in the other clinical cluster, 12/17 patients were associated with Endotype B (correlation analysis p-value = 0.0037). Although a much larger cohort is required for validation, the data suggest that these underlying biologic mechanisms (endotypes) might each associate with a unique clustering of patients according to clinical symptoms.

Endotype A was associated with pathways reflecting neutrophil degranulation while Endotype B demonstrated a gene expression pattern indicative of T cell activation. In AAV, both innate and adaptive immune processes are, as the name suggests, involved in disease pathogenesis (i.e., by the action of autoantibodies against neutrophil proteins). Our evidence however, suggests that these different arms of the immune system predominate in different subsets of patients, as opposed to operating in concert across all individuals regardless of subtype, and seemingly independent of ANCA specificity.

Neutrophil degranulation, a common feature of many inflammatory disorders, including severe asphyxia in asthma, acute lung injury, rheumatoid arthritis, and septic shock (32), was associated with Endotype A. The gene encoding a protein from the neutrophil degranulation pathway, glycogen phosphorylase L (PYGL), was one of the top 20 most significantly DE genes between the endotypes (p-value = 1.85×10^{-07}, mean fold change of genes in pathway = 2.17). Consistent with the role of neutrophil degranulation in lung disease, Endotype A patients had higher component PVAS for respiratory involvement (chest score: **Supplementary Table 2**). In contrast, there was no significant association between either endotype and renal-specific PVAS despite a large majority of patients with GPA and MPA having renal involvement. Moreover, pulmonary disease, and specifically, the involvement of granulomatous inflammation, occurs more frequently (twice as often) in GPA compared to MPA. Neutrophil degranulation can lead to neutrophil extracellular traps (NETs) (32, 33) that play a role in the capture and killing of bacteria and have been described in adult AAV (34, 35). NETs contain a variety of biomolecules (36) including DNA, histone proteins, S100 proteins, MPO and PR3. Consistent with the role of NETs in bacterial infections, genes involved in the recognition and uptake of bacteria, including TLR- and NOD-like receptor signaling, and FcγR-mediated phagocytosis were enriched in Endotype A; notably FcγRIIA and the phagocytic pathway downstream of this receptor were significantly (p-value = 0.025) enriched in Endotype A.

Endotype B contained all MPA/PAN patients, which is noteworthy given that MPA and PAN, prior to the identification of ANCA, were considered manifestations of the same disease in different sized blood vessels. Patients within Endotype B differentially expressed many genes associated with T cell receptor (TCR) signaling and the differentiation and regulation of T cells and NK cells. T cell surface protein CD4 was one of the top 20 DE genes showing higher expression in Endotype B. The expression of genes in the MHC class II pathway responsible for $CD4^+$ T helper cell activation were also elevated. The MHC class II pathway can be triggered either by endocytosed antigens or misfolded proteins (autoantigens) as is the case with certain types of arthritis (37, 38). These are interesting observations, since Th2 and Treg cells are involved in the prevention of autoimmune diseases (39), suggesting that the mechanism of pathogenesis in Endotype B might trigger immune regulatory responses to a greater extent. Given that specific Th2 populations can play a role in several inflammatory diseases [e.g., ulcerative colitis (40), chronic allergic inflammation (41) and eosinophilic gastrointestinal disease (42)], inflammation in patients associated with this Endotype could also be driven by a similar mechanism involving highly activated T cells, and an imbalance of Th2 cells that is pathogenic.

Our results also demonstrated that pediatric gene expression patterns involving neutrophil degranulation and T cell activation were present in blood samples from adults with vasculitis and showed significant overlap with findings from a published study of gene expression in nasal tissue from adults with GPA (28). The latter observation is consistent with the notion that inflammatory mechanisms in AAV are independent of the organ systems involved (43).

It is important to note that therapy was initiated in the majority of patients prior to sample collection and many treatments, notably corticosteroids, can influence gene expression. The nature of treatment, dose and route of administration varied considerably among patients and was consistent with our previous report of treatment variability in clinical practice (44). Despite this, it should be noted that no association was observed between endotype and receipt of corticosteroids by multi-factor analysis (31) (**Supplementary Table 2**). In contrast, we found a significant correlation between patients in Endotype B and the receipt of non-biologic immunosuppressive treatment. However, it would be difficult to conclude that these treatments are driving differences in gene expression between groups given that factors other than the nature of the disease, including cost, accessibility of drugs, and physician experience influence treatment choice (44). It is also equally likely that inflammatory mechanisms, as observed by differences in gene expression, respond to different pharmacological agents, leading to improved patient outcomes and preferred use by physicians.

In summary, we have classified pediatric and adult patients with small-to-medium sized vessel vasculitis (AAV, PAN and unclassifiable disease) into two distinct endotypes based on whole blood gene expression profiling. These data may argue for categorization of vasculitides based on biologic mechanism, however it remains to be proven if transcriptome-defined groups have clinical utility. Exploration of links between treatment outcomes particularly relapsing or refractory disease, and the pathogenic mechanisms identified by transcriptomics, will be the subject of future analyses of pediatric and adult vasculitis patients.

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.

ETHICS STATEMENT

The study protocol was approved by the Children's and Women's Research Ethics Board of the University of British Columbia [H12-00894] and the respective ethical committees or IRBs at participating PedVas sites. Written informed consent and assent/consent, respectively, was obtained from parents and from pediatric patients. Adults with chronic primary vasculitis were first enrolled in DCVAS, the Diagnostic and Classification Criteria for Vasculitis study. The DCVAS study consent form explicitly included an invitation for patients to participate in other research studies, subject to the patients consenting to those other studies, as was the case with inclusion of any adult samples in the pediatric vasculitis study.

AUTHOR CONTRIBUTIONS

EG and MS: bioinformatics analysis and manuscript preparation. KG and AL: data analysis and final revision of manuscript. RF: RNA sequencing. CR and JG: experimental design and final revision of manuscript. DF, SB, and KM: clinical care of patients, clinical data collection and entry, and final revision of the manuscript. PedVas Initiative Investigators: clinical care of patients and clinical data collection and entry. RL: clinical care of patients, clinical data collection and interpretation, and final revision of the manuscript. DC: study design, clinical care of patients, clinical data collection and interpretation, and final revision of the manuscript. RH: study design and bioinformatics data oversight and interpretation and manuscript preparation. KB: study design, sample collection, and clinical and bioinformatic data interpretation and manuscript preparation. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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The Immunopathology of Giant Cell Arteritis Across Disease Spectra

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Giant cell arteritis (GCA) is a granulomatous systemic vasculitis of large- and medium-sized arteries that affects the elderly. In recent years, advances in diagnostic imaging have revealed a greater degree of large vessel involvement than previously recognized, distinguishing classical cranial- from large vessel (LV)- GCA. GCA often co-occurs with the poorly understood inflammatory arthritis/bursitis condition polymyalgia rheumatica (PMR) and has overlapping features with other non-infectious granulomatous vasculitides that affect the aorta, namely Takayasu Arteritis (TAK) and the more recently described clinically isolated aortitis (CIA). Here, we review the literature focused on the immunopathology of GCA on the background of the three settings in which comparisons are informative: LV and cranial variants of GCA; PMR and GCA; the three granulomatous vasculitides (GCA, TAK, and CIA). We discuss overlapping and unique features between these conditions across clinical presentation, epidemiology, imaging, and conventional histology. We propose a model of GCA where abnormally activated circulating cells, especially monocytes and $CD4^+$ T cells, enter arteries after an unknown stimulus and cooperate to destroy it and review the evidence for how this mechanistically occurs in active disease and improves with treatment.

Keywords: vasculitis, CIA, LVV, Takayasu, PMR, temporal arteritis, GCA, giant cell arteritis

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INTRODUCTION

Giant cell arteritis (GCA) is a granulomatous systemic vasculitis of people age 50 or older that affects large- and medium-size arteries (1, 2). Vascular inflammation has two major patterns, which overlap in a clinical spectrum. The first and classic pattern, originally described by Horton in 1932, involves inflammation of the extracranial branches of the carotid artery with predilection for the temporal artery and is called cranial GCA. The second pattern involves the aorta and its proximal branches, particularly the axillary, subclavian, and proximal brachial branches, and is called large-vessel GCA (LV-GCA) (3). While autopsy studies in the 1970s demonstrated LV involvement in patients with cranial-GCA (4, 5), advances in imaging in the past decade have reemphasized the frequent co-occurrence of subclinical LV with cranial disease and have identified the less common entities of isolated cranial- and LV-GCA (6). Along with Takayasu arteritis (TAK), a systemic vasculitis that occurs mostly in women under age 50, and clinically isolated aortitis (CIA), a vasculitis restricted to the aorta, GCA is one of three non-infectious granulomatous vasculitides with prominent aortic involvement (1).

GCA is medical emergency due to its ability to cause irreversible vision loss and requires prompt diagnosis and initiation of treatment. Individual patient presentations vary depending on the complement of cranial or large vessels that are involved, yet patients often share common systemic

features. These include laboratory evidence of systemic inflammation, constitutional symptoms, and polymyalgia rheumatica (PMR), a condition characterized by pain and stiffness in the neck, shoulders, and pelvic girdle that often co-occurs with GCA (7, 8). Mechanistic understanding of both GCA and PMR has been limited by the lack of consensus diagnostic criteria. However, GCA is better characterized than PMR due to the historical *de facto* diagnostic gold standard being temporal artery biopsy (TAB), which has created a more homogenous clinical group and also provided a vital source of tissue for research purposes. Immunosuppression with glucocorticoids (GC) is the cornerstone of treatment for both GCA and PMR. As most patients have disease flares with GC tapering and require prolonged treatment, steroid sparing agents have been sought, with methotrexate identified as providing benefit in PMR and likely some in GCA, and targeted blockade of IL-6R with tocilizumab (TCZ) providing benefit in GCA. Multiple other drugs are being studied in clinical trials in GCA (9–12).

Here, we review the current understanding of the immunopathology of GCA on the background of the three settings in which comparisons are informative: LV and cranial variants of GCA; PMR and GCA; and the three granulomatous vasculitides (GCA, TAK, and CIA). We also discuss clinical presentation and epidemiology of disease, and the growing role of advanced imaging for clinical and research use. We identify areas of uncertainty and discuss possible mechanisms of disease pathogenesis.

CLINICAL PRESENTATION

Systemic inflammation is a cardinal feature of GCA, as well as PMR and TAK. Clinically, many patients experience non-specific constitutional symptoms including fatigue, anorexia, weight loss, fever, and night sweats. Laboratory evidence of inflammation includes anemia, thrombocytosis, and elevations in the inflammatory markers erythrocyte sedimentation rate (ESR) and/or C-reactive protein (CRP). Patients with CIA lack systemic features, according to the most commonly used definition of CIA (7, 8, 13, 14).

Cranial symptoms of GCA are the classic presentation of disease and account for the majority of the 1990 ACR classification criteria (7). Inflammation of medium-size arteries causes pain and tenderness in the artery wall itself and leads to vascular stenosis and ultimately occlusion, causing symptomatic ischemia. Ischemic symptoms include headache, jaw claudication, and acute onset visual disturbances (7), and are inversely correlated with the degree of systemic inflammation (15, 16). More rarely, scalp or tongue necrosis, sensorineural hearing loss, and even vertebrobasilar stroke can occur. The most commonly feared complication is irreversible vision loss, which occurred in 15–35% of patients prior to widespread recognition of GCA and emergent use of GC (2, 17, 18).

LV-GCA often presents with non-specific systemic symptoms, leading to delayed diagnosis (19, 20). Features suggestive of LV-GCA in patients with PMR include the need for unusually high doses of GC, bilateral diffuse lower extremity pain, pelvic girdle pain, and inflammatory low back pain (20). LV-GCA can also cause ischemic symptoms corresponding to supra-aortic vessel stenosis with resultant limb claudication or dizziness. Physical signs can include vascular bruits, loss of carotid or radial pulses, and/or discordant blood pressures (19, 21). These overlap with the symptoms and classification criteria for TAK (13). Rather than causing ischemia in downstream organs, inflammation of the aorta under the stress of high-pressure gradients generated by the heart leads to dilatation in 32% of patients with GCA, aneurysm formation in 2–10% patients, and ultimately may progress to dissection (22–24). Thus, LV-GCA is typically identified on imaging or in surgical specimens from repairs of aneurysms or dissections. In the case of surgical tissue, GCA must further be differentiated from CIA by evidence of systemic features or evidence of disease in arteries other than the aorta.

EPIDEMIOLOGY

GCA is the most common form of vasculitis in patients over age 50 with most being much older. PMR is 3–10 times more common than GCA and is the second most frequent rheumatic disease of elderly after rheumatoid arthritis (2). Forty–sixty percent of patients with GCA have symptoms of PMR while 16–21% PMR have GCA (25, 26). Age >50 is a defining feature of both GCA and PMR, and both peak around age 75, with the exception that patients with LV-GCA are typically younger between 50 and 65 (2, 3, 24, 27, 28). Other granulomatous vasculitides affecting the aorta also occur earlier; CIA has a mean diagnosis of age 65 while TAK peaks between 15 and 29 (14, 29). All conditions are more common in women, with increasing frequency from CIA and PMR (2:1), to cranial-GCA (almost 3:1), to LV-GCA (3:1), and finally to TAK (9:1) (3, 14, 26–30).

The incidence of cranial-GCA and PMR is most frequent in patients of Northern European ancestry. Overlapping incidence of GCA between Northern Europe at 14.6–43.5/ 100,000 and the ancestrally similar Olmstead County, Minnesota at 19.8/ 100,000 suggest a genetic predisposition (26, 27). In other populations, GCA occurs between 1.1 and 11.1/100,000 (26, 31–33), though there are no studies from Africa, South America, or the majority of continental Asia and the Middle East. It was previously thought that GCA was uncommon in African Americans (31). However, this has not consistently been shown in the literature, likely reflecting the ancestral heterogeneity within racial groups within the United States and perhaps under recognition of GCA in African Americans due to the misconception they are not affected (34–38). TAK is less common than GCA, with highest incidence in Asia, South America, and Turkey at 1–2/1,000,000 (39). The true incidence and demographics of LV-GCA and CIA are unknown but appear to be intermediate between cranial-GCA and TAK, at least in the United States (40).

While the increased frequency of GCA in patients of Northern European ancestry suggests a genetic predisposition, genetic studies have generated limited insight into pathophysiology of disease. An early reported and consistently reproduced finding is the association with MHC class II HLA DRB04, specifically

the *0401 and *0404 alleles, with cranial- and LV-GCA as well as PMR (3, 41–44). Indeed, large immune-focused genotyping arrays performed on patients with TAB-confirmed cranial-GCA and TAK identified the HLA locus as the only locus to achieve genome-wide significance for association with GCA, and one of two loci with genome-wide significance in TAK (45, 46). In GCA, the majority of this association was due to HLA-DRB1 and HLA-DQA1, with a minor contribution from MHC class I HLA-B. The opposite pattern was found for TAK (47). Strong class II associations suggest a key role for antigen presentation by MHC class II to helper $CD4^+$ T cells in GCA, and multiple studies have suggested changes to the MHC class II peptide-binding groove, however, the specific antigens recognized by $CD4^+$ T cells in GCA remain unclear (41, 46). Likewise, TAK has more cytotoxic $CD8^+$ T cell infiltration than GCA that may explain its association with class I (48). When data from GCA and TAK studies were combined in a meta-analysis, the only non-HLA SNP that reached significance was in IL12B, encoding the p40 portion of the IL-12 (p35p40)/IL-23 (p19p40) heterodimeric proteins that is shared by both cytokines (47). Yet, clinically targeting p40 with ustekinumab in two open-label trials has shown mixed results in GCA (49, 50). Collectively, epidemiologic data emphasizes the importance of old age, female sex, and genetics with GCA though how these factors contribute to disease pathogenesis remains largely unclear.

IMAGING

In 2018 the European League Against Rheumatism (EULAR) issued guidelines for use of imaging in LVV for the first time, recommending early imaging as the diagnostic test of choice to replace TAB in all cases of clinically suspected GCA (51). Currently, there are four major imaging modalities used in clinical practice (**Table 1**): ultrasound, MRI, CT, and [^{18}F]-fluorodeoxyglucose (FDG) positron emission tomography (PET) (6, 51). PET is combined with another technique, most often CT. All four modalities assess vascular wall thickness and a marker of inflammation that differs between techniques (**Table 1**). Ultrasound assessment is limited to superficial arteries and patients with GCA have non-compressible hypoechoic wall thickening called the "halo sign" (51). Compared to ultrasound, MRI angiography and CT angiography have increased vascular resolution, facilitating assessment of luminal irregularities such as vascular stenosis, aneurysm, and occlusion. Special MRI contrast sequences can also assess cranial vasculature (55, 56).

PET is a very sensitive technique that detects inflammation through the surrogate marker of increased glucose metabolism *via* FDG uptake and has been particularly important to define GCA and PMR. In 1999, a small prospective PET study first demonstrated LV enhancement in patients with GCA and, surprisingly, equally in those with PMR (57). Most subsequent studies are retrospective raising the possibility of selection bias. However, additional small prospective PET studies have demonstrated LV FDG uptake in 66.7–83% of patients with cranial GCA (54, 58) and 14–31% of patients with PMR (59, 60). Corresponding to limb girdle symptoms, patients with PMR show additional FDG uptake in periarticular regions to the hip and shoulder, ischial tuberosities, sternoclavicular joints, and trochanteric and interspinous bursa (61). Pathologic correlates to large vessel imaging studies are not intentionally obtained. Supporting the concept that imaging findings do reflect active vascular inflammation, some studies have reported that inflammatory markers correspond to the degree of LV FDG uptake (54, 62, 63), which is also reduced with treatment (58, 64, 65). However, low grade enhancement may persist with normal inflammatory markers (66). Multiple prospective serial studies have now shown this does not appear to predict clinical relapse and may rather represent vascular remodeling (58, 65, 67). Preliminary data suggests there may be an imaging cut-off that can distinguish ongoing inflammation from vascular remodeling, as well as from LVV mimics such as atherosclerosis, and is an ongoing area of research (68, 69).

Large vessel imaging patterns can also help differentiate between TAK and LV-GCA in patients who are at the border of age around 50 (**Figure 1A**). Indeed, a large imaging cohort study recently identified six patterns of LV involvement that were different between diseases (70). Favoring TAK were involvement of the abdominal aorta, renal, and mesenteric arteries; bilateral carotid and subclavian arteries; and isolated left subclavian artery. Favoring GCA were involvement of bilateral axillary and subclavian arteries; diffuse disease including the aorta and its proximal branches; and minimal disease without clear pattern. Additionally, vascular damage with stenosis, aneurysm, or occlusion is more common in TAK while vascular inflammation alone is more common in GCA (70). Extracranial carotid arteries involved in cranial-GCA are rarely affected by TAK. Scans obtained for other reasons may also incidentally reveal CIA in the arch and descending thoracic aorta (14).

Imaging has been instrumental to define GCA but provides little insight into pathophysiology. EULAR recommends using ultrasound and MRI to diagnose cranial-GCA, with no preference in technique for LV-GCA. The optimal use and interpretation of LV imaging in clinical practice is rapidly evolving and is thus far uncertain.

HISTOPATHOLOGY

Normal arteries have three layers separated by dense elastic fibers (**Figure 1B**). From the lumen outward, these include the tunica intima, internal elastic lamina (IEL), tunica media, external elastic lamina, and tunic adventitia. Intima and media are predominantly composed of endothelial cells and vascular smooth muscle cells (VSCM), respectively (71). Their thickness and complexity increases in large elastic vessels with proportional increase in stromal cells and extracellular matrix, especially within the elastic lamellae-rich media (71). The adventitia contains a dense network of elastin and collagen connective tissue produced by fibroblasts and is interdigitated with progenitor cells, adrenergic nerves, and immunosurveillant tissue resident myeloid cells called vascular dendritic cells (vasDC) (72, 73). It is also the site of the vasa vasorum, a microvascular network composed of endothelial cells and pericytes that supplies oxygen and other nutrients to the vascular wall (72). As large arteries require increased nutritional support due to their size, vasa vasorum extend further into the media in these vessels. Outside

TABLE 1 | Characteristics of imaging modalities clinically used to support a GCA diagnosis.

	Ultrasound	MRI	CT	FDG-PET
Vasculature examined	Superficial cranial, carotid, and axillary arteries	Cranial arteries, all large arteries	All large arteries	All large arteries; emerging use in cranial arteries
Marker of inflammation	"Halo sign" —vascular edema	Contrast enhancement	Contrast enhancement	FDG uptake—glucose metabolism
Advantages	Low cost, non-radiating	Vascular resolution, non-radiating	Vascular resolution, second lowest cost	Exam sequence not limited to vasculature and may detect mimics such as cancer, emerging use in flare
Disadvantages	Operator dependent, limited to superficial arteries	Reduced accessibility, high cost, highest number of patient contraindications	Reduced accessibility, radiation	Lowest accessibility, highest cost, radiation when combined with CT
EULAR GCA recommendation	Cranial-GCA, LV-GCA	Cranial-GCA, LV-GCA	LV-GCA	LV-GCA
Sensitivity[a]	Pooled: 77%[b] (95% CI: 62–87%)	Pooled: 73%[b] (95% CI: 57–85%)	73%[c]	67–71%[d]
Specificity[a]	Pooled: 96%[b] (95% CI: 85–99%)	Pooled: 88%[b] (95% CI: 81–92%)	85%[c]	91–100%[d]

[a] *Compared to clinical diagnosis of GCA. Caveats include that clinical diagnosis by ACR criteria favors cranial-GCA and there are fewer studies prospectively assessing sensitivity and specificity of CTA and PET.*
[b] *Data from a recent meta-analysis (52).*
[c] *(53).*
[d] *(53, 54).*

the artery proper lie more connective tissues supported by a network of small non-muscular blood vessels (74).

Temporal Arteries

GCA is a multi-focal, segmental destructive panarteritis (75–78) (**Figure 1B**). There is a transmural inflammatory infiltrate with greatest density between the adventitia and media that is composed predominantly of $CD4^+$ T cells and macrophages, with few B cells and eosinophils; mature neutrophils are rare and when abundant suggest an alternative diagnosis (79, 80). Despite the name, giant cells themselves occur to a variable degree and are prominent in ∼50% cases at the intima-medial junction around a deranged IEL. In the media there is laminar necrosis with loss of VSCMs and neoangiogensis; fibrinoid necrosis does not occur (79). The intima has features of vascular remodeling with hyperplasia and fibrosis, and occasionally thrombosis and recanalization, especially above sites of active inflammation. More active disease has a more diffuse and intense inflammatory infiltrate and greater number of giant cells, while quiescent disease has a scant infiltrate with fewer giant cells (81). At the end of this spectrum is "healed arteritis," when the features of vascular damage and remodeling are seen in the absence of inflammatory cells (78).

Three other patterns of inflammation associated with GCA and PMR have also been described, together referred to as "restricted inflammation" (RI). These include small vessel vasculitis (SVV) involving the vessels in connective tissue beyond the adventitia, vasa vasorum vasculitis (VVV), and inflammation limited to adventitia (ILA) (82). Giant cells and granulomas are not present. In their limited description, SVV consisted of slightly more T than B cells and few macrophages (74), while VVV showed approximately equivalent infiltration of T cells, B cells, and macrophages (83). The extent to which these patterns are reported and their role in GCA diagnosis is controversial (82, 84). Recently, a retrospective clinicopathologic study with advanced imaging found that patients with RI had fewer cranial symptoms, less systemic inflammation, and less halo sign on ultrasound (82). However, there was no difference in visual symptoms including permanent vision loss or the degree of LV involvement between RI and classic GCA. In an accompanying systematic literature review, the positive predictive value for RI was 23%, highest for ILA with 67% for GCA and 95% for PMR (82). Notably, other forms of vasculitis, infection, and certain hematologic malignancies can present with RI and particularly with SVV (85, 86).

Large Vessels

Large vessel pathology in GCA is less well-studied. Historically, patients with LVV have often been described to have TAK and patients with CIA may be aberrantly reported as having GCA (87). However, pathologic characteristics of GCA can be disentangled by the few studies that concurrently report TAB, which confirm histopathology is largely the same across vessel sizes (4, 81, 88, 89). Compared to TAB, the aorta has reduced adventitial inflammation with the majority of inflammatory infiltrate in the media; mild adventitial fibrosis is also occasionally seen (4, 88) (**Figure 1C**). Interestingly, patients with aortic dissection tend to have more diffuse involvement than seen on necropsy, suggesting more robust aortic inflammation in these patients (4, 89). CIA is histopathologically identical to GCA (14, 88). Few studies have directly compared TAK and GCA histopathology, but TAK has more inflammation and fibrosis in the adventitia and media, resulting in thicker walls (40, 48, 88, 90). There is also increased invasion of $CD8^+$ T cells, B cells, and γδT cells and more giant cells compared with GCA (48, 91) (**Figure 1C**).

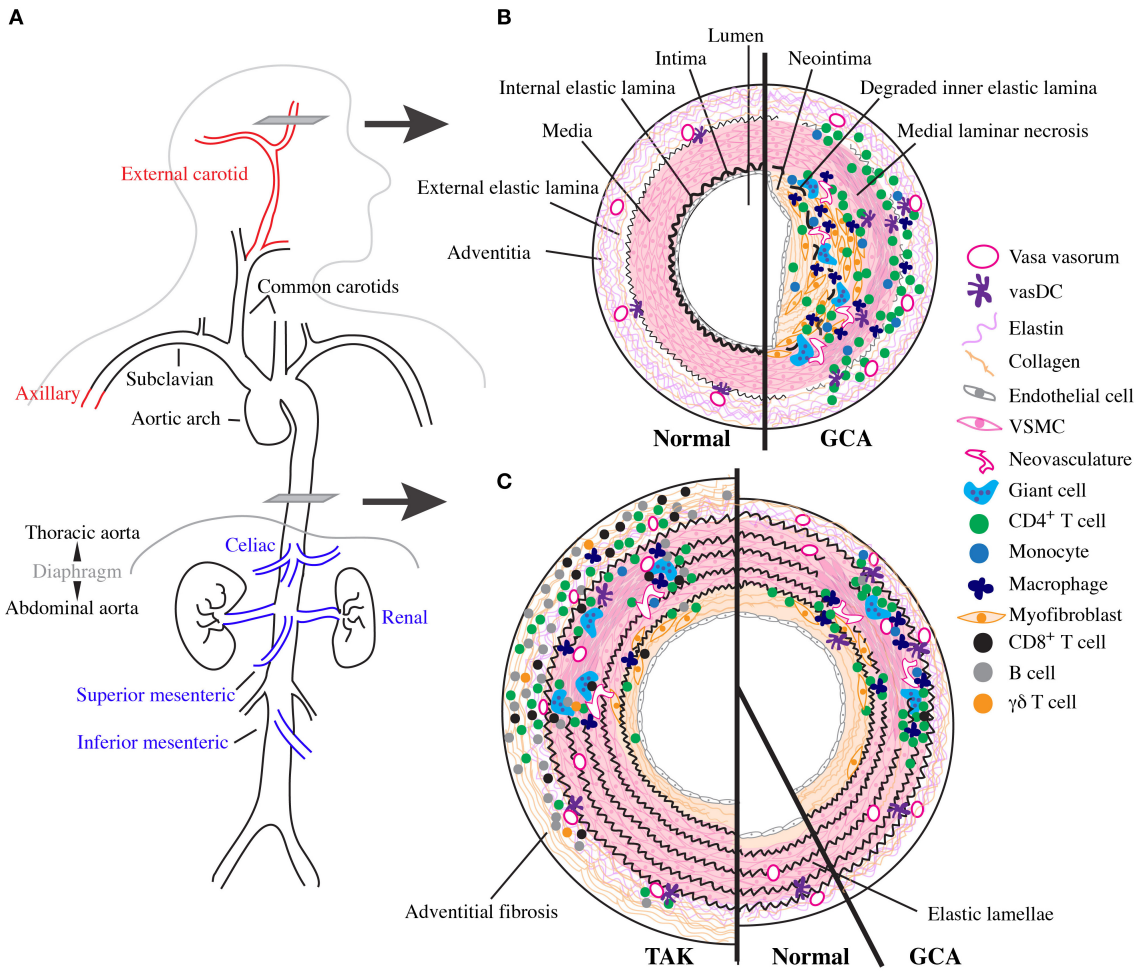

FIGURE 1 | Vascular topology and schematic cross sections of GCA, TAK, and normal arteries. **(A)** Depiction of the normal human large vessel vasculature. Vascular involvement with greatest specificity for GCA shown in red and with greatest specificity for TAK in blue. **(B)** Cartoon representing a cross section though the temporal artery as indicated in **(A)** with normal vessel on the left and the inflammatory infiltrate and vascular remodeling found in cranial-GCA on the right. **(C)** Cartoon representing a cross section through the thoracic aorta as indicated in **(A)** with TAK on the left, normal in the middle, and LV-GCA on the right.

PATHOPHYSIOLOGY OF GCA

Clinical features, epidemiology, imaging, and conventional histology give important information about GCA, PMR, TAK, and CIA, but little insight into pathophysiology. For that, we must rely on a small number of techniques, each with its own strengths and limitations, predominantly based on the characterization and manipulation of patient-derived peripheral blood mononuclear cells (PBMCs) and TAB tissue. Based on our review of data from these studies, here we envision the sequence of events that occurs in GCA, starting with initial immune activation, followed by arterial infiltration, damage, and repair response. We propose the following general model of the pathophysiology of GCA: Overlapping patterns of activation in circulating PBMCs seen between GCA and PMR suggest that immune activation precedes vascular damage. Pathologic analysis suggests vascular damage initiates in the adventitial vasa vasorum microvasculature because inflammation is never restricted to intima (85). The initial trigger for vascular injury in GCA is unknown but appears to involve interactions between pathologically activated circulating cells, especially $CD4^+$ helper T cells and monocytes, and multiple vascular cell types. Upon breach of vascular immunoprivilege, recruitment of these abnormal monocytes and $CD4^+$ T cells, especially IFN-γ-producing Th1 cells, cooperate to mediate vascular injury and repair. The sequence of recruitment is also unknown, but once initiated, multiple interconnected positive feedback loops sustain it in the vasculature and also likely feedback to amplify systemic immune activation.

Immune Activation and Circulating Leukocytes

Systemic inflammation is a core feature of GCA and PMR that is largely driven by IL-6, with elevated plasma levels in both conditions (92, 93). IL-6 is a pleotropic cytokine professionally

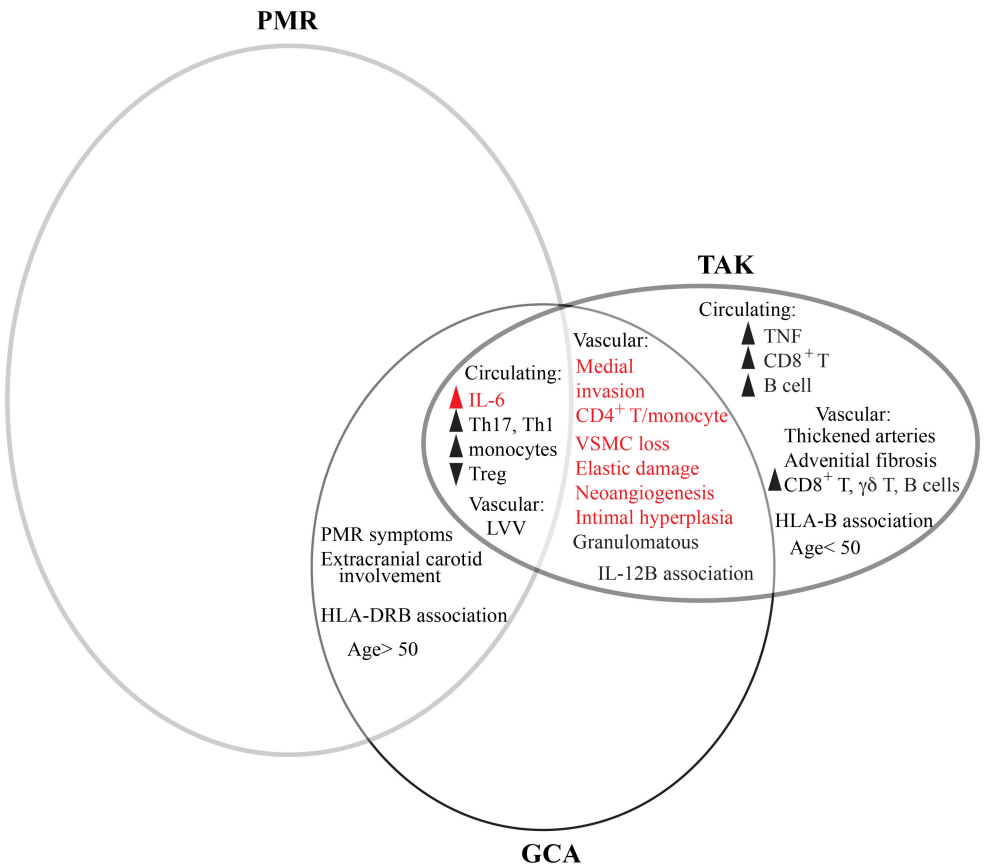

FIGURE 2 | Comparative features between GCA, PMR, and TAK. PMR and GCA are overlapping clinical entities as patients can concurrently have both conditions. However, more patients with GCA have PMR than do patients with PMR have GCA. Meanwhile, GCA and TAK share features of vascular inflammation in large vessels. PMR, GCA, and TAK all share elevated levels of IL-6 and changes to circulating immune cells. However, distinguishing TAK from GCA are some circulating lymphocytes and cytokines, vascular pathology, and patient age. Many features are common to other types of vascular disease, such as AAA (red). There is not currently enough information to determine the extent to which CIA overlaps with other conditions.

produced by monocytes, macrophages, and dendritic cells within the immune system—as well as by other cells including endothelial cells, VSMCs, fibroblasts, and B cells—as an early signal of tissue damage (94, 95). Monocytes appear to be the primary source of IL-6 among PBMCs of patients with GCA and PMR (93, 96), though the contribution from other non-circulating cell types has not been assessed and is likely significant. In the immune system, IL-6 has a key role in $CD4^+$ helper T cell differentiation, promoting the development of Th17 and T follicular helper (T_{FH}) cells, while inhibiting that of regulatory T cells (T_{reg}) due to opposite effects of the IL-6-induced pioneering transcription factor STAT3 in the generation of these cell types during inflammation (94, 95, 97). In the liver, IL-6 stimulates production of acute phase response proteins including CRP and fibrinogen, with resultant elevation of ESR. IL-6 levels are tied closely to clinical symptoms of GCA and are higher in patients who experience more relapses, with levels rising concurrently with symptoms during relapse (93, 98). Consistent with the negative association between systemic inflammation and cranial symptoms, patients with higher serum IL-6 have fewer ischemic complications even during relapse (98–100). Whether this reflects a biologic difference or increased clinical detection remains unclear. Beyond IL-6, other cytokines are not reproducibly systemically elevated across studies; multiple studies have shown circulating TNF and IFN-γ levels are unchanged (15, 92, 93, 101).

Patients with GCA and PMR have abnormally activated PBMCs, particularly among $CD4^+$ T cells, which are skewed toward effector cells. Although unchanged in number, polarization of $CD4^+$ T cells is aberrant. Both conditions share increased frequency of IFN-γ^+ Th1 cells and a STAT3-activation pattern with increased IL-17^+ Th17 cells and reduced Treg (92, 101–103); IL-21^+ T_{FH} cells are also elevated in GCA but untested in PMR. Th17 cells are stimulated by the cytokines IL-23 and IL-1β and are pathologically associated with multiple autoimmune diseases. Th1 cells develop downstream of STAT4-activating IL-12, which also stimulates their production of the signature cytokine IFN-γ, a well-known driver of granulomatous inflammation in infections such as *M. tuberculosis* (104, 105). In humans, the majority of T_{FH} cells also respond to IL-12 and

can co-produce IFN-γ, like Th1 cells; these cells accumulate in a multitude of inflammatory diseases and provide B cell help. T cell production of IL-21 can also enhance cytotoxicity of $CD8^+$ T cells and NK cells (106). In GCA, *in vitro* culture of patient T cells with IL-21 further engenders more Th1 and Th17 differentiation (102). Beyond polarization, GCA and PMR share an increased frequency of senescent T cells (107). Studies in PMR are more limited, but GCA patients have other evidence of increased activation. These include a shift from central memory $CD4^+$ T cells to effector memory and terminally differentiated effector memory cells; higher expression of HLA-DR and NOTCH1, which has a pleomorphic pro-inflammatory function in mature T cells; and a gene expression signature enriched for T cell receptor signaling (102, 108–110).

In comparison, other circulating lymphocytes appear to be less impacted, though comprehensive assessment using high-dimensional analyses is lacking. $CD8^+$ T cells from GCA and PMR patients have increased oligoclonality, and many but not all studies report lower numbers; at least in GCA, they also express higher HLA-DR (102, 111–115). In some studies, there is a global reduction in the number of B cells, while NK cell numbers are unchanged (102, 116).

In the myeloid compartment, prominent changes include increased numbers of circulating monocytes and immature neutrophils. Monocytosis of classical $CD14^{bright}$ $CD16^{lo}$ cells is present in both GCA and PMR (93, 117). In GCA, these cells are phenotypically identical to healthy controls (118, 119). However, they are transcriptionally primed in circulation, expressing higher levels of pro-inflammatory cytokines *IL6*, *IL1B,* and IL-12/23 components *IL23A* (p19), *IL12A* (p35), and *IL12B* (p40) as well as extracellular matrix-degrading (ECM) gelatinases *MMP2* and *MMP9* (96, 101, 118). Surprisingly, left shift with increased circulating immature neutrophils was recently shown to be the major cellular difference by mass cytometry (CyToF) between untreated GCA patient and healthy control PBMCs (80). Collectively, these data suggest there is increased bone marrow myelopoiesis and/or recruitment in active disease. Whether PMR patients have the same transcriptional changes to monocytes or cellular distribution remains to be seen.

Initiating Arterial Inflammation

Two major challenges to understanding GCA pathogenesis are the absence of a commonly used mouse model and the lack of availability of sequential patient samples. Given these challenges, mechanistic insights rely on three human systems based on temporal artery: (1) observations from TAB; (2) manipulation of TAB or normal arteries in Matrigel (120); or (3) manipulation of TAB or normal arteries in chimeric mouse systems (121). The chimeric systems are the most complex and have evolved over time. One currently used system involves three sequential steps to produce inflammation, which may rely in part on alloreactivity ("subcutaneous-chimera"): (1) implantation of a human artery segment as a subcutaneous graft on the lower midback of a highly immunocompromised NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mouse that lacks all lymphocytes and has severely defective myeloid cells; (2) stimulation with lipopolysaccharide (LPS), activating xenograft vasDC to adopt a pro-inflammatory $CD83^+$ $CD86^+$ phenotype and produce T-cell recruiting chemokines; and (3) adoptive transfer of PBMCs from treatment-naïve allogeneic GCA patients, generating immune infiltration that histologically and transcriptionally resembles GCA (103, 121). Additional mechanistic insights can also be learned from another chimeric vascular allograft rejection model, where human coronary artery xenograft surgically replaces the mouse infrarenal aorta ("interposition-chimera") (122). Similar to subcutaneous-chimeras, this model uses highly immunocompromised CB17.Cg-*Prkdc*^{scid}*Lyst*$^{bg-J}$/Crl mice that lack T and B cells through the same *Prkdc* mutation but differ in the mechanism of impaired NK and granulocyte function. After adoptive transfer of PBMCs from allogeneic blood donors, these mice develop xenograft vascular inflammation even in the absence of LPS over a similar time course. However, inflammation is more histopathologically similar to TAK, with prominent adventitial and intimal $CD4^+$ and $CD8^+$ T cell invasion and hyperplasia; unlike TAK and GCA, very few leukocytes invade the media, myeloid infiltration is rare, IEL are preserved, and neovascularization does not occur (123).

Arterial invasion requires activation of both circulating and vascular cells. Emphasizing the importance of pathogenic circulating cells, in subcutaneous-chimeras, normal human PBMCs cannot typically invade artery grafts even in the presence of LPS (109). Meanwhile, GCA-derived alloreactive T cells cannot invade artery grafts in the absence of LPS (121). Some of these effects may be caused by recruitment and duration of contact with the subcutaneous graft because normal human T cells—but not myeloid cells—can invade the interposition-chimera allograft without further stimulus downstream of endothelial antigen presentation to $CD4^+$ and $CD8^+$ T cells (122, 124). Interestingly, upon co-implantation of TAB fragments from GCA, PMR, or control patients into NSG mice, T cells recirculate from GCA arteries and invade PMR but not normal arteries in the absence of LPS, suggesting PMR vessels have lost immunoprivilege (121). PMR vasDC have a partially activated $CD83^+CD86^-$ phenotype on TAB. However, whether vasDC cause the arterial leakiness in PMR or GCA vessels is unclear because adoptive transfer of GCA T cell alloreactive clones in subcutaneous-chimeras in the absence of LPS also induces a $CD83^+$ vasDC phenotype, but T cells do not invade (121). Thus, while LPS stimulation can breach immunoprivilege in transplanted arterial sections *via* vasDC activation and perhaps prolonged contact with allogeneic T cells, this may not be the initiating event of vascular damage in GCA. These data also suggest—despite some overlapping phenotypes in PBMCs—that pathogenic differences occur between GCA and PMR cells that can facilitate entry into PMR primed vessels.

IFN-γ can independently break vascular immunoprivilege. Incubation of normal artery in Matrigel with IFN-γ induces VSMC expression of several chemokines including monocyte-recruiting CCL2 as well as Th1- and $CD8^+$ T cell-recruiting CXCL9, CXCL10, and CXCL11 (125). It also induces VSMC ICAM-1 expression, an adhesion molecule that binds leukocyte integrins and, when expressed by endothelial cells, facilitates vascular transmigration. Remarkably, addition of healthy control PBMCs results in invasion of macrophages—but not healthy T cells—that subsequently become giant cells (125). Endothelial

expression of HLA-DR is also induced by IFN-γ (122, 124), but whether pathogenic GCA PBMCs facilitate T cell entry has not been tested. This axis may also be important in early vascular injury in GCA, as TAB shows increased ICAM-1 expression by VSMCs in regions of structurally normal skip lesions and as well as by endothelial cells in the vasa vasorum (126, 127). Notably, other cytokines such as macrophage-derived TNF and IL-1β can also induce ICAM-1 expression on endothelial cells *in vitro* as well as enhance its upregulation by IFN-γ (128). Interestingly, *IL1B* is increased in PMR TAB compared to controls (129). Thus, local induction of cytokines from activated circulating cells may similarly prime segments of vasculature for inflammatory cell entry, though the mechanism of tissue tropism to the LV vasculature with this lens remains to be explored.

The Feed-Forward Inflammatory Infiltrate

On TAB, activated memory $CD4^+$ T cells massively invade GCA arteries, where they polarize even further into effector cells compared to PBMC, homing mostly to the adventitial-medial border but present in all three layers. These express a broad repertoire of T cell receptors with a minimal degree of clonal expansion (130, 131). While a comprehensive assessment of infiltrating T cells is lacking, there are varying degrees of IFN-γ, IL-17, IL-21, and IL-9 produced. The balance of polarization differs between patients and is functionally relevant because cranial ischemic symptoms correspond to increased Th1 function on TAB (99, 132, 133). Indeed, ischemia positively correlations with: (1) the Th1 signature cytokine IFN-γ, (2) its activator IL-12p35, and (3) the downstream number of giant cells (99, 132, 133). In contrast, patients with higher expression of the Th17 signature cytokine IL-17A have fewer relapses and more systemic symptoms (134, 135). Consistent with this, in interposition-chimeras, IL-17 blockade does not impact intimal hyperplasia but does reduce *IL6* (136). Though minimally described, TAB with RI also reflect different T cell composition. Compared to transmural inflammation, SVV has low levels of IL-17 and intermediate levels of IL-9 while VVV/ILA has the opposite pattern (135); the distribution of IFN-γ has not been described. VVV further lacks $NOTCH1^+$ infiltrating T cells (109). Thus, T cell polarization differs between clinical and pathologic phenotypes, but how different signature cytokines affect pathogenesis largely remains to be explored.

Myeloid cells also diffusely infiltrate all three layers of the artery on TAB and densely populate granulomas around the IEL. These include three populations: a smaller $CD16^-CCR2^+$ $CX3CR1^-$ cells that produce IL-6 and IL-1ß and phenotypically resemble circulating monocytes; $CD16^+CCR2^-CX3CR1^+$ macrophages that produce MMP9, MMP2, VEGF, and the potent mesenchymal mitogen PDGF; and giant cells that functionally overlap with $CX3CR1^+$ macrophages but express the above effectors to an even greater degree by immunohistochemistry (96, 117, 137, 138). Other myeloid generated cytokines elevated in TAB that contribute to the pro-inflammatory environment include TNF, IL-12, and IL-23 (101, 139, 140). Co-culture of human peripheral blood monocytes with aortic adventitial fibroblasts induces their differentiation into macrophages that produce MMP9 (141). In other biologic conditions, various cytokines can stimulate macrophage fusion into giant cells—including IFNγ, IL-1β, and IL-6—but correlation between IFN-γ levels and number of giant cells on TAB suggest this is the primary mechanism in GCA (99, 142). Collectively, these data suggest monocytes that are transcriptionally primed to produce pro-inflammatory cytokines and gelatinases in circulation are recruited from the peripheral blood, differentiate in the inflamed vessel into macrophages, and further combine to form giant cells in response to IFN-γ. However, it is also possible that monocytes and macrophages are independently recruited to inflamed vessels from the circulation.

Multiple cell types generate positive feedback chemokine loops that enhance T cell and myeloid recruitment. In subcutaneous-chimeras, vasDC produce CCL19 and CCL21 as well as its receptor CCR7, trapping them in the artery upon activation (73). They also produce CCL20 and attract cells expressing CCR6, a phenotype shared by many infiltrating T cells on TAB as well as by Th17 and Th1/Th17 precursors in GCA patient peripheral blood (92, 143, 144). Notably, a variety of cells including DC, macrophages, Th17 cells, and VSMC can produce CCL20 and while it is overexpressed on TAB, the cellular source has not been shown (134, 136, 145). VSMC are a nexus for accentuating inflammatory signals: macrophage-generated TNF stimulates macrophage-attracting CX3CL1 *in vitro*; macrophage-expressed PDGF induces monocyte-recruiting CCL2 in Matrigel; Th17-produced IL-17 causes Th1/Th17-recruiting CCL20 *in vitro* and in interposition-chimeras; and Th1-derived IFN-γ provokes CX3CL1 plus Th1-, $CD8^+$ T cell- and monocyte-recruiting chemokines, as previously described (125, 136, 146, 147). Finally, when co-cultured, fibroblasts induce monocyte expression of monocyte-recruiting CCL2 (141). Thus, upon entry of T cells and monocytes in the blood vessel, interactions with resident vascular cells perpetuate inflammation.

T cell interactions with other vascular cells also enhance inflammation. Endothelial cells in the vasa vasorum pathologically express Jagged1 on TAB. This can be experimentally reproduced *in vitro* by GCA plasma and mitigated by anti-VEGF, consistent with the increased systemic levels of VEGF in patients with GCA. *In vitro* and in subcutaneous-chimeras, Jagged1 ligates NOTCH1 expressed by circulating T cells and enhances their polarization to Th1 cells and, to a lesser extent, Th17 cells (103, 109). Upon entry into the vessel, T cells interact with vasDC. In normal artery specimens, these constitutively express PD-L1, a molecule that restrains $PD\text{-}1^+$ T cells generated during chronic immune stimulation (148). In GCA TAB, vasDC upregulate antigen presentation machinery of HLA-DR, CD83, and CD86 but downregulate PD-L1; meanwhile, vascular invasive but not circulating T cells highly express PD-1 (73, 121, 148). Blocking PD-L1 in subcutaneous-chimeras results in exuberant inflammation, suggesting physiologic $PD\text{-}1L^+$ vasDC restraint is lost in GCA immunopathology (148). Thus, inflammatory changes to other cell types augment the pathogenicity of pre-activated T cells.

Vascular Injury and Repair

Macrophages drive vascular injury largely *via* gelatinases. In normal vessels, VSMC constitutively produce pro-MMP2 and

its inhibitor TIMP2 resulting in a quiescent vessel without proteolysis (149). With inflammation, macrophage- and giant cell-derived MMP2 and MMP9 outpace inhibitors, resulting in progressive degradation of ECM that is consequently more easily infiltrated by T cells (118, 149). Destruction occurs locally around macrophages as demonstrated by the restriction of MMP9 and proteolysis to the adventitia in ILA on TAB (118). Giant cells are gelatinase factories and, taking residence along the IEL, cleave and destroy it. The mechanisms that drive VSMC laminar necrosis are poorly described, but likely also involve myeloid mediators because, like IEL degradation, it does not occur in interposition-chimeras that lack myeloid recruitment (122, 123).

Macrophages and Th1 inflammation launch vascular remodeling, resulting in intimal hypertrophy and neovascularization. In response to a variety of mitogenic signals in Matrigel but most robustly to PDGF, healthy contractile VSMC become proliferative, invasive myointimal cells (146). These leave the media and invade the intima where they produce the vascular ECM proteins collagen I and III, generating the hypertrophic neointima (138, 146). TAB levels of PDGF and IFN-γ correspond to the degree of intimal hyperplasia, which in turn correlates with ischemic symptoms as the macrolumen becomes progressively stenotic (138). While macrophages and giant cells at the media/intimal border both produce PDGF, recombinant IFN-γ can also directly stimulate VSMC to produce PDGF as well as upregulate its receptor in interposition-chimeras that lack PBMC adoptive transfer, resulting in neointimal hyperplasia even in the absence of cellular infiltration (150). Both macrophage and T cell pathways are likely active in GCA. Patients with ischemic symptoms also have higher plasma levels of endothelin 1 (ET-1), a potent vasoconstrictor physiologically generated by endothelial cells. Interestingly, ET-1 is expressed by infiltrating immune cells on TAB and can redundantly generate intimal-invasive myointimal cells from VSMC in Matrigel (151). The degree of intimal hyperplasia further correlates with the degree of neovascularization in the intima and media, and in turn, to levels of VEGF on TAB, suggesting this process is driven by hypoxia (152). However, neovascularization co-localizes with macrophage- and giant cell-rich areas on TAB (152). Thus, the extent of neovascularization likely reflects the degree of macrophage and giant cell activation through multiple mechanisms including their production of VEGF. Collectively, vascular remodeling results in thickened blood vessels that cause symptomatic ischemia and generates a conduit for further inflammatory cell entry through leaky neovasculature (127).

Treatment Effects

Glucocorticoids are the standard therapy for GCA and PMR. Consistent with the need for higher doses in GCA than PMR, systemic changes occur first while local changes seen in TAB generally take much longer. Plasma IL-6 is strongly inhibited after a single dose of GC, but the median time to normalization is 4 weeks (93). Though systematic sequential immunophenotyping of PBMCs during treatment has not been reported, B cells appear to be the first to respond and normalize after 2 weeks, a time course consistent with changes in mobilization (116). After 3 months of treatment, Th17 cell frequency, and $CD4^+$ but not $CD8^+$ T cell HLA-DR expression return to normal (92, 101, 102). Monocyte numbers are also reduced at this time but remain higher than healthy controls (117). Furthermore, after 3–9 months of treatment, monocyte expression of *IL6* and Th17-activating *IL1B* and *IL23A* normalize while expression of Th1-inducing and activating *IL12A* and *IL12B* remain elevated (101). Consistent with this, among $CD4^+$ T cells, Th1 take longer to respond, normalizing with full disease remission (101, 102). Finally, $CD8^+$ T cell numbers take up to 2 years to return to baseline numbers (111). Indeed, in patients diagnosed with GCA for at least 2 years, increased circulating $CD4^+$ T cells, reduced $CD8^+$ T cells, and the corresponding increased $CD4^+/CD8^+$ ratio but not inflammatory markers or monocytes numbers have recently been shown to be associated with thoracic aortic dilatation compared to controls (22).

Reports of TAB re-biopsy after GC treatment reveal similar results to PBMCs with initial control of Th17 pathways, and later reduction in Th1 pathways, as well as a prolonged timecourse of vascular healing. Compared to TAB with active GCA, re-biopsies at 3-9 months phenocopy peripheral blood and show profound reduction in *IL6*, *IL1B*, *IL23A,* and *IL17* while *IL12A IL12B*, and *INFG* are unchanged (101). In another study, patients with paired re-biopsy at 1 year demonstrated a global reduction in all tested cytokines including *IL1B*, *IL6, IL23A, IL12A, IL12B,* and *IFNG* as well as *MMP9*, though patients with more relapses showed higher levels of *IL12B* and *IFNG* (139). Consistent with this prolonged time course, a prospective study of 40 patients re-biopsied at 3, 6, 9, and 12 months found active arteritis in 7/10, 9/12, 4/9, and 4/9 samples, respectively, despite normalization of inflammatory markers and clinical symptoms. There was also a time-dependent increase in vascular remodeling (153). Thus, GC quickly control Th17 signatures in circulation and TAB, likely reflecting loss of STAT3 activation from monocyte-derived IL-6. Meanwhile, Th1 pathway takes longer to respond, consistent with prolonged monocyte production of STAT4-activating IL-12, which may drive relapse and ongoing vasculitis in some individuals. Finally, vascular remodeling continues after active inflammation resolves, like the prolonged FDG signal on PET imaging.

Multiple other treatment modalities have been tested for GCA in randomized clinical trials. In the GiACTA trial, targeted blockade of IL-6R with TCZ demonstrated superiority to a course of GC alone in achieving steroid-free remission at 1 year, as defined by lack of clinical flare and normal level of IL-6-induced CRP, becoming the first non-steroid FDA-approved treatment for GCA (11). Interestingly, fewer patients with relapsing disease responded to TCZ than patients with untreated disease, raising the possibility that these patients may have more Th1 driven disease. Furthermore, one patient in the TCZ every-other-week arm developed the ischemic complication of anterior ischemic optic neuropathy. In another study, a patient with highly active disease that normalized on TCZ—but who died unrelated to GCA after 6 months of therapy—had widely active vasculitis on autopsy (154). Though GCA-related adverse events were not statistically different between groups in GiACTA, these raise the question if TCZ controls vascular-level inflammation or if it blocks systemic manifestations of flare, which may further differ between newly diagnosed and relapsed patients.

Further insights to this question are suggested by the differing results of the two open-label studies of anti-p40 ustekinumab. In the initial promising Irish trial, all patients recruited had relapsing disease and ustekinumab was successful in achieving GC-reduction without flare, albeit with persistent low GC dose in the majority of patients. Notably, of 10 patients with LV disease in this study, eight underwent reimaging by CT angiography, which demonstrated not only a halt to further vascular damage but improvement of wall thickening in all patients and complete resolution in four (49). In the American trial, both newly diagnosed and relapsing patients were recruited and all patients were required to end GC at 6 months, resulting in clinical flare across the majority of patients with elevated inflammatory markers and PMR symptoms; though data was not shown for relapse between newly diagnosed vs/ relapsing patients they were stated not to be different (50). Vascular imaging follow up was not reported (50). Consistent with molecular studies, these differing trial results suggest a degree of independence between systemic symptoms of flare downstream of IL-6 and vascular damage in relapsing patients downstream of IL-12. While GC controls both endotypes, targeted therapies directed at either can fail; however, only low dose GC may be required to control the IL-6 axis, as in PMR, at least in patients with relapsing disease. These data also emphasize that long-term follow up of TCZ-treated patients and further clinicopathologic correlation will be important and the utility of a randomized trial for ustekinumab in GCA patients with relapsing disease. Similar to GC, blocking STAT activation directly with JAK inhibitors would allow combinatorial blockade of IL-6 and IL-12/23 without GC side effects and is theoretically compelling. Indeed, multiple JAK inhibitors are currently in clinical trials (12).

Beyond IL-6, another potential emerging treatment is to target T cell overactivation directly. Indeed, abatacept (CTLA-4:Fc) was superior in achieving relapse-free survival at 1 year in a phase 2 trial (155). Other targeted therapies using TNF blockade with infliximab, adalimumab, or etanercept have been ineffective (156–158).

Comparison to TAK and CIA

Due to relative lack of tissue compared to GCA, less is known about the immunopathology of TAK, and that of the more recently-described entity CIA remains virtually unexplored. Consistent with overlapping but distinct pathology, TAK shares several features in common with GCA but differs in cytotoxic mediators (**Figure 2**). Like GCA, changes to circulating inflammatory cells also reflect those in the tissue. Systemically, patients with TAK share elevated systemic levels of IL-6 and increased circulating classical monocytes, Th17 cells, and Th1 cells with fewer Treg (159–161). In tissue, memory $CD4^+$ T cells—including Th1 and Th17 subsets—are likewise the most prevalent invasive cell type, with equal macrophage infiltration between conditions (48, 162). Interestingly, in the opposite pattern of GCA, peripheral Th1 cells respond better to steroids than Th17 cells, which remain elevated despite clinical remission (162). Unlike GCA, patients with TAK also have elevated systemic TNF and consistent with this, TNF inhibitors are at least modestly clinically effective (161, 163). The major difference between TAK and GCA is among non-$CD4^+$ lymphocytes, as B cells and $CD8^+$ T cells are elevated in peripheral blood and tissue. As suggested by genetic HLA class I associations, $CD8^+$ T cells seem particularly relevant, rising in circulation during flares and found actively killing vascular cells on electron microscopy (91, 159). Interestingly, GCA patients with relatively higher levels of $CD8^+$ T cell invasion also have more severe disease, though in this condition it may also reflect the degree of Th1 inflammation given mutual dependence of $CD8^+$ T cells on the positive-feedback IFN-γ-CXCR3 recruitment loop (114).

PERSPECTIVES AND FUTURE DIRECTIONS

GCA is a complex disease because it lies at the interface of two clinical spectra—the pathologically similar granulomatous vasculitides and the clinically overlapping GCA and PMR—each of which have historically been imprecisely defined based on clinical phenotypes and therefore often overlap in the literature (**Figure 2**). Additionally, emerging results from advanced imaging and pathologic analysis show two additional spectra—LV- and cranial-GCA and histologic RI—that demonstrate even greater overlap with PMR than previously recognized. Compounding this complexity is the clinical need to treat GCA emergently and the recent transition from pathology to imaging for diagnosis, which respectively limit the availability of untreated patient PBMCs and tissue specimens for research.

Despite phenotypic similarities between TAK and GCA, the multiple differences between affected patients—in age, demographics, vascular distribution, genetics, histopathology, and immunophenotype—suggest that these are distinct disease entities with some degree of convergence (**Figure 2**). Furthermore, most shared features between TAK and GCA are not unique to vasculitis. In fact, the common vascular condition of abdominal aortic aneurysm (AAA), a permanent dilatation to the aorta that affects 1–2% of men age 65 and 0.5% of women age 70, shares most features: elevated systemic levels of IL-6; monocytosis; medial invasion of memory $CD4^+$ T cell and macrophages; and vascular remodeling with dissolution of the elastic lamellae, loss of VSCM, and neoangiogenesis (164–169) (**Figure 2**). This suggests despite different triggers of vascular injury, many pathways of arterial damage converge, though some differences persist and may inform our understanding of disease mechanisms. For example, granulomatous inflammation likely reflects the higher vascular IFN-γ and macrophage invasion in GCA, CIA, and TAK compared to AAA, while changes to circulating T cells reflect higher systemic levels of IL-6 (164, 167, 169, 170). Likewise, the prominent fibrosis in TAK appears to be an important distinction and may represent a novel disease target. Interestingly, the comparison of AAA is particularly relevant for GCA as they share several other epidemiologic features, including old age with rare incidence below age 50, increased prevalence in Northern Europe, and smoking as a core risk factor for aneurysm development (24, 166). Thus, these unexplained risk factors in GCA may represent common mechanisms of vascular risk.

The heterogeneous and overlapping patterns of pathologic RI, LV-involvement, and PMR with GCA remain a mystery. One possibility is that RI and PMR represent more subtle degrees of vascular injury that jointly affect the microvasculature, including that of large arteries. In some patients who experience an unknown stimulus, this may progress to more fulminant disease. Supporting this, a recent report demonstrated a key role for NOTCH3 signaling between the arterial microvasculature and synovial sublining fibroblasts to generate synovitis in rheumatoid arthritis (171). Indeed, microvascular endothelial cells upregulate the NOTCH3 ligand Jagged1 in GCA, though whether this also occurs in PMR synovitis has not been tested (103). Furthermore, subtle microvascular changes may explain the ability of GCA T cells to recirculate into PMR arteries in early experiments (121). Alternatively, the regulatory logic of $CD4^+$ T cells may differ in LV-GCA and/or RI. For example, several lines of evidence suggest that a Th1 signature favors vascular damage and ischemia. Mechanistically, this is an especially feed-forward module in the vasculature through cyclical recruitment of Th1, myeloid cells, and $CD8^+$ T cells that ultimately propagates stenotic tissue remodeling through macrophage activation and giant cell formation. However, the role of other helper T cell modules such as Th17 in GCA is less clear—despite the evidence that they are also present systemically and in vascular tissue. Though it is possible circulating Th17 and T_{FH} cells may simply represent off-target STAT3 activation of IL-6, another possibility is that this module corresponds more to LV inflammation. Supporting this, patients with LV disease typically have more systemic symptoms, as do patients with increased IL-17 on TAB. Furthermore, mice lacking the Rac activator Def6, a negative regulator of IRF4, spontaneously develop granulomatous aortitis due to aberrant T cell production of IL-21 and IL-17 (172). Given the more recent emphasis on LV-GCA, radio-pathologic correlation has not yet been performed but would be interesting. Comprehensive assessment of the immune infiltrate in RI is more easily achieved.

Since the discovery of GCA, the trigger for vascular inflammation has been questioned. Here, we propose a model where systemic changes in the circulation precede vascular injury and are required for disease initiation. In this model, systemic activation likely initiates in myeloid cells—perhaps monocytes—leads to circulating $CD4^+$ T cell polarization downstream of the pioneering transcription factors STAT3 and STAT4. However, recently published data suggests myeloid activation may be even further upstream, as early as the bone marrow, given the prominent left shift seen by CyToF in patients with untreated GCA (80). Supporting this, GCA can occur as a paraneoplastic phenomenon to myelodysplastic/myeloproliferative neoplasms as well as in the recently described somatic, myeloid-activating autoinflammatory condition VEXAS (173, 174). Upon breach of vascular immunoprivilege, pre-activated monocytes and $CD4^+$ T cells mutually enter the vessel and cooperate to destroy it. In chimeric systems, T cell invasion likely relies on allorecognition, but in GCA, HLA associations suggest an antigenic driver that is thus far elusive and may describe tissue tropism to the microvasculature of large vessels. With GC treatment, the IL-6-STAT3 axis regulating systemic symptoms is more quickly controlled while IL-12-STAT4 axis mediating vascular damage, at least in TAB, requires prolonged treatment, consistent with the different time course needed to control these cytokines in circulating monocytes. In the absence of IL-6, Th1 cells may paradoxically initially increase as the cytokine microenvironment favors their generation and persistence (105), consistent with the weaker performance of TCZ in relapsed patients and potentially better performance by ustekinumab (11, 49). Whether flares represent reemergence of abnormally activated circulating myeloid cells, lack of control in the vessel, or some other unexpected mechanism is unclear, but comprehensive longitudinal phenotyping is likely to be informative to this end, as recently described in rheumatoid arthritis (175). In the future, integration of such longitudinal data with imaging will be particularly useful to define clinically relevant entities such as persistent subacute inflammation, flare, and remission. Ultimately, clinical trials of various immune modulators in patients with GCA will provide further insights into proposed disease mechanisms and should include dual assessment of clinical flare as well as vascular damage.

AUTHOR CONTRIBUTIONS

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REVIEW
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Innate and Adaptive Immunity in Giant Cell Arteritis

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Autoimmune diseases can afflict every organ system, including blood vessels that are critically important for host survival. The most frequent autoimmune vasculitis is giant cell arteritis (GCA), which causes aggressive wall inflammation in medium and large arteries and results in vaso-occlusive wall remodeling. GCA shares with other autoimmune diseases that it occurs in genetically predisposed individuals, that females are at higher risk, and that environmental triggers are suspected to beget the loss of immunological tolerance. GCA has features that distinguish it from other autoimmune diseases and predict the need for tailored diagnostic and therapeutic approaches. At the core of GCA pathology are $CD4^+$ T cells that gain access to the protected tissue niche of the vessel wall, differentiate into cytokine producers, attain tissue residency, and enforce macrophages differentiation into tissue-destructive effector cells. Several signaling pathways have been implicated in initiating and sustaining pathogenic $CD4^+$ T cell function, including the NOTCH1-Jagged1 pathway, the CD28 co-stimulatory pathway, the PD-1/PD-L1 co-inhibitory pathway, and the JAK/STAT signaling pathway. Inadequacy of mechanisms that normally dampen immune responses, such as defective expression of the PD-L1 ligand and malfunction of immunosuppressive $CD8^+$ T regulatory cells are a common theme in GCA immunopathology. Recent studies are providing a string of novel mechanisms that will permit more precise pathogenic modeling and therapeutic targeting in GCA and will fundamentally inform how abnormal immune responses in blood vessels lead to disease.

Keywords: T cell, macrophage, vasculitis, NOTCH, endothelial cell, PD-L1, CD8 Treg, exosome

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INTRODUCTION

Giant cell arteritis (GCA), also known as "temporal arteritis," is an autoimmune disease that exclusively affects the elderly host (1). The disease preferentially involves the thoracic aorta and its major branch vessels, including the temporal artery and vessels supplying the optic nerve and the retina. Accordingly, the clinical manifestations of GCA include life-threatening complications, such as aortic dissection, aortic aneurysm, and blindness due to ischemia of the optic nerve. Globally, the highest incidence rates of GCA occur in Northern Europe, including Iceland, Norway, Sweden, and Denmark. High disease risk in Northern European populations has supported the concept that both genetic and environmental factors shape disease susceptibility. Genome-wide association studies

have confirmed earlier data that polymorphisms in the major histocompatibility complex (MHC), specifically the *Human leukocyte antigen (HLA)-DR* region confers the highest risk (2, 3). Amongst non-HLA regions, *PLG* and *P4HA2* appear to play a role as risk determinants (**Table 1**) (4). PLG (plasminogen) and P4HA2 (Prolyl 4-hydroxylase subunit alpha-2) are involved in vascular remodeling and neoangiogenesis, suggesting relevance of these processes in GCA pathogenesis. Of interest, a distinct set of genetic polymorphisms have been implicated in Takayasu arteritis (TAK) (5, 6), an autoimmune large vessel vasculitis that shares many similarities with GCA but preferentially affects young Asian women. In TAK, *HLA-B* has been shown to have the strongest disease association (**Table 1**). Like in GCA, patients with TAK have enrichment of genetic polymorphism in non-HLA regions; include such functionally related to activation of cytotoxic lymphocytes, e.g. natural killer cells and $CD8^+$ T cells. Differences in disease risk genes in GCA and TAK indicate that different pathomechanisms may contribute to autoimmune and auto-inflammatory diseases of the large arteries (7–9).

The vasculitic lesions of GCA are composed of tissue-infiltrating and tissue-resident innate and adaptive immune cells; mostly, $CD4^+$ T cells, dendritic cells, macrophages, histiocytes, and multinucleated giant cells (10, 11) (**Figure 1**). Recent advances in understanding the pathogenesis of GCA have provided important insights into disease-inducing and -sustaining mechanisms. Key pathogenic elements include a

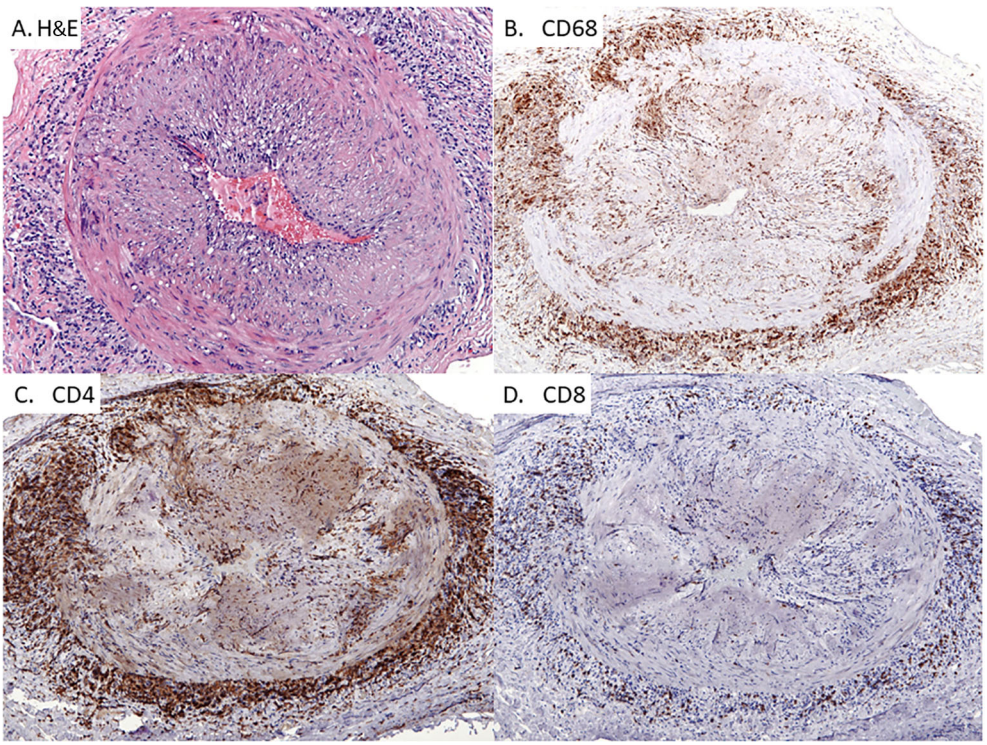

FIGURE 1 | Giant Cell Arteritis in the Temporal Artery. Classic histopathologic and immunophenotypic findings of temporal arteritis in a 78-year old woman presenting with headaches. Panel **(A)** Transmural arterial inflammation with marked luminal narrowing caused by intimal proliferation which creates a slit-like lumen. Multinucleated giant cells are concentrated in the medial layer (H&E x125) Panel **(B)** CD68-positive histiocytes accumulating within the medial and adventitial layers of the artery with only scattered histiocytes in the intimal layer (x100). Panel **(C)**: $CD4^+$ T cells with a similar pattern of distribution as the $CD68^+$ macrophages (x100). Panel **(D)** Infrequent $CD8^+$ T cells within the T cell infiltrates (x100).

TABLE 1 | Gene regions associated with large vessel vasculitis.

Reported year	Population	The number of participants	Gene region
2017	European ancestries	2134 GCA patients 9125 controls	*HLA-DRA/HLA-DRB1, PLG, P4HA2*
2018	Japan	415 TAK patients 2170 controls	*HLA-B, FCGR3A, IL12B, DUSP22, PTK2B, KLHL33, LILRA3, chr21q22*
2015	Turkey and North America	693 TAK patients 1536 controls	*HLA-B/MICA, IL6, RPS9/LILRB3, chr21q22*

vascular and an extravascular disease component, with site-specific immune processes relevant for disease inside and outside of the vascular wall. Also, it is now appreciated that the vessel wall has unique structural barrier features that make it an immuno-privileged tissue site, protecting it from unwanted immune responses. Breakdown of this immune privilege require aggressive immune responses that first must overcome the natural protection inherent to life-sustaining arteries. Studies of persistent vasculitis in GCA patients have stressed the autonomy of tissue-residing inflammatory infiltrates, building significant challenges for the elimination of vascular wall inflammation. Another hurdle in treating GCA relates to the functional heterogeneity of vasculitic effector cells, which lends stability to the inflammatory lesions and renders them resistant to targeted immunosuppression. The granulomatous nature of the vessel wall lesion has nurtured discussions that infectious microorganisms may serve as the vasculitogenic antigen, but reproducible data implicating a viral or bacterial antigen are missing (9). Other environmental triggers, such as air pollutants etc are insufficiently explored. The extravascular component of GCA is poorly understood. Clinically and diagnostically, it is characterized by intense acute phase responses, resulting in elevated Erythrocyte Sedimentation Rate (ESR) and C-reactive protein (CRP). Patients also complain about constitutional symptoms and proximal myalgias that are promptly responsive to glucocorticoids and the recently approved anti-interleukin (IL)-6 receptor antibody tocilizumab (12). However, fluctuations in acute phase reactants, as captured by measurement of ESR and CRP, can occur despite persistence of vessel wall inflammation (13). The lack of reliable disease biomarkers capturing vessel wall inflammation is problematic in managing GCA.

The present review will focus on recent advances in understanding the dysfunctional innate and adaptive immune responses that cause autoimmune vasculitis, with a focus on how the arterial wall immuno-privilege is broken, how vascular inflammation is sustained and how abnormal immunity maintains vascular remodeling. Progress in understanding pathogenic cascades will inevitably broaden the therapeutic armamentarium that is so urgently needed to improve management of vasculitis.

INNATE IMMUNITY IN GCA

Monocytes and Macrophages as Disease Drivers in GCA

The three-layered walls of the muscular and elastic arteries are free of inflammatory cells and protected by immune privilege. Inaccessible tissue sites, e.g. in the testis and the eye, prioritize the integrity of life-sustaining organs over localized immunity. Maintenance of such privileged sites involve a combination of mechanisms, including physical barriers, lack of antigen-presenting cells and counter regulatory processes dampening immune stimulation. In the case of GCA, the immune privilege is lost and both, innate as well as adaptive immune cells enter the privileged site (**Figure 1**). In the three-layered arteries, composed of the intimal layer, the medial smooth muscle cell layer and the supportive adventitial layer, access to the vessel wall occurs through the adventitial vasa vasorum network.

Recent work has described a molecular defect in circulating blood monocytes from GCA patients, which endows these cells with tissue invasive capabilities. Specifically, GCA monocytes spontaneously produce high amounts of the metalloproteinase MMP-9 and digest the basement membrane to overcome the barrier between vasa vasora capillaries and extracellular tissue. By blocking the proteinase activity of MMP-9 with a monoclonal antibody, Watanabe et al (14). implicated GCA monocytes in the breakdown of the basement membrane and in paving the way for both innate and adaptive immune cells into the wall (**Figure 2**). Remarkably, T cells failed to invade the tissue site unless they were accompanied by MMP-9-producing monocytes. Besides MMP-9, GCA monocytes produced high amounts of MMP-2 and -7 transcripts, while MMP-1, -3, -8, -10, or -12 transcripts were indistinguishable in GCA and control monocytes. Metalloproteinases are critically important in several physiologic and pathologic processes (15, 16) and it likely that inappropriate MMP-9 production is not an exclusive problem in GCA. However, the upregulation of MMP-9 already in monocytes clearly distinguishes GCA patients from healthy individuals.

Once circulating monocytes have differentiated into macrophages, the propensity to produce excess MMP-9 continuously is a distinguishing feature of macrophages that reach the adventitial and medial layer of the artery (14). Also, multinucleated giant cells (MNG), the hallmark cell of GCA lesions, have a particularly strong signal for MMP-9, indicating that the metalloproteinase critically affects events that lead to the formation and the destructive potential of the granulomatous lesions (**Figure 2**). A typical feature of GCA is the thinning of the medial layer, presumably by injuring vascular smooth muscle cells and the fragmentation of the elastic laminae that separate the media from the intima. Here, local delivery of MMP-9 by tissue-invasive monocytes/macrophages emerges as a pinnacle event. The media of a healthy artery is essentially impenetrable, a barrier that must be overcome by pathogenic macrophages endowed with MMP-9-dependent elastolytic and gelatinolytic activities (17, 18). Thus, abnormal programming of monocytes represents an upstream disease element, facilitating initial entrance and maneuvering of inflammatory cells in the arterial wall. This pathomechanism may be particularly important in the aorta of GCA patients, prone to dissection and aneurysm formation (**Figure 3**).

How aberrant MMP-9 production in GCA monocytes and macrophages is induced has remained unresolved, but this is a defect that is a prerequisite of disease and is present early in the disease process. MMP-9 continues to participate in the granulomatous inflammation in established and in late disease (**Figure 2**). The aberrant production of MMP-9 in monocytes and macrophages of GCA patients seems to be combined with upregulation of MMP-2, suggesting a coordinated pathomechanism that affects families of enzymes (14, 19).

Macrophage populations that settle in the granulomatous lesions are highly heterogenous. Early studies have given rise

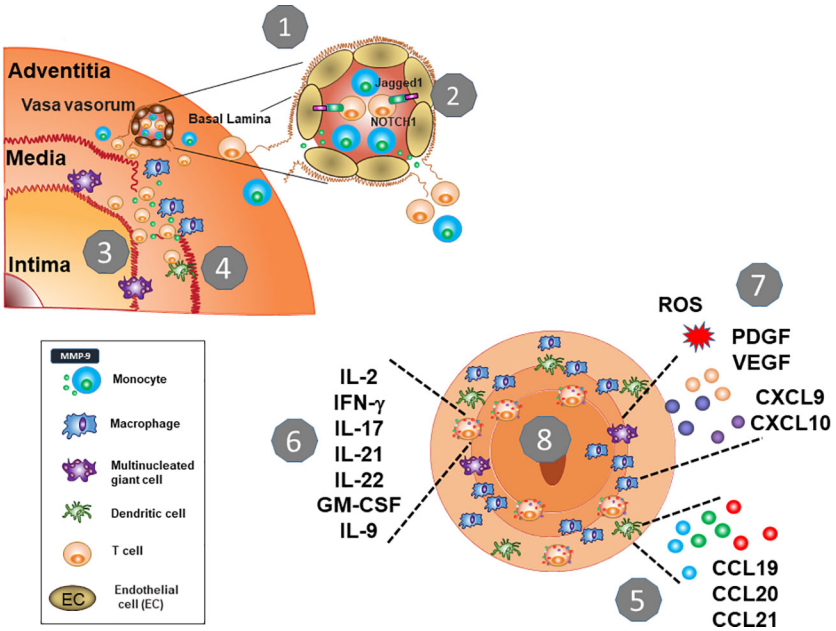

FIGURE 2 | Key Pathogenic Steps in Giant Cell Arteritis. **1.** The protective shield of the artery wall breaks when immune cells leave the vasa vasorum and invade into the tissue. An essential checkpoint is the digestion of the vascular basal lamina, facilitated by MMP-9-producing monocytes. **2.** Vasculitic T cells follow, and macrophages (histocytes) and T cells form granulomatous infiltrates in the adventitia and media. **3.** MMP-9-producing macrophages destructs the elastic laminae and eventually, tissue-damaging multinucleated giant cells emerge. **4.** T cells encounter DC that lack the immunoinhibitory ligand PD-L1 and enter unopposed and persistent activation. **5.** Wall-residing DC provide chemokines and cytokines to enhance immune cell recruitment. **6.** Chronically stimulated T cells differentiate into multifunctional effector cells providing an array of effector cytokines. They also acquire tissue residence and replenish the lesion from within. **7.** Macrophages are functionally heterogenous, but their functional commitment is directly related to their positioning in the vessel wall. Macrophage products include chemokines and cytokines, tissue-damaging mediators (ROS, MMP-9) as well as growth factors (VEGF, PDGF). **8.** Continuously stimulated T cells and macrophages elicit a maladaptive response-to-injury presenting as vessel wall remodeling, with wall vascularization and intimal hyperplasia.

to the concept that geographical mapping of the macrophages and functional commitment are linked. Macrophages in the media and at the media-intima border are most disease relevant. The fragmentation of the lamina elastica interna, the formal landmark separating the vascular smooth muscle cell (VSMC)-rich media from the intima, remains a hallmark of disease. The tissue-destructive potential of medial macrophages rests on the production of MMP-9, but also on the release of reactive oxygen species (ROS) (20). The tissue-damaging features of medial macrophages are counterbalanced by their ability to provide growth factors and angiogenic cytokines (**Figure 2**). Platelet-derived growth factor (PDGF)-producing macrophages and multinucleated giant cells sitting at the fragmented elastic lamina are critically important in driving the wall remodeling process, including the growth of neointima (21). Several cell types, including myofibroblasts, dedifferentiated VSMCs, and mesenchymal stem cells fuel the formation of the neo-tissue that occupies the artery's lumen and blocks blood flow (22). PDGF has been implicated in facilitating proliferation and directed migration of precursor cells. This maladaptive wound healing process is only possible with sufficient neoangiogenic activity. While intramural vessels are usually restricted to the adventitia, inflamed temporal arteries contain networks of newly formed capillaries, penetrating the media as well as the intima (21). Here, macrophage-derived vascular endothelial growth factor (VEGF) is instrumental in providing a potent growth factor for endothelial cells (23). Interestingly, multinucleated giant cells possess the ability to synthesize VEGF. VEGF is elevated markedly in the circulating blood of GCA patients (24–26), where this angiogenesis factor functions as a regulator of endothelial cells and promotes endothelial cell-T cell interaction (24). However, the precise cellular source of the circulating VEGF in GCA patients has not been determined. A close correlation between tissue site and macrophage functional commitment in the vasculitic lesions has recently been confirmed by Jiemy et al, who showed that MMP-9$^+$ macrophages were placed at areas of tissue destruction, while FRβ+ macrophages were positioned in the hyperplastic intima (27).

GCA is not the only vasculitis in which macrophages are key pathogenic effector cells. Rather, accumulation of highly activated macrophages in the disease lesions is a feature of other granulomatous diseases, including the small vessel vasculitis granulomatosis with polyangiitis (GPA). Specifically, overrepresentation of MMP-9 producing macrophages appears to be common between GCA and GPA (28). GPA is an autoimmune vasculitis of small blood vessels, typically associated with tissue destruction due to granuloma formation. Neutrophils forming neutrophil extracellular traps (NETs) are

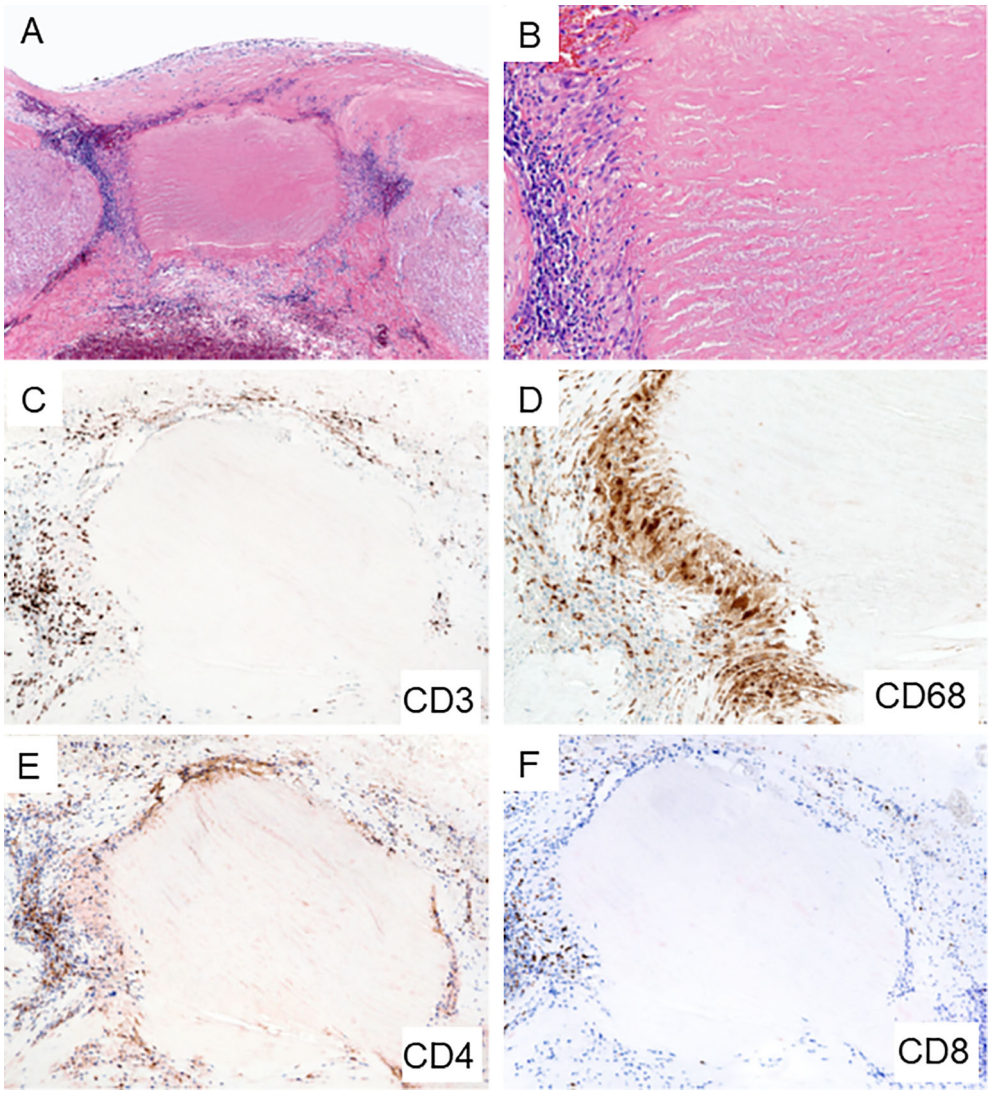

FIGURE 3 | Giant Cell Arteritis in the Aorta. Biopsy sample from surgically removed aortic wall of a patient undergoing emergency aortic repair. **(A, B)** Hematoxylin and eosin staining showing typical granulomatous inflammation with rings of predominantly lymphocytes and macrophages around necrotic medial tissue (A x60; B x200). **(C)** $CD3^+$ T cells form a collarette of inflammation enclosing the necrotic aortic wall (x100). **(D)** $CD68^+$ histiocytes palisade at the edge of the damaged tissue (x200). **(E)** $CD4^+$ T cells are the dominant T cell subset within the granulomatous infiltrates (x100). **(F)** Infrequent $CD8^+$ T cells in the aortic wall (x100).

believed to function as an inflammatory nidus. In a subset of GPA patients the disease predominantly manifests in the head and neck (H&N), presenting with bony erosions of the orbital and sinus walls, septal perforations, saddle-nose deformities, middle ear damage and epiglottitis, all related to uncontrolled destruction of bone, cartilage, and connective tissues. In mechanistic studies, NETs released from H&N GPA neutrophils functioned as powerful stimulators of macrophages, inducing MMP-9 production (28). Such MMP-9 high-producing macrophages possess tissue-destructive capabilities (28), and MMP-9-producing macrophages and multinucleated giant cells dominate the granulomatous tissue infiltrates in naso-sinal biopsies from H&N GPA patients (28). These data implicate degradation of collagen IV in basement membranes and digestion of extracellular matrix in the pathologic events leading to GPA.

Given the similarities in GCA and GPA, dysregulation of MMP-9 production may be a fundamental pathomechanism, shared amongst vasculitides and shared by diseases with granulomatous inflammation. So far, none of the genetic polymorphisms predisposing to either GCA or GPA have been connected to the functional domain of metalloproteinases.

Macrophages from GCA patients have been functionally compared to those of another vasculopathy, namely coronary artery disease (CAD). CAD is now accepted as an inflammatory blood vessel condition that progresses over decades and causes the formation of atherosclerotic plaques in the subendothelial

space of susceptible arteries (29). In the atherosclerotic plaque, highly activated macrophages take up deposited lipoproteins and modified lipids to transform into the pathognomonic foam cells. Giant cell formation occurs in just a subset of patients with atherosclerotic lesions. Thus, in GCA and CAD macrophages perform fundamentally different functions. Comparison of monocytes and monocyte-derived macrophages from GCA and CAD patients has demonstrated that these myeloid cells have distinct molecular signatures. CAD macrophages are prone to produce high amounts of inflammatory cytokines, such as IL-1β and IL-6, even more so than GCA macrophages (30). Another distinguishing features between the two diseases is the expression of the co-inhibitory ligand PD-L1, which is distinctly low in GCA, but high in CAD (30). Notably, macrophages from both patient populations abundantly produced chemokines (CXCL9, CXCL10), supporting a role in cell recruitment and assembly of the vessel wall lesions (**Figure 1B**). Metabolic conditioning was identified as the underlying mechanism. While CAD macrophages were programmed to uptake and utilize glucose, this was not the case for GCA macrophages. Addiction to glucose is one of the driving forces in CAD macrophages, dictating the dynamics of the glycolytic pathway, the setting of mitochondrial activity, the production of reactive oxygen species and ultimately, the secretion of IL-6 (31). The low glycolytic activity in GCA monocytes may be part of a broader metabolic program, as fasting blood glucose, cholesterol and triglyceride levels have been described to be negatively associated with the development of giant cell arteritis (32).

In summary, "trained immunity" in GCA leads to monocyte instruction, changing their metabolic circuitry and their functional differentiation. The concept of "trained immunity" is well understood in non-vasculitic cardiovascular disease (33–36) and relates to the concept that monocytes, macrophages, dendritic cells, and NK cells can be imprinted by encountering inflammatory stimuli, undergoing a priming process that changes their response to subsequent challenges. It is now recognized that the "training" is imprinted into the epigenome. In GCA monocytes, a lead abnormality is the high expression of MMP-9, a protease that takes center stage when inflammatory cells leave the blood stream and enter the "forbidden territory" of the vessel wall. Also affected is the expression of co-inhibitory ligands and the commitment to cytokine production. The training of monocytes has profound consequences for their later life as macrophages. They continue to produce MMP-9, now enabling them to destroy the tissue microenvironment. Functional analysis of lesional macrophages has emphasized their tissue repair capabilities, including the production of growth and angiogenesis factor, all promoting the maladaptive remodeling process in the GCA-affected artery (**Figure 2**).

Vascular Dendritic Cells (DC) as Presenters of Vasculitogenic Antigens

DCs are part of the innate immune system and are indispensable for the induction of adaptive immune responses. Specifically, DCs are needed to present antigen for T cell priming and are thought to be the principal initiators of T cell immunity (37, 38). Besides their role in presenting exogenous antigens, such as microbial antigens and allergens, DCs are also instrumental in the handling of self-antigens and thus determine the fate of auto-reactive T cells. In addition, activated DCs are an important source of cytokines and chemokines, orchestrating the assembly of inflammatory infiltrates. Finally, they finetune T cell activation by providing both co-stimulatory and co-inhibitory signals for T cells (37, 38). Critically involved in activating naïve T cells, DCs function in secondary lymphoid organs, such as lymph nodes and the bone marrow. In the case of medium and large arteries, they possess their own tissue residing DCs, so-called vasDCs (39, 40) (**Figure 4**). Such vasDCs are believed to have two disease relevant functions in GCA; (a) guarding the vessel wall immune privilege, possibly by providing tolerogenic signals, and (b) presenting vasculitogenic antigens in the vessel wall infiltrates (39, 40). Healthy temporal arteries possess vasDCs positioned at the adventitia-media junction (**Figure 4**). In the inflamed artery, DCs may move into other tissue niches to join macrophages in presenting antigen to T cells that are distributed throughout the vessel wall. vasDCs placed in the granulomatous infiltrates produce chemokines, such as CCL19, 20, and 21, and strongly express the co-stimulatory molecule CD86 (41–43) (**Figure 5**). The disease relevance of the CD28-CD86 co-stimulatory pathway was recently demonstrated in a study exploring CD28-blocking antibodies (44). Lesional T cells were found to be dependent on CD28-mediated co-stimulation, even more so than normal control T cells. Blocking the CD28-CD86 receptor ligand interaction had profound inhibitory effects on the vasculitic process (44). Not only was co-stimulation relevant in determining the strengths of T cell activation, it regulated the amount of pro-inflammatory effector cytokines produced in the vasculitic lesions. Most importantly, inflammation-induced remodeling of the vessel wall, involving intimal hyperplasia and neoangiogenesis, required crosslinking of CD28 by CD80/CD86 (44). Taken together, by controlling *in situ* co-stimulatory signals, vasDC ultimately shape several dimensions of the vasculitic process (**Figures 2** and **4**).

The dynamics and intensities of T cell activation not only depend on co-stimulation but are equally shaped by co-inhibitory signals. In healthy arteries, vasDC express the inhibitory ligand PD-L1, effectively dampening T cell triggering. It is now recognized that a defect in this co-inhibitory pathway is a hallmark of GCA (45). Specifically, DC-Sign$^+$ vasDC in healthy temporal arteries not only express CD80/CD86, but also PD-L1 (**Figures 2** and **5**). Crosslinking of the PD-1 receptor on T cells may indeed be one of the mechanisms through which vasDC protect the tissue niche and interrupt *in situ* immune activation. In GCA, vasDC lack PD-L1, suspending a negative feedback mechanism that halts inappropriate T cell stimulation. The PD-L1^{lo} phenotype is shared amongst patient-derived DC and macrophages (30), indicating a fundamental breakdown of this important immune checkpoint. The lack of PD-L1 in inflamed arteries does not explain why essentially all CD4$^+$ T cells in the lesions are strongly positive for PD-1 (45). Possible explanations are that under physiologic conditions negative signaling induced by

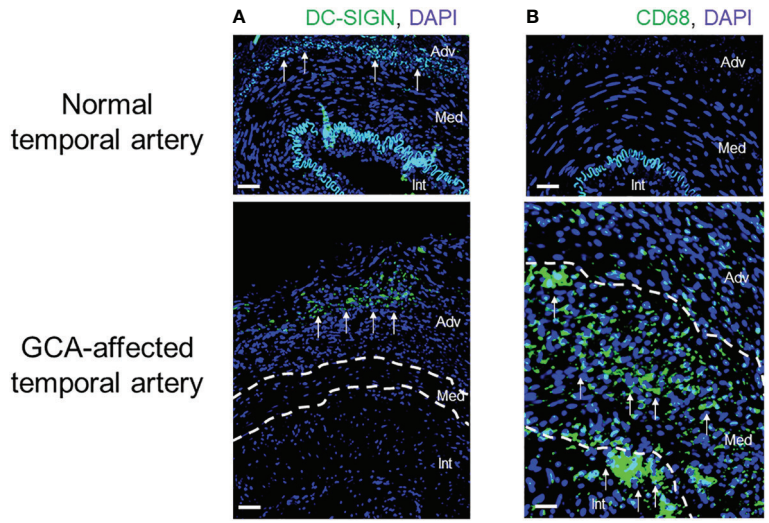

FIGURE 4 | Innate Immune Cells in Giant Cell Arteritis. Tissue sections from temporal artery biopsies were stained for the dendritic cell (DC) marker DC-SIGN **(A)** and the macrophage marker CD68 **(B)** and visualized by immunofluorescence imaging. Nuclei marked by DAPI. In the healthy artery, the autofluorescent lamina elastica interna separates the media and intima. DC-SIGN$^+$ dendritic cells are positioned at the adventitial-medial border. In the vasculitis-affected artery, DC-SIGN$^+$ dendritic cells expand in the adventitia. CD68$^+$ macrophages are essentially undetectable in the healthy artery but occupy all wall layers of the GCA artery. Int, intima; Med, media; Adv, adventitia. Scale Bar; 50 μm.

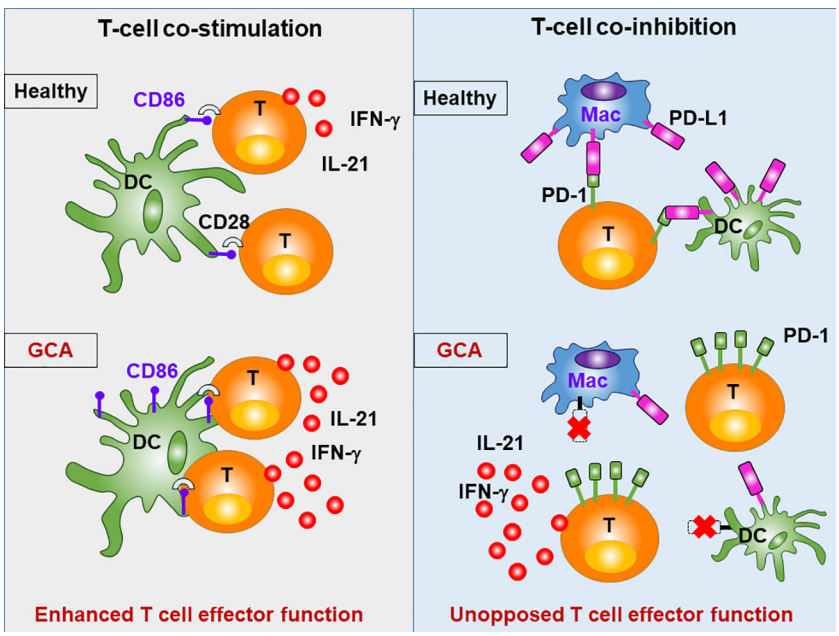

FIGURE 5 | Abnormal T cell Activation in GCA. The intensity and duration of adaptive immunity depends on the availability of specific antigen, but also on a mixture of positive (co-stimulatory) and negative (co-inhibitory) signals, that modulate the T cell receptor activation cascade. Patients with GCA have abnormalities in the CD28 co-stimulatory pathway and in the co-inhibitory PD-1/PD-L1 pathway, resulting in sustained and unopposed activation of pathogenic T cells. Under physiologic conditions, CD28 on T cells recognizes CD80/86 on antigen-presenting cells (e.g. dendritic cells; DC), prolonging and intensifying T cell activation. Signaling through this pathway is intensified in GCA. Under physiologic conditions, PD-1 on T cells recognizes PD-L1 on antigen presenting cells (e.g. macrophages; Mac), resulting in dampening of T cell activation. In GCA, PD-L1 is expressed at very low levels, disrupting this negative signal, and boosting T cell effector functions.

tissue-expressed PD-L1 prevents access of T cells to the tissue niche and that this mechanism is defective in PD-L1^{lo} hosts.

The PD-1/PD-L1 immune checkpoint is a critical regulator of immunity and is now one of the most important therapeutic targets in cancer patients (46). PD-1/PD-L1 deficiency in GCA has two clinically relevant consequences (47). First, excessive activity of this checkpoint is linked to insufficient anti-tumor immune responses. Tumors aberrantly express PD-L1 and utilize this mechanism to escape from anti-tumor T cell immunity. The defect of PD-1/PD-L1 signaling in GCA and the excess of PD-1/PD-L1 signaling in cancer patients raises the question whether GCA patients have a natural protection from malignancy. Epidemiological studies support the concept that GCA patients die from cancer less than expected (48, 49). If the broken PD-1/PD-L1 checkpoint has beneficial effects for GCA patients, then therapeutic efforts to restore the checkpoint could be effective to inhibit vasculitis while enhancing the risk for malignancy. This is not a trivial consideration, given the advanced age and the age-related cancer risk in patients with GCA. Vice versa, weakening the PD-1/PD-L1 checkpoint is the therapeutic goal in the widespread application of checkpoint inhibitors in patients with malignancies. This therapeutic intervention should place the host at risk to develop vasculitis. In support of this concept, numerous case reports have described aggressive aortitis and vasculitis in checkpoint inhibitor treated individuals (50, 51). In a human artery-SCID mouse chimeric system, in which human arteries are engrafted into NSG mice and vasculitis is induced by adoptive transfer of peripheral blood mononuclear cells of GCA patients, injection of a PD-1 blocking antibody produced aggressive vessel wall inflammation and vascular remodeling (45). More importantly, healthy mononuclear cells were able to induce vasculitis, if the checkpoint was blocked. This mimics conditions in checkpoint inhibitor treated cancer patients and emphasizes the risk of such cancer patients to come down with iatrogenic vasculitis.

Recent data suggest that a second immunoinhibitory checkpoint involving V-domain Immuno-globulin-containing suppressor of T cell activation (VISTA) may be less functional in GCA (52). Hid Cadina et al. have reported that VISTA+ Th cells are reduced in the blood of GCA patients but enriched in the inflamed temporal arteries.

Taken together, DC and other antigen-presenting cells make critical contributions to GCA, not only by *in situ* antigen presentation, but by distorting the threshold settings for T cell activation (**Figures 2**, **4** and **5**). GCA DC drive vasculitis by expressing CD86, amplifying disease-relevant enhancement of T cell immunity. At the same time, they fail to dampen lesional T cells by lacking PD-L1 on their surface. PD-L1^{lo} DC allow tissue entrance and persistence of highly activated effector T cells. The disbalance between robust co-stimulation and ineffective co-inhibition sets the stage for uncontrolled T cell immunity, with all the sequelae of a maladaptive response-to-injury. This scenario also presents an untapped therapeutic opportunity: treating GCA by interrupting excess co-stimulation or by reinstating co-inhibition (**Figure 5**). To which degree DC participate in the extravascular disease pathways of GCA is currently unknown. It is possible that DC in the circulation or in non-vascular tissues also have disease relevance and that they collude with abnormal T cells to render individuals susceptible to vasculitis (40).

Other Innate Cell Types

Other types of innate immune cells, such as neutrophils, eosinophils and NK cells are typically scarce or absent in the vasculitic lesions of GCA (53). Indeed, eosinophilic inflammation should prompt the search for an alternative diagnosis, such as eosinophilic granulomatosis with polyangiitis. In rare cases, temporal arteritis can be attributed to an alternative vasculitis and atypical features on histologic examination are often the first clue (54, 55). Neutrophils may have a role in extravascular GCA. In the peripheral blood of GCA patients, decrease of suppressor neutrophils has been reported to accelerate effector T cell proliferation (56). GCA shares with other vasculitides the presence of immature neutrophils in the peripheral blood, which tightly correlated with inflammatory activity. In an *in vitro* co-culture system, such immature neutrophils produced abundant reactive oxygen species that caused protein damage and injured the endothelial barrier permeability (57). Mast cells may play an active role in vessel wall inflammation and have been described as one of the cellular sources of VEGF in temporal arteritis lesions (58).

ADAPTIVE IMMUNITY IN GCA

GCA is an HLA class II associated disease and the dominant cell type in the vasculitic lesions are CD4$^+$ T cells, moving the adaptive immune system into center stage (**Figures 1** and **3**). Immunophenotyping of inflamed arteries demonstrates that CD4$^+$ T cells outnumber CD8$^+$ T cells (**Figures 1** and **3**), a feature which distinguishes GCA and Takayasu arteritis (9). In line with the observation that HLA class I molecules seem to be important as disease risk markers in Takayasu arteritis, the cytotoxic functions of CD8$^+$ T cells have been implicated as a relevant disease mechanism in this vasculitis (9). However, recent data have assigned a disease relevant role to CD8$^+$ T cells in the periphery. Specifically, CD8$^+$ T cells with regulatory function, CD8$^+$ Treg cells, are defective in GCA patients, failing to dampen CD4$^+$ T cell function in vasculitis (59, 60).

Granulomatous infiltrates are typically composed of CD4$^+$ T cells and macrophages and contain few B cells (61). Accordingly, autoantibodies seem to play no role in GCA. Cellular accumulations reminiscent of tertiary lymphoid aggregates, including B cells and plasma cells, have been seen in GCA affected aortic tissues (62). Whether they have functional relevance remains to be determined, but B cells are not recognized as drivers of the typical granulomatous reaction causing GCA. It is to be expected that systemic inflammation and the acute phase reaction typical for extravascular GCA leads to shifts in the distribution of circulating B cells (63). However, the biological relevance is unknown.

Sharing of T cell receptor sequences in independent tissues sites affected by GCA has nurtured the concept that antigen

recognition is central in the emergence of the granulomatous lesions (64, 65). The nature of a causative antigen, however, has remained speculative. A tempting speculation is the proposal to implicate viral infections. Elderly individuals harbor a spectrum of chronic viral infections and the immune aging process makes them more susceptible to reactivation (66). Thus, it has been proposed that varicella zoster may be the underlying trigger of GCA, but carefully designed studies have refuted this theory (67–69). Recent observations that cancer patients treated with immune checkpoint inhibitors are at high risk to develop therapy-induced vasculitis (9, 50, 51) have emphasized the role of antigen-nonspecific mechanisms. If unleashed, polyclonal T cell populations appear to be able to promote vasculitis.

At the current stage of knowledge, T cells in GCA patients make several mistakes that culminate in loss of tolerance and the establishment of chronic-persistent inflammatory infiltrates in the wall of susceptible arteries.

Peripheral CD4+ T Cells in GCA Patients

The hallmark abnormality in circulating $CD4^+$ T cells from GCA patients is the aberrant expression of NOTCH1 (24, 70) (**Figure 2**). *NOTCH1* is an oncogene, most notably, *NOTCH1* mutations are present in the majority of patients with T cell acute lymphocytic leukemia (71). NOTCH signaling controls cell fate decisions and is needed for the specification of T cells; directs cell proliferation, differentiation, and cell death (72, 73). In GCA, NOTCH1 expression on circulating $CD4^+$ T cells has been implicated in enabling their transition from the blood into the tissue, representing a major tolerance defect in this disease. $CD4^+NOTCH1^+$ T cells from GCA patients recognize aberrantly expressed JAGGED1 on the surface of vasa vasorum endothelial cells (24), facilitating their invasion into the vessel wall (**Figure 2**). Targeting the NOTCH1-JAGGED1 interaction was sufficient to suppress vasculitic activity (24), placing this receptor-ligand interaction at the top of GCA pathophysiology. A prerequisite for the transmigration from the capillary lumen into the perivascular space is the action of MMP-9-expressing monocytes, which first must digest the basal lamina to pave the way for T cells (14) (**Figure 2**). In the absence of monocytes or macrophages, GCA T cells fail to invade into 3D extracellular matrix. The dependence of T cells on pathogenic monocytes/macrophages exemplifies the co-occurrence of abnormalities in the innate and adaptive immune system steering inflammatory cells into an immunoprivileged tissue site.

Tissue-Residing $CD4^+$ T Cells in GCA Lesions

Lesional $CD4^+$ T cells in the vasculitic wall have two major abnormalities; they are pluripotent effector cells, supporting a multitude of inflammatory effector pathways and they can self-renew to sustain the wall infiltrates and turn acute vasculitis into chronic-persistent disease (**Figures 5** and **6**).

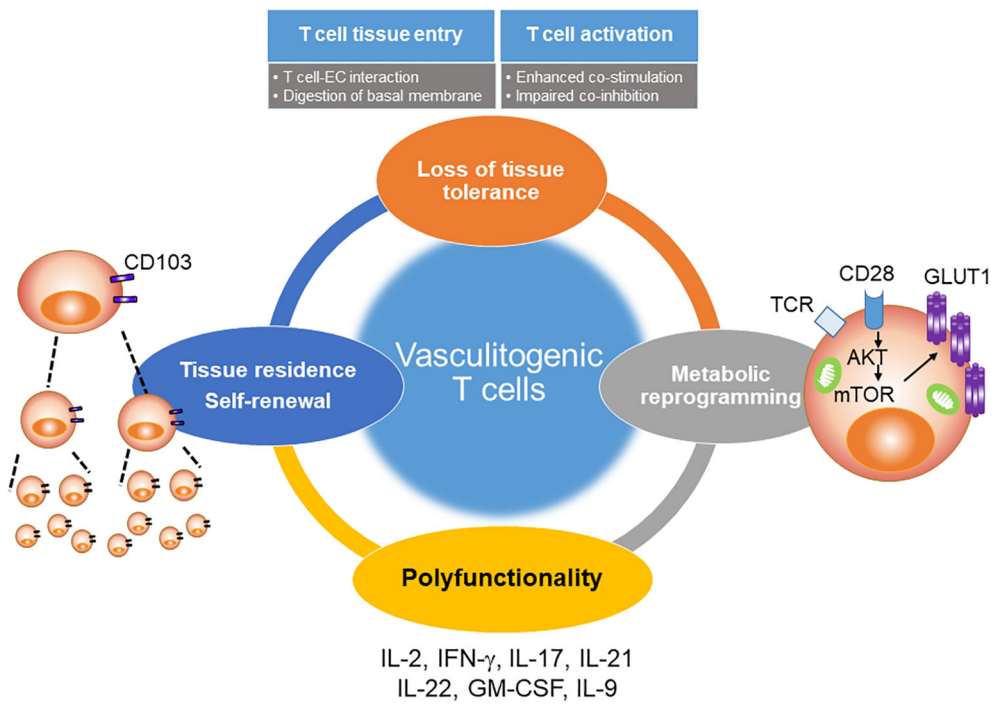

FIGURE 6 | Pathogenic role of T cells in GCA. The shift from protective to pathogenic immunity in GCA involves multiple processes. Four major abnormalities have been molecularly defined (1). In GCA patients, CD4+ T cells aberrantly express NOTCH1, which facilitates T cell-endothelial communication, tissue entry and uncontrolled T cell expansion (2). Excess CD28-dependent signaling imprints a metabolic signature that sustains pro-inflammatory T cells (3). Such T cells supply a multitude of effector cytokine to stimulate macrophages and vascular cells (4). A critical feature of disease-promoting $CD4^+$ T cells is the ability to establish tissue residency in the vessel wall. Tissue-resident memory $CD4^+$ T cells render the lesion autonomous and ensure chronicity of disease.

The vast majority of lesion-residing $CD4^+$ T cells are strongly positive for PD-1 (45) (**Figure 5**). On human T cells, PD-1 is an activation marker, but more importantly, has been implicated in tumor evasion mechanisms and in exhaustion of chronically stimulated T cells (74). $CD4^+$ T cells trapped in the arterial wall are not exhausted, nor are they senescent. Rather, their accumulation is a consequence of insufficiency in PD-L1 expression (see above). Both, vascular DC, and macrophages are distinctly low for PD-L1, disrupting a negative signal to $PD\text{-}1^+$ T cells. The entry of T cells into the wall and the accumulation/retention of T cells in the wall are both dependent on PD-L1 (45).

Besides expressing surface PD-1, lesional $CD4^+$ T cells are polyfunctional (45) (**Figure 6**). A multitude of T cell effector cytokines have been mapped to the lesions, including IL-2, IFN-γ, IL-17, IL-21, IL-9, IL-22, and GM-CSF (**Figure 6**). It has not been clarified whether the polyfunctionality occurs on the level of individual cells or the T cell population. IFN-γ-producing $CD4^+$ T cells represent the dominant T cell subset in inflamed temporal arteries (11, 75). IFN-γ-producing $CD4^+$ T cells are expanded in the peripheral blood of GCA patients and are resistant to corticosteroid therapy (75). IFN-γ has all the characteristics of a critical effector cytokine, as it activates macrophages, DC, and endothelial cells. Interestingly, $IFN\text{-}\gamma^+$ $CD4^+$ T cells map preferentially to the adventitia of GCA-affected temporal arteries (76). How they guide the activity of the granulomatous infiltrates needs to be clarified. However, the geographical distance to migrating myofibroblasts may be important, as IFN-γ is considered to inhibit proliferation of mesenchymal cells.

In contrast to their resistance to corticosteroids, $IFN\text{-}\gamma^+$ T cells are dependent on Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling and this dependence creates a vulnerability that can be therapeutically exploited. Inhibiting the JAK/STAT signaling pathway with a small molecule inhibitor targeting JAK1/3 is highly effective in suppressing vasculitis, including the IFN-γ-producing $CD4^+$ T cells (77). These data have raised the possibility that IFN-γ production is part of a feed forward loop, as IFN type II is a potent inducer of JAK/STAT signaling (78). Tissue transcriptomic studies have indicated that STAT target genes are strongly upregulated in the lesions, including target genes of IFN type 1 and type 2.

IFN-γ-producing $CD4^+$ T cells are accompanied by subsets of lesional T cells that produce IL-17, IL-21, and IL-9 (79, 80). Likely, each of these T cell lineages makes a specialized contribution to the disease process, but mechanistic studies detailing this are not yet available. $IL\text{-}17^+$ T cells in GCA lesions have been reported to be highly sensitive to corticosteroid therapy, disappearing upon initiation of this immunosuppressant (75) and are thus different from $IFN\text{-}\gamma^+$ T cells, that persist over prolonged periods despite steroid therapy (75). Th17 cells may thus be easily controllable and may not have much value as a therapeutic target.

IL-21-producing $CD4^+$ T cells are abundant in the tissue lesions and in the blood of patients with GCA and appears to be sensitive to glucocorticoid treatment (79). IL-21 is reported to play a role in supporting Th1 and Th17 responses and suppressing $FOXP3^+$ T regulatory cells in GCA (79), but the precise pathogenic role of IL-21 remains unclear.

IL-9 is a pleiotropic cytokine, with the potential to drive both pro-inflammatory and anti-inflammatory responses (81). High expression of IL-9 was reported in temporal artery biopsies (80), but how this cytokine influences vasculitic immune responses is unknown.

IL-22 is believed to mediate the crosstalk between immune cells and stromal cells (82). IL-22 has been encountered in temporal artery biopsies and is strongly linked to vasculitis (83). Little is known so far how stromal cells are involved in the disease process, but they are ultimately important in wall remodeling. Whether IL-22-dependent immunity is relevant in the maladaptive wound healing response awaits clarification.

The T cell effector cytokine GM-CSF is considered an important regulator of macrophages (84) and could provide effective T cell-macrophage communication in the granulomatous infiltrates (84). Indeed, macrophages activated by GM-CSF acquire numerous effector functions, enabling them to amplify tissue inflammation. GM-CSF is the product of a specialized T cell subset that has high disease relevance in multiple sclerosis (85, 86).

Tissue-Resident Memory T Cells in the Inflamed Artery

Despite the physiological ability of host immune protection by trafficking of memory T cells around the body, recent studies have revealed that specialization of pathogenic memory T cells into unique tissue-resident subsets may drive regional autoimmunity (87, 88). Long-lasting immunity causing temporal artery damage is mediated by tissue resident memory T cells (44, 77). Data from re-engraftment studies have revealed that vasculitis-causing T cells acquire tissue residency and build autonomous, self-sufficient inflammatory lesions (77), where repopulation of inflammatory $CD4^+$ T cells is maintained from tissue-resident memory populations (**Figure 6**). Further, metabolic analysis of tissue resident memory T cells in the vasculitic wall lesions has yielded evidence for high glycolytic activity resulting from CD28-dependent signals and fulfilling the energy demand of repopulating effector T cells (44). Those tissue-residing T cells are polyfunctional and steroid therapy resistant. In fact, a study analyzing temporal artery biopsies before and up to 12 months after steroid therapy found that half of GCA patients still have ongoing vessel wall inflammation after one year of immunosuppression (13).

$CD8^+$ T Cells in GCA

Early studies examining frequencies of circulating $CD8^+$ T cells in GCA gave rise to the hypothesis that a reduction of $CD8^+$ T cells is typical for active untreated GCA (89). This hypothesis was called into question by later studies (90). A recent manuscript described altered gene expression profiles in blood CD4 and in CD8 T cells in a cohort of 16 GCA patients that were monitored by longitudinal expression profiling (91).

A clue towards an entirely new disease mechanism in GCA CD8 T cells has come from studying the T cell aging process. T cell aging leads to a maladaptive response that directly contributes to chronic

TABLE 2 | Potential therapeutic targets in giant cell arteritis.

Targets	Pathogenic role in vessel wall inflammation	Drugs
mTOR signaling	T cell proliferation and survival; Metabolic control of T cell effector differentiation and of T cell functions;	Rapamycin
VEGF signaling	Endothelial cell homeostasis; Maintenance of vasa vasora; Pathogenic wall vascularization; Induction of co-stimulatory ligands (Jagged1);	Bevacizumab
NOTCH signaling	T cell fate decisions; T cell co-stimulation; T cell clonal expansion and survival; T cell tissue invasion; Trafficking of intracellular vesicles;	DAPT
JAK-STAT signaling	Type I and type II IFN-dependent responses;	Tofacitinib Baricitinib
CD28-AKT signaling	Uncontrolled co-stimulation; Metabolic programming of effector T cells;	Abatacept Anti-CD28
PD-1/PD-L1 signaling	Deficient co-inhibition; Failure of negative signaling; Inappropriate T cell expansion, survival and effector functions;	PD-L1 Fc PD1 agonists
MMP-9 production	Destruction of the arterial wall tissue barrier; Structural damage to the vessel wall;	MMP-9 blockade

inflammatory disease (92). $CD8^+$ T cells are well-known to age faster than $CD4^+$ T cells and a hallmark of T cell aging is the loss of naïve $CD8^+$ T cells (93). In fact, older individuals fail to generate $CD8^+$ $CCR7^+$ T regulatory cells, rendering them susceptible to unopposed immune reactivity (59). Age-dependent decline of protective immunity and rise of dysfunctional immunity may be one of the reasons that GCA occurs exclusively in individuals older than 50 years of age. Indeed, loss-of-function of protective $CD8^+$ Treg cells is associated with aging (59). Mechanistically, $CD8^+$ T regulatory cells suppress activation and expansion of $CD4^+$ T cells by releasing exosomes that contain preassembled NOX2 membrane clusters which are taken up by $CD4^+$ T cells (59). Defective $CD8^+$ T regulatory cells in GCA patients lose the ability to package NADPH oxidase into immunosuppressive exosomes. A recent study has identified the molecular mechanism leading to $CD8^+$ Treg cell failure in GCA patients (60). The inability of GCA $CD8^+$ Treg cells to release NOX2-containg, immunosuppressive exosomes was mechanistically connected to abnormalities in endosomal trafficking. Specifically, due to aberrant NOTCH4 signaling, GCA $CD8^+$ Treg cells changed the profile of RAB GTPases, which promoted NOX2 trapping in an intracellular compartment of early and recycling endosomes (60).

These studies have identified a novel molecular abnormality linking T cell aging, Treg cell failure and susceptibility to vasculitis. Implicating RAB GTPases and intracellular vesicular trafficking in disease pathogenesis opens new conceptual and therapeutic opportunities.

FROM BENCH TO BEDSIDE: POTENTIAL THERAPEUTIC TARGETS IN GCA

Glucocorticoids (GC) remain the standard therapy, possibly because of their untargeted immunosuppression and the multiplicity of pathogenic pathways contributing to GCA (**Figures 2**, **5**, **6**). GC are highly effective in suppressing extravascular GCA, flattening the acute phase response, clinical symptoms, and abnormal laboratory parameters (94). To examine the remission-inducing potential for the vessel wall component of the disease, we have utilized a dual-biopsy approach. 40 patients with a positive temporal artery biopsy received standard doses of prednisone and were re-biopsied on the collateral side at 3, 6, 9 or 12 months (13). About 50% of patients had active vasculitis after 12 months of GC therapy (13). Patients with a positive second biopsy had excellent clinical and laboratory responses and were clinically indistinguishable from patients in whom the second biopsy was negative. Thus, GC therapy is highly efficient for extravascular GCA and insufficiently treats vascular GCA. Also, clinical assessment and monitoring of sedimentation rate and CRP are not able to assess the inflammatory load in the vessel wall. Overall, new therapeutic approaches are needed to treat GCA, probably in form of combination therapy. The resistance of the vascular component, likely a consequence of the ability of the disease lesions to become autonomous, emphasizes the need for more efficient immunosuppression that can be given over extended time periods in elderly individuals. New therapeutic strategies need to go hand-in-hand with the development of diagnostic tools that allow quantification of vessel wall inflammation.

Tocilizumab, an anti-IL-6 receptor antibody, has shown efficacy in suppressing ESR and CRP, helping to spare GC dosing to manage the acute phase response (95). However, it remains unknown whether inhibiting IL-6 signaling has beneficial effects on vessel wall inflammation itself. In fact, discrepancies between vascular and extravascular inflammation in large vessel vasculitis has been increasingly recognized and represents the most challenging problem in the management of this autoimmune vasculopathy (96). Disease flares are frequently observed even in GCA patients treated with tocilizumab plus GC

that have reached normal acute-phase reactant levels. Further, disease progression of local vessel wall inflammation has been reported in patients with Takayasu arteritis on tocilizumab treatment although they were clinically asymptomatic and had normal laboratory findings (97–99). Patients with Kawasaki disease on tocilizumab treatment have been reported to develop giant coronary aneurysms despite clinical and laboratory improvements (100). These data are in line with the concept that correcting downstream inflammatory parameters is insufficient to reset upstream abnormalities in the immune system of the patients. Here, progress made in understanding the immune signaling networks underlying vascular inflammation needs to guide the exploration of novel therapeutic interventions, including those intended to control the inflammatory attack of the vessel wall. One possible approach is to target key effector cells in the vascular lesions, e.g. macrophages. Currently ongoing trials with the GM-CSF receptor blocker mavrilimumab are designed to disrupt the inappropriate macrophage activation in the lesions. An alternative approach is to interfere with disease relevant signaling pathways, which may be shared by several cell populations relevant in the disease process.

Here, we have summarized the signaling pathways that are now understood to contribute to the immunopathogenesis of GCA and may serve as therapeutic targets (**Table 2**).

mTOR Signaling

The serine/threonine kinase mTOR (mechanistic target of rapamycin) is designed to integrate environmental signals to coordinate cellular response patterns. mTOR is a critical signaling hub in all cell types relevant for vasculitis, including T cells, which rely on mTOR activity for their development, differentiation functional fitness. mTOR signaling guides effector cell fate decisions, a fundamental abnormality in T cells from GCA patients. mTOR has also been implicated in controlling the suppressive activity of regulatory T cells and regulates the process of T cell exhaustion. Aberrant mTOR activation is a hallmark abnormality in $CD4^+$ T cells from GCA patients, both in circulating as well as lesional T cells (24, 101). mTOR signaling may also be a driving force in endothelial cells of microvessels that provide access to the vessel wall and function as partners of effector T cells (101). mTOR functions as a sensor of nutrient resources, particularly amino acid supply (102). GCA T cells utilize a highly activated glycolytic program to support their effector functions and sustain their self-replicative potential (44). Inhibiting mTOR signaling may therefore disrupt an array of disease-relevant T cell functions. The wall remodeling process is dependent on cellular growth and expansion, with multiple cell types involved. mTOR activity may represent a common denominator driving cellular activity of diverse pathogenic population and as such represent a unifying target to treat vasculitis.

VEGF-NOTCH Signaling

Serum VEGF levels are highly elevated in patients with GCA, indicating the critical role of angiogenesis and endothelial cell function in this autoimmune vasculitis (21, 24). Endothelial cells lining the vasa vasorum are the gate keeper of the vessel wall and this barrier has been overcome to enter the tissue niche (8). Also, the remodeling process is particurly dependent on neoangiogenesis within the wall layers. Macrophages, multinucleated giant cells, and mast cells have been identified as a cellular source of VEGF in GCA. VEGF functions not only as an angiogenic factor but also activates endothelial cells, upregulating Jagged1 expression on adventitial vasa vasorum endothelial cells, thus turning the EC into an engaged partner to interact with NOTCH 1 receptor-expressing T cells (24). The surplus of VEGF promotes endothelial cell proliferation and sustains formation of new capillaries (103, 104). Anti-VEGF treatment is widely applied in the therapy of malignant tumors and has become a promising treatment to inhibit aberrant neovascularization in ocular disease (105). The NOTCH signaling pathway has been investigated as a therapeutic target to block proliferative activity in malignant cells (106). Here, GCA displays abnormalities that are shared between cancer and autoimmunity, encouraging the exploration of anti-angiogenic and anti-proliferative interventions in GCA. Stopping angiogenesis may have benefit in dampening wall remodeling. Inhibiting NOTCH signaling may prevent aberrant cellular activation for multiple disease relevant cell populations.

JAK-STAT Signaling

Transcriptomic analysis has shed light on ongoing JAK-STAT signaling in inflamed temporal arteries, implicating mostly Type I and Type II IFN-dependent responses (77). Type II IFN-regulated inflammation is in line with the critical position of IFN-γ in disease pathogenesis. Little is known about a potential role of IFN type I. Notably, upregulation of Type I and Type II IFN as upstream inducers of pathogenic immunity has also been reported for Takayasu arteritis (107). We have published a proof-of-principle study showing that treatment with tofacitinib, a selective JAK1 and JAK3 inhibitor, is highly efficient in suppressing vessel wall inflammation (77). Unexpectedly, interfering with JAK-STAT signaling was highly successful in interrupting both intimal hyperplasia and wall capillarization in the human artery -SCID chimera model (77). Recently reports suggest that tofacitinib may have a place in managing patients with refractory Takayasu arteritis (108). These data support the concept that autoimmunity in the wall of large arteries relies disproportionally on JAK-STAT signaling and that therapeutic opportunities lie in dampening excessive activity in these fundamental signaling pathways.

T-Cell Co-stimulation and Co-inhibition

GCA is a granulomatous vasculitis, defining T cells and macrophages as the key pathogenic drivers. The intensity and duration of T cell activation is not only dependent on antigen recognition, but equally important are co-stimulatory and co-inhibitory signals. Recent data support a role for CD28-mediated co-stimulation in several domains of the disease process, including T cell expansion, survival, and metabolic fitness. Randomized controlled trials have reported the efficacy and safety of abatacept, CTLA-4Ig, that blocks T cell co-stimulation in patients with GCA (109). However, these clinical trials have

focused on assessing inflammatory markers (ESR, CRP) and clinical relapses, which all reflect activity in the extravascular arm of GCA. Data are needed to understand whether blocking CD28 co-stimulation has beneficial effects for chronicity of vascular inflammation and the adverse remodeling process of the vascular wall. Proof-of-principle studies in humanized mice are encouraging, emphasizing the dependence of wall inflammation on CD28-mediated co-stimulatory input (44).

Under physiologic conditions, co-stimulatory signals are offset by co-inhibitory signaling. Amongst the inhibitory pathways, the PD-1/PD-L1 pathway is best known due to the aberrant expression of PD-L1 on tumor cells, which paralyses anti-tumor T cell responses. The PD-1/PD-L1 pathway is deficient in GCA due to the low PD-L1 expression on the patients' dendritic cells and macrophages (45). Numerous therapeutic antibodies are in use to disrupt PD-1/PD-L1 signaling in cancer patients, but so far, no therapeutics are available to strengthen PD-1 signaling. Options include agonistic anti-PD-1 antibodies, transferring negative signals into T cells or replacing the lacking PD-L1 with soluble PD-L1 fused to an Fc domain.

Excess Production of the Metalloproteinase MMP-9

Breakdown of the basal lamina, enabling the transition of macrophages and T cells out of the blood stream into the extracellular space of the vessel wall, is an early pathogenic event in GCA and depends on MMP-9-mediating digestion of the protective basal membrane (14). In a preclinical model system, treatment with an antibody blocking MMP9 activity was sufficient to halt vasculitis and prevent vessel wall remodeling (14). An appealing aspect of targeting MMP-9 lies in the potential to stop invasion of the vessel wall while protecting the immunocompetence of the host. MMP-9 was detected in three cell populations: monocytes, macrophages, and multinucleated giant cells. Interfering with the activity of MMP-9 would thus provide opportunities to target innate immunity in GCA, while preserving adaptive immunity. Also, MMP-9 participates in very early steps of autoimmune vasculitis and may be able to terminate invasion of the artery. At the same time, MMP-9 is a key molecule in the destruction of elastic membranes and may be particurly important in complication of GCA aortitis, such as wall dissection and aneurysm formation (14). Finally, MMP-9 blocking agents may be best placed in combination therapies that use a two-pronged approach to have an impact on the complex pathogenesis of GCA.

IL-12/IL-23 Signaling

A hallmark of GCA is the recruitment and retention of highly differentiated effector T cells that become part of the granulomas (**Figure 5**). The differentiation process depends upon lineage-inducing cytokines, such as IL-12 and IL-23, which are major regulators of T cell fate. IL-12 and IL-23 have been implicated in promoting Th1 and Th17 lineage commitment in both GCA and Takayasu arteritis (75, 110). In situ IL-12 and IL-23 heterodimers have been reported in temporal arteritis lesions (111). In addition, genome-wide association studies have categorized IL-12B as a susceptibility gene for Takayasu arteritis (112, 113). Ustekinumab, a monoclonal antibody that inhibits both IL-12 and IL-23 signaling by binding to the common p40 subunit, has been tested in patients with GCA and Takayasu arteritis (114, 115). A prospective, open-label trial of ustekinumab in 13 patients with active new-onset or relapsing GCA was prematurely closed because patients could not reach prednisone-free remission (116). Blocking IL-12/IL-23 should interfere with the differentiation program of naïve into memory/effector T cells, a process that may precede the onset of vasculitis. Given the autonomy of the vascular lesions (see above tissue-resident memory T cells), interfering with the IL-12/IL-23 pathway may need to be combined with blocking the primary seeding of the vessel wall.

CONCLUSIONS

Autoimmune disease infrequently targets arteries, but autoimmune vasculitis is a dangerous disease due to the high potential for life-threatening complications. Large arteries, such as the aorta, respond to autoimmune attack with loss of wall integrity, clinically presenting as dissection, aneurysm formation of rupture (**Figure 3**). In medium arteries, wall inflammation results in a maladaptive remodeling process that occludes the lumen and causes tissue ischemia (**Figure 1**). The pathologic lesion is a granulomatous reaction, often with formation of multinucleated giant cells (**Figures 1** and **3**). The molecular signature of disease-relevant monocytes and macrophages includes the aberrant production of the metalloproteinase MMP-9 and the selective loss-of-function of the inhibitory ligand PD-L1. In the vasculitic lesions, macrophages are critical effector cells, supplying cytokines, metalloproteinases and angiogenic factors. The therapeutic targeting of pathogenic macrophage functions is only superficially explored but holds promise to provide entirely new strategies for anti-vasculitic immunotherapy (**Figure 2**).

As documented by the granulomatous nature of autoimmune vasculitis, GCA is ultimately a disease of misdirected adaptive immunity. The master regulators of the faulty immune response are $CD4^+$ T cells that enter a protected tissue niche, take tissue residence, gain autonomy, and differentiate into multiple classes of differentiated effector T cells (**Figure 6**). Accordingly, the vasculitic lesions are rich in a spectrum of effector cytokines, including IL-2, IL-9, IL-17, IL-22, GM-CSF, IFN-γ, and IL-21. Each of these effector cytokines contributes in its own right, multiplying the pathogenic potential of T cell accumulations forming within the layers of the arterial wall. The multiplicity of effector T cell populations makes a single causative antigen highly unlikely.

The molecular signature of pathogenic $CD4^+$ T cells in GCA includes the aberrant expression of the NOTCH1 receptor, and the reliance on CD28 costimulatory signaling unopposed by PD-1 inhibitory signaling (**Figures 2** and **6**). GCA patients have metabolically active $CD4^+$ T cells with persistent mTORC1

activation. These T cells are powerful drivers of pathogenic cascades that finally lead to wall destruction or to intimal hyperplasia and luminal occlusion. The complexity of GCA pathogenesis offers multiple intersection points that should allow to broaden the diagnostic and therapeutic approach to this difficult-to-manage autoimmune disease (**Figure 6**; **Table2**). A major hurdle lies in the split of the disease process into an extravascular and a vascular component which are at least to a large extent independent of each other. Extravascular and vascular GCA follow different trajectories, relate to different pathogenic mechanisms and ultimately, require different diagnostic and therapeutic schemes.

Several unanswered questions remain. How does the aging process of the vessel wall and the immune system conjoin to render the host susceptible to GCA? How does the tissue microenvironment create the stringent tissue tropism of this autoimmune disease? Are there vasculitogenic antigens or is the fundamental abnormality solely a defect in threshold setting of $CD4^+$ T cells? How do $CD4^+$ T cells engage vascular stromal cells to cause intimal hyperplasia? What is the underlying mechanism driving T cell polyfunctionality? What are the pathogenic processes underlying extravascular GCA? A new conceptual approach to this autoimmune and autoinflammatory condition will pave the way to the development of novel diagnostic and therapeutic modalities.

AUTHOR CONTRIBUTIONS

MA, JG, and CW wrote the manuscript. SO and GB contributed figures. The concept presented in the manuscript were developed by CW, JG, GB, and DL. All authors contributed to the article and approved the submitted version.

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Phenotype, Susceptibility, Autoimmunity, and Immunotherapy Between Kawasaki Disease and Coronavirus Disease-19 Associated Multisystem Inflammatory Syndrome in Children

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Coronavirus disease-19 (COVID-19) in children is usually mild but some are susceptible to a Kawasaki disease (KD)-like multisystem inflammatory syndrome in children (MIS-C) in the convalescent stage, posing a need to differentiate the phenotype, susceptibility, autoimmunity, and immunotherapy between KD and MIS-C, particularly in the upcoming mass vaccination of COVID-19. Patients with MIS-C are prone to gastrointestinal symptoms, coagulopathy, and shock in addition to atypical KD syndrome with fever, mucocutaneous lesions, lymphadenopathy, and/or cardiovascular events. MIS-C manifests KD-like symptoms that alert physicians to early recognize and adopt the KD treatment regimen for patients with MIS-C. MIS-C linked to COVID-19 teaches us infection-associated autoimmune vasculitis and vice versa. Studies on genetic susceptibility have identified certain human leukocyte antigen (HLA) locus and toll-like receptor (TLR) associated with KD and/or COVID-19. Certain HLA subtypes, such as HLA-DRB1 and HLA-MICA A4 are associated with KD. HLA-B*46:01 is proposed to be the risk allele of severe COVID-19 infection, and blood group O type is a protective factor of COVID-19. The autoimmune vasculitis of KD, KD shock syndrome (KDSS), or MIS-C is mediated by a genetic variant of HLA, FcγR, and/or antibody-dependent enhancement (ADE) resulting in hyperinflammation with T helper 17 (Th17)/Treg imbalance with augmented Th17/Th1 mediators: interleukin-6 (IL-6), IL-10, inducible protein-10 (IP-10), Interferon (IFNγ), and IL-17A, and lower expression of Treg-signaling molecules, FoxP3, and transforming growth factor (TGF-β). There are certain similarities and differences in phenotypes, susceptibility, and pathogenesis of KD, KDSS, and MIS-C, by which a physician can make early protection, prevention, and precision treatment of the diseases. The evolution of immunotherapies for the diseases has shown that intravenous immunoglobulin (IVIG) alone or combined with corticosteroids is the standard treatment for KD, KDSS, and MIS-C. However, a certain portion of patients

who revealed a treatment resistance to IVIG or IVIG plus corticosteroids, posing a need to early identify the immunopathogenesis, to protect hosts with genetic susceptibility, and to combat Th17/Treg imbalance by anti-cytokine or pro-Treg for reversal of the hyperinflammation and IVIG resistance. Based on physiological and pathological immunity of the diseases under genetic susceptibility and host milieu conditions, a series of sequential regimens are provided to develop a so-called "Know thyself, enemy (pathogen), and ever-victorious" strategy for the prevention and immunotherapy of KD and/or MIS-C.

Keywords: Kawasaki disease, multisystem inflammatory syndrome in children, susceptibility, autoimmunity, immunotherapy, coronavirus disease-19

PHENOTYPE OF KAWASAKI DISEASE-LIKE MULTISYSTEM INFLAMMATORY SYNDROME IN CHILDREN DIFFERENT FROM KD

Coronavirus disease-19 (COVID-19) is usually mild in children (1–3). However, 3–6 weeks after the disease or exposure to persons with COVID-19, some children are affected by multisystem inflammatory syndrome in children (MIS-C) (4–9). Those with MIS-C frequently have gastrointestinal symptoms, coagulopathy, and shock in addition to atypical Kawasaki disease (KD) symptoms with intractable fever, mucocutaneous lesions, lymphadenopathy, and/or cardiovascular events (4–8), which alert physicians to early recognition and adopt the KD treatment regimen for them (4–9). MIS-C occurring 3–6 weeks after contracting COVID-19 suggests that MIS-C is an infection-associated autoimmunity. The life-threatening infection-associated hyperinflammatory syndrome does not completely respond to intravenous immunoglobulin (IVIG) therapy, which is a standard treatment for KD. IVIG plus corticosteroids (4–8) or/and blockade of interleukin-1 (IL-1) or IL-6 action (9) have been used to treat patients with MIS-C. Now the questions remains, who are susceptible, what is (are) the trigger(s), how to predict and differentiate between KD and MIS-C, and which regimen is the optimal therapy for KD or MIS-C based on mechanistic infection immunity?

Kawasaki disease (KD) is a hyperinflammatory febrile vasculitis in children below 5 years of age, with at least four of the five clinical symptoms/signs: skin rashes (>90%), bilateral conjunctival injection (>90%), oral mucosal changes (>90%), peripheral extremity changes, and cervical lymphadenopathy (at least 1.5 cm in diameter), which might develop weeks after a mild respiratory or gastrointestinal symptom (10–12). Those with <4 criteria for KD are classified as incomplete or atypical KD. Children in the extremes of the age spectrum (≤6 months old, or ≥5 years old) tend to have atypical KD associated with delayed diagnosis and treatment (13, 14). Atypical presentation of KD in children may be associated with a higher risk of coronary arteritis because of a delayed diagnosis and treatment (14, 15). KD is previously called mucocutaneous lymph node syndrome by *Tamisaku Kawasaki* in 1974 (10), in regard to vasculitis including coronary arteritis and aneurysm (10–15). The hyperinflammatory response of KD is related to infection, autoimmunity, and/or genetic susceptibility (10–12). KD is more prevalent in East Asia, such as Japan, China, Korea, and Taiwan (10–12, 16–18). The incidence of KD varies from country to country, e.g., 4.5 per 100,000 children younger than 5 years of age in India, 25 per 100,000 in the USA, 56 per 100,000 in Taiwan, and over 250 per 100,000 people in Japan (17, 18).

Recently, a surge in the prevalence of KD-like illness in children has been found with the COVID-19 outbreak in the USA, UK, France, Spain, and Italy (4–9, 19). COVID-19 can cause acute respiratory distress syndrome (ARDS), carditis, thrombosis, and/or shock in adults, but generally, induce mild symptoms in infants and children (1–3). The COVID-19-associated MIS-C occurs in older children and tends to manifest with gastrointestinal symptoms, coagulopathy, and shock in addition to the KD symptoms. Patients with KD usually have thrombocytosis (10–15), but patients with MIS-C have high, normal, or low platelets (4–9), which may be related to coagulopathy or microangiopathy. The MIS-C is similar to KD shock syndrome (KDSS) occurring in relatively older children with atypical KD, showing shock, thrombosis, and IVIG resistance (20–22). The immune response in MIS-C is different from that in COVID-19. COVID-19 is contagious but MIS-C is not. MIS-C is due to post-infection autoimmunity because it occurs 3–6 weeks after the exposure to COVID-19 or persons with COVID-19. Patients with MIS-C have a unique serology with anti-S antibodies (IgG, IgM, and IgA) but not anti-N antibodies, in contrast, patients with COVID-19 have both anti-S and anti-N antibodies (23). The skin, gastrointestinal, and shock symptoms in MIS-C are sometimes undifferentiated from those in toxic shock syndrome (TSS), but the medical history is different because TSS is related to superantigens of bacteria which are usually associated with bacterial infections, surgical wound, or usage of tampons (24).

The COVID-19-related MIS-C, representing atypical KD syndrome in older children at a median age of 8 years (6, 9), is prone to IVIG resistance and life-threatening cardiovascular events, such as myocardial infarction, thrombosis, and/or shock (4–9, 19), which is the most life-threatening morbidity in children during the COVID-19 pandemic.

SIMILARITIES AND DIFFERENCES AMONG KD, KDSS, AND MIS-C

There are some overlapped and different symptoms and signs among KD, KDSS, and MIS-C in regard to age, sex, race, severity, and treatment responses (**Table 1**). Patients with KD usually have vasculitis in mucocutaneous regions (>90% of eyes, lips, and/or skin symptoms) and coronary arteritis, but few patients have myocarditis (∼5%) and shock (∼7%) (10–12). In contrast, KDSS frequently shows myocarditis, thrombosis, and shock (20–22). The KDSS is associated with a higher rate of IVIG treatment resistance, with older age and more serious hypotension, skin rash, leukocytosis, neutrophilia, and hypoalbuminemia, especially frequently found in Hispanics (20, 22). Patients with MIS-C more frequently have a shock, myocarditis, thrombosis, and gastrointestinal symptoms (4–9). Both patients with KDSS and MIS-C more frequently require intensive care supports, such as inotropic agents, ventilation support, anti-thrombotic therapy, and additional anti-inflammatory therapies (4–9, 19–22). KD is prevalent in Eastern Asia (10–12) but MIS-C is more frequently found in Western countries, especially Afro-Caribbean (4–9, 19). The mean age of patients with KD is 2.5 years, and that of patients with KDSS is 3.7 years (22). In the largest cohort report of 186 Afro-Caribbean patients with MIS-C, the median age is 8.3 years (9), showing prominent gastrointestinal symptoms and thrombosis with variable platelet counts (high, normal, or low).

In fact, KDSS is a severe form of KD with hypotension, coagulopathy, more cardiovascular involvement, and IVIG resistance, initially recognized in 2009 (20). In a retrospective analysis of 103 patients with KDSS, abnormalities in the coronary arteries were 65% and the mortality rate was 6.8% (22). Before the institution of IVIG therapy for KD, the KD mortality and cardiac morbidity were 2 and 20%, respectively. After the institution of the IVIG treatment for KD, the mortality decreased below 0.1% and coronary artery aneurysm downed to <4%. The MIS-C mortality ranges between 0 and 10% (average, 2%). KD is not contagious although many infections, such as *Staphylococcus aureus*, streptococci, rhinovirus, coronavirus, enterovirus, chlamydia, or Epstein-Barr (EB) virus had been associated with KD. About 40% of patients with KD have reactive skin erythema and/or scaling at the Bacillus Calmette–Guérin (BCG) inoculation site (11), suggesting the autoreactive antigen for KD may cross-react with the antigen of BCG, or the BCG reactivation is a bystander of hyperinflammatory reaction of KD. It is highly suspicious that the MIS-C is a severe KD-like vasculitis mediated by a COVID-19-induced autoimmune reaction. This is not the first time that human coronavirus (HCoV) is correlated to KD. In 2005, Esper et al. (25) reported that a novel human coronavirus called human coronavirus New Haven (HCoV-NH) was associated with an outbreak of KD in New Haven, showing RT-PCR detection of the positivity at 8/11 vs. 1/22 in a case-control study. This association of KD with HCoV was not replicated in a study at Taiwan using 53 consecutive KD samples in which no detectable HCoV-NH or HCoV-NL63 was observed in nasopharyngeal secretions (26). They, however, did not measure the serum antibodies against HCoV-NH or HCoV-NL63. These studies suggest that children in Western countries are susceptible to coronavirus-related KD-like vasculitis and children in Asian countries are susceptible to non-coronavirus-related KD vasculitis.

In laboratory data as shown in **Table 2**, patients with KD tend to have thrombocytosis, and patients with KDSS or MIS-C tend to have varied platelet counts. C-reactive protein (CRP) and procalcitonin levels are much higher in patients with KDSS or MIS-C. The ferritin levels are also higher in patients with KDSS or MIS-C. Lymphopenia is often prominent in patients with COVID-19 or MIS-C (1–9, 19), but not in patients with KD or KDSS (10–12, 20–22). Coagulopathy is also more often in KDSS or MIS-C (9, 19–22, 27, 28). Ferritin (>500–1,000 ng/ml) and D-dimer (>1,000–4,000 ng/ml) levels are much higher in children with KDSS and MIS-C (9, 19–22, 27, 28). Cytokine storm in the blood is quite similar between KD and MIS-C, showing augmented hypercytokinemia in IL-6, IL-10, IL-17, inducible protein-10 (IP-10) (CXCL10), and MCP-1 (CCL2),

TABLE 1 | Demographic data and phenotypes of Kawasaki disease (KD) and multisystem inflammatory syndrome in children (MIS-C).

Phenotypes	KD		COVID-19 Mild in Children	MIS-C Multisystem
	KD	KDSS		
Skin/mucosa (%)	>90	>90	Few	50–60
CAL (%)	20	65	Rare	29–80
Myocarditis (%)	5	20	Rare	65
Shock (%)	7	100	Rare	60
Age range (Median) (year)	0.5–5.0 (2.0)	2–12 (3.5)	0–18	2–18 (8.3)
Sex	Male	Female	Both	Both
Race	Asian	Hispanic	All races	Afro-Caribbean
IVIG resistance	15%	40%	–	80% (add on steroid)
Fatality (%)	<0.1	0–6.8	<0.1	0–10 (2)
Pathogen	Unknown	Unknown	SARS-CoV-2	SARS-CoV-2 associated

KDSS, Kawasaki disease shock syndrome; COVID-19, coronavirus disease-19; CAL, coronary artery aneurysm; IVIG, intravenous immunoglobulin; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

TABLE 2 | Laboratory data of KD, COVID-19, and MIS-C.

Laboratory data	Kawasaki disease		COVID-19 mild in children	MIS-C multisystem
	KD	KDSS		
Platelets	>350,000/μl	High or Low	Normal	High, normal, low
CRP (mg/dl)	50–150	>100	<50	>100
Procalcitonin (ng/ml)	>0.5	>1.0	<0.25	>1.0
Ferritin (ng/ml)	100–200	500	<100	>1,000
Lymphopenia	Rare	Rare	Some	Moderate
Coagulopathy	No	Some	Rare	Often
D-dimer (ng/ml)	<1,000	>1,000	<1,000	>4,000
Cytokines	IL-6, IL-17, IP-10	IL-6, INFγ, IL-10	IL-6, IFNγ, IL-8	IL-6, IP-10, IL-10
Anti-S antibody (%)	NT	NT	100	>80

KDSS, Kawasaki disease shock syndrome; IL, interleukin; IFN, Interferon; IP, inducible protein; NT, not tested.

especially higher IL-6 and IL-10 levels in KDSS (27), and IL-10 and TNF-α levels in MIS-C (28). Over 80% of patients with MIS-C have detectable anti-S antibody against spike (S) antigen of severe acute respiratory syndrome coronavirus-2 (SARS-Cov-2), but less than one-third have detectable RNA of the virus (4–9). Apparently, MIS-C is mediated by a skewed immune response toward T helper 17 (Th17) reaction in the convalescent stage of COVID-19.

IMMUNOPATHOGENESIS OF KD AND MIS-C

A patient with a viral infection usually has normal or lower leukocyte counts, and low CRP and procalcitonin levels unless he/she also has superimposed bacterial infections. However, regardless of whether COVID-19 is contagious or MIS-C is not contagious, lymphopenia and elevated CRP are found in both conditions in children. Apparently, SARS-CoV-2 induces a proinflammatory reaction in the acute stage of COVID-19 and a hyperinflammatory reaction of vasculitis (4–9, 19) with augmented levels of Th17 and Th1 mediators in MIS-C (28). There are many unsolved issues on the immunopathogenesis of KD and MIS-C. It is debatable whether MIS-C and KD are post-infectious hyperinflammation, autoinflammatory, or autoimmune disorders (29–34). Current autoimmune concepts have limitations to explain the pathogenesis of variant systemic vasculitis syndrome, which is not contagious but infection-associated hyperinflammation in the convalescent stage (29). Inflammation-inducing substances, not only those originating from pathogens, including toxins and pathogen-associated molecular patterns (PAMPs), but also those originating from injured or infected-host cells including pathogenic proteins, pathogenic peptides, and damage-associated molecular patterns (DAMPs), especially in intracellular pathogen infections, such as virus, chlamydia, BCG, and SARS-CoV-2, may alter the immune responses based on "the protein-homeostasis-system hypothesis" (30). Given the fact that marked different incidences in KD and MIS-C across the populations may be explained by colonization states of pathogens (31), and an imbalance of regulatory and cytotoxic SARS-CoV-2-reactive CD4 T cells in COVID-19 (32), we focused on the imbalanced Th17/Treg regulation for explanations of the same and different manifestations among KD, KDSS, COVID-19, and MIS-C in this perspective article.

We have studied the immune responses of KD for over two decades and look into those related to COVID-19 in the literature in order to explore the link and implication for effective prediction, prevention, and treatment of KD and MIS-C. Literature and clinical experience of KD management prompt early recognition of the KD-like vasculitis in MIS-C for IVIG immunotherapy and beyond, vice versa, information accumulated in MIS-C suggest that KD-like vasculitis is an infection-associated autoimmunity (32–34). Another possibility is an antibody-dependent enhancement (ADE) of FcγR-mediated autoimmunity that has been reported in SARS-CoV-2 infection (35) and also reported in SARS-CoV-1 infection (36). In a genetic association study of FcγRIIA polymorphisms with the severity of SARS-CoV-1 infections, Yuan et al. (37) found that variant FcγRIIA-R/R131 in the intensive care unit (ICU) subgroup of patients with SARS was significantly more frequent than in normal controls. We have previously studied immunopathogenesis of SARS-CoV-1 infection and found that SARS-CoV-1 infection caused an early innate augmentation with adaptive immunosuppression and then induced a late exacerbation (38, 39). To address the hyperinflammatory reaction of KD, we first demonstrate that overexpression of inducible nitric oxide (NO) synthase associated with elevated blood NO levels is present in patients with KD before IVIG therapy (40), and that the T-cell activation marker CD40L is prominently expressed on T cells and platelets in children with KD, which is reversed after IVIG therapy (41). This is comparative to the dynamic time course of immune responses of KD validated by a kinetic immunopathology in a series of autopsy classifications of early necrotizing vasculitis with innate phagocyte activation followed by a remodeling of adaptive immunity with lymphocyte infiltration in the convalescent stage (42).

Before the era of IVIG therapy, the rate of coronary artery aneurysm was 25% and mortality was 1–2% (10, 42–44) in patients with KD, after the institution of IVIG therapy the rate of coronary artery aneurysm downs to 3–4% and mortality to <0.1% (42, 43). Now the challenge for the KD treatment is IVIG resistance in 15–20% of KD patients, which requires additional immunotherapy. Similarly, KDSS and MIS-C have even higher IVIG resistance rates and more frequently require combined therapy with IVIG and steroid pulse therapy (19–22, 28). Interestingly, corticosteroids treatment before the IVIG institution era showed an exacerbated morbidity of coronary artery aneurysms in 64.7 vs. 20% treated with antibiotic alone or 11% treated with aspirin alone (44). Parameters in patients with IVIG resistance are persistent fever and elevated IL-6 levels (45). Furthermore, skewed T-cell polarization toward Th2 response favors the outcomes of IVIG therapy. A higher eosinophil count associated with a higher IL-5 level is a favorable marker for the success of IVIG treatment. In contrast, lower initial eosinophil counts and lower IL-4 and IL-5 levels are associated with IVIG-resistance (46). Patients with KD have prominent Th17 immune responses with augmented IL-6, IL-10, G-CSF, and IL-17A levels, and lower Treg pathway transcription factor FoxP3 expression before IVIG treatment (47). The augmented cytokine storm declines and the Treg cell increases after IVIG treatment.

The Th17 polarization with elevated IL-6, IL-17A, and G-CSF levels is correlated to a higher neutrophils vs. lymphocytes (N/L) ratio in KD patients complicated with IVIG resistance and coronary arteritis (48), which is similar to the severity of COVID-19 associated with an increase in neutrophils and decrease in lymphocytes and elevated INF-γ, IL-6, and IL-8 levels (49, 50). The cytokine profile in MIS-C is different from that in severe COVID-19 in higher IL-10 and TNF-α levels (28). It is shown that IL-6 together with TGF-β induces Th17 differentiation from naïve T cells (51), whereas IL-6 inhibits TGF-β-induced Treg differentiation *via* degradation of FoxP3 (52), suggesting that higher IL-6, IL-10, and IL-17A but lower FoxP3 and TGF-β expression in patients with KD or MIS-C is involved in the Th17/Treg imbalance. This is further supported by our finding that DNA polymorphisms of TGF-β-signaling pathway genes, e.g., TGF-β2 and SMAD3, were associated with the susceptibility of KD (53), and the Th17/Treg imbalance could also be mediated by epigenetic regulation of DNA methylation and/or micro RNAs (miRNAs) on innate and adaptive immune genes as a biomarker of KD (54–59). In HumanMethylation450 BeadChips assay, we have found that DNA hypomethylation on the promoter CG sites of many immune activation genes in leukocytes of patients with KD before IVIG treatment (54–57). The hypomethylated genes were associated with augmented gene (mRNA) expression, particularly the toll-like receptors (TLRs). The TLR1, 2, 4, 5, 8, and 9 receptor genes were significantly hypomethylated and associated with augmented mRNA expression (55). Similarly, other innate immunity genes, e.g., FcγR2A, IL-10, and S100A8 were also hypomethylated before IVIG treatment (54–57). Importantly, we found that the CpG site methylation changes >20% in the acute stage of KD were mainly hypomethylated (97%) genes but only 3% hypermethylated genes (56). After IVIG treatment, the hypomethylated genes and augmented mRNA expression reversed (54–57).

Moreover, it is found that miRNA expression is also a good biomarker of KD, which differentiated KD from other febrile diseases by a set of 4 miRNA expression at C_T (miR-1246)-C_T (miR-4436b-5p) and C_T (miR-197-3p)-C_T (miR-671-5p) (58). The miRNA control of Treg expression in patients with KD has been characterized before and after IVIG treatment (59). The epigenetic control of Treg development and maintenance has been defined predominantly *via* FoxP3 expression (60). These epigenetic profiles and functional markers of different Treg population (tTreg, iTreg, and pTreg) tend to have a promising role as specific mechanistic biomarkers for the prediction and prevention of Th17-mediated autoimmunity (61–63). The study model can be applied to study the epigenetic biomarkers and therapeutic targets of MIS-C and KD with and without shock syndrome by potential immunotherapy of cytokine inhibitor, DNA methylation, and/or miRNA expression in addition to IVIG with and without corticosteroids. The immunopathogenesis of KD and MIS-C probably progresses from an early Th17 response, followed by a later T-regulatory response. In the early Th17 response before IVIG treatment, the Treg pathway signals are depressed, and the reciprocal Th17/Treg imbalance reverses after IVIG treatment (47, 54–59). This is supported by the fact that the corticosteroids treatment alone in the acute stage was useless and even harmful in the 1970s (10, 44); instead, the combination of IVIG with corticosteroids showed a better response than the IVIG therapy alone in the 2010s, especially in the patients with IVIG resistance (64). Based on the rationales described above, we postulate that there are two phases during the development of KD or MIS-C syndrome; the early Th17 reaction and late Treg resolution stage have different immunopathogenic processes with individual biomarkers and require different immunotherapies.

BOTH KD AND MIS-C OCCUR IN CHILDREN AND ADULTS

A population-based surveillance system called COVID-19–associated hospitalization surveillance network (COVID-NET) analyzed 576 hospitalized COVID-19 pediatric patients and showed the prevalence of COVID-19 in children increased from 0.1 per 100,000 to 8 per 100,000 with the progress of COVID-19 pandemic, in which a race disparity in hospitalization deviated to Hispanic children, and nine (10.8%) of 83 admitted children had MIS-C (65), suggesting MIS-C may attribute to one-tenth of the admitted severe COVID-19 in children. MIS-C is rare or sporadic in adults (66, 67). COVID-19 deserves further studies on the autoimmunity under endogenous or exogenous milieu because it might directly trigger autoinflammatory conditions by molecular mimicry or cause autoimmunity in predisposed individuals in other environmental conditions (68). The algorithm for diagnosis and treatment of complete and incomplete KD in children has been proposed to diagnose and treat KD in adults (67, 68), and MIS-C in adults (67, 68).

Some adults have been diagnosed with atypical or incomplete KD, contemporarily or retrospectively (69, 70), and occasionally

caused sudden death (71) or sequelae of the KD from Children (72). MIS-C has also been demonstrated in certain adults (67, 68). The autopsy of patients with COVID-19 showed a severe endothelial injury associated with the detectable intracellular virus, disrupted cell membranes, and widespread thrombosis with microangiopathy in the lungs (73, 74). The alveolar microthrombi were nine times more in patients with COVID-19 than in patients with influenza (75), and viral particles were detected in epithelial cells and endothelial cells of the lungs (73, 74). Micro-embolism and thrombosis indicating vasculitis and coagulopathy are similar between fatal patients with COVID-19 with ARDS and fatal patients with SARS (75, 76). Pathological findings of autopsy in KD are vasculitis with leukocyte infiltration, called periarteritis nodosa-like arteritis, coronary thrombosis with macrophage and lymphocyte infiltration, and aneurysm, but not pulmonary vasculitis (42, 77, 78). Autopsy features in MIS-C have not been characterized yet. We anticipate that abnormal proinflammatory insults with skewed Th17/Treg imbalance in MIS-C will be seen in the lesions of the cardiovascular system but not in the lesions of pulmonary vessels. The pathology in the COVID-19-induced ARDS showing pulmonary involvement with detectable viral RNA and thrombo-emboli in autopsy is different from the pathological finding in KD showing sterile vasculitis, leukocyte infiltration, and aneurysm, indicating immunity to infections in the former vs. autoimmunity in the latter, which could occur in both children and adults.

GENETIC SUSCEPTIBILITY OF KD AND MIS-C

No specific genes have been linked to the susceptibility of MIS-C. The siblings of patients with KD are 10 times more likely to have KD. Several susceptibility genes (e.g., *ITPKC*, *CASP3*, *CD40*, and *ORAI1*) have been linked to KD (79), and KD is also associated with the human leukocyte antigen (HLA)-BW22J2 subtype, which is found specifically in Japanese and not in Caucasians (80). In patients with KD with coronary artery lesions (CAL), the frequency of HLA-DRB1*11 is significantly increased and that of HLA-DRB1*09 is decreased (81); In fact, HLA subtypes linked to KD are different between children in Asia and those in Western countries (82). We have previously found that HLA-DRB1 was associated with KD susceptibility (83). To search for the risk allele(s) of major histocompatibility complex (MHC) class 1, HLA-MICA (MHC class I chain-related gene A) locus, we found that the HLA-MICA A4 allele was significantly associated with the coronary artery aneurysm in patients with KD (84), and it has been validated in a genome-wide association case-control study in a Taiwanese population (85). We also found that DNA polymorphisms of TGF-β2 and SMAD3 are associated with the susceptibility of KD (53), and a dominant T allele of rs2243250 in the IL-4 gene conferred a great protective effect against the development of CAL in patients with KD ($p = 0.006$) (86). Taken together, this suggests that a specific HLA subtype could present a viral antigen peptide to T cells and induce a skewed Th17-Th1/Th2-Treg development involved in the altered hyperinflammation in KD. Although there is no any genetic association with MIS-C in the literature, the HLA-B*46:01 has been proposed to be associated with the severity of COVID-19 in a computational simulation by using SARS-CoV-2 whole-genome peptides for simulating their binding to 145 MHC class I HLA-A, -B, and -C genotypes, in which HLA-B*15:03 shows the greatest capacity to present highly conserved SARS-CoV-2 peptide which is shared among common human coronaviruses, suggesting that it could enable cross-protective T-cell-based immunity (87). Deletion or mutation of TLR7, which is a single-stranded RNA virus sensor in endosomes for induction of interferons (IFNs), contributes to the severity of COVID-19 in young adults (88). In contrast, imiquimod, a TLR agonist is proposed to enhance the defense against COVID-19 (89). Taken together, we would propose to clarify whether an HLA subtype, such as HLA-B*46:01 together with a TLR7 variant induces an augmented proinflammatory reaction under conditional milieu and alters the epigenetic control of FoxP3 expression resulting in the Th17/Treg imbalance in KD and/or MIS-C.

Interestingly, different blood group subtypes have also been shown to be associated with the susceptibility of COVID-19 (90–92). Initially, a report from Wuhan, China described blood group A subjects were more susceptible to COVID-19, and the presence of anti-A antibody was probably protective (90). Furthermore, another report from China showed that females but not males, with blood group A are susceptible to COVID-19 (91). In contrast, later in the USA, the other report described that B and AB blood groups were susceptible to the infection but not severity (92). The reproducible result among the studies is that the O blood group population is less susceptible to COVID-19 (90–92), but it remains controversial whether blood groups A and/or B population are susceptible to and/or vulnerable to severity. Perhaps, the differences are related to different studies in different races.

We are currently studying the association of MHC genotypes, haplotypes, and antigen presenting pocket prediction with KDSS and/or MIS-C *via* a consortium in Taiwan. Hopefully, the more the cases of MIS-C identified the more the opportunity to identify the association of KDSS or MIS-C with HLA subtypes in the COVID-19 outbreak or the COVID-19 mass vaccination. We could also study whether different H2 subtypes interacting with viral antigen under host situations lead to altered immunity contributing to KDSS or MIS-C in a mouse model using vaccine antigens with and without adjuvant. This experimental study would test whether the HCoV associated KD-like hyperinflammation is related to different races with varied HLAs by which different antigens induce altered immune responses because of different HLA subtypes and environments.

EVOLUTION OF IMMUNOTHERAPIES FOR KD AND MIS-C

Since MIS-C reveals KD-like syndrome fulfilling complete or incomplete criteria, a physician could rapidly recognize and adopt the treatment regimen of KD for MIS-C, and mitigate the life-threatening disease. The treatment of KD in the acute febrile stage has evolved from corticosteroids, IVIG, and aspirin

to a combination of IVIG, aspirin, and steroids through the past 50 years (10, 44, 64). Although long-term aspirin, whether it is high (anti-inflammatory) or low (antiplatelet) dose, does not appear to lower the frequency of coronary abnormalities (42), a low dose aspirin and/or antithrombotic treatment with low molecular weight heparin or warfarin is prescribed according to the progress of coronary aneurysm in the convalescent stage (42, 93). A combination of IVIG and corticosteroids significantly reduced the risk for coronary artery lesions compared with IVIG alone (7.6 vs. 18.9%; OR: 0.3; 95% CI 0.20–0.46) in a meta-analysis (64). Different dosing of IVIG for KD has been clarified (94), and different dosing of corticosteroids in the clinical trials at different countries explained the overall varied benefits on the outcomes of IVIG and corticosteroids in coronary artery aneurysm (64, 95). In pneumonia-associated ARDS, early treatment with corticosteroids and/or IVIG may reduce the aberrant immune responses that have been described (30). This may be also applicable to the treatment of COVID-19-related ARDS. For instance, a combination of pulse corticosteroids and IVIG therapy has been shown to rescue patients with tocilizumab-resistant severe COVID-19 (96). High dose IVIG regimen (2 gm/Kg) is largely demonstrated as more effective (42, 94), however, early IVIG therapy for KD within 4 days did not provide better protection from the development of CAL (97, 98). Whether the earlier and higher doses of immunotherapy for MIS-C and KD responsible for better outcomes deserves further studies.

Intravenous immunoglobulin resistance in the acute stage is frequently associated with the development of CAL in patients with KD. A few different scoring systems have been developed to predict IVIG resistance (99–101), and to provide a precise anti-inflammatory regimen, such as to infliximab (or anakinra) in addition to aspirin, IVIG, and corticosteroids therapy (101). Unfortunately, a scoring system (Kobayashi Score) which is successfully used to predict and prevent CAL in Japan (99) performs poorly in sensitivity and specificity in Western countries (100). MIS-C is IVIG resistant in most patients, therefore IVIG plus corticosteroids is used to treat the life-threatening condition (4–9, 96).

Certain unique complications of KD, such as shock, macrophage activation syndrome (MAS), or coronary aneurysm are usually associated with IVIG resistance and require additional anti-inflammatory regimens, such as cyclosporin, anti-IL1, or anti-IL6 treatment (101, 102). It is reasonable to add anti-IL6 for KD or MIS-C for IVIG resistance because serum IL6 levels correlate with IVIG resistance (45, 103). However, in a study of four patients with IVIG-resistant KD who are responsive to anti-IL6 treatment but affected by coronary artery aneurysms in 2 of them (104). The patients with KD with IVIG-resistance usually respond to anti-TNF, anti-IL-1, or steroid pulse therapy (101–104). Furthermore, anti-cytokines, such as tocilizumab and anakinra or anti-coagulopathy regimens have been used for COVID-19 hyperinflammatory syndrome in adults and resulted in favorable outcomes (105, 106), and are suggested to be used in MIS-C with IVIG resistance (9, 19, 28). Apparently, a scoring system based on symptoms and biochemistry to predict IVIG resistance may not be enough. Some studies had identified IVIG resistance associated with elevated IL-6, IL-10, and/or TNF-α levels in KD with and without shock syndrome (27, 45, 48), and we showed that lower IL-5 levels associated with lower eosinophil number was correlated to IVIG resistance (46), and allele rs2243250T of the IL-4 gene conferred protection against coronary artery lesions in KD (85). Patients with KD or MIS-C in different countries or races may require varied criteria for the prediction of the resistance to IVIG or anti-cytokine treatment. A new scoring system should include conventional symptoms and individuals' immunological parameters to provide a better guide to decrease IVIG resistance and increase effectiveness of the additional anti-inflammatory therapy. In addition to hyperinflammation and shock syndrome, patients with KDSS (20–22) or MIS-C (9, 19) usually manifest with coagulopathy, embolism, and thrombosis. It remains to be determined whether the embolism, thrombosis, and/or coagulopathy in MIS-C require certain anti-thrombotic therapy, and whether patients with MIS-C with coronary involvement require a long term aspirin treatment, which is a regimen for treating patients with KD who develop coronary abnormalities (42, 93).

MECHANISTIC IMMUNOTHERAPIES OF MIS-C BASED ON INFECTION IMMUNITY AND AUTOIMMUNITY

Until July 2020, more than 1,000 cases of MIS-C had been reported (107). However, the definition and treatment regimens are not standardized yet! While COVID-19 remains pandemic, MIS-C cases will increase further. It is also a concern that this augmentation of Th17/Treg imbalance in MIS-C after COVID-19 may be extended by a COVID-19 mass vaccination in which the vaccine antigen with adjuvant may increase the risk of MIS-C. Both KD and MIS-C are non-contagious but potentially virus or antigen- (PAMP-) induced autoimmunity in genetically susceptible individuals. Patients from different genetic backgrounds and environments including pre-existing subneutralized antibodies or abnormal autoantibodies directed against different compartments, such as endoglin, EDIL3, and or casein kinases of endothelial cells, and so on, may influence the development of MIS-C or KD (108). Both patients with MIS-C and KD have augmented IL-6, IL-17A, and IP-10 production (27, 28, 45, 108), but the levels of SCF, TWEAK, and ADA significantly decreased in patients with KD but not in patients with MIS-C (108), suggesting they have similar immune activation pathways but different regulatory (suppressive) pathways. Taken together, we postulate immunopathogenesis of the COVID-19 associated MIS-C begins with innate immunity of SARS-CoV-2 infection to cells *via* ACE2 (**Figure 1A**), followed by adaptive immunity of COVID-19 with antigen presentation through HLA for T-cell differentiation toward an effective immunity or altered Th17 response (**Figure 1B**), and leading to individual autoimmunity with MIS-C in the convalescent stage (**Figure 1C**).

As shown in **Figure 1A**, the SARS-CoV-2 virus enters the host cells *via* ACE2 where a serine protease cleaves the viral spike (S) protein and allows the virus to fuse with the plasma membrane for internalization (109). Host RNA sensing receptors,

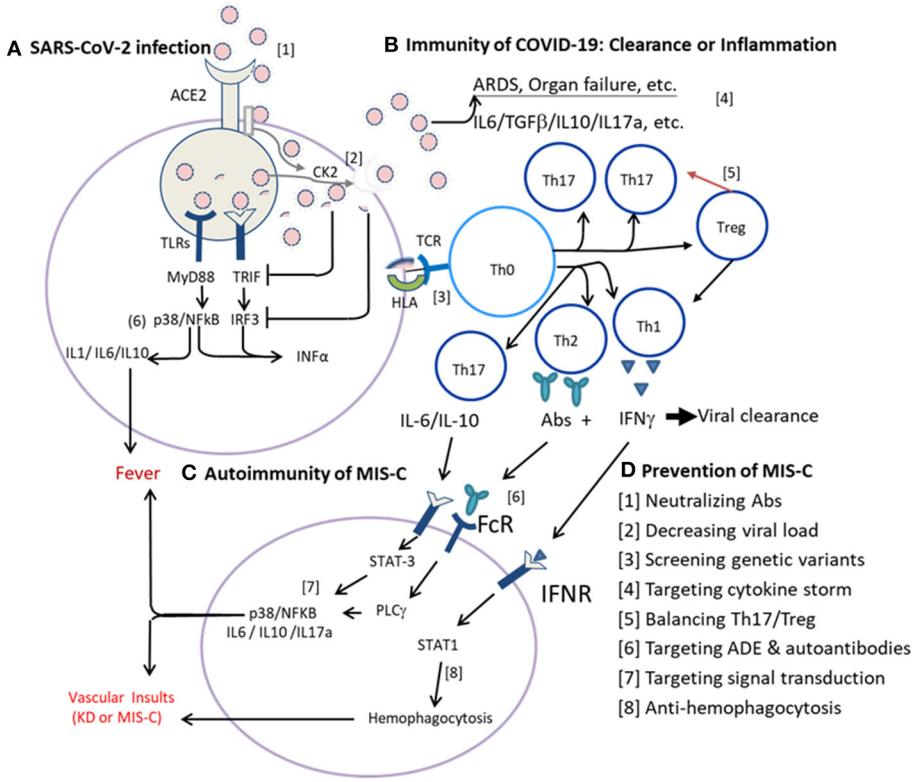

FIGURE 1 | Immunotherapies of multisystem inflammatory syndrome in children (MIS-C) based on infection immunity and autoimmunity. **(A)** Innate immunity of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection to cells *via* ACE2. The SARS-CoV-2 virus enters host cells *via* ACE2 where a serine protease cleaves the viral spike (S) protein and allows the virus to fuse with the plasma membrane for internalization. Host RNA sensing receptors, such as RIG-1 (DDX58), Toll-like receptor-3 (TLR3), TLR7, and/or TLR8, detect the internalized virus and induce the production of interferons (IFNs) *via* MyD88, TRIF (TICAM1), IRF3, and/or IRF7 pathways, promote production of proinflammatory cytokines *via* MAPK (e.g., p38) and NFkB pathways. Normally, the host RNA sensing receptor(s) of RIG-1 and TLR7 signaling pathways will mediate an effective induction of IFNs for virus clearance. While the virus hijacks RNA sensing receptors and pathways or activates casein kinase 2 (CK2) for filopodial protrusion of budding viral particles, the virus multiplies rapidly and the infection spreads systemically. **(B)** Adaptive immunity of coronavirus disease-19 (COVID-19). Upon antigen presentation for T-cell adaptive immunity *via* human leukocyte antigen (HLA), an optimal adaptive immunity with T-cell immunity and B-cell production of neutralizing antibodies (Abs) for virus clearance is normally elicited. While the initial virus load is high, or the TLR is either of congenital deficit or of acquired deficit as in the elderly, or the viral glycoproteins (antigens) could suppress the MyD88, TRIF, IRF3, and/or IRF7 signaling pathways, the antigen-presenting cells (APCs) are hijacked and present the viral antigen with an altered signal for the polarization of naïve T helper cells (Tho) toward Th17 response with inflammatory cytokines production, but not Treg regulation for a proper Th1 cell immunity and/or Th2 humoral (B cell) response for neutralizing antibody production. **(C)** Individual autoimmunity with MIS-C. In convalescence, most patients recovered due to efficient adaptive immunity of Th1 cell immunity and Th2 neutralizing Abs unless individual subjects with genetic susceptibility or altered endogenous milieu. For instance, the viral antigens (PAMPs) interact with individual HLA subtype(s) of APCs and induce altered autoimmunity with Th17/Treg imbalance and augmented cytokine storm of IL-6, IL-17A, and/or IL-10 expression or abnormal autoantibodies resulting in MIS-C with systemic vasculitis, thrombosis, and shock, as seen in MIS-C. In addition, certain host milieu, e.g., abnormal homeostasis of vitamins and microbiota which could compromise Treg responses and enhance Th17/Treg imbalance. Alternatively, altered FcγR or subneutralized IgG antibodies might induce antibody-dependent enhancement (ADE) of immune reaction, and autoantibodies to form immune complex or to bind endothelial cells and induce abnormal hyperinflammation. In summary, the autoimmune vasculitis of KD or MIS-C can be mediated by a genetic variant of HLA, FcγR, and/or ADE resulting in hyperinflammation with Th17/Treg imbalance. **(D)** Prevention of MIS-C can be made based on infection immunity and autoimmunity described above. A series of sequential steps ([1]–[8]) as indicated can be utilized to prevent the life-threatening MIS-C.

such as RIG-1 (DDX58), TLR3, TLR7, and/or TLR8, detect the internalized virus, induce IFN production *via* MyD88, TRIF (TICAM1), IRF3, and/or IRF7 pathways, and promote the production of proinflammatory cytokines *via* MAPK (e.g., p38) and NFkB pathways (110, 111). A virulent virus can hijack RNA sensing receptors and pathways or activate casein kinase 2 (CK2) for the enhancement of budding viral particles (112). While the initial virus load is low or the virus belongs to a less virulent strain, the host RNA sensing receptor(s) of RIG-1 and TLR7 signaling pathways will mediate an effective induction of IFNs and antigen presentation for an optimal adaptive immunity with T-cell immunity and B-cell production of neutralizing antibodies (Abs) for virus clearance. Alternatively, while the virus load is high, or the TLR7 is either of congenital deficit (88) or of acquired deficit as in the elderly (113), or the viral glycoproteins (antigens) could suppress the MyD88, TRIF, IRF3, and/or IRF7 signaling pathways (111, 114), the antigen-presenting cells (APCs) are hijacked and present the viral antigen with an altered signal for the polarization of naïve Th cells (Tho) toward Th17 response with inflammatory cytokines production, but not Treg

regulation for a proper Th1 cell immunity and/or Th2 humoral (B cell) response for neutralizing antibody production. The virus will multiply effectively, and a large number of antigens (PAMPs) will be released to augment Th17/Treg imbalance and promote cytokine storm, leading to ARDS with neutrophilia and lymphopenia, epithelial cell damage, vascular leakage, and/or coagulopathy in COVID-19 (**Figure 1B**).

In convalescence (**Figure 1C**), most patients recovered due to efficient adaptive immunity of Th1 cell immunity and Th2 neutralizing antibodies. While the viral antigens (PAMPs) interact with certain HLA subtype(s) of APCs and induce altered autoimmunity with Th17/Treg imbalance, in which an augmented cytokine storm of IL-6, IL-17A, and/or IL-10 expression and abnormal autoantibodies could result in MIS-C with systemic vasculitis, thrombosis, and shock as seen in MIS-C. The abnormal virus-host response may not only depend on genetic variants, e.g., HLA subtypes (87) and TLR7 variants (88), but also host milieu, e.g., homeostasis of vitamins and microbiota which could maintain better Treg responses for anti-inflammatory reactions (115–117). Alternatively, altered FcγR or subneutralized IgG antibodies might induce ADE of immune reaction. ADE resulting from the interaction of the FcγRIIA with a variant polymorphism had been found in SARS-CoV-1 infections (118), and a similar mechanism has been demonstrated *in vitro* in COVID-19 (119). It is possible that the autoimmune vasculitis of KD or MIS-C is mediated by a genetic variant of HLA, FcγR, and/or ADE resulting in hyperinflammation with Th17/Treg imbalance. However, the Th17/Treg imbalance may be different between MIS-C and KD because the Th17 mediators were elevated in both the diseases but the immunosuppressive mediators: SCF, TWEAK, and ADA were lower in KD than in MIS-C (108). In patients with KD or MIS-C with failure of IVIG and corticosteroids treatment, additional immunotherapies might be applicable by targeting different Th17/Treg imbalances.

Therapeutic Perspectives of MIS-C

Based on the postulated immunopathogenesis of COVID-19 associated MIS-C described above, we could make a series of sequential steps ([1]–[8]) to prevent the life-threatening MIS-C as indicated in **Figure 1D** and as described below:

[1] *Blocking virus entry by neutralizing Abs*. In a meta-analysis of 12 controlled trials with more than 4,000 participants, transfusions of convalescent plasma with neutralizing Abs interrupted the virus-ACE2 interaction. The treatment in hospitalized COVID-19 patients reduced the mortality rate by 57% (10 vs. 22%; OR: 0.43, $p < 0.001$) (120). Similarly, convalescent plasma or neutralizing monoclonal antibodies (MoAbs) have also rescued patients with Ebola (121), SARS (122), and Middle East respiratory syndrome (MERS) (123). Thus, early administration of hyperimmune or recombinant MoAbs with neutralizing Abs directed against SARS-CoV-2 should be able to decrease virus load and raise better immune response toward balanced Th17/Treg reaction resulting in less severity and less autoimmunity.

[2] *Decreasing viral load*. There are many *in vitro* studies showing that several potential anti-COVID-19 agents could block the entry, replication, and/or shedding of SARS-CoV-2 (112, 124). The decrease of viral replication and shedding could be made by the inhibition of virus-cell fusion, virus and host proteases, lysosome acidification, RNA synthetase, and virus budding (124, 125). A proper regimen (e.g., remdesivir, avigan, or silmitasertib) to decrease the virus transmission between the infected and non-infected cells may enhance immune response and mitigate the possible autoimmunity. A combination of neutralizing MoAbs and anti-virus agent may induce a synergistic effect.

[3] *Screening genetic variants*. Similar to KD which has been linked to certain alleles of HLA subtypes in regard to disease susceptibility and severity, the severity of COVID-19 has been proposed to be associated with HLA-B*46:01 in a computational simulation by using SARS-CoV-2 whole-genome peptides for simulating their binding to 145 MHC class I HLA-A, -B, and -C genotypes (87). Moreover, a recent report showed that a mutant (D839Y/N/E) from a European strain of SARS-CoV-2 could serve as a superantigen to induce T-cell receptor activation, resulting in hyperinflammatory response, which may be implicated in the development of MIS-C as well as cytokine storm in adult patients with COVID-19 (126). Deletion or mutation of TLR7 has also been attributed to more severity of COVID-19 in young adults (88). Further studies to identify the risk genetic variants for severity and/or autoimmunity of COVID-19 would help develop a screening genetic test for protecting susceptible children from contacts of COVID-19 and incubate better Th17/Treg balance by nurturing internal milieu with proper homeostasis of vitamin D, vitamin A, and microbiota (104–106).

[4] *Targeting cytokine storm*. In an early trial with anti-IL6R for patients with COVID-19 hospitalized with cardiopulmonary exacerbation showed potential benefits in decreasing CRP levels, fever, and severity (127). However, later randomized trials demonstrated no significant effects on the severity or fatality of COVID-19 (128). Taken together, aiming at a single target of one cytokine action may be ineffective but a combined regimen or sequential targeting may be required for eliminating the cytokine storm mediated by a couple of hyperinflammatory cytokines in COVID-19 or MIS-C. Th17 mediators, Il-6 and IL-17A and Th1 down-stream mediators, TNF-α and IP-10, more prominently increased in KD than in MIS-C (27, 28, 45, 108), suggesting that targeting IL-17A by Secukinumab or anti-TNFα could be considered in patients with KD with IVIG resistance or with KDSS. Children with MIS-C, who did not have IL-17A or TNFα overexpression (108), may be treated with a combination of IVIG, corticosteroids, and recombinant IL-1-receptor antagonist, Anakinra.

[5] *Balancing Th17/Treg immune response*. Abnormal immune regulation has been shown in patients with KD or MIS-C (46–50). Both genetic and epigenetic alterations in Treg pathways have been demonstrated in patients with KD (53–56). The induction and/or stabilization of Treg cell development is affected by endogenous milieu, such as vitamins and metabolites from microbiota

(115–117, 129–131). Vitamin D levels have been shown lower in many patients with COVID-19 and associated with increased inflammatory cytokines and an increased risk of pneumonia (129). The lower vitamin D concentration is not only linked to higher severity of COVID-19 (130, 131), but also associated with an increase in thrombotic episodes (132, 133), which are frequently observed in COVID-19 associated MIS-C (4–9). Vitamin D deficiency has been also shown to be associated with KD with IVIG resistance (134). Moreover, microbiota have recently been shown to coordinate adipocyte-derived mesenchymal stem cells to combat autoimmunity of Type 1 diabetes in mice (135), and mesenchymal stem cells (MSC) or their exosomes have been proposed to eliminate hyperinflammation of COVID-19 (136, 137). We have also recently shown that exosomes derived from MSCs (MSC-EVs) could rescue inflammatory neuropathic pain (138). Evidence accumulated has demonstrated that the effects of MSCs and exosomes derived from MSCs are useful in treating inflammatory diseases and fibrosis (139, 140). This regimen may be suitable for not only cytokine storm but also post-infectious pulmonary fibrosis. In addition, epigenetic modulations of FOXP3 expression by DNA methylation and/or miRNA expression (54–60), may also be applicable to correct the Th17/Treg imbalance.

[6] *Targeting ADE and autoantibodies*. ADE of immunopathology has been concerned to potentially happen in dengue virus, Zika virus, Ebola virus, respiratory syncytial virus (RSV), and coronaviruses (119). The potential ADE in MIS-C could be treated by using IVIG with and without corticosteroids as shown in patients with MIS-C (4–9), or might be rescued by the elimination of the glycosylation site at N297 of the IgG Fc portion or by a mutation in the Fc region resulting in an effective antibody neutralization but not ADE (119). Several autoantibodies, such as autoantibodies to MAP2K2, CSNK1A1, CSNK2A1, and CSNK1E1 were notably found in patients with MIS-C, and autoantibodies directed against EDIL3 were exclusively found in patients with KD (108), suggesting these autoantibodies might be used as biomarkers for differential diagnosis and their anti-idiotypic antibodies might be used for prevention of autoimmune vasculitis.

[7] *Targeting signal transduction pathways*. COVID-19 has been shown to induce hyperactivation of TLR-mediated MAPK pathway and CK2-mediated filopodial protrusion of viral shedding (112). Inhibition of p38 activation has been shown to decrease viral replication and cytokine induction in *in vitro* cell model (112). This is further supported by a recent report showing autoantibodies to MAP2K2, and three members of the casein kinase family (CSNK1A1, CSNK2A1, and CSNK1E1) are notable in children with MIS-C (108). Inhibitors of the phosphokinases which are activated in an *in vitro* model of SARS-CoV-2 infection, including CK2, CDK, AXL, and PIKFYVE kinases, may possess antiviral efficacy. A combination of different inhibitors of the kinases may have a synergistic effect on anti-viral and anti-inflammatory responses. A recent study showed a combination of a viral protease inhibitor, GC376, and the RNA-dependent RNA synthetase inhibitor, remdesivir, offered sterilizing additive effects (125). In addition, a proteasome inhibitor MG132 which could inhibit IL-6/TGF-β-mediated downregulation of FOXP3 protein may potentially raise the Treg activity (60).

[8] *Anti-hemophagocytosis*. Hemophagocytosis syndrome also called MAS usually occurs in patients with autoimmune disorders (141, 142). Interestingly, patients with KDSS (20–22) or MIS-C (9, 19) can have IVIG resistance associated with the hemophagocytosis with anemia, elevated Th1 mediator, such as IFNγ, associated with hyperferritinemia and hypertriglyceridemia. In this situation, a combination of IVIG with cyclosporin-A, anti-TNF-α, and/or MSC administration may be required (9, 30, 98–101).

In summary, the autoimmune vasculitis of KD, KDSS, or MIS-C is mediated by a genetic variant of HLA, FcγR, and/or ADE resulting in hyperinflammation with Th17/Treg imbalance with augmented Th17/Th1 mediators: IL-6, IL-10, IP-10, IFNγ, and IL-17A, and a lower expression of Treg-signaling molecules, FoxP3 and TGF-β, and other suppressive immune mediators. Th17/Treg imbalances among them share similar activation pathways but different regulatory (suppressive) pathway. Based on the similar and different immunopathogenesis, we can make early protection, prevention, and precision treatment of the diseases beyond IVIG and corticosteroids therapies. Evolution of immunotherapies for the diseases has shown that IVIG alone or combined with corticosteroids is the standard treatment for KD, KDSS, and MIS-C. However, some patients are resistant to these therapies, and these susceptible individuals must be detected and given the treatment which can render an early block of viral entry, viral replication and/or shedding, and combat Th17/Treg imbalance by anti-cytokine or pro-Treg for reversing the hyperinflammation and IVIG resistance. Clarifying phenotypes, genetic susceptibility, and hyperinflammatory mechanisms of KD, KDSS, and MIS-C with and without IVIG resistance may help develop a so-called "Know thyself, enemy (pathogen) and ever-victorious" strategy for prevention and immunotherapy for KD and/or MISC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are cited properly in the references, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

M-RC collected the references and summarized the KD studies in Mackay Children's Hospital for drafting the manuscript. H-CK collected the references and summarized the KD studies in Kaohsiung Chang Gung Memorial Hospital for drafting the manuscript. Y-JL drafted the scheme and revised the manuscript. HC provided the information and references regarding KDSS and compared the phenotypes among KD, KDSS, and MIS-C. SL provided the references and suggestions for the section regarding epigenetic controls of Th17/Treg balance on the autoimmunity of KD and MIS-C. H-CL drafted the section of Treg balance influenced by milieu conditions, such as homeostasis of vitamin

D and microbiota. KY designed the article scheme and organized the information for the completion of the article approved by all authors before submission. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neutrophil Extracellular Traps: A Potential Therapeutic Target in MPO-ANCA Associated Vasculitis?

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Our understanding of immune recognition and response to infection and non-infectious forms of cell damage and death is rapidly increasing. The major focus is on host immunity and microbiological invasion. However, it is also clear that these same pathways are important in the initiation and maintenance of autoimmunity and the damage caused to targeted organs. Understanding the involvement of cell death in autoimmune disease is likely to help define critical pathways in the immunopathogenesis of autoimmune disease and new therapeutic targets. An important immune responder cell population in host defense and autoimmunity is the neutrophil. One autoimmune disease where neutrophils play important roles is MPO-ANCA Microscopic Vasculitis. This a severe disease that results from inflammation to small blood vessels in the kidney, the glomeruli (high blood flow and pressure filters). One of the best studied ways in which neutrophils participate in this disease is by cell death through NETosis resulting in the discharge of proinflammatory enzymes and nuclear fragments. In host defense against infection this process helps neutralize pathogens however in auto immunity NETosis results in injury and death to the surrounding healthy tissues. The major autoimmune target in this disease is myeloperoxidase (MPO) which is found uniquely in the cytoplasm of neutrophils. Although the kidney is the major organ targeted in this disease MPO is not expressed in the kidney. Autoantibodies target surface MPO on activated circulating neutrophils resulting in their lodgment in glomerular capillaries where they NETose releasing extracellularly MPO and nuclear fragments initiating injury and planting the key autoantigen MPO. It is the cell death of neutrophils that changes the kidney from innocent bystander to major autoimmune target. Defining the immunopathogenesis of this autoimmune disease and recognizing critical injurious pathways will allow therapeutic intervention to block these pathways and attenuate autoimmune injury. The insights (regarding mechanisms of injury and potential therapeutic targets) are likely to be highly relevant to many other autoimmune diseases.

Keywords: ANCA, glomerulonephritis, cell death, NETs, MPO

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INTRODUCTION

Anti Neutrophil Cytoplasmic Antibody (ANCA) associated vasculitis (AAV) is an autoimmune disease characterized by inflammation of the small blood vessels. AAV can be divided into three different clinical categories: Microscopic polyangiitis (MPA), Granulamotisis with polyangiitis (GPA), and Eosinophilic granulomatosis with polyangiitis (EGPA). A common clinical feature between all 3 vasculitides is a loss of tolerance to neutrophil enzymes, myeloperoxidase (MPO), and proteinase 3 (PR3) which results in the generation of ANCA to either MPO or PR3. MPA patients have a high incidence of MPO-ANCA development and is more prevalent in the Asia pacific region. GPA is the dominant clinical subtype of AAV north of the equator. GPA patients have a higher incidence of PR3-ANCA.

The major organ effected is the kidney (more frequently observed in MPA disease) often resulting in rapidly progressive crescentic glomerulonephritis (RPCGN), which has the worst outcome. If left untreated 90% of patients will progress to renal failure and eventual death. Patients present with general fatigue, elevated temperature, flu-like symptoms accompanied with weight loss and diminished appetite (1, 2). These symptoms result from systemic inflammation and loss of kidney function. Extra renal symptoms are common and often characteristic and particular to their specific disease.

Until the introduction of nitrogen mustard based immunosuppression most notably cyclophosphamide, treatment was primarily based on high doses of glucocorticoids. Prior to this regimen most patients would progress to end stage renal failure within 5 months (3–6). With discovery of ANCA as a diagnostic marker in the 1980s and with current treatment, remission can now be induced within 6 months and survival rates have increased to approximately 75% of patients at 5 years. Although these treatment regimens are largely successful, relapse rates remain high at around 50% at 5 years. Despite maintenance therapy, the high percentage of relapses suggest that standard treatment does not actually completely delete the pathogenic autoimmunity. The toxicity of the treatment itself contributes significantly to morbidity and mortality (3–6). Drug-related toxicity and adverse effects will cause difficulty in over 90% of patients. The most common form of death within the first year of contracting the disease will be infection associated with the immunosuppressed state of the patient. Prolonged use of cyclophosphamide is also associated with an increase rate of malignancy (7).

There is an unmet need for more specific treatment to treat the underlying immunopathology in AAV. Teasing out the underlying pathogenic mechanisms of directing AAV is essential for the development of effective treatment strategies. A key pathological feature of MPO-AAV is the accumulation of dying neutrophils. Neutrophil cell death releases the key autoantigens in ANCA associated vasculitis (AAV); myeloperoxidase (MPO) and proteinase 3 (PR3). Alongside MPO and PR3 one of the key molecules released during cell death is double stranded deoxyribonuclease acid (dsDNA). Without the protection of the cell membrane dsDNA activates multiple sensors and signaling pathways, amplifying inflammation. This review will evaluate the contributing role of Neutrophil Extracellular Traps (NETs) to the pathogenesis of MPO-AAV, with a focus on the activation of NETs, the deposition of NET derived antigens and danger associated molecular patterns (DAMPS) and the development of new experimental NET inhibitors which show promise in attenuating pathological damage to the kidney.

OVERVIEW OF THE PATHOLOGY OF ANCA ASSOCIATED VASCULITIS

AAV is a disease of unknown etiology. The pathogenesis of AAV is complex and involves a wide range of pathogenic processes. In the case of MPO-AAV and PR3-AAV there is a loss of immunological tolerance to neutrophil enzymes MPO and PR3, this in turn generates ANCA and MPO specific T cells. Neutrophil priming and activation via C5a and C5aR interactions exteriorise MPO (or PR3) on their cells surface which MPO-ANCA (or PR3 ANCA) binds to, these neutrophils home to the microvasculature of the glomeruli via selectins, P, E, and L which play a role by reducing the speed of the neutrophil in the circulation which allows it to roll. The second signal comes from the neutrophil surface integrins β_1 and β_2 which interact with the ICAM-1 and ICAM-2 ligands on the inflamed endothelium, the rolling neutrophils slow down, and crawl along the endothelium mediated by integrins to the site of transmigration from the capillaries into the interstitium. Neutrophils either degranulate or release neutrophil extracellular traps (NETs, to be discussed in detail in a later section), causing direct injury through the release of enzymes, reactive oxygen species (ROS), and proteases. Excess neutrophils, MPO specific T cells, and macrophages [via a delayed type hypersensitivity (DTH) mechanism] are recruited resulting in a vicious cycle of injury which causes glomerular injury (8) (see **Figure 1**).

NEUTROPHIL ACTIVATION IN MPO-AAV

The first *in vitro* experiments demonstrating the potential pathogenicity of ANCA came from the laboratory of Falk et al., where it was demonstrated that neutrophils can be activated by ANCA (9). Purified ANCA sera from patients' with pauci-immune necrotizing and crescentic GN incubated with normal

Abbreviations: AAV, Anti Neutrophil Cytoplasmic Antibody; ANCA, Anti Neutrophil Cytoplasmic Antibody Associated Vasculitis; Cl, Chlorine; DAMPS, Danger Associated Molecular Associated Patterns; DNA, Deoxyribonuclease; dsDNA, Double Stranded Deoxyribonuclease; DTH, Delayed Type Hypersensitivity; EPA, Eosinophilic Granulomatosis with Polyangiitis; GN, Glomerulonephritis; GPA, Granulamotisis with Polyangiitis; H2O2, Hydrogen Peroxidise; HMGB1, High Mobility Group Box 1; IgG, Immunoglobulin; IRI, Ischemia Reperfusion Injury; LPS, Liposaccharide; MLKL, Mixed Linked Kinase Domain-like; MPA, Microscopic Polyangiitis; MPO, Myeloperoxidase; MPO-AAV, Myeloperoxidase ANCA associated vasculitis; mtDNA, Mitochondrial Deoxyribonuclease; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; NE, Neutrophil Elastase; Nec 1, Necrostatin 1; NETs, Neutrophil Extracellular Traps; PAD4, Peptidyl Arginine Deiminase; PMNCs, Polymorphonuclear cells; PR3, Proteinase 3; PTU, Propylthiouracil; RIPK1, Receptor interacting Protein Kinases-3; ROS, Reactive Oxygen Species; RPCGN, Rapidly Progressive Crescentic Glomerulonephritis; SLE, Systemic Lupus Erythematosus.

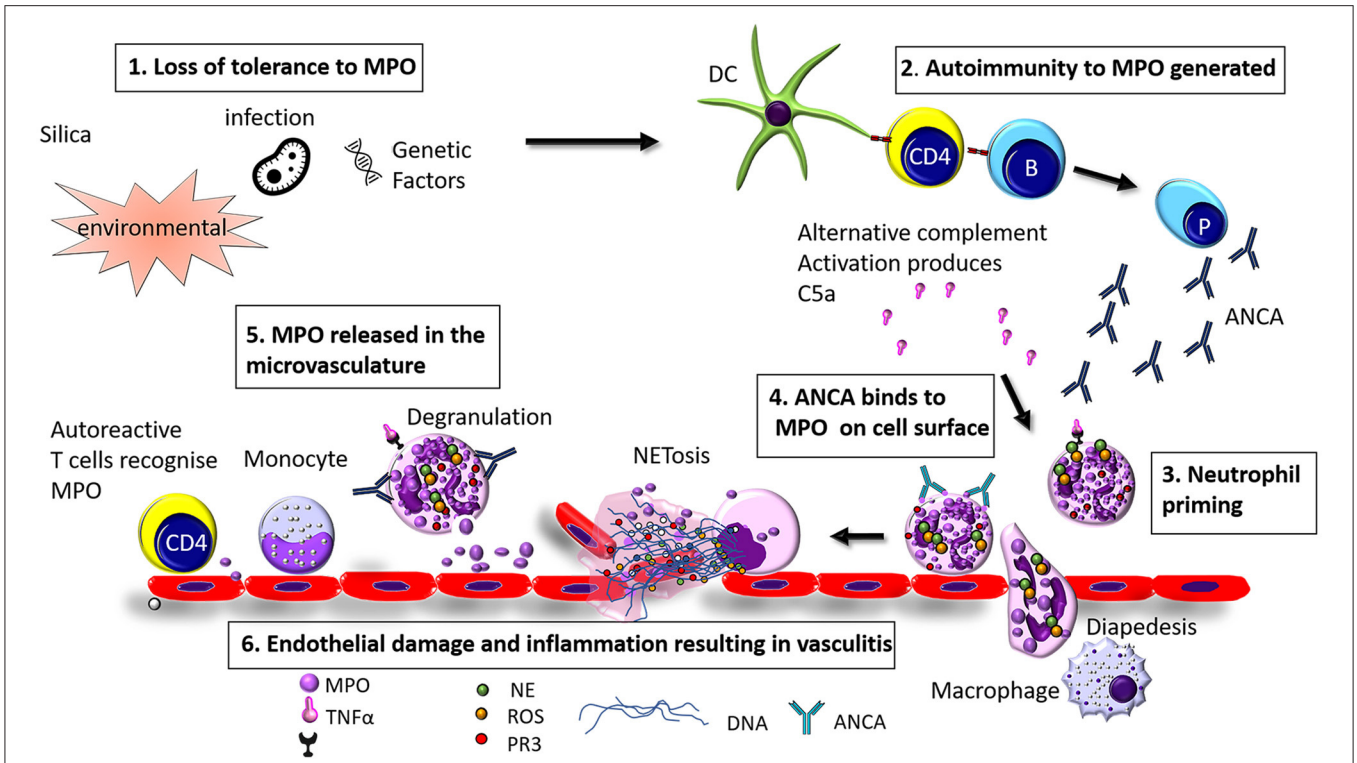

FIGURE 1 | Hypothesized pathogenesis of Myeloperoxidase anti neutrophil cytoplasmic antibody vasculitis. (1) Loss of tolerance of MPO can be caused by infection, environmental factors (such as exposure to silica), or genetic factors (2) Loss of tolerance to MPO results in the production of MPO-ANCA and anti MPO specific T cells (3) Neutrophils are primed by inflammatory mediators such as TNFα, LPS, IL-8, or C5a (4) ANCA binds to MPO exteriorised on the cell surface and (5) MPO is released in to the glomerular vasculature through degranulation or NETosis (6) Endothelial cells are injured resulting in inflammation and vasculitis.

human neutrophils, induced a reactive oxygen species burst (ROS) followed by degranulation of the neutrophil. Further flow cytometry studies demonstrated that after priming neutrophils with the cytokine tumor necrosis factor (TNF) neutrophils exteriorized MPO to the cell surface. The surface location of MPO allowed interaction with ANCA.

Brouwer et al. (10) further demonstrated both *in vitro* and *in vivo* that the numbers of activated neutrophils within kidney biopsies of patients with Wegener's granulomatosis correlated significantly with serum creatinine levels taken at the time of biopsy (22 PR3 positive and 5 MPO positive) (10). All ANCA samples from patients ($n = 19$) were capable of activating primed neutrophils, however no correlation was observed between the ANCA titer of patients and the numbers of activated neutrophils within renal biopsies. A major deficiency of the hypothesis that ANCA induced renal disease was the fact that the key autoantigens, MPO and PR3 were not expressed in the kidney, the major organ damaged by autoimmunity to these antigens. This was also the first study to demonstrate the presence of neutrophil enzymes MPO, PR3 and elastase (HLE) extracellularly within renal biopsies. Their deposition occurred when neutrophils were trapped in the glomerular capillaries where degranulation and other forms of neutrophil cell death released these autoantigens extra cellularly into the kidney. This initiates injury, attracting anti MPO/PR3 T cells driving Delayed Type Hypersensitivity (DTH) effector responses which also induces further neutrophil attraction. In summary neutrophils play an essential role in converting the kidney from innocent bystander status to secondary target status where deposited MPO within the kidney is recognized as an antigen by MPO specific T and B cells.

A major advance in confirming the unique pathway and role of renal targeting by neutrophils was an experiment by the Falk and Jennette group using neutrophil depleted mice. These mice could develop anti-MPO autoimmunity but they could not develop GN unless neutrophils were infused into their circulation. Exogenous neutrophil infusions allowed the development of anti MPO vasculitis and GN confirming the non-redundant role played by neutrophils in the disease (11, 12).

Studies in human ANCA demonstrated that ANCA IgG binds to the MPO or PR3 expressed on the surface of human neutrophils and ligates the Fragment Crystalline gamma RIIa receptor (FCγRIIa). Porges et al. demonstrated that blockade of the FCγRIIa with a monoclonal antibody significantly reduced the production of ROS of ANCA activated neutrophils (13). Binding of ANCA to the FCγRIIa receptor may be one mechanism in which human neutrophils activate signal transduction pathways which result in inflammation (13).

In addition to FCγRIIa other studies have also shown a role for FcγRIIIb in ANCA activation of neutrophils. Kocher et al.

demonstrated that FCγRIIa requires a high density of ANCA binding whereas FcγRIIIb is expressed at 10x higher density than FcγRIIa on the cell surface of neutrophils (14). These results are indicative of FcγRIIIb being involved in the initial engagement by ANCA and that cross linking of the FcγRIIIb favors adhesion of activated neutrophils to the endothelium.

Further *in vitro* studies demonstrated the role of cytokines in priming neutrophils for ANCA activation. Kettritz et al. further confirmed the requirement of cytokine priming of neutrophils with TNFα showing the translocation of both MPO and PR3 to the cell surface. Studies of both the binding and activating properties of ANCA, indicated that both intact ANCA and the $F(ab)_2$ portion of the Ig could cross link MPO or PR3 on the membrane surface of neutrophils (15). ANCA binding alone was not sufficient enough to activate neutrophils, crosslinking of either MPO or PR3 on the cell surface was required to induce a ROS burst.

Complement Activation of Neutrophils in AAV

The complement system is comprised of 3 distinct pathways, the classical, lectin, and alternative pathway all of which produce effector molecules used to fight infection. All 3 pathways initiate a complement cascade converging to form C3 and C5 convertases. The resulting complement cascade generates C5a which binds to the C5aR1 and C5aR2. C5aR1 is expressed on multiple immune cells, including neutrophils and monocyte/macrophages. Cleavage products of the complement cascade (C3a and C5a) are seen by the immune system as danger signals, and recruit further cells of the immune system, in particular neutrophils.

In the context of AAV, evidence suggests that C5a-C5aR interactions are pathological. C5a can prime neutrophils for NET formation, recruit neutrophils to the vascular bed of the glomeruli, and enhance Dendritic cell activation (16). Multiple animal studies of AAV have shown that the alternative pathway plays a critical role in priming neutrophils for activation by ANCA via signaling through C5a-C5aR1 interactions (16–18). Complement activation factors are increased in patients with active AAV disease. These observations suggested that C5a may be a therapeutic target in AAV. Inhibition of the C5aR1 with the antagonist CCX168 (Avacopan) has been demonstrated in phase II and III clinical trials to be effective in modifying disease severity in patients with active AAV (19–22).

NEUTROPHIL EXTRACELLULAR TRAPS (NETS)

A novel mechanism by which neutrophils kill microorganisms was discovered by Brinkmann et al. (23). This study demonstrated that activated neutrophils were able to produce extracellular traps containing DNA fibers, coated with neutrophil proteins from the primary granules (MPO, NE, cathepsin G), secondary granules (lactoferrin), tertiary granules (MMP9), and histones (H1, H2A, H2B, H3 and H4 and the H2A-H2B DNA complex). This seminal study suggested that neutrophils produce NETs to amplify the effectiveness of their antimicrobial granules by producing a large net in a concentrated area, which formed both an antimicrobial and physical barrier to prevent the spread of microorganisms. The authors of this study very insightfully suggested that this mechanism may also have a detrimental effect on the host that could stimulate autoimmunity (23). This initial work, opened a new field in neutrophil biology that in the last 16 years has expanded to define, the signaling pathways for NET production, identification methods of NETs, and a role for NETs in perpetuating inflammation in autoimmune diseases. NETosis is a powerful inducer of injury and therefore a major potential therapeutic target.

Sequence of Events Required for NETosis

NET formation is a unique form of neutrophil cell death that it is not instigated by either necrosis or apoptosis. Live cell imaging of NETs by Fuchs et al. revealed the basic steps required for NET formation (24). PMA stimulated peripheral neutrophils from healthy donors were imaged for 240 min, and the steps in NET formation were outlined to follow a pattern: Firstly 60 min after stimulation, neutrophil nuclear lobules begin to lose their shape, the nuclear envelope begins to disintegrate into small vesicles and chromatin decondensation begins with segregation of eu- and heterochromatin, 180 min after stimulation the nuclear envelop has completely disintegrated, granular membranes rupture and the decondensed chromatin mixes freely with both the contents of the granules and cytoplasmic material, then the outer cell membrane ruptures and a protrusion of a net like structure comprised of DNA, histones, MPO, and NE is extruded (see **Figure 2**). The authors through elegant experiments determined that this form of cell death was neither apoptosis nor necrosis as DNA fragmentation a key marker in apoptosis was absent via TUNEL staining in NET formation. Necrotic cells did not make NETs, their nuclear membranes did not rupture, and the nucleus just fused into a homogenous mass with no apparent segregation of eu- and heterochromatin (24).

NETosis Requires ROS

Reactive oxygen species (ROS) are required for the formation of NETs. The most compelling evidence comes from patients with Chronic Granulomatous Disease (CGD) who have genetic deficiencies that cause mutations in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. CGD patients are unable to form ROS and as a result are unable to make NETs (24). CGD patients suffer from recurrent bacterial and fungal infections that they are unable to clear infection efficiently due to the lack of NADPH which is required for phagocytosis by granulocytes. Addition of H_2O_2 to CGD patients' neutrophils *in vitro* enables neutrophils to produce NETs, suggesting that the sequence of events that allows NET formation can be restored downstream of NADPH (24).

Role of Peptidyl Arginine Deiminase (PAD4) in the Citrullination of Histones

One of the determining factors that distinguishes between neutrophil NET formation and either apoptosis or necrosis is the

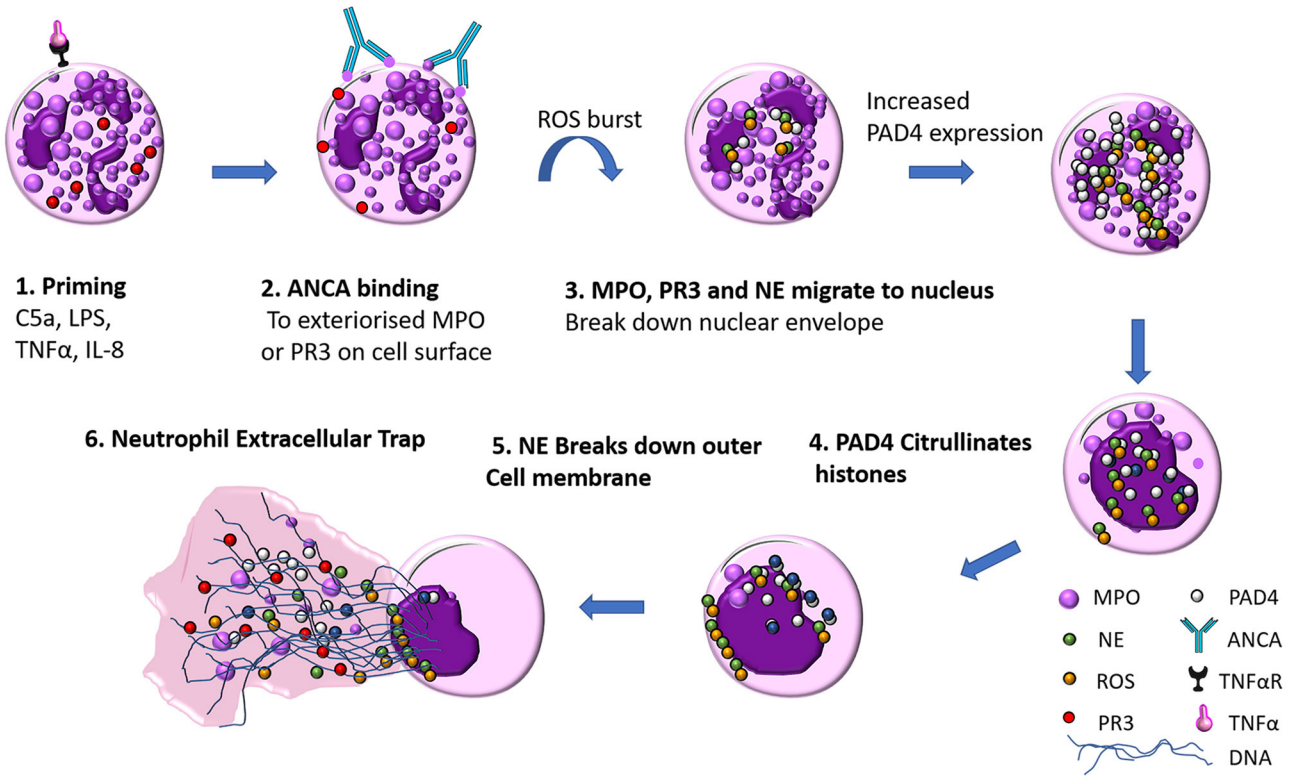

FIGURE 2 | ANCA mediated neutrophil extracellular trap (NET) formation. (1) Neutrophils are primed by inflammatory mediators (such as TNFα, LPS, IL-8, or C5a), which exteriorises MPO on the cell surface (2) ANCA antibody recognize MPO as their cognate antigen and bind to the surface of the neutrophil which initiates a ROS burst (3) NE, PR3, and MPO migrate to the nucleus where they break down the nuclear envelope (4) Increased PAD4 expression facilitates citrullination of histones, the contents of the nucleus and cytoplasm mix as DNA decondenses (5) Neutrophil elastase breaks down actin fibers of the neutrophil cytoskeleton (6) DNA is released with MPO, PR3, ROS and citrullinated histones.

citrullination of histones mediated by PAD4. Initial observations of the role of PAD4 and its association with deimination of histones in neutrophils came from experiments in animal models of rheumatoid arthritis where the expression of PAD4 and citrullinated histones correlated with an increase in disease severity (25). Neeli et al. observed in experiments with stimulated human neutrophils that NET formation was associated with deimination of H3, whereas apoptotic cells produced no deimination of H3, making citrullination of H3 a novel marker to distinguish between NET formation and apoptosis (26). To investigate further the requirement of PAD4 in the citrullination of histones in NET formation Wang et al. demonstrated that the inhibition of PAD4 by the pan PAD inhibitor Cl-amidine failed to produce NETs in HL-60 granulocytes, through the inhibition of H3 citrullination (27). The same group followed up these experiments in $PAD4^{-/-}$ mice and demonstrated that PAD4 was required for NET mediated efficient bacterial killing. Neutrophils stimulated to form NETs by LPS, PMA, and H_2O_2 *in vitro* all required PAD4. $PAD4^{-/-}$ neutrophils were unable to form NETs in response to any of the stimuli. Using a model of necrotizing fasciitis the authors demonstrated that $PAD4^{-/-}$ mice were unable to produce NETs and had a reduced capacity to clear bacterial infection compared with PAD4+/+ mice (28).

Critical Enzymes and Molecules Required for NETosis

Both NE and MPO are stored in the primary (azurophilic granules) of neutrophils, and are both found abundantly adhered to NET fibers. Experiments by Papayannopoulos et al. demonstrated that translocation of NE to the nuclear envelope was required for the decondensation of chromatin (29). After the initial ROS burst NE is released from primary granules where it migrates to the nucleus and digests nucleosomal histones, ultimately instigating the decondensation of the chromatin. MPO was also found to bind to chromatin and enhance decondensation independent of its enzymatic functions. The role of MPO in NET formation was further explored in subsequent experiments and neutrophils from 100% MPO deficient individuals were shown to be unable to form NETs (30). Interestingly neutrophils from individuals with only a partial MPO deficiency were still able to make NETs. MPO contributes 5% of the dry weight of neutrophils and is the most abundant neutrophil granzyme. MPO generates hypochlorous acid through the oxidation of chloride and halide ions in the presence of H_2O_2, this is highly antimicrobial. Despite the requirement of MPO to generate NETs and its highly effective antimicrobial properties, a genetic deficiency in MPO does not appear to have a major

impact on individuals with this defect, they generally live healthy lives unless they have co-morbidities such as diabetes (31).

The importance of MPO in regulating ROS was determined by demonstrating that MPO inhibitors successfully blocked PMA induced NET formation, and that this process could not be rescued downstream by adding extracellular MPO (32). Observations by other laboratories suggest the requirement for MPO in NET formation may be dependent on the activating stimulus. Neither ROS nor MPO is required for NET formation when triggered by *Leishmania* parasites, but NE was required (33). These findings were in agreement with experiments by Parker et al., who found that MPO was required for NET formation when stimulated by PMA but not by *S. aureus* and *E. coli* (34). Overall these experiments highlight the need for physiological relevant experiments, although PMA is a potent NET inducer, it is not found physiologically in either humans or mice and caution should probably be used when using it to study signaling pathways, and assessing requirements for enzymes and ROS, as they may not be representative of what happens *in vivo.*

Role of Gasdermin D in NET Formation

Pyroptosis is a lytic form of inflammatory cell death where Gasdermin D (GSDMD) plays a critical role in forming pores that allow the influx of water resulting in swelling of the cell and osmotic lysis (35). Recently GSDMD has been implicated in having a crucial role in the formation of NETs.

During a large screening of small molecule inhibitors of NETosis Sollberger et al. identified a compound LDC7559 with a pyrazolo-oxazepane scaffold that binds to GSDMD preventing NETosis (36). Using LDC7559 to inhibit NET formation it was demonstrated that neutrophil elastase (NE) and GSDMD work together to break down the nuclear envelope and again to break down the cell membrane at point of expulsion of the DNA webs. LDC7559 inhibits NET formation at low concentrations (1 μM) without blocking phagocytosis, ROS production, NE or MPO activity. These attributes of LDC7559 have potential as therapeutic treatment of diseases with excessive NET burden as it will prevent NETosis whilst keeping host defense intact. This work further consolidated the critical role of NE in NETosis as NE is essential for the activation of GSDMD. This highlights both NE inhibitors and GSDMD as potential therapeutic benefit in inhibition of NETs in inflammatory diseases (29, 30, 36).

Amongst the first hypothesized steps in NETosis we can now add the role of GSDMD (24). The accepted chain of events are: (1) the neutrophil undergoes a ROS burst, followed by (2) migration of neutrophil enzymes NE and MPO to the nucleus where NE induces break down of the nuclear envelope via cleavage and activation of gasdermin-D that punches holes in the nuclear membrane, (3) increased PAD4 expression facilitates citrullination of histones and decondenses DNA, (4) NE destabilizes the structure of chromatin and modifies histones and cleaves actin within the cytoskeleton weakening the outer cell membrane to allow, (5) expulsion of the NET.

Caspase 11 Driven NETosis

Traditionally defined NETosis requires citrullination of histones via PAD4, neutrophil elastase and to a limited extent MPO. A recently discovered form of NETosis driven by detection of cytosolic LPS activates caspase 11 and occurs independently of the requirement of traditional neutrophil enzymes. Inhibition of MPO, NE and PAD4 does not inhibit caspase 11 mediated NETosis (37). This mechanism is thought to have evolved to enhance gram negative bacteria killing by releasing antimicrobial NETs of DNA. Inhibition of both GSDMD and caspase 11 blocks NETosis. Release of MPO and NE is inhibited, which suggests that GSDMD is most likely to be involved in creating pores in the membranes of lysosomes which are responsible for liberating MPO and NE (37). Caspase 11 and GSDMD work in partnership to facilitate NETosis in two stages of NETosis. In stage 1 GSDMD creates pores in the nuclear membrane allowing caspase 11 to enter and cleave histones allowing chromatin relaxation for NET extrusion. In stage two GSDMD creates pore in the cell membrane to allow release of the DNA (37).

Although this type of NETosis is thought to have evolved to defend neutrophils against the invasion of gram negative bacteria, there are other implications where this may be relevant in other diseases. Both GSDMD and caspase 11 may be potential therapeutic targets.

Signaling Pathways in NET Formation

Several different pathways have been reported to play functional roles in NET formation. The first of these pathways is known as the Raf (rapidly accelerated fibrosarcoma),- MEK-(mitogen-activated protein kinase), -ERK (extracellular signal-regulated kinase pathway). The Raf-MEK-ERK pathway is responsible for controlling the expression of the anti-apoptotic protein Mcl-1. PMA stimulated neutrophils downregulate Mcl-1 immediately after stimulation, which blocks apoptosis and favors NET formation. PKC, cRaf, and MEK inhibitors can block this downregulation, suggesting that the raf-MEK-ERK pathway is responsible for controlling NET formation. The authors of this study suggest this pathway could be a potential therapeutic target in diseases where aberrant NET formation occurs (38).

There has been speculation that NET formation could also be activated by the same pathway that causes necroptosis in neutrophils. Necroptosis is another form of cell death distinct from apoptosis. It is considered to be a programmed form of necrosis. The simplified version of the complex signaling pathway is that once activated a series of signals cause interaction between RIPK3 (receptor interacting protein kinases-3) which phosphorylates MLKL (mixed lineage kinase domain-like), which creates a conformational structural change allowing the oligomerization of MLKL to form a pore, which results in increased osmotic pressure within the cell as ion and water enter the cell resulting in rupture of the cell membrane. Experiments using *Ripk3*$^{-/-}$ mice and MLKL inhibition demonstrated that this pathway is used downstream of ROS production (39). Nec-1 an inhibitor of necroptosis, which prevents the formation of the necrosome through the modulation of RIPK1 and the subsequent phosphorylation of RIPK3 and MLKL, prevented the formation of NETs induced by PMA or monosodium urate (MSU) crystals in both human and mouse neutrophils. In direct contrast, Amini et al. found that NET formation occurred independent of signaling through RIPK3 and MLKL (40). Using

the neutrophils from the genetically modified mice deficient in *Ripk3* and human neutrophils chemically inhibited with the MLKL inhibitor necrosulfonamide (NSA), the authors conclude that NET formation is independent of a necroptotic form of death. The notable difference between these 2 studies could be dependent on the different stimuli used to activate and stimulate NET formation. Desai et al. used MSU crystals, and PMA whereas Amini et al. used GM-CSF primed neutrophils with C5a, LPS, or *E. coli*.

ANCA mediated NETosis can be mediated by the RIKP1/3 MLKL pathway (22). *In vivo* NETs can be pharmacologically inhibited using the RIPK1 inhibitor Nec-1s, and the MLKL inhibitor necorusulfonamide (22). RIPK3 and MLKL deficient mice are protected from the development of necrotizing crescentic glomerulonephritis induced by anti-MPO antibody. RIPK3 deficient bone marrow (BM) transfers, in the same animal model were protected suggestive of necroptosis playing a critical role in the progression of necrotizing crescentic glomerulonephritis.

MPO Is Biologically Active in NETs

The bactericidal capacity of MPO in NETs has been shown *in vivo* to be biologically active when adhered to DNA remnants from NET formation. Parker et al. measured the peroxidase activity of MPO released from NETs, and found that over 80% of the MPO released from the neutrophil came from NET formation, and was still biologically active (34). The findings of these experiments have two implications. Firstly, MPO adhered to NETs may aid in the bactericidal capacity of NETs and increase the role of neutrophils role in reducing the spread of bacteria at sites of infection. The second implication, is that the extracellular NET MPO that is deposited is biologically active and may cause direct injury to the surrounding tissues and also become available as an autoantigen in autoimmunity in AAV.

NETs Transfer Neutrophil Antigens in AAV

Although the role of NETs in enhancing host defense against bacterial infection has been well-established, the production of NETs also has potential to expose the immune system to potential self-antigens, and therefore may play a role in perpetuating inflammation by triggering and enhancing injurious autoimmunity to extracellular MPO.

Proof of concept that NETs can initiate autoimmunity in AAV comes from animal models of the disease. Sangaletti et al. demonstrated that neutrophil antigens from NETs can be transferred to dendritic cells (DCs) (41). Live cell imaging of stimulated pro inflammatory neutrophils co-cultured with myeloid DCs (mDCs) showed that NET forming neutrophils make stable connection with mDCs, whilst apoptotic neutrophils only form infrequent contact with mDCs. To examine the possibility of the transfer of the auto-antigens MPO and PR3 from dying neutrophils to mDCs, co- cultures of neutrophils and mDCs were observed and the efficacy of neutrophil antigen transfer characterized. Necrotic cells failed to transfer either MPO or PR3. These auto-antigens were internalized by the DCs with apoptotic bodies less frequently than MPO or PR3 from NET forming neutrophils. Addition of DNase I to the co-culture media prevented the transfer of NET associated antigens, by disintegrating the DNA back bone of the NETs, indicating that the intact DNA structure is required for successful transfer of the autoantigens. Based on these findings the authors of this study transferred the mDCs cultured with either NETs or apoptotic neutrophils into mice via intraperitoneal injection, weekly for a period of 6 weeks, collected serum and examined both the kidneys and lungs for pathology at the termination of the experiment 4 months later. Results demonstrated that the mDCs co-cultured with the NET forming neutrophils had an increased production of circulating ANCA, IgG, and C3 deposition, and an increased kidney pathology score compared to the apoptotic neutrophils co-cultured with mDCs, which had a reduced amount of ANCA production and no evidence of vasculitis in either the lungs or kidneys.

This study provided proof of concept that NET forming neutrophils can initiate vasculitis. The authors conclude that AAV skews neutrophil cell death toward NETosis rather that apoptosis. They also confirm that apoptotic cell death is more likely to protect from excessive inflammation and the development of autoimmunity. The authors do not discuss the possibility that apoptotic neutrophils co-cultured with mDCs may actually have a protective effect. This has been reported in other diseases such as type 1 diabetes (42). DCs co-cultured with apoptotic bodies from β cells and injected into mice have been shown to induce a reduction in co-stimulatory molecules and production of cytokines in NOD diabetic mice (42).

NETs not only exposed the autoantigens MPO and PR3 but also make histones available for interaction with the immune system. Histones themselves elicit strong responses from the immune system by activating danger associated molecular patterns (DAMPS), recognized in particular by toll-like receptors (TLR) 2, TLR4, and nucleotide-binding oligomerisation domain like (NOD) receptors. Histones have been implicated in enhancing injury in many other forms of disease such as sepsis, trauma associated lung injury, sterile liver injury, and kidney injury (43–47).

Histone capacity to induce inflammation and injury is supported by the actions of PAD4. A recent study by Kumar et al. found that inhibition with the PAD inhibitor Cl-amide or histone antibody depletion, reduced glomerulonephritis in a mouse model of anti GBM (48). A reduction in glomerular crescents, and leukocyte infiltration was significant, indicating that targeting extracellular histones could be an effective treatment in glomerulonephritis.

NET Interactions With T Cells

NETs have an established role in innate immunity, but less is known about their interactions with the adaptive immune system. *In vitro* studies of human NETs and T cells have indicated that NETs can prime CD4+ T cells by decreasing their activation threshold (49). Co-culture of T cells and NETs alone increases the upregulation of the T cell activation markers CD25 and CD69, but does not increase T cell proliferation. Co-cultures of DCs, T cells and NETs, results in an increase in the activation of T cells, measured by the increased production of T cell

proliferation cytokines IFNγ, IL-17A, IL-4, IL-10, and IL-2. This effect however is not seen when NETs alone are cultured with T cells, or in transwell plates where the DCs and NETs are separated from the T cells, indicating that T cell direct contact with both DCs and NETs is required for T cell activation (49).

In vivo studies of NETs indicate they can activate Th17 cells, indirectly by priming human macrophages and in a murine model of atherosclerosis (50). Co-cultures of monocytes with NETs activated by cholesterol crystals resulted in an increased production of monocyte cytokines (including IL-1β). In a model of murine atherosclerosis, NETs were found associated with atherosclerotic lesions, which could be reduced when disease was triggered in NET deficient mice (APoE, PR3, and NE deficient) to prevent NET formation. The production of IL1β by macrophages increased the secretion of T cell derived IL17, and subsequent neutrophil recruitment to vascular endothelia (50).

Complement and NET Interactions

Neutrophils from either $C3^{-/-}$ or C3a $receptor^{-/-}$ (C3aR) mice are unable to form NETs (51, 52). This indicates that complement is non-redundant in initiating NETosis. Activated neutrophils express complement factors such as C3 and properdin and Factor B on their cell surface- all components of the alternative complement pathway (53). In host defense neutrophils form NETs to increase bactericidal killing by increasing their surface area in which they ensnare bacteria. Complement opsonization of bacteria have an enhanced ability to induce NETosis (54). C5a both recruits' neutrophils and primes them for NET induction through upregulation of Toll-like and complement receptors (55). Neutrophil enzymes contained with the DNA fibers of NETs have the capacity to cleave C3 and C5 into active fragments. For example MPO can cleave C5a and C5b, whereas Neutrophil Elastase can cleave C3 in to C3a and C3b (56, 57).

OBSERVATIONS OF NETS IN CLINICAL AAV

Kessenbrock et al. provided the first data that indicated a possible pathological role for NETs in AAV. ANCA IgG from patients with small vessel vasculitis (SVV) were able to trigger NET formation in isolated human neutrophils from healthy individuals primed with TNFα (23% of neutrophils formed NETs), whereas IgG from normal healthy individual was only able to stimulate 11% of neutrophils to form NETs. The main autoantigen targets MPO and PR3 were both present upon the NET DNA fibers and were still accessible for ANCA Ig to bind to. After establishing that ANCA could activate NET formation and that the auto antigenic targets MPO and PR3 were accessible within the NETs the authors sought evidence of *in vivo* NET formation in the biopsies of AAV patients. Using antibodies to NET components (DNA, histones, MPO, NE, PR3, and LL37), they found that NETs were present in 9/15 biopsies examined (58).

We have demonstrated that NET formation is closely associated with the deposition of MPO in glomeruli in a large clinical study of MPO-AAV patients ($n = 47$) (59). Extracellular MPO was significantly higher in glomeruli with NETs. This was also the first study to demonstrate the presence of macrophage extracellular traps (METs) that were MPO positive and frequently observed within the glomeruli of MPO-AAV patients (59). Considering macrophages are one of the prevalent leukocyte subtypes infiltrating glomeruli in AAV biopsies, potential therapies targeting NETs should also extend to METs.

Further studies of NET involvement in AAV patients include case reports demonstrating NET formation through the use of antibodies against MPO, Citrullinated Histones and PAD4 or using DAPI in combination with MPO to identify NETs (60, 61). A larger study containing 15 AAV patients (for peripheral neutrophil studies) and 6 kidney biopsies in China showed similar results to the Kessenbrock et al. study, demonstrating enhanced NET formation in patients with AAV compared to healthy controls. There was evidence of increased NET formation in the biopsies of patients with active crescentic GN but minimal NET evidence in the non-crescentic patients providing evidence that NETosis maybe injurious.

Attempts have been made by several research groups to show an association between circulating NET markers and active disease. In a large cohort of 93 AAV patients Soderberg et al. (62), found that patients with active AAV had significantly more remnants of NETs than healthy controls within their circulation. No difference was found between patients in remission and healthy controls. In contrast, Wang et al. found the level of circulating Neutrophil extracellular traps did not correlate with disease activity. In this cohort there were 34 patients with active AAV and 63 patients with AAV in remission (63). A significant difference in the numbers of circulating NETs between AAV patients and healthy controls was determined using circulating cell free DNA (cfDNA) as a marker of NETs. There was no correlation between NET formation in the circulation of patients with active disease when compared to patients in remission.

Wang et al. investigated the levels of DNase I an enzyme that specifically degrades DNA and found that patients with AAV had significantly higher circulating levels of DNase I than healthy controls whereas there was no observed difference between the active AAV and remission group. Taken together current data does not suggest that the presence of NET remnants in the serum is a reliable indicator for disease severity or activity (63).

More recently it has been shown that NET formation can occur in AAV independent of ANCA (64). In a cohort of 99 AAV patients, patients' neutrophils isolated from whole blood demonstrated excessive NET formation *ex vivo* when stimulated with ANCA serum depleted of IgG. Serum levels of ANCA did not correlate with NET remnants, however increased NET formation was observed in patients with active disease. Of interest patients with MPO-AAV had significantly more NET formation than those of PR3-AAV patients. Neither C5aR inhibitors or eculizumab were able to prevent formation of NETs *ex vivo,* indicating that C5a-C5aR interactions are not critical for human NET formation *ex vivo.* Whereas, a genetic deficiency in either C3a or C3aR prevents NET formation in murine neutrophils (as discussed earlier).

NET formation in AAV is distinct from NET formation observed in systemic lupus erythematosus (SLE). Serum from $n = 80$ AAV patients and $n = 59$ SLE patients, were incubated with healthy circulating neutrophils, and induced excessive NET formation. AAV patient serum significantly induced more NETs compared to serum from both healthy controls and SLE patients (65). The qualitative appearance of NETs from AAV patients also differed from SLE patients. AAV induced NETs were lytic in nature with large expulsion of DNA, and occurred much slower than that of SLE induced NETs which were faster, with less expelled DNA, a higher concentration of mitochondrial DNA (mtDNA) and less lysis of the cell membrane. These results should be interpreted with caution as they were performed *ex vivo* by incubating patient serum with healthy neutrophils. A more efficient way of determining the difference between AAV and SLE NETs would be to conduct the same experiment with the neutrophils from the patients. As previously mentioned AAV neutrophils have a higher propensity to NET than those of healthy neutrophils, therefore data collected from neutrophils form healthy patients and SLE patients may not behave in the same manner (62).

Collectively these disparate results indicate using circulating NETs is not a good biomarker for AAV disease activity. Observations from *ex vivo* experiments may not be representative of what happens *in vivo*. Detection of NETs in kidney biopsies would provide more relevant evidence of pathogenicity as these are the sites of direct injury opposed to measuring NET remnants from the circulation in serum, or inducing NET formation *ex vivo*.

VITAL NETS AND MITOCHONDRIAL DNA RELEASE

Neutrophils are also able to produce NETs which do not ultimately end in death of the neutrophil. This has been termed "vital NETosis," and uses some of the same pathways as traditional "suicidal NETosis," such as the translocation of NE to the nucleus, activation of PAD4 and decondensation of chromatin. Instead of extruding all the nuclear contents as in suicidal NETosis, in vital NETosis, the DNA is released within vesicles, and the neutrophils phagocytic functions remain intact (51). *In vivo* imaging of neutrophils during gram-positive skin infections, have demonstrated that NET forming neutrophils can still effectively crawl despite being anuclear, and still have the ability to kill bacteria via phagocytosis (51).

Mitochondrial (mt) NETosis has also been observed, like normal NETosis it is ROS dependent. Mitochondrial nets have been formed *in vitro* in response to stimulation by GM-CSF and C5a (66). Recently mitochondrial NETosis has been demonstrated in low density neutrophils from lupus nephritis patients (67). The mitochondria formed NETs were pro-inflammatory and were able to induce production of type 1 interferons. Using MRL/*lpr (l*upus prone mice), it was shown that the *in vivo* administration of MitoTEMPO an inhibitor of mitochondrial ROS was able to reduce NETosis, and ameliorate autoimmunity in the MRL/*lpr* mice (67).

RELEASE OF DNA FROM OTHER LEUKOCYTES OTHER THAN NEUTROPHILS IS PROINFLAMMATORY

Neutrophils are not the only cells that release DNA. It has been recently described that activated CD4 T cells can release extracellular DNA traps (68). *Ex vivo* cultured Human CD4 T cells and naïve CD4+T cells from mice produced DNA threads when activated with anti-CD3/antiCD28 antibodies for 24 h. The DNA released is highly oxidized, coated with histones similar to what is see in the generation of NETs. The expelled DNA from CD4 T cells, was also detectable *in vivo* in the lymph nodes of EAE mice. The generation of these T cells is dependent on the generation of reactive oxygen species (ROS). Using the mitochondrial ROS inhibitor SkQ1 at a low dose *in vivo* reduces excess mitochondrial ROS and is protective in a model of EAE. Antigen specific (MOG_{35-55}) *ex vivo* stimulation of lymph node cells from mice treated with SkQ1 had reduced immune responses and reduction in pro-inflammatory cytokines.

B cells release mitochondrial DNA in response to stimulation with CpG and non-CpG oligonucleotides (ODN) but no other known NET stimulators (69). This is independent of typical cell death such as necrosis or apoptosis. Unlike NETs this process is rapid, happening within minutes of stimulation, does not rely on ROS or NADPH. B cell expulsion of DNA is of mitochondrial origin and released in full size rather than fragments. B cell mt DNA release, acts like a DAMP and induces the production of Type 1 IFNs in co-cultures PBMCs. Inhibition of B cell mt DNA is not accomplished by inhibitors of other types of cell death, or by blocking ROS. The only treatment effective in blocking the release of B cell mt DNA was $ZnCl_2$.

This data indicates when considering the inhibition of NETs, the effectiveness should be assessed also in other cells leukocytes and lymphocytes which may be contributing to inflammation through the release of ecDNA.

NET DERIVED EXTRACELLULAR DNA IS PRO-INFLAMMATORY

One of the major components of NETs is the backbone DNA structure. Within NETs this backbone of DNA provides the substrate that the neutrophil enzymes, proteases, and histones adhere to. Although the production of NETs helps to fight infection by containing the spread of infectious bacteria, the DNA itself can cause inflammation through activation of DNA sensors, and histone exposure (as discussed in previous section). DNA is released from dying cells through several different mechanisms including apoptosis, necrosis, necroptosis, and pyroptosis. Besides the different signaling pathways involved and pathological appearance of these types of cell deaths, the size of the DNA and quantity released during death differs also.

DNA derived from NETs can activate the immune system by acting as a danger signal. The nuclear location of DNA offers protection from exposure to the immune system under normal healthy conditions. Self-DNA which is released extracellularly during either pathogenic processes or autoimmunity is seen

by the immune system as a Danger Associated Molecular Pattern (DAMP) where as non-host DNA is recognized as a Pathogen Associated Molecular Pattern (PAMPS) by Pattern Recognition Receptors (PRRs). DNA which escapes the nucleus, is detected by several different sensors. TLR9 recognition of DNA occurs within endosomes. Within the cytoplasm extra nuclear DNA is detected by either three prime repair enzymes, exonuclease 1 (TREX1), or cyclic GMP-AMP synthase (cGAS). This recognition of extra nuclear DNA activates the adapter protein Stimulator of Interferon Genes (STING). The different DNA sensors can also control which pathway of cell death may occur. For instance, extracellular DNA activation of TLR9 will induce apoptosis, activation of RIP3 will activate necrosis and NETosis, caspase 9 will trigger apoptosis, and activation of AIM2 will induce pyroptosis.

TARGETING NETS AS THERAPY IN AAV

There are currently no alternative proven safe therapies that can replace immunosuppressive cyclophosphamide or rituximab for the treatment of AAV. Both these treatments render patients susceptible to infection (70, 71). Cyclophosphamide suppresses bone marrow production of all leukocytes and therefore immunity is unable to be restored quickly. White blood cell counts can take weeks to return to an acceptable level leaving patients vulnerable to infection, and with a worsened prognosis upon pathogen insult. While non-targeted immunosuppression can attenuate AAV, critical functions for maintaining host immunity are compromised, resulting in severe and life-threatening side effects, specifically increased susceptibility to opportunistic infections and malignancy.

Potential Strategies to Prevent NETosis

Recent evidence suggests targeting some cell death pathways, particularly NETosis may be safer than conventional therapies. This is because emerging inhibitors of some critical NETosis specific enzymes have few off target effects. However, many of the potential NETosis inhibitors are reversible or have short half-lives. Early infection detection and rapid withdrawal would minimize the risk of increased susceptibility to infection. A summary table of inflammatory mediators released by NETs and pathways with potential therapeutic avenues can be found in **Table 1** and experimental evidence demonstrating a pathogenic role of NETS in disease initiation/progression are summarized in **Table 2**.

Peptidyl Arginase Deiminases

There are several potential candidates for targeting NETosis as therapy in autoimmune diseases, including Cl-amidine (and the second generation BB-Cl amidine), both pan PAD inhibitors, DNase I which specifically targets and clears DNA and gasdermin D inhibitors which will prevent both pyroptosis and NETosis. Multiple studies have shown a protective effect in autoimmune diseases when PAD4$^{-/-}$ animals have been used, so it would be likely that a PAD inhibitor would have some effect in ameliorating autoimmunity in diseases in which NETs are likely to play a role such as lupus, atherosclerosis, arthritis, diabetes, and AAV (86, 87, 92–95). Although Cl-amidine and BB-Cl amidine which has a longer half-life *in vivo* has been beneficial in animal models, there are concerns that in human diseases that the inhibition of all the PADs not just PAD4 which is implicated in NET formation could have a detrimental effect in patients by increasing their susceptibility to infections. Specific inhibitors for PAD4 with no off target effects are emerging. Our laboratory has shown the use of GSK484 a specific PAD4 inhibitor and the pan inhibitor BB-CLA are protective in animal model of AAV. With a reduction in NETs, extracellular MPO and neutrophils recruitment to glomeruli. Interestingly BB-CLA is also able to induce an increase in numbers of MPO specific T regulatory cells (88).

Deoxyribonucleases (DNase)

The deoxyribonuclease family can be divided into two broad classes based on their biochemical structure and functional properties. The DNase I family consists of DNase I, DNase 1L1, DNase 1L2, and DNase1L3. Little is known about the functions of DNase L1, and DNase 1L2. DNase L1 is produced mainly by muscle and myocardium whereas DNase 1L2 is produced in varied sites including the brain lungs, placenta and skin. All of the DNases cleave DNA, for the purposes of this review we will concentrate on DNase I and DNase 1L3 which have both been implicated in regulating ecDNA in autoimmunity.

DNase I family enzymes require bivalent magnesium and calcium ions for activation. Without magnesium DNase I is unable to efficiently cleave DNA phosphodiester bonds. DNase I is mainly produced by the pancreas, and salivary and parotid glands and released into the blood stream. G-Actin forms a complex with DNase I and inactivates it, the biological significance of this is not yet known, however this does suggest that DNase I is subjected to regulation *in vivo*. DNase I preferentially degrades double stranded DNA (dsDNA) over single stranded DNA (ssDNA). The primary role of DNase I is to degrade extracellular nuclear proteins- any disruption in this process has the potential to cause autoimmunity.

DNase1l3 is expressed highly in the lymphoid organs, and has a role in promoting plasma ecDNA homeostasis. DNase1l3 enhances fragmentation of DNA and targets nucleosomes and DNA protein complexes. DNase1l3 plays a role both in intra and extracellular degradation of DNA. It has two nuclear localization signals unlike DNase 1 which only has one in the N-terminal signal. It is proposed that this is why DNase1l3 may have a role in apoptosis where it enhances chromatin cleavage.

DNase I and DNase 1l3 both degrade NETs *in vitro* and *in vivo*, providing dual protection against excess NET formation (80). Mice genetically deficient in either DNase I or DNase 1l3 are protected against chronic neutrophilia induced by G-CSF. This indicates that in the absence of one DNase the other has the ability to compensate. However, if mice are both DNase I and DNase 1l3 deficient, mice die within 5 days. Mutations in both DNase I and DNase1l3 are associated with autoimmune diseases (96, 97). Mice deficient in DNase I and DNase 1l3 spontaneously develop SLE (98–100).

TABLE 1 | Inflammatory mediators released by Neutrophil Extracellular Traps and potential inhibitors.

Molecule	Model of investigation	Inhibitor	References
Inflammatory mediators released by Neutrophil extracellular traps			
Myeloperoxidase (MPO)	Experimental AAV, clinical MPO	AZM198	(59, 72)
Proteinase 3 (PR3)			
Neutrophil Elastase (NE)	Lung disease	Alvelestat	(73–76)
		BAY 85-8501	(77–79)
Histones	Experimental model of GN	Anti-histone antibody	(48)
DNA	Animal models of AAV, Animal model of chronic neutrophilia	DNase 1 DNase 1L3	(22, 80, 81)
mtDNA	Animal model of EAE	SkQ1	(68)
Reactive oxygen species (ROS)	Animal model of EAE	SkQ1	(68)
High Mobility Group Box 1 (HMGB1)	Animal models of sepsis, and IRI	Glycyrrhizin Gabexate mesilate HMGB1 specific ab	(82, 83)
S100A8/A9	SLE, T1D Clinical trials	Quinoline-3-carboxamide	(84, 85)
Molecular targets in the initiation of NETS			
Peptidyl arginine deiminase 4 (PAD4)	PTU induced AAV NET inhibition in Lupus prone MRL/lpr mice	Cl amidine Cl amidine	(86) (87)
	Experimental AAV Experimental AAV	BB-Cl amidine GSK484	(88) (88)
Gasdermin D	Human and murine neutrophils	LDC7559	(36)
RIPk1	Mouse NETs	Necrostatin (NEC)	(89)
RIPk3	Mouse model of AAV	Necrostatin (NEC1)	(22)
MLKL	Mouse model of AAV	Necorusulfonamide	(22)

TABLE 2 | Experimental evidence of the pathogenic role of NETs in AAV.

Model	Experimental evidence	References
AAV PBMC and kidney biopsies	PR3-ANCA and MPO-ANCA stimulate NET formation *ex vivo*, glomerular NETs in AAV patient biopsies	(58)
AAV kidney biopsies	Extracellular MPO associated with glomerular NET formation, description of macrophage extracellular traps	(59)
AAV PBMCs	AAV patient neutrophils more likely to spontaneously NET than healthy controls	(62)
Animal model of AAV	NET formation can be inhibited by blocking the Necroptosis pathway	(22)
Animal model of AAV	NETs transfer neutrophil antigens in AAV	(41)
AAV PBMCs	NET formation can occur independent of ANCA, and complement pathway.	(62, 64)
AAV serum with healthy donor neutrophils	AAV NETs distinct morphology compare to SLE NETs	(65)
AAV Case Reports	NETs present in glomeruli of biopsy	(60, 61)
PTU induced animal model of AAV	Cl amidine prevented MPO-ANCA production	(86)
PTU induced Wistar-Kyoto Rat model of AAV	Pharmaceutical Immunoglobulins reduce NETs and attenuated MPO-AAV	(90)
Animal model of AAV	DNase I reduces NET formation and ameliorates AAV	(81)
Animal model of AAV	$NE^{-/-}$ mice are NET depleted and protected from development of AAV	(91)
Animal model of AAV	$PAD4^{-/-}$ mice have significantly less glomerular NETs and attenuated AAV	(88)
Animal model of AAV	PAD inhibitors BB-Cl amidine and GSK484 inhibit NET formation and protect attenuate development of AAV	(88)

DNase I, has been shown to be safe and tolerable in a clinical trial in lupus, although not effective in treating the disease possibly due to the heterogeneity of the disease pathogenesis in Lupus, and the disease progression not reliant on immunoreactivity to DNA alone. However, valuable information on the safety and side effects can be taken from the trial, and applied to other forms of autoimmunity where extracellular DNA may play more of an injurious role (101).

Experimental evidence indicates that DNase I is therapeutic in two different models of ANCA vasculitis. Schreiber et al. demonstrated in a model of AAV that DNase I prevents ANCA induced NET formation, glomerular injury and protects mice from development of disease. MPO-ANCA activated NETs incubated with endothelial cells *in vitro* increased albumin permeability, which pre-treatment with DNase I prevented (22). Our own laboratory has shown that DNase I prevents the

development of AAV, through a reduction in ecDNA released during cell death, reduced NET formation and an associated decrease in the deposition of the autoantigen MPO. DNase I is able to reduce autoimmunity to MPO with a reduced number of MPO specific lymphocytes (IL17a and IFNγ) in the draining lymph nodes and an increase in the number of MPO specific T regulatory cells, suggesting that DNase I may have an immunomodulatory role. DNase I given at the time of antigen presentation reduced the numbers of CD11c positive DCs, and activation markers MHCII, CD40, CD80, CD86, and the numbers of activated CD4+ CD69+ T cells (81).

Treatment with DNase I is not without difficulties. To achieve a therapeutic benefit in animal models of AAV, DNase I is administered intravenously twice daily to combat the short half-life (3–4 h) of DNase I in the circulation (22, 81). Recombinant DNase I is rapidly inactivated by G actin therefore the benefits of an inhaled actin resistant DNase is being currently investigated in Phase I and II clinical trials for cystic fibrosis patients (NCT02605590, NCT02722122). Other avenues to combat the limited half-life of DNase I in the circulation involve using gene delivery vectors derived from adeno-associated virus vectors (AAvec). AAvec would overcome the critical issue of the short half-life of the enzyme to provide a "one shot" therapeutic for SLE and ANCA-vasculitis through continuous delivery of DNase I. AAvec technology has already been used successfully to treat blindness, clotting disorders, and neuromuscular disease in humans (102). Over expression of both DNase I and DNase Il3 protein with pLIVE plasmids enables the liver to produce long lasting over expression of both proteins in mice. In a model of sepsis in DNase $I^{-/-}$ and DNase $1l3^{-/-}$ mice, plasmid expression of DNase I and DNase 1l3 restores endogenous circulating levels of both types of DNase and prevents vascular occlusion from NETosis in a model of chronic neutrophilia (80).

DNase I coated melanin-like nanospheres have been used successfully to control NET dysregulation in a model of sepsis (103). To circumvent the relative short half-life of DNase I in the circulation bare bioinspired melanin-like nanospheres (bMNSs) are coated with polydopamine which immobilizes DNase I on the surface of the bMNSs. The DNase I bMNSs retain biological activity for 36 h. Intravenous injection of DNase I bMNSs significantly reduced NETosis, extracellular DNA levels, neutrophil counts, reduced MPO and NE activity, and proinflammatory cytokines in a murine model of sepsis. Mice given DNase I bMNSs had improved survival rates compared to mice given endogenous DNase I.

In addition to clearing NET DNA DNase I can clear DNA from other types of cell death where DNA is released into the extracellular environment such as apoptosis, necrosis, and pyroptosis. One limitation of DNase I is that it only dismantles formed NETs. Some NET remnants (histones and proteases) are left behind in vessel walls (104). DNase I as adjunctive therapy may allow reduction in the dose of currently used effective drugs particularly steroids, where high dose related side effects are common.

Neutrophil Elastase Inhibitors

The role of neutrophil elastase (NE) as a major constituent of NETs is well-accepted however the role of NE in instigating NETosis is controversial. NE was first shown in 2010 to work synergistically with MPO to break down the nuclear envelope and help cleave histones as discussed in a previous section (29). These findings were confounded when it was found that $NE^{-/-}$ mice were able to form NETs in a model of deep vein thrombosis, putting into dispute the role of NE in forming NETs (105). Mice with a genetic deficiency in NE only had a modest reduction in NET formation compared to WT controls when ionomycin was used to stimulate NET formation. The conflict in these results suggests that NE plays a different role in instigating NETosis dependent on the stimulus. It is also possible in the absence of NE another protease takes over the role of regulation of NETosis. Recently it has been shown that GSDMD and NE work synergistically to break down the nuclear envelope and the outer cell membrane during NETosis highlighting again the importance of NE in NETosis and its relevance as a therapeutic target, both in instigating NETs and neutralizing once it has been exteriorized thorough NETosis (36).

Two promising candidates for neutrophil elastase inhibitors are Alvelestat and BAY 85-8501, both have been shown to be safe and tolerable in clinical trials for the treatment of airway diseases so is poised for clinical translation to target NETosis in AAV.

Alvelestat (AZD9668), is a 3rd generation neutrophil elastase inhibitor which is reversible and highly selective for Neutrophil Elastase and to a lesser extent PR3 (77). It has been utilized in Phase II clinical trials for the treatment of air way diseases at a dose of 60 mg b.i.d (twice daily) including cystic fibrosis (73), chronic obstructive pulmonary disease (74), and bronchiectasis (75). Alvelestat has been effective in inhibiting NETs in a model of acute lung injury/acute respiratory distress syndrome in mice and significantly reducing the inflammatory response (76).

BAY 85-8501 is a 5th generation neutrophil elastase inhibitor with potency similar to that of endogenous antiproteases, it has improved metabolic stability over the previous generation of inhibitors with an improved half-life. Phase I clinical trials with healthy males showed that a dose of up to 1 mg is well-tolerated with no evidence of adverse reactions (78). Phase II clinical trials have shown Bay 85-8501 to be safe for the treatment of non-cystic fibrosis bronchiectasis (79), and bronchiectasis (75). BAY 85-8501 reduces human primary neutrophil NET formation *in vitro* when stimulated with PMA (36). Based on the results of the safety profile of BAY 85-8501 and the *in vitro* capability of inhibiting NET formation, it is a promising candidate to block NET formation in AAV.

Inhibition of NE raises the possibility that host defense will be compromised. This is minimized with the use of the most recent generation of NE inhibitors as the inhibitors are reversible and have been shown to be dose dependent in *ex vivo* blood zymosan assays of neutrophils to determine the phagocytosis capability after use of the NE inhibitors (77). Of clinical relevance is preliminary data that they can inhibit NE activity to a level that has clinical benefit (between 50 and 90%) but does not

reduce the innate capacity of neutrophils to engulf and kill bacteria (77).

Our laboratory has shown that neutrophil elastase knock mice (*Elane*$^{-/-}$) are protected from the development of murine MPO-AAV with a significant reduction in albuminuria/creatine ratio, glomerular segmental necrosis, glomerular NETs and associated MPO deposition, recruitment of glomerular CD4+ T cells, macrophages and neutrophils (91). Both Alvelestat and BAY 85-8501 have been used to inhibit NETs in a model of MPO-AAV with favorable results. Daily administration of the NE inhibitors via oral gavage reduced glomerular injury, recruitment of glomerular CD4 T cells, CD8 T cells, macrophages and neutrophils. Glomerular NET accumulation and MPO deposition was significantly reduced. ANCA was significantly reduced in the BAY 85-8501 treated mice but not in the Alvelestat treated mice.

Collectively these experimental results demonstrate that NE inhibition is protective in experimental MPO-AAV. Favorable results from clinical trials in terms of safety and efficacy are encouraging for the investigation of clinical translation of Alvelestat and BAY 85-8501 for the treatment of MPO-AAV (106).

CONCLUDING REMARKS

Current treatment for AAV is suboptimal with unavoidable adverse effects. A targeted more specific treatment is required. NETs are a common feature within glomeruli of kidneys from MPO-AAV patients. The key autoantigens in AAV MPO and PR3 are released during NET formation along with other potential injurious molecules that cause direct injury to glomerular endothelial cells. Therapeutically targeting NETs will prevent the release of these autoantigens, reducing glomerular damage, with the potential to ameliorate the progression of AAV.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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CD8+ T Cells in GCA and GPA: Bystanders or Active Contributors?

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Vasculitis refers to inflammation of blood vessels and can cause a variety of serious complications depending on which vessels are affected. Two different forms of vasculitis are Giant Cell Arteritis (GCA) and Granulomatosis with Polyangiitis (GPA). GCA is the most common form of vasculitis in adults affecting the large arteries and can lead to visual impairment and development of aneurysms. GPA affects small- and medium-sized blood vessels predominantly in the lungs and kidneys resulting in organ failure. Both diseases can potentially be fatal. Although the pathogenesis of GCA and GPA are incompletely understood, a prominent role for CD4+ T cells has been implicated in both diseases. More recently, the role of CD8+ T cells has gained renewed interest. CD8+ T cells are important players in the adaptive immune response against intracellular microorganisms. After a general introduction on the different forms of vasculitis and their association with infections and CD8+ T cells, we review the current knowledge on CD8+ T-cell involvement in the immunopathogenesis of GCA and GPA focusing on phenotypic and functional features of circulating and lesional CD8+ T cells. Furthermore, we discuss to which extent aging is associated with CD8+ T-cell phenotype and function in GCA and GPA.

Keywords: giant cell arteritis, granulomatosis with polyangiitis, CD8+ T cells, vasculitis, aging

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INTRODUCTION

The vasculitides are a heterogeneous group of disorders characterized by inflammation of blood vessels. Classification of the different forms of primary vasculitis is defined by the 2012 International Chapel Hill Consensus conference (1) and is based primarily on the size of the inflamed vessels. The distinct forms of vasculitis also differ from each other regarding age of onset, genetic predisposition, pathogenesis and affected organs.

The onset of some forms of vasculitis has been linked to infectious triggers. A prime example is granulomatosis with polyangiitis (GPA), which has been associated with various microbial agents, in particular *Staphylococcus aureus*. GPA is a severe systemic autoimmune disease that predominantly affects the elderly. The disease is characterized by necrotizing vasculitis of small- to medium-sized blood vessels and the presence of anti-neutrophil cytoplasmic antibodies (ANCA) mainly directed against proteinase 3 (PR3). Due to inflammation of small blood vessels, several

organs and tissues can be severely affected. In GPA, especially upper and lower respiratory tract and kidney involvement are common. Besides necrotizing vasculitis, GPA is characterized by granulomatous inflammation of the respiratory tract. Studies have shown that the majority of GPA patients are nasal carriers of *S. aureus* which correlated with higher relapse rates although a direct link between *S. aureus* carriage and disease activity or relapse risk remains to be established (2, 3). Moreover, in a subset of GPA patients a clinical benefit from treatment with antibiotics has been demonstrated adding to the notion that a microbial factor may trigger the disease (4).

Giant cell arteritis (GCA) is the most common form of large vessel vasculitis and affects females twice as often as men. GCA is strongly age-related as it only affects people older than 50 years of age (5). The symptoms experienced by GCA patients are largely dependent on the anatomic localization and type of the affected vessels. Different studies reported an association between disease onset and infections with specific pathogens such as *Mycoplasma pneumoniae*, Parvovirus B19, Herpes Zoster and Parainfluenza virus but none of these associations could be conclusively validated in follow-up studies (6–8). This does not exclude a role for infections in the onset of GCA, but rather suggests the involvement of shared inflammatory pathways that can be activated by various infectious agents.

Other forms of vasculitis in which infections have been strongly implicated include Kawasaki disease (KD) (9–11), a medium vessel vasculitis affecting young Asian populations, Polyarteritis nodosa (PAN), a medium-sized vessel vasculitis affecting adults (12–15) and Takayasu Arteritis (TA), which affects younger adults from Asian populations (16).

Our immune system is designed to protect our body against infectious agents and utilizes specialized immune cells for this purpose. CD8+ T cells are key in the anti-viral defense by clearance of virus-infected cells, but also act against intracellular bacterial infections. It is therefore not surprising that the role of CD8+ T cells has been investigated in several forms of vasculitis that have been associated with infectious agents. The forms of vasculitis in which the role of CD8+ T cells is most pronounced, are diseases affecting children (KD) or younger adults (TA) (summarized in **Table 1**). CD8+ T cells are highly prevalent in inflammatory infiltrates of KD patients and upregulated CD8+ and interferon pathway genes were detected in post mortem coronary artery biopsies (17, 18). In TA CD8+ T cells are also abundantly present in affected arteries and perforin was suggested to induce vascular cell injury (25). Regarding older adults with PAN, available data is limited to a small number of case series, which revealed the presence of CD8+ T cells at the site of vascular inflammation, mostly outnumbering the CD4+ T cells (29–32).

Although disease onset and progression in GCA and GPA have also been linked to infectious agents, the contribution of CD8+ T cells to the pathogenesis of these diseases is largely unknown. Studies on immune mechanisms involved in disease pathogenesis have mainly focused on the roles of macrophages and CD4+ T cells in GCA, and additionally on neutrophils and autoantibody producing B cells in GPA. Recent studies however, have clearly demonstrated the presence of CD8+ T cells in vasculitis lesions in both GCA and GPA (33, 34). We deemed this observation of particular interest due to the possible role of infectious triggers in both diseases and the notion that aging, having a profound influence on especially CD8+ T-cell functions, presents a risk factor for both GCA and GPA.

Therefore, we here review and discuss the studies on CD8+ T-cell involvement in the pathogenesis of GCA and GPA to determine whether CD8+ T cells are active contributors to disease pathogenesis or just bystanders with limited pathogenic functions, and to determine to which extent aging affects the function and phenotype of CD8+ T cells in GCA and GPA. After a general overview of CD8+ T-cell function in health, disease and aging we discuss the current knowledge on CD8+ T cells in GCA and GPA with respect to circulating and lesional phenotypes, transcriptomic profiles and function. Paired medical subject (MeSH) headings used for our literature search included giant cell arteritis, temporal arteritis, granulomatosis with polyangiitis, Wegener's, CD8+ T cells and ANCA-associated vasculitis. The reference lists of the articles selected with these keywords were also used to include additional relevant articles. Single case reports, reports in <5 patients, studies without any clear

TABLE 1 | Evidence suggesting CD8+ T cell involvement in Kawasaki disease and Takayasu Arteritis.

Kawasaki disease	Takayasu Arteritis
* Strongly associated with (viral) infections (9–11). * Transmural infiltration of more memory CD8+ T cells than CD4+ T cells in biopsies of coronary artery aneurysms (17). * Genes related to CD8+ T-cell activation and type 1 interferon induced genes are upregulated in biopsies (18). * CD8+ T cells, but not CD4+ T cells, are required for KD development in a murine model of KD (19). * The frequency of activated CD8+ T cells, defined by HLA-DR expression, was higher in peripheral blood of active KD patients compared to controls (20). * Treatment with intravenous immunoglobulin (IVIG) inhibits CD8+ T-cell activation (20). * Patients that were IVIG resistant had a higher percentage of peripheral blood CD8+ HLA-DR+ T cells compared to responders (20). * Percentages of the costimulatory receptor NKG2D-expressing CD8+ T cells were lower in peripheral blood of acute KD than in HCs (21). NKG2D-expressing CD8+ T cells could also have migrated to tissues or NKG2D could be downregulated in response to ligand-bound activation.	* MHC-I genes, especially HLA-B52 is strongly associated with TA, suggesting involvement of CD8+ T cells (22–24). * CD8+ T cells are present in aortic tissues (25, 26). * A study in a small group of rather old TA patients suggests that TA aorta biopsies have more infiltrating CD8+ T cells than temporal artery biopsies of GCA patients (26). * Perforin was expressed in CD8+ T cells in aortic tissues of TA patients, which was suggested to induce vascular cell injury (25) * 248 genes in CD4+ and 432 genes in CD8+ T-cell samples differed between TA and HCs. TA patients had upregulation of type I interferon genes (27) * Strong expression of NKG2D, a costimulatory receptor for CD8 + T cells and NK cells, and its ligand MICA in aortic lesions of TA patients (28).

patient characteristics, and inaccessible whole text of studies were excluded.

CD8+ T CELLS IN HEALTH AND DISEASE; ACTIVATION AND SUBSET DIFFERENTIATION

The primary function of CD8+ T cells is to identify and eliminate virus- or bacteria-infected, malignant and damaged cells. Upon recognition of their cognate peptide presented by MHC class I, CD8+ T cells can kill their target cells via perforin and granzymes and release cytokines such as Interferon (IFN)-γ and tumor necrosis factor alpha (TNF-α). Some cytokines are directly cytotoxic, but others recruit and activate other effector cells. Fas-Fas-Ligand interactions between target cells and CD8+ T cells, respectively, result in death of target cells as well.

Next to these protective functions, CD8+ T cells can also be detrimental and contribute to autoimmune diseases. For instance, auto-reactive CD8+ T cells seem to contribute to autoimmune pathology in type 1 diabetes, alopecia areata, multiple sclerosis and inflammatory bowel disease (35–38). In healthy individuals, several mechanisms are in place that prevent the activation of CD8+ T cells upon autoantigen presentation. In patients with an autoimmune disease however, failing regulatory mechanisms and other factors can result in activation of autoreactive CD8+ T cells. These factors include among others, the activation state of the dendritic cells (DCs), the number of antigen-MHC complexes, TCR affinity, the local cytokine milieu and the functionality of regulatory CD4+ and CD8+ T cells (38).

Activation of CD8+ T cells, in either the context of health or disease, leads to a sequence of events that results in differentiation of naive CD8+ T cells into memory cells and thus the generation of a pool of antigen-specific memory CD8+ T cells (**Figure 1**). Upon proper antigen presentation and appropriate co-stimulation and cytokine involvement, naive CD8+ T cells proliferate and give rise to clonal expansions of antigen-specific effector CD8+ T cells. Contraction of the effector pool takes place after antigen clearance via apoptosis of most short-lived effector cells. Long-term memory is generated by the survival of a small subset of long-lived antigen-specific memory CD8+ T cells. The memory CD8+ T cell pool generated in response to antigen recognition consists of several subsets. Central memory CD8+ T cells (T_{CM}) are hardly found in the circulation as these home to secondary lymphoid organs whereas effector memory CD8+ T cells (T_{EM}) cells are more abundant in the circulation and often migrate to the (inflamed) tissues (39, 40). Nowadays it is clear that tissue residing memory CD8+ T cells (T_{RM}) have a role in instant protection of the host tissues to both internal and external imminent threats. Indeed, CD8+ T_{RM} cells are thought to reside in the tissues permanently and do not seem to circulate. Antigen recognition by T_{RM} cells also promotes the recruitment and activation of other innate and

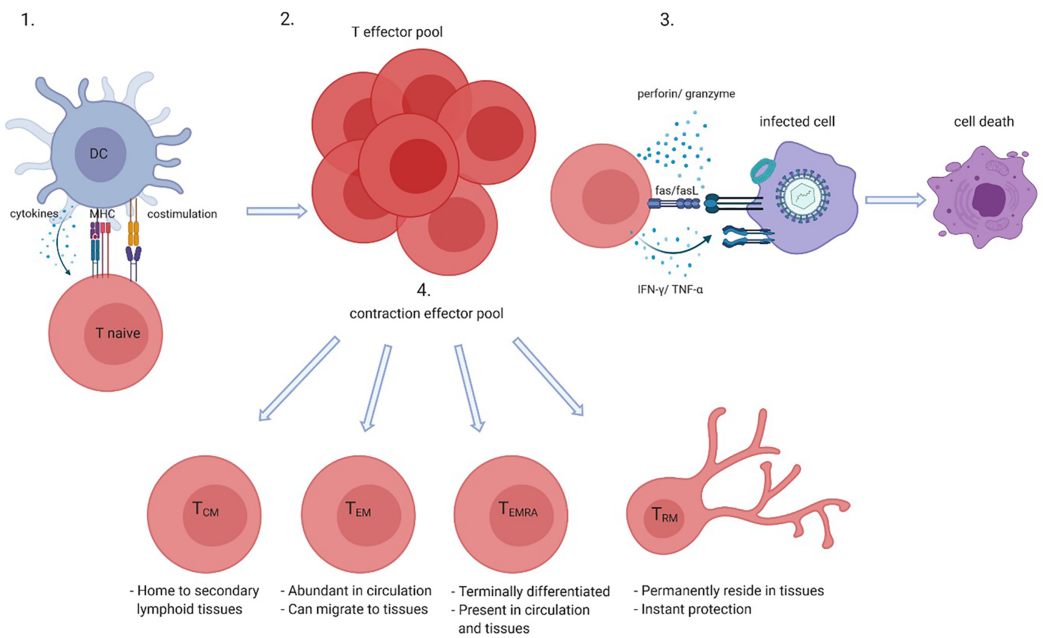

FIGURE 1 | CD8+ T-cell activation and differentiation. Naive CD8+ T cells when presented with their cognate antigen by antigen presenting cells such as dendritic cells (DCs) in the context of co-stimulation (CD28-CD80/86) and in the presence of cytokines become activated (1) which gives rise to an antigen-specific CD8+ effector pool (2). Effector CD8+ T cells kill infected, tumor or damaged cells by perforin and granzyme, Fas and Fas ligand (FasL) interactions and directly or indirectly via cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)- α (3). After antigen clearance most effector cells go into apoptosis which leads to contraction of the effector pool, but long-lived memory cells such as central memory (T_{CM}), effector memory (T_{EM}), memory cells that re-express CD45RA T_{EMRA}, and tissue residing memory (T_{RM}) cells survive (4). Created with BioRender.com.

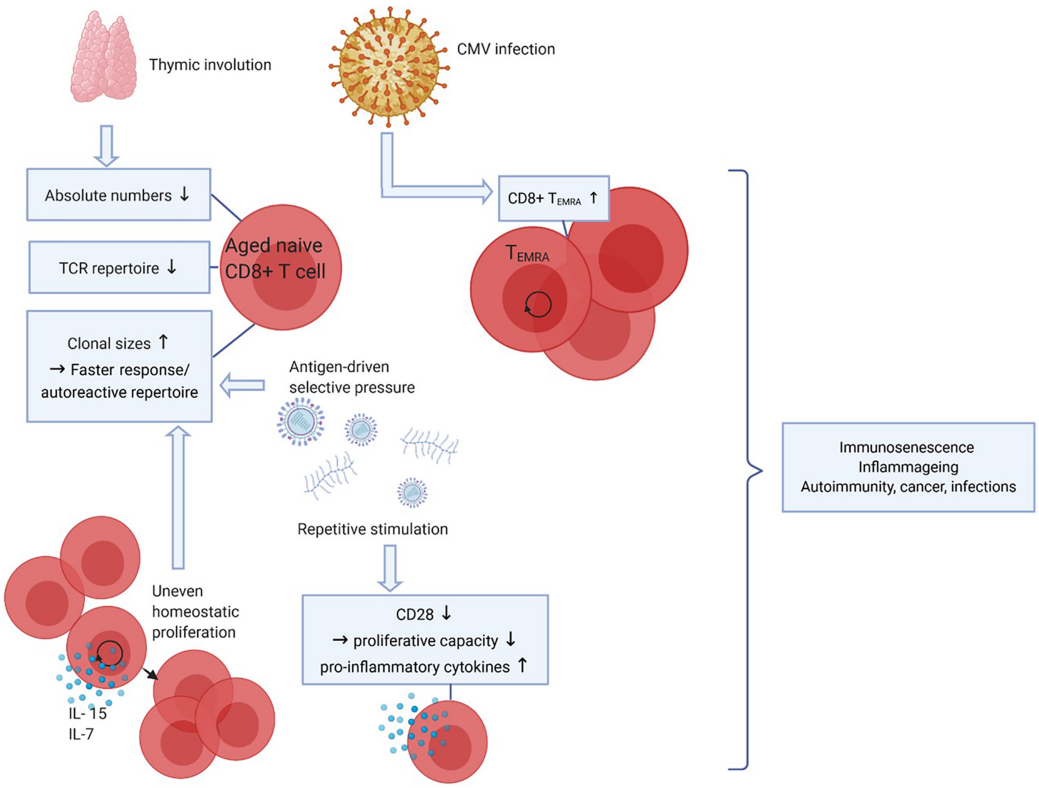

FIGURE 2 | Effects of aging on CD8+ T cells. Upon aging, the thymus size decreases which leads to a reduced output of naive CD8+ T cells, thereby affecting the diversity of the T-cell receptor (TCR) repertoire. Also, due to antigen-driven selective pressure and uneven homeostatic proliferation naive clonal sizes increase, which leads to a faster response to certain antigens but may also favor an autoreactive repertoire. Repetitive stimulation by (e.g. latent viral) antigens decreases CD28 expression, CD8+ T cells lacking CD28 have decreased proliferative capacity but produce more pro-inflammatory cytokines. CMV infection leads to increasing CD8+ T_{EMRA} cell numbers. Together, these phenomena lead to decreased immune cell functions (immunosenescence), low-grade inflammation (inflammaging) and a higher risk to develop autoimmunity, cancer and infections. Created with BioRender.com.

adaptive immune cells to the tissue. Another memory CD8+ T-cell subset detected in both the circulation and the (inflamed) tissues is the T_{EMRA} subset. T_{EMRA} cells are late-stage memory CD8+ T cells that re-express CD45RA and are likely terminally differentiated (40). The latter subset is characteristic of aging and latent CMV infection.

CD8+ T CELLS, AGING, CMV AND INFLAMMAGING

Upon aging, many changes in the immune system occur, but the decline of naive CD8+ T-cell numbers is the most profound hallmark of aging (**Figure 2**). The thymus involutes early in life around puberty, and T-cell homeostatic proliferation of naive T cells is necessary to maintain the pool of naive T cells. This process causes absolute numbers of naive CD4+ T cells to remain stable in elderly but naive CD8+ T-cell numbers still profoundly decrease upon aging (41–43). In both CD4+ and CD8+ naive T cells the size of the TCR repertoire declines between the ages of 30 and 70 years. Although this may limit the size of the TCR repertoire, the TCR repertoire still remains very large and the decrease in diversity in elderly is predicted to unlikely have functional consequences (44, 45).

Aging is often associated with T-cell clonal expansions especially in the naive repertoire. Clonal sizes increase due to antigen-driven selective pressures or by uneven homeostatic proliferation in response to homeostatic cytokines. The latter seems to be the cause of increased clonal sizes within both the CD4+ and the CD8+ naive pools, as the clonally expanded naive CD8 T cells had different TCR sequences compared to memory CD8+ T cells. Increases in clonal sizes within the naive T-cell pool upon aging might be beneficial for the host as expanded clones may result in a faster response to antigenic challenges (44, 45). Conversely, this may also generate an auto-reactive repertoire and contribute to development of autoimmune diseases with aging.

Latent infections with viruses such as varicella zoster virus, Epstein-barr virus (EBV) and CMV are well known to have an impact on the T-cell composition in the blood. This is likely caused by continuous stimulation of the immune system. As CMV is a relatively large virus expressing many proteins, the

CD8+ T-cell immune repertoire controlling the virus is quite broad. CMV seropositivity is well known to increase with age and 50-85% of adults of 40 years and older are seropositive (46). As a substantial repertoire of CD8+ T cells needs to control this infection the development and expansion of highly differentiated CD8+ T_{EMRA} cells have been documented (42, 47). As a result CD8+ T_{EMRA} cells accumulate with age, a progress called memory inflation (48, 49). This process is kept in balance to some extent because CD8+ T_{EMRA} cells have a lower proliferative capacity. At the same time, CD8+ T_{EMRA} cells are highly cytotoxic and can produce many cytokines, which is beneficial in combatting infections, and thus may compensate for their lower proliferative potential (45).

Another important marker of T-cell aging and of highly differentiated cells such as CD8+ T_{EMRA} cells is the lack of CD28 expression. CD28 is the most important co-stimulatory receptor expressed by T cells as it interacts with CD80/86 on professional antigen presenting cells. Repetitive stimulation of CD8+ T cells, however, leads to downregulation of CD28 (50). CD8+CD28- cells are highly differentiated, produce pro-inflammatory factors and proliferate poorly to TCR stimulation but are still able to proliferate in response to cytokines like interleukin (IL)-15 (51). As senescent cells are unable to proliferate in response to any signal, CD8+CD28- cells are not considered truly senescent but instead described as "senescent-like". The higher proportion of CD8+CD28- cells in aged adults has been linked to decreased responses to infectious agents and vaccines (52).

Clonally expanded naive CD8+ T cells and accumulation of highly differentiated CD8+ T cells with high cytotoxic capacity are normal phenomena that are related to aging. However, these phenomena alongside decreased function of other immune cells such as B cells can lead to decreased immune clearance and lower ability to maintain self-tolerance. Immunosenescence is a general term used to describe the waning of immune functions with aging. Immunosenescence may lead to increased occurrence of infections, cancer and autoimmunity. Furthermore, high cytokine production, for instance by differentiated CD8+ T cells, can lead to a state of low-grade inflammation in the elderly, also known as inflammaging (53). Immunosenescence and inflammaging have been associated with several autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (54, 55).

PATHOGENESIS OF GCA

GCA is a granulomatous vasculitis that can affect the aorta and its proximal branches, including cranial vessels. Symptoms include severe headaches, scalp tenderness or necrosis, visual disturbances or even visual loss (56). Most GCA patients have a relapsing disease course. Studies indicated that human leukocyte antigen (HLA) class II genes, in particular *HLA-DRB1*04*, are associated with disease susceptibility, visual loss and glucocorticoid resistance (57–59), HLA class I genes have been associated with genetic susceptibility as well (57, 60).

It is unclear what causes the disease although several infectious agents have been proposed to be involved in triggering the immune response that results in severe inflammation of the vessel wall. However, an unknown endogenous factor (e.g. danger associated molecular pattern (DAMP)) should not be excluded and may function as a trigger for initiation of the disease in a susceptible host. Activation of tissue-residing dendritic cells (DCs) bearing toll-like receptors (TLRs) in the adventitia of the vessel wall by such an unknown trigger may start the cascade of disease-specific inflammatory processes.

The proposed sequences of events thus start with activated DCs producing cytokines and chemokines which attract additional DCs and activate T cells. Especially T helper (Th)-1 and Th-17 cells that produce IFN-γ and IL-17, respectively, seem to be involved in the perpetuation of the inflammatory response in GCA. These cells and their cytokines contribute to both local and systemic inflammation by instigating pleotropic effects on various immune cells. IFN-γ induces the production of chemokines by vascular smooth muscle cells (VSMCs). Monocytes and macrophages migrate to the vessel wall in response to these chemokines. In addition, IFN-γ activates macrophages, which are crucial in the destruction and remodeling of the vessel wall and may fuse to form multinucleated giant cells, which is one of the pathological hallmarks of GCA. IL-17 has pro-inflammatory effects on macrophages, neutrophils, endothelial cells and fibroblasts (61).

Following the activation of the adaptive arm of the immune system, an amplification of the local immune response takes place, causing vascular wall remodeling and disruption including neoangiogenesis fueling the inflammation and facilitating further immune cell recruitment to the vascular wall. Vessel-infiltrated macrophages appear to be primarily responsible for these effects, since they produce chemokines, damaging reactive oxygen species (ROS) and matrix metalloproteinases. Furthermore, macrophages produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-7 and IL-33 of which local production may translate into systemic effects. Giant cells and macrophages produce platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) which can activate VSMCs (62–64). Activated VSMCS differentiate into myofibroblasts after migrating to the intima of the vascular wall. This results in intimal hyperplasia and luminal occlusion (62). The different phases in the pathogenesis of GCA have been nicely illustrated previously in a review paper by Samson et al. (61).

In the 1980's and 90's many studies were directed toward elucidating if circulating CD8+ T cells could act as a possible biomarker for GCA disease activity. However, during that period many studies did not differentiate between GCA and polymyalgia rheumatica (PMR), a rheumatic disease that often overlaps with GCA. Nevertheless, some studies found that CD8+ T cells were decreased in these patients (65–67), but it is highly likely that these studies were confounded by glucocorticoid treatment (68–72). It was not until recently that the possible role of CD8+ T cells gained more interest (34), as CD8+ T cells were detected at the site of inflammation in GCA-affected

lesions. To which extent CD8+ T cells contribute to disease pathogenesis will be addressed in this review (**Supplementary Table 1**).

PHENOTYPE OF CD8+ T CELLS IN CIRCULATION AND AFFECTED TISSUES OF GCA PATIENTS

As described previously, infectious triggers have been associated with the onset of GCA pathogenesis, but endogenous triggers could be involved as well. To assess whether a specific (viral or self) antigen causes activation and differentiation of CD8+ T cells in GCA, several studies assessed the clonality and TCR repertoire of these cells. Whereas one study found that circulating CD8+ T cells were clonally expanded in GCA patients (34), other studies found no differences in clonal expansions between HCs and GCA/PMR patients (73, 74). Clearly, these data are not conclusive on the involvement of cognate antigens in the development of GCA. However, one of the latter studies reported that even though no differences in clonal expansions were found between patients and controls, the TCR repertoire with regard to Jβ gene segments itself seemed to differ (74). In addition to this, another study found that the expression of TCR Vα and Vβ genes were different between T cells in peripheral blood and vascular tissues. The authors state that this difference may be due to local expansion of T cells caused by either recruitment of specific T cells or local proliferation of these T cells. In this study, no distinction could be made between TCR genes of CD4+ and CD8+ T cells in vascular tissues (75). Most importantly, patients and controls in these studies investigating TCR diversity were not controlled for CMV status or matched for HLA polymorphisms, factors associated with TCR diversity (76). Thus, it remains to be identified whether CD8+ T cells are clonally expanded by a response toward shared self or foreign antigens, such as derived from infectious agents. Knowledge on CD8+ T-cell clonality and TCR repertoire can aid our understanding of selective CD8+ T-cell expansion during the early phases of the disease or may inform on risk factors for development of the disease.

In a recent study, Samson and co-workers performed a detailed analysis of the phenotype of circulating CD8+ T cells in GCA (34). In this study, higher percentages of circulating cytotoxic CD8+ T cells, defined by perforin and granzyme B expression, and higher systemic levels of soluble granzyme A and B were observed. Furthermore, Tc-17 cell frequencies were found increased in GCA patients as well. Since GCA patients had higher frequencies of CXCR3+ CD8+ T cells and higher systemic levels of the ligands CXCL9, -10 and -11, Samson et al. hypothesized that CXCR3-expressing CD8+ T cells migrate to the tissue in response to those chemokines, and subsequently become activated by an unknown trigger.

Several studies identified CD8+ T cells at the site of tissue inflammation in GCA by immunohistochemistry on temporal artery biopsies (TAB). TAB tissues are often taken for diagnostic purposes, and provide an important source of information to unravel the disease pathogenesis of GCA. Three vessel wall layers can be distinguished in a TAB; from outside in: the adventitia, the media and the intima. CD8+ T cells are present in TABs, especially in the adventitia and media layers, but are less abundant than CD4+ T cells (69). Schaufelberger et al. reported that CD8+ T cells comprised 12%-46% of total T cells in TABs (75).

As mentioned above, Samson et al. hypothesized that CXCR3-expressing CD8+ T cells migrate to the tissue in response to CXCL9, -10 and -11. CXCR3+ CD8+ T cells were indeed found in TAB tissue, as well as the CXCR3 ligands CXCL9 and CXCL10. These findings led the authors to propose an adaptation of the hypothetical pathogenic model of GCA, one where CD8+ T cells have a role in aggravating the local immune response. IFN-γ produced by Th1 cells can trigger the release of, among others, CXCL9, -10 and -11 by VSMCs. This in turn can lead to recruitment of CXCR3-expressing cells, such as CXCR3+CD8+ T cells. After activation by an unknown trigger, CD8+ T cells start to produce cytokines such as IL-17 and IFN-γ. IFN-γ production by CD8+ T cells triggers additional release of chemokines, creating a feed-forward loop of additional recruited and activated CD4+ and CD8+ T cells (34). Interestingly, in this study it was also found that strong CD8+ T-cell infiltration in TABs was associated with a more severe disease course and associated with more visual disturbances. This finding, however, has not yet been confirmed by other studies in an independent cohort of patients.

Taken together, although it remains to be elucidated which factors activate CD8+ T cells in the vascular wall, it is clear that CD8+ T cells are present in GCA inflamed tissue. Studies on chemokine expression suggest involvement of the CXCL9/10/11-CXCR3 axis. Although granzyme B and perforin- expressing CD8+ T cells are elevated in blood of GCA patients, it remains to be elucidated whether these cells contribute to vasculitis development.

EFFECT OF AGE ON CD8+ T-CELL PHENOTYPE IN CIRCULATION AND AFFECTED TISSUES OF GCA PATIENTS

Several studies looked into the aging-related phenotype of CD8+ T cells in GCA. Aging is strongly associated with an increase of CD8+CD28- and to a lesser extent also CD4+CD28- (41, 77). In addition, T cells lacking the co-stimulatory receptor CD28, often upregulate other co-stimulatory receptors, such as natural killer group 2 member D (NKG2D). Studies on CD8+CD28- frequencies in GCA are inconsistent. Whereas one study described similar absolute and relative values of CD8+CD28- cells in GCA/PMR patients compared to HCs (73), another study reported higher frequencies of CD3+CD8+CD28- cells in GCA/PMR patients (78). However, the first study included treatment-naive patients only whereas the second study reported on patients of whom almost all were on glucocorticoid treatment. Furthermore, both studies did not report the CMV status of their study cohort, even though CMV status is well-known to increase the numbers and percentages of CD8+CD28- significantly. Also

the finding that the frequency of circulating NKG2D-expressing CD8+CD28+ T cells was higher in GCA/PMR patients than HCs (78) could be confounded by glucocorticoid treatment. Indeed, the authors also report that patients on high-dose glucocorticoid treatment had higher NKG2D expression on CD8+CD28+ T cells than patients on low-dose glucocorticoids.

In TAB tissues of GCA patients, both NKG2D-expressing T cells and expression of one of its ligands MHC class I polypeptide-related sequence A (MICA) were detected. MICA was present on endothelial cells in the intima and on endothelial cells surrounding the vasa vasorum. Furthermore, other cells in the intima and adventitia showed moderate MICA expression whereas VSMCs in the media showed strong expression. Lymphocytes and giant cells were MICA positive as well. Although the authors did not formally proof expression of NKG2D by CD4+ or CD8+ T cells in TABs, staining of consecutive sections suggested that NKG2D was predominantly expressed by CD4+CD28- T cells, because the majority of T cells in TABS were CD4+ and CD28- (78). However, double-staining of NKG2D and CD8 and/or NKG2D and CD3/CD4 should be performed to confirm this finding, as NKG2D is generally mostly expressed by CD8+ T cells and NK cells.

Together, these findings do not support the contention that CD8+ T cells of GCA patients have a more age-associated phenotype than HCs, for instance by upregulation of NKG2D to compensate for downregulation of CD28. Even though NKG2D-expressing T cells were found in the vascular wall, we cannot yet conclude whether these cells are CD4+ or CD8+. However, the finding that MICA was expressed throughout the whole tissue suggests that MICA could be one of the ligands that activates NKG2D+CD8+ T cells in GCA lesions. Importantly, as before, none of the studies described in this section controlled for CMV serostatus, which is crucial when investigating age-associated CD8+ phenotypes.

FAILING REGULATION: REGULATORY CD8+ T CELLS IN GCA AND AGING

Studies focusing on the functionality of CD8+ T cells in GCA are scarce. The only functional data available on CD8+ T cells is of a particular subset of CD8+ T cells, the CD8+ Tregs. Sufficient regulatory function of T cells is necessary to prevent the activation of autoreactive T cells. It has been postulated that CD8+ Tregs are essential in peripheral tissues such as secondary lymphoid organs (79). Circulating CD8+ FoxP3+ Tregs indeed co-express CCR7+, involved in lymphocyte homing to secondary lymphoid organs. CD8+ Tregs are thus present in secondary lymphoid organs, where they produce NADPH oxidase 2 (NOX2) in vesicles to suppress activation and expansion of CD4+ T cells. In aged individuals, the CD8+ Tregs demonstrated NOX2 deficiency which may underly the failing suppression of the immune response. This effect was even more pronounced in GCA patients (80). This was taken to suggest that CD8+ Tregs are important in the starting phase of the disease, because dysfunctional CD8+ Treg could cause unopposed CD4+ T-cell priming in secondary lymphoid organs. Unopposed T-cell priming could result in excessive inflammatory T-cell responses. Indeed, frequencies of CD8+CCR7+ Tregs expressing NOX2 were lowered in GCA, independent of glucocorticoid use, when compared to age matched controls (6% vs 23%). Interestingly, frequencies of NOX2+CD8 Tregs did not differ between patients with small-vessel vasculitis and age-matched controls: 46% of their CD8+CCR7+ Tregs expressed NOX2, against 40-50% in young healthy donors (80). Notably, the small vessel vasculitis patients had no antibodies against PR3 and MPO and were on glucocorticoid treatment.

Thus, it cannot be excluded that glucocorticoid treatment may have preserved CD8+CCR7+NOX2+ Treg frequencies. The suggestion that frequencies of CD8+ Tregs differ between different forms of vasculitis are interesting but, as this notion is based on a single report, these findings require independent confirmation.

In a follow-up study the molecular mechanisms underlying the aberrant function of CD8+ Tregs in GCA were studied. Here the authors used a mouse model in which vasculitis was induced in engrafted human arteries. Transfer of CD8+ Tregs from HCs prevented CD4+ T-cell expansion in the spleens of these mice and inhibited vessel wall invasion of CD3+ T cells. In contrast, transfer of CD8+ Tregs from GCA patients had no beneficial effects. This was caused by aberrant signaling through the NOTCH4 receptor leading to dysfunctional CD8+ Tregs in GCA (81).

Together these studies suggest a role for this rare CD8+ Treg subset in prevention of disease onset in GCA patients. Although this is an interesting notion it would require further substantiation and may await technological advances as the frequency of CD8+CCR7+ Tregs is very low even in HC.

FUNCTION OF CD8+ T CELLS IN GCA: INSIGHTS FROM TRANSCRIPTOME STUDIES

In GCA, a transcriptome study has been performed on CD4+ and CD8+ T cells of 16 GCA patients with the aim to identify gene expression profiles that could aid in confirming diagnosis and in defining predictive biomarkers. In this study, transcription profiles of CD4+ and CD8+ T cells were assessed at six timepoints in GCA patients, from acute phase to 12 months, and at two time points in HCs. In the acute phase (T1), 288 genes were differentially expressed by CD8+ T cells and 196 by CD4+ T cells compared to HCs. The authors hypothesized that gene expression profiles would normalize after 12 months, and that genes that remain differentially expressed compared to HCs may be of clinical interest. In CD8+ T cells, two genes were differentially expressed at 12 months compared to HCs. These genes were *SGTB* which is associated with neuronal apoptosis and *FCGR3A* which is associated with susceptibility to another large vessel vasculitis: Takayasu arteritis. The implications of these differentially expressed genes for the pathogenesis of GCA are still unclear.

However, the authors also correlated gene expression to disease symptoms and found that *IL32* was associated with a history of PMR, visual disturbance and raised neutrophils in the acute phase, and bilateral blindness and death within 12 months (82).

PATHOGENESIS OF GPA

GPA is also an aging-associated form of vasculitis as the typical age of onset is around 45 to 65 years of age. Notably, GPA not only affects adults, but also children – albeit rarer than adults. GPA affects especially the small- to medium-sized vessels. In GPA, the onset of the disease is most likely the result of a complex interplay between genetic background and environmental factors (4, 83, 84). In PR3-AAV, genome-wide association studies have revealed an association with HLA class II genes, in particular with *HLA-DPB1*04:01* (85, 86).

Before the onset of symptoms, central and peripheral T and B cell tolerance toward PR3 is lost which leads to the generation of autoreactive T and B cells and results in the production of PR3-ANCAs that are characteristic for this disease. Although there is some evidence that defective Treg function and lower numbers of Bregs may contribute to loss of tolerance toward PR3 in GPA, the immunopathogenesis of the disease is far from understood.

More knowledge exists on the effector phase of the disease in which ANCA-mediated activation of primed neutrophils causing blood vessel injury is considered to be a central event (4, 87).

One of the most severe disease manifestations of GPA is the development of necrotizing crescentic glomerulonephritis (NCGN). Besides GPA, NCGN is also a frequent manifestation in microscopic polyangiitis (MPA), another form of ANCA-associated vasculitis (AAV) characterized by an autoimmune response against myeloperoxidase (MPO). Here, we will mainly focus on GPA, but studies often include both GPA and MPA patients, especially those focusing on renal disease manifestations. NCGN is characterized by necrosis of the glomerular capillary loops, after which fibrin, red blood cells, lymphocytes and macrophages seep into the urinary space surrounded by Bowman's capsule. Subsequently, parietal epithelial cells start to proliferate which leads to glomerular crescent formation. Due to excessive inflammation, Bowman's capsule is destructed, and glomerulosclerosis may develop leading to renal function loss. Moreover, interstitial infiltrates surrounding the necrotic lesions of glomeruli as well as inflammation of small arteries in the tubulointerstitium are commonly observed as well (4).

Studies on renal biopsies of GPA patients have demonstrated the presence of CD8+ T cells in periglomerular areas, the majority of which were located adjacent to Bowman's capsule (88). Similarly, CD8+ T-cell infiltration has been documented in renal tissues of untreated MPO-ANCA positive MPA patients as part of the inflammatory infiltrate in the interstitium. Interestingly, in these studies interstitial CD8+ T-cell numbers, as well as those of CD4+ T cells and macrophages correlated inversely with renal function (89) an observation that has been corroborated by others (90). Collectively, these observations suggest that CD8 T cells are active contributors to disease pathogenesis in GPA. In the next sections, we will review the current knowledge on CD8+ T-cell phenotypes and function in GPA and discuss how these may link to disease development and progression (**Supplementary Table 2**).

PHENOTYPE OF CD8+ T CELLS IN CIRCULATION AND AFFECTED TISSUES OF GPA PATIENTS

In GPA several studies have interrogated the phenotype of CD8+ T cells in the circulation and in affected organs such as the kidneys and lungs. The frequencies of circulating CD8+ T-cell differentiation subsets did not differ between GPA patients and HCs (91). In lung biopsies of untreated newly-diagnosed GPA patients, CD4+CD45RO+ and to a lesser extent CD8+CD45RO+ cells were found (33). Interestingly, in renal biopsies it has been reported that two-thirds of the total T-cell infiltrates is comprised of CD8+ T cells, suggesting differences in infiltrating CD4/CD8 ratios between affected tissues in GPA (88).

To assess whether CD8+ T cells are active contributors to disease pathogenesis, cytokine production and expression of activation markers as well as mechanisms of cell migration have been studied. Circulating CD8+ T cells in GPA patients were found to produce more IFN-γ compared to those from HCs (92). Moreover, in lung tissues of GPA patients increased IFN-γ gene expression has been reported compared to disease control tissue although in this study it was not established whether CD4+ or CD8+ T cells are the main producers of IFN-γ in GPA-affected tissues (33).

In 2008, Iking-Konert and colleagues provided evidence for the activation of CD8+ T cells during active disease indicated by the presence of CD11b-expressing CD8+ T cells in GPA and MPA patients. CD11b was exclusively expressed by CD8+CD28- cells in patients in remission, whereas in active disease a population of CD11b+CD28+ within the CD8+ T cell population appeared that was less prevalent in healthy donors (mean 8.9% versus 1.2% in HCs). Expression of CD11b, the α-chain of the β2 integrin Mac-1, is upregulated upon activation of T cells. Yet, whereas CD11b expression persisted on activated T cells, these cells were found to show progressive loss of CD28 expression. Therefore, the authors concluded that the CD8+ CD28+CD11b+ cells must be a transient phenotype of activated T cells (93). However, as absolute numbers were not reported, it cannot be concluded that there is an actual shift of CD11b+ CD28+CD8+ toward CD11b+CD28-CD8+ T cells in GPA/ MPA patients.

Besides activation markers, additional studies have demonstrated that circulating CD8+CD45RO+ T cells in GPA display increased expression levels of the chemokine receptors CCR3 and CCR5 on CD8+CD45RO+ cells suggesting their readiness to respond to chemotactic gradients (94).

Furthermore, the expression of XCL1, a chemokine specifically targeting lymphocytes, was found to be increased in circulating CD4+ and CD8+ T cells in GPA (95) as well as in the

renal interstitium of affected kidneys. In these renal tissues, XCL1 was co-expressed by CD4+ and CD8+ T cells (95). As XCL1 is a strong attractor for T cells, XCL1 expression might induce more interstitial T-cell infiltration.

In short, in GPA circulating CD8+ T cells appear to have an activated phenotype defined by CD11b expression, but it remains unclear if these circulating cells infiltrate the tissues despite the fact that CD8+ T cells can readily be detected in renal and lung biopsies. Further studies should investigate whether CD8+ or CD4+ T cells are the major producers of IFN-γ in GPA affected tissues, as this could aid in unraveling their contribution to disease pathogenesis. In addition, XCL1 expression could act as an amplifier of CD4+ and CD8+ T-cell migration to the renal tissues.

EFFECT OF AGE ON CD8+ T-CELL PHENOTYPE IN CIRCULATION AND AFFECTED TISSUES OF GPA PATIENTS

Given that GPA is predominantly a disease of the elderly, there has been an increased interest in studying the impact of immune aging on disease pathogenesis. As described previously, immune aging is associated with a decrease in CD28 expression by CD8+ T cells especially. A number of studies have now confirmed increased frequencies of CD28- T cells in GPA, particularly within the CD8+ T-cell compartment (96–99).

Loss of CD28 expression is associated with a poor response to TCR stimulation, and has therefore been associated with senescence. Consequently, the telomere length of circulating T cells in GPA patients has been assessed as well. T cells of GPA patients demonstrated indeed shorter telomere lengths than age-matched HCs. However, lack of CD28 and shorter telomere length was especially observed in GPA patients with long lasting disease suggesting recurring activation of the same T cells. Also, since these studies were performed on total T cells, it remains unclear whether shorter telomere lengths are characteristic of either CD4+ and CD8+ T cells lacking CD28, or both (96). Compared to disease controls, increased proportions of CD28 negative cells have also been detected in bronchoalveolar lavage (BAL) fluid and in biopsies from the upper respiratory tract of GPA patients. Again, however, it is unclear whether the CD28- cells in these biopsies were CD4+ or CD8+ T cells (99).

As previously described, the co-stimulatory receptor NKG2D has been implicated in the disease pathogenesis of several forms of vasculitis including KD, TA and GCA. As CD8+ T cells lacking CD28 often upregulate NK-like co-stimulatory receptors, NKG2D and its ligand MICA have been considered important markers in age-associated vasculitides such as GPA. In one study investigating kidney biopsies of active untreated GPA patients, MICA expression was detected on peritubular and glomerular capillaries as well as on epithelial cells. In this study, CD8+ T cells and NKG2D-expressing cells were also found around tubular and glomerular capillaries although it was not determined whether the NKG2D-expressing cells were also CD8+ (100).

Late-stage differentiated cells such as CD8+ T_{EMRA} cells often express CD57. CD57 expression is increased upon aging and CD57+ cells can produce pro-inflammatory cytokines. In GPA and MPA patients younger than 40 years of age, the frequency of circulating CD8+CD57+ cells was found increased compared to age-matched healthy donors. Increases in CD8+CD57+ cells were associated with severe disease and multiple organ involvement (101). However, another study found no differences in percentages of CD28- and CD57+ cells in CD4+ and CD8+ T cells in GPA and MPA patients versus HCs (102).

When interpreting data on phenotypes of immune cells in general, and CD8+ T cells in particular, it is important to take CMV serostatus into account. Importantly, the studies described above did not correct for CMV serostatus, even though CMV infections generally lead to increased numbers and frequencies of CD4+CD28- and CD8+CD28- T cells and late stage differentiated cells (42, 47, 103, 104). Indeed, also in GPA, CMV serostatus has been associated with high frequencies of CD28- T cells and CD57+ T cells (102). Regarding CD28 expression, concomitant infections with CMV and EBV, as determined by the presence of antigen-specific memory T cells, have been associated with a loss of CD28 expression by circulating CD8+ and CD4+ T cells in GPA patients. Interestingly, cellular positivity for CMV or EBV only was not associated with this phenotype in GPA patients, nor was CMV and EBV negativity. However, no differences in frequencies of CMV or EBV antigen-specific cells were found within the total CD8+ T-cell and CD8+CD28- population in GPA patients and HCs. This suggests that CMV and EBV infections exert indirect effects on CD8+ T cells which causes the expansion of CD8+CD28- cells in GPA patients, such as through bystander activation and/or cytokine mediated expansion (97). Expansion of non-antigen-specific cells by inflammatory processes are especially pronounced during later stages of disease or infection, whereas initial immune responses are caused by antigen-specific cells (105).

Another study reported on lower CD28 expression on CMV-specific CD8+ T cells in GPA patients than in HCs (106). The CMV-specific CD8+ T cells were either CD28-CD27+ or late stage memory cells defined by loss of CD28-CD27- expression. However, frequencies of CD28- cells were also lower in the non CMV-specific CD8+ T-cell repertoire of GPA patients. The authors suggested that higher CD28- frequencies in GPA patients could be a result of the disease itself rather than CMV status. However, as mentioned before, CMV and EBV infection could have an indirect effect on the expansion of CD8+CD28- cells in GPA as well.

In summary, it remains unclear whether markers of aging are more frequently expressed in GPA patients, as CMV and EBV status may modulate the expression of these markers, confounding data interpretation and comparison, especially with regard to CD28 expression. Also, methodological differences between studies such as the use of fresh whole blood versus freshly isolated or cryopreserved PBMCs may have influenced the reported CD28 expression data (107). Since NKG2D has been implicated in several forms of vasculitis, additional studies into the spatial temporal expression and functional role of this receptor in the inflammatory response in GPA are warranted.

FUNCTION OF CD8+ T CELLS IN GPA: INSIGHTS FROM *IN VITRO* STUDIES

To better understand if and how CD8+ T cells contribute to GPA pathogenesis, studies investigating their functionality are imperative. However, data on the function of CD8+ T cells in GPA is limited. As mentioned earlier, CD8+CD28+CD11b+ cells were found to be increased in the circulation of active GPA patients. These cells are also capable of producing IFN-γ *in vitro*. Co-cultures with polymorphonuclear neutrophils (PMN) showed that IFN-γ-producing CD8+C11b+ T cells can activate PMN from GPA patients to express MHC class II (93). Previously, it was found that PMN of GPA patients acquire characteristics of antigen presenting cells by expressing MHC class II, a phenotype not present in HCs (108). As described earlier as well, circulating CD8+ T cells expressing the chemokine XCL1 were elevated in GPA patients and XCL1 was also expressed in the renal interstitium. *In vitro* stimulation of PMN with XCL1 led to increased production of the pro-inflammatory cytokine IL-8 (95). Collectively, these studies imply that CD8+CD28+CD11b+ and CD8+CXCL1+ cells could exert pro-inflammatory effects on PMN. However, to which extent these processes contribute to the disease pathogenesis in GPA is currently unknown and requires further study.

FUNCTION OF CD8+ T CELLS IN GPA: INSIGHTS FROM TRANSCRIPTOME STUDIES

Two transcriptome studies from the same group provide evidence that CD8+ T cells could play a role in GPA/AAV based on the transcriptional profile of these cells during active disease. These studies were originally designed to understand the molecular basis of the considerable variation that exists between autoimmune patients regarding clinical course and outcome of their disease. Furthermore, these transcriptome studies aimed to discover biomarkers that could aid in developing strategies for personalized medicine. In the first study, microarray analysis of purified total CD8+ T cells from patients with active disease revealed that two distinct CD8+ expression signatures may serve to predict the clinical course of AAV and other autoimmune diseases such as SLE. Patients with a relapsing course of their diseases and a poor prognosis were found to have a CD8 transcriptional profile enriched in genes involved in the IL-7R and TCR pathway and effector memory cells. Further analysis showed that these patients also had an expanded memory CD8+ T-cell population. Based on these results, the authors postulated that via enhanced IL-7R and TCR signaling, CD8+ T-cell proliferation in response to antigens increases leading to an expanded memory population. In autoimmune diseases, autoreactive cells with enhanced IL-7R and TCR signaling pathways expand more easily upon restimulation, which leads to more effector cells thereby promoting tissue damage (109). In a subsequent study, McKinney and colleagues demonstrated that a transcriptome profile of CD8+ T cells resembling an exhausted signature correlates with good outcome in autoimmune diseases such as AAV (110). Based on these results, the authors suggested that targeted induction of T-cell exhaustion could be a novel treatment strategy for autoimmune diseases.

FUNCTION OF CD8+ T CELLS IN GPA: INSIGHTS FROM ANIMAL MODELS

Unlike GCA, animal models have been developed for MPO-ANCA-associated vasculitis that have been instrumental in dissecting the various effector mechanisms involved in disease development. In most of these models the kidney is the main organ affected mimicking human focal necrotizing crescentic glomerulonephritis (FNGN).

In one such model, autoimmunity to MPO is induced in mice by immunization with human MPO which results in a humoral (MPO-ANCA) as well as a cellular immune response to autologous mouse MPO. In this model, an additional challenge with heterologous anti-mouse glomerular basement membrane antibodies recruits neutrophils to glomeruli causing local deposition of MPO in glomerular capillaries where it can be recognized by effector T cells triggering glomerulonephritis (GN) development. Initial studies in this model demonstrated an important role for CD4+ effector T cells as CD4+ T-cell depletion in the effector phase markedly attenuated GN development (111). More recently, this model has been employed to study the role of CD8+ T cells in the development of tissue injury as well. Systemic depletion of CD8+ T cells in the effector phase reduced GN development accompanied by diminished renal production of IFN-γ and TNF-α and less glomerular macrophages. In the same study, the authors generated MPO-specific CD8+ T-cells clones which upon transfer mediated glomerular injury when MPO was deposited in glomerular capillaries (112). Collectively, these studies support a pathogenic role for antigen-specific CD8+ T cells in AAV pathogenesis

In an attempt to assess the role of CD8+ T cells in glomerular crescent formation more directly, Chen and colleagues recently generated mice that express the model antigen EGFP on podocytes. Upon transfer of EGFP-specific CD8+ T cells these mice developed crescentic glomerulonephritis but only when injury to the glomerular filtration barrier was induced concomitantly by injection of a nephrotoxic serum. These observations imply that the nephrotoxic serum disrupts the physical barrier that would otherwise prevent recognition of EGFP by CD8+ T cells. From these data a model emerges in which antigen-specific CD8+ T cells can infiltrate the urinary space through Bowman's capsule when it is breached. In turn, these CD8+ T cells may interact with podocytes bearing their cognate antigen, which accelerates kidney injury and further stimulates the formation of crescents (113).

However, whether these observations can be translated to the situation in humans is as yet unclear. Theoretically,

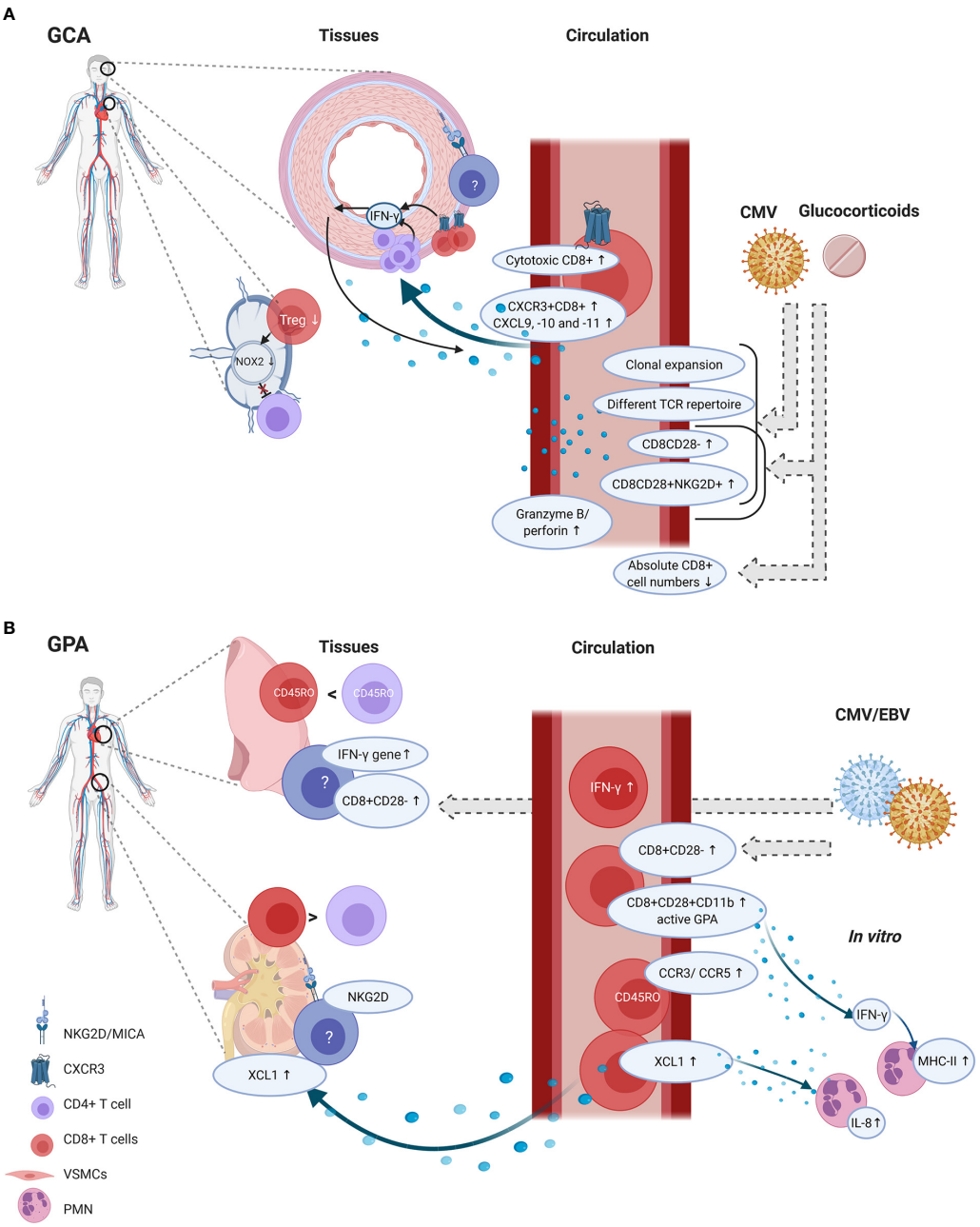

FIGURE 3 | CD8+ T-cell functions and phenotype in tissue and circulation of GCA and GPA patients. GCA **(A)** In disease onset, CD8+ Tregs residing in lymph nodes could be involved, as their function decreases which could lead to unopposed CD4+ T-cell activation. In the circulation of GCA patients several phenotypic changes are present in CD8+ T cells. The percentages of cytotoxic T cells are increased alongside elevated serum levels of granzyme B and perforin. In vascular tissues, the hypothesis is that first CD4+ T cells produce IFN-γ. IFN-γ production leads to CXCL9, -10 and -11 production by vascular smooth muscle cells (VSMCs). This attracts CXCR3+CD8+ T cells which contribute to the feed forward loop by producing IFN-γ. Some studies reported on age-associated changes in CD8+ T cells, such as increased clonal expansions, a different T-cell receptor repertoire (TCR), increased CD8CD28- and CD8CD28+NKG2D+ frequencies and decreased absolute numbers CD8+ T cells. But these findings could be confounded by glucocorticoid use and/or CMV infection. GPA **(B)** CD8+ T cells are present in lung biopsies but their frequency with regard to CD4+ T cells is higher in renal tissue. In renal tissues, NKG2D expression has been found but it is unclear which cells express this marker. Furthermore, MICA expression was found. CD8+ T cells could migrate to the tissues by XCL1, and XCL1 also induces IL-8 production by polymorphonuclear neutrophils (PMN). In the circulation, also CCR3 and CCR5 was upregulated on memory CD8+ T cells, but it remains unclear how this relates to tissue migration. CD8+ T cells in the circulation show an activated phenotype as CD11b was elevated, and these cells are able to produce IFN-γ. IFN-γ levels were also elevated in the circulation and the IFN-γ gene was elevated in lung biopsies. From in vitro studies it is known that IFN-γ can induce PMN to express MHC-II molecules. Although CD8+CD28- frequencies seem increased in tissues and circulation of GPA patients, it is likely that these findings are confounded by CMV and/or EBV infections. Created with BioRender.com.

podocytes could present ANCA antigens as at least MPO has been detected in and around podocytes in renal biopsies of AAV patients (89). Moreover, focal endocapillary inflammation is commonly observed in early lesions in GPA as well. This suggests that an initial inflammatory response in the glomerular capillaries may lead to leakage of ANCA antigen-specific CD8+ T cells through Bowman's capsule where they may interact with the PR3 or MPO bearing podocytes and accelerate crescent formation (114).

DISCUSSION

Are CD8+ T Cells Active Contributors to Disease Pathogenesis or Just Bystanders?

In GCA, several findings suggest that CD8+ T cells are active contributors to disease pathogenesis (**Figure 3A**). First and foremost, CD8+ T cells are clearly present in TAB tissues and one study suggested that strong CD8+ T-cell infiltration in TABs might be associated with a more severe disease course and also with more visual disturbances. Secondly, *in vitro* data showed the importance of CD8+ Tregs in inhibiting CD4+ T-cell activation. As GCA patients demonstrated impaired functioning of CD8+ Tregs, these cells could render aged adults more susceptible to disease development. Lastly, possible migratory mechanisms involving CXCR3+CD8+ T cells have been proposed. However, none of these findings conclusively confirm a pathogenic role of CD8+ T cells in GCA pathogenesis.

CD8+ T cells appear to be active contributors to GPA pathogenesis, especially in the development of glomerulonephritis (**Figure 3B**). Several observations support this contention. Firstly, CD8+ T cells are present in renal biopsies, adjacent to Bowman's capsule. Secondly, CD8+ T cells seem to outnumber CD4+ T cells in renal biopsies. Thirdly, CD8+ T cells correlate with decreased renal function. Circulating CD8+ T cells bear a more activated phenotype and possible mechanisms involving direct effects on PMNs have been described. Finally, data from mouse models suggest that CD8+ T cells can interact with antigen-bearing podocytes in glomeruli, which accelerates kidney injury. However, the evidence that immune cells can gain access through Bowman's capsule is still correlative, as it was only found that CD8+ T cells were more commonly present within the glomeruli when Bowman's capsule was ruptured (113). No direct proof exists that CD8+ T cells breach Bowman's capsule. Furthermore, it remains uncertain whether podocytes cross-present neo-epitopes or circulating antigens to CD8+ T cells (115).

Both GCA and GPA susceptibility have been associated with HLA class II genes. In GCA, associations with HLA-B, a class I molecule, have been reported as well. However, associations with HLA class I genes in GCA do not appear to be as strong as in TA. How HLA class I and/or II genes contribute to disease susceptibility in GCA and GPA is still unclear. At least in GPA, associations with HLA class II genes could also just reflect the role of the HLA molecules in peptide presentation, such as PR3 (4).

To What Extent Does Aging Affect the Function and Phenotype of CD8+ T Cells in GCA and GPA?

Although several studies have reported that typical aging-associated phenotypes of CD8+ T cells are more frequent in GCA and GPA patients than in controls, it cannot be excluded that data have been confounded by CMV and/or EBV infection, glucocorticoid treatment and methodological differences (**Figure 3**). Also, data on clonality and TCR diversity in GCA are likely confounded by CMV serostatus and/or HLA polymorphisms. In GPA, the effect of treatment on phenotype and function of CD8+ T cells is always difficult to assess, as most studies looked into a heterogeneous group of MPA and GPA patients with large age-ranges and in different disease states. Whereas in most studies in GCA the patients were newly diagnosed and not yet on treatment, in most GPA studies patients received immunomodulatory therapy. These limitations make it difficult to compare studies and draw firm conclusions.

In this review, we discussed NKG2D as a possible marker of aging, as T cells that downregulate CD28 expression often upregulate other co-stimulatory NK markers. However, NKG2D is not an exclusive marker of aging as it is also involved in other forms of vasculitis that affect younger adults and children, such as KD and TA. Although it is still unclear to what extent NKG2D and its ligand MICA contribute to disease pathogenesis in GCA and GPA, it is interesting that NKG2D and MICA have been implicated in these other forms of vasculitis. This underlines the possible importance of NKG2D and MICA and the vascular inflammatory environment and suggests that binding of CD8+ T cells to MICA by virtue of their NKG2D receptor activates these cells to become proinflammatory.

Future Outlook

To truly understand the role of CD8+ T cells in the pathogenesis of primary vasculitides, more integrated and in-depth analyses of CD8+ T cells in both GCA and GPA are required. As in both GCA and GPA several transcriptome studies have been performed, a comprehensive analysis of all differentially expressed genes could open up novel avenues for research on shared or distinct disease mechanisms and the exploration of new targets for disease monitoring and therapy. As an example, longitudinal profiling of GCA patients, revealed differential expression of the IL-32 gene in CD8+ T cells whereas elevated serum levels of IL-32 that correlated with disease activity have been reported in AAV (116). Thus, IL-32 could perhaps constitute an interesting lead for further study in relation to disease severity in various vasculitides. In GPA, an exhaustion profile of CD8+ T cells has been associated with favorable clinical outcome. More research into this exhausted profile would teach us more about the involvement of CD8+ T cells in disease pathogenesis and potentially uncover novel targets for therapy.

Although the standard therapies for GCA and GPA are not designed to directly target CD8+ T cells, studying the effects of these treatments on CD8+ T cell function could aid in unraveling their role in these diseases. As an example, one study in AAV found that whereas rituximab treatment did not affect CD4+ and

Treg frequencies, it was associated with reduced CD8+ T_{EMRA} frequencies and circulating chemokine and cytokine levels. Interestingly, co-cultures of CD8+ T cells and autologous B cells from AAV patients resulted in enhanced production of proinflammatory cytokines indicating a pathogenic crosstalk between B cells and CD8+ T cells (117).

Studies on CD8+ Tregs in GCA should be confirmed by other research groups possibly aided by advanced technologies such as single cell sequencing. If confirmed, revival of these non-functional CD8+ Tregs would certainly be of interest in prevention of aging-associated pathologies such as GCA.

In both GCA and GPA, more detailed interrogation of vasculitic tissues is required ideally employing state of the art technologies such as imaging mass cytometry. Single cell RNA sequencing of TAB tissue digests from microdissected lymphocyte infiltrates can also be performed to investigate CD8 + T-cell heterogeneity at the single cell level to obtain better insights into CD8+ T-cell function in the lesional environment. Furthermore, this would also help to investigate whether CD8+ T_{RM} cells are present and involved in disease pathogenesis.

Aberrant DNA methylation and microRNA expression in CD8+ T cells have been linked to autoimmune diseases such as multiple sclerosis, type 1 diabetes and SLE. Thus, for both GCA and GPA it would be interesting to investigate the epigenetic profile of CD8+ T cells as well, especially because emerging evidence suggests that microRNAs, histone modifications and DNA methylations can lead to dysfunctional CD8+ T cells (118).

Another area of research relevant to delineate the effects of CD8+ T cells on GCA and GPA includes the microbiome. It is well known that the microbiome has profound effects on the wellbeing of people, for instance by regulating immune homeostasis. Alterations of the microbiome, generally referred to as dysbiosis, have been linked to states of aberrant immune activation implicated in various chronic autoimmune diseases (119). Many studies on the effects of the microbiome on the immune response are focused on finding anti-tumor properties of certain bacterial strains. For instance, a recent study in a mouse model of inflammation-associated tumorigenesis found that gut microbiota can have direct effects on CD8+ T-cell responses (120). Furthermore, another study demonstrated that a combination of several bacterial strains isolated from healthy human feces promoted the development of IFN-γ-producing CD8+ T cells in mice and enhanced the efficacy of immune checkpoint blockade therapy in tumor models (121). However, these observations also suggest that dysbiosis of the gut microbiome may enhance auto-inflammatory effects, for instance by boosting IFN-γ production by CD8+ T cells. So far, data on the microbiome of the gut or other body niches in GCA and GPA is limited but this certainly warrants further investigation.

CONCLUSION

Taken together, in vasculitic diseases, CD8+ T cells may be active contributors to disease pathogenesis via their effector function, likely to enhance local inflammation and tissue damage, and/or via their failing regulatory function. Both aspects deserve further exploration employing novel technologies in concerted actions involving well-described patient cohorts.

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

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Glomerular Immune Deposition in MPO-ANCA Associated Glomerulonephritis Is Associated With Poor Renal Survival

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Background: Rapidly progressive glomerulonephritis caused by antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is typically characterized as pauci-immune glomerulonephritis. However, immune complex (IC) deposition in the glomerulus has been reported in a growing number of studies. Here, we assess the presence of glomerular immune deposits alongside renal outcome in myeloperoxidase (MPO)-ANCA associated glomerulonephritis (MPO-ANCA GN).

Methods: Clinical and histopathologic characteristics of 97 patients with MPO-ANCA GN classified by renal biopsy from January 2008 to December 2019 were extracted retrospectively from electronic medical records. The extent of immune deposits in the kidney (C3, C4, C1q, IgA, IgG, IgM) at diagnosis were analyzed by immunofluorescence (IF). Patients were followed up for a median period of 15 months. The response to treatment and outcomes of renal and histological lesion changes were also assessed.

Results: In our study, 41% (40/97) of patients showed positive IF (≥2+) for at least one of the six immunoglobulin or complement components tested. Patients with IC deposits showed higher levels of serum creatinine (p=0.025), lower platelet counts (p=0.009), lower serum complement C3 (sC3) (≤790 ml/L) (p=0.013) and serum IgG (p=0.018) than patients with pauci-immune (PI) deposition at diagnosis. End-stage renal disease was negatively associated with eGFR (HR 0.885, 95% CI 0.837 to 0.935, p<0.0001), platelet count (HR 0.996, 95% CI 0.992 to 1.000, p=0.046) and serum globulin (HR 0.905, 95% CI 0.854 to 0.959, p=0.001). Patients with lower sC3 levels showed a worse renal outcome than the patients with normal sC3 at diagnosis (p=0.003). Analysis of the components of the renal deposits found that patients with IgG deposits exhibited a poorer renal outcome compared to patients that were IgG negative (p=0.028). Moreover, Bowman's capsule rupture occurred less frequently in patients with IgM deposition

compared with IgM negative counterparts (p=0.028). Vascular lesions and granuloma-like lesions had been seen more frequently in cases with IgA deposition than those without IgA deposition (p=0.03 and 0.015, respectively).

Conclusion: In conclusion, patients with immune complex deposits in the kidney showed less platelet count, lower sC3 and sIgG levels, and higher serum creatinine levels. Patients with low sC3 at initial and with continued low sC3 during the treatment displayed a trend toward poorer kidney survival. Moreover, the IC group showed a worse renal outcome than the PI group, further enforcing the present strategy of introducing complement targeted therapies in AAV.

Keywords: immune deposits, pauci-immune, ANCA, MPO – myeloperoxidase, AAV (ANCA-associated vasculitis)

INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis (ANCA-GN) is typically characterized by no or little immune deposition in the glomerulus, which is defined as pauci-immune glomerulonephritis (GN) (1). However, glomerular immune-complex (IC) deposits were also reported in the renal biopsy of patients with ANCA-GN in some studies (2, 3). IC GN is characterized by granular deposits of polyclonal immunoglobulin (Ig) by immunofluorescence (IF) or immunohistochemistry (IHC), and complement is often co-deposited along with the Ig. Immune deposits were also found by electron microscopy in over half of the renal biopsies with ANCA-associated crescentic GN (2).

Analysis of the features of patients with ANCA-associated vasculitis (AAV) with immune complex deposition in the kidney showed more proteinuria, greater hypocomplementaemia, and greater glomerular hypercellularity (3, 4). Additionally, the type and location of the immune deposits often indicate the underlying etiology and display different histological lesions in ANCA-GN. Renal complement deposition and decreased serum complement levels observed in ANCA-GN patients indicated that ANCA-GN patients usually undergo complement activation (5, 6). Renal IgA deposition had also been reported in previous studies. Haas et al. and Chen et al. found that patients with necrotizing and/or crescentic GN with glomerular IgA deposits responded well to aggressive therapy (7, 8). Dudreuilh et al. reported that the appearance of IgG deposits in renal biopsy did not affect the renal outcome or probability of relapse (9).

Myeloperoxidase ANCA-associated vasculitis (MPO-AAV) often manifests as rapidly progressive GN (RPGN) and is much more common in China than PR3-AAV (10, 11). In this study, we focused on immune-complex deposits in renal biopsy specimens from MPO-AAV patients. Also, the clinical and histologic features and outcomes of patients were studied retrospectively.

METHODS

Patients

The patients diagnosed with primary MPO-AAV and received a renal biopsy in Xiangya Hospital from January 2008 to December 2019 were enrolled in this study. All patients fulfilled 2012 revised International Chapel Hill Consensus Conference nomenclature of vasculitides. The clinical data and the pathological information of the biopsy were reviewed. Patients excluded from the study included those with anti-GBM antibody nephritis, lupus nephritis, IgA nephropathy, secondary vasculitis, such as drugs, infections. A total of 97 biopsies meeting these criteria were identified. The detail for recruitment was displayed in **Supplemental Figure 1**. The study protocol was in accordance with the ethical standards of the Ethics Committee of Xiangya Hospital of Central South University (IRB approval number 200901008) and with the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

Clinical and Laboratory Findings

Patient age and gender were recorded. Vasculitis disease activity was evaluated by the Birmingham Vasculitis Activity Score (BVAS) (12). Laboratory data included the following: routine blood test, serum albumin, serum creatinine (Scr), erythrocyte sedimentation rate (ESR), 24 hours urine protein, C reactive protein (CRP), serum Complement 3 (sC3) and 4 (sC4), serum IgA (sIgA), serum IgG (sIgG) and serum IgM (sIgM) levels. All these parameters were collected at the approximate time of biopsy, and during follow-up. The estimated glomerular filtration rate (eGFR) was calculated according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (13).

Renal Biopsy

All study patients received a renal biopsy when first hospitalized with newly-diagnosed MPO-ANCA GN, as well as before the commencement of immunosuppressive therapy. All 97 renal biopsies were reviewed using light microscopy by two pathologists who were blinded to the patient clinical data. The classification of MPO-ANCA GN was based on the Berden classification (14). The proportion of cellular crescent and global glomerulosclerosis, histological characteristics, including fibrinoid necrosis, Bowman's capsule rupture, periglomerular inflammation, granuloma-like lesions, vascular lesions, thrombotic microangiopathy (TMA), and the scores of interstitial infiltrate and tubulointerstitial injury were all evaluated. Interstitial infiltrate and tubulointerstitial lesions

were described as: mild (score of 1) for < 25% involvement, moderate (score of 2) for 25-50% involvement and severe (score of 3) for > 50% involvement (15).

Immunofluorescence and Immunohistochemical

Glomerular immunofluorescence for IgA, IgM, IgG, C3, C1q, and C4 were scored by the intensity of immunostaining: negative (–), trace(±), mild (1+), moderate (2+), and strong (3+) (16). A biopsy with no or few immune deposits (pauci-immune) was defined as less than 2+ intensity of immunostaining (on an intensity scale 0, ±, 1+, 2+, 3+). The immune complex deposition was defined as a score of 2+ or higher in staining for any kind of immunoglobulin (Ig) and/or complement observed by immunofluorescence microscopy (2). Cases of positive for fibrin in immunofluorescence were calculated. Macrophages, neutrophils, and T cells infiltrated in renal tissue were assessed by probed with CD68, CD15, CD3 antibodies Macrophages, neutrophils, and T cells infiltrated in renal tissue were assessed by probed with CD68, CD15, CD3 antibodies in 51 cases of PI group and 39 cases of IC group, respectively. The frequencies of leukocytes cells were determined as the sum of expressing cells in all glomeruli divided by the number of glomerular cross-sections.

Treatment and Follow-Up

As described previously, all 97 patients received the standard induction therapy including glucocorticoids combined with cyclophosphamide (17). Treatment resistance was defined as having an increasing and/or unchanged disease activity in patients with acute AAV after 4 weeks of treatment with standard induction therapy, or a reduction of 50% in BVAS after 6 weeks of treatment, or a chronic, persistent disease defined as the presence of at least 1 major or 3 minor items on the BVAS list after 12 weeks of therapy (18). End-stage renal disease (ESRD) was defined as eGFR < 15 mL/min/1.73 m^2 and requiring permanent renal replacement therapy (RRT), or kidney transplantation. We assessed the response to the treatment and the development of ESRD during follow-up every 3 months. The patients were followed up until the death or the last follow-up date (June 30, 2020). All the patients were followed-up for a median period of 15 months, ranging from 1 month in those cases which died to 137 months.

Statistical Analysis

Results are expressed as a percentage or mean ± SD for normally distributed variables and median (range) for non-normally distributed variables. All parameters were compared by the $\chi 2$ test or Fisher's exact test for categorical data and Kruskal–Wallis test and Mann-Whitney U test for normally or non-normally distributed continuous data. Predictors of ESRD were evaluated in all patients using multivariate cox regression analysis, and the results are expressed as hazard ratios (HRs) with 95% confidence intervals (95% CIs) and p values. We evaluated baseline characteristics as potential covariates and transformed these to proper function forms to fit the models best and meet the inferential goal of finding predictors for the outcome of ESRD. Hemoglobin, blood platelet count, serum creatine, ESR, serum C3, C4 and serum immunoglobulin, and tubulointerstitial scores were used as continuous variables. Age, eGFR, CRP, and urine protein indexes were categorized as quartiles. The percentage of different histological classification were used as categorical covariates and changed contrast by using indicator method. The presence of IC deposits (≥2+) and gender, histological characteristics (fibrinoid necrosis, and Bowman's capsule rupture, TMA, periglomerular inflammatory, granulomatous lesions) on biopsy were used as binary covariates. Each regression model was conducted with a selection criterion of $P<0.05$. Then, significant predictor variables were chosen by backward elimination using anαlevel of 0.05 until all variables remaining in the model show significant association with ESRD. Renal survival and patient survival were evaluated by Kaplan-Meier curves using log-rank and generalized Wilcoxon-Breslow tests. A P-value<0.05 was considered to be statistically significant. Analyses were performed using SPSS statistical software (version 23.0).

RESULTS

Baseline Patient Characteristics

Among all 97 patients with MPO-ANCA GN, 59% (57/97) of patients had no or few deposits in their glomeruli from renal biopsy, which were classified as the "pauci-immune" (PI) group, while the other 41% (40/97) of cases were classified as the "immune complex" (IC) group. The clinical and histopathological characteristics of the two groups are shown in **Table 1**.

Immune complex deposits in the glomeruli were more frequently found in males than females with MPO-ANCA GN. Patients with immune complex deposits in the kidney showed less platelet count, lower sC3, sIgA and sIgG level, and higher serum creatinine levels than the PI group (p=0.009, 0.013, 0.018, 0.023, 0.025, respectively). CRP and ESR levels were higher in the PI group compared with the IC group (p=0.001 and 0.036, respectively). It is noteworthy that we also found more glomerular fibrin deposition in patients in the IC group than in the PI group, although the differences observed did not reach statistical significance (p=0.139). We also assessed the frequency of neutrophils, macrophages and T cells in the glomerular, periglomerular and interstitial compartments by immunostaining. The immune cells were detected in similar numbers in PI and IC groups as shown in **Table 1**.

Risk Factors for ESRD

Of the 97 patients, ESRD occurred in 42 (43.30%) patients during follow-up. Furthermore, 55% (22/40) patients in IC group and 35.09% (20/57) patients in PI group experienced ESRD during follow-up. In the multivariate analysis (**Table 2**), ESRD was shown to be negatively associated with eGFR (HR 0.885, 95% CI 0.837 to 0.935, p<0.0001), platelet count (HR 0.996, 95% CI 0.992 to 1.000, p=0.046) and serum globulin (HR 0.905, 95% CI 0.854 to 0.959, p=0.001).

TABLE 1 | Comparison of clinicopathologic parameters between MPO-ANCA-GN patients with and without immune complex deposits.

	PI[a] group (*N = 57*)	IC[b] group (*N = 40*)	Total (*N = 97*)	*P*-value
Age (year)	58 (51.50, 65.00)	58 (45.33, 65.00)	58 (49.5, 65.0)	0.424
Sex (male/female)	26/31	27/13	53/44	0.033*
Hemoglobin (g/dl) (mean, SD)	78.28 ± 15.43	81.10 ± 19.22	79.44 ± 17.05	0.426
Platelet (×10^3/mm3) (mean, SD)	276.02 ± 95.43	224.15 ± 93.48	254.63 ± 97.58	0.009*
Serum albumin (g/L) (mean, SD)	44.02 ± 17.29	43.60 ± 16.39	43.85 ± 16.84	0.903
Serum globulin (g/L) (mean, SD)	31.78 ± 7.06	31.58 ± 6.13	31.69 ± 6.66	0.885
Blood urea nitrogen (mmol/L) (median, IQR)	14.13 (9.51, 21.33)	18.29 (9.77, 23.55)	15.99 (9.51, 21.79)	0.160
Serum creatinine (mg/dl) (median, IQR)	3.33 (2.38, 5.51)	4.63 (2.85, 8.26)	4.08 (2.45, 6.19)	0.025*
eGFR[c] (ml/min per 1.73 m2) (median, IQR)	15.14 (8.55, 25.70)	11.04 (6.31, 22.34)	13.88 (7.43, 24.94)	0.079
Urinary protein (g/24 h) (median, IQR)	1.28 (0.48, 3.05)	1.24 (0.58, 2.98)	1.28 (0.49, 3.00)	0.849
Urinary blood cell count (/UL) (median, IQR)	300.00 (60.50, 496.08)	283.95 (91.4, 580.9)	300.00 (85.25, 513.43)	0.819
CRP[d], mg/dL (media, IQR)	24.15 (8.76, 71.15)	7.97 (2.91, 22.9)	16.05 (5.05, 42.85)	0.001*
ESR[e] (mm/h) (mean, SD)	76.77 ± 34.27	60.30 ± 39.81	70.22 ± 37.26	0.036*
MPO-ANCA[f] titer (U/ml) (mean, SD)	95.01 ± 42.89	83.92 ± 45.08	90.16 ± 43.86	0.319
Serum immunological indexes				
sC3 (mg/L) (mean, SD)	829.66 ± 248.23	699.62 ± 241.18	776.27 ± 252.40	0.013*
sC4 (mg/L) (mean, SD)	245.61 ± 99.32	259.09 ± 128.35	251.14 ± 111.69	0.565
sIgA (mg/L) (median, IQR)	2655.0 (1790.0, 3692.5)	1790.0 (1462.5, 2972.5)	2490.0 (1550.0, 3180.0)	0.023*
sIgM (mg/L) (median, IQR)	978.5 (748.7, 1540.0)	982.0 (754.5, 1220.0)	979 (754.0, 1520.0)	0.736
sIgG (g/L) (mean, SD)	15.40 ± 4.85	12.97 ± 4.85	14.40 ± 4.97	0.018*
Classfication, n%				
Focal	5, 8.77%	7, 17.95%	12, 12.50%	0.096
Mixed	27, 47.37%	9, 23.08%	36, 37.50%	
Crescentic	14, 24.56%	12, 30.77%	26, 27.08%	
Sclerotic	11, 19.30%	11, 28.21%	22, 22.92%	
Histological characteristics				
Fibrinoid necrosis, n%	29, 50.88%	19, 47.50%	48, 49.48%	0.837
Bowman's capsule rupture, n%	25, 43.86%	10, 25%	35, 36.08%	0.085
Periglomerular inflammatory, n%	19, 33.33%	8, 20%	27, 27.84%	0.173
Granulomatous lesions, n%	5, 8.77%	7, 17.50%	12, 12.37%	0.224
TMA[g], n%	13, 22.81%	13, 32.50%	26, 26.80%	0.354
Interstitial infiltrates (n, 0/1/2/3)	0/23/29/5	0/20/14/6	0/43/43/11	0.580
Tubulointerstitial lesions (n, 0/1/2/3)	0/24/27/6	0/14/19/7	0/38/46/13	0.215
Positive of fibrin, n%	17, 29.82%	18, 45.00%	35, 36.08%	0.139
BVAS[h]	16.98 ± 6.42	18.23 ± 5.39	17.49 ± 6.02	0.319
RPGN[i], n %	14, 24.56%	16, 40.00%	30, 30.92%	0.082
Treatment-resistant to the treatment, n%	12, 26.09%	11, 42.31%	23, 31.94%	0.156

*[a]PI, pauci-immune; [b]IC, immune complex; [c]eGFR, estimated glomerular filtration rate; [d]CRP, C-reactive protein; [e]ESR, Erythrocyte Sedimentation Rate; [f]MPO-ANCA, myeloperoxidase anti-neutrophil cytoplasmic antibody; [g]TMA, thrombotic microangiopathy; [h]BVAS, Birmingham vasculitis activity score; [i]RPGN, rapidly progressive glomerulonephritis. *P < 0.05.*

TABLE 2 | Multivariable predictors of ESRD[a] by multivariate COX regression analysis.

Predictor	*P*-value	HR[b] (95% CI[c])
Platelet (×10^3/mm^3)	0.046	0.996 (0.992,1.000)
Serum globulin (g/L)	0.001	0.905 (0.854, 0.959)
eGFR	<0.0001	0.885 (0.837, 0.935)
sIgG	0.051	0.917 (0.837, 1.000)
Interstitial infiltrates	0.065	1.529 (0.974, 2.400)

[a]ESRD, end stage renal disease; [b]HR, hazard ratio; [c]CI, confidence interval.

Renal Histopathology and IC Deposits

As shown in **Table 3**, C3 was the most common complement component found in the glomeruli of kidney biopsy specimens. C3 IF staining of 1+ was seen in 18 (18.56%) cases, while there were only 19 cases (19.59%) that were more intensively stained. C1q was also seen in 10 (10.75%) renal biopsies with IF staining ≥1+, but only 4 (4.30%) cases showed stronger staining (IF≥2+). Only 7 patients (13.73%) showed a weak stain (1+) in C4 deposits. For renal immunoglobulin deposition, IgA, IgM and IgG showed an IF intensity of 1+ in 18.75%, 17.71%, 13.54% cases, respectively and strong intensity IF (≥2+) was found in 5.21%, 28.12%, 18.75% cases, respectively (**Table 3**).

As shown in **Table 3**, Bowman's capsule rupture (p=0.028) and periglomerular inflammation (p=0.034) were less commonly seen in biopsies with IgM deposition.

Besides, in the renal biopsies with stronger IgA deposition (IF≥2+), granuloma-like lesions and vascular lesions were more frequently seen than those without IgA deposition (p=0.03 and 0.015, respectively).

Renal Survival Analysis

Analysis of renal survival showed patients which had lower sC3 levels had higher rates of ESRD compared to cases with normal serum C3 values (p=0.003, **Figure 1A**). Furthermore, the patients with continued low sC3 during therapy showed worse renal outcomes than the patients with sC3 levels returning to normal during treatment (p=0.013, **Figure 1B**). Also, patients with low sC3 both at initial and with continued low sC3 during the

TABLE 3 | Immunofluorescence findings in biopsies and histopathological features in MPO-ANCA-GN.

		The proportion of crescentic glomeruli, %			Fibrinoid necrosis		Bowman's capsule Rupture		Periglomerular inflammation		Granulomatous lesions		Vascular lesions		Thrombotic microangiopathy		Proportion of sclerotic glomeruli, %		Interstitial infiltrates (score≥2)		Interstitial fibrosis/tubular atrophy lesions (score≥2)	
		n (%)	Mean ± STD	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	Mean ± SD	*P*	n (%)	*P*	n (%)	*P*
C3	Number of negative	60 (61.86)	39.98 ± 26.05	0.788	29 (48.33)	0.492	27 (45.00)	0.066	20 (33.33)	0.278	7 (11.67)	0.831	18 (30.00)	0.496	16 (26.67)	0.813	32.84 ± 22.98	0.939	33 (55.00)	0.491	35 (58.33)	0.735
	Number of 1+	18 (18.55)	43.77 ± 23.48		11 (61.11)		4 (22.22)		4 (22.22)		3 (16.67)		7 (38.89)		4 (22.22)		32.35 ± 27.27		12 (66.67)		11 (61.11)	
	Number ≥2+	19 (19.59)	37.93 ± 25.83		8 (42.11)		4 (21.05)		3 (15.79)		2 (10.53)		4 (21.05)		6 (31.58)		39.50 ± 37.31		9 (47.37)		13 (68.42)	
C4	Number negative	44 (86.27)	44.21 ± 27.31	0.805	21 (47.73)	0.703	20 (45.45)	0.393	16 (36.36)	0.401	8 (18.18)	0.797	18 (40.91)	0.690	16 (36.36)	1.000	35.21 ± 25.18	0.132	28 (63.64)	0.411	25 (56.82)	1.000
	Number of 1+	7 (13.73)	45.75 ± 17.67		4 (57.14)		2 (28.57)		1 (14.29)		1 (14.29)		2 (28.57)		3 (42.86)		19.64 ± 12.30		3 (42.86)		4 (57.14)	
C1q	Number of negative	83 (89.25)	41.39 ± 25.06	0.351	42 (50.60)	0.370	33 (39.76)	0.087	27 (32.53)	0.032*	12 (14.46)	0.430	24 (28.92)	0.318	24 (28.92)	0.825	33.02 ± 26.04	0.138	49 (59.04)	0.835	49 (59.04)	0.735
	Number of 1+	6 (6.45)	26.46 ± 29.69		1 (16.67)		0 (0)		0 (0)		0 (0)		2 (33.33)		1 (16.67)		47.87 ± 34.24		3 (50)		3 (50)	
	Number ≥2+	4 (4.30)	34.14 ± 33.45		2 (50)		1 (25)		0 (0)		0 (0)		3 (75)		1 (25)		54.32 ± 34.79		2 (50)		4 (100)	
IgA	Number of negative	73 (76.04)	40.83 ± 24.70	0.272	36 (49.32)	0.917	31 (42.47)	0.089	24 (32.88)	0.032*	8 (10.96)	0.015*	22 (30.14)	0.03*	19 (26.03)	0.757	32.82 ± 25.96	0.685	42 (57.53)	0.862	43 (58.90)	0.858
	Number of 1+	18 (18.75)	42.54 ± 24.86		9 (50.00)		3 (16.67)		1 (5.56)		1 (5.56)		3 (16.67)		5 (27.78)		40.69 ± 30.40		9 (50.00)		12 (66.67)	
	Number ≥2+	5 (5.21)	20.45 ± 40.91		2 (40.00)		1 (20.00)		2 (40.00)		3 (60.00)		4 (80.00)		2 (40.00)		34.22 ± 31.00		3 (60.00)		3 (60.00)	
IgM	Numberof negative	52 (54.17)	26.71 ± 43.22	0.022*	31 (59.62)	0.075	25 (48.08)	0.028*	20 (38.46)	0.034*	7 (13.46)	1.000	14 (26.92)	0.741	13 (25.00)	0.700	27.00 ± 35.28	0.454	32 (61.54)	0.155	30 (57.69)	0.432
	Number of 1+	17 (17.71)	19.55 ± 28.86		6 (35.29)		5 (29.41)		4 (23.53)		2 (11.76)		6 (35.29)		6 (35.29)		22.01 ± 38.82		11 (64.71)		9 (52.94)	
	Number ≥2+	27 (28.12)	23.51 ± 40.30		10 (37.04)		5 (18.52)		3 (11.11)		3 (11.11)		9 (33.33)		7 (25.93)		29.67 ± 34.37		11 (40.74)		19 (70.37)	
IgG	Number of negative	65 (67.71)	40.98 ± 24.93	0.786	30 (46.15)	0.725	24 (36.92)	0.571	18 (27.69)	0.613	7 (10.77)	0.457	18 (27.69)	0.711	14 (21.54)	0.185	31.03 ± 24.27	0.342	36 (55.38)	0.550	37 (56.92)	0.242
	Number of 1+	13 (13.54)	36.81 ± 30.74		7 (53.85)		6 (46.15)		5 (38.46)		1 (7.69)		5 (38.46)		5 (38.46)		36.16 ± 24.99		9 (69.23)		7 (53.85)	
	Number ≥2+	18 (18.75)	40.39 ± 24.82		10 (55.56)		5 (27.78)		4 (22.22)		4 (22.22)		6 (33.33)		7 (38.89)		44.94 ± 34.86		9 (50.00)		14 (77.78)	

*$P < 0.05$, differences in three groups were compared by analysis of the Kruskal-Wallis test or Fisher's Exact Test.

treatment all displayed a trend toward decreased patient survival (**Figures 1C, D**) without reaching statistical significance.

The IC group showed a worse renal outcome than the PI group (Breslow p=0.030) (**Figure 2A**), but there was no difference in patient survival (**Figure 2B**). In further analysis of the outcome of patients with different types of immune deposits, no differences in renal survival were observed between patients with kidney IF intensities ≥2+ for C3, C1q, IgA, IgM and patients with kidney IF intensities ≤1+ for C3, C1q, IgA, IgM (**Figures 3A–D**). However, patients with IgG deposits (≥2+ in IF) showed a poorer renal outcome than the patients with no or low level deposits (≤1+ in IF) (p=0.028) (**Figure 3E**).

DISCUSSION

Immune complex deposition in MPO-AAV does not seem to be as uncommon as previously considered. We found that 41% of cases had at least one Ig or complement component deposited in the glomeruli (IF≥2+ on a scale of 0 to 4). Though the absence or paucity of IC localization in the glomeruli is classically defined as a characteristic of ANCA-GN, similar to our study, there have been some previous studies that reported ANCA-GN as not always being pauci-immune. Hass et al. reported that 54% of 126 cases showed immune complex deposits by electron microscopy (2). Another study also showed 26.4% of ANCA-GN cases exhibited immunostaining of ≥2+ intensity in renal biopsies (19). The prevalence of stronger IF findings in our study was higher than previously reported, which may be ascribed to recruiting only MPO-ANCA GN patients rather than all the ANCA-GN patients including PR3-ANCA-GN, and there were no comparative data in detail about IC deposition between MPO-ANCA GN and PR3-ANCA-GN.

In our study, the serological tests at diagnosis, namely lower platelet count, sC3, sIgG, and higher sCr can be seen in patients with IC deposits compared with the PI group. The most plausible reason for that sCr is significantly higher in the patients with IC deposits but eGFR is not significantly different between the two groups is that there were more males in the IC group compared with PI group. Low levels of platelets suggest active coagulation particularly the renal deposition of fibrin which has been shown to a prominent mediator of inflammatory kidney injury (20). Although there was a trend of increased glomerular fibrin deposition in MPO-AAV-GN patients with immune complex deposits in the kidney, this did not reach statistical significance. Further studies with a large number of patients are needed to quantitate the level of renal fibrin deposition in the kidney of MPO-ANCA-GN patients, which may suggest the renal presence of another important potential therapeutic target.

Meaningfully, platelet counts, serum globulin and eGFR were shown to be risk factors for ESRD in our multifactorial analysis. In line with previous reports, patients with lower sC3 at diagnosis displayed poorer renal survival (6). In addition, treatment resistance was observed to be negatively associated with platelet count and sC3 level in our previous study (21). Our data here is consistent with the findings of others (15, 22–26).

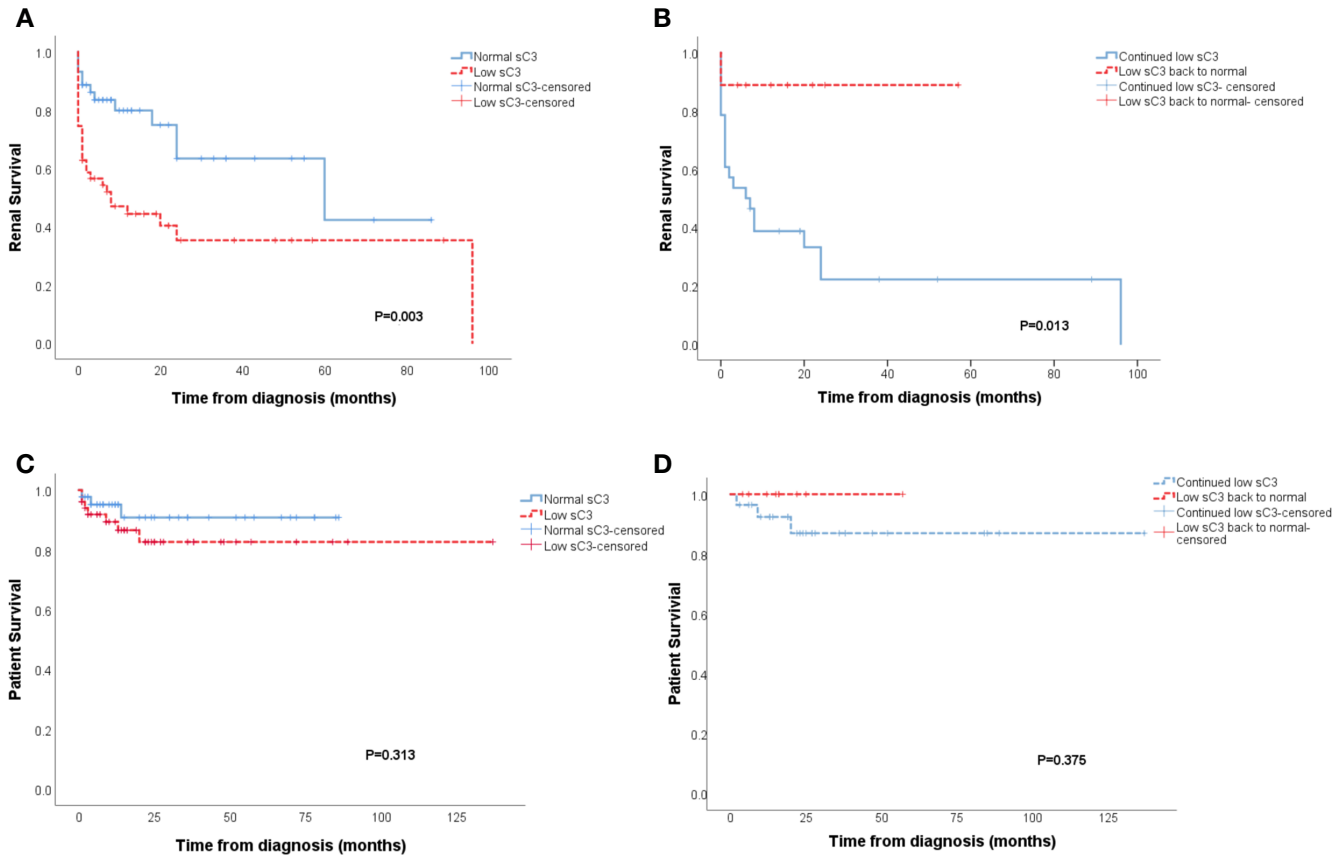

FIGURE 1 | Patients with low sC3 showed worse renal outcomes than patients with normal sC3 level at diagnosis (p = 0.003) **(A)**. Patients who persist with low sC3 exhibit a substantially higher degree of ESRD than the patients whose sC3 level returned to normal during the treatment process (p = 0.013) **(B)**. The patient survival of patients with low sC3 at diagnosis and persists with low sC3 in the treatment **(C, D)**.

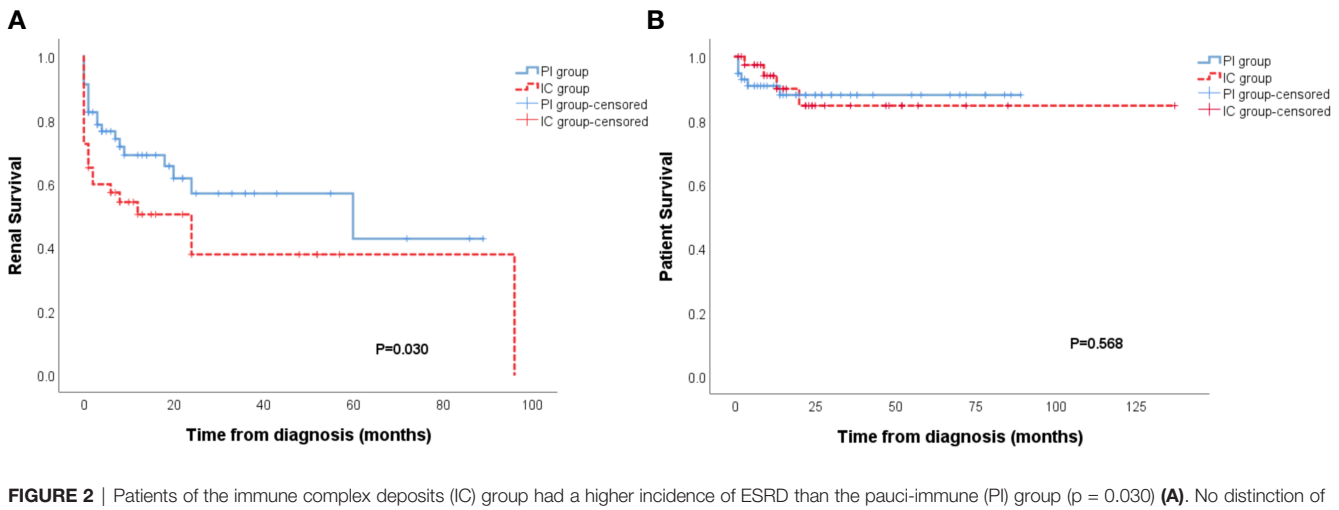

FIGURE 2 | Patients of the immune complex deposits (IC) group had a higher incidence of ESRD than the pauci-immune (PI) group (p = 0.030) **(A)**. No distinction of patient survival had been observed between PI and IC group **(B)**.

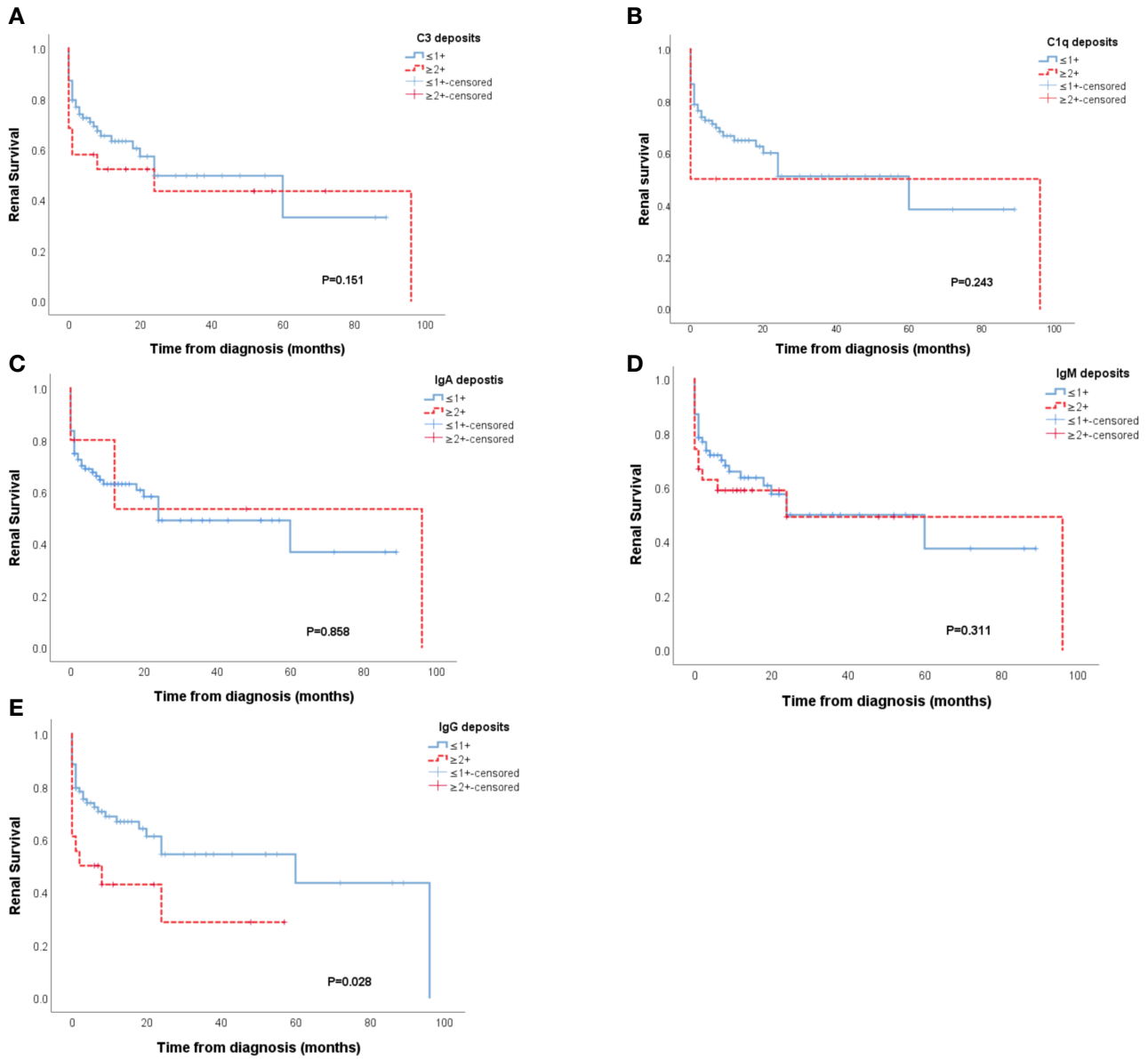

FIGURE 3 | Renal survival of patients with and without C3, C1q, IgA, IgM deposits **(A–D)**. Patients with IgG deposits showed poorer renal prognosis than patients that had no or low IgG deposits in the glomeruli (p = 0.028) **(E)**.

We found that there was a significantly poorer renal outcome in patients with persistently lower sC3 levels than those whose sC3 levels recovered to normal during therapy upon follow-up. The documented role of sC3 reduction in treatment resistance and renal prognosis in the MPO-AAV patients, as shown in our data, support the use of complement targeted therapies in AAV, such as the C5a receptor inhibitor CCX168 (21, 27–30).

However, we did not observe significant differences in BVAS. The Berden classification of renal injury in ANCA-GN is widely accepted (14). This classification does allow the intensity of immune inflammation and damage to be assessed. Nonetheless, we find no differences of the four classes of MPO-ANCA-GN, active and chronic lesions between PI and IC groups. The reason for this is unclear. We suspect that one plausible explanation for this is the small number of patients in the present study. It is now widely accepted that cell mediated immunity is prominent in MPO-AAV patients and has been shown to be a major components in inducing inflammatory injury in many models of this disease (31). The immune cells were detected in similar numbers in the glomerular, periglomerular and interstitial compartments in PI and IC groups (**Table S1**). Furthermore, ESR and CPR levels, which

are routinely used as biomarkers of disease activity in clinical work, were lower in the group with IC than the pauci-immune group. This data is consistent with that obtained by other investigators (32, 33), but our data were in contrast to the study by Brons et al. showing immune deposits in skin lesions of patients with Wegener's granulomatosis during active disease (34). The exact reason for this remains unknown, which might be associated with the production and deposition of ICs in the kidney in MPO-AAV patients. It suggested that IC in the glomeruli might have no relation to systemic disease activity. On the contrary, IC in the glomeruli might be associated with the chronic process in MPO-ANCA GN.

Moreover, different types of deposition also have various implications in MPO-ANCA GN. In agreement with the results of previous studies, the deposition of complement in our study has suggested again that complement activation usually occurs in MPO-ANCA GN. Gou et al. detected the alternative complement pathway activation fragment Bb deposited both in the kidney and in urine which confirmed complement activation through the alternative pathway that occurred in the development of AAV (35). In our study, more commonly C3 and less commonly C1q had been seen deposited in the kidney, similar to previous reports (36), suggesting that the complement system may be commonly activated by the alternative pathway but also by the classical complement pathway less often (5). However, contrary to previous studies (29, 36), both C3 and C1q deposition in the kidney didn't show a special connection with any histopathological signs of MPO-ANCA-GN.

The findings of immunoglobulin in kidney specimens were accompanied by C3 deposition. IgG deposits had no relation with histological changes, but it was associated with a higher risk for ESRD. The patients with IgG deposition demonstrated a higher rate of ESRD, which was in contrast to another retrospective study (9). The basis for this discrepancy needs to be investigated in future studies. Differences in participants and an insufficient number of cases in both studies may partly explain this discrepancy. Different from IgG deposits, IgA seems to be more related to histological characteristics of ANCA-GN. Granuloma-like and vascular lesions had been more frequently seen in the cases with IgA deposition. As reported previously, IgA nephropathy (IgAN) with ANCA positivity showed less typical mild, focal, and segmental mesangial and endocapillary hypercellularity and more severe clinical features than the cases that were ANCA negative (7, 37). Also, some reports proposed that patients both with IgA deposition and ANCA positivity responded well to aggressive immunosuppressive therapy (37, 38). But, there were few studies about ANCA-GN with IgA deposition (32). In our study, there was no difference in renal outcome between patients with and without IgA deposition. However, granuloma-like and vascular lesions, as the characteristic pathological changes of MPO-ANCA GN, were closely associated with IgA deposition.

Unexpectedly, on histological sections, we also discovered that Bowman's capsule rupture and periglomerular inflammation occurred infrequently in the cases with IgA and IgM deposits. Bowman's capsule is implicated in functionally isolating potential immune effectors thereby preventing injury to the glomerulus. So, the rupture was considered as a trigger of inflammatory cell migration (such as CD8+ T cells and macrophagocytes) and crescentic formation (39). The inverse negative relationship between IC deposits and Bowman's capsule rupture and periglomerular inflammation suggests that Bowman's capsule rupture and periglomerular inflammation with subsequent inflammatory cell infiltration into the glomerulus might be related to the clearance of IC in MPO-ANCA GN. This needs more clinical observation and animal studies to further confirm.

Our study has several limitations as it is retrospective and has a small case load, which may have introduced information and recall bias. Moreover, the number of glomeruli in some biopsies is relatively small, which might lead to some bias and reduce the power to demonstrate associations between histopathological findings and immune deposits. The relatively short period of follow-up was another limitation in our study, which also influenced the analysis of the prognosis of patients.

In conclusion, patients with immune complex deposits in the kidney showed less platelet count, lower sC3 and sIgG levels and higher serum creatinine levels. Patients with low sC3 at initial and with continued low sC3 during treatment all displayed a trend toward poorer kidney survival. Moreover, the IC group showed worse renal outcome than the PI group, further enforcing the present orientation to introduce complement targeted therapies in AAV. Also, patients with IgG deposits showed a poorer renal outcome, which can help to evaluate prognosis to some extent. IgA deposits were associated with vascular and granuloma-like lesions in MPO-ANCA GN, suggesting the connection between the immune deposits and histological features in ANCA-GN. Our findings suggest MPO-AAV-associated renal damage can be immune complex-induced and could assist in guiding clinical work.

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 the Ethics Committee of Xiangya Hospital of Central South University (IRB approval number 200901008). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YZ, PX, XX, HL, and QZ contributed to the conception of the study. WL, YZ, and JO performed the analysis with constructive

discussions. CS, J-BC, and XA analyzed and interpreted the data. HY, WL, and ZX completed the pathological analysis. RT, TW, WP, JH, and Y-OZ contributed significantly to the clinical follow-up. WL finished the manuscript. YZ and JO supervised and edited the manuscript. PE edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.625672/full#supplementary-material

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Antineutrophil Cytoplasmic Antibody-Associated Vasculitis Update: Genetic Pathogenesis

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Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterized by the inflammation of small and medium vessels and presence of proteinase 3-ANCA or myeloperoxidase-ANCA in the circulation. AAV comprises three clinical subtypes: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic GPA (EGPA). Although the pathogenesis of AAV is still unclear, genetic and environmental factors and the immune system are thought to be involved. Genetic factors have been confirmed to play an important role in AAV. Genome-wide association studies have identified numerous genetic variants in MHC and non-MHC regions associated with AAV. The strongest evidence of MHC association in AAV is human leukocyte antigen (HLA)-DP. A significant association between AAV and genetic variations in non-MHC regions, such as *CTLA-4*, *FCGR2A*, *PTPN22*, *SERPINA1*, and *TLR9* has also been found. Moreover, different clinical subtypes of AAV have distinct genetic backgrounds. GPA is associated with *HLA-DP1*, MPA with *HLA-DQ*, and EGPA with *HLA-DRB4*. These findings could help elucidate the etiology of AAV and develop new biomarkers for diagnosis and targeted therapy. Herein, we briefly summarize the updates on the genetic pathogenesis and biomarkers of AAV.

Keywords: vasculitis, antineutrophil cytoplasmic antibody, genetic, genome-wide association studies, variation

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INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a complex systemic autoimmune disease presenting with the inflammation of small and medium vessels that results in vascular destruction and tissue necrosis (1, 2). AAV is divided into three groups according to clinical features: granulomatosis with polyangiitis (GPA, formerly called Wegener's granulomatosis), microscopic polyangiitis (MPA), and eosinophilic GPA (EGPA, formerly called Churg-Strauss syndrome) (3). The disease is characterized by the presence of proteinase 3 (PR3)-ANCA or myeloperoxidase (MPO)-ANCA in the serum. GPA is predominantly associated with PR3-ANCA,

while MPA and EGPA are predominantly associated with MPO-ANCA, but are also occasionally ANCA-negative (4). EGPA is often divided into two subtypes, MPO-ANCA+ EGPA and ANCA– EGPA. Vasculitis can occur in any organ or tissue, commonly affecting the respiratory tract and kidneys, causing life-threatening kidney failure or pulmonary hemorrhage (3).

AAV has an estimated prevalence of 48–184/1,000,000 individuals worldwide, while the incidence and prevalence in Europe are higher than those in other regions (2). The annual incidence in Europe is 4.9–10.6/1,000,000 individuals for GPA, 2.7–11.6/1,000,000 individuals for MPA, and 0.5–3.1/1,000,000 individuals for EGPA. The epidemiological manifestations of AAV differ among geographical regions; GPA and PR3-ANCA AAV are more common in Europeans, while MPA and MPO-ANCA AAV are more common in Asians (5).

Although the pathogenesis of AAV remains elusive, it is believed that both genetic and environmental components are involved. Environmental factors, including silica exposure, bacterial or viral infections, and drugs suggest an association with the occurrence and relapse of AAV (6). Familial studies and genetic association studies have demonstrated that AAV has a background of genetic susceptibility (7–9). It has been estimated that 20% of AAV risk is due to genetic factors (10).

In this review, we mainly discuss the genetic studies on AAV, focusing on the identified susceptibility genes or loci to enrich our understanding of the disease.

GENETIC APPROACHES FOR AAV

Different genetic approaches have revealed that a large number of genes are related to AAV. First, several candidate gene association studies have been used to identify susceptibility genes associated with AAV. Although these approaches are easy to carry out, they are prone to false-positive results. Human leukocyte antigen *(HLA)*, *PTPN22*, *CTLA-4*, *IL-10*, and *TLR9* have been found to be associated with AAV (11, 12). The emergence of genome-wide association studies (GWAS) has led to their wide use in exploring the genetic factors involved in various diseases. GWAS is a powerful approach to identify the genetic architecture of complex diseases, and it has made major contributions towards a better understanding of AAV genetics. Four GWAS have been performed in patients with AAV since 2012 (two for MPO and GPA (13, 14), one for GPA (15), and one for EGPA (16)). The latest GWAS was on EGPA in 676 cases and 6809 controls with the identification of 11 loci associated with EGPA. The study also revealed that EGPA comprises genetically distinct subgroups, that MPO-ANCA+ subgroup was strongly associated with HLA-DQ and MPO-ANCA- subgroup was associated with non-HLA regions, such as GPA33 and IL5/IRF1 (16). All these GWAS studies were conducted in populations with European ancestry and have identified more than 20 genes associated with AAV. The findings of GWAS are listed in **Tables 1** and **2**. In addition, meta-analysis and fine mapping have also been used to identify new gene variations and analyze the effect of the variants (17–21).

AAV SUSCEPTIBILITY GENES

HLA Region

HLA

HLA, located on chromosome 6p21, is the most gene-dense region of the human genome and contains diverse genes involved in key immune responses (22). Molecules encoded by *HLA* participate in a variety of immune and inflammatory pathways. *HLA* single-nucleotide polymorphisms (SNPs) are associated with numerous human diseases, especially autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (23). Since AAV is considered an autoimmune disease, *HLA* may also be a potential predisposing factor for AAV. Candidate gene studies in *HLA* regions were performed in different populations, including the Swedish, Germans, and Italians in Europe and Japanese and Chinese in Asia. *HLA-DPA1*, *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*, and *HLA-DRB1* were found to be involved in AAV susceptibility.

(1). HLA and AAV

Two GWAS were performed to identify the genetic factors associated with AAV. The first GWAS in a European population in 2012 demonstrated that *HLA-DP* rs3117242 (G) was the strongest signal in the HLA region ($P = 1.5\times10^{-71}$, OR = 3.67) (13). Another GWAS in Canadian and American populations conducted in 2017 showed that SNPs rs141530233 and rs1042169 at *HLA-DPB1* had the largest effect on the risk of developing AAV ($P = 1.13\times10^{-89}$, OR = 2.99; $P = 1.12\times10^{-84}$, OR = 2.82, respectively). *HLA-DPA1*, *HLA-DQA1*, and *HLA-DQB1* are also risk alleles that may cause AAV (14). These studies not only indicated a highly significant association between AAV and *HLA* regions but also showed genetic distinctions between different clinical phenotypes and ANCA specificity. GPA and PR3-ANCA AAV are associated with *HLA-DPB1* and *HLA-DPA1*, while MPA and MPO-ANCA AAV are associated with *HLA-DQB1* and *HLA-DQA2*.

(2). HLA and GPA

GWAS for GPA with 492 patients and 1,506 healthy individuals of European descent identified 32 SNPs across the HLA region; among them, *HLA-DPB1* rs9277554 and *HLA-DPA1* rs9277341 were significantly associated with GPA in the combined cohort (15). In another study with 150 GPA patients and 100 healthy controls conducted in northern Germany, *HLA-DPB1**0401 was identified to be associated with GPA ($P = 1.51 \times 10^{-10}$, OR = 3.91), and *DPB1**0401/RXRB03 haplotype frequency was significantly increased in patients with GPA ($P = 7.13 \times 10^{-17}$, OR = 6.41) (18). The result was replicated in an independent German cohort with 108 patients with GPA ($P = 6.4 \times 10^{-8}$) (20). *HLA-DPB1* was also considered a genetic risk factor for GPA in a cohort of 176 Han Chinese patients with AAV (100 with GPA, 76 with MPA) and 485 healthy controls ($P = 1.83 \times 10^{-5}$, OR = 2.57). In this study, the result also showed that the *HLA-DPB1* SNP rs3117242 variant T allele was significantly associated with GPA patients ($P = 6.24 \times 10^{-5}$, OR = 2.09), but not with MPA patients (24).

TABLE 1 | A summary of main genetic associations with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis through genome-wide association studies, according to Clinical subgroups.

Subgroups	Chr	Reported Gene(s)	SNP	Population	Cases-Controls	P-value	OR	Reference (PMID)
GPA and MPA	1p13.2	*PTPN22*	rs2476601	European	1986-4723	1.86×10^{-7}	1.36	28029757
	1p13.2	*PTPN22*	rs6679677	European	1986-4723	1.88×10^{-8}	1.4	28029757
	6p21.32	*HLA-DP*	rs3117242	European	2267-6858	1.5×10^{-71}	3.67	22808956
	6p21.32	*COL11A2*	rs3130233	European	2267-6858	7.8×10^{-15}	1.51	22808956
	6p21.32	*COL11A2*	rs3117016	European	2267-6858	6.4×10^{-24}	1.83	22808956
	6p21.32	*SERPINA1*	rs7151526	European	2267-6858	2.4×10^{-9}	0.59	22808956
	19p13.3	*PRTN3*	rs62132295	European	1353-1599	7.1×10^{-5}	0.78	22808956
	6p21.32	*HLA–DPB1*	rs141530233	European	1986-4723	1.13×10^{-89}	2.99	28029757
	6p21.32	*HLA–DPB1*	rs1042169	European	1986-4723	1.12×10^{-84}	2.82	28029757
	6p21.32	*HLA–DPA1*	rs9277341	European	1986-4723	6.09×10^{-71}	2.44	28029757
	6p21.32	*HLA–DQA1*	rs35242582	European	1986-4723	6.34×10^{-23}	1.6	28029757
	6p21.32	*HLA–DQB1*	rs1049072	European	1986-4723	6.46×10^{-13}	1.4	28029757
	6p21.32	*SERPINA1*	rs28929474	European	1986-4723	3.09×10^{-12}	2.18	28029757
	19p13.3	*PRTN3*	rs62132293	European	1986-4723	8.60×10^{-11}	1.29	28029757
GPA	5q23.1	*SEMA6A*	rs26595	European	987-2731	2.9×10^{-8}	0.74	23740775
	6p21.3	*HLA–DPA1*	rs9277341	European	750-1820	2.18×10^{-39}	0.33	23740775
	6p21.3	*HLA–DPB1*	rs9277554	European	750-1820	1.92×10^{-50}	0.24	23740775
	6p21.32	*HLA-DP*	rs3117242 (G)	European	1683-6858	3.1×10^{-85}	5.39	22808956
	6p21.32	*HLA–DPB1*	rs141530233	European	1556-4723	3.8×10^{-93}	3.82	28029757
	6p21.32	*HLA–DPB1*	rs1042169	European	1556-4723	1.09×10^{-90}	3.66	28029757
	6p21.32	*HLA–DPA1*	rs9277341	European	1556-4723	2.78×10^{-73}	2.86	28029757
	6p21.32	*HLA–DQA1*	rs35242582	European	1556-4723	1.6×10^{-20}	1.63	28029757
	6p21.32	*SERPINA1*	rs7151526	European	1683-6858	4.4×10^{-10}	0.54	22808956
	6p21.32	*SERPINA1*	rs28929474	European	1556-4723	3.53×10^{-13}	2.35	28029757
	19p13.3	*PRTN3*	rs62132293	European	1556-4723	7.06×10^{-11}	1.32	28029757
	Xp22.2	*MOSPD2*	rs6628825	European	1683-6858	2.6×10^{-5}	0.8	22808956
MPA	6p21.32	*HLA–DQB1*	rs1049072	European	236-4723	4.16×10^{-9}	1.89	28029757
EGPA	2q13	*BCL2L11*	rs72946301	European	534-6809	9.0×10^{-11}	1.81	31719529
	5q22.1	*TSLP*	rs1837253	European	534-6809	5.2×10^{-11}	1.52	31719529
	6p21.32	*HLA-DQ*	rs9274704	European	534-6809	1.2×10^{-20}	2.01	31719529
	10p14	*10p14*	rs34574566	European	534-6809	2.9×10^{-8}	0.7	31719529

TABLE 2 | Summary of main genetic associations with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis through genome-wide association studies, according to ANCA subgroups.

Subgroups	Chr	Reported Gene(s)	SNP	Population	Cases-Controls	P-value	OR	Reference
PR3+ AAV	6p21.32	*HLA–DP*	rs3117242	European	1521-6858	6.2×10^{-89}	7.03	22808956
	6p21.32	*HLA–DPB1*	rs141530233	European	1361-4723	1.33×10^{-106}	6.19	28029757
	6p21.32	*HLA–DPB1*	rs1042169	European	1361-4723	6.53×10^{-106}	6.09	28029757
	6p21.32	*HLA–DPA1*	rs9277341	European	1361-4723	4.52×10^{-84}	3.69	28029757
	6p21.32	*HLA–DQA1*	rs35242582	European	1361-4723	5.78×10^{-18}	1.62	28029757
	6p21.32	*SERPINA1*	rs28929474	European	1361-4723	1.29×10^{-13}	2.43	28029757
	19p13.3	*PRTN3*	rs62132295(A)	European	1521-1599	2.6×10^{-7}	0.73	22808956
	19p13.3	*PRTN3*	rs62132293	European	1361-4723	3.59×10^{-13}	1.39	28029757
	6q22.33	*ARHGAP18*	rs1705767	European	1521-6858	5.2×10^{-8}	0.73	22808956
	14q32.13	*SERPINA1*	rs7151526	European	1521-6858	5.6×10^{-12}	0.53	22808956
	Xp22.2	*MOSPD2*	rs6628825	European	1521-6858	6.1×10^{-7}	0.77	22808956
MPO+ AAV	6p21.32	*HLA-DQ*	rs5000634	European	556-6858	2.1×10^{-8}	0.65	22808956
	6p21.32	*HLA–DQA2*	rs3998159	European	378-4723	5.24×10^{-25}	2.72	28029757
	6p21.32	*HLA–DQA2*	rs7454108	European	378-4723	5.03×10^{-25}	2.73	28029757
MPO- AAV	6p21.32	*HLA–DQB1*	rs1049072	European	378-4723	2.13×10^{-24}	2.37	28029757

(3). HLA and MPA

There was no specific GWAS study for MPA, but the clinical subgroup analysis of AAV GWAS demonstrated that *HLA-DQB1* was significantly associated with MPA ($P = 4.16\times10^{-9}$, OR = 1.89) (14). A significant association of *HLA-DRB1**0901 with MPA ($P = 0.0037$, OR = 2.44) and MPO-AAV ($P = 0.0014$, OR = 2.44) was demonstrated in 50 MPA and 64 MPO-ANCA AAV cases compared with 265 controls in a Japanese cohort (25). The result was confirmed with new observations that *DRB1**13:02 was associated with protection against MPA and MPO-ANCA AAV in 468 Japanese patients with AAV and 596 controls($P = 2.1\times10^{-4}$, OR = 1.57) (26). In a small cohort of 50 patients with MPA and 77 Japanese controls, *DQB1**0303 was significantly associated with MPA ($P = 0.017$, OR = 2.35) (27). A recent study genotyped *HLA-DRB1*, *DQA1*, *DQB1*, *DPB1*, and *HLA-DP* in 258 patients with MPO-AAV and 597 healthy controls in the Chinese population and found that *HLA-DQA1**0302 ($P = 3.45\times10^{-9}$,

OR = 2.34) and *DQB1**0303 ($P = 3.26 \times 10^{-9}$, OR = 1.89) were risk alleles for MPO-ANCA AAV (28).

(4). HLA and EGPA

A recent GWAS for EGPA including 676 EGPA cases and 6,809 controls in European populations showed that *HLA-DQ* was strongly associated with EGPA at genome-wide significance ($P = 1.2 \times 10^{-20}$, OR = 2.01), especially MPO -ANCA EGPA ($P = 1.1 \times 10^{-28}$, OR = 5.68) (16). This result was consistent with a study comprising 102 German patients with EGPA ($P = 2.00 \times 10^{-5}$, OR = 1.87) (29) as well as another study with 48 Italian patients ($P = 2.32 \times 10^{-4}$, OR = 2.49) (30). The Italian study also revealed that *HLA-DRB1**07 allele frequency was significantly higher in patients with EGPA than in controls ($P = 0.42 \times 10^{-2}$, OR = 2.42) (30).

Furthermore, *HLA* alleles were associated with the severity, prognosis, and relapse of AAV. *HLA-DRB1**04:05 was associated with poor renal prognosis; *HLA-DRB1**04:02 was associated with high mortality (31); and *DPB1**04:01 was significantly associated with an increased risk of relapse (32).

AAV is an autoimmune disease. The identification of HLA-alleles associated with AAV confirm the centrality of auto-reactivity in the development of AAV, help us make a better understanding about its autoimmune pathogenesis, and point to logical therapeutic strategies. The identification of HLA-alleles not only can help us to distinguish from cases of GPA, MPA and EGPA and their subtypes, but also to evaluate and predict the severity, prognosis, and relapse of AAV.

Retinoid X Receptor Beta

RXRB, located on 6p21.32, encodes a member of the retinoid X receptor family of nuclear receptors. Strong associations of *RXRB* with GPA were revealed through the markers located in the *HLA* region and *RXRB* loci. Because *RXRB* located in the *HLA* region near *HLA-DPB1*, *HLA-DPB1*0401/RXRB03* haplotype was found to be strongly associated with GPA ($P = 7.13 \times 10^{-17}$, OR = 6.41) (18). *RXRB* SNP rs6531 ($P = 5.20 \times 10^{-5}$, OR = 1.88) was significantly increased among patients with GPA compared to controls (19). The result was confirmed in a later meta-analysis, which included 140 genetic variants associated with AAV (17).

Ring Finger Protein 1

RING is also located in the *HLA* region, near *HLA-DPB1*. RING is a member of the polycomb repressive complex 1 that mediates histone H2A polyubiquitination and monoubiquitination, regulating its gene expression. Three SNPs in the region near *RING1* (rs213210, rs213209, and rs213208) were found to be associated with GPA in German patients (20). The *HLA-DPB1/RING1* haplotype is strongly associated with GPA in ANCA-positive subjects.

Non-HLA Region

Genes Encoding ANCA Associated Proteins

Serpin family A Member 1

SERPINA1, located on 14q32.13, encodes α1-antitrypsin, which is the major inhibitor of PR3 and is thought to limit the damage to local tissues (33). In AAV, the genetic variants of *SERPINA1* may lead to the decreased function of α1-antitrypsin, potentially resulting in ANCA generation as PR3 accumulates in tissues, causing the inflammation of blood vessels. The first AAV GWAS reported that the *SERPINA1* Z allele along with the rs7151526 risk allele were significantly associated with AAV and were the most prominent non-*HLA* regions associated with both PR3-ANCA and GPA subgroups (13). Another GWAS confirmed the result with the identification of *SERPINA1* rs28929474, which was associated with AAV ($P = 3.09 \times 10^{-12}$, OR = 2.18), especially associated with GPA ($P = 3.53 \times 10^{-13}$, OR = 2.35) (14). Several studies have investigated the role of the Z allele in AAV and showed that heterozygous patients for the Z variant of *SERPINA1* have an increased risk of developing GPA than the general population (34, 35).

Proteinase 3 (PRTN3)

PRTN3, located on 19p13.3, encodes PR3. PR3 is a neutrophil intracellular protease that is the main antigen of ANCA autoantibodies. PR3 is located on the plasma membrane of a subset of neutrophils and stored in the neutrophil azurophilic granules. The membrane-bound form interacts directly with ANCA (33), decreasing neutrophil activation and endothelial adhesion.

In a Swedish cohort of 79 patients with GPA and 129 controls, the coding and promoter sequences of *PRTN3* were investigated. An association with GPA was demonstrated by *PRTN3* A564G variation in the promoter region affecting a transcription factor-binding site ($P < 0.00001$, OR = 4.2) (36). The association of *PRTN3* has been reported in two GWAS of AAV. Subgroup analysis revealed that *PRTN3* SNP rs62132295 and rs62132293 were significantly associated with AAV, especially PR3-AAV or GPA ($P = 2.6 \times 10^{-5}$, OR = 0.78; $P = 8.60 \times 10^{-11}$, OR = 1.29) (13, 14). As observed for the above-mentioned *HLA* association, the strength of the *PRTN3* SNP signal increased in the PR3-ANCA-positive subgroup, independent of the clinical diagnosis ($P = 2.6 \times 10^{-7}$, OR = 0.73). No association between *PRTN3* and patients with MPA or MPO-AAV was found, which indicates that *PRTN3* only plays a crucial role in the pathogenesis of anti-PR3-positive AAV.

Immunoregulatory Genes for AAV

Protein Tyrosine Phosphatase, Non-Receptor Type 22

PTPN22, located on 1p13.2, encodes lymphoid tyrosine phosphatase (Lyp), a member of the non-receptor class 4 subfamily of the protein tyrosine phosphatase family. *PTPN22* 620W variation causes the substitution of arginine with tryptophan at amino acid residue 620, disturbing the function of *PTPN22*, which is involved in B-cell receptor and T cell receptor signaling, causing autoimmune diseases. It is associated with type 1 diabetes, RA, SLE, and other autoimmune disorders (37, 38).

The 620W allele conferred susceptibility to AAV in a German cohort ($P = 0.002$, OR = 1.75), and the allele frequency was significantly increased in ANCA-positive GPA ($P < 0.0002$) (39). This result has been confirmed in a British cohort ($P = 1.4 \times 10^{-4}$, OR = 1.40) (40) and an Italian cohort ($P = 0.005$, OR = 1. 91)

(41). A meta-analysis of four studies in 1,399 patients of European descent found the association between the *PTPN22* 620W allele and the occurrence and development of AAV (42).

Cytotoxic T Lymphocyte-Associated Antigen-4

CTLA4, located on 2q33.2, encodes an inhibitory glycoprotein expressed on activated T cells, which transmits an inhibitory signal to T cells. CTLA4 competes with CD28 to bind to CD80 and CD86, which function antagonistically. CTLA4 is a negative regulator of T cell activation and inhibits the immune response, while CD28 transmits a stimulatory signal. The expression of CTLA4 on CD4 T cells is increased in GPA (43). Mutations on this gene have been associated with Graves' disease, celiac disease, SLE, and other autoimmune diseases.

Although the *CTLA4* SNP did not show genome-wide association in the GWAS of AAV, the association with AAV was found in candidate gene studies in different populations. *CTLA4* rs3087243 showed an association with AAV *in* 641 British AAV cases and 9,115 controls ($P = 6.4 \times 10^{-3}$, OR=0.84). *CTLA4* exon 1 (+49) and 4 (CT60) polymorphisms showed an association with AAV in another independent British cohorts with 222 cases and 629 controls($P_{(+49)} = 0.004$, $P_{(CT60)} = 0.002$, respectively) (40, 44). The similar result was confirmed in a Swedish cohort with 101 cases and 200 controls ($P = 0.049$) (45).

Toll-Like Receptors

TLRs, located on chromosome 3p21.2, encode a family of innate receptors whose specificities are predetermined in the germline. TLRs can recognize microbiological structures and activate immune responses that participate in the development of autoimmune diseases (46). Infection, particularly *Staphylococcus aureus* infection, is considered a potential trigger of AAV, and TLR9 signaling may be involved in disease pathology, favoring models of infectious agents triggering AAV development (47). SNPs in *TLR9* were genotyped in a German cohort comprising 863 patients with AAV and 1,344 healthy controls to investigate the contribution of genetic polymorphisms of *TLR9* to the susceptibility and clinical manifestation. Strong association of *TLR9* genotypes and haplotypes with the subgroups of GPA and MPA was observed, but no association was observed with EGPA. In a cohort of 426 Dutch and British AAV cases, the findings were not replicated (21). TLR9 signaling may be involved in disease pathology, favoring models of infectious agents triggering AAV development.

Fc Gamma Receptors

FCGRs are a group of proteins expressed on the surface of different cell types with different affinities for the Fc portions of different IgG subclasses. The key role of Fc receptors is the regulation of inflammatory and immune responses. *FCGR3B* encodes FcgRIIIB, which is expressed by neutrophils and eosinophils and is important in the tethering of neutrophils to immune complexes and the clearance of immune complexes (48). The *FCGR3B* copy number may play a role in the development of SLE or other autoimmune diseases (49).

In a study consisted with two independent cohorts of individuals with GPA from Britain and France, strong association between *FCGR3B* copy numbers and risk of GPA was shown. In British patients, low *FCGR3B* copy number was found to be associated with GPA ($P = 0.015$, OR = 2.46), but in French GPA patients, the association with high *FCGR3B* copy number was identified ($P = 0.002$, OR = 0.28) (50). The study also explored the relationship between *FCGR3B* and MPA in a separate British cohort and observed a significant association between low *FCGR3B* copy number and MPA($P = 0.013$, OR = 2.56). Later, the results were replicated in a large Chinese cohort with 139 AAV patients and 564 controls ($P = 0.040$, OR = 1.72) with the result that low *FCGR3A* CNVs were significantly associated with AAV susceptibility ($P = 0.042$, OR = 2.64) (51).

Possible associations of SNPs of FCGR2A, FCGR2B, FCGR3A and the FCGR3B haplotype NA1/NA2 and EGPA have been discussed in a recent study, which included 130 EGPA patients and 181 controls. FCGR3B haplotype NA1/NA2 was found to be associated with relapse-free survival of EGPA that relapse-free survival was significantly lower in carriers of FCGR3B haplotype NA1-NA1 than the others (P = 0.029). MPO-ANCA+ EGPA subgroup was also associated with relapse(P = 0.032) while no association was observed in MPO-ANCA- EGPA subgroup(P = 0.68) (52).

Interleukin-10

IL-10 is an anti-inflammatory cytokine produced by T helper cells, and its expression increases in patients with AAV (53). Two small studies in German and Caucasian cohorts proposed an association of *IL-10* SNP (–1082) in GPA (54); interestingly, one study also demonstrated a signal for the *IL-10* SNP in 36 patients with MPA ($P < 1.00 \times 10^{-6}$) (55). In a large German cohort of 403 patients with GPA, 103 patients with EGPA and 507 controls, none of the *IL-10* polymorphisms were associated with GPA, but *IL-10* -3575/-1082/-592 TAC haplotype was highly significantly associated with ANCA-negative EGPA ($P = 3.00 \times 10^{-5}$, OR = 2.16) (56).

Interleukin 2 Receptor Subunit Alpha

IL2RA encodes the high-affinity IL-2 receptor, which is represented not only on the surface of T cells, but also on activated B cells, NK cells, and monocytes (57). The IL2-IL2RA pathway is critical for the physiological function of the immune system; survival, proliferation, and activation of T cells and development of a normal Treg repertoire (58). An association has been reported in a UK cohort of 670 patients with AAV for the *IL2RA* SNP rs41295061 ($P = 1.22 \times 10^{-2}$) (59), but no correlation between *IL12RA* and AAV was found during the replication stage of the first GWAS validation.

Other Susceptible Genes

Defensin Beta 4A

DEFB4 is an antibiotic peptide. The copy number variation of *DEFB4* reportedly causes susceptibility to inflammatory disorders (60). Higher *DEFB4* copy number was demonstrated to be significantly higher in a small cohort of 112 Chinese patients with AAV than in controls, which supported the role of the defensin system in autoimmunity (61).

Rho GTPase-Activating Protein 18

ARHGAP18 belongs to a family of Rho GTPase-activating proteins that modulate cell signaling. The subgroup analysis of

the first GWAS for AAV revealed that *ARHGAP18* was associated with GPA ($P = 3.3 \times 10^{-7}$, OR = 0.84) and PR3-ANCA AAV ($P = 5.2 \times 10^{-8}$, OR = 0.87) (13).

BCL2-Like11 and MIR4435-2HG(MORRBID)

BCL2L11 encodes BIM, which belongs to the BCL-2 protein family and is crucial for controlling apoptosis, immune homeostasis, and autoimmune diseases (62, 63). *MORRBID* encodes a long non-coding RNA that regulates BIM transcription, controls eosinophil apoptosis, and is dysregulated in hypereosinophilic syndrome (64). In the GWAS of EGPA, SNPs near *BCL2L11* and *MORRBID* were identified to be associated with EGPA ($P = 9 \times 10^{-11}$, OR = 1.66) (16).

Thymic Stromal Lymphopoietin

TSLP is located on 5q22.1. Its product, TSLP, is released by stromal and epithelial cells in response to inflammatory stimuli, which drives eosinophilia and enhances TH2 responses that are associated with immunity in various inflammatory diseases, including asthma, allergic inflammation, and chronic obstructive pulmonary disease (65). *TSLP* SNP rs1837253 which lies immediately upstream of TSLP was observed to be EGPA susceptibility variant ($P = 5.2 \times 10^{-11}$, OR = 1.42) (16).

ETS Proto-Oncogene 1

ETS1, located on chromosome 11q24.3, encodes a member of the ETS family of transcription factors. ETS1 inhibits the differentiation of Th17 cells and induces the development of Tregs. Genetic variation may cause an increase in Th17 in patients with GPA, causing inflammation of small vessels. A GWAS in a Chinese population reported that *ETS1* SNP rs1128334 was significantly associated with SLE (66). In order to observe whether this gene was associated with other autoimmune diseases, a Japanese study which contained 466 patients with AAV and 1099 healthy controls was carried out. *ETS1* SNP rs1128334 was genotyped and found to be associated with GPA ($P = 0.030$, OR = 1.54) and PR3-ANCA AAV ($P = 0.021$, OR = 1.72) (67).

Telomerase Reverse Transcriptase and Desmoplakin

TERT is the catalytic subunit of telomerase and contributes to the maintenance of telomere length (68). *TERT* rs2736100A is associated with shorter leukocyte telomere length (69). TERT also has an anti-apoptotic effect, and a decreased expression of TERT associated with the risk allele may result in the enhancement of apoptosis (70). With the evidence that a proportion of T cells show short telomeres in patients with GPA and an enhancement in neutrophil apoptosis in patients with AAV, *TERT* has been proposed as a susceptibility gene for AAV (71). In a Japanese cohort comprising 544 patients with AAV and 5558 controls, the frequency of *TERT* rs2736100A alleles was significantly increased in MPA ($P = 2.3 \times 10^{-2}$, OR= 1.38) and MPO-AAV ($P = 0.15\times 10^{-2}$, OR = 1.33) (72). *DSP* rs2076295G showed similar results to *TERT* rs2736100A ($P = 0.69\times 10^{-2}$, OR = 1.32) in MPO-AAV ($P = 0.011$, OR = 1.26) (72). DSP reportedly modulates Wnt/β-catenin signaling, which is involved in cell proliferation, differentiation, immune response, and carcinogenesis. Wnt signaling plays a role in autoimmune diseases and may also be associated with the onset of AAV.

TABLE 3 | Summary of genetic associations with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis excluding those derived from genome-wide association studies.

Gene	Variation	Disease	Populations
HLA	DQA1*0302, DQB1*0303, DPB1*04:01, DRB1*0901, DRB1*1101, DRB1*13:02, HLA–DPA1, HLA–DQA2	AAV (GPA, MPA, EGPA)	Worldwide
RING	rs213210, rs213209, and rs213208	GPA	German
RXRB	rs6531	GPA	German
CD226	rs763361	GPA	German
CTLA4	rs3087243, CT60	GPA, MPA and EGPA	British, Swedish
DEFB4	High CNVs	AAV	Chinese
DSP	rs2076295G	MPA	Japanese
ETS1	rs1128334	GPA	Japanese
FCGR3B	Low CNVs, High CNVs	AAV	French, British, Chinese
IL10	IL10 -3575/-1082/-592 TAC haplotype	EGPA(ANCA-)	German
IL2RA	rs41295061	AAV	British
MUC5B	rs35705950	AAV-ILD	Japanese
PTPN22	rs2476601, C1858T	GPA, MPA	German, Italian, British
TLR-9	rs352162, rs352140, rs352139 CTC, TCT	GPA, MPA, EGPA	German, British, Dutch
TERT	rs2736100A	MPA	Japanese

All the genes associated with AAV were summarized in **Table 3**.

CONCLUSION

In the past 20 years, genetic studies, including GWAS and candidate gene studies, have identified numerous key loci that are associated with the risk of AAV. These genes have not only provided novel etiological clues to deepen our understanding of AAV but also novel therapeutic and prophylactic biomarkers for new approaches to treat and prevent AVV. Biomarker-targeting therapies and preventing recurrence in the future may be more effective. With the increasing number of GWAS being conducted, it is desirable to combine these findings and improve the statistical power. To date, GWAS for AAV have been performed in the European population; in the future, more GWAS with large samples in different populations and different subgroups of AAV are required to reveal the pathogenesis of the disease and identify reliable biomarkers for precision medicine.

AUTHOR CONTRIBUTIONS

YS is the investigator for the review article and has contributed to the concept and planning of the article, collection of data, and reporting of the work described. WL, HH, MC, and TY

contributed to the collection of data, and reporting of the work described. All authors contributed to the article and approved the submitted version.

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Risk of Stroke in Systemic Necrotizing Vasculitis: A Nationwide Study Using the National Claims Database

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Objective: Evidences indicate that the risk of stroke is increased in autoimmune rheumatic diseases. This study aimed to investigate the incidence of stroke in patients with systemic necrotizing vasculitis (SNV) using the national health database.

Methods: Data were obtained from the Korean National Claims database between 2010 and 2018 to identify incident SNV [anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV) and polyarteritis nodosa (PAN)] cases. The standardized incidence ratio (SIR) and incidence rate ratio (IRR) were calculated to estimate the risk of stroke in patients with SNV compared to the general population and among disease subgroups. Time-dependent Cox's regression analysis was performed to identify risk factors for stroke.

Results: Among 2644 incident SNV cases, 159 patients (6.0%) were affected by stroke. The overall risk of stroke was significantly higher in patients with SNV compared to the general population (SIR 8.42). Stroke event rates were the highest within the first year of SNV diagnosis (67.3%). Among disease subgroups, patients with microscopic polyangiitis (MPA) exhibited higher IRR compared to PAN (adjusted IRR 1.98). In Cox's hazard analysis, older age and MPA were associated with higher risk of stroke [hazard ratio (HR) 1.05 and 1.88], whereas the administration of cyclophosphamide, azathioprine/mizoribine, methotrexate, and statins were protective in stroke (HR 0.26, 0.34, 0.49, and 0.50, respectively).

Conclusion: A considerable number of SNV patients experienced stroke, especially in the early phase of disease. Older age and MPA diagnosis were associated with elevated risk of stroke, while the administration of immunosuppressive agents and statins was beneficial in preventing stroke.

Keywords: systemic necrotizing vasculitis, anti-neutrophil cytoplasmic antibody-associated vasculitis, polyarteritis nodosa, stroke, incidence, microscopic polyangiitis

INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a rare systemic inflammatory disorder causing necrotizing organ injury within small vessels, which is typically associated with myeloperoxidase-ANCA or proteinase 3-ANCA (1). AAV is divided into three distinct diseases according to the clinical, laboratory, and histological characteristics: microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (2, 3). AAV can affect any organ of the body and various clinical symptoms occur depending on the organs affected. On the other hand, polyarteritis nodosa (PAN), which is classified as systemic vasculitis involving the medium-sized vessels, also causes necrotizing vasculitis (4). Owing to the pathologic similarities, AAV and PAN are traditionally considered to comprise a group of systemic necrotizing vasculitis (SNV) (5).

Stroke is defined as a medical condition of acute and focal functional impairment of the brain, retina, or spinal cord; it is associated with significant mortality and disability (6, 7). Stroke is generally divided into hemorrhagic and ischemic subtypes; ischemic stroke is the most common type, accounting for up to 70% of cases (8, 9). Several common risk factors for the occurrence of stroke, such as older age, sex, hypertension, dyslipidemia, diabetes mellitus, atrial fibrillation, and smoking, have been suggested (9, 10). Besides, a growing body of evidence has suggested that chronic inflammation and high degree of inflammation are crucial factors associated with increased vascular thrombosis in autoimmune rheumatic diseases by triggering the coagulation cascade (11, 12). In this context, previous studies have reported an increased risk of stroke in patients with large vessel vasculitis, such as Takayasu arteritis (TA) and giant cell arteritis (GCA). A retrospective study by Hwang et al. demonstrated that more than 10% of patients with TA experienced ischemic stroke (13), and Nesher et al. showed that cranial ischemic complications are common in patients with GCA, implying that patients with systemic vasculitis are at a high risk of developing stroke (14). Mechanistically, the development of atherosclerotic lesions is considered an important cause of stroke (15). Similarly, in the pathogenesis of SNV, subclinical atherosclerosis is accelerated as a consequence of systemic and localized inflammation and deterioration of helper T (Th) cell balance (16). Based on these findings, even though it is possible that the risk of stroke is increased in patients with SNV, the incidence of stroke in patients with SNV has not been investigated in detail. Therefore, this study was conducted to investigate the incidence of stroke in SNV using a nationwide database.

MATERIALS AND METHODS

Data Extraction From the Health Insurance and Review Agency Database

The principal diagnosis and comorbidities [based on International Classification of Diseases (ICD)-10 codes], clinical information (age, sex, geographic area), and the administered medication of the patients were obtained from the Health Insurance and Review Agency (HIRA) database. The HIRA database is a nationwide claims data repository that includes information of medical service utilization of an individual, which is comprised of: general and providers' information, the use of healthcare services, diagnosis, and the data of drug prescription (17). This information is submitted by medical institutions to the Korean government in order to request monetary reimbursement and is integrated and recorded in the HIRA database. A schematic figure depicting the generation and utilization of HIRA data is described in **Supplementary Figure 1**. By using this database, it is possible to access the information of the entire population (nearly 50 million patients) that is enrolled in the National Health Insurance System of Korea (18).

To identify SNV patients, the respective ICD-10 codes for MPA (M31.7), GPA (M31.3), EGPA (M30.1), and PAN (M30.0) were used. Patients were diagnosed with SNV when they were first registered with the corresponding ICD-10 codes for AAV or PAN in a general or tertiary hospital and were prescribed with glucocorticoids (betamethasone, dexamethasone, methylprednisolone, prednisone, prednisolone, hydrocortisone, triamcinolone, budesonide, and deflazacort) during the follow-up period (19). The date on which the diagnosis of SNV was first registered in the HIRA database was defined as the index date, and the medications of immunosuppressive agents (glucocorticoids, cyclophosphamide, rituximab, azathioprine/mizoribine, and methotrexate), antiplatelet agents (aspirin, clopidogrel), and statins that were prescribed after the diagnosis of SNV were also counted.

The entire data of the study population were first extracted from the HIRA database between January 2008 and December 2018, and a 2-year washout period was given to exclude patients with the diagnosis of SNV prior to the study period. This study was approved by the ethics review board of Severance Hospital, and the requirement to obtain informed consent was waived owing to the retrospective study design (IRB approval number: 4-2019-0177).

Identification of SNV Cases With Stroke and Comorbidities

SNV patients with incident stroke were defined as those admitted to a hospital and newly registered with ICD-10 codes for stroke (I60-I64) in the HIRA database after the diagnosis of SNV (20, 21). The follow-up duration was defined as the index date of SNV diagnosis to the date of stroke occurrence in patients with stroke and until the last follow-up date for patients without stroke. Comorbidities for stroke searched included hypertension (I10-15), diabetes mellitus (E10-14), atrial fibrillation/flutter (I48), and dyslipidemia (E78) within 1 year of the index date of SNV diagnosis.

Estimation of Stroke Incidence in Patients With AAV Using In-Hospital Data

For internal validation purposes, the incidence of stroke was estimated by reviewing the medical records of 193 patients first diagnosed with AAV in Severance Hospital between December 2000 and December 2018. All patients were classified into AAV

subgroups according to the 2007 European Medicines Agency algorithm for AAV and the descriptions provided by the 2012 Chapel Hill Consensus Conference. Demographic data, including age, sex, body mass index, and ANCA serotypes, and laboratory data, as well as the diagnosis and stroke subtypes were investigated. Birmingham Vasculitis Activity Score (version 3) (BVAS) and Five Factor Score (2009) were calculated as previously described (22, 23).

Statistical Analysis

Continuous and categorical variables are presented as mean ± standard deviation and frequencies (percentage), respectively, and were compared using Student's t-test and the chi-square test. To compare the incidence of stroke between SNV patients and the general population, the standardized incidence ratio (SIR) adjusted by age was calculated using data from the 2006 Korean Center for Disease Control & Prevention Report for the general population (24). The risk of stroke among the disease subgroups of SNV was compared by calculating age- and sex- adjusted incidence rate ratio (IRR) and 95% confidence interval (CI) using Poisson regression analysis with an offset for person-years. The cumulative incidence of stroke was calculated using the Kaplan–Meier method, and differences among disease subgroups were determined by the log-rank test. Factors associated with the incidence of stroke were investigated using Cox's regression model by including medication usage as time-dependent covariates. SAS Enterprise Guide version 9.4 (SAS Institute Inc., Cary, NC) was used for all statistical analyses, and a p-value < 0.05 was considered statistically significant.

RESULTS

Baseline Characteristics of SNV Patients With and Without Stroke

A total of 2984 AAV and PAN cases were found in the HIRA database between 2010 and 2018. Of these, 340 patients who had been previously diagnosed with stoke (I60-I64) were excluded. In the remaining 2644, 159 (6.0%) patients were identified to have stroke after the diagnosis of SNV was established (**Figure 1**). The mean follow-up duration was 1.2 years for patients with stroke and 3.4 years for patients without stroke. When comparing the clinical characteristics between patients with stroke and those without, the age at diagnosis was higher (mean age 66.50 and 56.02, p<0.001) in patients with stroke. In addition, the diagnosis of MPA was more frequent in patients with stroke than in those without (49.7% vs. 33.2%, p<0.001). Regarding comorbidities, hypertension, diabetes mellitus, and dyslipidemia were more common in SNV patients with stroke, whereas the proportion of patients administered glucocorticoids ≥ 1 year, azathioprine/mizoribine, methotrexate, and statins was significantly higher in

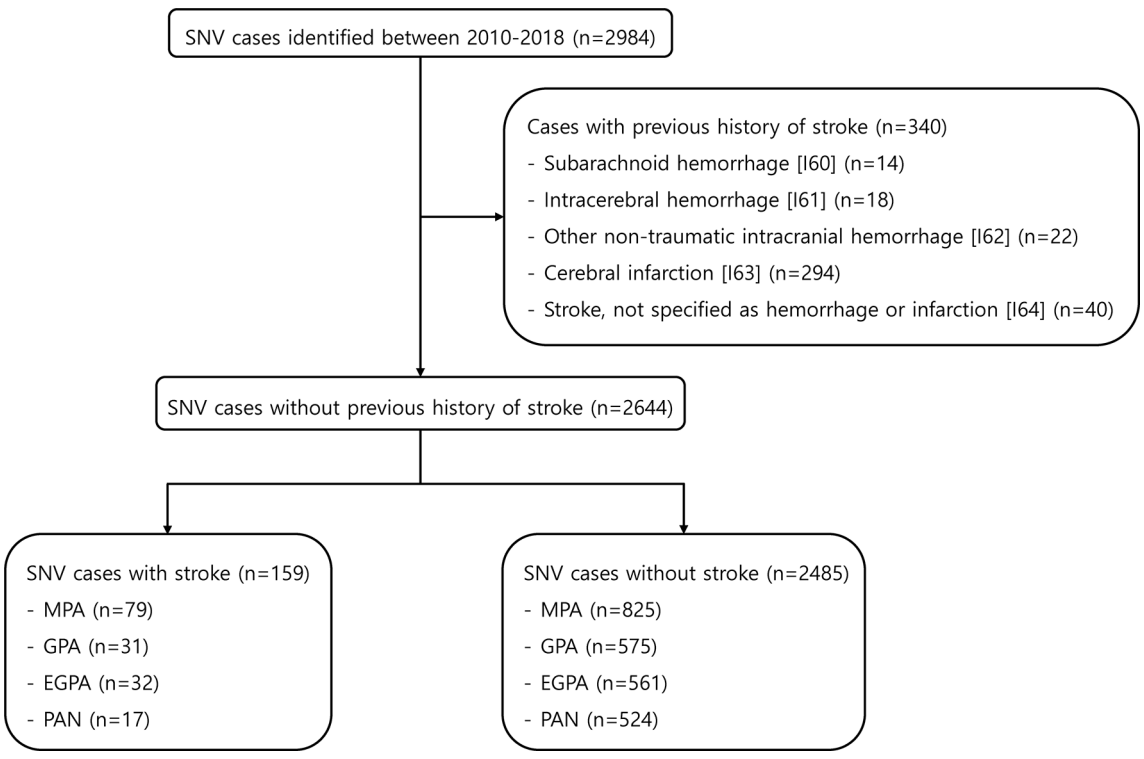

FIGURE 1 | Selection of the study population from the HIRA database. HIRA, Health Insurance and Review Agency; SNV, systemic necrotizing vasculitis; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; PAN, polyarteritis nodosa.

patients without stroke (**Table 1**). Significant differences were present regarding baseline clinical characteristics of SNV subgroups. In particular, patients with MPA were older and the proportion of patients with comorbidities at baseline was higher in MPA compared to other subgroups of SNV (**Supplementary Table 1**).

Risk of Stroke in SNV Patients Compared to the General Population

The age distribution of SNV patients when they experienced stroke is shown in **Figure 2**. The total number of stroke events increased with age, and the peak was observed at 65-74 years (n=59). In those aged ≥75 years, it was slightly lower than that in patients aged 65-74 years (n=52).

Compared to the general population, the overall risk of stroke was significantly higher in patients with SNV (SIR 8.42, 95% CI 7.16-9.84); this was found to be the same in MPA, GPA, EGPA, and PAN. The overall risk of stroke was highest in patients with MPA (SIR 16.26, 95% CI 12.87-20.26). Moreover, when patients were divided according to age, such as age ≤ 54 years, age 55-74 years, and age ≥ 75 years, the SIR for stroke was highest in patients aged ≤ 54 years; this decreased as the age of patients increased (**Supplementary Figure 2**).

Type and Incidence of Stroke After SNV Diagnosis

Among the 159 patients with stroke, cerebral infarction [I63] (n=115) was the most common stroke subtype, accounting for 72.3% of patients. Furthermore, the incidence of stroke was highest in patients with MPA (n=79, 49.7%), followed by those with EGPA, GPA, and PAN. Regarding the time for stroke occurrence after SNV diagnosis, 107 (67.3%) patients developed

TABLE 1 | Baseline clinical characteristics of SNV patients with stroke and those without stroke.

	Total, n=2644	Patients with stroke, n=159	Patients without stroke, n=2485	p-value
Age at diagnosis (years)	56.65 ± 16.84	66.50 ± 13.34	56.02 ± 16.85	<0.001
≤54	1052 (39.8)	28 (17.6)	1024 (41.2)	<0.001
55-74	1225 (46.3)	84 (52.8)	1141 (45.9)	
≥75	367 (13.9)	47 (29.6)	320 (12.9)	
Sex, n (%)				0.885
Male	1191 (45.1)	73 (45.9)	1118 (45.0)	
Female	1453 (54.9)	86 (54.1)	1367 (55.0)	
Diagnosis [ICD-10 code], n (%)				<0.001
MPA (M31.7)	904 (34.2)	79 (49.7)	825 (33.2)	
GPA (M31.3)	606 (22.9)	31 (19.5)	575 (23.1)	
EGPA (M30.1)	593 (22.4)	32 (20.1)	561 (22.6)	
PAN (M30.0)	541 (20.5)	17 (10.7)	524 (21.1)	
Geographic Area, n (%)				0.219
Seoul	1197 (45.3)	64 (40.3)	1133 (45.6)	
Outside Seoul	1447 (54.7)	95 (59.7)	1352 (54.4)	
Comorbidities [ICD-10 code]				
Hypertension [I10-15]				<0.001
No	1477 (55.9)	60 (37.7)	1417 (57.0)	
Yes	1167 (44.1)	99 (62.3)	1068 (43.0)	
Diabetes mellitus [E10-14]				<0.001
No	1813 (68.6)	87 (54.7)	1726 (69.5)	
Yes	831 (31.4)	72 (45.3)	759 (30.5)	
Atrial fibrillation/flutter [I48]				0.299
No	2576 (97.4)	153 (96.2)	2423 (97.5)	
Yes	68 (2.6)	6 (3.8)	62 (2.5)	
Dyslipidemia [E78]				0.005
No	1409 (53.3)	67 (42.1)	1342 (54.0)	
Yes	1235 (46.7)	92 (57.9)	1143 (46.0)	
Medication usage, n (%)				
Immunosuppressive agents				
Glucocorticoid usage ≥ 1 year	1225 (46.3)	31 (19.5)	1194 (48.1)	<0.001
Cyclophosphamide	1124 (42.5)	62 (39.0)	1062 (42.7)	0.399
Rituximab	252 (9.5)	8 (5.0)	244 (9.8)	0.064
Azathioprine/mizoribine	991 (37.5)	29 (18.2)	962 (38.7)	<0.001
Methotrexate	431 (16.3)	8 (5.0)	423 (17.0)	<0.001
Antiplatelet agents				
Aspirin	608 (23.0)	32 (20.1)	576 (23.2)	0.430
Clopidogrel	232 (8.8)	10 (6.3)	222 (8.9)	0.318
Statins	996 (37.7)	45 (28.3)	951 (38.3)	0.015

Values are expressed as mean (standard deviation) or number (percentages).
SNV, systemic necrotizing vasculitis; ICD, International classification of diseases; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; PAN, polyarteritis nodosa.

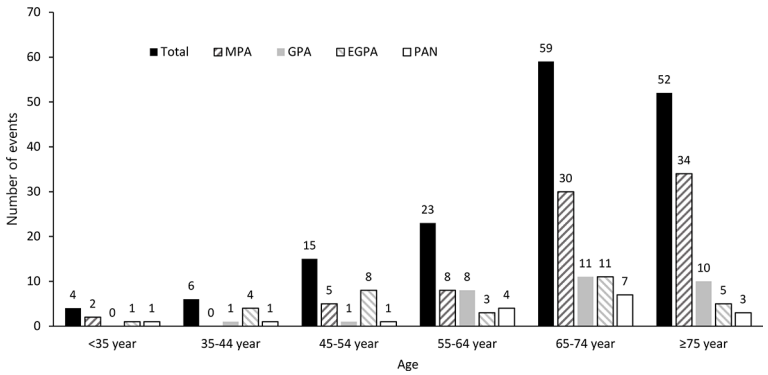

FIGURE 2 | Age distribution of SNV patients on stroke occurrence. The incidence of stroke events was estimated by dividing age into 10-year intervals. SNV, systemic necrotizing vasculitis; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; PAN, polyarteritis nodosa.

stroke within 1 year of SNV diagnosis. Meanwhile, the incidence of stroke gradually decreased over time after SNV was diagnosed, showing a similar pattern in all disease subgroups (**Table 2**).

When we compared the cumulative incidence rate of stroke according to disease subgroups, patients with MPA, GPA, and EGPA had a higher incidence of stroke than those with PAN (all p<0.001). The cumulative incidence rate of stroke was highest in patients with MPA; it was 6.79 (95% CI 5.06-8.52), 10.02 (95% CI 7.65-12.39), and 19.46 (95% CI 11.56-27.36) at 1, 4, and 9 years, respectively (**Figure 3**). On comparing the incidence rates among the SNV subgroups, MPA patients exhibited the highest risk (crude IRR 4.85 vs. PAN, 95% CI 2.87-8.19); the risk of stroke was consistently higher in MPA patients than in PAN even after adjusting for age and sex (adjusted IRR 1.98, 95% CI 1.15-3.40) (**Supplementary Table 2**).

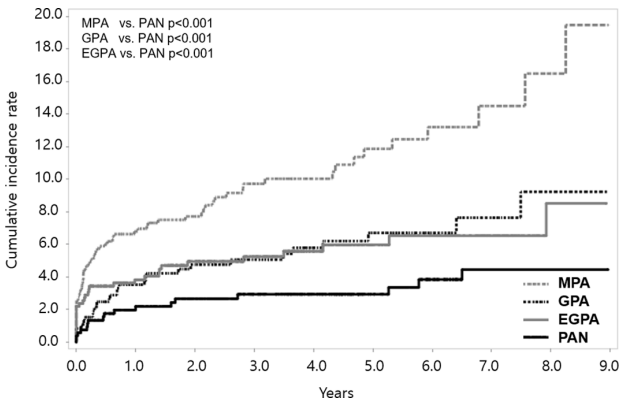

FIGURE 3 | Cumulative incidence rate of stroke according to SNV subgroups. Among the SNV subgroups, the cumulative incidence rate of stroke was found to be highest in MPA. SNV, systemic necrotizing vasculitis; MPA, microscopic polyangiitis; PAN, polyarteritis nodosa; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis.

Risk Factors Associated With the Occurrence of Stroke in SNV

In Cox's hazard regression analysis, older age, the diagnosis of MPA, GPA, and EGPA, and comorbidities of hypertension,

TABLE 2 | Type and incidence of stroke events after SNV diagnosis.

Type of stroke	Total	MPA	GPA	EGPA	PAN
Subarachnoid hemorrhage [I60]	14 (8.8)	7 (8.9)	3 (9.7)	4 (12.5)	0 (0.0)
Intracerebral hemorrhage [I61]	21 (13.2)	11 (13.9)	2 (6.5)	6 (18.7)	2 (11.8)
Other non-traumatic intracranial hemorrhage [I62]	6 (3.8)	2 (2.5)	1 (3.2)	2 (6.3)	1 (5.9)
Cerebral infarction [I63]	115 (72.3)	57 (72.1)	25 (80.6)	20 (62.5)	13 (76.5)
Stroke, not specified as hemorrhage or infarction [I64]	3 (1.9)	2 (2.5)	0 (0.0)	0 (0.0)	1 (5.9)
Total	159 (100.0)	79 (100.0)	31 (100.0)	32 (100.0)	17 (100.0)
Time of stroke occurrence after SNV diagnosis					
<1 year	107 (67.3)	56 (70.9)	19 (61.3)	22 (68.8)	10 (58.8)
1-3 years	29 (18.2)	13 (16.5)	6 (19.4)	6 (18.8)	4 (23.5)
>3 years	23 (14.5)	10 (12.7)	6 (19.4)	4 (12.5)	3 (17.7)

Values are expressed in number (percentages).

SNV, systemic necrotizing vasculitis; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; PAN, polyarteritis nodosa.

diabetes mellitus, and dyslipidemia were associated with increased risk of stroke in the unadjusted analysis. On the other hand, the administration of cyclophosphamide, azathioprine/mizoribine, and methotrexate were inversely associated with stroke occurrence. However, in an adjusted analysis, age at diagnosis [hazard ratio (HR) 1.05, 95% CI 1.03-1.06, p<0.001], the diagnosis of MPA (HR 1.88, 95% CI 1.08-3.26, p=0.025), and cyclophosphamide (HR 0.26, 95% CI 0.14-0.49, p<0.001), azathioprine/mizoribine (HR 0.34, 95% CI 0.18-0.65, p=0.001), methotrexate (HR 0.49, 95% CI 0.24-0.99, p=0.046), and statin (HR 0.50, 95% CI 0.32-0.80, p=0.004) administration were independent risk factors of stroke (**Table 3**).

Clinical Characteristics of AAV Patients With Stroke in the Hospital Database

We reviewed the medical records of patients diagnosed with AAV in the hospital and who experienced stroke. Among the 193 patients included, 12 (6.2%) experienced stroke during the follow-up. The baseline age and BVAS at diagnosis among patients with stroke were 64.5 years and 15.6, respectively, and the follow-up duration was 18.7 months. MPA (58.3%) was the most common diagnosis, followed by GPA (33.3%) and EGPA (8.3%). The disease duration after AAV diagnosis was less than 1 year in 9 (75.0%) patients, and ischemic subtype (91.7%) accounted for the majority of stroke events (**Table 4**).

DISCUSSION

Even though the incidence of stroke is increased in patients with autoimmune rheumatic diseases (12), in patients with SNV, the incidence is unclear. In this study, using a nationwide claims database, we showed that a considerable number of patients with SNV (6.0%) are affected with stroke after disease diagnosis and

TABLE 3 | Factors associated with the risk of stroke in SNV patients.

	Crude hazard ratio			Adjusted hazard ratio		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
Age at diagnosis	1.06	(1.05-1.07)	<0.001	1.05	(1.03-1.06)	<0.001
Sex						
Male	1.08	(0.79-1.47)	0.646	0.99	(0.73-1.37)	0.977
Female		1.00 (ref)			1.00 (ref)	
Diagnosis						
MPA	3.69	(2.18-6.25)	<0.001	1.88	(1.08-3.26)	0.025
GPA	1.88	(1.04-3.41)	0.036	1.20	(0.65-2.19)	0.559
EGPA	1.89	(1.05-3.40)	0.035	1.25	(0.69-2.27)	0.460
PAN		1.00 (ref)			1.00 (ref)	
Geographic area						
Seoul		1.00 (ref)			1.00 (ref)	
Outside Seoul	1.26	(0.92-1.73)	0.149	1.25	(0.90-1.72)	0.179
Comorbidities						
Hypertension						
No		1.00 (ref)			1.00 (ref)	
Yes	2.42	(1.76-3.34)	<0.001	1.30	(0.91-1.87)	0.151
Diabetes mellitus						
No		1.00 (ref)			1.00 (ref)	
Yes	2.02	(1.48-2.76)	<0.001	1.26	(0.91-1.77)	0.170
Atrial fibrillation/flutter						
No		1.00 (ref)			1.00 (ref)	
Yes	1.59	(0.71-3.60)	0.263	0.95	(0.43-2.11)	0.898
Dyslipidemia						
No		1.00 (ref)			1.00 (ref)	
Yes	1.83	(1.33-2.51)	<0.001	1.29	(0.91-1.83)	0.149
Medication usage[†]						
Immunosuppressive agents						
Glucocorticoid usage ≥ 1 year	0.82	(0.47-1.41)	0.467	1.15	(0.65-2.02)	0.628
Cyclophosphamide	0.34	(0.18-0.63)	<0.001	0.26	(0.14-0.49)	<0.001
Rituximab	0.21	(0.01-3.35)	0.266	0.12	(0.01-1.97)	0.136
Azathioprine/mizoribine	0.36	(0.19-0.69)	0.002	0.34	(0.18-0.65)	0.001
Methotrexate	0.40	(0.20-0.82)	0.012	0.49	(0.24-0.99)	0.046
Antiplatelet agents						
Aspirin	1.33	(0.82-2.14)	0.249	1.20	(0.73-1.98)	0.480
Clopidogrel	1.56	(0.73-3.34)	0.250	1.12	(0.52-2.42)	0.767
Statins	0.77	(0.50-1.19)	0.243	0.50	(0.32-0.80)	0.004

[†]*Medication usage was selected as time-dependent covariates.*

SNV, systemic necrotizing vasculitis; CI, confidence interval; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; PAN, polyarteritis nodosa.

TABLE 4 | Baseline characteristics of AAV patients with stroke using in-hospital data.

Patient number	Age at initial diagnosis	Sex	BMI, kg/m2	BVAS	FFS (2009)	ANCA serotypes	ESR, mm/h	CRP, mg/L	Total cholesterol, mg/dL	Diagnosis	AAV disease duration (months)	Subtype of stroke
# 1	75	Male	26.0	24	3	ANCA (-)	90	95.8	150	MPA	1.0	Ischemic
# 2	56	Female	23.1	18	2	MPO-ANCA or p-ANCA (+)	120	94.3	165	MPA	0.0	Ischemic
# 3	76	Female	21.6	19	3	MPO-ANCA or p-ANCA (+)	116	178.7	119	MPA	4.0	Ischemic
# 4	73	Female	24.0	18	2	MPO-ANCA or p-ANCA (+)	110	216.5	113	GPA	0.0	Ischemic
# 5	77	Female	17.1	24	3	MPO-ANCA or p-ANCA (+)	120	137.5	152	MPA	1.0	Ischemic
# 6	57	Female	17.0	22	2	MPO-ANCA or p-ANCA (+)	62	146	134	MPA	1.0	Ischemic
# 7	69	Female	25.2	19	3	MPO-ANCA or p-ANCA (+)	94	83.4	99	MPA	46.0	Ischemic
# 8	71	Female	23.4	12	1	MPO-ANCA or p-ANCA (+)	61	100	146	GPA	4.0	Ischemic
# 9	75	Female	21.1	6	3	MPO-ANCA or p-ANCA (+)	90	109	117	GPA	0.0	Ischemic
# 10	31	Male	20.2	3	1	MPO-ANCA or p-ANCA (+)	48	12.03	202	MPA	0.0	Ischemic
# 11	60	Female	21.4	7	0	ANCA (-)	6	1	269	EGPA	109.0	Hemorrhage
# 12	54	Male	23.0	15	2	ANCA (-)	25	12.8	308	GPA	58.0	Ischemic

AAV, anti-neutrophil cytoplasmic antibody-associated vasculitis; BMI, body mass index; BVAS, Birmingham vasculitis activity score; FFS, five factor score; ANCA, anti-neutrophil cytoplasmic antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MPA, microscopic polyangiitis; MPO, myeloperoxidase; P, perinuclear; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis.

the risk of stroke was significantly higher than that among the general population (SIR 8.42). Most stroke events in patients with SNV presented as ischemic subtypes, similar to the finding in the general population. In addition, among the disease subgroups, patients with MPA were most commonly affected with stroke. Importantly, these findings were reproduced through the in-hospital data, which revealed comparable results. Finally, the administration of immunosuppressive agents and statins showed clinical benefits in the prevention of stroke.

The risk of stroke is increased in autoimmune rheumatic diseases, such as rheumatoid arthritis and systemic lupus erythematosus (25). Likewise, the risk of stroke was significantly higher in SNV patients than in the general population. Even though the exact cause of this finding is unclear, the elevated risk of stroke may be attributable to the development of premature atherosclerosis in chronic inflammatory diseases (26). Otherwise, the acceleration of atherosclerosis can be also facilitated by direct vascular involvement in inflammation, which is mediated by antibodies or immune complexes (27). Additionally, the overproduction of proinflammatory cytokines, chemokines, and coagulation proteins may promote endothelial dysfunction and activation, resulting in atherosclerosis promotion (28). Finally, the activation of macrophages in autoimmune rheumatic diseases, which plays an important role in the progression of atherosclerotic lesions and the formation of foam cells, could be relevant to increased risk of stroke in SNV (29).

Age is an important factor contributing to stroke in the general population (9). According to the 2006 data from the Korean Center for Disease Control & Prevention Report, the incidence of stroke increases with age in the general population (21). Consistently, the baseline age of SNV patients with stroke was 66.5 years, which was approximately 10 years higher than that of SNV patients without stroke. Moreover, in patients with SNV, the incidence of stroke was much higher than that of the normal population of the same age group, implying that age and disease itself are independent risk factors of stroke. Collectively, it could be suggested that careful observation for the occurrence of stroke and the implementation of preventive measures of stroke are required in patients with SNV.

Several studies have investigated the risk of stroke in patients with SNV, but with inconsistent results. Although Mourguet et al. reported that patients with GPA and MPA experienced ischemic stroke four times more frequently than the general population, the observations by Berti et al. revealed that stroke incidence was eight times higher in patients with AAV than in the general population (30, 31). On the contrary, the risk of stroke was not apparent in patients with GPA (32). The discordant results could be related to the difference in disease subgroups analyzed, the number of patients included, and the type of stroke investigated. The results from our study are noteworthy as we observed the increased risk of stroke in SNV using a largest dataset, and the incidence of stroke in Asia has not been described in the literature. Nevertheless, given that geographic variations are present regarding the epidemiology

of vasculitis and that racial and ethnic disparity should be considered in the incidence of stroke (33, 34), further research is necessary to validate the findings from our study.

Intriguingly, the observations from our study have demonstrated that 67.3% of patients have experienced stroke within 1 year of diagnosis, and the incidence of stroke gradually decrease during the disease course, which was found to be similar to the data from the United Kingdom (35). In addition, in a time-dependent Cox's hazards regression analysis, treatment with immunosuppressive agents of cyclophosphamide, azathioprine/mizoribine, and methotrexate was inversely correlated with the occurrence of stroke. Of note, even though statistical significance was not demonstrated, the adjusted HR of developing stroke in patients treated with rituximab, which is now considered as a first line therapy for AAV, was found to be remarkably low. Owing to the fact that patients generally have the highest disease activity on initial presentation, and the reason to prescribe immunosuppressive drugs are used to maintain adequate disease control, it could be suggested that tight disease control is essential to reduce the occurrence of stroke in patients with SNV. This assumption could be supported by the analysis of in-hospital data, which showed that patients who developed stroke in the early disease phase generally had a higher disease activity (BVAS) and inflammatory markers of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Nonetheless, given that patients with SNV are usually followed-up more shortly after initial diagnosis, a possibility of detection bias from closer follow-up could not be excluded.

Among the disease subgroups, the incidence of stroke was the highest in MPA and lowest in PAN, and Cox's hazards regression analysis revealed that the risk of stroke was increased in MPA compared to PAN. Although the direct cause of the difference of stroke according to disease subgroups is unknown, previous reports have described that stroke is known to be a relatively rare complication of PAN (36). Moreover, the higher incidence of stroke, especially in MPA, could be associated with the fact that MPA is an aggressive disease with severe renal and pulmonary symptoms and has a poor prognosis compared to other SNV subtypes (37, 38). Accordingly, considering the high inflammatory burden in MPA, it could be speculated that the occurrence of stroke is higher in MPA than in other disease subtypes. Meanwhile, it is reported that patients with MPA in South Korea and Japan most often manifest with renal involvement (39, 40), and a characteristic pathologic lesion in renal biopsy in MPA is rapidly progressive glomerulonephritis (5, 41). Based on the fact that impaired renal function is also a potential risk factor of stroke, the higher incidence of renal involvement in MPA could be associated with the increased risk of stroke (42).

Comorbidities that could influence in the occurrence of stroke include hypertension, diabetes mellitus, dyslipidemia, and atrial fibrillation (24). In this study, no significant association between the comorbidities investigated and stroke was observed in Cox's hazards regression analysis. Surprisingly, we found that the use of statins was associated with reduced risk of stroke. Several different effects of statins could be considered in attenuating the risk of stroke in SNV. First, statins are effective in preventing atherosclerosis by reducing circulating low-density lipoproteins, and the attenuation of atherosclerotic lesions by statins could lead to a decreased incidence of stroke (43). Second, statins are able to provide non-lipid dependent vascular protective effects. Statins could maintain endothelial function through upregulation of endothelial nitric oxide synthase or antioxidant defense systems (44). Furthermore, statin inhibits recruitment, adhesion, and migration of inflammatory cells to help stabilize the inflamed vasculature (45). Third, statins could also provide immunomodulatory effects by suppressing T cell activation, which plays a crucial role in vascular inflammatory disorders (46). Statins could act as inhibitors of MHC-II-mediated T-cell activation by influencing MHC-II expression through IFN-γ and suppress Th1 responses (47). Furthermore, it was also shown that statins affect Th17 cell differentiation and directly inhibit IL-17 production in CD4+ cells (48). Finally, it was shown that treatment with statin could hamper macrophage activation, as well as reducing the expression of inflammatory cytokines and chemokines (45, 49). Therefore, the preventive effect of statins in stroke could be also mediated by controlling vascular dysfunction and providing immunomodulatory effects.

Although this is the first study to evaluate the risk of stroke in patients with SNV using a nationwide database, several drawbacks are present in this study. First, due to the inherent limitations of the HIRA data, we failed to analyze the effect of common modifiable risk factors for stroke, such as smoking, alcohol, and body mass index. Second, besides age, sex, and medication usage, other clinical data, such as disease activity and organ involvement, were not identifiable through the HIRA database. In addition, laboratory data regarding cholesterol profiles and its changes, as well as ANCA serotypes and inflammatory markers of ESR and CRP, were also not accessible. Third, even though a Cox's hazard regression analysis was performed to identify risk factors associated with stroke in SNV, large disparities of baseline characteristics were present between the disease subgroups. Fourth, the risk of stroke in SNV compared to the general population was assessed using a nationwide report as a reference, and the comorbidities of patients were investigated only by using the ICD-10 codes. Thus, the relationship between comorbidities in SNV and stroke should be better verified in future studies. Fifth, in this study, both the occurrence of ischemic and hemorrhagic stroke was analyzed as stroke event. However, as the underlying pathogenesis of ischemic and hemorrhagic stroke is different, the precise mechanism leading to increased stroke in SNV remains to be further investigated. Finally, owing to the relatively small number of patients with stroke and those treated with rituximab, the effect of rituximab therapy in reducing stroke might not have reached statistical significance.

CONCLUSION

In conclusion, the results of our study demonstrated that the overall incidence of stroke is elevated in patients with SNV,

especially in the early phase of disease diagnosis. In addition, differences were found regarding the incidence of stroke according to disease subgroups. Moreover, the use of immunosuppressive agents and statins was associated with decreased risk of stroke. Our results indicate that special attention should be given regarding the incidence of stroke in patients with SNV, and adequate disease control and use of statins could be beneficial in minimizing the risk of stroke.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics review board of Severance Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SA and S-WL designed the report and wrote the paper. SA, MH, and JY participated in data acquisition and interpretation. SA, Y-BP, and S-WL drafted and revised the manuscript. SA, JY, and S-WL designed the concept and approved the final paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.629902/full#supplementary-material

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REVIEW
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New Insights Into Novel Therapeutic Targets in ANCA-Associated Vasculitis

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Biologics targeting inflammation-related molecules in the immune system have been developed to treat rheumatoid arthritis (RA), and these RA treatments have provided revolutionary advances. Biologics may also be an effective treatment for anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis, particularly in patients with resistance to standard treatments. Despite the accumulation of clinical experience and the increasing understanding of the pathogenesis of vasculitis, it is becoming more difficult to cure vasculitis. The treatment of vasculitis with biologics has been examined in clinical trials, and this has also enhanced our understanding of the pathogenesis of vasculitis. A humanized anti-interleukin-5 monoclonal antibody known as mepolizumab was recently demonstrated to provide clinical benefit in the management of eosinophilic granulomatosis with polyangiitis in refractory and relapsing disease, and additional new drugs for vasculitis are being tested in clinical trials, while others are in abeyance. This review presents the new findings regarding biologics in addition to the conventional immunosuppressive therapy for ANCA-associated vasculitis.

Keywords: anti-neutrophil cytoplasmic autoantibody, anti-neutrophil cytoplasmic autoantibody-associated vasculitis, biologics, cytokine, cytokine-immunological terms

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INTRODUCTION

Anti-neutrophil cytoplasmic autoantibodies (ANCAs) are the major serological markers of primary systemic necrotizing small vessel inflammation or ANCA-associated vasculitis (AAV), including granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (1, 2). ANCAs for proteinase 3 (PR3) are more prevalent in patients with GPA, and ANCAs for myeloperoxidase (MPO) are more prevalent in patients with MPA, but there is substantial overlap. Regarding the clinical utility of ANCAs' specificity in the classification of the forms of AAV, ANCA specificity is more likely to be associated with the patient's genetic predisposition (3), treatment effect(s) (4), the risk of recurrence (5, 6), and the prognosis (7) than the clinical diagnosis. Distinct cytokine profiles were identified for PR3-AAV and MPO-AAV with GPA, MPA, and EGPA (8).

These differences in circulating immuno-mediators are strongly associated with ANCA specificity, not clinical diagnosis, and the heterogeneity of AAV subtypes is associated with the clinical phenotypes identified in the traditional clinical classification of GPA and MPA (8). Clinical trial results and clinical practice data have formed the foundation of the management of AAV, which is based on the disease

severity. In 2016, the European League Against Rheumatism (EULAR) updated their recommendations for the management of primary small- and medium-vessel vasculitis, including the management of AAV (9). Glucocorticoids are a central component of the management of AAV in induction and maintenance therapy and are not sufficient by themselves, especially in the context of organ invasion. For active AAV, the current treatment recommendation is to first administer high doses of glucocorticoids, followed by a gradual decrease in steroids (10).

Cyclophosphamide is also used in combination with steroids to induce remission in AAV, but its metabolites are toxic to the bladder and reproductive organs and may cause malignancy and infertility in the long term (11). In non-immediately life-threatening AAV, there was no significant difference in remission rates between treatment groups receiving daily oral versus intravenous pulsed cyclophosphamide therapy regimens, and the total dose of cyclophosphamide was reduced in the intravenous pulse group (11).

Advances in induction therapy have transformed AAV from a life-threatening disease to a chronic disease with relapse. Relapse is not uncommon, occurring in 30%–50% of patients with AAV within 5 years of onset, and often 12–18 months after treatment with immunosuppressive agents is discontinued (12). In AAV, maintenance therapy is recommended to prevent relapse after achieving remission by the induction therapy. Maintenance immunosuppressive agents such as cyclophosphamide, mycophenolate mofetil, and azathioprine are used in combination to prevent relapse after the successful induction of remission. More recently, biologic agents have begun to play an important role in the induction of clinical remission and the maintenance of remission in severe AAV. The biologic rituximab is indicated for remission induction and the management of severe and relapsed GPA and MPA, and data suggest a role for pre-emptive fixed-interval rituximab maintenance therapy in remission treatment (13, 14). Treatment with the biologic mepolizumab also provided a significantly greater number of weeks of remission and higher remission rates than a placebo when it was used as maintenance therapy for EGPA (15). Other biologics are either being tested in clinical trials or have failed because their effectiveness could not be verified, or they produced unacceptable side effects. However, it is possible that using biologics could reduce the rate of side effects caused by steroids in the treatment of AAV, by providing a new mechanism of action. This review presents new insights into novel therapeutic targets in AAV.

REVIEW

ANCAs

ANCAs are autoantibodies against cytoplasmic antigens expressed on the primary granules of neutrophils and the lysosomes of monocytes. The primary granules of neutrophils contain a series of antimicrobial proteins, including lysozyme, MPO, neutral serine proteinases (PR3, elastase, and cathepsin G), and acid hydrolases (cathepsins B and D). Autoantibodies can develop against any of these proteins, but the most clinically important antibodies are against MPO and PR3. During the active phase of AAV, ANCA is usually immunoglobulin G (IgG), but other immunoglobulin classes (IgM and IgA) have also been reported. PR3-ANCA is most frequently associated with GPA (75%), and MPO-ANCA is associated with MPA (60%) and EGPA (30%). MPO-ANCA is also associated with renal limited vasculitis (80%) (**Table 1**) (16, 17).

Atypical ANCAs, which do not react with either PR3 or MPO (positive by indirect immunofluorescence and negative by enzyme-linked immunosorbent assay), have been identified in a range of nonvasculitic conditions: inflammatory bowel diseases, autoimmune diseases, and malignancies.

PR3- and MPO-ANCA have also been found in chronic infections such as endocarditis, tuberculosis, human immunodeficiency virus, hepatitis C, and bartonellosis. The presence of both anti-MPO and anti-PR3 antibodies in the same patient is very rare and suggests drug-induced vasculitis (16). In a subgroup of patients (10%) whose clinical and pathological features are consistent with AAV, the test result for ANCA remains negative. Although these patients may have a similar clinical course and response to treatment, ANCA-negative patients are more likely to have renal-limited disease or less severe systemic disease (16).

Cytokine Production

Th1/Th2

Berti et al. reported that the circulating cytokine profiles were significantly different between patients with PR3-ANCA and those with MPO-ANCA (8), and they noted that compared to the PR3-ANCA group, nine biomarkers were higher in their MPO-AAV group: interleukin (IL)-6, -15, and -18, granulocyte-macrophage colony-stimulating factor, chemokine (C-X-C) ligand 8/IL-8, chemokine (C-C motif) ligand 17/THYMUS, activation-regulated chemokine, and IL-18 binding protein. In contrast, four biomarkers were higher in MPO-AAV than in

TABLE 1 | The positive rate of ANCA in vasculitis.

ANCA-associated vasculitis	MPO-ANCA	PR3-ANCA
GPA	20%	75%
MPA	60%	30%
EGPA	30%	5%
Renal limited vasculitis	80%	10%
Drug-induced vasculitis	90%	10%

ANCA, antineutrophil cytoplasmic autoantibodies; EGPA, eosinophilic granulomatosis with polyangiitis; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; MPO, myeloperoxidase; PR3, proteinase 3.

PR3-AAV: soluble IL-6 receptor, soluble tumor necrosis factor (TNF) receptor II, neutrophil gelatinase-associated lipocalin, and soluble intercellular adhesion molecule-1. In active AAV, the cellular infiltration in kidney, lung, and nasal tissues is composed mainly of macrophages, T cells, and B cells (18). In a validation study of T-cell markers in 38 renal biopsies from patients with AAV, Kidder et al. observed an increased number of CD8+ T cells in the periglomerular and interstitial areas in kidneys with a crescent-shaped histology. They also reported a significant correlation between the number of CD8+ T cells and the glomerular filtration rate. Despite the low number of T lymphocytes infiltrating the glomerulus, CD8+ T cells were more predominant than CD4+ T cells (19). In contrast, T cells stimulated with MPO showed much less or no proliferative response in both patients and healthy controls (20). Thus, at least in GPA, the pathogenic role of T cells at the effector stage of AAV is not fully established, because CD4+ T cells, based on their cytokine profile and associated functions, have been shown to have two distinct types: Th1 and Th2 cells (**Figure 1**). In localized GPA, T cells in nasal inflammatory infiltrates were shown to express the Th1 marker CD26 (21). In addition, more interferon-alpha (IFNα)-positive cells were detected in the nasal inflammatory infiltrate in localized GPA than in generalized GPA. The tissues of eosinophilic infiltration and eosinophilia are also common in GPA and EGPA, and thus polarization to the Th2 profile would be expected in EGPA.

Those findings were accompanied by increases in spontaneous IFNα and IL-10 produced by peripheral blood mononuclear cells in patients with localized GPA compared to generalized GPA, whereas high levels of IL-4 mRNA were detected in the nasal inflammatory infiltrates of the patients with generalized GPA. A Th2 environment in this phenomenon was confirmed in nasal granulomas of systemic GPA. In immunohistochemistry staining, IFNα was not detected in nasal biopsies of 10 patients with systemic active GPA, but IL-4 was upregulated (22). These cells are likely to be T cells, especially Th2 cells and eosinophils. These data thus support potential differences in Th responses between local and systemic GPA in nasal granulomatous lesions. Csernok et al. reported the expression of IFNα mRNA in nasal granulomas with systemic GPA, but IL-4 mRNA was only expressed in two of five patients (23). Komocsi et al. also demonstrated Th1-like cytokine production and features suggestive of CD4+ T-cell-mediated cytotoxicity. In GPA, CD4+CD28- T cells are recruited from the blood to granulomatous lesions *via* interaction with CD18, followed by cytokine secretion to promote monocyte accumulation and granuloma formation (24).

Th17

The Th17 T helper subset is involved in the defense against extracellular bacteria and fungi and has been implicated in autoimmune diseases (25). Th17 cells express the transcription

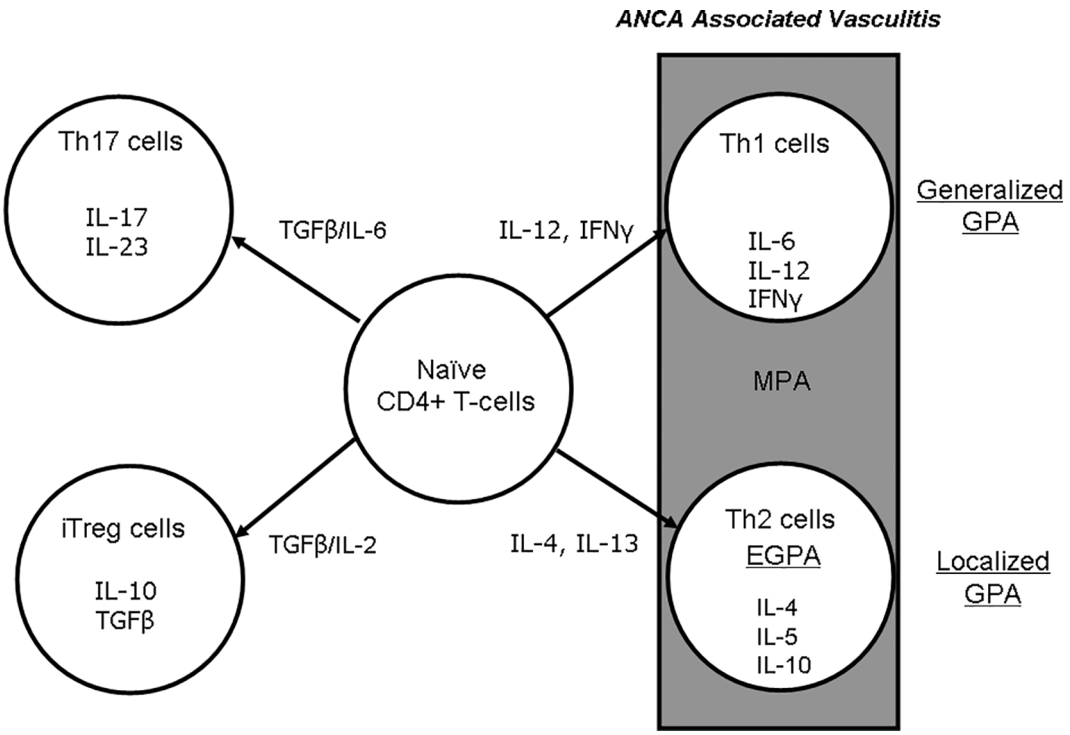

FIGURE 1 | Differentiation of naïve CD4+ T cells to Th1, -2, -17, and iTreg cells and effects on cytokine production in AAV. AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; EGPA, eosinophilic granulomatosis with polyangiitis; GPA, granulomatosis with polyangiitis; IFNγ, interferon gamma; IL, interleukin; iTreg, inducible regulatory T cell; MPA, microscopic polyangiitis; TGFβ, transforming growth factor beta.

factor RORγt (retinoic acid-related orphan receptor gamma t) and produce IL-17A–F, which are centered on IL-17A (26). IL-23 is responsible for the expansion and maturation of the Th17 subset (27). Th17 cells are also inhibited by Th1 and Th2 cytokines.

IL-17A acts on monocytes/macrophages and functions directly *via* ligation to the receptors expressed on monocytes (28). In peripheral blood, IL-17A induces the release of pro-inflammatory mediators from macrophages, and IL-17A induces a high percentage of Th17 cells in patients with AAV compared to healthy controls (29). Thus, IL-17A, as an important regulator and initiator of inflammation, mediates both innate and adaptive immunity. IL-17A not only defines the Th17 subset; it is also a logical therapeutic target for diseases induced by Th17-dominant autoimmune responses.

Nogueira et al. described that in a cohort of 28 patients with acute AAV and 65 patients in AAV, serum IL-17A levels were significantly higher than in healthy controls, with a similar pattern for serum IL-23 levels (30). Other researchers have found no evidence of this expansion Th17 response in AAV (31, 32). For example, Krohn et al. detected no difference in serum IL-17A levels between 70 AAV patients and the healthy controls, but interestingly, they observed a significant increase in IL-17C levels (33). Velden et al. demonstrated the presence of IL-17 in kidney tissues by immunohistochemical staining, and they reported that the percentage of Th1-like Th17 cells was higher in patients in the acute phase of the disease or in untreated remission compared to healthy controls (34). Thus, IL-17A, as an important regulator and initiator of inflammation, mediates both innate and adaptive immunity. Neutrophils can also induce Th17 cell chemotaxis, making this cell axis even more interesting as a potential target in the treatment of AAV (35).

C5 and C5a Receptor

C5a, an anaphylatoxin of complement, is a potent inflammatory mediator (36). Alternative classical and lectin pathways converge on the activation of C5, releasing C5a and C5b. C5a is a potent chemoattractant for neutrophils, and ligation of C5aR/CD88 by C5a activates neutrophils. Neutrophil priming increases the availability of ANCA antigens at the surface, where they interact with ANCA and activate neutrophils. When stimulated with proinflammatory cytokines, the terminal C5a/C5aR axis is activated, generating an automatic amplification loop that triggers an acute necrotizing vasculitis process from the primed neutrophils (resulting from their interaction with ANCA) (37). C5a acting on C5aR is a potent neutrophil chemoattractant and agonist (38), increases neutrophil adhesion, induces neutrophil degranulation, and generates reactive oxygen intermediates; C5a activates vascular endothelial cells *via* the C5aR, promoting cell retraction and increasing vascular permeability (39). Historically, the role of complement was thought to be limited in AAV, as renal biopsies rarely showed complement deposition and the absence of hypocomplementemia. Other clinical studies have supported these findings by showing activation of the alternative pathway in the cardiovascular system and deposition of complement components of the alternative pathway in tissues. Active AAV patients with renal involvement had higher levels of C3a and C5a in the circulation (40). These results could be the potential target in the treatment of AAV.

Treatment Strategy for AAV

Immunosuppressive Agents

Cyclophosphamide

Cyclophosphamide is associated with reduced ovarian reserve, ovarian failure, and male infertility (41–45). A review of the prednisolone reduction regimens published in major trials has shown that on average, the cyclophosphamide doses 10 mg (after 19 weeks) and 7.5 mg (after 21 weeks) have been achieved (11, 46–51). Cyclophosphamide is usually administered orally or as pulse therapy for 3–6 months, and after remission is achieved, the cyclophosphamide is changed to a less-toxic agent. Intravenous cyclophosphamide pulse therapy may allow the reduction of the cumulative dose and consequently the toxicity. The strategy was demonstrated in the CYCLOPS study (11). Remission rates in patients with systemic but not immediately life-threatening AAV were not significantly different between those who received daily oral cyclophosphamide and those who received pulsed cyclophosphamide. With the pulsed cyclophosphamide regimen, patients could receive a cumulative dose of cyclophosphamide half that of the daily oral cyclophosphamide regimen, but the dose of corticosteroids remained the same. However, pulsed cyclophosphamide is associated with a higher risk of recurrence than daily oral cyclophosphamide (52).

Methotrexate and Mycophenolate Mofetil

Methotrexate (20-25 mg/week, orally or parenterally) can be used in place of cyclophosphamide in patients with less severe disease and normal renal function (53–62). There have been trials using either methotrexate or mycophenolate mofetil as remission-inducing agents in patients with AAV (60–62). Therefore, methotrexate should only be considered in cases of non-organ-threatening disease. So far, the induction trials using methotrexate are generally larger and have a higher grade of evidence than trials using mycophenolate mofetil. The two randomized controlled trials (RCTs) with mycophenolate mofetil to date have been conducted primarily in patients with MPA (61, 62). MPA often affects renal function, and methotrexate is not indicated in such situations. These studies did not include patients with pulmonary hemorrhage or central nervous system involvement, and mycophenolate mofetil should not be used routinely in life-threatening situations.

Biological Agents

Representative clinical trials and the pathogenesis of AAV by the inhibition of binding of biological agents for AAV with biologics are shown in **Table 2** and **Figure 2**.

Anti-CD20 Monoclonal Antibody

A rationale for B-cell activation in AAV has been based on the pathogenicity of ANCA (63). B cells also acts as antigen-presenting cells for T lymphocytes (64), and they produce pro-inflammatory cytokines that are useful for T-cell hyperactivity and neutrophil priming (**Figure 1**). These mechanisms suggest that B-cell depletion could be a potential target for AAV therapy.

TABLE 2 | Clinical trials with biological agents in ANCA associated vasculitis.

Drug(s)	Status	Allocation	n	Inclusion criteria	Primary endpoint	Trial no.	Last Update Posted	Public-ation results
Rituximab	Completed	RCT	197	GPA	Disease remission for 6 mos.	NCT00104299	4/21/2017	13
Rituximab	Completed	RCT	44	AAV with renal involvement	Disease remission and rates of relapse at 24 mos.	ISRCTN28528813	3/6/2015	65
Abatacept	Terminated	RCT	7	AAV	Relapse rate over 24 mos.	NCT00482066	3/29/2015	
Abatacept	Recruiting	RCT	63	GPA	Reduce the treatment failure rate for 12 mos.	NCT02108860	3/3/2019	
Abatacept	Completed	N/A	20	GPA	Adverse events up to 3 yrs + 4 mos.	NCT00468208	1/18/2016	86
Belimumab	Completed	RCT	106	GPA and MPA	Time to first relapse up to 4 yrs	NCT01663623	4/17/2018	75
Belimumab + Rituximab	Recruiting	RCT	30	AAV with PR3 ANCA positivity	Time to PR3 ANCA negativity	NCT03967925	6/9/2020	
Infliximab/ Rituximab	Completed	N/A	20	AAV	Partial or complete remission of the vasculitis	NCT00307593	11/19/2007	
Infliximab	Completed	Non-RCT	37	GPA, MPA, and renal limited vasculitis	Disease remission for 52 wks	NCT00753103	9/16/2008	
Alemtuzumab	Unknown	RCT	24	GPA	Response and a severe adverse event for 6 mos.	NCT01405807	7/29/2011	
Etanercept	Completed	RCT	180	GPA	Sustained remissions for 27mos.	NCT00005007	12/28/2007	79
Avacopan	Completed	RCT	300	AAV	Disease remission for 26 wks	NCT02994927	6/22/2020	97
Avacopan	Completed	RCT	42	AAV	Disease remission at 12 wks	NCT02222155	11/16/2016	95
Avacopan	Completed	RCT	67	AAV	Achieving ≥50% reduction in disease activity at 12 wks	NCT01363388	6/27/2020	96
Eculizumab	Withdrawn	RCT	0	AAV	Change in disease activity as measured at 12 wks	**NCT01275287**	2/23/2017	
Mepolizumab	Active, not recruiting	Case Only	300	EGPA	Adverse events up to 2 yrs	NCT03557060	9/10/2018	
Mepolizumab	Completed	RCT	136	EGPA	Each category of accrued duration of remission for 52 wks	NCT02020889	1/31/2018	15
Mepolizumab	Completed	N/A	10	EGPA	Attain remission rate for 52 wks	NCT00716651	6/15/2012	
Mepolizumab	Completed	N/A	10	EGPA	Adverse events for approx. 44 wks	NCT00527566	3/22/2017	
Benralizumab/ Mepolizumab	Recruiting	RCT	140	EGPA	Remission rate at 36 and 48 wks	NCT04157348	10/8/2020	

AAV, ANCA-associated vasculitis; EGPA, eosinophilic granulomatosis with polyangiitis; GPA, granulomatosis with polyangiitis; mos., months; MPA, microscopic polyangiitis; N/A, not available; PR3 ANCA, proteinase 3 antineutrophil cytoplasmic antibody; RCT, randomized controlled trial; wks, weeks; yrs, years.

Rituximab

Rituximab, an anti-CD20 IgG1 chimeric mouse/human monoclonal antibody, was approved by the U.S. Food and Drug Administration in 2011 for the management of AAV. Rituximab in AAV has been used in two RCTs, the RAVE (Rituximab for the Treatment of Wegener's Granulomatosis and Microscopic Polyangiitis) trial and the RITUXVAS trial, an international, randomized, open-label trial comparing rituximab-based regimens with standard cyclophosphamide/azathioprine regimens in the treatment of active "generalized" AAV (13, 14). There were several differences between these two trials: the RITUXVAS trial included new patients with severe renal disease, whereas the RAVE trial included new and recurrent patients with well-maintained renal function. Oral cyclophosphamide was used as a comparator in RAVE, and pulsed cyclophosphamide was used in the RITUXVAS trial. In both trials, rituximab was given as four infusions with a body surface area of 375 mg/m^2, but in the RITUXVAS trial, cyclophosphamide was given in addition to rituximab for two to three cycles. Prednisolone was tapered and discontinued by 5 months in the RAVE trial, but was reduced to 5 mg by 6 months in the RITUXVAS study and continued for the remainder of the study. In the RAVE trial, after 18 months, 39% of patients in the rituximab group and 33% of those in the control group maintained complete remission (65). In that study, there was no significant difference in the total number of serious adverse events between the two groups. In the RITUXVAS trial, the rate of sustained remission was 76% in the rituximab group and 82% in the control group (14). A long-term analysis of these patients showed that relapse occurred at 24 months in 42% of the rituximab group and 36% of the cyclophosphamide group (66). Rituximab can be used in patients who are intolerant to cyclophosphamide, in patients of reproductive age, and in patients who have had substantial prior exposure to cyclophosphamide. However, the efficacy of rituximab monotherapy in severe disease has not been established and there is no consensus on the appropriate dosing regimen.

Anti-IL-5 Monoclonal Antibody

One of the hallmarks of EGPA is eosinophilic inflammation. IL-5, a major cytokine that activates eosinophils, has been postulated to be involved in the pathogenesis of EGPA. IL-5 is produced by Th2 cells and induces differentiation and maturation of human eosinophils (67). IL-5 also inhibits eosinophil apoptosis (68).

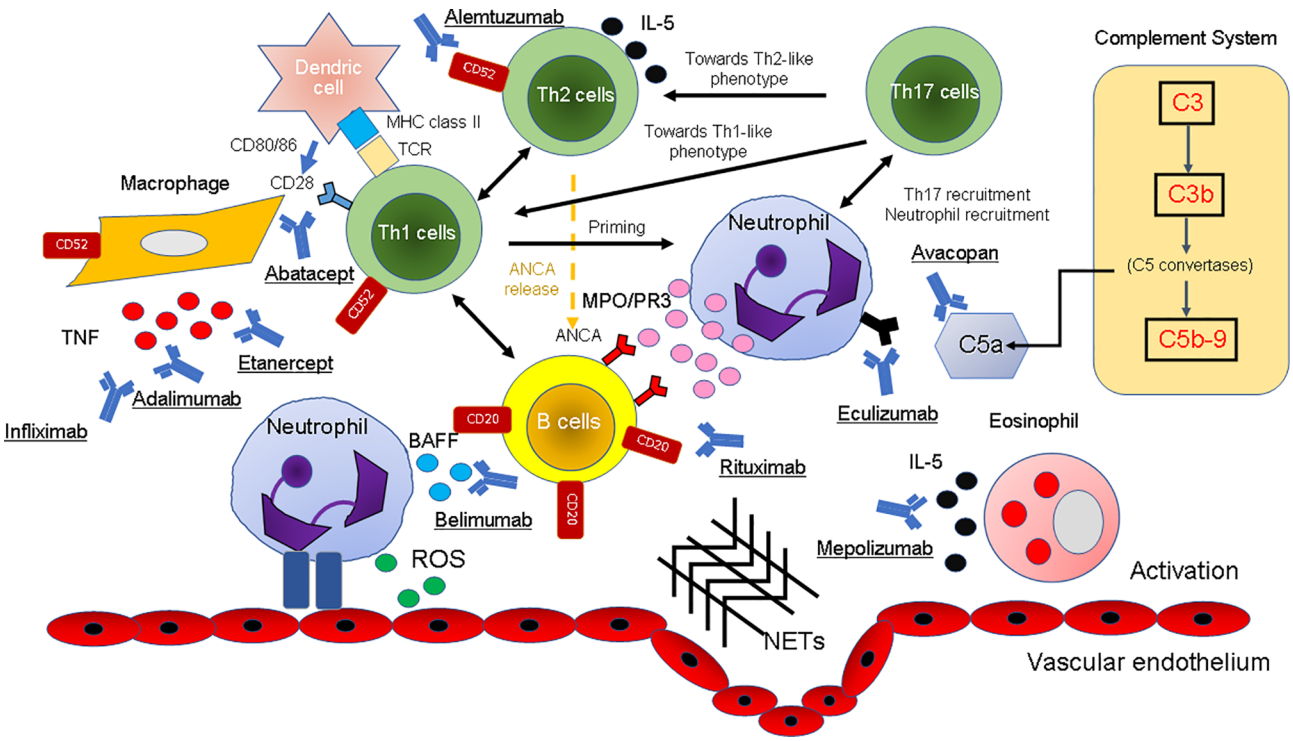

FIGURE 2 | The pathogenesis of AAV and the inhibition of the binding of biological agents. The mechanism of the onset of AAV in the immune and complement systems in various cell types is illustrated. *T cells:* Etanercept, adalimumab, and infliximab as TNF-α inhibitors and alemtuzumab as an anti-CD52 inhibitor have been used for the treatment of AAV because they block the cytokine signaling pathway. By containing CTLA4, abatacept blocks the engagement of CD28 on the surface of T cells by B7.1 (CD80) or B7.2 (CD86) on the surface of APCs or B cells with its ligand, thereby inhibiting T-cell activation. *B cells:* B cells also act as APCs for T lymphocytes, and they produce pro-inflammatory cytokines that are useful for T-cell hyperactivity and neutrophil priming. Immunotherapies targeting B cells (depletion of B cells with rituximab and blockade of BLyS by belimumab) reduce the recruitment of these effector cells at the site of immune complex deposition, thereby reducing inflammation and tissue damage. *Eosinophils:* IL-5 is produced by Th2 cells and induces differentiation and maturation of human eosinophils. By neutralizing IL-5, mepolizumab inhibits the IL-5 signaling pathway and may be a therapeutic option for patients with EGPA. *Complement system:* Eculizumab and avacopan bind C5 and C5aR with picomolar affinity and inhibit the enzymatic activation by C5 convertases. *Others:* The binding of ANCA to these autoantigens activates neutrophils attached to the vascular endothelium. Degranulation of neutrophils releases ROS and NETs that damage the vascular endothelium. AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; APCs, antigen-presenting cells; BAFF, B-cell activating factor inhibitor; BLys, B-lymphocyte stimulator; NETs, neutrophil extracellular traps; ROS, reactive oxygen species; EGPA, eosinophilic granulomatosis with polyangiitis; IL, interleukin; MPO, myeloperoxidase; PR3, proteinase 3; TNF, tumor necrosis factor.

Mepolizumab

Mepolizumab is a humanized anti-IL-5 monoclonal antibody that shows clinical benefit in the management of refractory and relapsing EGPA (15). In patients with EGPA, mepolizumab resulted in a significantly greater number of weeks in remission and a higher proportion of patients in remission compared to a placebo, thus allowing for reduced glucocorticoid use. In 2015, the U.S. Food and Administration expanded the approved use of mepolizumab to treat EGPA. Limitations of the study on which the approval was based were that less than 10% of patients were ANCA positive at baseline and that no analysis of outcomes according to ANCA status was performed.

B-Cell Activating Factor Inhibitor (BAFF)

BAFF is also involved in Ig class switching and subsequent antibody production *in vivo*. BAFF promotes B-cell proliferation and splenic B-cell survival *in vitro* (69, 70). Soluble BAFF binds to three different TNF receptors: B cell maturation antigen (BCMA), transmembrane activator, calcium regulator, and cyclophilin ligand interactor (TACI), and BAFF-R (BR3). BCMA and TACI, but not BAFF-R, bind to another B receptor for a proliferation-inducing ligand that is also a cell survival ligand (71). When BAFF binds to its high affinity BAFF-R, the NF-κB pathway (both classical and non-classical pathways) and the MAPK pathway are activated, leading to the expression of genes essential for B cell survival (72).

Belimumab

Belimumab is a human monoclonal IgG1 antibody against B-lymphocyte-stimulating factor (B-Lys) against BAFF and is being investigated as a therapeutic option for AAV. B-lymphocyte-stimulating (B-Lys) factor is a cytokine that promotes B-cell survival, maturation, and differentiation, and B-Lys has been observed to be elevated in the serum of patients with AAV, particularly patients with GPA (73, 74). Belimumab has been used as a treatment for Lupus. The BREVAS clinical trial is a

phase III multi-center, multinational, randomized and double-blind study evaluating the efficacy and safety of belimumab in combination with azathioprine for the maintenance of remission in GPA and MPA patients (75). This trial demonstrated that the addition of belimumab to a regimen of azathioprine plus low-dose glucocorticoids to maintain remission of AAV did not reduce the risk of recurrent vasculitis. However, patients who received belimumab after remission with rituximab did not experience recurrence of vasculitis. the BREVAS trial has limitations, including sample size (placebo n=52, belimumab n=54). In addition, the number of patients in remission with rituximab who received belimumab was quite low (n=14). Investigation of the maintenance of remission of AAV with belimumab as monotherapy is needed to determine the potential therapeutic benefit of this biologic. Currently, the combination of belimumab and rituximab is being investigated in patients with PR3 ANCA-positive AAV, and the primary endpoint is to compare the time to PR3 ANCA negativity with rituximab alone.

Anti-TNF-α Antibody

TNF-α is a multifaceted cytokine that plays a central role in inflammation and leads to the production of a wide range of other pro-inflammatory cytokines and chemokines in the kidney disease (76). In AAV, TNF-α mRNA expression is increased in leukocytes and renal tissue, indicating its involvement in the pathophysiology of the disease (77, 78). These data suggested that TNF-α induces the expression of autoantigens involved in vasculitis on the leukocyte cell membrane, preparing the cells for the effects of ANCAs.

Etanercept

Etanercept, a p75 Fc fusion protein against TNF-α, is a biologic agent that has been studied in AAV. A randomized controlled trial (Wegener's Granulomatosis Etanercept Trial [WGET]) compared the ability of treatment with etanercept 25 mg 2×/week plus standard care with that of placebo plus standard care to maintain clinical remission in patients with GPA (79). Of the 174 evaluable patients, 126 (72.4%) achieved sustained remission, while only 86 (49.4%) remained in remission. There was no significant difference between the etanercept and control groups in the rates of sustained remission (69.7% vs. 75.3%) and sustained low-level disease activity (86.5% vs. 90.6%). Analysis of the relative risk of disease relapse during follow-up also showed that there was no significant difference in the incidence of disease relapse between the etanercept and control groups.

Infliximab

Infliximab is a chimeric mouse/human monoclonal antibody against TNF-α and has been used for the treatment of AAV (GPA and MPA). A prospective randomized controlled trial comparing infliximab with rituximab for remission induction in patients with severe refractory GPA showed that both agents were effective in inducing remission, but the general data tended to favor rituximab over infliximab; all non-responders to infliximab were thus switched to rituximab (80). Although no significant results were obtained, the trial's analyses suggested that (*i*) rituximab has a higher response rate and a higher sustained remission rate, but (*ii*) there are some cases in which infliximab is useful.

Adalimumab

Adalimumab is a humanized anti-TNF-α monoclonal antibody that is being studied in a phase II open-label, prospective study in patients with AAV with renal impairment. Adalimumab (40 mg every 2 weeks) plus cyclophosphamide pulsed infusion (compared to cyclophosphamide pulsed infusion alone) showed similar remission-inducing effects and adverse events, but it reduced the dose of glucocorticosteroids in the adalimumab group (81). However, that study had several limitations including a small sample size and the lack of a control group and randomization. At present, all clinical trials of anti-TNF-α antibodies as treatments for AAV have been suspended.

Concerns have been raised regarding the risk of malignancy with anti-TNF-α treatment in AAV, and the results of a subsequent analysis of the WGET trial with an extended period of follow-up (82) suggested that the increased risk of cancer was not significantly different between the intervention and placebo groups compared to the general population, and that this risk could not be attributed solely to etanercept treatment. However, all of the solid malignancies in the etanercept group occurred in patients who received cyclophosphamide, suggesting that etanercept should not be administered after cyclophosphamide because of the increased incidence of malignancy.

CTLA4-Ig

CTLA4-Ig, a soluble fusion protein consisting of the extracellular domain of CTLA4 and the CH2-CH3 domain of IgG modified not to bind the Fc receptor, binds to B7.1 and B7.2 with much higher affinity than CD28 and thus serves as an efficient competitive antagonist of the important B7/CD28 When CD28 on the surface of T cells binds to B7.1 (CD80) or B7.2 (CD86) on the surface of activated antigen-presenting cells (APCs) or B cells (83). It activates signaling pathways that promote T cell survival, leading to the formation of a CD40L (CD40+) receptor on T cells. CD40L (CD154) expression is induced in T cells; CD40L interacts with CD40 on APCs, resulting in further upregulation of MHC and B7 and release of cytokines and other inflammatory mediators (84). In addition, CD40L on activated T cells interacts with CD40 on antigen-specific B cells, which induces B cell proliferation and germinal center formation (85, 86). Costimulation-dependent cell-cell interactions within the germinal center lead to B cell maturation through immunoglobulin isotype switching, somatic mutations, clonal expansion of high-affinity B cells, terminal differentiation into plasma cells, and formation of memory B cells that further activate T cells by expressing B7 and acting as APCs (85, 86). which further activate T cells by expressing B7 and acting as APCs (85–87).

Abatacept

Abatacept is a fusion protein that fuses the Fc region of IgG1 with the extracellular domain of CTLA4 and inhibits the intracellular co-stimulation of T cells. By containing CTLA4, abatacept blocks the engagement of CD28 with its ligand, thereby

inhibiting T-cell activation. Based on the rationale that the blockage of T-cell activation might impact the disease pathogenesis of GPA (88), an open-label trial was conducted to investigate the safety and efficacy of abatacept in patients with non-severe relapsing GPA. The trial's results demonstrated that abatacept treatment induced remission in the majority of patients (80%) and was well tolerated overall (89). Eleven of the 15 patients (73%) were able to withdraw from prednisone. However, the trial had limitations including the small sample size (n=20) and uncontrolled design. The results cannot be generalized to all GPA patients, and because the study excluded patients with severe disease, no conclusions can be reached about the efficacy of abatacept at the current stage.

Anti-CD52 Monoclonal Antibody

Alemtuzumab

Alemtuzumab is a humanized anti-CD52 monoclonal antibody that selectively reduces the peripheral blood concentrations of T lymphocytes, monocytes, and macrophages. Alemtuzumab has been used to treat AAV patients who have discontinued all immunosuppressive drugs except prednisolone 10 mg/day. A small, uncontrolled study of alemtuzumab administered at doses of 4, 10, and 40 mg on consecutive days was reported, and the patients were followed for an average of 5 years (90). The majority of patients (85%) achieved clinical remission, but a significant proportion of these patients (72%) relapsed at a median period of 9 months after the treatment ended. Adverse events such as severe infections, malignancies, and Grave's disease have been reported in patients treated with alemtuzumab (90, 91). Alemtuzumab appears to be able to bring refractory and relapsing AAV into remission. Patients treated in the above-described study had a high incidence of adverse events, particularly serious infections, and many received treatment after suffering significant morbidity with a very poor pre-treatment prognosis. Young patients with relapsing or refractory disease before suffering major vital organ damage may benefit from alemtuzumab. Careful monitoring for infections, thyroid disease (in the long term), and malignancies (over the long term) should be performed in all patients treated with alemtuzumab. In order to recommend alemtuzumab as the standard of care for refractory AAV, randomized controlled trials testing the efficacy of alemtuzumab are needed. However, clinical trials of AAV in alemtuzumab have also been suspended.

Th17 Inhibitors

Antibodies directed against IL-17 and IL-23 (secukinumab and ustekinumab, respectively) are effectively used in other autoimmune conditions such as psoriasis (92), but no studies of these monoclonal antibodies for the treatment of vasculitis have been conducted despite the existence of a clear rationale.

C5 and C5a Receptor Blocking Antibodies

Eculizumab

Eculizumab, a commercial C5 blocking antibody, binds C5 with picomolar affinity and inhibits its enzymatic activation by C5 convertases, possibly through steric hindrance (93). Antibodies directed against C5 have shown remarkable clinical benefits for the diseases paroxysmal nocturnal hemoglobinuria (94) and atypical hemolytic uremic syndrome (95), but a study in AAV was withdrawn without a detailed description (no reference).

Avacopan

Avacopan (formerly known as CCX168) is a novel, orally available, highly selective human C5aR antagonist with no other known pharmacological effects (93). Avacopan does not inhibit the interaction of C5a with its associated receptor C5L2 (also called C5aR2), and avacopan is thought to have anti-inflammatory properties (96). Abacopan has also been shown to exert a protective effect in a mouse model of anti-MPO-induced glomerulonephritis (97). Abacopan, a specific C5aR antagonist, does not inhibit the formation of the terminal complement complex or the membrane attack complex C5b-9, which are required for the elimination of pathogenic endophytic bacteria such as *Neisseria meningitidis*.

The CLEAR and CLASSIC Phase II trials confirmed that treatment with avacopan 30 mg 2×/day is safe and can be used in place of glucocorticoids for the induction of AAV remission (98, 99). The results of the ADVOCATE trial, a large randomized trial comparing avacopan+placebo with standard glucocorticoid therapy, both in combination with cyclophosphamide or rituximab, are awaited for approval and will probably lead to a major breakthrough in the treatment of AAV (100).

CONCLUSION

Biologics play an important role in inducing and maintaining clinical remission in severe ANCA-associated vasculitis. The improved understanding of the disease process gained over the past decade has led to the identification of multiple new targets and strategies to treat this deadly disease. The development of tools to assess the pathogenesis of vasculitis and extensive experience with a series of clinical trials has established a foundation from which new agents can be evaluated.

Rituximab is indicated for the induction of remission and the management of severe recurrent GPA/MPA, although the role of preemptive, fixed-interval therapy with rituximab in maintaining remission has been suggested. Mepolizumab has shown efficacy in the management of severe refractory and relapsing EGPA. Belimumab and avacopan are undergoing clinical trials testing their efficacy and safety in AAV. Although anti-TNF-α biologics are not currently recommended as remission-inducing therapy for AAV, case reports and nonrandomized, open-label trials have provided data that justify the use of these biologics in certain relapsed and refractory cases. However, concerns remain regarding the incidence of malignancies. Abatacept has been well tolerated and a high percentage of patients have achieved disease remission and have been able to discontinue prednisone. Nevertheless, further studies are required to establish the safety and efficacy of these biologics. The clinical efficacy of alemtuzumab has been suggested, but a significant proportion of relapses and a high rate of adverse events such as severe infections were reported for this biologic. All clinical trials for AAV have been suspended at this time.

Strategies to reduce the toxicity associated with treatment without compromising efficacy are important goals for future research. Treatments need to be optimized and customized according to the severity of the patient's disease and the risk of recurrence. This will require a better understanding of the etiology of AAV and the development of powerful biomarkers.

In conclusion, the potential benefits, adverse effects, and risks of biologic agents in the management of AAV should be considered prior to the administration of these drugs for the induction and maintenance of disease remission.

AUTHOR CONTRIBUTIONS

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

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Inhibition of IL-6 in the LCWE Mouse Model of Kawasaki Disease Inhibits Acute Phase Reactant Serum Amyloid A but Fails to Attenuate Vasculitis

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Objective: Kawasaki disease (KD) is the most common cause of acquired pediatric heart disease in the developed world. 10% of KD patients are resistant to front-line therapy, and no interventions exist to address secondary complications such as myocardial fibrosis. We sought to identify proteins and pathways associated with disease and anti-IL-1 treatment in a mouse model of KD.

Methods: Vasculitis was induced *via* Lactobacillus casei cell wall extract (LCWE) injection in 5-week-old male mice. Groups of mice were injected with LCWE alone, LCWE and IL-1 receptor antagonist anakinra, or saline for controls. Upper heart tissue was assessed by quantitative mass spectrometry analysis. Expression and activation of STAT3 was assessed by immunohistochemistry, immunofluorescence and Western blot, and IL-6 expression by RNA-seq and ELISA. A STAT3 small molecular inhibitor and anti-IL-6R antibody were used to evaluate the role of STAT3 and IL-6 in disease development.

Results: STAT3 was highly expressed and phosphorylated in cardiac tissue of LCWE-injected mice, and reduced following anakinra treatment. *Il6* and *Stat*3 gene expression was enhanced in abdominal aorta of LCWE-injected mice and reduced with Anakinra treatment. IL-6 serum levels were enhanced in LCWE-injected mice and normalized by anakinra. However, neither inhibition of STAT3 nor blockade of IL-6 altered disease development.

Conclusion: Proteomic analysis of cardiac tissues demonstrates differential protein expression between KD-like, control and anakinra treated cardiac tissue. STAT3 and IL-6 were highly upregulated with LCWE and normalized by anakinra treatment.

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However, both STAT3 and IL-6 were dispensable for disease development indicating they may be bystanders of inflammation.

Keywords: Kawasaki disease, vasculitis, LCWE, magnetic and microwave proteomics, IL-6, STAT3, anakinra, IL-1

INTRODUCTION

Kawasaki disease (KD) is a relatively common febrile disease and vasculitis of childhood (10-20/100,000 incidence in North America) of unknown etiology, and typically presents as a febrile illness with features including rash, mucositis and conjunctivitis in children from ages 6 months to 5 years (1). While the acute process is self-limited, many patients with KD subsequently develop inflammatory vasculitic lesions of the coronary vasculature, resulting in coronary artery aneurysms (CAA). An emerging concern in the KD field is the recognition some patients develop late-onset myocardial fibrosis, which can lead to heart failure and death (1, 2).

Current management of KD is focused on preventing coronary artery aneurysms, and is anchored by the use of intravenous immunoglobulin (IVIG). IVIG suppresses the inflammatory response and reduces the number of patients afflicted with persistent CAA from 20% to around 5% if administered within the first 10 days of illness (3, 4). However, beyond surveillance for sequelae, there is no current management or therapy for myocardial fibrosis in KD (5). Despite current treatment and practices, KD remains the most common cause of acquired heart disease in children in the US and developed world (1). In addition, approximately 10% of all KD patients are resistant to initial IVIG therapy, and require adjunctive treatment (6).

While there is some understanding of the immunologic interplay that occurs in the acute phase of KD (7), many of the molecular pathological mechanisms that result in acute cardiac inflammation remain unclear. The current general consensus is that an unknown infectious agent promotes both innate and adaptive immunologic responses, resulting in an autoimmune attack on cardiac tissue (7). Co-localization of T cells and dendritic cells in and around lesions supports this hypothesis (7). Histopathological studies of autopsy specimens demonstrate infiltration of the vascular wall and adventitia by cells believed to be myofibroblasts or myofibroblast-like cells (8, 9). These cells may be responsible for the remodeling that results in coronary aneurysms and cardiac fibrosis (9).

Interleukin-1 (IL-1) plays a crucial role in mediating KD. Serum levels of IL-1β (10) and IL-1 related proteins are upregulated in the peripheral blood of KD patients during the acute phase of illness (11). IL-1 is required for pathogenesis in the *Lactobacillus casei* (LCWE) induced murine model of KD vasculitis (12–14), as evidenced by the observation that the IL-1 receptor antagonist anakinra can suppress disease in the model (12, 13). Recent studies also support an essential role for IL-1 in the *Candida albicans* model of KD (CAWS) (15–17).

The LCWE-induced murine model of KD vasculitis captures many of the features of human KD (18, 19). Like human KD, the murine model develops a much more vigorous disease phenotype in young animals and a greater severity in males. Importantly, the LCWE-induced KD murine model replicates most of the salient histopathological features of human KD (8, 18), such as the presence of intimal hyperplasia, adventitial and myocardial fibrosis, as well as elastin destruction and neo-elastic lamina formation (8). Additionally, many immunologic features and similarities of the disease are shared by both humans and mice (20, 21). The cytokine profile of the acute phase of the mouse model also bears strong similarity to that of the human disease, with elevations of IL-1β, TNF-α, IL-6, MCP-1, and IL-10 in the serum, as well as clinical symptoms of dysregulated temperature (20, 22). Human transcriptome, human genetic data and experimental murine models of KD all support a key role for IL-1β in the pathogenesis of KD, and also show the effectiveness of the LCWE model in terms of translational features to humans, such as histopathologic and even echocardiographic changes (10–17, 23–26).. Furthermore, the mouse model reliably predicts treatment efficacy, including of IVIG treatment (22).

We hypothesized that changes in cardiac protein expression after anakinra therapy of LCWE-injected mice would highlight disease- and treatment-relevant proteins. Here, we used a method of quantitative high-throughput sample preparation by magnetic-assisted digestion and tandem mass tag labelling (27) to analyze cardiac lysates from control and LCWE-injected mice developing KD vasculitis. By this method, we identified proteins expressed in the cardiac tissue including STAT3 that are responsive to anakinra treatment. However, despite increased STAT3 and IL-6 in the acute phase of disease, and the suppression of the acute phase reactant serum amyloid A (SAA) (28) by IL-6 blockade, their inhibition had no effect on the development of coronary and aortic vasculitis in the LCWE mouse model.

MATERIAL AND METHODS

Mice

WT C57BL/6 mice were obtained from the Jackson Laboratory and housed under specific pathogen-free (SPF) conditions in the American Association for Laboratory Animal Science (AALAS)-accredited facility at the Texas Biomedical Research Institute or Cedars Sinai Medical Center. All procedures involving animals were reviewed and approved by the institutional animal use and care committees (IACUC) of the Texas Biomedical Research Institute or Cedars Sinai Medical Center. Protocols were compliant with IACUC goals to reduce pain, suffering and distress. No adverse events occurred. Humane endpoints were euthanasia by IACUC approved methods.

Abbreviations: KD, Kawasaki Disease; LCWE, *lactobacillus casei cell wall extract;* MS, mass spectroscopy; IHC, immunohistochemistry; PBS, phosphate buffered saline; ELISA, enzyme linked immunosorbent assay.

LCWE-Induced KD Vasculitis Mouse Model

Lactobacillus casei (ATCC 11578) cell wall extract (LCWE) was prepared as previously described (29). Male mice aged 5 weeks old were i.p. injected with 500 μg of LCWE or 500μl PBS. For anakinra treatment, anakinra was donated from unused patient samples (SOBI, Sweden) and injected i.p. at a dose of 25mg/kg/dose (approximately 500 μg per mouse) daily for five days, beginning one day before LCWE injection, as previously described (12). For Stattic experiments, Stattic (Selleckchem, Catalog No.S7024, 100μg in 50% DMSO : PBS solution) or vehicle control (50% DMSO : PBS solution) was administered by i.p. injection every three days starting 1 day prior to LCWE injection. Experiment was performed twice in two different laboratories, once with 13 male mice total, 6 in control group, and 7 in treated group; for the repeat, the experiment was performed with 10 male mice per group. For IL6R antagonism experiments, 600μg of anti-IL6R (MR-16-1, Genentech) or isotype control antibody were administered by i.p. injection every three days starting 1 day prior to LCWE injection. Experiments were performed with 13 male mice per group, with several repeat experiments. Sample sizes were based on number needed to see 50% differences at 80% power at a 0.05 significance level, given penetrance of Kawasaki model of 80%. All group assignments were randomized. No animals were excluded from analysis. Order of treatments, measurements and caging of animal was random to minimize confounders. All analysts (pathologists) and technicians were blinded. Mice were euthanized for tissue harvest at day 7 or 14 post LCWE (as indicated) and perfused with PBS. Hearts were removed and embedded in Optimal Cutting Temperature (OCT) compound for histological examination. Abdominal aortas were photographed, the abdominal aorta diameter (measured at 5 points) and abdominal aorta area was quantified with the software Image J. Serial cryosections (7μm) of heart tissue were stained with hematoxylin and eosin (H&E) and histopathological scoring of coronary arteritis, aortic root vasculitis, and myocarditis were performed blinded (12, 18). Images were obtained using a Biorevo BZ-X710 (Keyence) microscope.

Proteomics Analysis

The upper portion of the heart (50%, including the aortic root) was placed directly into RIPA buffer (Milipore, Rockville MD) and then into liquid nitrogen for storage for subsequent homogenization. For isobaric TMT labeling, homogenized protein was pooled from representative specimens as reference material prior to sample preparation from individual specimens. Whole protein lysate was incubated and bound to C8 magnetic beads (BcMg, Bioclone, Inc.). The proteins were reduced with 10mM dithiothreitol (DTT) and alkylated with 50mM iodoacetamide. Overnight proteolysis was performed in a 1:25 trypsin-to-protein ratio at 37°C. Released tryptic peptides from digested proteins, including reference material described above, were normalized using the Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific) and then modified at the N-terminus and at lysine residues with tandem mass tag (TMT)-6plex isobaric labeling reagents (Thermo Scientific). Each individual specimen was encoded with one of the $TMT_{127-131}$ reagents, while reference material was encoded with the TMT_{126} reagent. To normalize across all specimens, TMT-encoded lysates from individual specimens were mixed with the reference material in equal ratios. These TMT peptides were cleaned with C18 ZipTips (Milipore).

Mass Spectrometry

The trypsin-digested TMT-labeled extracts were analyzed by nanoLC-MS consisting of an UltiMate 3000 Nano LC System and an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher). Reversed-phase liquid chromatography was performed using a 20 cm × 75 μm ID column packed with XBridgeTM BEH C18 media (2.5 μm, 130 Å). The flow rate was maintained at 200 nL/min. A full scan MS acquired in the range $300 \leq m/z \leq 2000$ was followed by 10 data-dependent MS/MS events on the 10 most intense ions. The mass resolution was set at 60000 for full MS. The dynamic exclusion function was set as follows: repeat count, 1; repeat duration, 30 s; exclusion duration, 30s. HCD was performed using normalized collision energy of 35% and the activation time was set as 0.1 ms. Probability based protein database searching of MS/MS spectra against the Trembl protein database (release April 25, 2018; 114,759,640 entries) was performed with a 10-node MASCOT cluster (v.2.3.02, Matrix Science) with the following search criteria: 10 ppm precursor ion mass tolerance, 0.05 Da product ion mass tolerance, 3 missed cleavages, trypsin, carbamidomethyl cysteines as a static modification, oxidized methionines and deamidated asparagines as variable modification, an ion score threshold of 20 and TMT-6plex for quantification.

Immunohistochemistry

Heart tissues were fixed in 10% neutral buffered formalin, processed conventionally, embedded in paraffin, cut at 5 microns transversely through the aortic root, H&E stained and scored by board-certified veterinary pathologists blinded to the grouping. Routine immunohistochemistry for STAT3 (Invitrogen, Thermo Fisher cat# MA1-13042, 1:1600 dilution) was performed on serial sections adjacent to the H&E sections. Briefly, slides were cut at 5 microns, dried (60°C for 1 hr), deparaffinized through 3 changes of xylene, 2 changes of absolute alcohol, 2 changes of 95% alcohol, and rinsed in distilled water. Antigen retrieval was conducted using Target Retrieval Solution pH 9 (Dako cat# S2367) in a decloaking chamber (Biocare Medical). Slides were allowed to cool, rinsed with Tris Buffered Saline with Tween (Dako cat# S3006) and treated with Dual Endogenous Enzyme block (Dako cat# S2003). Slides were incubated with anti-STAT3, as well as isotype control antibodies (Rabbit Isotype Control, Invitrogen, cat# 02-6102. 1:1600 dilution). Slides were then treated with ADVANCE HRP Link (Dako cat# K4069), ADVANCE HRP Enzyme (Dako cat# K4069), DAB substrate-chromagen (Dako cat# K3467), and Hematoxylin counterstain (Statlab cat# SL200), dehydrated, cleared, and cover-slipped. For scoring immunohistochemistry, a scale of 0 – 5 (Normal= 0, Minimal= 1, Mild= 2, Moderate= 3, Moderately Severe= 4, Severe= 5), was used for degree of

positively stained cells in and around the coronary artery, aortic root, valve and coronary arteries.

Immunofluorescence

OCT frozen 7μm heart tissue sections were fixed in acetone, washed in PBS, and stained overnight with 1 μg/ml of the following antibodies: Phospho-STAT3 (9145; Cell Signaling Technologies) or control Rabbit IgG (ab17180; Abcam). Sections were incubated with 2μg/ml Donkey Anti-Rabbit Alexa Fluor 555 (A-31572, Invitrogen). Nuclei were counterstained with Mounting Medium with DAPI (ab104139, Abcam). Images were obtained using a Biorevo BZ-9000 (Keyence) fluorescent microscope and mean fluorescence intensity (MFI) were quantified using Image J software.

Serum Analysis

Quantification of IL-6 in serum collected 2 weeks post-LCWE injection in mice treated with or without anakinra was performed with a highly sensitive multiplex enzyme-linked immunosorbent assay (ELISA) using electrochemiluminescence detection technology (Meso Scale Discovery [MSD], Rockville, Maryland, USA). Briefly, serum was prepared according to MSD protocol and were measured in duplicate: 50μl of 2-fold diluted samples and calibrators were pipetted in each well and incubated for 2 hours at room temperature with shaking. Following washing the plates three times with wash buffer (1xPBS and 0.05% Tween20), 25μl of MSD detection antibody solution was added to each well and incubated for 2 hours at room temperature with shaking. Plates were washed three times with wash buffer and then 150μl of MSD Read Buffer was added to each well. The plates then were read and analyzed by MSD instrument (MESO Quicklex S 120). Serum SAA levels were determined by ELISA (mouse SAA Elisa kit, Tridelta Development Ltd.) according to the manufacturer's instructions.

Western Blot Analysis

Frozen mouse hearts (-80°C) were immersed into RIPA lysis buffer and homogenized using a Polytron. Protein content of whole lysate was quantified, and samples were prepared for western blot using 5x Laemmli buffer. The membranes were incubated with antibodies against STAT3 (1:1000, Cell Signaling Technology, #9139) and phospho(Y705)STAT3 (1:1000, Cell Signaling Technology, #9145). Protein content was normalized to GAPDH (1:1000, Cell Signaling Technology, #5174). Due to space limitation we show the cropped blots in the manuscript. The complete gels are included in the supplementary data.

Statistics

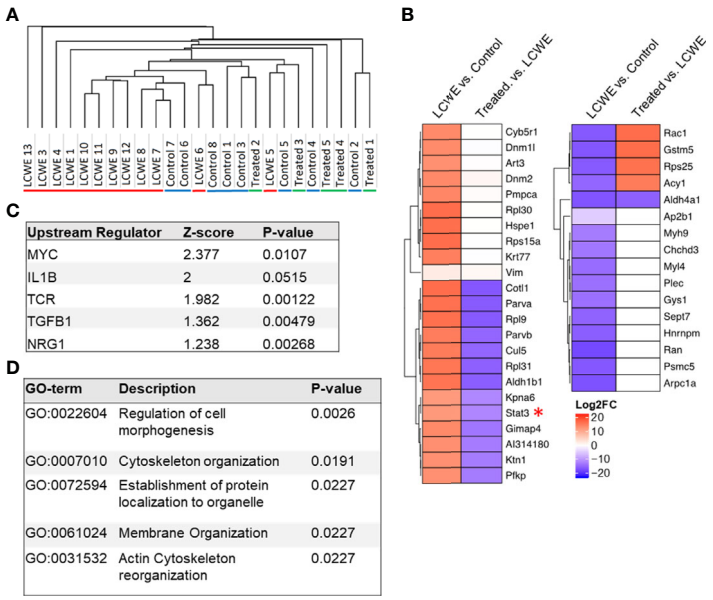
For proteomics protein expression evaluation, Scaffold Q+ (Scaffold_4.6.2, Proteome Software Inc.) was used to quantitate Label Based Quantitation (TMT) peptide and protein identification. Differentially expressed proteins were determined by applying Mann-Whitney Test with unadjusted significance level $p<0.05$ corrected by Benjamini-Hochberg. Principal component analysis (PCA) was performed using XLSTAT Software (Addinsoft, Paris, France). Heat map construction and hierarchical clustering using one minus Pearson's correlation was performed using Morpheus (http://software.broadinstitute.org/morpheus). Pathway analysis performed using STRING (30) (https://string-db.org/). All other data were analyzed by Student's t test for single comparisons or one-way ANOVA with Tukey's multiple testing correction for multiple comparisons. Differences between groups were considered to be significant at a P value of <0.05. Graphical representation of data and statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Receiver operator curve was constructed using STATA version 11 (STATA Corp, College Station, TX).

RESULTS

The Myocardial Proteome in the LCWE-Induced KD Vasculitis Model Shows a Distinct Protein Expression Profile Which Is Partially Normalized by Anakinra Treatment

The LCWE model induces a profound inflammatory response in the tissue surrounding the aortic root and vasculitis of the coronary arteries which extends well into the mid portion of the mouse heart. We performed proteomics analysis to evaluate the changes in cardiac tissue protein expression during LCWE-induced KD vasculitis and the response to anakinra treatment. Five-week old male WT mice were injected with either PBS or LCWE, and a subset of LCWE-injected mice were treated with anakinra daily, for five days. Two weeks later, cardiac tissues were collected and analyzed as previously described (31). We visualized protein expression differences between the groups using Pearson's hierarchical clustering (**Figure 1A**). LCWE-injected mice formed a distinct and minimally overlapping cluster from PBS controls. Two of the thirteen mice injected with LCWE clustered with the PBS controls, reflective of the incomplete penetrance of the LCWE-model (70-80% penetrance), as previously described (32) (**Figure 1A**). Furthermore, LCWE-injected mice treated with anakinra clustered with PBS control mice, indicating that IL-1Ra treatment suppressed the LCWE-induced proteomic signature (**Figure 1A**). Principal component analysis (PCA) also suggested the presence of three distinct and minimally overlapping groups, differentiating LCWE-injected KD mice, LCWE-injected mice treated with anakinra, and control mice (**Supplementary Figure 1**). Next, we performed differential protein expression analysis between the groups. We found 23 proteins were significantly upregulated (FC >2 and p-value <0.05) and 16 proteins were significantly downregulated (FC >2 and p-value <0.05) in cardiac tissue between LCWE-injected KD mice and PBS-injected controls (**Figure 1B**). Notably, expression of the transcription factor STAT3 was greatly increased in cardiac tissue of LCWE-injected mice. Approximately half of the proteins differentially regulated in LCWE-injected KD mice were normalized by anakinra treatment, including STAT3 (**Figure 1B**). Overall, these data indicate that LCWE induces changes in cardiac protein expression which are partially normalized by anakinra treatment.

The differentially expressed proteins (LCWE vs. PBS control) were then analyzed using IPA upstream regulator



Upstream Regulator	Z-score	P-value
MYC	2.377	0.0107
IL1B	2	0.0515
TCR	1.982	0.00122
TGFB1	1.362	0.00479
NRG1	1.238	0.00268

GO-term	Description	P-value
GO:0022604	Regulation of cell morphogenesis	0.0026
GO:0007010	Cytoskeleton organization	0.0191
GO:0072594	Establishment of protein localization to organelle	0.0227
GO:0061024	Membrane Organization	0.0227
GO:0031532	Actin Cytoskeleton reorganization	0.0227

FIGURE 1 | Proteomics analysis of cardiac tissue using the LCWE induced mouse model of KD. **(A)** Hierarchical one-minus Pearson's clustering. "LCWE" indicates LCWE-injected mice (n=13), "Control" indicates PBS injected mice (n=8), and "Treated" indicates LCWE injected mice treated with anakinra (n=5). **(B)** Heatmap of fold change for proteins found differentially expressed (FC > 2, p<0.05) between LCWE-injected mice and PBS controls or between anakinra-treated LCWE and untreated LCWE mice. **(C)** Top regulators (ranked by z-score) identified by IPA Upstream Analyses of proteins differentially expressed (FC > 2, p<0.05) between KD mice and PBS controls. D) STRING Gene Ontology Biological Process analyses of proteins differentially expressed (FC > 2, p<0.05) between KD mice and PBS controls.

analysis (**Figure 1C**) and STRING database analysis of Gene Ontology (**Figure 1D**). IL-1β was amongst the top upstream regulators when ranked by Z-score, in line with previous investigations in this model (12–14, 19), and our findings that anakinra treatment suppressed the LCWE-induced proteome changes. STRING analysis identified pathways involved in cellular morphogenesis and cytoskeletal organization (**Figure 1D**; **Supplementary Table 1**). These pathways may be reflective of the signals driving the pronounced myofibroblast proliferation that occurs in cardiac tissues and especially around inflamed vascular tissue during KD (8).

Expression of STAT3 Is Increased in Cardiac Tissue in the LCWE-Induced KD Mouse Model of Vasculitis

Given the known roles of STAT3 in the immune response (33), we next sought to validate the upregulation of STAT3 in the LCWE model by immunohistochemistry. Five-week old WT mice were injected with either PBS, LCWE or LCWE in combination with anakinra treatment. Two weeks later, cardiac tissues were collected, and sections were stained with isotype control or anti-STAT3 antibody. STAT3 was significantly upregulated in the cardiac tissue of LCWE-injected mice (**Figures 2A, B**), confirming our proteomics data. The majority of the increased STAT3 expression was observed in the peri-vascular and peri-aortic root inflammatory zones, and not in the myocardial tissue of LCWE-injected mice (**Figure 2A**). Importantly, treatment with anakinra normalized STAT3 staining (**Figures 2A, B**).

STAT3 tyrosine phosphorylation (Y705) results in STAT3 activation and transcriptional activity. To determine if the STAT3 protein present in cardiac tissue of LCWE-injected mice was phosphorylated, we performed immunofluorescence staining for p(Y705)STAT3 on heart sections, and found significantly greater expression of pSTAT3 in LCWE-injected mice compared to PBS controls (**Figures 2C, D**). Western blot analysis of whole heart lysates from PBS or LCWE injected mice confirmed an increased ratio of p(Y705)STAT3 to total STAT3 in cardiac tissue from LCWE injected mice (**Figures 2E, F**), indicating STAT3 is activated in LCWE-injected mice.

The STAT3 Activator, IL-6, Is Enhanced in LCWE-Injected Mice and Regulated by Anakinra

STAT3 can be activated by a range of cytokines and growth factors including IL-6, IFNγ, TNFα, CSF2, CSF3, IL-10, TGF-β, VEGF and EGF. To determine the possible upstream regulators of STAT3 in the LCWE-induced mouse model of KD vasculitis, we utilized our previously published RNA-seq data (GSE141072) (19) from the abdominal aorta of mice treated with PBS, LCWE or LCWE and anakinra. We analyzed this dataset for the expression of *Stat3* and the genes encoding the cytokines and growth factors listed above (**Figure 3A**). We found significantly

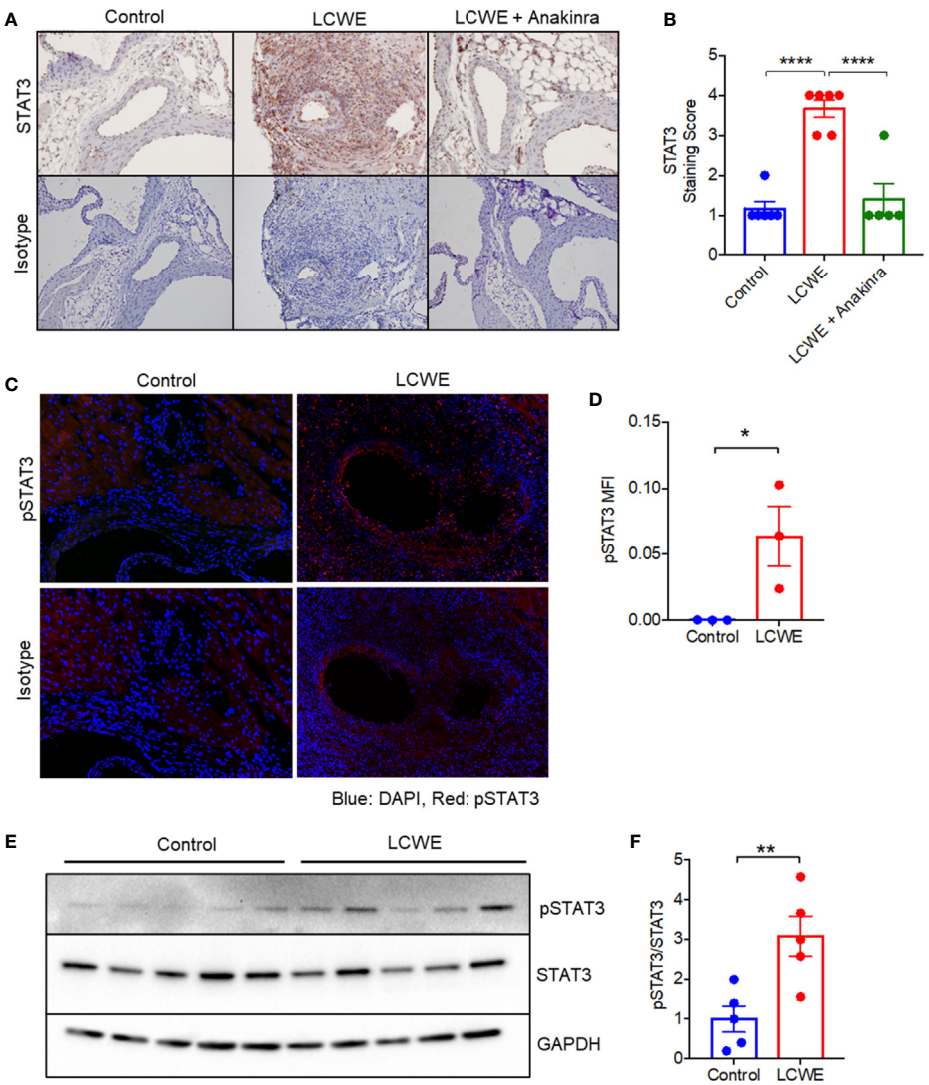


FIGURE 2 | STAT3 expression in the LCWE-induced model of vasculitis and response to anakinra treatment. **(A)** Representative images of immunohistochemistry staining for STAT3 in murine heart of PBS control, LCWE injected or LCWE injected mice treated with anakinra. **(B)** STAT3 staining score in PBS control, LCWE injected and LCWE injected mice treated with anakinra. **(C)** Representative images of pSTAT3 immunofluorescence staining in murine heart tissue from PBS control and LCWE injected mice. **(D)** Quantification of pSTAT3 immunofluorescence staining in murine heart tissue. **(E)** Western blot of pSTAT3 and STAT3 in murine heart tissue from PBS control and LCWE injected mice. **(F)** Quantification of pSTAT3/STAT3 ratio from Western Blot. Data was analyzed by one-way ANOVA with Tukey's multiple testing comparison **(B)** or Student's t test **(D, F)**. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

(FDR < 0.05, FC > 2) enhanced expression of *Stat3*, *Il6*, *Csf3* and *Tgfb1* in abdominal aorta of mice treated with LCWE, and these were all downregulated with anakinra treatment (**Figure 3A**). Of the candidate STAT3 activators, the most highly upregulated was *Il6*. IL-6 is a well characterized inducer of STAT3 activation and expression, and high serum IL-6 levels are found in the acute phase of KD (34, 35). We therefore analyzed serum levels of IL-6 in this model. We found high levels of IL-6 in the serum of LCWE-injected mice which were reduced to control levels by anakinra treatment (**Figure 3B**). These data suggest STAT3 activation and phosphorylation may be regulated, in part, by IL-6 in the LCWE model.

Treatment With the Small Molecule STAT3 inhibitor, Stattic, Fails to Attenuate LCWE-Induced KD Vasculitis Despite a Reduction of STAT3 Tyrosine Phosphorylation in Heart Tissue

Given STAT3 expression and phosphorylation was enhanced in the LCWE induced mouse model, we hypothesized that STAT3 may contribute to disease pathogenesis. To test the role of STAT3 in LCWE-induced KD vasculitis, we examined the impact of treatment with the small molecule STAT3 inhibitor, Stattic, on disease pathogenesis. LCWE-injected mice were

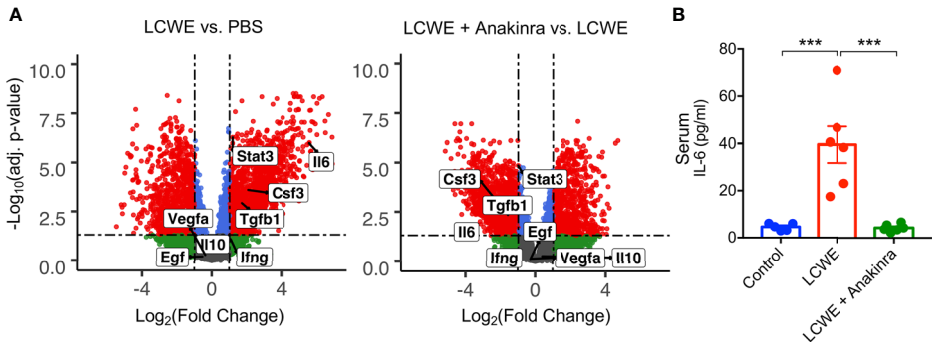


FIGURE 3 | IL-6 expression in the LCWE-induced KD mouse model. **(A)** Volcano Plots demonstrating differential expression of *Stat3*, *Il6, Csf3, Tgfb1, Ifng, Il10, Vegfa and Egf* in the abdominal aorta of PBS vs. LCWE treated mice and LCWE vs. LCWE + anakinra treated mice. *Csf2* was not detected. Red represents FDR < 0.05 and FC > 2, blue represents FDR < 0.05 and FC < 2, green represents FDR > 0.05 and FC < 2. **(B)** Serum levels of IL-6 in PBS, LCWE and LCWE + anakinra treated mice. Data was analyzed by one-way ANOVA with Tukey's multiple testing comparison **(B)**. ***p < 0.0001.

treated with Stattic or vehicle control (**Figure 4A**). Immunofluorescence of heart sections confirmed that Stattic inhibited the LCWE-induction of STAT3 phosphorylation (**Figures 4B, C**). However, Stattic treatment had no significant effect on heart vessel inflammation (**Figures 4D, E**) or AAA development (**Figures 4F–H**; **Supplementary Figure 3**). These results indicate that STAT3 does not play a pathogenic role in the acute phase of LCWE-induced KD vasculitis.

Treatment With an IL6R Antagonist Fails to Attenuate LCWE-Induced KD Vasculitis Despite Suppression of Acute Phase Reactant, Serum Amyloid A

Next, to determine the role of IL-6 in LCWE-induced KD vasculitis, we examined the impact of treatment with an IL-6R antagonist antibody on disease pathogenesis. LCWE-injected mice were treated with anti-IL6R or isotype control (**Figure 5A**). Antibody treatment suppressed LCWE-induced serum amyloid A (SAA) expression is serum (**Figure 5B**). However, IL6R antagonism had no significant effect on heart vessel inflammation (**Figures 5C, D**) or AAA development (**Figures 5E, G**; **Supplementary Figure 4**). These results indicate that IL6 does not play a pathogenic role in the acute phase of the LCWE model, and that despite attenuation of acute phase reactant (SAA), IL-6 blockade does not rescue the focal vasculitis in this model.

DISCUSSION

In this study, we show that the myocardial proteome undergoes significant remodeling during LCWE-induced murine KD vasculitis, and that this expression pattern is partially normalized by anakinra blockade of the IL-1 receptor. We identified STAT3 and IL-6 as highly expressed in cardiac tissue of LCWE-injected mice, and normalized by anakinra. However, blockade of STAT3 or IL-6 did not alter acute disease in the LCWE-induced mouse model of KD vasculitis. Despite a decline in acute phase reactants, histologic evidence of vascular inflammation remained unchanged

Using proteomic analysis of myocardial tissue, we identified a set of proteins differentially expressed during LCWE-induced KD vasculitis in mice, which were partially normalized by anakinra treatment. STAT3 was amongst the most highly up-regulated proteins, and was suppressed by anakinra treatment. Furthermore, we found STAT3 tyrosine phosphorylation was enhanced in cardiac tissue from LCWE-injected mice compared to controls, indicating activation of the pathway. STAT3 is a transcription factor of major importance in both cardiac function and the immune system (36). STAT3 can be expressed by many different cell types and has a diverse set of functions including regulation of inflammation as well as cell growth, proliferation, differentiation, migration, and apoptosis. The multi-faceted role of STAT3, in particular its role both in regulation of inflammation and promotion of immune response, may be the reason that blockade of the IL-6 pathway did not result in resolution of local inflammation (33). STAT3 gain of function mutation is associated with autoimmune disease, potentially by impairing development of regulatory T cells and promoting activation of T helper type 17 cells (37). However, STAT3 signaling also plays a role in attenuating acute inflammatory responses in phagocytes and dendritic cells (38).

IL-6 is a pleiotropic inflammatory cytokine that can affect multiple cell types either through binding to the membrane bound IL-6 receptor (classical IL-6 signaling), which is believed to mediate the anti-inflammatory and regenerative activities, or through binding to soluble IL-6 receptors (IL-6 trans-signaling), which mediates the pro-inflammatory responses (39, 40). IL-6 participates in the pathogenesis of several inflammatory diseases, and inhibitors of IL-6 are commonly used to treat rheumatoid arthritis and other classical inflammatory diseases such as Crohn's disease and psoriasis (40). IL-6 is also a potent inducer of STAT3 activation and expression. Like STAT3, we found that IL-6 was enhanced in the LCWE model of KD vasculitis and its expression was reduced with anakinra treatment. This is in line with KD patient data, which shows

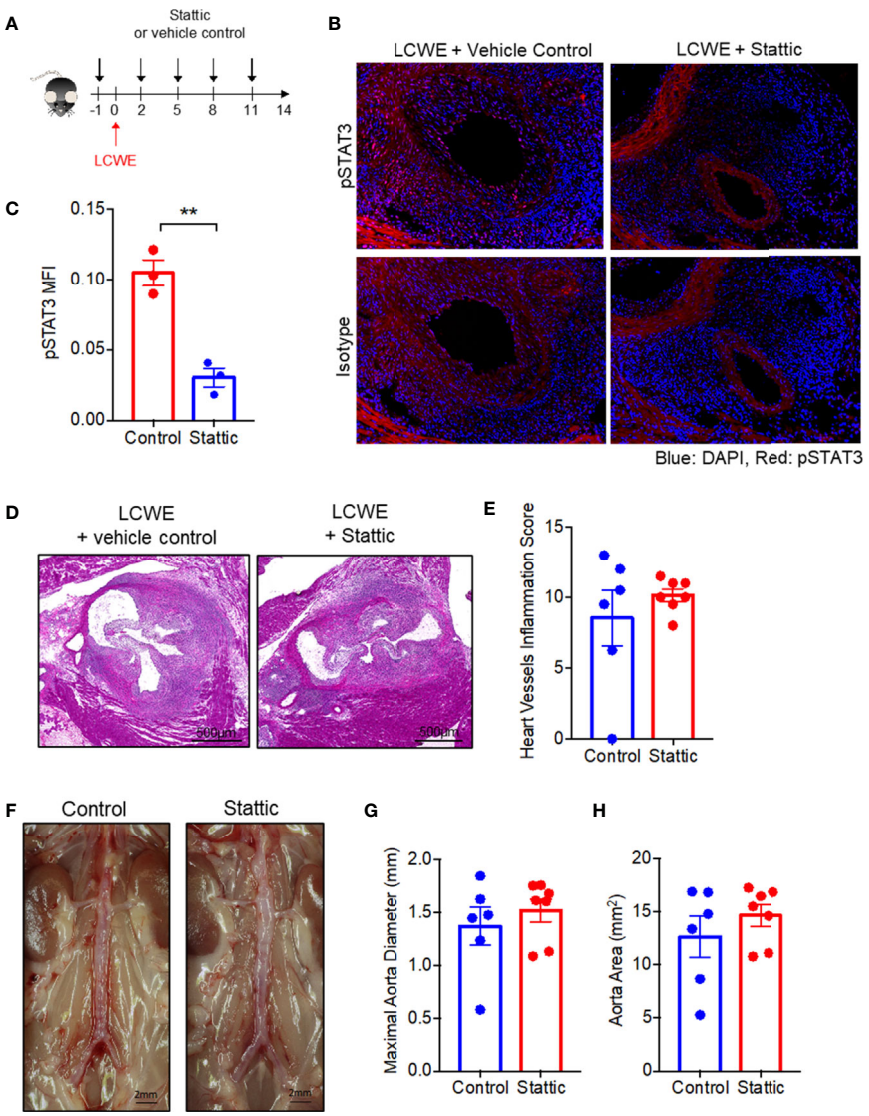


FIGURE 4 | STAT3 inhibition fails to attenuate LCWE-induced KD vasculitis. **(A)** Schematic of the experimental design. **(B)** Representative images of pSTAT3 immunofluorescence staining in murine heart tissue from LCWE-injected mice treated with vehicle control of Stattic. **(C)** Quantification of pSTAT3 immunofluorescence staining in murine heart tissue. **(D, E)** Representative H&E stained heart sections **(D)** and heart vessel inflammation scores **(E)** of LCWE-injected WT mice treated with either vehicle control or the STAT3 inhibitor Stattic at 2 weeks post-LCWE injection. Scale bars, 500μm. **(F)** Pictures of the abdominal aorta of LCWE-injected WT mice treated with either vehicle control or Stattic at 2 weeks post-LCWE injection. **(G, H)** Maximal abdominal aorta diameter **(G)** and abdominal aorta area **(H)** of LCWE-injected WT mice treated with either vehicle control or Stattic at 2 weeks post-LCWE injection. Results are representative of two independent experiments (n = 6-7 mice per group). Data was analyzed by Student's t test. ** $p < 0.01$.

IL-6 is expressed during the acute phase of disease and is decreased during convalescence (34, 35). IL-6 plays a pathogenic role in a number of inflammatory and autoimmune diseases. Tocilizumab, a recombinant humanized monoclonal antibody that targets the soluble and membrane-bound IL-6 receptor to inhibit IL-6 signaling, is used for the treatment of various inflammatory diseases and more recently COVID-19 and MIS-C (41, 42). The high serum IL-6 levels during the acute phase of KD led to the hypothesis that tocilizumab may be therapeutically beneficial for the treatment of KD. However, similar elevations in IL-6 are also observed in febrile controls (34) and there appeared to be no correlation of serum IL-6 with development of coronary aneurysm or dilatation (35). In a small trial involving four IVIG-resistant patients, 2 developed giant coronary artery aneurysms following tocilizumab treatment despite reduced fever and CRP levels (43). This study indicated that IL-6R antagonism had no beneficial effect for the treatment of KD, however lacked statistical power to conclude a worsening of disease may occur. Using the LCWE-induced mouse model of KD vasculitis, we show that despite high IL-6 and STAT3 expression, suppression of these factors does not reduce the development of the cardiovascular lesions.

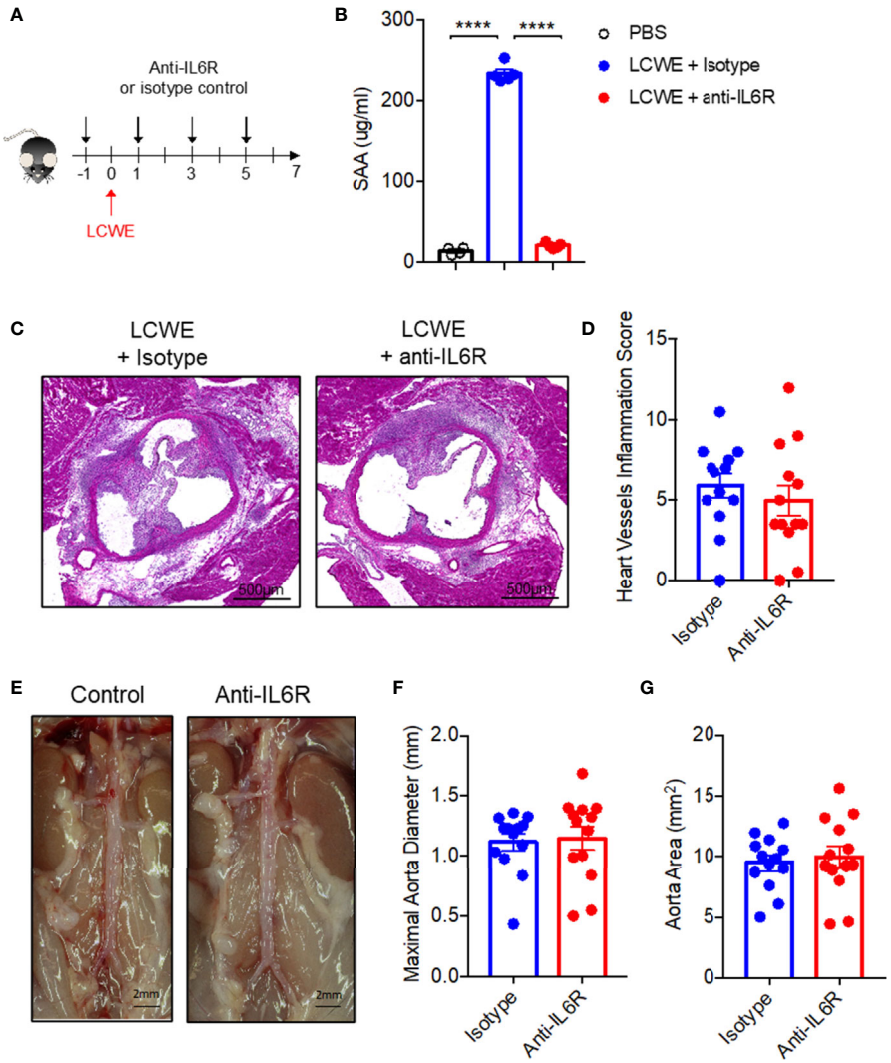


FIGURE 5 | Blocking IL-6R signaling fails to attenuate LCWE-induced KD vasculitis. **(A)** Schematic of the experimental design. **(B)** SAA concentration in serum from PBS or LCWE mice treated with isotype control or anti-IL6R antibody. **(C, D)** Representative H&E stained heart sections **(C)** and heart vessel inflammation scores **(D)** of LCWE-injected WT mice treated with either isotype control or anti-IL6R antibody at 1-week post-LCWE injection. Scale bars, 500μm. **(E)** Pictures of the abdominal aorta area of LCWE-injected WT mice treated with either isotype control or anti-IL6R antibody at 1-week post-LCWE injection. **(F, G)** Maximal abdominal aorta diameter **(F)** and abdominal aorta area **(G)** of LCWE-injected WT mice treated with either isotype control or anti-IL6R antibody at 1-week post-LCWE injection. Results are from two independent experiments combined (n = 13 mice per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple testing comparison **(B)** or Student's t test **(D, F, G)**. ****$p < 0.0001$.

The complex role of IL-6 and STAT3 in both inflammation and autoimmunity could explain the lack of effect.

IL-1β activates transcription factors that regulate IL-6 production (44). Indeed, IL-1β, the target of anakinra or canakinumab, strongly induces IL-6 production by several cell types, including vascular endothelial and smooth muscle cells (45, 46). We therefore propose that in the context of KD, IL-6 may be a bystander cytokine induced by IL-1β signaling, but plays no inflammatory role in the pathogenesis of cardiovascular lesion formation in the disease. Notably, we did observe that IL-6 inhibition resulted in reduced levels of SAA. SAA is a robust, clinically utilized acute phase reactant which is arguably as or more sensitive for infection and inflammation as the C-reactive protein, and plays active roles in innate immunity (28). The inhibition of SAA by tocilizumab with clear failure to inhibit vascular inflammation is of clinical importance. While measurement of acute phase reactants are one tool for diagnosis of rheumatic diseases including vasculitis (47), it has been recognized in the rheumatology literature that declines in acute phase reactants may not correlate with resolution of direct vascular injury in vasculitis (47, 48). Tocilizumab has been studied as a therapeutic agent in several vasculitis syndromes, including giant cell arteritis (49) (GCA) and Takayasu arteritis (50). While tocilizumab showed efficacy for GCA (49), it showed a trend of improvement but did

not meet the study endpoint for Takayasu arteritis despite improvement in acute phase reactants (50). Recently, case reports of progression of vasculitis in patients with Takayasu arteritis despite treatment with tocilizumab have been published (51, 52). Our finding of failure of IL-6 blockade in the LCWE-model, along with lack of success in studies in human KD and potentially Takayasu arteritis, may suggest that more study is warranted for the use of tocilizumab in certain vasculitides.

Of the other proteins that were upregulated in myocardial tissue in the LCWE-induced KD murine model in our studies, a number appeared to be related to changes in cell morphogenesis. We posit that these proteins are reflective of the signals driving the pronounced myofibroblast proliferation that occurs in cardiac tissues and especially around inflamed vascular tissue during KD (8). Vimentin, a marker of mesenchymal transition (53), plays an active role in regulation of protein trafficking and gene expression, and has recently been described as being directly involved in angiogenesis *via* regulation of the Notch pathway (54). Other proteins that were upregulated in our study include Parva, Parvb, and Dnm2, which are also involved in cell morphogenesis (55). A second set of upregulated proteins of interest included Dnm1l and Dnm2. These proteins are involved in regulation of calcium signaling and mitochondrial fusion in cardiomyocytes and the development of cardiac hypertrophy (56, 57).

Myofibroblasts play a key role in the pathogenesis of remodeling and fibrosis in KD by proliferating and secreting matrix products that obstruct the coronary lumen (8). STAT3 has recently been identified as a putative mediator of dermal fibrosis in patients with scleroderma and its murine models, acting downstream of TGF-β to drive pro-fibrotic fibroblast responses (58, 59). Interestingly, the TGF-β signaling pathway has been linked to KD pathogenesis and is speculated to play a role in the generation of myofibroblasts in the disease (2, 9). Indeed, our data show TGF-β is an upstream regulator of proteins differentially expressed in cardiac tissue of LCWE-injected mice. Furthermore, we found *Tgfb* gene expression is enhanced with LCWE in abdominal aorta tissue and suppressed by anakinra. While we did not find a role for STAT3 in the acute phase of KD, it is possible that it does play a role in mediating fibrosis. Long-term studies examining fibrotic pathways are needed to investigate this possibility.

Limitations in the study are the inherent differences in murine vs. human biology; however, here the mouse model offers the most robust method to investigate details of pathology not typically available with human disease, and the LCWE model, as discussed, has played an important role in understanding pathophysiology of KD. Bias in the study was attenuated by the blinding of direct analysts such as pathologic interpretation of images, and of technicians during assays, as well as primary investigators, but cannot be completely eliminated.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ProteomeXchange, PXD024310.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional animal use and care committees (IACUC) of the Texas Biomedical Research Institute and Cedars Sinai Medical Center.

AUTHOR CONTRIBUTIONS

MG, MA, RP, CCH, and TF conceived, designed, acquired and interpreted the data, drafted the manuscript ED, SK, RE, AG, and SM-I made substantial contributions to the acquisition and interpretation of data RP, MNR, JP, MG, MA, and TF made substantial contributions to the design of the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.630196/full#supplementary-material

Supplementary Figure 1 | Principal component analysis of proteomics data. Proteomics data clustering based on two primary factors, with the majority of mice in each group forming clusters as shown in circles.

Supplementary Figure 2 | Western Blot data. Full western blot data from **Figures 2E, F**.

Supplementary Figure 3 | Whole photographs of images used on **Figure 4F**.

Supplementary Figure 4 | Whole photographs of images used on **Figure 5E**.

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A Partial Picture of the Single-Cell Transcriptomics of Human IgA Nephropathy

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The molecular mechanisms underlying renal damage of IgA nephropathy (IgAN) remain incompletely defined. Here, single-cell RNA sequencing (scRNA-seq) was applied to kidney biopsies from IgAN and control subjects to define the transcriptomic landscape at single-cell resolution. We presented a comprehensive scRNA-seq analysis of human renal biopsies from IgAN. We showed for the first time that IgAN mesangial cells displayed increased expression of several novel genes including MALAT1, GADD45B, SOX4, and EDIL3, which were related to cell proliferation and matrix accumulation. The overexpressed genes in tubule cells of IgAN were mainly enriched in inflammatory pathways including TNF signaling, IL-17 signaling, and NOD-like receptor signaling. Furthermore, we compared the results of 4 IgAN patients with the published scRNA-Seq data of healthy kidney tissues of three human donors in order to further validate the findings in our study. The results also verified that the overexpressed genes in tubule cells from IgAN patients were mainly enriched in inflammatory pathways including TNF signaling, IL-17 signaling, and NOD-like receptor signaling. The receptor-ligand crosstalk analysis revealed potential interactions between mesangial cells and other cells in IgAN. IgAN patients with overt proteinuria displayed elevated genes participating in several signaling pathways compared with microproteinuria group. It needs to be mentioned that based on number of mesangial cells and other kidney cells analyzed in this study, the results of our study are preliminary and needs to be confirmed on larger number of cells from larger number of patients and controls in future studies. Therefore, these results offer new insight into pathogenesis and identify new therapeutic targets for IgAN.

Keywords: IgA nephropathy, single-cell RNA sequencing, kidney, TNF and IL-17 signaling, NOD-like receptor signaling

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INTRODUCTION

IgA nephropathy (IgAN) is the most common primary glomerular disease worldwide (1, 2). IgAN takes a slow but relentless clinical course which eventually progresses to end-stage renal disease (ESRD) in 30–40% of patients within 20–30 years of diagnosis (3).

There is now strong evidence that IgAN is an autoimmune disease, and a multi-hit hypothesis has been proposed to explain the immunopathogenesis of IgAN (4–6). Other mechanisms including mesangial- podocytic-tubular crosstalk, genetic factors, and complement activation also participate in pathogenesis of IgAN (6, 7). However, comprehensive analysis of the cell types and molecular pathways that promote disease progression is lacking.

Traditional bulk RNA sequencing (RNA-seq) is a powerful tool to profile transcriptomic variations in different diseases. However, bulk RNA-seq only evaluates the average gene expression of a large population of cells in the tissue (8). The fast development of single-cell RNA sequencing (scRNA-seq) technology allows the inquiry of transcriptomic profiles and signaling pathways in diverse cell types from a given sample simultaneously, and unlike bulk RNA-seq, it can define comprehensive gene sets at the single-cell level (9, 10). Recently, this new methodology has been employed in various renal diseases (11–14). In the present study, we applied scRNA-seq to kidney samples from patients with IgAN to identify gene expression at the single cell level, and explore novel cellular interactions and crucial molecular pathways contributing to the disease development.

METHODS

Ethical Approval and Consent

Samples were collected as part of the Kidney Precision Medicine Study which was approved by the Medical Ethics Committee of the Xiangya Hospital of Central South University for Human Studies (approval number 201711836). All subjects provided written informed consent, and all experiments were performed in accordance with study protocol.

Public Dataset Acquisition and Processing

The scRNA-Seq data of healthy kidney tissues of three human donors were downloaded from the Gene Expression Omnibus (GSE131685). We reproduced the downstream analysis using the code provided by the author in the original paper. We applied Harmony to integrate samples and performed downstream analysis using Seurat (version 3.1, Satija Lab, https://satijalab.org/seurat/). All gene expression was normalized and scaled using NormalizeData and ScaleData. Top 2,000 variable genes were selected by FindVariableFeautres for PCA analysis. Clustering analysis using FindClusters was performed by first reducing the gene expression matrix to the first 20 principal components and then using a resolution of 0.3 for graph-based clustering.

Clinical Sample Procurement

Kidney specimens from four newly diagnosed IgAN patients were obtained from department of nephrology in Xiangya Hospital, Central South University. Kidney sample as a portion of renal core needle biopsies by 18-gauge needles indispensable for clinical diagnosis was obtained from consented IgAN patients. Control kidney tissues were collected at one site by needle biopsy of living donor kidneys after removal from the donor and before implantation to the recipient. Only small amount (2–3 mg) of renal biopsy was acquired for the scRNA-seq procedure. Kidney tissues were cleaned with sterile PBS after obtainment.

Kidney Sample Processing and Single-Cell Dissociation

The fresh kidney sample was placed into the GEXSCOPE Tissue Preservation Solution (Singleron Biotechnologies) immediately at 2–8°C (15). After washed by Hanks Balanced Salt Solution (HBSS) for three times, biopsy sample was cut into small pieces (1–2 mm). Subsequently, sample was digested in 2 ml GEXSCOPE Tissue Dissociation Solution (Singleron Biotechnologies) in a 15 ml centrifuge tube by continuous agitation maintained at 37°C for 15 min. Sample was then filtered through a 40-μm sterile cell strainer (Corning). After centrifuged at 1,000 rpm for 5 min at 4°C, cell pellets were resuspended with 1 ml phosphate buffer saline (PBS) (HyClone). The cell suspension was incubated with 2 ml GEXSCOPE Red Blood Cell Lysis Buffer (Singleron Biotechnologies) for 10 min at 25°C to remove red blood cell. After centrifuged at 1,000 rpm for 5 min, the cell pellet was resuspended with PBS. Then cells were counted using a TC20 automated cell counter (Bio-Rad). Live cells were determined by trypan blue staining (Gibco). If the cell viability exceeded 85%, the subsequent sample processing was performed.

Library Preparation and Preprocessing of scRNA-Seq Data

The single-cell suspension was adjusted to a concentration of 1×10^5 cells/ml in PBS. Subsequently, single cell suspension was loaded onto the microfluidic chip. The single cell RNA-seq libraries were prepared following the manufacturer's protocols (Singleron GEXSCOPE Single Cell RNA-seq Library Kit, Singleron Biotechnologies) (15). The captured single cell RNA-seq libraries were sequenced were sequenced by using an Illumina HiSeq X10 instrument and 150 bp paired-end reads.

Marker Genes Analysis in Different Cell Types

After cell cluster identification, the marker genes of each cell population in kidney were determined relative to other cell clusters with the "Wilcox" (Likelihood-ratio test) using the FindAllMarkers function in Seurat. The selected marker genes were expressed in over 10% of the cells in one cluster and average log (Fold Change) was more than 0.25. The heatmap was completed by the top 20 marker genes of each cell cluster.

Differentially Expressed Genes Identification Between Groups

We identified differentially expressed genes (DEGs) in each cell cluster of kidney by comparing the transcriptional profile of IgAN and control subjects. The DEGs of each cell cluster between the two groups were defined with the "wilcox" (Likelihood-ratio test) by the FindAllMarkers function in Seurat. Avg.exp replied the average expression of the gene in the cell type. The gene with absolute value of average log (Fold Change) exceeded 0.25 and *P* value smaller than 0.05 was identified as differentially expressed gene.

Enrichment and Cell Interaction Analysis

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were performed by "cluster Profiler" R package. We also conducted cell the analysis of cell interactions *via* Cellphone DB.

RESULTS

A total of five renal biopsy specimens were collected from patients with IgAN (n = 4), and healthy control (n = 1) obtained from living donor kidney. All four IgAN patients had proteinuria ranged from 0.27 to 2.57 g per 24 h. IgAN subjects were divided into microproteinuria and overt proteinuria group. Microproteinuria which was determined as daily urinary protein excretion amount 20–300 mg (16), was observed in one IgAN patient (25%). Overt proteinuria which was defined as daily urinary protein excretion exceeding 300 mg (16), was observed in three IgAN subjects (75%). The estimated glomerular filtration rate (eGFR) of IgAN patients ranged from 32.72 to 114.09 ml/min/1.73m^2 (81.83 ± 38.07 ml/min/1.73m^2). Serum IgA concentration was elevated in one patient (**Supplemental Table 1**). IgAN subjects had no coexistent diseases such as diabetes and lupus. Other secondary kidney diseases were also excluded. At the time of biopsy, all IgAN patients have not received any medications including RAAS blockers in the past. The information about blood pressure was shown in **Supplemental Table 1**. The living relative donor was a 41-year-old male, with no history of diseases including hypertension and diabetes. His creatinine was 0.94 mg/dl, and eGFR was 100.47 ml/min/1.73m^2. He has not been prescribed any medications in the past.

Cell Lineage in Kidney Identified by scRNA-seq

A total of 20,570 cells were isolated and sequenced in five specimens. The cell viability ranged from 85 and 91% (**Supplemental Table 2**). We performed data pre-processing and quality control, then got the transcriptomic data. Fourteen distinct cell clusters were defined using unsupervised clustering analysis, and labeled according to lineage-specific marker gene expression (**Figure 1A**). Enrichment of different cell clusters was calculated for each subject respectively (**Figure 1B**). The numbers of cells in each cluster in kidney from individual patients and control were provided in **Supplemental Table 8**. Bar plots represented frequency of cell clusters in kidney of each subject (**Figure 1C**). The expression of top 20 marker genes for each cell population among 14 clusters proved the reliability of cell classification method (**Figure 1D** and **Supplemental Table 7**). **Figure 1E** and **Table 1** showed each cell population identified by selected cell lineage-specific marker gene expression.

Identification of Gene Expression Changes in Glomerulus of IgAN Subjects

We next compared transcriptomes of intrinsic renal cells from glomerulus in IgAN patients with healthy counterpart. Entire lists of cell-type-specific DEGs were displayed in **Supplemental Table 3**. We defined representative DEGs in mesangial cells, endothelial cells, and podocytes through comparing the transcriptional profile of IgAN and control subjects (**Figure 2A**). Detailed information about DEGs in each cell cluster in kidney from IgAN and control subjects were displayed in **Supplemental Table 9**.

Overexpression of several genes (*MALAT1*, *GADD45B*, *SOX4*, and *EDIL3*) in IgAN mesangial cells were reported here for the first time. *MALAT1*, a highly conserved nuclear long non-coding RNA molecule, which is implicated in extracellular matrix (ECM) production, oxidative stress, and fibrosis (17), was upregulated in IgAN mesangial cells. *GADD45B*, a member of the growth arrest and DNA damage related gene family, has been proved to play significant roles in DNA damage, cell growth, and apoptosis (18). *GADD45B* probably participated in podocytes injure of focal segmental glomerular sclerosis (FSGS) (19). The effects of tumor related genes (*SOX4* and *EDIL3*) in IgAN need further clarification. *FOS* is a member of the *FOS* gene family which acts as a regulator of cell proliferation, differentiation, and apoptosis (20). One recent study demonstrated that *FOS* expression was enriched in IgAN patients with important roles in mesangial proliferation and glomerular sclerosis (21). Consistently, gene expression profiles showed *FOS* expression was elevated in mesangial cells of IgAN. GO enrichment analysis showed DEGs in the mesangial cells were enriched for biologic processes including integrin binding and ion channel binding (**Figure 2C**).

In order to further validate the findings in our study, we compared the results of four IgAN patients with the published kidney cell atlas data downloaded from the Gene Expression Omnibus (GSE131685). As shown in **Supplemental Table 10**, compared with our previous data, there were more highly expressed DEGs in IgAN mesangial cells and endothelial cells. Consistent with our previous findings, we also detected overexpression of several genes (GADD45B, FOS, ID2, and MT-RNR1) in IgAN mesangial cells. Furthermore, several genes (SOX4, MT-RNR1, PECAM1, UTRN, MTATP6P1, and MT-ND4L) were also found to be upregulated in IgAN endothelial cells.

As shown in **Figure 2A**, IgAN podocytes had increased expression of the serine protease *PRSS23*, which has been

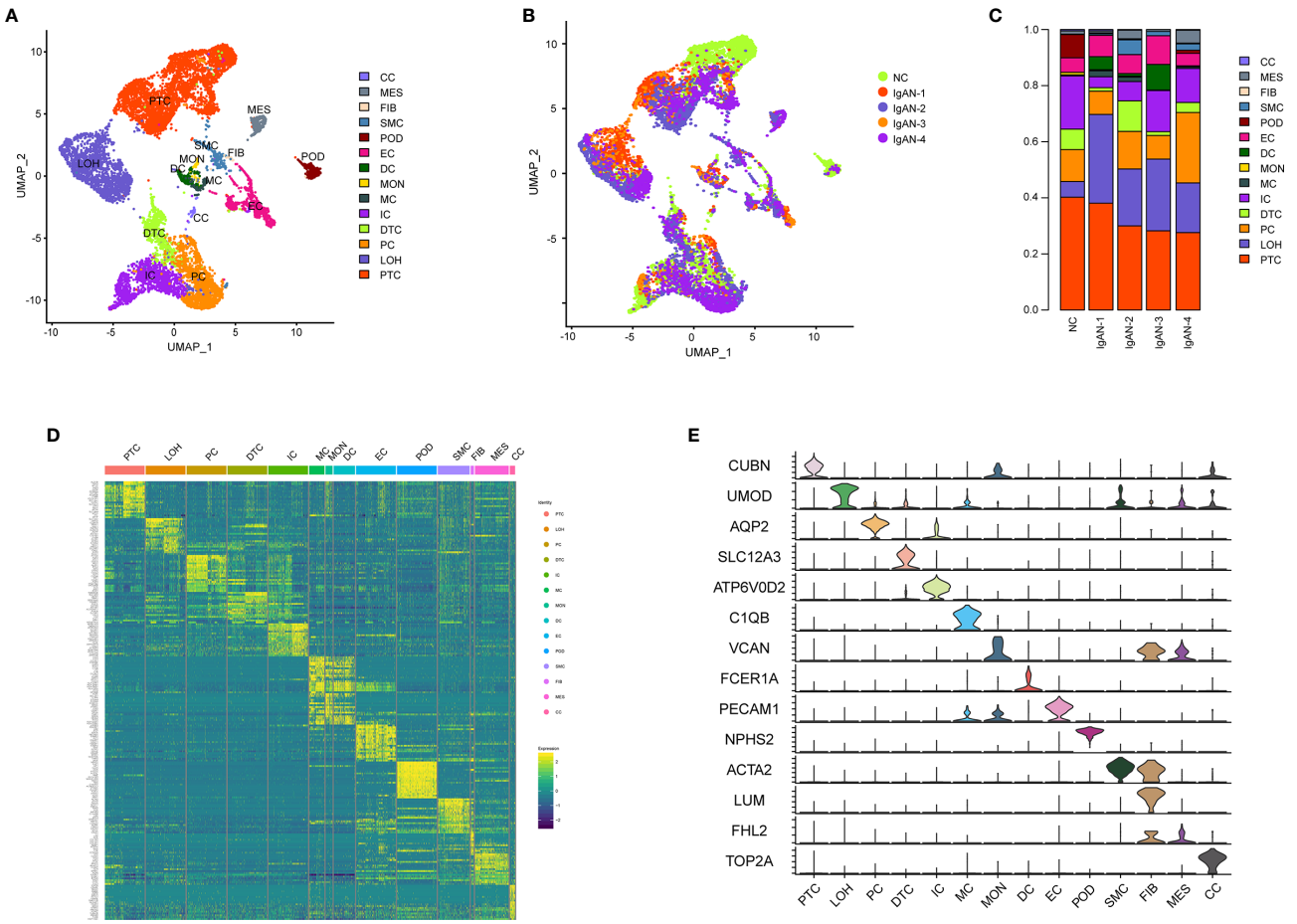

FIGURE 1 | Cell lineage analysis by comprehensive single-cell RNA-sequencing in IgAN and control subjects. **(A)** Fourteen distinct cell clusters identified by UMAP plotting, and clusters were colored and labeled distinctively. The color of cells represented the group origin. **(B)** UMAP plot of cell clusters from different subjects of IgAN patients (n = 4) and control (n = 1). The color of cells reflected the individual origin. **(C)** Bar plots represented frequency of cell clusters in kidneys from different subjects. Blocks represented different subjects, and block height was in proportion to the number of cells. **(D)** Heatmap of top 20 specific marker genes for each cluster from kidney. Each column represented a kind of cell cluster, and each row corresponded to marker gene for individual cluster. **(E)** Violin plot of selected marker genes that identified the clusters generated by UMAP plotting. It was colored by different cell subtypes. Abbreviations were as follows: PTC, proximal tubule cells; LOH, loop of Henle cells; PC, principal cells; IC, intercalated cells; DTC, distal tubule cells; EC, endothelial cells; POD, podocytes; MES, mesangial cell; SMC, smooth muscle cells; DC, dendritic cells; MC, macrophages; MON, monocytes; CC, cycling cells; FIB, fibroblasts.

TABLE 1 | Cell-lineage-specific marker genes of different cell types.

Cell type	Marker genes
Mesangial cells (MES)	FN1, FHL2, CTGF, MYL9
Endothelial cells (EC)	PECAM1, VWF, CLDN5, ACKR1
Phagocytes (POD)	NPHS2, PODXL, PTPRO
Proximal tubular cells (PT)	CUBN, SLC13A1, LRP2, ALDOB
Distal tubular cells (DT)	SLC12A3, CALB1, SLC8A1
Loop of Henle cells (LOH)	UMOD, SLC12A1, CLDN16
Principal cells (PC)	AQP2, AQP3
Intercalated cells (IC)	SLC26A7, SLC4A1, ATP6V1G3
Macrophages (MC)	LYZ, CD68, MRC1, C1QA, C1QB
Monocytes (MON)	LYZ, VCAN, CD14, FCN1
Dendritic cells (DC)	CD1C, CD1E, FCER1A, CLEC10A
Fibroblasts (FIB)	LUM, COL1A1, DCN
Smooth muscle cells (SMC)	ACTA2, TAGLN, MYH11, MYLK
Cycling cells (CC)	TOP2A, MKI67

demonstrated to be highly expressed in the glomeruli of human fibrotic kidneys and may play a pathogenic role in renal fibrosis (22). *NGF*, a protective factor overexpressed in the kidney of various renal disorders (23), was elevated in podocytes of IgAN. As a growth factor, *NGF* protects immune and non-immune cells from apoptotic cell death and might be involved in kidney physiopathology in previous findings (23). IgAN podocytes expressed increased *HES1*, which acts as an effector of Notch signaling pathway. It was reported previously that *HES1* expression in podocytes mediated epithelial to mesenchymal transition (EMT) (24). We found that DEGs included overexpressed cellular adhesion gene (*SELP* and *PECAM1*) and angiogenesis (*XIPS*) in glomerular endothelial cells of IgAN. IgAN endothelial cells also expressed increased levels of the transcription factor *SOX4*, a central component of TGF-β

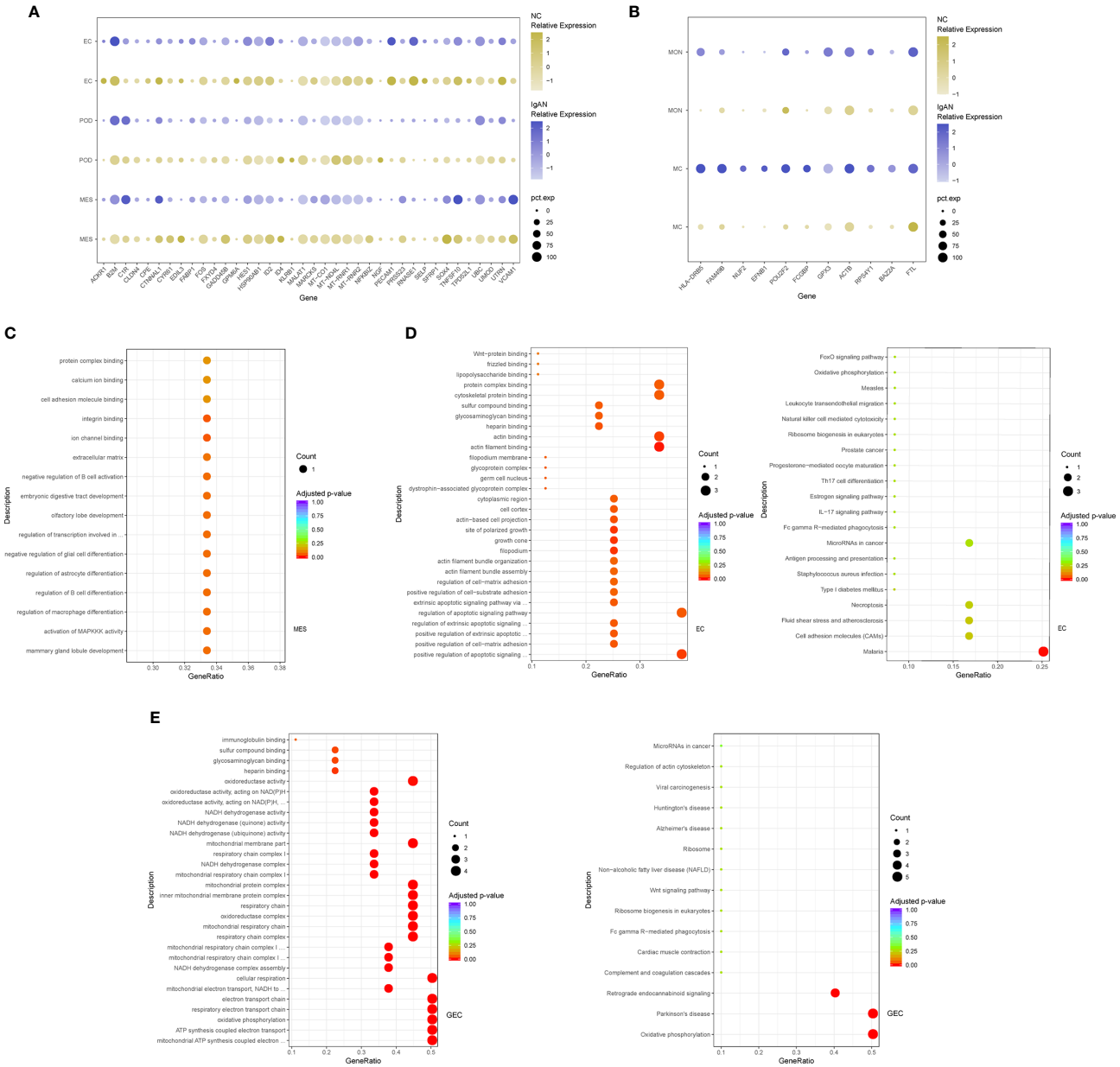

FIGURE 2 | DEGs and intercellular signaling in glomerulus and immune cells of IgAN and control subjects. **(A)** Representative DEGs in mesangial cells, endothelial cells and podocytes in kidney by comparing the transcriptional profile of IgAN and control subjects. **(B)** Representative DEGs in monocytes and macrophages by comparing the transcriptional profile of IgAN and control subjects. **(C)** GO enrichment analysis displayed that upregulated DEGs were involved in some biological processes in mesangial cells comparison with control subject. **(D, E)** GO and KEGG enrichment analysis revealed DEGs were mainly enriched in some biological processes and pathways in endothelial cells and glomerular endothelial cells respectively. Abbreviations were as follows: pct.exp, percentage of cells expressing gene; count, number of genes annotated to GO terms or KEGG pathway.

signaling to mediate EMT in various types of cancer (25), which has not been reported in IgAN yet. The elevated expression of atypical chemokine receptor *ACKR1* was displayed in IgAN endothelial cells. *ACKR1*, which is enriched within endothelial junction, has a role in supporting leukocyte recruitment (26). Interestingly, endothelial cell cluster highly expressed the endothelial extracellular endonuclease *RNASE1*, acting as a key regulator to maintain endothelial homeostasis and protect the endothelial layer (27). Furthermore, endothelial cells were divided into glomerular endothelial cells and vascular endothelial cells. GO and KEGG enrichment analysis showed DEGs in the endothelial cells were enriched for the biologic processes or signaling including positive regulation of cell-matrix adhesion, apoptotic signaling, and oxidation (**Figures 2D, E**).

Tubular Cells From IgAN Patients Were Enriched With Inflammatory, Matrix Remodeling and Related Signatures

The epithelial cells were obtained and sub-clustered into five groups based on marker genes. We also identified representative DEGs in renal tubules in IgAN and control subjects (**Figure 3A** and **Supplemental Table 4**). Altered signaling networks in the tubular cells were identified in IgAN subjects. Comparison of proximal tubule cells from IgAN and control subjects revealed upregulation of genes participating in TNF signaling, IL-17 signaling, NOD-like receptor signaling, and regulating leukocyte transendothelial migration (**Figure 3B**). Comparison of the DEGs in the distal tubule cells revealed enrichment of genes participating in MAPK cascade, p38MAPK cascade in IgAN (**Figure 3C**). The loop of Henle cells in IgAN had increased genes participating in TNF signaling, IL-17 signaling, Th17 cell differentiation, NOD-like receptor signaling. Moreover, genes participating in PI3K-Akt signaling, including *ITGB6*, *ITGB8*, and *YWHAH*, were increased in the loop of Henle cells in IgAN. Also, genes participating in Toll-like receptor signaling, including *SPP1*, *JUN*, and *FOS* were increased in the loop of Henle cells in IgAN (**Figure 3D**).

As illustrated in **Figures 3E, F**, principal cells and intercalated cells were enriched for genes participating in TNF signaling, IL-17 signaling, Th17 cell differentiation in IgAN. Furthermore, *NFKBIA*, *TXNIP*, *CXCL3*, and *CXCL2*, which were all get involved in NOD-like receptor signaling regulation (28), were increased both in principal cells and intercalated cells. Further studies are needed to elucidate NOD-like receptor signaling in pathogenesis of IgAN.

As shown in **Supplemental Tables 11** and **12**, we compared the results of four IgAN patients with the published kidney cell atlas data in order to further validate the findings in our study. The tubular epithelial cells were sub-clustered into five groups. Many altered signaling networks in renal tubular cells were also identified and validated in IgAN subjects. Interestingly, comparison of the DEGs in the proximal tubular cells from IgAN patients also revealed enrichment of genes participating in TNF signaling, IL-17 signaling, and NOD-like receptor signaling. Distal tubular cells from IgAN subjects had elevated expression of genes participating in IL-17 signaling. The loop of Henle cells in IgAN also had increased genes participating in TNF signaling, IL-17 signaling, Th17 cell differentiation, NOD-like receptor signaling, and PI3K-Akt signaling. Consistently, principal cells were also enriched for genes participating in TNF signaling, IL-17 signaling, and NOD-like receptor signaling in IgAN.

Identification of Gene Expression Changes in Immune Cells in Kidney of IgAN Subjects

We identified leukocytes which were composed of macrophages, monocytes, and dendritic cells in kidney sample of IgAN patients, which was consistent with previous research (29). However, significant numbers of some leukocyte subpopulations including T cells in kidney were not detected, that might be a potential limitation due to either dissociation technical limitation and/or the cell number below our detection limit, as the infiltration and effect of renal T cells in pathogenesis of IgAN is well-proven (30). Because few numbers of leukocytes appear in control, we used two peripheral blood mononuclear cell (PBMC) datasets specimens commonly available as control (31, 32).

Dataset integration showed DEGs of monocyte and macrophage subsets (**Supplemental Table 5**). *GPX3*, a member of glutathione peroxidase family, which could protect cells against oxidative stress (33), was reduced in renal macrophages of IgAN. As illustrated in **Figure 2B**, macrophages from kidney in IgAN displayed decreased expression of *FAM49B*, which is primarily localized in the mitochondria and its downregulation leads to increased fission and mitochondrial reactive oxygen species (ROS) production (34). *FCGBP*, a negative regulator of EMT, which is proved to have the function of cell protection and anti-inflammation in tissues (35), was under-expressed in macrophages from kidney of IgAN.

Comparative Analysis Identify Cell-Cell Crosstalk in IgAN Through Ligand-Receptor Interactions Analysis

Exploring the intercellular communication and signaling network may provide new opportunities to identifying new therapeutic targets in IgAN. Potential interactions of receptors and ligands in distinct cell types of kidney were presented (**Figure 4A**). IgAN mesangial cells expressed *CXCL1* and a potent monocyte-attracting chemokine *CCL2*. *CXCL1*, a member of the CXC chemokine family, serves as a mesangial-derived adjuster to induce damage of other cells in IgAN, other than its neutrophil chemoattractant ability (36). We found *CXCL1* and *CCL2* expressed in mesangial cells might both interact with the chemokine receptor *ACKR1* expressed at endothelial junctions. In addition, mesangial cells expressed *JAGGED1*, and its receptor *NOTCH4*, was expressed in endothelial cells, indicating involvement of Jagged/Notch signaling pathways in pathogenesis of IgAN (**Figure 4B**). Mesangial cells expressed the growth factors *FGF2* and *PDGFD*, which promote proliferation and matrix-production of mesangial cells by interacting with their respective receptors *FGFR1* and *PDGFRB* expressed in podocytes or Loop of Henle (**Figures 4C, D**). *PDGFRB*, indicating a potential mechanism for mesangial proliferation and renal fibrosis (37), was also expressed in fibroblasts (**Figure 4E**). Similarly, ligand-receptor pair analysis could be performed for the remaining interactions between different cells of interest for further study.

DEGs and Pathway Analysis in Kidney From IgAN Patients With Overt Proteinuria Compared With IgAN Patients With Microproteinuria

The gene expression profile was compared between IgAN subjects with microproteinuria and overt proteinuria. Detailed information of cell-type-specific DEGs in two groups of IgAN was shown in **Supplemental Table 6**. The gene expression profile

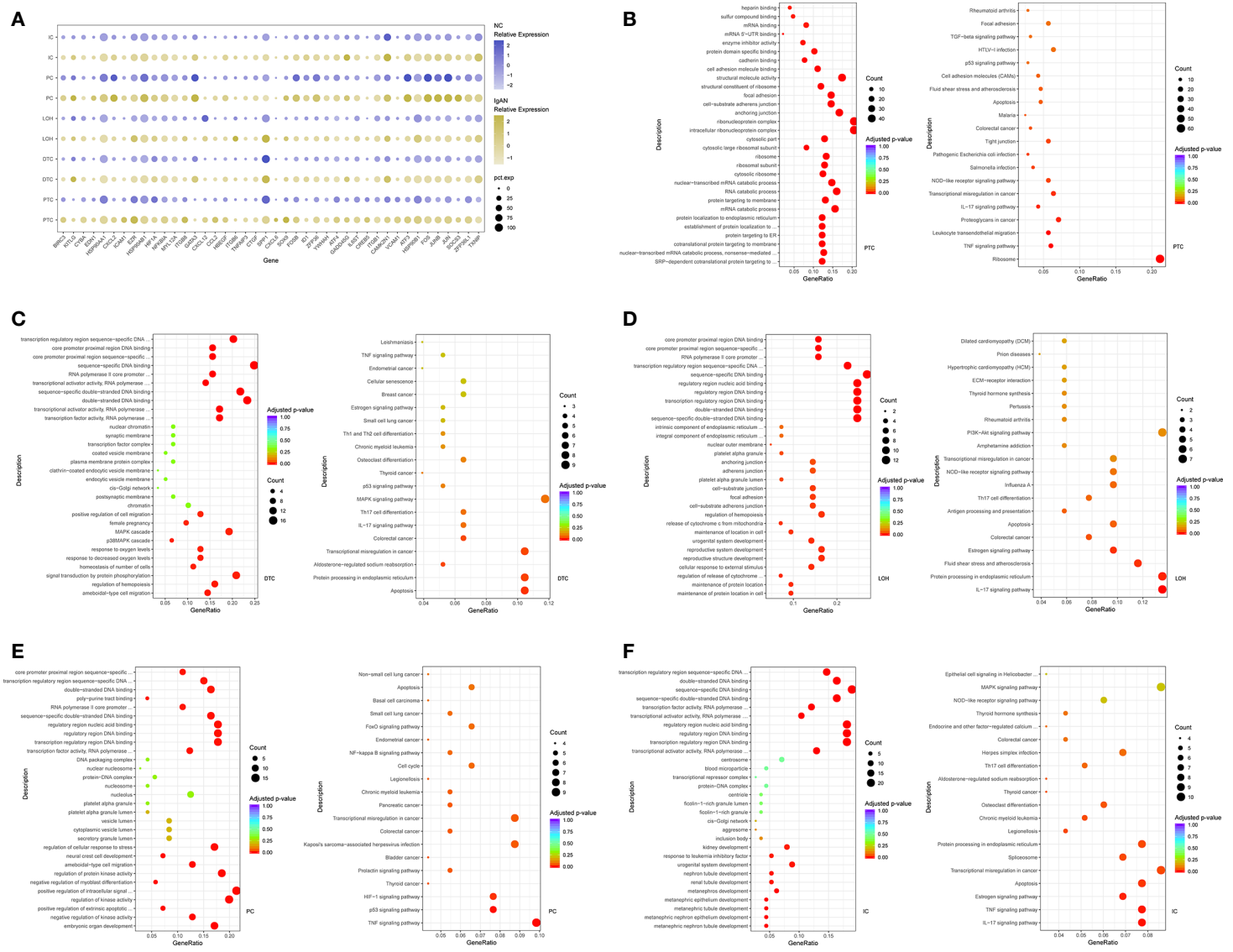

FIGURE 3 | DEGs and intercellular signaling in tubules of IgAN and control subjects. **(A)** Representative DEGs in proximal tubule cells, distal tubule cells, loop of Henle cells, principal cells, and intercalated cells by comparing the transcriptional profile of IgAN and control subjects. **(B)** GO enrichment analysis displayed that upregulated DEGs in proximal tubule cells were enriched in some biological processes, KEGG analysis showed these genes in proximal tubule cells were mainly involved in the pathways, such as TNF signaling, IL-17 signaling, and NOD-like receptor signaling pathway. **(C–F)** GO and KEGG enrichment analysis of DEGs in distal tubule cells, loop of Henle cells, principal cells, and intercalated cells respectively comparing IgAN to control subjects. Abbreviations were as follows: pct.exp, percentage of cells expressing gene; count, number of genes annotated to GO terms or KEGG pathway.

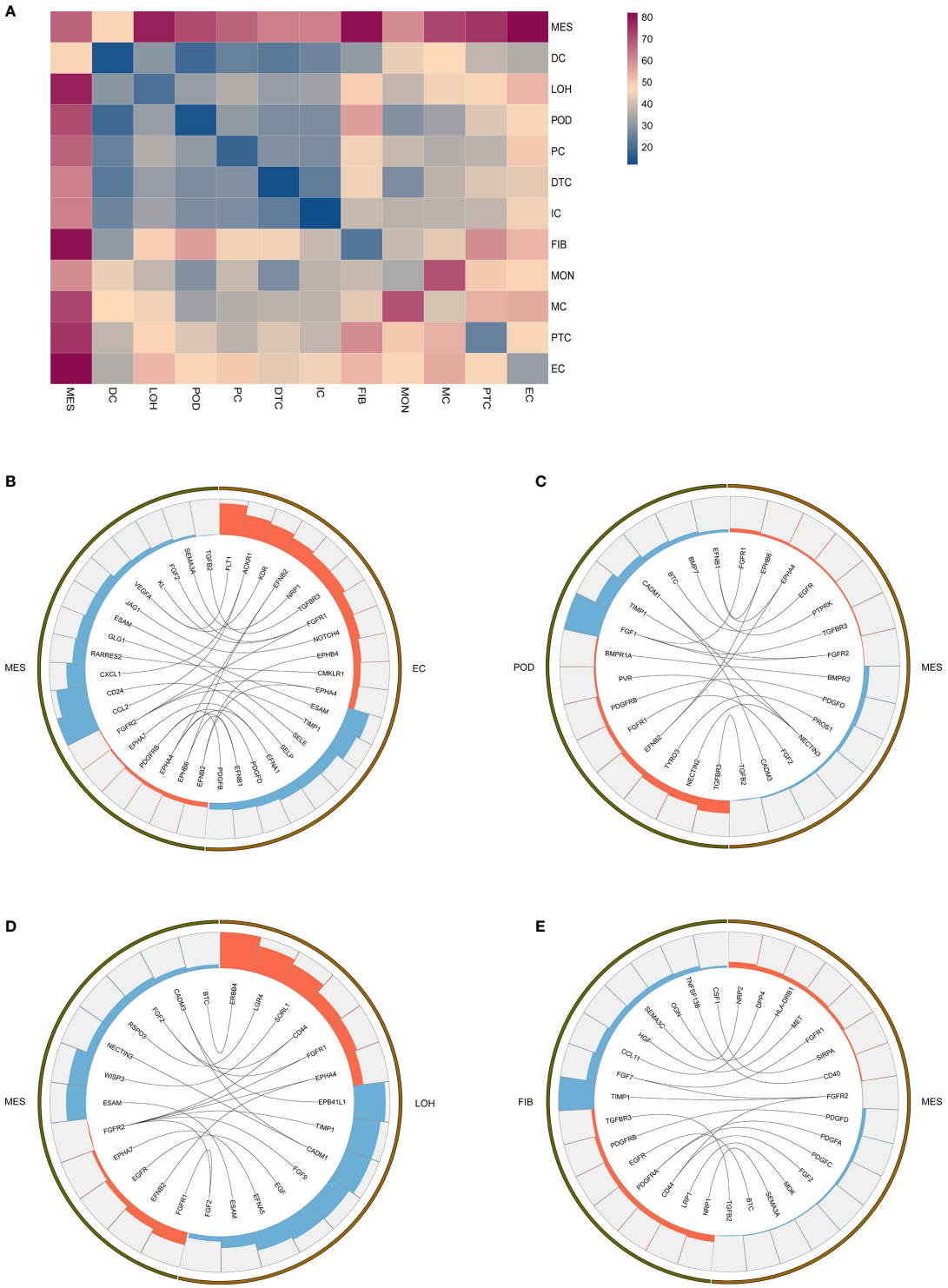

FIGURE 4 | Possible ligand-receptor interactions between different cell types in the kidney of IgAN patients. **(A)** Ligand-receptor signaling pathways between cell clusters in the kidney were analyzed and shown. **(B)** Representative ligand-receptor interactions between mesangial cells and endothelial cells. **(C)** Representative ligand-receptor interactions between mesangial cells and podocytes. **(D)** Representative ligand-receptor interactions between mesangial cells and loop of Henle cells. **(E)** Representative ligand-receptor interactions between mesangial cells and fibroblasts. Lines represented interrelations between the mesangial cells and other cells. Lines between the ligand and conjuncted receptor were shown. Each cell type was depicted by colors. Only IgAN patients (n = 4) were analyzed.

in kidney of microproteinuria group was different from that of overt proteinuria group. *SPARC*, an extracellular matrix-associated glycoprotein, was significantly increased in mesangial cells of overt proteinuria group, compared to microproteinuria group. Overexpression of *SPARC* has been shown to colocalize with collagen, and participate in extracellular matrix deposition and renal fibrosis (38). IgAN patients with overt proteinuria exhibited higher mesangial expression of *ROCK2*, compared with microproteinuria group, indicating it might be a new potential therapeutic target for IgAN. Previously, *ROCK2* has been proved to promote mesangial proliferation and ECM production by strengthening the inflammatory process and fibrotic circuitry in diabetic nephropathy (39). Furthermore, in comparison to microproteinuria group, overt proteinuria group showed upregulated endothelial *TXNIP* expression, which facilitates oxidative stress and inflammatory response (40). Also, *SPARCL1* and *CD74*, which are involved in the regulation of cell adhesion, migration, and proliferation, were higher in endothelial cells of the overt proteinuria group compared to the microproteinuria group (41, 42).

Comparison of the DEGs in mesangial cells displayed enrichment of genes participating in complement activation and alternative pathway in IgAN with overt proteinuria in comparison to microproteinuria (**Figure 5A**). Endothelial cells in IgAN with overt proteinuria had increased genes involved in extracellular matrix binding and cell-substrate adherens junction (**Figure 5B**). Furthermore, genes involved in many pathways including leukocyte transendothelial migration, chemokine signaling, and type I interferon signaling pathway were increased in proximal tubule cells of IgAN subjects with overt proteinuria (**Figures 5C, D**). The loop of Henle cells had elevated genes participating in ECM-receptor interaction, interferon-gamma-mediated signaling, and neutrophil activation (**Figures 5E, F**). Correspondingly, principal cells had elevated genes involved in granulocyte activation and p38MAPK cascade (**Figure 5G**). Intercalated cells had increased genes involved in interferon-gamma-mediated signaling pathway and T cell costimulation in IgAN with overt proteinuria compared with microproteinuria group (**Figure 5H**).

DISCUSSION

In this study, we comprehensively generated profiles of distinct cell types and gene expression in kidney biopsy specimen using scRNA-seq in IgAN subjects and living donor control. We also verified that scRNA-seq analysis of kidney sample obtained from IgAN patients was a feasible and effective technique. We presented a comprehensive scRNA-seq analysis of human renal biopsy tissue from IgAN. Our findings revealed upregulation of cell proliferation and cell adhesion related genes and activation of inflammatory pathways by comparing DEGs in different cell populations. Our study provided several novel insights into pathogenesis of human IgAN through detecting and analyzing cell subpopulations, cell-type-specific gene expression, and distinct signaling pathways.

Although the exact pathogenesis has not been fully elucidated, increased polymeric IgA deposition in the mesangium within the kidney is the characteristic of IgAN (2). Also, it is considered universally that hypercellularity of mesangial cells and mesangial expansions represent primary pathological mechanisms in the development of IgAN (43). In this study, we show for the first time, several upregulated genes (*MALAT1*, *GADD45B*, *SOX4*, and *EDIL3*) in IgAN mesangial cells. Their overexpression might be related to mesangial cells proliferation and matrix accumulation. Only few studies have reported that upregulation of *GADD45B* expression participating in podocytes injury of FSGS (19). There were no published studies about *MALAT1* in kidney diseases so far. These findings suggested mesangial expression of these genes might emerge as novel potential biomarker and therapeutic targets underlying IgAN.

Glomerular endothelial proliferation and adhesion are involved in progression of IgAN, and the upregulation of adhesion molecules in endothelial cells correlates with severity of glomerular inflammation in IgAN (44). The overexpressed genes in our investigation were mainly enriched in the processes of cell-matrix adhesion, leukocyte migration, and EMT. ScRNA-seq detected several genes which have not been reported in IgAN previously. Glomerular endothelial cells had upregulated genes of regulators of angiogenesis (*XIPS*), leukocyte adhesion molecules (*PECAM1*), EMT (*SOX4*), and leucocyte recruitment (*ACKR1*). Among them, *XIPS* was not reported previously in kidney pathologies. Together, these observations indicate that these glomerular endothelial cells alterations may be involved in pathogenesis of IgAN, the significance and specificity of these markers need further investigation to validate.

We also detected DEGs and related signaling pathways in tubule cells from IgAN subjects and control. A growing body of literature indicates that the tubulointerstitial inflammation and fibrosis are common features of chronicity and disease development, and renal prognosis correlates more closely with the degree of tubulointerstitial injury than the severity of glomerular lesions. The crucial role of tubular epithelial cells in IgAN has also been suggested (45). Our findings showed the overexpressed genes in proximal tubule cells were mainly enriched in TNF signaling, IL-17 signaling, and leukocyte transendothelial migration. TNF signaling pathway plays a crucial role in a variety of physiological and pathological processes, including cell proliferation, apoptosis, differentiation, induction of inflammation, and regulation of immune reactions, and it also acts as a pathogenic signaling in progression of IgAN (46, 47). T-cell-derived cytokine IL-17 and related signaling can activate downstream pathways including NF-kappa B and MAPKs to increase the expression of pro-inflammatory cytokines and chemokines (48). IL-17 signaling has been implicated in promoting inflammatory cytokines release, leukocytes recruitment, and progression of kidney injury in IgAN (49). Our group previously found that IgAN mice had increased elevated frequency of Th17 cells, and Th17-related

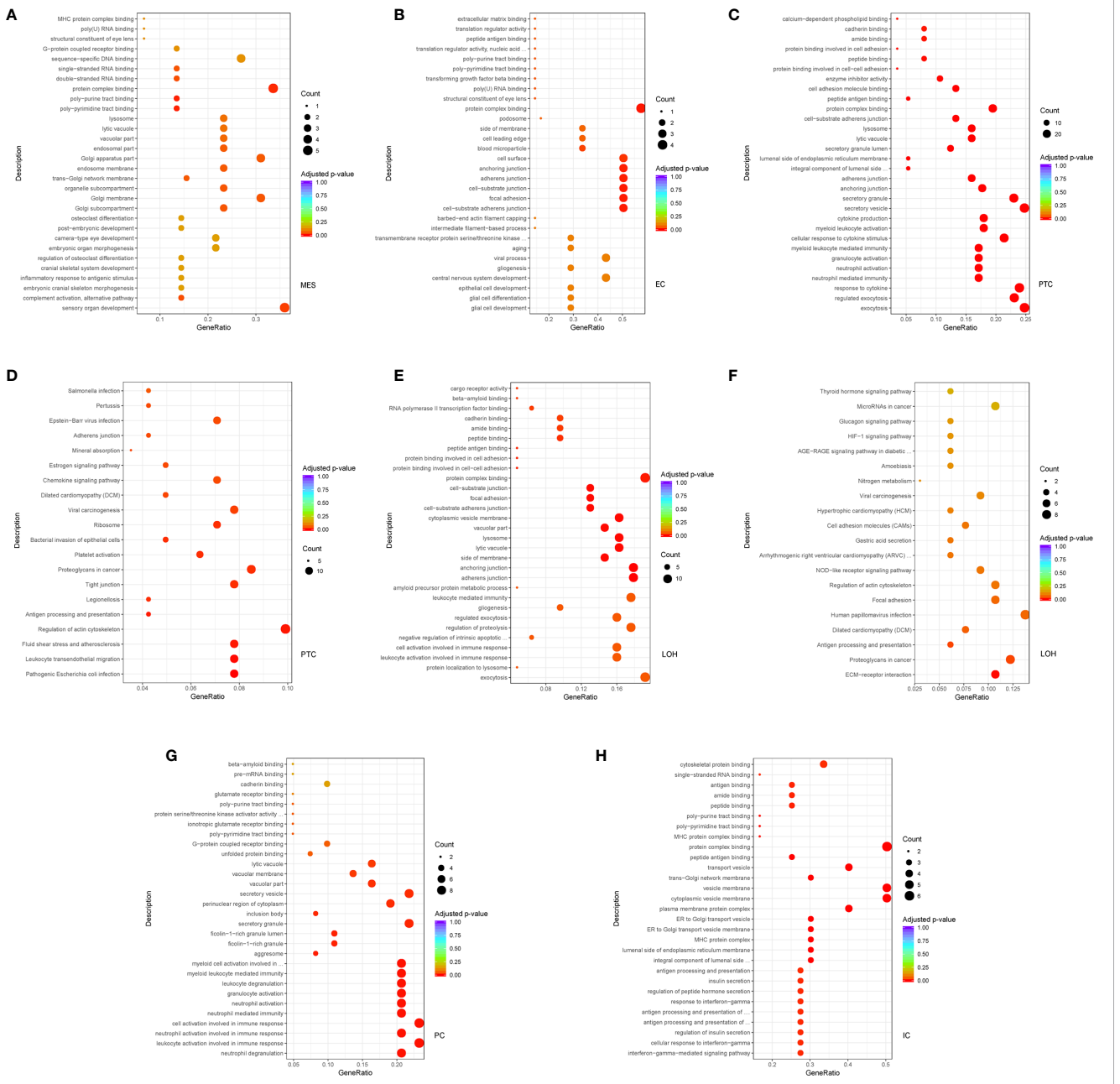

FIGURE 5 | DEGs involved in biological process and intercellular signaling in kidney from IgAN patients with overt proteinuria compared to microproteinuria. **(A, B)** GO enrichment analysis in mesangial cells and endothelial cells respectively between IgAN patients with overt proteinuria and with microproteinuria. **(C–F)** GO and KEGG enrichment analysis in proximal tubule cells and loop of Henle cells. **(G, H)** GO enrichment analysis in principal cells and intercalated cells between IgAN patients with overt proteinuria and with microproteinuria. Abbreviations were as follows: count, number of genes annotated to GO terms or KEGG pathway.

cytokines including IL-17A and CCL20 were all increased in the kidneys (50). In our study, TNF signaling and IL-17 signaling were also observed in other tubule cells including loop of Henle cells, principal cells, and intercalated cells, indicating the effects of these signaling may be extensive in IgAN. Interestingly, we identified elevated genes participating in NOD-like receptor signaling in tubule cells including proximal tubule cells, loop of Henle cells, principal cells, and intercalated cells. NOD-like receptor signaling can translate danger recognition into the production of proinflammatory cytokines and chemokines, and play a critical role in human diseases (28). One previous study has shown that NLRP3, a member of NLRs, localized mainly to the tubular epithelium, and increased NLRP3 expression correlates with better clinical prognosis in IgAN patients (51).

Furthermore, a prior study from our group showed that kidney tissue NLRC5 expression was significantly increased in the IgAN compared to that in the healthy control (52). Therefore, our novel finding about the upregulated genes involved in NOD-like receptor signaling will provide new insights into in pathogenesis of IgAN. Furthermore, in order to further validate the findings in our study, we compared the results of four IgAN patients with the published scRNA-Seq data of healthy kidney tissues of three human donors. The results also verified that the overexpressed genes in tubule cells from IgAN patients were mainly enriched in inflammatory pathways including TNF signaling, IL-17 signaling, and NOD-like receptor signaling.

By analyzing the receptor-ligand crosstalk among distinct cell types in kidney of IgAN, we determined intercellular signaling networks. For example, fibrotic related signaling and chemokine signaling pathway were observed in kidney of IgAN. We found mesangial cells expressed the growth factors *FGF2* and *PDGFD* indicative of a fibrotic reaction, which interacted with their respective receptors *FGFR1* and *PDGFRB* detected in resident kidney cells including podocytes and fibroblasts. The FGF and PDFG pathways are associated with ECM accumulation and renal fibrotic processes (37). We also found chemokines *CXCL1 and CCL2* in mesangial cells interacted with their chemokine receptor *ACKR1* expressed in endothelial junctions, implying renal resident cells cross-talk may serve as modulator for immune cells recruitment and infiltration in the kidney. Further confirmation of these interactions would be important for developing novel therapeutic targets.

In order to explore molecular signatures in IgAN patients with different degrees of proteinuria, we examined transcriptomics of different cell clusters in kidney. Genes involved in mesangial proliferation and ECM production (*SPARC*, *ROCK2*) were upregulated in mesangial cells from IgAN subjects with overt proteinuria compared to microproteinuria group. DEGs analysis of kidney also revealed that higher expression of genes (*TXNIP*, *SPARCL1*, and *CD74*), which participated in cell adhesion, migration, and inflammation, were also displayed in endothelial cells of kidney from overt proteinuria group than microproteinuria group. Interestingly, we also found genes participating in diverse biological processes and signaling pathways including complement activation, leukocyte transendothelial migration, chemokine signaling, and type I interferon signaling pathway, were increased in diverse cell clusters of kidney from IgAN with overt proteinuria. Our dataset may indicate new mechanisms correlating with different degrees of proteinuria in the progression of IgAN.

Infiltration of immune cells in renal tissue of IgAN patients have been found in clinicopathologic studies previously (30). It has been reported that interstitial or glomerular immune cells, especially macrophages/monocytes accumulation are related to proteinuria and renal damage in IgAN (29). Similarly, our research revealed an elevated number of macrophages, monocytes, and dendritic cells in kidney samples of IgAN subjects. Compared with publicly available PBMC control, infiltrating IgAN macrophages have decreased expression of genes (*GPX3*, *FAM49B*, and *FCGBP*), which are cell protective factors mainly related with anti-oxidation and anti-fibrosis. Therefore, these alterations may elicit the oxidative and inflammatory response in kidney of IgAN.

However, our study had several limitations. First, the sample size in this research was relatively small. Increasing number of samples is needed to reflect the disease severity and stages, and limit the heterogeneity and individual variation in IgAN in future study. Second, we detected relatively few leukocytes and some resident glomerular cells such as podocytes, which might not reflect whole gene expression status at different parts and times. It might reflect dissociation bias of scRNA-seq technology. Third, the above novel findings were only indicated at the transcriptomic level in IgAN. Fourth, as no other kidney disease control group was included in this study, the changes of DEGs are not specific for IgAN and the changes may be generic to being proteinuria or glomerular inflammation. Fifth, based on number of mesangial cells and other kidney cells analyzed (**Supplemental Table 8**), the results of our study are preliminary and needs to be confirmed on larger number of cells from larger number of patients and controls in future studies. Furthermore, the results discovered in our study need further validation using tissue staining, functional studies *in vitro* using cell lines or primary human cells, and animal models of IgAN.

Collectively, this study showed cell-specific transcriptional profiles in kidney, and identified several novel genes, involved signaling pathway and potential pathologic ligand-receptor interaction in IgAN patients. ScRNA-seq of kidney tissues revealed new insights into molecular signatures and provided potential targets for the treatment of IgAN.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: Gene Expression Omnibus, GSE171314.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YZ, RT, TM, WL, and XX conceived and designed the research; YZ, RT, JO, and XX wrote the paper; JO, PE, XA, and WP, QZ, and PX revised the paper; TM, WL, YZ, YT, and ZX carried out experiments; RT, TM, WL, and CS generated and provided analytical tools; JC, RT, YZ, XX, CS, PJ, and XD analyzed data.

All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.645988/full#supplementary-material

Supplemental Table 1 | Demographic and biochemical characteristics of patients with IgAN.

Supplemental Table 2 | Number and viability of cells in kidney from each sample.

Supplemental Table 3 | DEGs in cell subtypes of glomerulus comparing IgAN and control subjects. Abbreviations were as follows: pct.1, the percentage of cells where the gene is detected in the first group (IgAN patients); pct.2, the percentage of cells where the gene is detected in the second group (control subject); avg logFC, log fold-change of the average expression between the two groups, positive values indicate that the gene is more highly expressed in the first group.

Supplemental Table 4 | DEGs in tubular cells from IgAN and control subjects.

Supplemental Table 5 | DEGs in immune cells from IgAN and control subjects.

Supplemental Table 6 | DEGs in different cell clusters of kidney from IgAN patients with overt proteinuria compared to microproteinuria.

Supplemental Table 7 | Top 20 marker genes of each cell type shown in heatmap

Supplemental Table 8 | Cell number of distinct clusters in kidney from each subject

Supplemental Table 9 | Detailed information about DEGs in each cell cluster in kidney from IgAN and control subjects. Abbreviations were as follows: avg.exp, the average expression of the gene in the cell type; ave exp scaled, avg.exp after normalization; pct.exp, percentage of cells expressing gene; feature plot represented gene name.

Supplemental Table 10 | DEGs by cell type comparing four IgAN patients with the published scRNA-Seq data of healthy kidney tissues of three human donors downloaded from the Gene Expression Omnibus (GSE131685).

Supplemental Table 11 | Gene ontology enrichment analysis for individual cell types performed with the "cluster Profiler" R package using DEGs (**Supplementary Table 10**).

Supplementary Table 12 | Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis for individual cell types performed with the "cluster Profiler" R package using DEGs (**Supplementary Table 10**).

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Bile Acids Elevated in Chronic Periaortitis Could Activate Farnesoid-X-Receptor to Suppress IL-6 Production by Macrophages

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Chronic periaortitis (CP) is a rare autoimmune disease without effective treatment. By analyzing the serum bile acid spectrum in 28 CP patients with the ultra-performance liquid chromatography-tandem mass spectrometry, we found that the bile acids were significantly altered in CP patients, with significant increases in chenodeoxycholic acid (CDCA) and glycochenodeoxycholic acid (GCDCA) and decrease in deoxycholic acid (DCA). Signaling pathway enrichment analysis from the RNA sequencing results suggested that the altered gene sets in PBMC of CP patients were associated with bile acid metabolism. Furthermore, we found that pathological concentration of CDCA could significantly inhibited IL-6 expression in RAW 264.7 cells after LPS stimulation. Since CDCA is a well-known natural high-affinity ligand for the bile acid receptor farnesoid-x-receptor (FXR) while GW4064 is the synthetic specific agonist of this receptor, we then revealed that GW4064 significantly decreased IL-6 expression in RAW 264.7 cells and bone marrow-derived macrophages but not in $FXR^{-/-}$ macrophages upon LPS stimulation. The western blot results with the anti-FXR antibody showed significantly increased expression in the nuclear proportion, suggesting that FXR agonist promoted the transportation of FXR into the nucleus but did not increase the FXR expression in macrophages. Dual-luciferase report assay and ChIP assay demonstrated that upon activation, FXR could directly bind to the promoter site of IL-6, leading to the decreased expression of IL-6. Thus, bile acids, especially CDCA, may operate to damp inflammation *via* FXR-mediated downregulation of IL-6 in mononuclear cells and provide a protective mechanism for CP patients.

Keywords: bile acid, farnesoid-x-receptor, IL-6, chronic periaortitis, macrophages

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INTRODUCTION

Bile acids are a group of water-soluble, amphipathic molecules exclusively produced in the liver. Apart from their classically known role as lipid solubilizers and their functions in managing metabolic liver disorders, bile acids are now considered to be more and more involved in maintaining systemic metabolic and immune homeostasis, and even described as potential attractive therapeutic agents (1). Increasing recognition has been raised on their nonnegligible role as important signaling molecules in the regulation of metabolically driven inflammation (1, 2) as well as autoimmune diseases. Chenodeoxycholic acid (CDCA) was reported to have a treatment role in rheumatoid arthritis as early as 1976 (3). The immunosuppressive roles of CDCA and ursodeoxycholic acid (UDCA) were also discovered in an allogeneic immune response mouse model (4). As such, Farnesoid-X-receptor (FXR), a bile acid receptor, may play a pivotal role in these signaling pathways since bile acids, especially CDCA, are well-known as natural high-affinity ligands for this nuclear hormone receptor (5).

Farnesoid-X-receptor (FXR) is a member of the orphan nuclear receptor family and is widely expressed in the liver, small intestine, adipocytes and also macrophages. Function as a bile acid activated nuclear receptor and a transcription factor, FXR can regulate the gene expression of bile acid and lipid metabolism with the activation of its natural ligand, CDCA, and participate in the process of bile acid metabolism, lipid metabolism and glucose metabolism (5). In addition, it is also noteworthy that FXR now play more of its role in the anti-inflammatory and anti-fibrosis aspects. Recent experimental and clinical evidences have indicated that, FXR agonists can significantly alleviate the damage of target organs, weaken the activation of immune cells, promote the differentiation of Tregs (6) and reduce the release of IL-1β, TNFα and other inflammatory factors, indicating that FXR plays an important anti-inflammatory role in the innate and adaptive immunity (7). FXR agonists have also achieved several good curative effects in the treatment of primary biliary cholangitis (also known as primary biliary cirrhosis), revealing the outstanding role of FXR in anti-fibrosis aspect (8, 9). Furthermore, beneficial effect of FXR agonists has also been emerged in autoimmune disease (10, 11).

Chronic periaortitis (CP) is a rare disorder and characterized by chronic idiopathic fibrosis. It is mainly manifested by inflammatory adipose tissues and collagen fibers, spreads from the adventitia of the abdominal aorta or/and the iliac artery into the retroperitoneum, wrapping around the abdominal aorta and/or iliac artery as well as inferior vena cava (12, 13). Extension of the mass could also contribute to the adhesion or obstruction of the surrounding organs. According to the extent of disease progression, CP can be divided into idiopathic retroperitoneal fibrosis (IRF), inflammatory abdominal aortic aneurysms (IAAA) and perianeurysmal retroperitoneal fibrosis (PARF), of which IRF is most common. CP was originally identified as an immune response to local atherosclerosis (14), however, emerging evidences have unveiled the fundamental of CP as an autoimmune disease. Recent associations have been found between the susceptibility of CP with HLA-DRB1 * 03 and other immune disease-related alleles (15).

Although bile acids are known to affect host metabolism and innate immunity through the activation of FXR, however, less has been described about the bile acid spectrum in CP patients and it is still unknown whether the bile acid-FXR pathway has a role in the pathogenesis or as a potential treatment for the inflammation along with fibrosis in this disease. Moreover, it was reported that the serum level of the pro-inflammatory factor IL-6 was significantly higher in active CP patients rather than healthy controls, and the IL-6 expression was dominantly elevated in $CD68^+$ macrophages/monocytes (16). Since IL-6 is a very important cytokine that could be able to stimulate chronic inflammation in adipocytes and also central to pro-fibrotic interactions within fibroblasts, IL-6 may play a pivotal role contributing to the pathogenesis of CP. Therefore, we wonder if bile acid-FXR pathway could have a distinct function in the regulation of IL-6 expression in macrophages/monocytes. In our present study, we analyzed the serum bile acid spectrum of normal subjects, CP patients and disease controls. We also analyzed the transcriptomes in PBMCs of controls and CP patients and found that the pathways enriched in the analysis are highly associated with bile acid metabolism. We also demonstrated that the pathological but not physiological level of CDCA could significantly decrease the IL-6 expression in RAW264.7 after the LPS stimulation. Moreover, by using RAW 264.7 cells and bone marrow-derived macrophages (BMDMs) from wild type and $FXR^{-/-}$ mice, we demonstrated that FXR activation by GW4064 could be able to inhibit IL-6 production. Furthermore, we showed that FXR could directly bind to the promoter region of IL-6, leading to the inhibition of IL-6 in macrophages.

Abbreviations: CP, chronic periaortitis; FXR, farnesoid-X-receptor; IRF, idiopathic retroperitoneal fibrosis; IAAA, inflammatory abdominal aortic aneurysms; PARF, perianeurysmal retroperitoneal fibrosis; BMDM, bone marrow-derived macrophages; PBMC, peripheral blood mononuclear cell; TA, Takayasu's arteritis; PAD, peripheral arterial disease; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry; DEG, differentially expressed gene; KEGG, Kyoto encyclopedia of genes and genomes; GSEA, gene set enrichment analysis; DMARDs, disease-modifying anti-rheumatic drugs; CA, cholic acid; TCA, taurocholic acid; GCA, glycocholic acid; CDCA, chenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; DCA, deoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; LCA, lithocholic acid; TLCA, taurolithocholic acid; GLCA, glycolithocholic acid; UDCA, ursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; GUDCA, glycoursodeoxycholic acid.

MATERIALS AND METHODS

Human Subjects

Serum samples and peripheral blood mononuclear cells of naïve CP patients without any medical treatment were collected from Department of Rheumatology, Ren Ji Hospital, Shanghai Jiao Tong University, Department of Rheumatology, Zhongshan Hospital, Fudan University and Department of Rheumatology, the First Affiliated Hospital of Wenzhou Medical University

between November 2016 to July 2020, confirmed by pathology or by CT/MRI examination in line with the previous diagnostic criteria of CP (16) and exclusion of tumors and other secondary causes. Blood samples were collected after 10 h of overnight fasting. A total of 28 CP cases were finally confirmed. Meanwhile, 10 naïve Takayasu's arteritis (TA) patients were also recruited from Department of Rheumatology, Ren Ji Hospital, Shanghai Jiao Tong University and Department of Rheumatology, Zhongshan Hospital, Fudan University. The general information, clinical symptoms and laboratory examinations of CP and TA patients were collected and documented (**Table 1**). In addition, 47 patients diagnosed with peripheral arterial disease (PAD) were recruited from the Department of Vascular Surgery, Ren Ji Hospital, Shanghai Jiao Tong University. Serums of 88 healthy controls were collected from the medical examination center of Ren Ji hospital. The study protocol was guided and approved by the Ethics Committee of Renji Hospital, and the study was performed in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent.

Examination of Serum Bile Acid

Serum samples of the patients were set at room temperature for 30 min, centrifuged at 6,000g for 10 min to obtain the serum and then stored in a refrigerator at -80°C. Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) standard box (manufactured by Guangzhou Ke Li Mass Spectrometer Medical Instrument Co., Ltd.) was used and the examination of serum CDCA was conducted following the manufacturer's instructions. API 3200 instrument from SCIEX Corporation was used for the examination.

Gene Expression Profiling

Total RNA was isolated from 1×10^6 peripheral blood mononuclear cells from CP or healthy controls, using the RNeasy Mini Kit (QIAGEN). RNA-seq was carried out by Genergy Biotech (Shanghai, China) and the following data analysis as well as cluster enrichment with KEGG and GSEA were then performed based on the website of Networkanalyst (17). Data were log2 transformed, followed by normalization to the 75th percentile, and corrected to the median of all samples. Features passing the quality check (variance percentile rank above 10 and read count over 5) and showing changes in expression levels equal to or more than 2-fold were selected for further analysis. A volcano plot was utilized to identify statistically significance ($P < 0.05$). The differentially expressed genes (DEGs) screened from the previous procedure were then put into the pathway enrichment analysis using KEGG to elucidate the biological interpretation. GSEA was also applied to enrich the pathway analysis.

TABLE 1 | Demographics, clinical characteristics of CP and TA patients.

Feature	Chronic Periaortitis	Takayasu's Arteritis
Age, years, median (IQR)	62.5 (55.8-68)	45 (38.5-56.3)
Gender, n (%)		
Male	24 (85.7%)	3 (30%)
Female	4 (14.3%)	7 (70%)
Comorbidity, n (%)		
Sjögren's syndrome	1 (3.6%)	0
Hypertension	4 (14.3%)	3 (30%)
Diabetes mellitus	3 (10.7%)	1 (10%)
Dyslipidemia	2 (7.1%)	2 (20%)
Laboratory examination, median (IQR)		
ESR (mm/h)	66 (26.0-101.0)	23 (9.3–51.5)
CRP (mg/L)	10.9 (3.4-30.2)	12.8 (3.2–66.9)
ALT (U/L)	18 (9.5-40.5)	20 (16.5-26)
γ-GT (U/L)	23 (17-92)	26.5 (20.8-117)
Total bile acid (μmol/L)	3.8 (2.3-10.6)	1.7 (0.8-3.9)
Creatinine (μmol/L)	87.5 (79-145.5)	66.2 (54.8-77.4)
IgG4 (g/L)	2.4 (0.8-3.5)	1.2(1.0-1.3)

Cell Preparation

Murine macrophage cell line RAW264.7 (source from ATCC, TIB-71, male) and murine fibroblast cell line L929 (source from ATCC, CCL-1, male) were obtained from the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 5% CO_2 at 37°C and grown in DMEM medium for RAW264.7 and DMEM/F12 medium for L929 containing 10% FCS and 1% (v/v) penicillin/streptomycin (All medium and supplements were obtained from GIBCO). Bone marrow-derived macrophages were generated from total bone marrow cells flushed out from 9-14 weeks old wild type C57/B6 mice or FXR knockout ($FXR^{-/-}$) mice and the littermates and then incubated in DMEM and GlutaMAX (Gibco, Thermo Fisher Scientific) supplemented with 10% (v/v) FCS (Gibco, Thermo Fisher Scientific), 1% (v/v) penicillin/streptomycin, and 15% (v/v) L929 conditioned medium for 7 days.

Flow Cytometry

On Day7 of the differentiation, bone marrow-derived macrophages from FXR-/- mice and the littermates were harvested and stained with CD11b-PerCP-Cy 5.5, F4/80-APC, CD11c-FITC and CD206-PE. The cells were then measured by a BD FACSCalibur Flow Cytometer. The percentage of the differentiated macrophages (CD11b+ F4/80+), M1 macrophages (CD11b+ F4/80+ CD11c+ CD206-) and M2 macrophages (CD11b+ F4/80+ CD11c- CD206+) were then calculated. All antibodies for flow cytometry were from BD Biosciences.

Real-Time PCR Analysis

A standard phenol-chloroform extraction was performed with Trizol reagent to isolate total RNA from RAW 264.7 cells as with bone marrow-derived macrophages from wild type and $FXR^{-/-}$ mice and the littermates after diverse treatments. cDNA was synthesized from 1 μg of total RNA with a Reverse Transcription Kit (PrimeScript RT reagent Kit Perfect Real Time, Takara). Real-time PCR analysis was then obtained and analyzed with a SYBR label (SYBR Premix Ex Taq™, Tli RNaseH Plus, Takara) in QuantStudio™ 6 Flex Real-Time PCR System. The real-time PCR primer sequences are *Il-1β* for, GGACATGAGCACCT TCTTTTC; *Il-1β* rev, CTAATGGGAACGTCACACACC; *Tnfα* for, AAACACAAGATGCTGGGACA; *Tnfα* rev, TTGATGGTG GTGCATGAGAG; *Il-6* for, TAGTCCTTCCTACCCCAA TTTCC; *Il-6* rev, TTGGTCCTTAGCCACTCCTTC; *Gapdh* for,

CAGAACATCATCCCTGCATC; *Gapdh* rev, CTGCTTCAC CACCTTCTTGA; *Nr1h4* for, TAGTCTTCACCACAGCCACC; *Nr1h4* rev, CAGGTTGGAATAGTAAGACGAGG. The relative count of genes was calculated by normalizing to *Gapdh* mRNA and expressed as fold change relative to the control/ vehicle group.

ELISA

The concentrations of TNFα, IL-1β and IL-6 in the relevant supernatant were performed with the Mouse TNF-alpha DuoSet ELISA Kit (DY410, R&D SYSTEMS), Mouse IL-1b DuoSet ELISA Kit (DY401, R&D SYSTEMS) and Mouse IL-6 ELISA MAX™ Kit (Cat. 431301, Biolegend) following the manufacturer's instructions.

Western Blot Analysis

RAW 264.7 cells with different treatments were homogenized in RIPA buffer with protease and phosphatase inhibitors. The nuclear and cytoplasmic extraction was conducted under the protocol of the Nuclear and Cytoplasmic Extraction Kit (CW0199, CWBIO, China). The protein extracts were then separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane. The membrane was incubated with antibodies against Nr1h4 (sc-13063, Santa-Cruz, 1: 1000), GAPDH (Cat#2118, Cell Signaling, 1:1000) and LaminB (GTX103292, GeneTex, 1:1000) overnight at 4°C.

Luciferase Reporter Assay

The putative FXR binding regions in the promoter of IL-6 were firstly predicted with online databases PROMO and NUBIscan. Then the regions were amplified by PCR from genomic DNA extracted from splenocytes in C57BL/6 WT mice and cloned using Kpn I and Sma I into the pGL-3 firefly reporter vector (Promega). All the constructs were verified by sequencing. Then respective luciferase reporter constructed or basic pGL-3 vector, as with Renilla plasmid and the FXR overexpression vector (Genecopoeia, Guangzhou, China) were co-transfected into 293T cells using lipofectamine 2000 (Thermo). Transfected cells were then lysed and luciferase activity was quantified with the Dual-Luciferase Reporter Assay (Promega) following the manufacturer's instructions and normalized to the activity of the co-transfected Renilla reporter gene.

ChIP

ChIP was performed with the EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (17-10086, Millipore) following the manufacturer's instructions. IL-6 promoter regions were amplified with the specific primer derived from the result of luciferase assay for the corresponding promoter sites by real-time PCR.

HE and Immunohistochemical Staining

HE staining was performed by the Department of Pathology as common procedure. Anti-IL-6 polyclonal antibody (ab6672, Abcam Corporation, 1: 200) and anti-Nr1h4 polyclonal antibody (sc-13063, Santa-Cruz, 1: 50) were used to explore the expression of IL-6 and FXR in the lesion location of the disease. The bound antibodies were then visualized using EnVision reagent (K500711 kit, Dako, Denmark). Immunohistochemical results were observed by Nikon ECLIPSE Ti-s inverted microscope and analyzed by NIS-Element software. An isotype control (ab27478, Abcam, 1: 400) was used instead of each primary antibody as the negative control.

Data Analysis

GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA) was used for statistical treatment. Experimental data were shown as the mean ± SEM. Two-tailed unpaired Student's t-test and ANOVA with multiple comparisons test were used as indicated in respective experiments.

RESULT

Bile Acids Are Altered in Chronic Periaortitis Patients

To investigate the levels of different bile acids in CP patients, we firstly performed ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) metabolite profiling to depict the bile acid spectrum in the serum of CP patients. Controls included healthy controls and the patients diagnosed as other vasculitis (Takayasu's arteritis) and vascular disease with atherosclerotic lesions (peripheral artery disease). Comparing to healthy controls, the level of CDCA, which is one of the two primary bile acids, and its glycine-conjugated secondary bile acid in human, glycochenodeoxycholic acid (GCDCA), was predominantly elevated in CP patients (**Figure 1**). While deoxycholic acid (DCA), a secondary bile acid converted from the other primary bile acid cholic acid (CA), along with its glycine-conjugated form glycodeoxycholic acid (GDCA), were significantly decreased in CP patients. Of note, the patients of Takayasu's arteritis showed much more increase in CA, GCA, TCA, TCDCA, DCA, TDCA and tauroursodeoxycholic acid (TUDCA) while the patients of peripheral artery disease showed a dominant increase in CDCA. These results suggest that the bile acid metabolism, especially the metabolism of CDCA and GCDCA, is obviously altered in individuals with CP than normal conditions. The bile acid spectrum of CP is also significantly different from other vascular diseases.

Altered Gene Sets in CP Patients Are Associated With Bile Acid Metabolism

In order to investigate the impact of bile acids changes in CP on immune cells, we collected the peripheral blood mononuclear cells from 8 CP patients along with 8 age-gender matched healthy controls and performed RNA sequencing. Principal component analysis showed that the gene sets in CP patients were significantly different from the healthy controls (**Figure 2A**). Differentially expressed transcript analysis revealed that 1336 genes were up-regulated in CP patients compared to healthy controls while 668 genes were down-regulated, as showed in the volcano plot (**Figure 2B**). Based on the

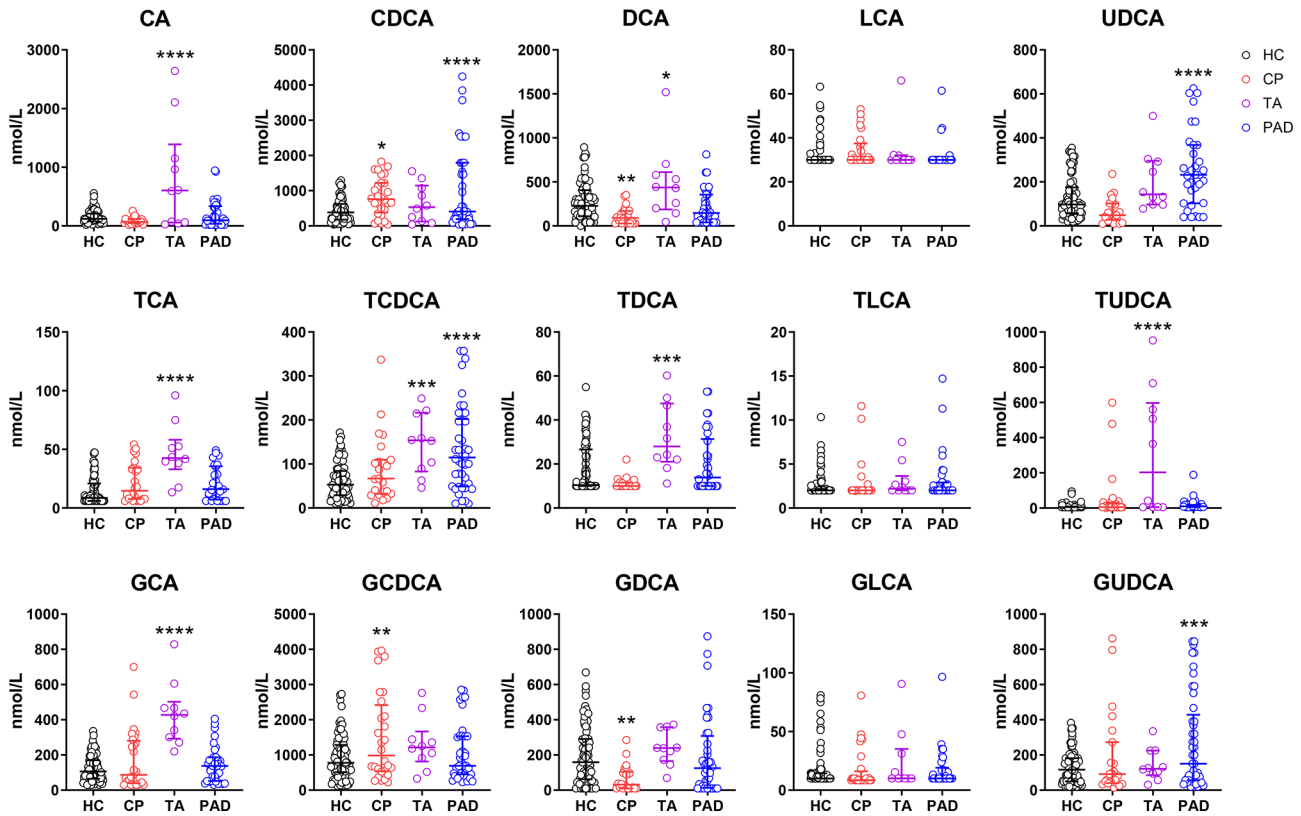

FIGURE 1 | Bile Acids are Altered in Chronic Periaortitis Patients. Serum was collected from healthy controls (n=88, shown in black) as well as the patients of chronic periaortitis (CP) (n=28, shown in red), Takayasu's arteritis (TA) (n=10, shown in purple) and peripheral artery disease (PAD) (n=47, shown in blue) and tested by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Bile acid spectrum in diverse groups were then analyzed. Data are shown as median (IQR). * means patients' data comparing to healthy controls, respectively. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, one-way ANOVA, followed by Dunnett's multiple comparisons test. CA, cholic acid; TCA, taurocholic acid; GCA, glycocholic acid; CDCA, chenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; DCA, deoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; LCA, lithocholic acid; TLCA, taurolithocholic acid; GLCA, glycolithocholic acid; UDCA, ursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; GUDCA, glycoursodeoxycholic acid.

differentially expressed genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was adopted to perform the signaling pathway enrichment. Interestingly, we found that the bile acid metabolism associated signaling pathways (18) were significantly enriched in up-regulated genes, named as phagosome, fatty acid metabolism and cytokine-cytokine receptor interaction (**Figure 2C**). GSEA results also revealed a significant upregulation of the gene sets in fatty acid metabolism as well as cytokine-cytokine receptor interaction (**Figure 2D**). Together, these results suggest that bile acids impact differently on immune cells in CP patients comparing to the healthy controls.

Pathological Level of CDCA Decreases IL-6 Expression in Macrophages

To investigate the effect of altered bile acids on immune cells, especially the elevation of CDCA, we checked the role of CDCA on macrophages with the murine cell line RAW 264.7. We treated the cells with different concentrations of CDCA for 24h, and then stimulated the cells with 100ng/ml LPS. As expected, we found CDCA at physiological concentration (1μM) did not have the inhibitory role on the expression of pro-inflammatory cytokines TNFα and IL-6, but slightly inhibited IL-1β expression. However, at pathological level (over 2.5μM) as in CP patients, CDCA could significantly decrease IL-6 expression (**Figure 3**).

FXR Agonist GW4064 Inhibits IL-6 Expression in Macrophages

Since CDCA is a well-known natural high-affinity ligand for farnesoid-x-receptor (FXR) (19), we then wondered if the inhibitory role of CDCA on macrophages could be mediated by FXR activation. As GW4064 is the synthetic specific agonist of bile acid receptor FXR, we investigated the impact of GW4064 on function of monocytes/macrophages, especially on the expression of IL-6, the cytokine significantly up-expressed in $CD68^+$ macrophages/monocytes of CP patients (16). Firstly, we incubated the RAW 264.7 macrophages with different

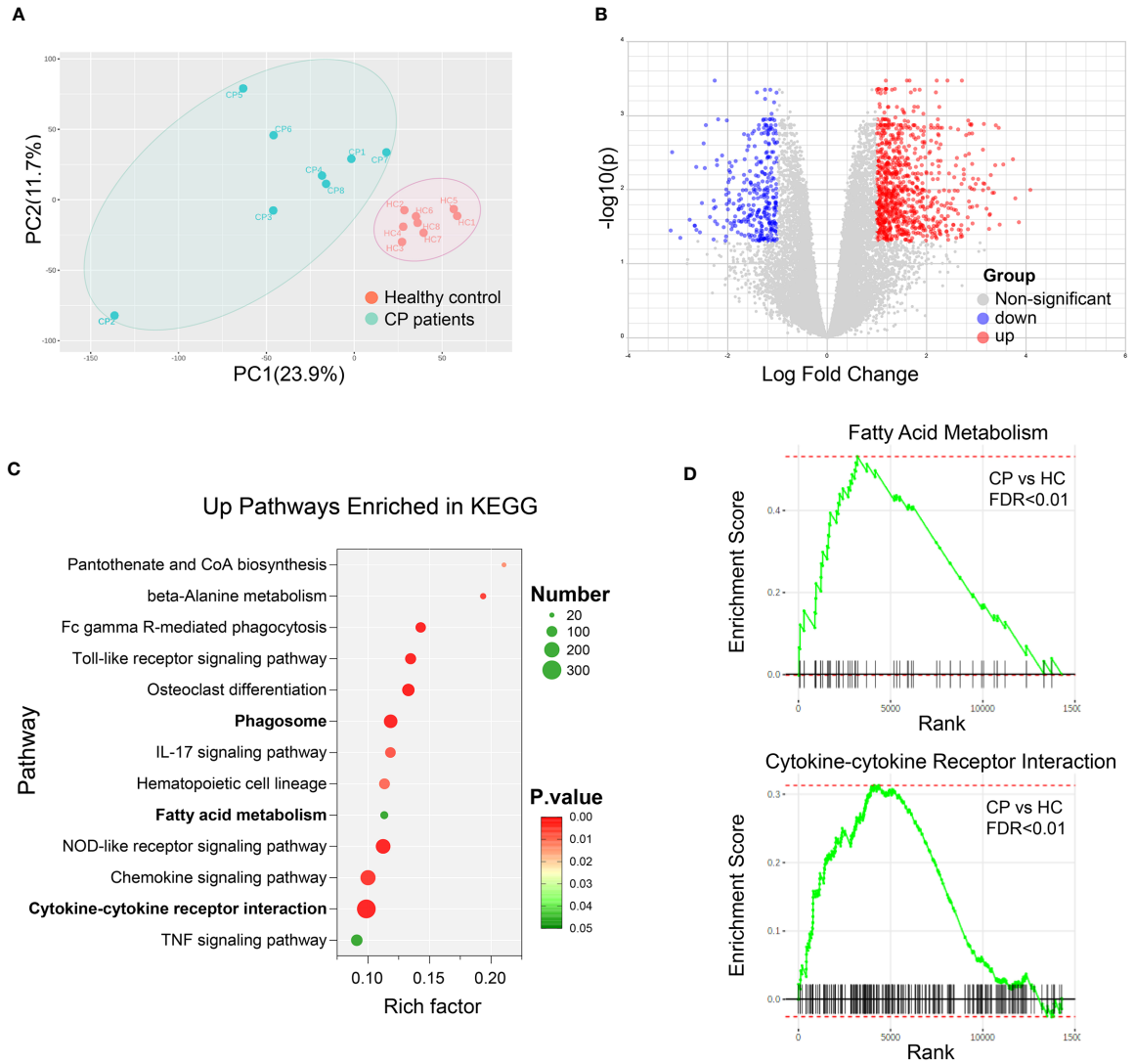

FIGURE 2 | Bile Acid Metabolism is Associated with the Altered Signalings in CP Patients. Peripheral blood mononuclear cells (PBMC) were collected from 8 CP patients along with 8 age-gender matched healthy controls. RNA sequencing and transcript analysis were then performed and gene sets were enriched in KEGG and GSEA. **(A)** Principal component analysis of healthy controls (HC) and CP patients. **(B)** Volcano plot of the differentially expressed transcript analysis in CP comparing to HC. **(C)** Signaling pathways enriched in KEGG with the up-regulated genes in CP compared to HC. **(D)** Representative signaling pathways, fatty acid metabolism and cytokine-cytokine receptor interaction, enriched in GSEA results comparing CP to HC.

concentrations of FXR agonist GW4064 for 24h, and then stimulated the cells with 100ng/ml LPS. As showed in **Figure 4A**, the mRNA levels of the pro-inflammatory factors TNFα, IL-1β and IL-6 were significantly up-regulated upon the LPS stimulation; however, GW4064 could significantly inhibit their expressions 6h after the stimulation, in a dose-dependent manner. The protein level of IL-6 in the supernatant of stimulated macrophages was also decreased after the GW4064 treatment (**Figure 4B**). We then consolidate the findings with bone marrow-derived macrophages. We observed a consistent phenotype with a significant decrease of pro-inflammatory factors in both mRNA level and protein level, especially for IL-6 (**Figures 4C, D**). In addition, the incubation of the antagonist of FXR Z-Guggulsterone with BMDM led to an increase of TNFα and IL-6 on mRNA level (**Figure 4C**). Furthermore, FXR knockout did not affect the macrophage differentiation (**Supplementary Figure 1**), however, the incubation of GW4064 with FXR knockout ($FXR^{-/-}$) BMDM revealed no significant inhibition of IL-6 in both mRNA and protein level (**Figures 4E, F**). Taken together, these data indicates that the activation of FXR has a specific inhibitory role on the IL-6 expression, and bile acids, especially CDCA may have an inhibitory role on the IL-6 expression through the activation of FXR.

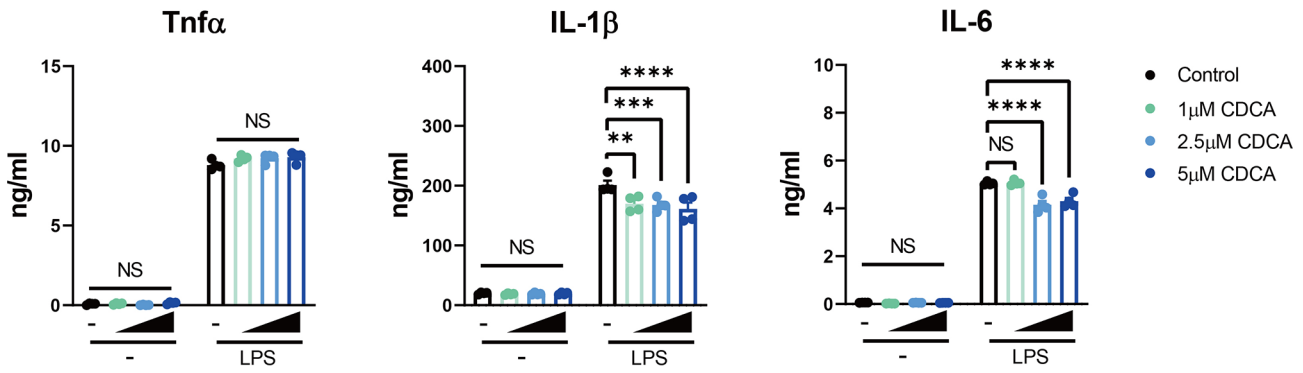

FIGURE 3 | Pathological Level of CDCA Decreases IL-6 Expression in Macrophages. RAW 264.7 macrophages were incubated with or without different concentrations of CDCA for 24h, and then stimulated with or without 100ng/ml LPS. TNFα, IL-1β and IL-6 levels in the supernatant from 24-hr cultures are shown. Data are shown as mean ± SEM. Experiments were repeated 3 times and representative data are shown. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; NS, no significant difference, two-way ANOVA, followed by Dunnett's multiple comparisons test.

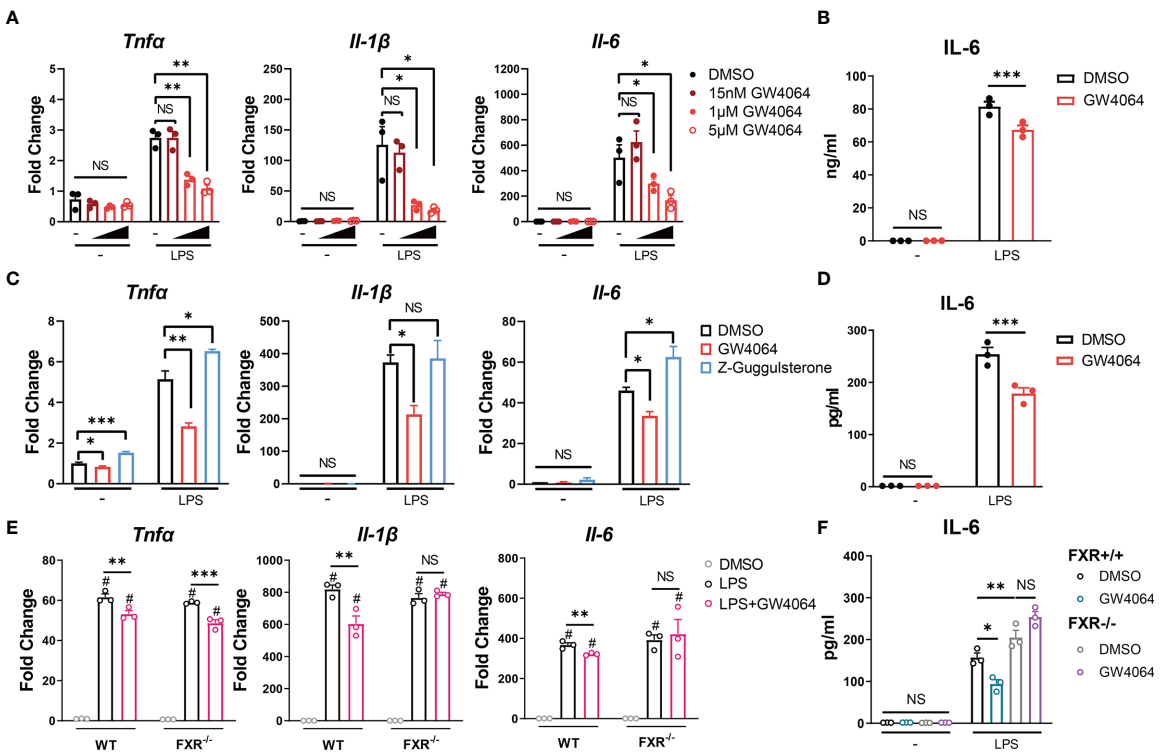

FIGURE 4 | FXR Agonist GW4064 Inhibits IL-6 Expression in Macrophages. **(A, B)** RAW 264.7 macrophages were incubated with or without different concentrations of FXR agonist GW4064 for 24h, and then stimulated with or without 100ng/ml LPS. **(A)** The mRNA levels of TNFα, IL-1β and IL-6 from harvested cells at 6h after the stimulation are shown. **(B)** IL-6 levels in the supernatant from 24-hr cultures are shown. **(C, D)** Bone marrow-derived macrophages were incubated in the presence of DMSO, 5μM GW4064 or 1μM FXR antagonist Z-Guggulsterone for 24h. Cells were then stimulated with or without 100ng/ml LPS. **(C)** The mRNA levels of TNFα, IL-1β and IL-6 from harvested cells at 6h after the stimulation are shown. **(D)** IL-6 levels in the supernatant from 24-hr cultures are shown. **(E, F)** Bone marrow-derived macrophages from FXR knockout ($FXR^{-/-}$) mice and the littermates were incubated with or without 5μM GW4064 for 24h, and then stimulated with or without 100ng/ml LPS. **(E)** The mRNA levels of TNFα, IL-1β and IL-6 from harvested cells at 6h after the stimulation are shown. **(F)** IL-6 levels in the supernatant from 24-hr cultures are shown. Data are shown as mean ± SEM. Experiments were repeated 3 times and representative data are shown. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS, no significant difference; # means compared to the relative unstimulated control group, two-way ANOVA, followed by Sidak's multiple comparisons test.

FXR Agonist Promotes the Translocation of FXR Into the Nucleus

We investigated how FXR agonist affects FXR in RAW 264.7 macrophages. We firstly evaluated the FXR expression in the macrophages. Incubation with GW4064 did not significantly change expression of *Nr1h4*, the gene encoding FXR (**Figure 5A**). Under LPS stimulation, cells incubated with GW4064 had significantly increased expression of *Nr1h4* (**Figure 5A**). Protein analysis by western blotting did not reveal a clear increase in total FXR (**Figure 5B**). However, FXR within the nucleus was significantly increased after LPS stimulation. In parallel, cytoplasm expression of FXR was reduced, particularly in GW4064-treated cells (**Figures 5B, C**). These results indicate that LPS stimulation together with GW4064 stimulation of FXR could impact on transcription as well as nuclear translocation of FXR, fitting to its function as a transcription factor.

FXR Binds to the Promoter Site of IL-6

We next investigated how GW4064 might regulate IL-6 production by macrophages. Considering that FXR is a transcription factor, we looked into the direct role for FXR in regulating IL-6 transcription. Based on the databases of PROMO and NUBIscan, we performed the binding site prediction of FXR within about 2000bp upstream of the transcription start site of IL-6, and found 4 potential binding sites in the promoter site of IL-6 (**Figure 6A** upper). According to the prediction results, we constructed several plasmids containing different numbers of the binding site based on the basic pGL-3 plasmid (**Figure 6A** bottom, left), performed the co-transfection of them respectively with FXR-overexpression plasmid as well as Renilla plasmid, and tested the luciferase activity with the dual-luciferase assay. The reporter assay showed that comparing to the basic pGL-3 plasmid, FXR could directly bind to the plasmid containing the whole 4 binding sites and significantly promoted the luciferase activity. Moreover, the successive deletion of the former 3 binding sites did not change a lot of the luciferase activity, while the deletion of the last binding site led to a disappear of the significance of the luciferase activity, indicating that the actual binding region of FXR on the promoter of IL-6 is located on the last predictive site (**Figure 6A** bottom, right). The additional ChIP assay was also conducted targeting the last predictive site, and the result depicted that FXR indeed possessed a higher binding activity to the fourth predictive binding site on the promoter of IL-6 under the activation of GW4064 upon LPS stimulation (**Figure 6B**).

IL-6 and FXR Are Expressed in CP Human Sample

To further investigate the FXR expression as well as IL-6 expression locally in CP patients, we performed a biopsy of the retroperitoneal lesion from one CP patient and conducted the following HE and immunohistochemical staining. The morphological observation from the HE staining revealed a typical manifestation of CP, with an abundant infiltration of foam-like cells and lymphocytes within the adipose tissues and collagen fibers (**Figures 7A, B**). IL-6 was broadly expressed within the lesion and especially within the site of inflammation

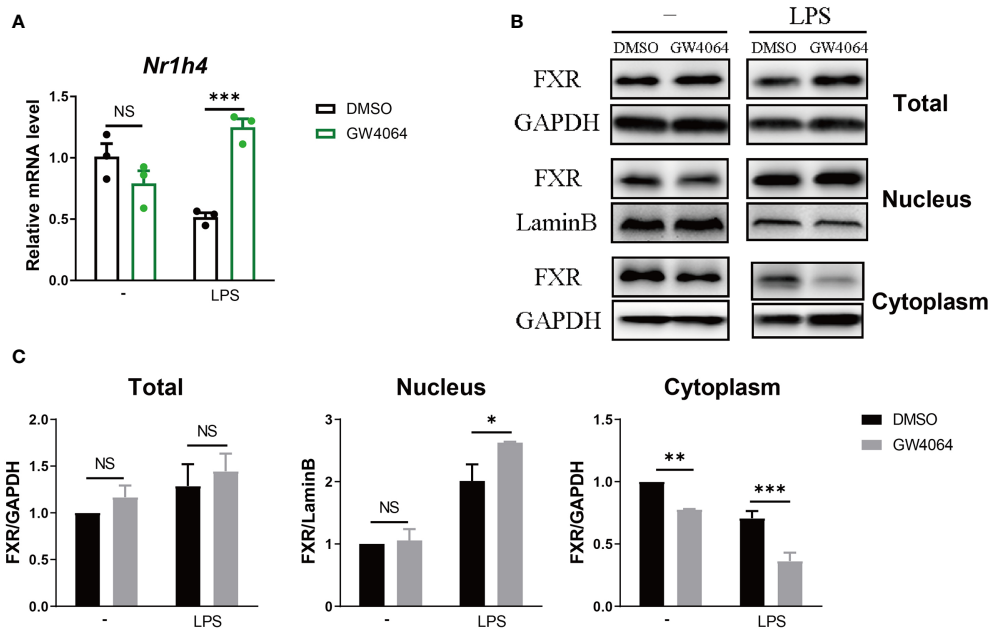

FIGURE 5 | FXR Agonist Facilitates the Translocation of FXR into the Nucleus. **(A, B)** RAW 264.7 macrophages were incubated with or without 5μM FXR agonist GW4064 for 24h, and then stimulated with or without 100ng/ml LPS. The mRNA levels of FXR within the cells **(A)** were tested 6h after the stimulation and total protein levels as well as nuclear and cytoplasmic protein levels were tested 24h **(B)** after the stimulation. **(C)** Quantification of the western results from **(B)**. Data are shown as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; NS, no significant difference, two-way ANOVA, followed by Sidak's multiple comparisons test.

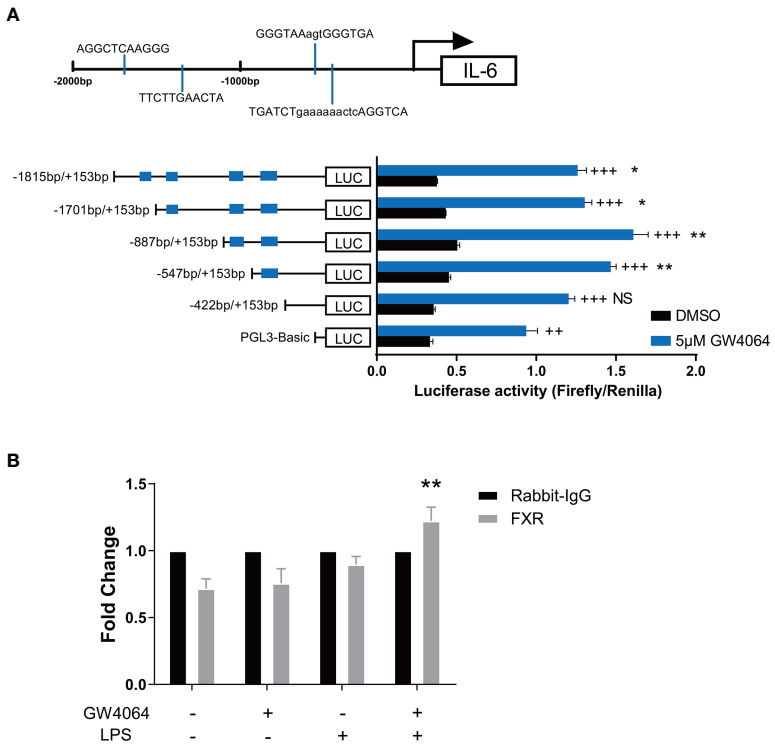

FIGURE 6 | FXR Binds to the Promoter Site of IL-6. **(A)** Four potential FXR binding sites in the promoter site of IL-6 were predicted based on the databases of PROMO and NUBIscan within about 2000bp upstream of the transcription start site of IL-6 (upper). Plasmids containing different numbers of the binding site based on the basic PGL-3 plasmid were constructed and co-transfected respectively with FXR-overexpression plasmids as well as Renilla plasmids together into 293T cells. 24h after the transfection, cells were then activated with or without 5μM GW4064 for extra 24h. Luciferase activity was then evaluated with the dual luciferase assay (bottom). Data are shown as mean ± SEM. + means compared to unstimulated control and * means comparing to the luciferase activity of PGL-3 basic vector. ++ $p < 0.01$; +++ $p < 0.001$. *$p < 0.05$; **$p < 0.01$; NS, no significant difference, two-way ANOVA, followed by Sidak's multiple comparisons test. **(B)** RAW 264.7 macrophages were incubated with or without 5μM FXR agonist GW4064 for 24h, and then stimulated with or without 100ng/ml LPS for 24h. Cells were then fixed with 1% w/v formaldehyde solution and chromatin from the cells was analyzed for recruitment of FXR to the last predictive binding site of the IL-6 promoter by ChIP assay. The quantification of DNA in the precipitation with FXR antibody was normalized to input chromatin and plotted relative to the Rabbit IgG. Data are shown as mean ± SEM. **$p < 0.01$, two-way ANOVA, followed by Sidak's multiple comparisons test.

(**Figure 7C**). FXR was also expressed within the lesion, especially in the nucleus of the cells (**Figure 7D**). These results suggest that FXR indeed exists in the inflammatory and fibrotic lesion of CP, and may benefit the therapy of CP with its inhibitory role on IL-6 expression in the local site.

DISCUSSION

In 1905, Albarran first reported three cases of ureteral obstruction caused by extensive retroperitoneal fibrosis. And then in 1972, inflammatory abdominal aortic aneurysm was first reported (20). Later in 1984, Mitchinson found the consistency in the pathogenesis of IRF and IAAA, further putting forward the concept of perianeurysmal retroperitoneal fibrosis (21). Then the three definitions were summarized as the same disease spectrum, collectively referred to as chronic periaortitis.

Incidence of CP is quite rare. No report has been published so far about the overall incidence of CP, while studies has showed the incidence of IRF was 1-1.38/10 million, males more than females, and the average onset age is 50-60 years old (12, 13). CP has an insidious onset and chronic disease progression, often discovered by routine medical examination or serious complications caused by the damage of corresponding organs. Meanwhile, the clinical symptoms as well as conventional laboratory examinations are mostly not specific, contributing to the difficulty with the early diagnosis. Even though the progress of CP is slow, the late diagnosis and even misdiagnose along with the late initiation of the treatment lead to the poor prognosis of the disease. Moreover, no standard treatment has been settled for CP so far and large amounts of glucocorticoid are still considered to be the first choice, with strong side effects and easy to relapse after drug reduction or withdrawal (22, 23). The treatment of conventional disease-modifying anti-rheumatic drugs (DMARDs) such as cyclophosphamide (24), methotrexate (23) is still under exploration, and tamoxifen as well as biological agents such as TNFα antagonists (25), CD20 monoclonal antibodies (26) and anti-IL-6 antibodies (16) are

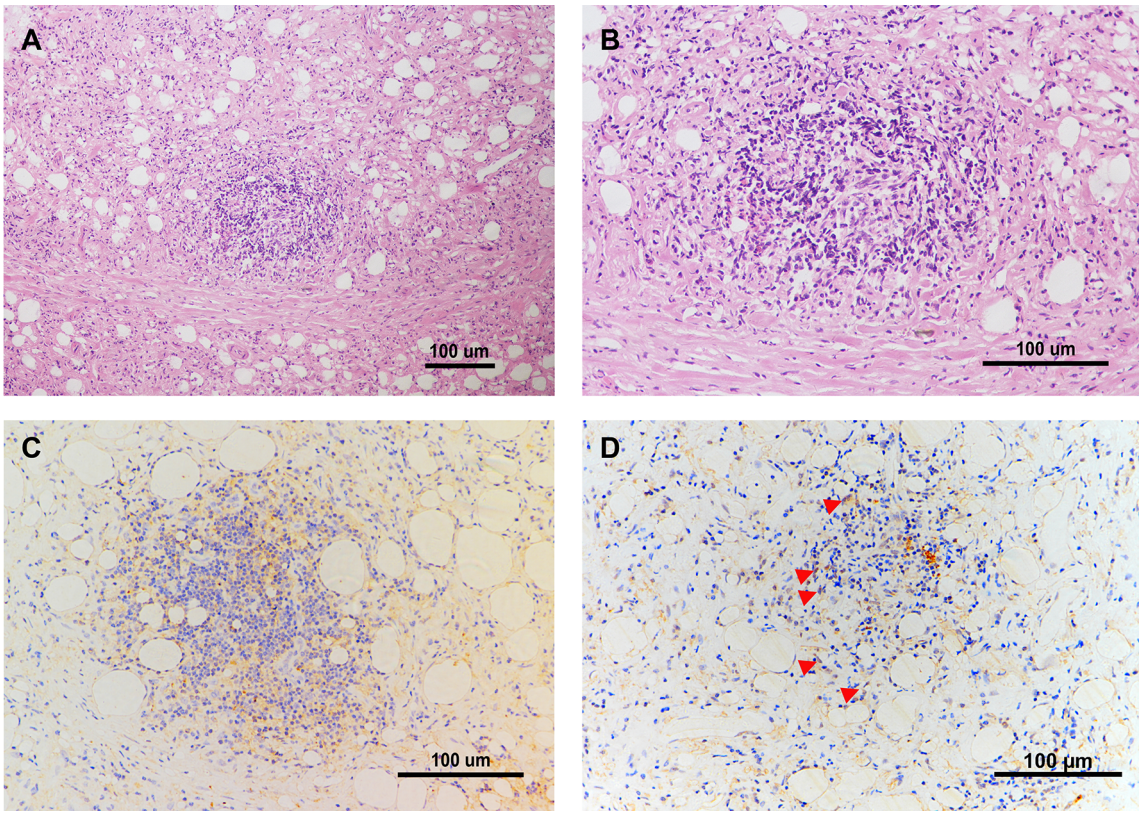

FIGURE 7 | IL-6 and FXR are Expressed in the Retroperitoneal Lesion of CP. **(A, B)** Representative images of HE staining in the biopsy of the retroperitoneal lesion from one CP patient. Scale bars: 100 μm. **(C)** Representative image of immunohistochemical staining of IL-6 in the biopsy of the retroperitoneal lesion. Scale bars: 100 μm. **(D)** Representative image of immunohistochemical staining of FXR in the biopsy of the retroperitoneal lesion. Scale bars: 100 μm. The red arrows indicate FXR positive cells especially within the nucleus.

gradually added to backup list, but their effect, however, is still limited. It seems like we are still pale and weak in front of this tough disease.

Therefore, it is worthy looking forward to some novel therapy for this complicated disease. In our present study, we for the first time explored the possibility of FXR agonist in the treatment of CP through the activation of FXR to function as a transcriptional inhibitor for the pro-inflammatory and pro-fibrotic cytokine IL-6, thus not only alleviate the local inflammatory response and also simultaneously ameliorate the fibrosis of retroperitoneal mass, reducing the damage to the surrounding organs. Furthermore, in comparison with healthy controls as with other vasculitis and metabolic vascular disease, the spectrum of bile acids in the serum of CP patients reveals a specific character with a significant increase of CDCA, indicating a spontaneous defense reaction of the body to inhibit the progression of this disease. Meanwhile, the signaling enrichment analysis of the transcripts also suggests the participation of bile acid metabolism in CP. Thus, increased CDCA may be a new biomarker of this disease, and CDCA supplementation or CDCA analogue as with FXR agonist may be a novel method in the treatment of CP. Recently, the rapid development of new FXR agonists such as obeticholic acid (9) have gained good results from a series of clinical studies, providing a bright future for the treatment of liver fibrosis, and new efficient FXR agonists could also make up for the liver toxicity of CDCA. Expansion of these drugs from the utility only in liver and intestine disease to CP could further provide perspective and promising treatment for CP, and the future randomized clinical trial is worthy to be considerate.

It is reported that level of serum IL-6 was significantly elevated in CP patients than the healthy controls ($p<0.05$) (16). Our immunohistochemical staining results also showed that IL-6 was highly expressed in the retroperitoneal mass of active CP patient, which indicates that IL-6 might be a vital cytokine involved in the inflammatory and pro-fibrotic response of CP. Moreover, although mainly expressed in liver and intestine, FXR was also observed for the first time existing in the retroperitoneal mass, and may function locally in the transcriptional regulation of IL-6. CDCA-FXR/IL-6 pathway may benefit for the resolution of CP by enhancing the inhibitory effect of FXR on IL-6.

However, there are still several problems worthy of improvement in this study. Due to the rare incidence of the disease, few CP patients were recruited and the data was still limited, thus the sample size should be further expanded in the future; serum CDCA level can be influenced by many factors such as diet, drugs, and diseases like hypertension, diabetes and

other metabolic diseases. In addition, urinary system is also involved in the metabolism of CDCA, whether the invasion of the retroperitoneal mass into the urinary system contributes to the accumulation of CDCA remains undetermined. In future studies, these confounding factors should be focused and controlled as much as possible. Furthermore, the mechanism underlying the spontaneous increase of CDCA in CP patients remains unknown. Since the metabolism of bile acids is often connected with microbiota (27), the alteration of the microbiota within the gut of CP patients remains to be further elucidated. This aspect seems quite interesting and warrants further investigation. What's more, until now there is still no appropriate animal model for the investigation of CP, which is also the direction we should figure out in the future.

In conclusion, our results suggest a reactive increase of CDCA in CP patients, which could help to downregulate the pro-inflammatory and pro-fibrotic factor IL-6 in macrophages by activating the transcription factor FXR and then directly binds to the promoter site of IL-6. Thus this could be a promising research direction and treatment strategy for CP in the future.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Renji Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of Renji Hospital. Written informed consent was obtained from the owners for the participation of their animals 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

SC and XC designed the study and wrote the manuscript. XM contributed to network reconstruction. SC performed sequencing analysis. SC performed *in vitro* experiments and analysis. XM and YL analyzed human samples. LS, LJ, HX, and XC collected human samples. XC supervised the study and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.632864/full#supplementary-material

Supplementary Figure 1 | FXR Knockout does not Affect the Macrophage Differentiation. On Day7 of the differentiation, the BMDM from $FXR^{-/-}$ mice and the littermates were harvested and stained. The percentage of the differentiated macrophages ($CD11b^+$ $F4/80^+$), M1 macrophages ($CD11b^+$ $F4/80^+$ $CD11c^+$ $CD206^-$) and M2 macrophages ($CD11b^+$ $F4/80^+$ $CD11c^-$ $CD206^+$) were then calculated. **(A)** Representative images of the gating strategy for the macrophages in two groups. **(B)** Percentage of the macrophages in two groups. Data are shown as mean ± SEM, NS means no significant difference, student t-test.

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ORIGINAL RESEARCH
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Potential Role of Macrophage Phenotypes and CCL2 in the Pathogenesis of Takayasu Arteritis

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Objectives: To investigate vascular macrophage phenotype as well as vascular and peripheral chemokine (C-C motif) ligand 2 (CCL2) expression during different stages of disease progression in patients with Takayasu Arteritis (TA).

Methods: In this study, 74 patients with TA and 50 controls were recruited. TA disease activity was evaluated with Kerr scores. Macrophage phenotype and CCL2 expression were examined by immunohistochemistry in vascular specimens from 8 untreated and 7 treated TA patients, along with 4 healthy controls. Serum CCL2 were quantified by enzyme-linked immune-absorbent assay from TA patients at baseline (n=59), at 6-months (n=38), and from 46 healthy volunteers. Vascular macrophage phenotype, vascular CCL2 expression and serum CCL2 levels during different stages, as well as the relationship between serum CCL2 and disease activity or other inflammatory parameters (erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and interleukin 6 (IL-6)) were investigated.

Results: In untreated patients, vascular M1 macrophages and CCL2 showed increased expression, mainly in the adventitia. In contrast, in treated patients, vascular adventitial M1 and CCL2 expression were decreased, while vascular medial M2 macrophages and CCL2 levels were increased. Distribution of macrophages and CCL2 was consistent within the TA vascular lesions regardless of the disease stage. Furthermore, peripheral CCL2 was elevated in patients with TA (TA: 160.30 ± 120.05 vs. Control: 65.58 ± 54.56 pg/ml, $P < 0.001$). CCL2 levels were found to correlate with ESR, CRP, and IL-6 (all R values between 0.55 and 0.6, all $P < 0.001$). Receiver operating curve analysis demonstrated that CCL2 (at the cut-off value of 100.36 pg/ml) was able to predict disease activity (area under the curve = 0.74, $P = 0.03$). Decrease in CCL2 level was observed in patients with clinical remission (CR), but not in patients without CR, after 6 months of treatment (CR patients: baseline 220.18 ± 222.69 vs. post-treatment 88.71 ± 55.89 pg/ml, $P = 0.04$; non-CR patients: baseline 142.45 ± 104.76 vs. post-treatment 279.49 ± 229.46 pg/ml, $P = 0.02$).

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Conclusions: Macrophages contribute to vascular pathological changes in TA by undergoing phenotype transformation. CCL2 is an important factor for recruiting macrophages and a potential biomarker for disease activity.

Keywords: Takayasu arteritis, macrophage phenotype, vascular fibrosis, biomarker, CCL2

INTRODUCTION

Takayasu arteritis (TA) is a type of chronic granulomatous arteritis that involves the aorta and its main branches. In Asian population, it predominantly occurs in young women aged less than 40 years old (1, 2). Histologically, TA is characterized by vascular inflammation in the active stage and vascular fibrosis and remodeling in the chronic stage, which leads to irreversible vascular stenosis or even occlusion (3). Although current treatment regimens including glucocorticoids and immunosuppressants can achieve rapid relief of systemic inflammation, they are not believed to effectively halt tissue fibrosis, which may eventually lead to a poor prognosis. Therefore, clarification of the pathogenesis of TA is critical to develop effective treatment strategies.

acrophages are crucial immune cells, which are highly heterogenic and can be polarized into M1 or M2 phenotypes according to their functions. Both phenotypes play distinct roles in the pathogenesis of inflammatory disorders (4–8). Macrophage has been studied in vascular lesions of TA, and M2 phenotype was found to be dominated in the vascular lesions, but the impact of treatment on the macrophage phenotype was not fully illustrated (9). Chemokine (C-C motif) ligand 2 (CCL2) is a major monocyte chemotactic protein produced by macrophages as well as other cells such as endothelial cells, smooth muscle cells and fibroblasts (10). Production of this protein can be induced by pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, growth factors, or other antigen stimulants (11). CCL2 is elevated in the peripheral blood in patients with TA (12). However, its expression in the vascular lesions at the different stages of TA remains unclear.

This study aimed to compare the vascular macrophage phenotype as well as the vascular and peripheral expression of CCL2 between untreated and treated patients with TA.

MATERIALS AND METHODS

Study Population

This study was performed in Zhongshan Hospital, Fudan University in China. Seventy-four patients with TA and 50 healthy controls were recruited from January 1, 2019 to May 31, 2020. Patients were diagnosed according to the 1990 American College of Rheumatology (ACR) classification criteria (13). Among them, 15 patients and 4 control subjects were enrolled for vascular tissue examination, while the remaining 59 patients with TA and 46 healthy control subjects were recruited for serum examination. Among the 15 patients who provided vascular tissue for histological examination, 8 were treatment-naive, and 7 had accepted medical treatment when they underwent surgery. Control samples of vascular tissue were obtained either from heart transplantation donors (normal aorta samples) or from patients undergoing nephrectomy (normal renal artery samples). Healthy controls for serum detection were included from the health examination center in our hospital. The study design and flowchart of this study were shown in **Supplementary Figure 1**.

The study and all its protocols were approved by the Institutional Review Board of Zhongshan Hospital, Fudan University, China (approval number: B2016-168), and conformed to the tenets of the Declaration of Helsinki. Written informed consents were obtained from all patients.

Data and Specimen Collection

All clinical data (including symptoms, laboratory results, and imaging features) were collected at diagnosis and after 6 months of treatment for patients who provided serum samples. After treatment, clinical remission (CR) was considered if patients' Kerr criteria was < 2. Otherwise, patients with Kerr ≥ 2 were assessed as non-CR. For patients who provided tissue samples, information about treatment regimen, clinical symptoms, laboratory reports, and imaging results were collected at the time of surgery.

Immunohistochemistry

Specimens obtained from TA patients and four vascular controls were subjected to immunohistochemical (IHC) staining using a previously described method (14). In brief, tissue sections were deparaffinized and rehydrated. Antigen retrieval was conducted using citrate buffer solution (0.01 mol/L, pH 6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2 (30 min, room temperature). Slides were blocked with 75 μL goat serum (30 min, RT). Subsequently, primary antibodies against CD68 (Abcam, ab955), HLA-DR (Abcam, ab92511), CD163 (Abcam ab182422) and CCL2 (Proteintech, 25542-1-AP) were added to each slide. The following day, the corresponding secondary antibodies were added to the slides and developed with 3,3′-diaminobenzidine (DAB). The tissue sections of each patient were also subjected to routine hematoxylin and eosin (H&E) staining.

For the H&E or IHC analysis, all the slides were digitally scanned using a 3DHISTECH scanning microscope, and images were viewed and selected using Pannoramic Viewer 1.15.3 (3DHISTECH Ltd, Hungary). Vascular thickness was measured and lymphocyte aggregation (LA) were evaluated on H&E staining. For aortic wall, the thickness ≤ 0.2 cm was considered normal (–); the thickness between 0.2 - 0.4 cm was graded as (+); the thickness between 0.4 - 0.6 cm was graded as (++); the thickness over 0.6 cm was graded as (+++). LA was

assessed semiquantitatively under 100x magnification: none (-), occasional (+), many (++); and dense clusters (+++). Cells that were double positive for CD68 and HLA-DR were considered as M1 macrophages, while those that were double positive for CD68 and CD163 were regarded as M2 macrophages (15). Positive M1 or M2 cells were counted from ten different squares (about 0.45mm^2) under 400x magnification, which were randomly selected from the areas with prominent inflammatory infiltrate (16). Different layers of each specimen were counted separately by two raters who were blinded to patients' clinical data. Average number of the ten squares by the two raters were calculated and used for analysis. Similarly, ten squares (about 0.45mm^2) within the greatest inflammatory infiltrates were chosen from different layers of each specimen for CCL2 quantification. Image pro-plus 7.0 (Media Cybernetics, Silver Spring, USA) was used in this process and the results were presented as the average ratio of the integrated optical density (IOD) to the area of the selected fields.

Enzyme-Linked Immunosorbent Assay

Peripheral CCL2 levels were measured in patients at baseline (n=59), at 6 months (n=38) after treatment and in healthy control subjects (n=46). Serum was collected from each patient and control subjects and stored at −80°C. The concentration of CCL2 in blood samples was analyzed using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D system, DCP00), according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as mean ± standard deviation for quantitative data following the normal distribution, as median and interquartile for quantitative data not following the normal distribution, or as frequencies (percentage) for categorical data. Comparisons between patients and controls were performed using the Student's *t*-test, Mann-Whitney test or chi-square test, as appropriate. Paired Student's *t*-test was used to compare serum CCL2 levels before and after treatment. Student's *t*-test was performed to compare peripheral CCL2 levels among patients with different activity status assessed by Kerr criteria (17). Correlation analysis of peripheral CCL2 and other inflammatory indexes including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and interleukin 6 (IL-6) were performed using Spearman or Pearson correlation analysis. Receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to evaluate the potential of CCL2 to serve as a biomarker for assessment of patients' disease activity. ROC was also performed for ESR and CRP and the efficacy between CCL2 and ESR or CRP in this performance was compared. A two-sided p value < 0.05 was considered to indicate statistical significance.

RESULTS

Current Treatments Exerting Minimal Effect on Vascular Fibrosis in TA

Vascular specimens from the 15 patients with TA included ascending aorta (n = 12), aortic valve (n = 1), abdominal aorta (n = 1) and renal artery (n = 1). Vascular specimens of the control subjects (n = 4) included normal aorta (n = 1), and renal arteries (n=3). Clinical characteristics of these patients are listed in **Table 1**. There were no significant differences in terms of onset age (35.85 ± 14.14 vs. 39.85 ± 11.81 years, P = 0.95) or disease duration (29.75 ± 23.19 vs. 32.43 ± 40.27 months, P = 0.88) between the treated and untreated patients for vascular tissue study. Based on the Kerr criteria, the majority (6/8, 75%) of untreated patients were in the active status of TA, whereas most treated patients (6/7, 85.71%) were in the inactive status. Levels of CRP and IL-6 were non-statistically higher in untreated patients than in treated patients (CRP: 50.63 ± 67.50 vs. 23.69 ± 40.97 mg/L, P = 0.12; IL-6: 24.99 ± 23.86 vs. 8.14 ± 9.55 pg/mL, P = 0.07).

In contrast to normal vascular tissue (**Figures 1A–D**) H&E staining revealed inflammatory cells to be prominent in all untreated patients (100%, **Table 1** and **Figures 1E–H**), predominantly noted in the vascular adventitia (**Figure 1H**). Vascular wall thickening and fibrosis without excess inflammation were observed in most treated patients (85.71%, **Table 1** and **Figures 1I–L**).

Increased M1 infiltration in the vascular adventitia of untreated patients

Significantly higher infiltration of macrophages was observed in all three layers of untreated vascular tissues than in control samples (**Figures 2A, B**). The infiltrating macrophages were identified as M1 owing to their CD68+HLA-DR+ phenotypes (**Figures 2A**q-t, C, P < 0.01). Most macrophages were distributed within the vascular adventitia (**Figures 2A**p, **B**), where the vasa vasorum was enriched and the inflammation was prominent.

Increased M2 Infiltration in the Vascular Media in Treated Patients

Compared with untreated tissue, macrophage infiltration was significantly lower in treated vascular tissue (**Figures 3A**a–d, **B**). However, analysis of macrophages in different layers of vascular tissue revealed a distinct pattern. Different from adventitia in untreated vascular tissue, macrophages mainly infiltrated in the media of treated vascular tissue (**Figures 3A**k, **B**). Moreover, the macrophages present in treated vascular media were predominantly M2 type (P = 0.002, **Figure 3C**). These results suggest a shift in the phenotype of infiltrated macrophages in vascular lesions from M1 to M2 following treatment for TA.

Consistent Distribution and Similar Changes of CCL2 as Macrophage in Vascular Lesions

Given that CCL2 is a major monocyte chemotactic protein, CCL2 expression in lesion tissue of TA patients was further evaluated. Compared with normal vascular tissue (**Figures 4A**a–d, **B**), CCL2 levels were significantly higher in untreated tissue of patients with TA (**Figures 4A**e–h, P = 0.003). In addition, CCL2 was mainly expressed in the vascular adventitia

TABLE 1 | Clinical characteristics of the patients enrolled for vascular tissue examination.

Patients	Gender (F/M)	Specimen	Thickness	Onset age (y)	Symptom duration (mo)	Treatment	ESR (mm/h)	CRP (mg/L)	IL-6 (pg/mL)	LA
Untreated patients (n = 8)										
1	F	Ascending aorta	+++	46	16	N	22	3	3.3	++ +
2	M	Renal artery	++	42	5	N	80	71.4	13.54	++
3	F	Ascending aorta	+	24	43	N	11	37.8	74.06	++
4	M	Ascending aorta	–	43	62	N	10	1	18.65	++ +
5	M	Ascending aorta	+	39	51	N	22	44.8	22.97	++
6	M	Aortic valve	+	56	46	N	7	24.9	34.11	++
7	M	Ascending aorta	+	44	3	N	92	207.4	/	++
8	F	Abdominal aorta	+++	28	12	N	117	14.7	8.33	++
Treated patients (n = 7)										
1	F	Ascending aorta	++	29	24	P + LEF	53	/	5.3	+
2	M	Ascending aorta	+++	33	24	P + CTX	5	1.9	4.8	-/+
3	M	Ascending aorta	++	39	10	P	/	14.7	/	++
4	F	Ascending aorta	+	40	12	P + CTX	79	8.7	27.5	-/+
5	F	Ascending aorta	+++	31	120	P	10	18.4	2.93	+
6	M	Ascending aorta	++	43	1	P + HCQ	36	2.6	5.3	++
7	F	Ascending aorta	++	64	36	P + LEF	35	4	3	+
Control subjects (n = 4)										
Con 1	M	Ascending aorta	–	25	/	/	/	/	/	–
Con 2	M	Renal artery	–	53	/	/	/	/	/	–
Con 3	F	Renal artery	–	39	/	/	/	/	/	–
Con 4	F	Renal artery	–	27	/	/	/	/	/	–

F, Female; M, male; N, no; mo, month; P, prednisone; LEF, leflunomide; CTX, cyclophosphamide; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; Thickness, ≤ 0.2 cm (-); 0.2 - 0.4 cm (+); 0.4 - 0.6 cm (++); ≥ 0.6 cm (+++). LA (lymphocyte aggregation), none (-), occasional (+), many (++); dense clusters (+++).

where macrophage infiltration was high (**Figures 4A**h, **B**, $P < 0.01$).

In contrast, the total expression of CCL2 throughout vascular tissue was significantly lower in treated patients (**Figures 4A**e, i, **B,** $P < 0.01$), especially in the adventitia where CCL2 expression was decreased (**Figures 4A**h,l, **B**, $P < 0.001$). However, in the vascular media of treated patients, CCL2 expression was significantly increased and distributed along the tissue cells (**Figures 4A**k, **B,** $P < 0.05$).

Correlation of Peripheral CCL2 Levels With Disease Activity in TA

Clinical characteristics of patients for whom serum estimation of CCL2 was performed are shown in **Table 2**. The results show that serum CCL2 level was remarkably and significantly higher in patients with TA than in healthy controls (160.30 ± 120.05 vs. 65.58 ± 54.56 pg/mL, $P < 0.001$, **Figure 5A**). Among patients with TA, serum CCL2 level was significantly higher in active patients (Kerr ≥ 2) than in inactive patients (178.20 ± 153.96 vs. 62.90 ± 22.74 pg/mL, $P = 0.01$, **Figure 5B**). No significant difference was observed in the serum CCL2 levels among patients with different imaging types ($P > 0.05$, **Figure 5C**).

Erythrocyte sedimentation rate (ESR), CRP, and IL-6 are common indicators of disease activity in TA. Correlation between peripheral CCL2 and these parameters were further evaluated. The results suggested that serum CCL2 levels were moderately correlated with ESR, CRP, and IL-6 ($P < 0.001$ for all; **Figures 5D–F**, respectively). To evaluate the efficacy of CCL2 to predict disease activity (Kerr ≥ 2), ROC curve analysis was performed. The results indicated that CCL2 cut-off value of 100.36 pg/mL was able to predict disease activity in TA (AUC as 0.74) with specificity of 91.67% and sensitivity as 51.78% ($P = 0.03$, **Figure 5G**). But its efficacy was lower than ESR (ESR cut-off value = 20mm/H, AUC = 0.93, Sensitivity = 88.37%, Specificity = 84.62%, $p < 0.01$; CCL2 vs. ESR: $P = 0.03$, **Figure 5G**). No significant difference was observed between CCL2 and CRP in this performance (CRP cut-off value = 2.1mg/dL, AUC = 0.83, Sensitivity = 86.05%, Specificity = 76.92%, $P < 0.001$; CCL2 vs. CRP: $P = 0.23$, **Figure 5G**).

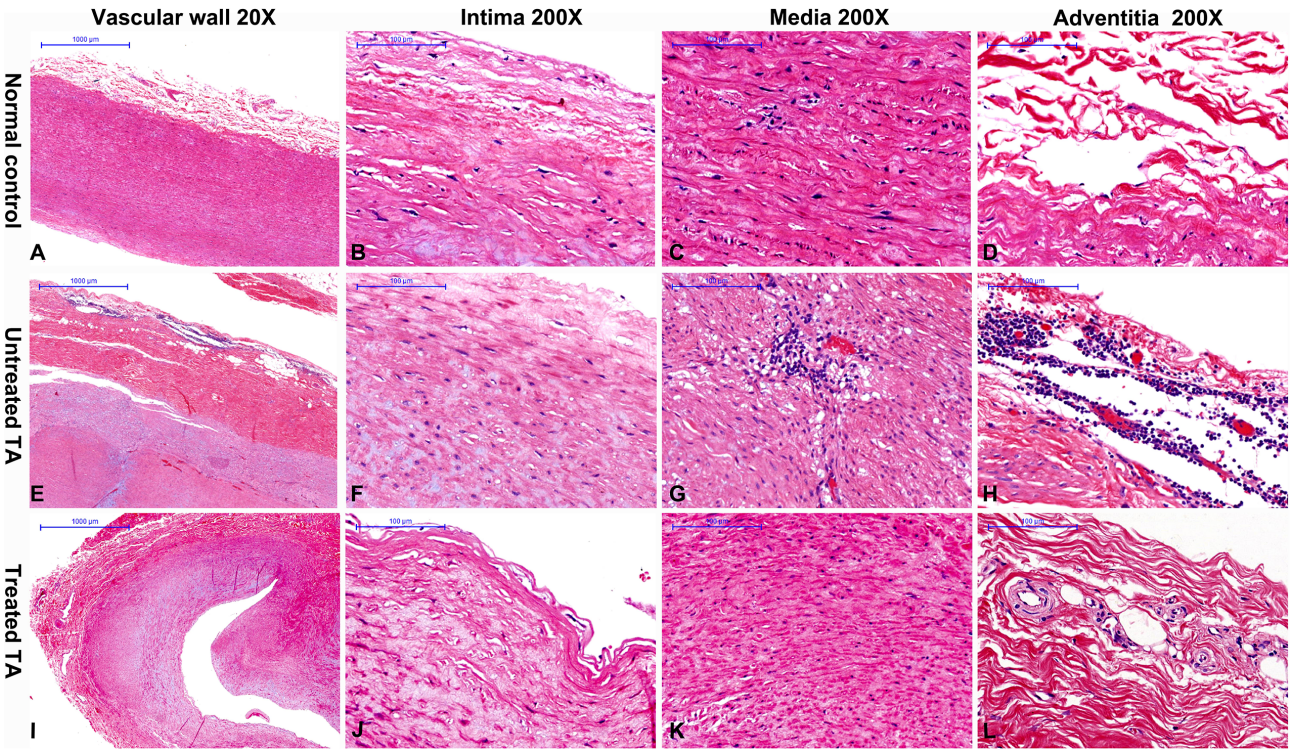

FIGURE 1 | Hematoxylin and eosin staining of vascular tissue from controls, untreated and treated patients with TA **(A)** Representative hematoxylin and eosin images of normal aorta **(A–D)**, untreated TA aorta **(E–H)** and treated TA renal artery **(I–L)**. A great amount of inflammatory cells are observed in untreated patients **(H)**. The inflammatory cells are decreased in treated patients **(I)**.

After the treatment, patients' disease status was evaluated. At 6 months, 15 patients were excluded due to lacking serum samples, whereas the other 6 patients were lost to follow-up. Thus, 38 patients were remained at 6 months. The average prednisone dose for them was 14.63 ± 4.84 mg per day. Among 38 patients, 24 (63.16%) patients achieved CR. Among the CR patients, the percentage of those with CCL2 of less than 100.36 pg/mL was 83.33%, while among non-CR patients, the percentage of those with CCL2 more than 100.36 pg/mL was 85.71%. Compared to the patients with CR, CCL2 levels in non-CR patients were higher at 6 months (88.71 ± 55.89 vs. 279.49 ± 229.46pg/mL, P = 0.02, **Figure 5H**). Moreover, compared to their baseline levels, CCL2 serum levels in CR patients were decreased (220.18 ± 222.69 vs.88.71 ± 55.89 pg/mL, P = 0.04, **Figure 5I**), whereas those in non-CR patients were increased after 6 months (142.45 ± 104.76 vs. 279.49 ± 229.46 pg/mL, P = 0.02, **Figure 5J**).

DISCUSSION

The present study investigated the potential role of macrophage and CCL2 in the vascular pathogenesis of TA. The distribution pattern and phenotype of macrophages and CCL2 expression in vascular tissue of patients with TA indicated that macrophage presented a phenotype shift (M1 to M2) and distribution change (adventitia to media) as the disease progressed from an active to an inactive phase. Vascular CCL2 expression was closely related with macrophage distribution during this process. Moreover, peripheral blood CCL2 levels were found to be elevated and correlated with patient disease activity, thus serving as a promising biomarker in the evaluation of patient treatment efficacy.

Macrophages are known to contribute to the progression of vascular lesions in TA (16). It is well known that macrophages with different phenotype can present distinct biological activity (18). M1 subset is believed to play a pro-inflammatory role by secreting IL-1, IL-6 and tumor necrosis factor-α (TNF-α), whereas M2 subset promote tissue fibrosis by producing pro-fibrotic factors such as transformation growth factor-β (TGF-β) (8, 19–22). Based on this concept, an M1-dominated macrophage population is expected to enhance vascular inflammation in the acute stage, while an M2-dominated macrophage population should contribute to vascular fibrosis in chronic stage in TA.

Excessive polarization of macrophages to M1 or M2 in different stages of disease implies an imbalance of macrophage differentiation regulators in vascular lesions of TA. As previously

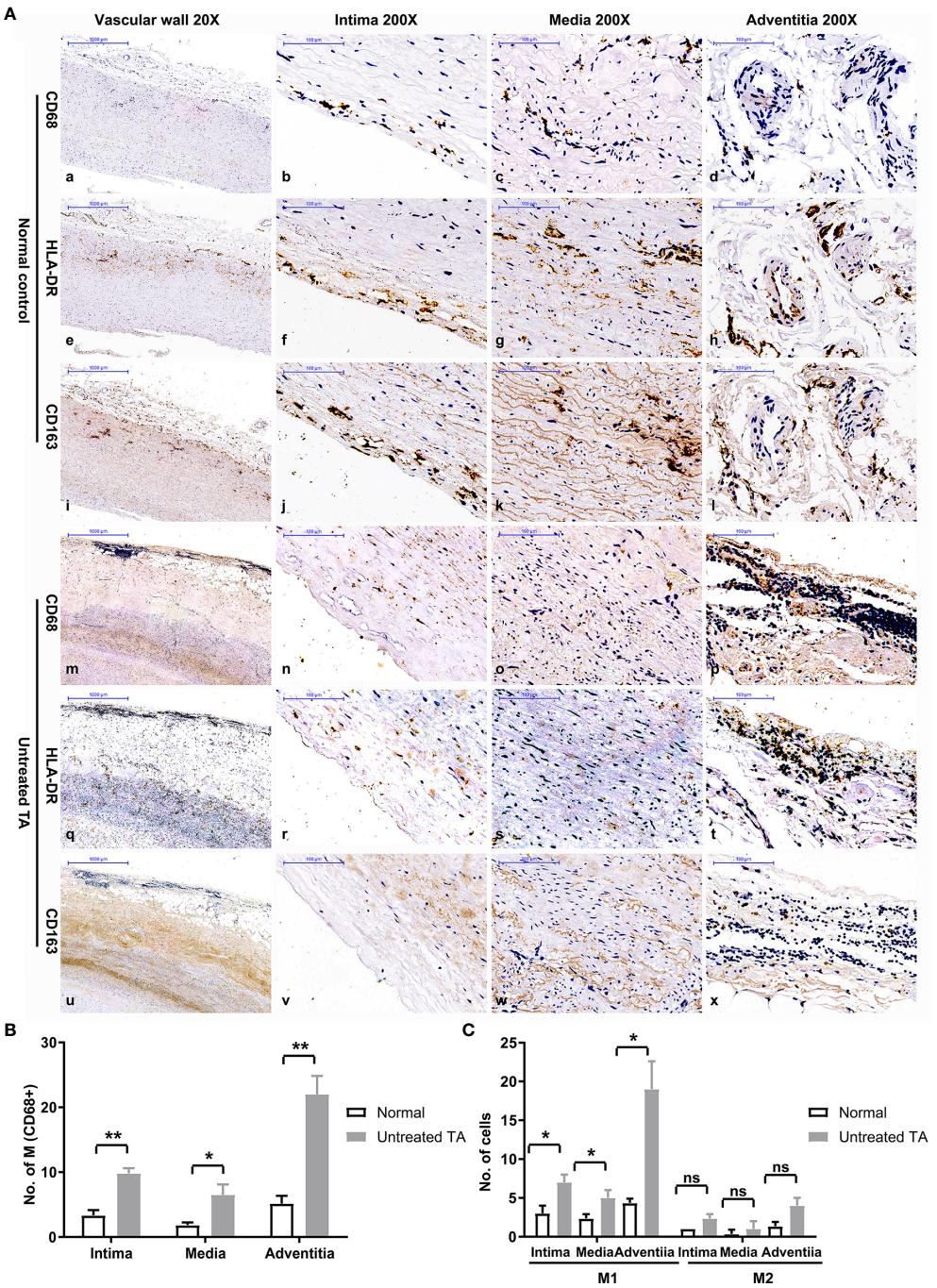

FIGURE 2 | Distribution and phenotype of macrophages in controls and untreated patients with TA. **(A)** Representative immunohistochemistry images of CD68, HLA-DR and CD163 positive cells in control arterial specimens (CD68:a-d; HLA-DR: e-h; CD163: i-l) and TA aortic specimens (CD68: m-p; HLA-DR: q-t; CD163: u-x); image a, e, i, m, q, u-20X, the remaining images-200X. **(B)** The number of macrophages in different layers of control arterial specimens and TA aortic specimens. **(C)** Number of M1 or M2 macrophages in different layers of control specimens and TA aortic specimens. *< 0.05; **< 0.01; ns, no significance.

reported (14), multiple pro-inflammatory cytokines were detected in active lesions of TA, such as interferon-γ (IFN-γ), IL-6, IL-12, and IL-17. $CD4^+$ T cells also mainly presented as pro-inflammatory phenotypes, Th1 and Th17. These factors probably promoted differentiation of macrophages to M1 subset in acute stage. However, mechanism of M2 polarization in the chronic stage is poorly understood. Common M2 polarization cytokines such as IL-4, IL-10 and IL-13 were less

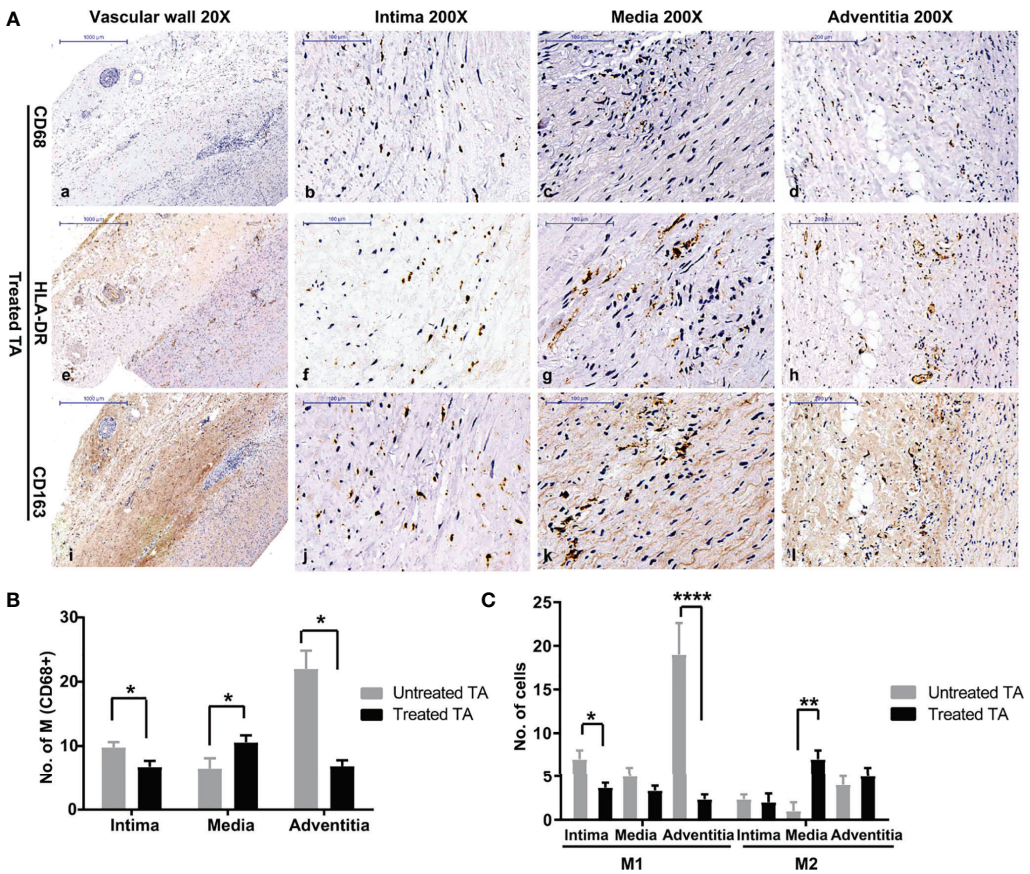

FIGURE 3 | Distribution and phenotype of macrophages in treated patients with TA **(A)** Representative images of CD68 (a–d), HLA-DR (e–h), and CD163 (i–l) positive cells in treated aorta. **(B)** Number of macrophages in different layers of untreated TA arteries, and treated TA arteries. **(C)** Number of M1 and M2 macrophages in vascular intima, media, and adventitia of untreated arteries, and treated arteries. *< 0.05; **< 0.01; ****< 0.0001.

frequently observed in vascular lesions (14). In the present study, the chronic stage vascular specimens were obtained from patients post treatment (mainly glucocorticoids and different immunosuppressants). Thus, the impact of medications on macrophage M2 polarization cannot be ruled out; this, however, further research is needed to validate this possibility.

Based on these observations, application of precise treatment strategies targeting M1 and M2 in acute and chronic stages, respectively, is expected to improve patient vascular lesions more effectively. Although current therapies have impacts on macrophage phenotype, they are not able to prevent vascular remodeling process. Thus, there is a need for exploring novel treatment regimens that can inhibit inflammation as well as fibrosis.

In the present study, we observed that CCL2 was expressed at the same sites as macrophage infiltration in the vascular lesions (despite the stage of disease), indicating the critical role of CCL2 in macrophage recruitment. CCL2 could be produced by immune cells as well as tissue cells such as myofibroblasts and smooth muscle cells (5, 23, 24). In a previous study, we have also shown that CCL2 can also be expressed by adventitial fibroblast after IL-6 stimulation (25). Therefore, multiple cells may be involved in macrophage recruitment *via* CCL2 expression in vascular lesions of TA.

In addition to the chemotactic function of CCL2, research has shown that CCL2was also involved in tissue fibrosis. It has been reported that CCL2 was able to promote proliferation and IL-6 production of vascular smooth muscle cells (26). In addition, CCL2 is reportedly capable of inducing collagen synthesis and TGF-β expression in lung fibroblasts (27). Since vascular lesions in TA are characterized by fibrosis in chronic stage, these studies suggest that CCL2 may play multiple roles in TA pathogenesis.

In the present study, we observed that peripheral CCL2 levels were correlated with Kerr score as well as with disease activity parameters such as ESR and CRP in TA. In vascular tissues, CCL2 was closely related to macrophage infiltration, thus peripheral CCL2 level may also reflect vascular tissue CCL2 expression and macrophage infiltration indirectly. Its levels were found to be significantly different in the same patient during disease progression, as well as in patients showing remission vs.

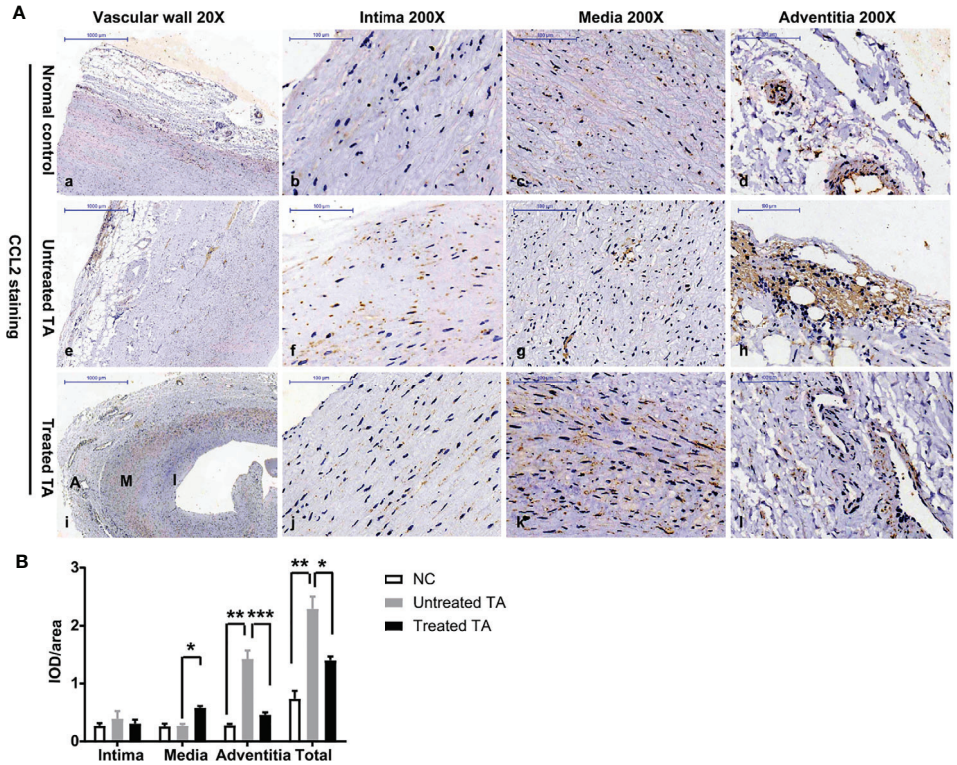

FIGURE 4 | CCL2 expression in arterial specimens from controls, untreated TA, and treated TA **(A)** Representative immunohistochemistry images of CCL2 in normal aorta (a–d), TA untreated aorta (e–h), and TA treated renal artery (i–l). **(B)** Quantification of CCL2 expressions in normal arteries, untreated, and treated TA arteries (intima, media and adventitia). *< 0.05; **< 0.01; ***< 0.001.

TABLE 2 | Baseline clinical characteristics of the patients recruited for serum detection.

Characteristics	TA group (N = 59)	Healthy group (N = 38)	P-value
Age (y, mean ± SD)	35.62 ± 14.03	38.04 ± 10.94	0.34
Gender ratio (F:M)	49:10	22:7	0.79
Disease duration (mo)	41.51 ± 96.29	/	/
Active status (n, %)	46 (77.97)		
Headache/Dizziness (n, %)	30 (50.8)	/	
Fever (n, %)	13 (22.00)	/	
Weakness (n, %)	18 (30.5)	/	
Hypertension (n, %)	20 (33.9)	/	
Pulselessness/decreased pulse (n, %)	16 (27.1)	/	
Neck murmur (n, %)	16 (27.1)	/	
Hemoglobin (g/L)	114.50 ± 16.97	110–150 (F), 120–160 (M)	/
WBC (× 10^9/L)	8.95 ± 7.50	3.5–9.5	/
PLT (× 10^9/L)	281.14 ± 88.42	100–300	/
ESR (mm/H)	47.86 ± 39.13	< 28 (F), < 40 (M)	/
CRP (mg/L)	29.71 ± 40.01	< 3	/

WBC, white blood cell; PLT, platelet; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

those that did not show remission. Therefore, CCL2 may serve as a promising biomarker in the assessment of disease activity and treatment effect.

This was a preliminary study of macrophages and CCL2 in TA. More detailed studies such assessing the specific phenotypes of macrophage in vascular tissue such as M2a, M2b, and M2c, are required. In addition, due to the low incidence of this disorder, the tissue sample size in this study was relatively small. Further research, with larger cohort of patients, is warranted to validate the results of this study.

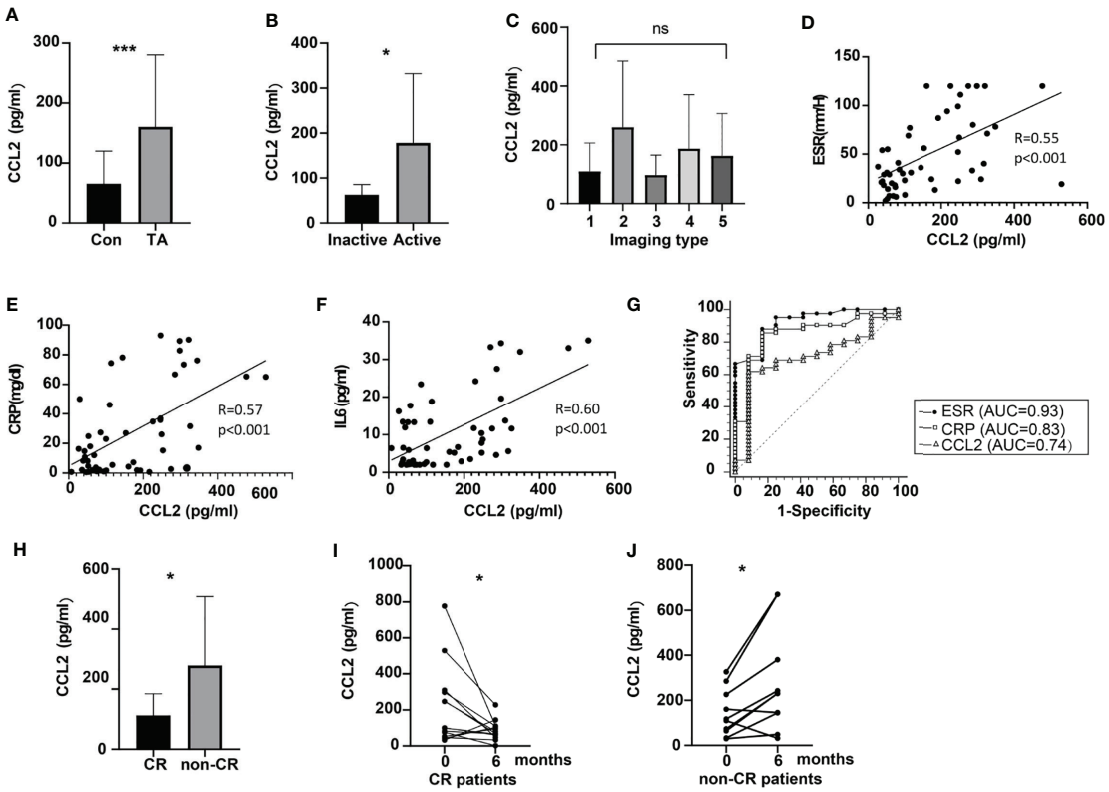

FIGURE 5 | Expression of peripheral CCL2 and the relationship of CCL2 levels with disease activity in patients with TA. **(A)** Levels of peripheral CCL2 in normal controls and patients with TA (n_{TA} = 59, $n_{control}$ = 46, P < 0.001). **(B)** Levels of peripheral CCL2 in active and inactive patients with TA (n_{active} = 46, $n_{inactive}$ = 13, P = 0.01). **(C)** Levels of peripheral CCL2 in patients with different imaging types ($n_{type\ I}$ = 20, $n_{type\ II}$ =8, $n_{type\ III}$ = 3, $n_{type\ IV}$ =2, $n_{type\ V}$=26). **(D–F)** Relationship between CCL2 levels and ESR, CRP or IL-6 levels in patients with TA (n = 59) **(G)**. ROC of CCL2, ESR and CRP to predict disease activity in patients with TA (n = 59). **(H)** CCL2 levels in CR and non-CR patients (n_{CR} = 24, $n_{non\text{-}CR}$ = 14, P = 0.02). **(I)** Changes of peripheral CCL2 levels in CR patients at baseline and the 6th month after treatment (n = 24, P = 0.04). **(J)** Changes of peripheral CCL2 levels in non-CR patients at baseline and the 6th month after treatment (n = 14, P = 0.02) AUC, area under the curve; CR, clinical remission; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein, IL-6, interleukin 6; CCL2, chemokine (C-C motif) ligand 2; ROC, receiver operatic characteristic; TA, Takayasu arteritis;
*< 0.05; ***< 0.001; ns, no significance.

CONCLUSION

Macrophages contribute to vascular pathological changes in TA by undergoing phenotype transformation and distribution changes. CCL2 is an important factor for recruiting macrophages and a potential biomarker for disease activity.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

XK was responsible for the data analysis and manuscript writing. MX provided the control vascular samples. XC participated in the IHC staining. LYM was responsible for collecting the clinical data of patients for serum CCL2 detection. HC and JH were responsible for the evaluation of macrophages from vascular specimens. XS provided part of control vascular specimens. LLM helped the statistical analysis. LJ designed the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CMTM6-Deficient Monocytes in ANCA-Associated Vasculitis Fail to Present the Immune Checkpoint PD-L1

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Objectives: ANCA-associated vasculitides (AAV) affect small- and medium-sized blood vessels. In active disease, vessel wall infiltrates are mainly composed of monocytes and macrophages. Immune checkpoint molecules are crucial for the maintenance of self-tolerance and the prevention of autoimmune diseases. After checkpoint inhibitor therapy, the development of autoimmune vasculitis has been observed. However, defects of immune checkpoint molecules in AAV patients have not been identified yet.

Methods: Monocytes and monocyte-derived macrophages from AAV patients and healthy age-matched controls were tested for surface expression of immunoinhibitory checkpoint programmed cell death ligand-1 (PD-L1). Using *in vitro* co-culture approaches, the effect of monocyte PD-L1 expression on $CD4^+$ T cell activation and proliferation was tested.

Results: Monocytes from AAV patients displayed lower PD-L1 expression and a defective PD-L1 presentation upon activation, an effect that was correlated with disease activity. Lower PD-L1 expression was due to increased lysosomal degradation of PD-L1 in AAV monocytes. We identified a reduced expression of CMTM6, a protein protecting PD-L1 from lysosomal breakdown, as the underlying molecular defect. PD-$L1^{low}$ AAV monocytes showed increased stimulatory capacity and induced T cell activation and proliferation. Inhibiting lysosomal function corrected this phenotype by increasing PD-L1, thus normalizing the pro-stimulatory behavior of AAV monocytes.

Conclusions: This study identifies a defect of the immunoinhibitory checkpoint PD-L1 in monocytes from patients with AAV. Low expression of CMTM6 results in enhanced lysosomal degradation of PD-L1, thus providing insufficient negative signaling to T cells. Correcting this defect by targeting lysosomal function may represent a novel strategy to treat AAV.

Keywords: ANCA vasculitis, PD-L1, macrophages, lysosomes, immune checkpoint, vasculitis < rheumatic diseases, monocytes

INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitides (AAV) are characterized by necrotizing inflammation of small blood vessels and the presence of ANCA with specificity for proteinase-3 (PR3) or myeloperoxidase (MPO). Frequent target tissues are the respiratory tract, kidneys, skin, and peripheral nerves. Immunopathologically, AAV present with an absence or paucity of immunoglobulin and complement deposition in affected vessels. Early vascular lesions of AAV consist of neutrophils with admixed monocytes. Within days, the initial inflammatory lesion is replaced by inflammation with a predominance of monocytes and macrophages (1). Also in renal biopsies, monocytes and macrophages are the predominant cells in glomeruli of patients with AAV (2, 3).

In AAV, systemic monocyte activation has been observed and their activation state persisted during remission (4–6). Transmembrane receptors that facilitate extracellular matrix adhesion are upregulated on AAV monocytes indicating an increased ability to interact with vascular endothelium (7). Monocytes also express antigens recognized by ANCA, and stimulation *in vitro* with ANCA promoted cytokine and reactive oxygen species (ROS) production (8, 9). Moreover, AAV monocytes recognize neutrophil extracellular traps (NETs) to upregulate the alarmin S100A9, which results in the induction of metalloproteinase-9 and enables monocytes to invade into the extracellular matrix (10).

Immune checkpoint molecules are crucial for the maintenance of self-tolerance. Programmed death ligand-1 (PD-L1) is an inhibitory checkpoint molecule that counterbalances the overwhelming activation of the immune system. PD-L1 is expressed on antigen-presenting cells (monocytes, macrophages, and dendritic cells) and endothelial cells (11). By engaging its ligand programmed death-1 (PD-1) on T cells, TCR- and CD28-mediated activation cascades are down-regulated. In animal studies, disruption of PD-L1 signals has been associated with inflammatory disease (12–14). In line with this, a defect in the PD-1/PD-L1 axis was reported in large vessel vasculitis (15, 16). In cancer immunotherapy, the use of checkpoint inhibitors that block the PD-1/PD-L1 pathway has proven to be very effective (17–19). In parallel, increasing reports of immune-related adverse events were noted (irAEs) (20). In fact, several forms of vasculitis have been reported to develop or re-activate after checkpoint inhibitor therapy, including AAV (21–24).

Here, we report that monocytes from AAV patients express low levels of the inhibitory checkpoint PD-L1. Patient-derived cells showed a defect in presenting PD-L1 in response to inflammatory stimuli, thus leading to increased activation of T cells in co-culture experiments. Mechanistically, inhibiting lysosome function in monocytes from AAV patients corrected their hyperstimulatory phenotype by upregulating PD-L1, indicating that increased lysosomal degradation of PD-L1 occurs in AAV monocytes. In line with this, AAV monocytes display a lower expression of chemokine-like factor-like MARVEL transmembrane domain containing family member 6 (CMTM6), a protein protecting PD-L1 from lysosomal breakdown (25, 26).

This study establishes a link between autoimmune small-vessel vasculitis and immunoinhibitory checkpoint deficiency, highlighting the potential role of uncontrolled innate immunity in promoting the development of autoimmune disease.

MATERIALS AND METHODS

Patients and Controls

The study population included 26 AAV patients classified as granulomatosis with polyangiitis (GPA, n=21) or microscopic polyangiitis (MPA, n=5) as defined by the Chapel Hill Consensus Conference nomenclature (27) and 29 age-matched healthy controls. All AAV patients tested positive for ANCA by indirect immunofluorescence and PR3 or MPO-ELISA and were enrolled between July 2019 and July 2020. Besides, seven RA patients fulfilling the 2010 EULAR/ACR diagnostic criteria were enrolled. Patient blood samples were provided by the Imm-Rheum Biobank of the Department of Rheumatology and Clinical Immunology, University Medical Center Freiburg. Clinical characteristics are given in **Table 1**. Demographically matched healthy individuals were obtained from the Institute for Cell and Gene Therapy at the University Medical Center Freiburg. They had no history of autoimmune disease, cancer, chronic viral infection, or any other inflammatory syndrome. The study was approved by the Institutional Review Board

TABLE 1 | Clinical characteristics of patients and controls.

	AAV	HC	RA
ANCA-pos. patients (n)	26	20	7
Proteinase 3 +	19 (73%)		
Myeloperoxidase +	7 (27%)		
Seropositive (RF and anti-CCP)			7 (100%)
Sex			
Female	17 (65%)	14 (70%)	5 (71%)
Male	9 (35%)	6 (30%)	2 (29%)
Age (years, mean ± SD)	61.7 ± 3.4	56,2 ± 5.2	68.2 ± 6.4
Disease duration (years, mean ± SD)	6.4 ± 1.3		
Mean CRP (mg/l, mean ± SD)	12.6 ± 2.8		7.3 ± 3.4
DAS28-CRP			2.5 ± 0.2
Organ involvement			
Ear, nose, and throat (ENT)	17 (66%)		
Lungs	18 (69%)		
Kidneys	17 (66%)		
Skin	5 (19%)		
Joints	8 (31%)		
Peripheral nerves	7 (27%)		
Medication at blood drawing			
untreated	2 (8%)		0 (0%)
Prednisone	17 (65%)		1 (14%)
Prednisone dose (median ± SD)	4.5 mg ± 4.1		0.2 mg ± 0.1
Rituximab	12 (46%)		0 (0%)
Methotrexate	5 (19%)		5 (71%)
Azathioprin	4 (15%)		0 (0%)
Cyclophosphamide	2 (8%)		0 (0%)
Leflunomide	2 (8%)		0 (0%)
Mycophenolat mofetil	2 (8%)		0 (0%)
Hydroxychloroquine	1 (4%)		2 (29%)

(Ek 218/20, Ek 383/19). Active disease was defined as new onset or recurrence of symptoms that are associated with AAV combined with an increase in C-reactive protein (CRP), which was not explained by an infection, and a Birmingham Vasculitis Activity Score (BVAS) ≥ 1 (28).

PR3- and MPO-ELISA

The ANCA staining pattern (cytoplasmatic or perinuclear) was assessed by indirect immunofluorescence. ANCA specificity for proteinase 3 (PR3, Organtec) or myeloperoxidase (MPO, Euroimmun) was measured by enzyme-linked immunosorbent assay (ELISA) and interpreted according to the manufacturers' reference ranges with the upper limit of the normal of <10 U/ml for PR3 and <20 U/ml for MPO.

Cells and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by density gradient centrifugation with Lymphoprep (anprotec). Monocytes were isolated using EasySep Human Monocyte Enrichment Kit without CD16 Depletion (Stemcell Technologies) and stimulated with IFNγ (100 IU/ml; PeproTech) or TNFα (5 ng/ml; BioLegend) for 24 hours. To inhibit lysosomal function, cells were treated with Bafilomycin A1 (20 nM; Cayman Chemicals) or chloroquine (10 μM; Sigma-Aldrich). For additional experiments, hydrocortisone (Sigma-Aldrich) was added or monocytes were pre-treated with TNFα (5ng/ml) for 30 minutes and stimulated with 5 μg/ml anti-PR3 (Santa Cruz Biotechnology) or anti-MPO (Miltenyi Biotec).

In co-culture experiments, monocytes were pretreated with IFNγ (100 IU/ml; PeproTech) for 24 hours and then co-cultured with $CD4^+$ T cells in a ratio of 1:3 (400,000 monocytes to 1.2 million T cells). $CD4^+$ T cells were derived from different HLA-mismatched healthy donors and were distributed equally throughout experiments in order to assess the T cell response based on the difference of the monocyte population. To assess early T cell activation, $CD4^+CD25^+$ T cells were quantified by flow cytometry after 48 hours of co-culture. To assess T cell proliferation, $CD4^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with monocytes for 5 days. Proliferation rates were analyzed by CFSE dilution. Monocyte-derived macrophages were differentiated in RPMI 1640 medium (Life technologies) supplemented with 10% FBS (Gibco) and 20 ng/ml M-CSF (BioLegend) or 20 ng/ml GM-CSF (BioLegend) for 6 days as reported previously (29). Cells were detached using StemPro Accutase Cell Dissociation (Life Technologies, Thermo Fisher).

Quantitative RT-PCR

Total RNA was extracted with Quick-RNA Microprep Kit (Zymo Research), cDNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Gene expression was determined using SYBR Select Master Mix (Applied Biosystems) on an OneStepPlus RT-PCR machine (Applied Biosystems). Used primers are listed below (**Table 2**). Gene transcript numbers were adjusted relative to β-actin transcripts.

Flow Cytometry

For intracellular staining, cells were permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences). Surface staining and intracellular protein evaluation were done using BD LSR Fortessa. Data were analyzed with FlowJo software (Tree Star). The following antibodies or stainings were used: CMTM6 (biorbyt), PD-L1, PD-L2, CD80, CD86, CD4, CD25 (all BioLegend), and CFSE (Molecular Probes).

Statistical Analysis

Statistical significance was assessed after data sets were tested for normal Gaussian distribution by D'Agostino normality testing. For data sets with normal distribution, parametric testing was applied (unpaired t-test and paired t-test). For other data sets, non-parametric testing was applied (Mann-Whitney test, Wilcoxon test, Kruskal-Wallis test with Dunn's multiple comparisons test, and Spearman correlation). All data were analyzed by Prism V.9.1.0 (GraphPad).

RESULTS

Reduced Frequency of PD-L1+ Monocytes in Peripheral Blood of AAV Patients

Monocytes from patients with PR3- or MPO-ANCA-positive AAV were tested for surface expression of the immune checkpoint molecule PD-L1. The frequency of PD-L1+ monocytes in AAV patients was reduced compared to cells from healthy control (HC) donors (**Figure 1A**). A reduction of PD-L1 expression on AAV monocytes was observed across all monocyte subsets (**Figure 1B**; for subset identification see **Supplementary Figure 1**). To evaluate whether this reduction in PD-L1 expression is associated with defects in the expression of other inhibitory or stimulatory molecules, we tested the surface expression of PD-L2, CD80, and CD86. While the inhibitory PD-L2 and the stimulatory CD80 were expressed only weakly, almost all monocytes expressed CD86. Overall, no differences between HC and AAV patients were detected (**Figures 1C–E**). Together, these data demonstrate that patient-derived monocytes have a defect in the expression of PD-L1.

AAV Monocytes Display a Defect in PD-L1 Presentation Upon Activation

Monocytes acquire PD-L1 expression through extracellular stimuli, e.g. when entering tissue sites and through cytokine stimulation. Analog to tissue infiltration, the culture of monocytes on tissue

TABLE 2 | Primer list.

Gene	Forward primer	Reverse primer
β-actin	GATCATTGCTCCTCCTGAGC	CGTCATACTCCTGCTTGCTG
PD-L1	TGGCATTTGCTGAACGCATTT	TGCAGCCAGGTCTAATTGTTTT
CMTM6	TTTCCACACATGACAGGACTTC	GGCTTCAGCCCTAGTGGTAT

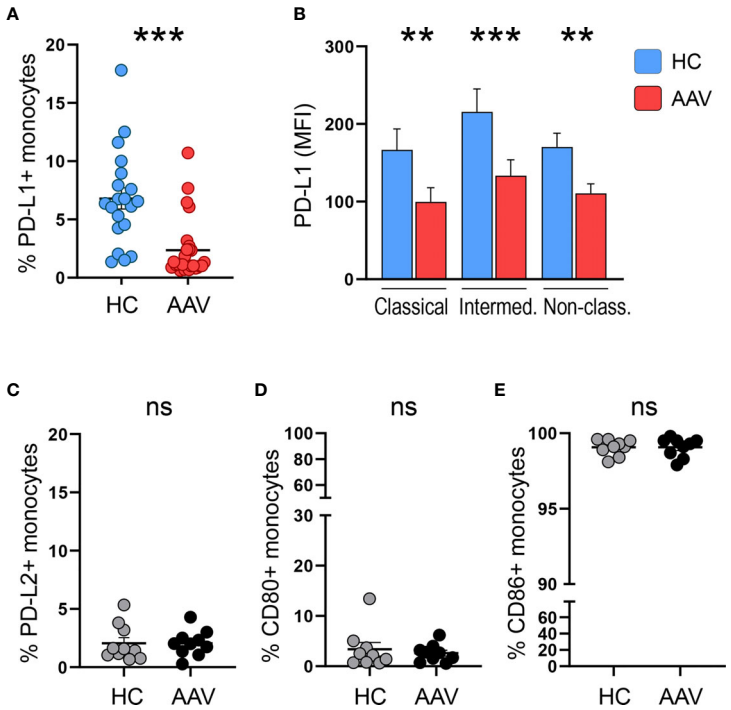

FIGURE 1 | Less PD-L1+ monocytes in patients with AAV. **(A)** Percentages of PD-L1+ monocytes in HC (n=20) and AAV patients (n=26). **(B)** Surface expression of PD-L1 (MFI) on classical (CD14+CD16-), intermediate (CD14+CD16+), and non-classical monocytes (CD14dim CD16++) in healthy control donors (n=20) and AAV patients (n=26). **(C–E)** Percentages of PD-L2+, CD80+, and CD86+ monocytes in HC and AAV patients (n=9-10 each cohort). Mann-Whitney **(A, B, D)** test or unpaired t-test **(C, E)** were applied. **P<0.01; ***P<0.001. Bar graph shows mean ± SEM. AAV, ANCA-associated vasculitis; HC, healthy control donors; MFI, mean fluorescence intensity; PD-L1, Programmed death-ligand 1; ns, statistically not significant.

culture plastic alone is enough to induce PD-L1 expression (30). Still, the strongest known inducer of PD-L1 is IFNγ (31, 32). We tested for PD-L1 induction on monocytes from HC and AAV patients after 24 hours of *in vitro* culture alone or with additional stimulation by IFNγ. As a control, we used monocytes from patients with another chronic inflammatory disease, rheumatoid arthritis (RA), to assess the impact of chronic inflammation on PD-L1 expression.

When left untreated for 24 hours, induction of PD-L1 was weaker in monocytes from AAV patients compared to monocytes from HC and RA patients. After 24 hours of stimulation, IFNγ potently induced PD-L1 expression. Again, monocytes from AAV patients displayed less PD-L1 than HC and RA monocytes. In general, control monocytes from RA patients did not differ from HC monocytes in their PD-L1 expression (**Figures 2A, B**). We further tested TNFα as this cytokine was reported to have at least some effect on PD-L1 expression (31, 32). Overall, its effect was rather weak compared to IFNγ, and also in this set of experiments AAV monocytes failed to properly upregulate PD-L1 (**Supplementary Figure 2**).

The defect in PD-L1 protein presentation of AAV monocytes was not accompanied by a reduction of *PD-L1* mRNA on the transcriptional level (**Figure 2C**). Thus, a global defect in enhancing PD-L1 expression upon activation was detected in AAV monocytes that appeared to be regulated at the protein level.

Current Medication of AAV Patients Is Not Associated With PD-L1 Expression

As the majority of AAV patients received medical treatment (**Table 1**), we performed a subgroup analysis and examined PD-L1 expression of AAV patients depending on their medication.

PD-L1 was low on monocytes from untreated patients on circulating cells (**Figure 3A**) and after activation with IFNγ (**Figure 3D**) but due to the low sample size (n=2) no statistical conclusion can be drawn. We further analyzed subgroups based on cortisone intake (**Figures 3B, E**) and rituximab infusion (**Figures 3C, F**) and observed no relevant effect.

Glucocorticoids (GC) have pleiotropic effects and can act directly on monocytes. We, therefore, treated GC-naïve monocytes with hydrocortisone in different concentrations, either in the presence or absence of additional IFNγ. In summary, hydrocortisone did not alter PD-L1 expression (**Supplementary Figure 3**).

These data indicate that the medication of AAV patients did not bias their monocytes towards low PD-L1 expression.

PD-L1 Expression on Monocytes Correlates With Disease Activity Markers

We hypothesized that the reduced frequency of PD-L1+ monocytes in the peripheral blood of AAV patients and the

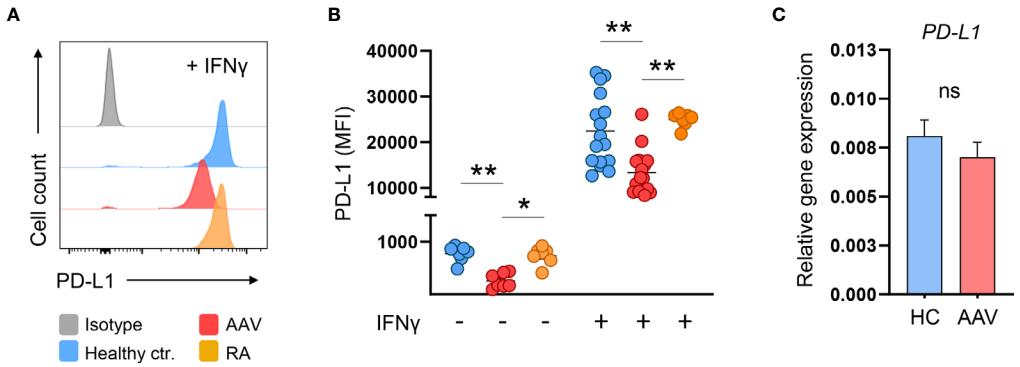

FIGURE 2 | AAV monocytes fail to upregulate PD-L1. **(A)** Representative histograms and **(B)** summarizing scatter plot of PD-L1 surface expression of monocytes left untreated for 24 hours (n=7 each group) or after stimulation with IFNγ for 24 hours (100 IU/ml; HC n=21, AAV n=26, and RA n=7). **(C)** Gene expression of *PD-L1* in monocytes from HC and AAV patients (n=14 each group) after stimulation with IFNγ for 24 hours measured by RT-PCR, relative to housekeeping gene β-actin. Kruskal-Wallis test with Dunn's multiple comparisons test **(B)** and Mann-Whitney test **(C)** were applied. *P<0.05; **P<0.01. Bar graphs show mean ± SEM. AAV, ANCA-associated vasculitis; HC, healthy control donors; MFI, mean fluorescence intensity; PD-L1, Programmed death-ligand 1; RA, Rheumatoid arthritis; ANCA, Anti-neutrophil cytoplasmic antibodies; ns, statistically not significant.

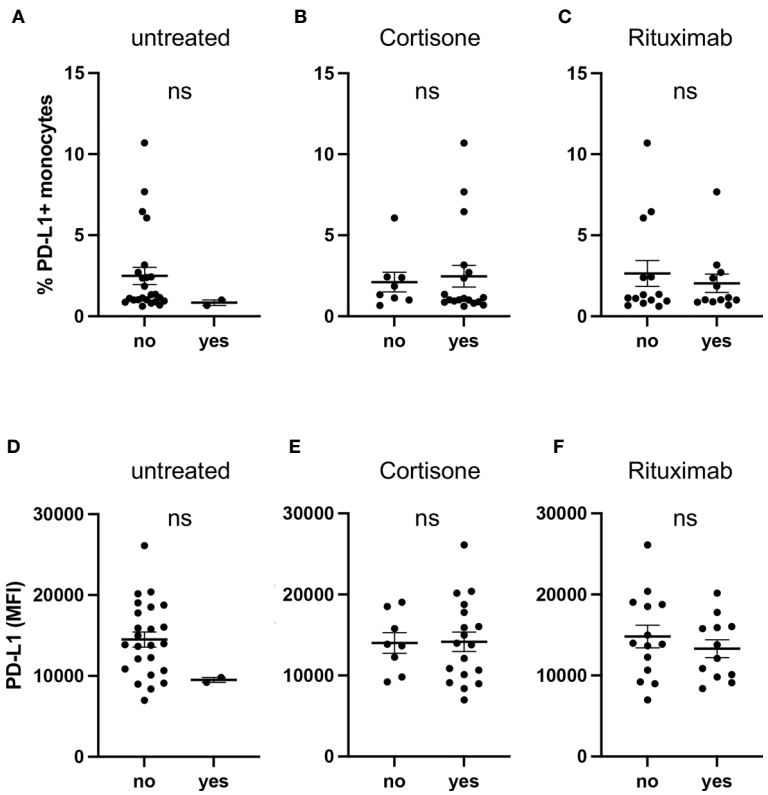

FIGURE 3 | Current medication of AAV patients is not associated with PD-L1 expression. Percentages of PD-L1+ monocytes **(A–C)** and PD-L1 expression of monocytes stimulated with IFNγ for 24 hours **(D–F)** from AAV patients subgrouped depending on their medication. Mann-Whitney test **(A–D)** and unpaired t-test **(E, F)** were applied. MFI, mean fluorescence intensity; PD- L1, Programmed death-ligand 1; ns, statistically not significant.

induced PD-L1 expression after cell activation are correlated with disease activity.

The low frequency of circulating PD-L1+ monocytes was correlated with high ANCA titers in AAV patients (**Supplementary Figure 4A**), while no correlation was observed between C-reactive protein (CRP) serum concentrations, blood monocyte counts, and renal organ involvement of AAV with the frequency of PD-L1+ monocytes (**Supplementary Figures 4B, C, E**). Although there was a trend for

patients with active disease to show lower frequencies of PD-L1+ monocytes, it did not reach statistical significance (**Supplementary Figure 4D**).

IFNγ-induced PD-L1 expression correlated inversely with ANCA titers as well as CRP serum concentrations of AAV patients (**Figures 4A, B**), while blood monocyte counts did not correlate with PD-L1 (**Figure 4C**). Moreover, PD-L1 expression after stimulation with IFNγ was lower in patients with active disease (**Figure 4D**) but similar between patients with or without kidney involvement of AAV (**Figure 4E**).

As high ANCA titers correlated with low PD-L1 expression, we tested whether direct stimulation of monocytes with anti-PR3 or anti-MPO antibodies could downregulate PD-L1. Short-term treatment (4 hours) did not alter PD-L1 expression in healthy or AAV monocytes (**Supplementary Figures 5A, B**). After 24 hours, anti-MPO antibodies did not affect PD-L1 expression, while there was a trend for anti-PR3 antibodies to upregulated PD-L1 at least in some of the healthy and patient samples tested (**Supplementary Figures 5C, D**). Also, adding anti-PR3 or anti-MPO antibodies when cells are stimulated with IFNγ did not change PD-L1 levels (**Supplementary Figure 6**).

These data indicate that lower surface presentation of PD-L1 on monocytes is associated with high ANCA titers and disease activity. This effect is not attributed to direct effects of ANCA antibodies on PD-L1.

Blocking Lysosomal Function Restores PD-L1 Expression in AAV Monocytes That Have Low Expression of CMTM6

PD-L1 mRNA was not downregulated in AAV monocytes, indicating a regulation on the post-transcriptional level. Breakdown and degradation of surface PD-L1 have been reported to occur in lysosomes (33). Thus, we hypothesized that lysosomal degradation contributes to PD-L1 deficiency in AAV monocytes. Indeed, Bafilomycin A1 treatment that inhibits lysosomal function restored PD-L1 expression on AAV monocytes without affecting PD-L1 expression on HC monocytes (**Figures 5A, B**). A similar effect was observed when AAV monocytes were treated with chloroquine (**Supplementary Figure 7B**). One of the functions of chloroquine is the inhibition of lysosomes.

The protein chemokine-like factor-like MARVEL transmembrane domain containing family member 6 (CMTM6) emerged as a master regulator of the PD-L1 protein pool by preventing PD-L1 from being targeted for lysosome-mediated degradation (25, 26). CMTM6 protein levels were reduced in circulating AAV monocytes (**Supplementary Figure 5B**) as wells as after activation with IFNγ (**Figures 5C, D**). CMTM6 protein levels correlated with PD-L1 expression (**Figure 5E**). Lower expression of CMTM6 in AAV monocytes corresponded to a reduction in *CMTM6* mRNA (**Figure 5F**).

Collectively, these data demonstrated that CMTM6 is reduced in AAV monocytes, thus facilitating lysosomal degradation of

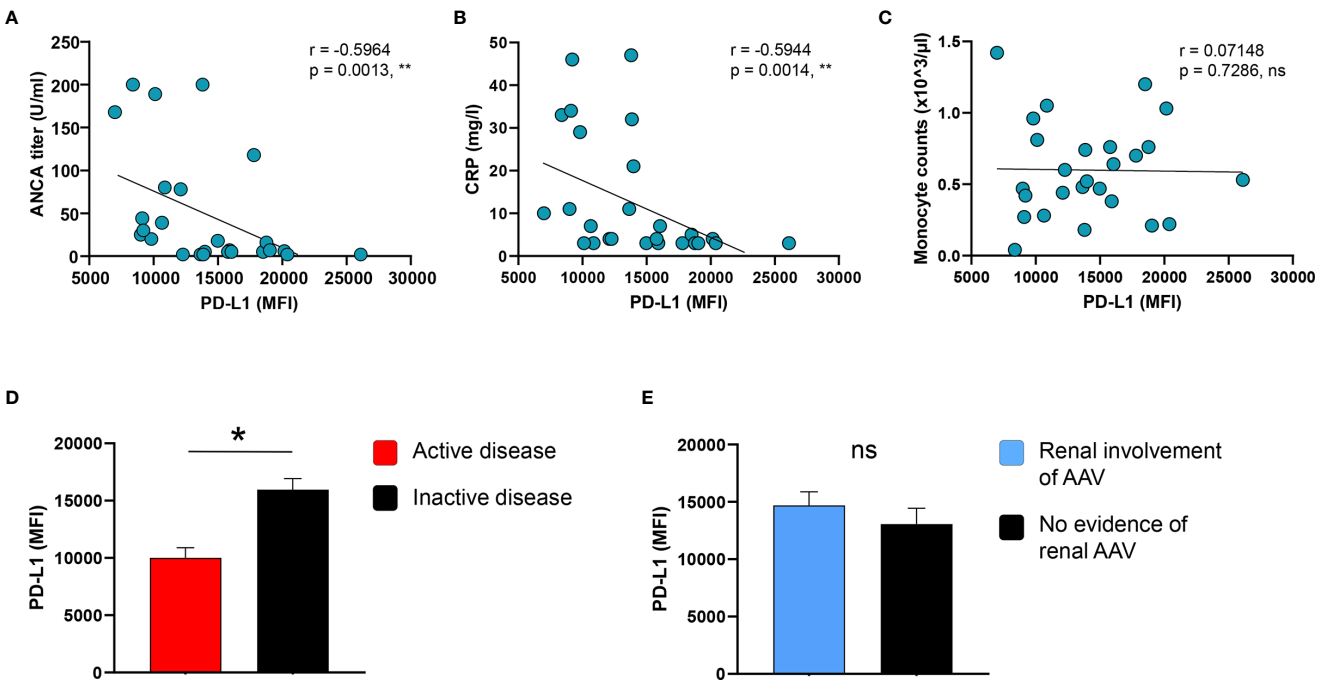

FIGURE 4 | PD-L1 induction on monocytes correlates with disease markers and activity. **(A–C)** Correlation of IFNγ-induced PD-L1 expression with ANCA titers, serum levels of CRP, and blood monocyte counts in patients with AAV (n=26). IFNγ-induced PD-L1 expression on monocytes compared between AAV patients with **(D)** either active or inactive disease (active n=8, inactive n=18) and **(E)** with or without renal AAV (renal AAV n=17, without renal AAV n=9). Spearman correlation **(A–C)** and unpaired t-test **(D, E)** were applied. *P<0.05; **P<0.01. Bar graph shows mean ± SEM. AAV, ANCA-associated vasculitis; HC, healthy control donors; MFI, mean fluorescence intensity; PD-L1, Programmed death-ligand 1; ANCA, Anti-neutrophil cytoplasmic antibodies; CRP, C-reactive protein; ns, statistically not significant.

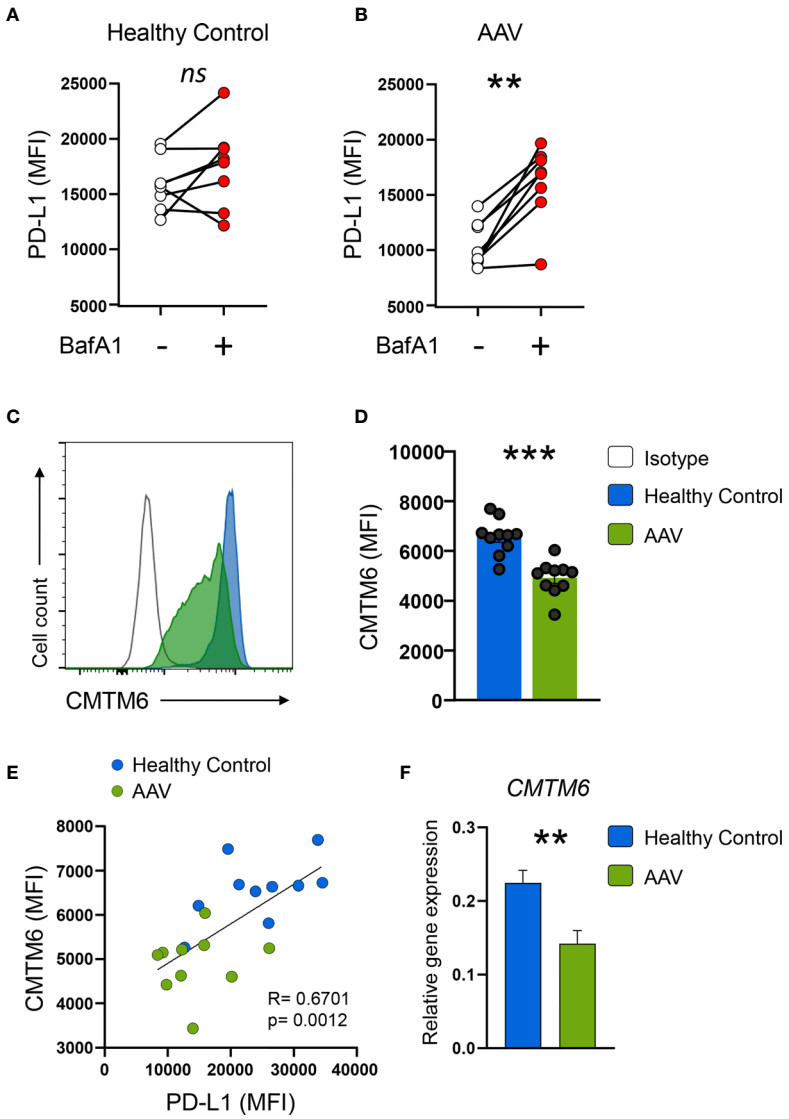

FIGURE 5 | AAV monocytes are deficient for CMTM6, blocking lysosomal function restores capacity to upregulate PD-L1. **(A, B)** Monocytes from either HC **(E)** or AAV patients **(F)** were stimulated with IFNγ for 24 hours in the presence or absence of the lysosomal inhibitor BafA1 (20nM). Monocytes not treated with BafA1 inhibitor were treated with the corresponding vehicle. **(C)** Representative histograms of CMTM6 protein expression in IFNγ-stimulated (24 hours) monocytes from HC and AAV patients. **(D)** Summarizing scatter dot plot showing results from 10 experiments. **(E)** Correlation of PD-L1 expression with CMTM6 in IFNγ-stimulated monocytes in HC and AAV patients (each group n=10). **(F)** Gene expression of *CMTM6* in monocytes from HC and AAV patients after stimulation with IFNγ for 24 hours measured by RT-PCR, relative to housekeeping gene β-actin. Paired t-test **(A)**, Wilcoxon test **(B)**, unpaired t-test **(D)**, Spearman correlation **(E)**, and Mann-Whitney test **(F)** were applied. ***P<0.01; ***P<0.001. Bar graph shows mean ± SEM. AAV, ANCA-associated vasculitis; HC, healthy control donors; MFI, mean fluorescence intensity; PD-L1, Programmed death-ligand 1; CMTM6, CKLF-like MARVEL transmembrane domain containing 6; BafA1, Bafilomycin A1; ns, statistically not significant.

PD-L1. Blocking lysosomal function restored their capacity to upregulate PD-L1.

PD-L1/CMTM6-Defect Is Preserved in Monocyte-Derived Macrophages

Monocytes can alter their phenotype based on environmental signals, e.g. they can differentiate into monocyte-derived macrophages. To study whether the defect in PD-L1 and CMTM6 expression was present also in macrophages, we differentiated monocytes with M-CSF or GM-CSF as previously described (29). After 6 days of differentiation, GM-CSF was a more potent inducer of PD-L1 than M-CSF. Monocyte-derived macrophages from AAV patients had lower expression of PD-L1 after differentiation with GM-CSF; after M-CSF differentiation there was a trend for lower PD-L1 expression in patients (**Figures 6A, B**) and CMTM6 (**Figure 6C**) compared to macrophages from HC

donors. This suggests that the underlying defect for PD-L1 deficiency is imprinted to monocytes from AAV patients and carried on to corresponding cells once they differentiate.

Monocytes From AAV Patients Show an Enhanced Stimulatory Capacity

To examine the ability of PD-L1^{lo} AAV monocytes to stimulate T cells, we measured monocyte-induced T cell activation and expansion (in HC-derived T cells) by adapting a previously published co-culture model (16). AAV monocytes and HC monocytes were cultured with CFSE-labeled CD4$^+$ T cells, after 5 days frequencies of dividing CD4$^+$ T cells were measured. Frequencies of proliferating CD4$^+$ T cells were higher in co-cultures with AAV monocytes (**Figures 7A, B**). Additionally, AAV monocytes enhanced early T cell activation as measured by the frequency of CD4$^+$ CD25$^+$ T cells after 48 hours (**Figures 7C, D**).

To understand whether the enhanced activation of T cells primed by monocytes is directly attributed to PD-L1 expression, anti–PD-L1 antibodies were added to co-cultures with monocytes from HC donors. Blocking PD-L1 on HC monocytes simulated PD-L1 deficiency and increased CD4$^+$ T-cell activation (**Figure 7E**), confirming published data (16). To further elucidate whether blockade of PD-L1 degradation in lysosomes normalizes the hyperstimulatory behavior of patient-derived cells, AAV monocytes were pre-treated with the lysosomal inhibitor Bafilomycin A1. Inhibiting lysosome function in AAV monocytes restored their ability to balance immune cell interaction with T cells, resulting in less CD4$^+$ T cell activation (**Figure 7F**).

In essence, PD-L1^{lo} monocytes from AAV patients cause enhanced stimulation of CD4$^+$ T cells. Targeting lysosomal degradation of PD-L1 in AAV monocytes corrected this phenotype.

DISCUSSION

The development of autoimmune small-vessel vasculitis has been observed after checkpoint inhibitor therapy (21–24), indicating a relevant role of immune checkpoint molecules in the disease process. However, no molecular alterations of such molecules in

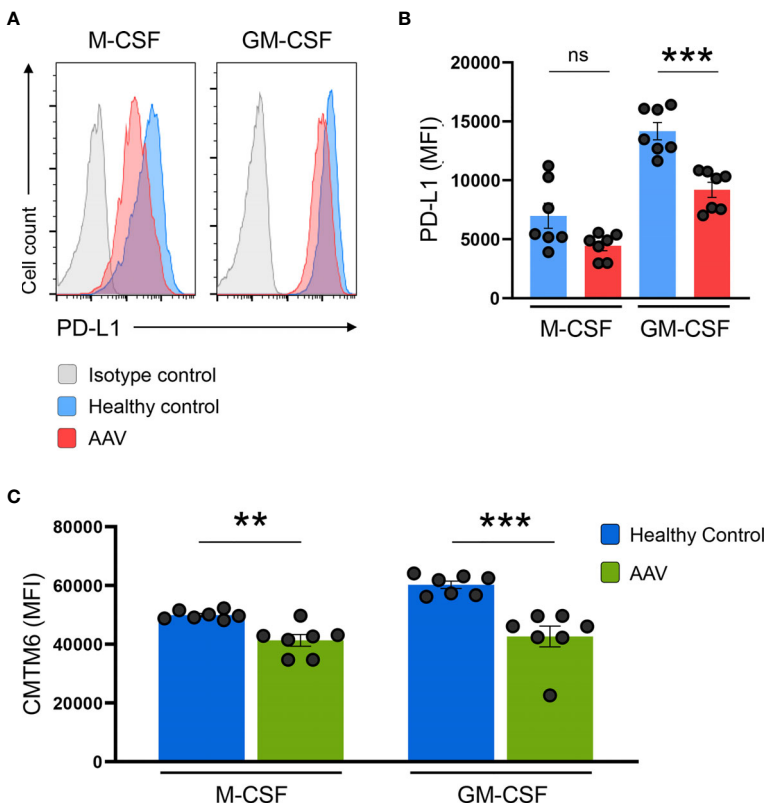

FIGURE 6 | PD-L1/CMTM6-defect is passed to monocyte-derived macrophages. **(A)** Representative histograms of PD-L1 expression (MFI) on monocyte-derived macrophages after differentiation with M-CSF or GM-CSF for 6 days. **(B)** Summarizing scatter dot plot presenting results from experiments with cells from HC or AAV patients (n=7 each group). **(C)** Expression of CMTM6 protein in monocyte-derived macrophages from HC or AAV patients (n=7 each group). Mann-Whitney test **(B, C)** was applied. **P<0.01; ***P<0.001. Bar graph shows mean ± SEM. Bar graph shows mean ± SEM. AAV, ANCA-associated vasculitis; HC, healthy control donors; MFI, mean fluorescence intensity; PD-L1, Programmed death-ligand 1; M-CSF, macrophage colony-stimulating factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; ns, statistically not significant.

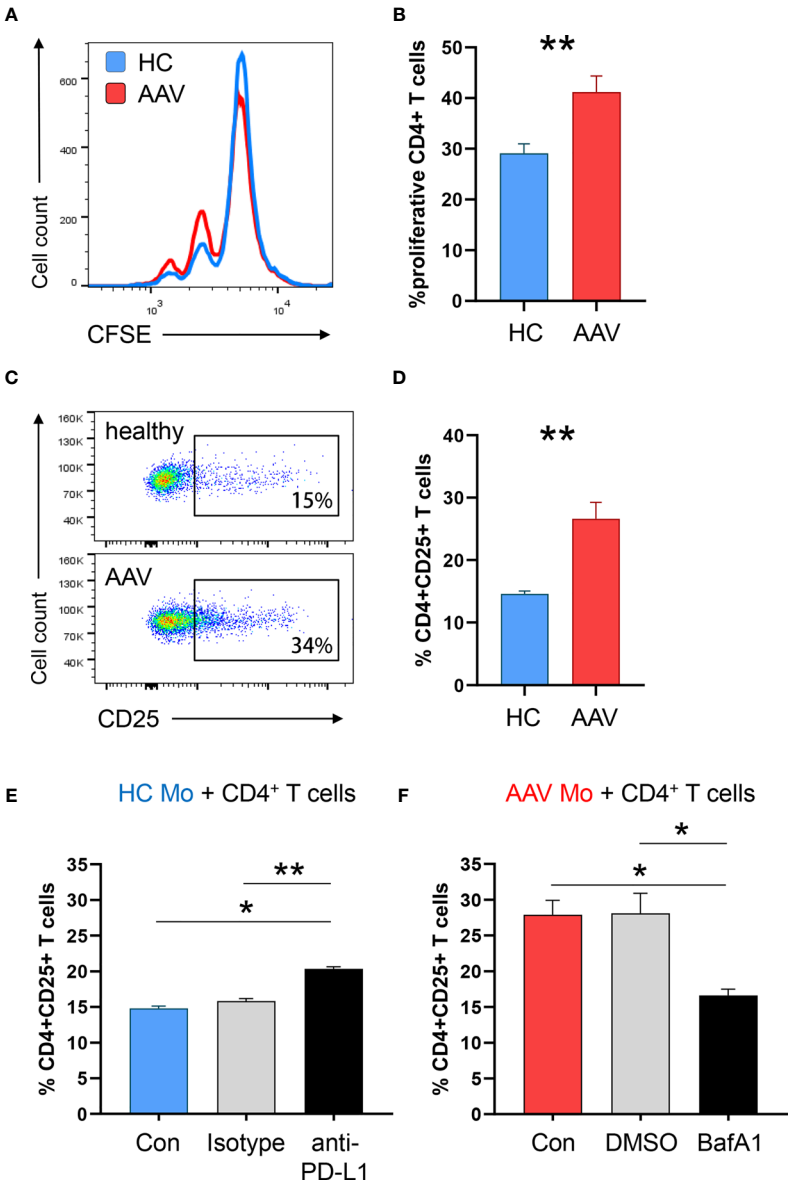

FIGURE 7 | PD-L1^{lo} monocytes from AAV patients show enhanced stimulatory capacity. Monocytes from healthy or AAV donors were pretreated with IFNγ for 24h. Then, their capacity to stimulate T cells was probed by coculturing them with CD4+ T cells from healthy donors (ratio monocytes/T cells 1:3). T-cell proliferation was determined through CFSE dilution and T-cell activation was quantified by the frequency of $CD4^+CD25^+$ T cells. **(A)** Proliferation of CD4+ T cells was measured by flow cytometry after 5 days of co-culture. Representative histograms of CSFE expression. **(B)** Frequencies of proliferating CD4+ T cells when cocultured with either HC or AAV patient-derived monocytes (n=8 each group). **(C)** Activated $CD4^+CD25^+$ T cells quantified by flow cytometry after 48 h. **(D)** Percentage of activated CD4+ T cells after coculture (n=6 each group). **(E)** Co-culture with monocytes from healthy donors was performed in the presence of anti-PD-L1 antibodies or isotype control. Frequencies of activated $CD4^+CD25^+$ T cells from 6 independent experiments (isotype n=3) were measured after 48 h by flow cytometry. **(F)** Co-culture with monocytes from AAV patients was performed after monocytes were pre-treated with the lysosomal inhibitor BafA1 (20nM) or DMSO vector control. Frequencies of activated $CD4^+CD25^+$ T cells from 6 independent experiments (DMSO n=3) were measured after 48 h by flow cytometry. Mann-Whitney test **(B)**, unpaired t-test **(D)** and Kruskal-Wallis test with Dunn's multiple comparisons test **(E, F)** were applied. *P<0.05; **P<0.01. Bar graph shows mean ± SEM. Bar graph shows mean ± SEM. AAV, ANCA-associated vasculitis; HC, healthy control donors; MFI, mean fluorescence intensity; PD-L1, Programmed death-ligand 1; M-CSF, macrophage colony-stimulating factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor.

the immune system of patients with AAV have been reported so far. In this study, we found that monocytes from AAV patients show a defect in presenting the immunoinhibitory checkpoint PD-L1 leading to enhanced stimulation of T cells.

Physiologically, the co-inhibitory ligand PD-L1 shows limited expression on circulating monocytes (30) and in normal tissues (11). Induction of PD-L1 on antigen-presenting cells occurs rapidly after cell activation to instigate a negative feedback loop thereby preventing

overactivation of the adaptive immune system, e.g. of $CD4^+$ T cells. Failure of PD-L1 induction promotes autoimmunity as reported for giant cell arteritis, an immune-mediated large vessel vasculitis, in which PD-L1-deficient dendritic cells facilitate inflammatory vascular damage (16). In AAV lesions, monocyte infiltration is a characteristic hallmark and vascular infiltrates show a predominance of monocytes and monocyte-derived macrophages. When entering tissue sites, monocytes encounter a multitude of pro- and anti-inflammatory stimuli and their response determines whether inflammation resolves or amplifies. In the case of AAV, PD-$L1^{lo}$ monocytes may disturb the immunomodulatory PD-L1/PD-1 axis, thereby contributing to enhanced activation of $CD4^+$ T cells and, thus, consolidating the chronic inflammatory process.

PD-L1 protein deficiency was not accompanied by decreased *PD-L1* mRNA transcripts in AAV monocytes, which indicated a regulation on the post-transcriptional level. Cleavage of PD-L1 by metalloproteinases (MMP) has been reported (34, 35) with tumor-derived MMP-13 potently degrading PD-L1 (36). In our experiments, inhibition of MMP-13 did not affect PD-L1 expression of monocytes (data not shown). Lysosomes are the cell's degradation center and are responsible for the breakdown of proteins, polysaccharides, and complex lipids (37). Recently, two independent reports identified CMTM6, a protein of previously unknown function, as a major regulator of the PD-L1 protein pool. CMTM6 co-localizes with PD-L1 and prevents PD-L1 from being targeted for lysosome-mediated degradation (25, 26). Blocking lysosomal function with a specific inhibitor corrected PD-L1 deficiency in AAV monocytes indicating increased lysosomal breakdown of PD-L1 due to low levels of CMTM6. A similar effect was observed when AAV monocytes were treated with chloroquine. Chloroquine and hydroxychloroquine (HCQ) also impair lysosomal function but are less specific as they interfere with toll-like receptors and intracellular nucleic acid sensors (38). Interestingly, some groups report the successful use of HCQ in patients with AAV (39), and currently, a phase-II study is ongoing that evaluates HCQ in the treatment of AAV (HAVEN: Hydroxychloroquine in ANCA Vasculitis Evaluation, NCT04316494).

Emphasizing the role of monocyte PD-L1 expression in the disease course of AAV, higher ANCA titers correlated with lower numbers of circulating PD-L1+ monocytes in vasculitis patients. Moreover, their diminished capacity to present PD-L1 upon cell activation predicted higher ANCA titers, higher CRP serum concentrations, and active disease.

An unexpected finding of this study was the influence of colony-stimulating factors (CSFs) on PD-L1 expression. Higher PD-L1 surface expression was observed when monocytes were differentiated to macrophages with GM-CSF instead of M-CSF. In the literature, only one report describes the effect of GM-CSF on PD-L1, showing that tumor-derived GM-CSF induced PD-L1 expression in neutrophils (40). PD-L1 induction by GM-CSF could be part of a negative feedback loop to prevent uncontrolled inflammation sparked by more pro-inflammatory GM-CSF-differentiated macrophages. Alternatively, lower PD-L1 expression by M-CSF may reflect the greater lysosomal activity of M-CSF-derived macrophages (41). Serum levels of M-CSF are increased in AAV patients with active nephritis (42) and renal M-CSF production is upregulated in vasculitic glomeruli where it associates with local macrophage proliferation (43), suggesting an M-CSF-skewed macrophage phenotype in renal disease in AAV.

The design of this study bears limitations. The majority of AAV patients receive immunosuppressive therapy, which could alter any kind of read-out. AAV is a rare disease and often presents with severe symptoms making an immediate start of therapy inevitable. Our subgroup analysis as well as experiments testing the direct effects of cortisone on PD-L1 expression did not indicate evidence for a medication bias towards lower PD-L1 expression. Although we cannot exclude other factors in the natural course of the disease, the correlation of PD-L1 with markers of inflammation and disease activity suggests that low PD-L1 expression on monocytes is associated with disease activity.

Another limitation is the use of RA monocytes as disease control. To exclude that chronic inflammation lowers PD-L1 expression, we tested monocytes from RA patients, a typical chronic-inflammatory disease. It could be argued that inflammation in those patients is localized mainly in the joints and not in the vessel wall. Still, RA patients do have a component of systemic inflammation, which results in their increased cardiovascular risk (44) - as such a vascular inflammatory process. Further studies in other autoimmune disease would be needed to clarify whether low PD-L1 expression is specific for vasculitis. In connective tissue disease, in SLE for example, contradictory results have been reported with one group finding low PD-L1 expression on monocytes in a pediatric cohort (45) and another study reporting upregulation of PD-L1 on SLE monocytes (46).

In summary, this study identified a defective immunoinhibitory PD-L1 checkpoint on monocytes and monocyte-derived macrophages from patients with AAV. CMTM6-deficient vasculitic monocytes degrade PD-L1 in lysosomes, thus providing insufficient negative signaling to $CD4^+$ cells, fostering the development of highly activated T cells in patients with autoimmune small-vessel vasculitis. Correcting this defect in monocytes by targeting lysosomal function may be a promising novel strategy to treat AAV, especially to maintain remission.

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 the Institutional Review Board (Ek 218/20, Ek 383/19) of the University of Freiburg. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MZ, JT, and RV conceived the study. MZ performed experiments. MZ and NV analyzed data. BS contributed technical expertise. NC and MR enrolled patients and oversaw patient recruitment. MZ, NV, JT, and RV wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.673912/full#supplementary-material

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Antineutrophil Cytoplasmic Antibody-Associated Vasculitis With Acute Kidney Injury: Short-Term Recovery Predicts Long-Term Outcome

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Background: Kidney involvement is common in antineutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV). It tends to be aggressive, and in some patients, the kidney involvement may reach the criteria of acute kidney injury (AKI). Here, we aim to describe the clinical characteristics of these patients and find risk factors for poor outcomes.

Methods: Patients diagnosed with AAV in our hospital from February 2003 to February 2017 were included. Those who reached the KDIGO AKI criteria were reclassified according to the KDIGO AKI stage. The clinical features of these patients were analyzed. Also, according to the variation of serum creatinine 3 months after AKI episode, patients were further divided into two groups: patients whose serum creatinine (Scr) level at the third month decreased by 30% or more from the peak Scr level was classified into G1 and others were classified into G2. Long-term renal and survival outcomes of these patients were analyzed with a Cox model. The renal endpoint was reaching end-stage renal disease (ESRD), and the survival endpoint was death. Nomograms were built based on cox models.

Results: Of 141 AAV patients included, during the median follow-up period of 64.0 (IQR 34.8, 85.4) months, 36 (25.5%) patients reached renal endpoints, and 22 (15.6%) patients died. The median renal survival time was 35.9 (IQR 21.3, 72.6) months and the median survival time was 48.4 (IQR 26.8, 82.8) months. Multivariate analysis showed that poor recovery of Scr level at 90 days ($P < 0.001$, RR = 9.150, 95%CI 4.163–20.113), BVAS score ($P = 0.014$, RR = 1.110, 95% CI1.021–1.207), and AKI stage 3 ($P = 0.012$ RR = 3.116, 95%CI 1.278–7.598) were independent risk factors for renal endpoints; poor recovery of Scr level at 90 days ($P = 0.010$, RR = 3.264, 95%CI 1.326–8.035), BVAS score ($P = 0.010$, RR = 1.171, 95%CI 1.038–1.320) and age ($P = 0.017$, RR = 1.046, 95% CI 1.008–1.086) were independent risk factors for all-cause death. The c-index of nomograms is 0.830 for the renal outcome and 0.763 for the survival outcome.

Conclusion: KDIGO AKI stage 3 is the risk factor for ESRD in AAV patients with AKI. The BVAS score and level of kidney function recovery at 90 days are the independent risk factors for both ESRD and all-cause death and are of predictive value for the outcome.

Keywords: ANCA associated vasculitis, acute kidney injury, end stage renal disease, mortality, outcome

INTRODUCTION

Kidney involvement is common in antineutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) (1, 2). It occurs in about 90% of patients with microscopic polyangiitis (MPA), and about 70% of patients with granulomatosis with polyangiitis (GPA) (3). A typical manifestation of renal involvement is aggressive kidney vasculitis (4). It can present with a rapid decline of kidney function and may need renal replacement therapy within a few days, and even lead to end-stage renal disease (ESRD). In some patients, the clinical course of kidney injury can be very aggressive and can reach the criteria of acute kidney injury (AKI).

AKI is characterized as sudden renal impairment. Studies have shown that AKI is a risk factor for ESRD, and is associated with poor survival outcomes (5). The criteria and staging system proposed by the Kidney Disease: Improving Global Outcome (KDIGO) group based on the time and level of increase of creatinine or decrease of urine output is recommended for evaluating the severity of AKI and predicting renal outcomes (6, 7).

There are limited approaches to evaluate the level of progression of renal injuries caused by AAV. Here we retrospectively analyzed AAV patients who reached the KDIGO AKI criteria in our center, trying to describe their characteristics, as well as finding out the associations between AKI stage or short-term kidney function recovery and long-term outcomes.

METHODS

Patients

Patients diagnosed with AAV in our hospital from February 2003 to February 2017 according to the Chapel Hill consensus (1) were retrieved. Those who were diagnosed with ESRD at admission were excluded. Patients who reached the KDIGO criteria for AKI, and followed up for more than 3 months or reached the primary endpoint within 3 months were included (**Figure 1**). Based on the KDIGO guideline, AKI is defined as serum creatinine (Scr) increased more than 0.3 mg/dl (26.5 μmol/l) within 48 h, Scr raised to a 1.5-fold baseline within 7 days, or a decreased urine output less than 0.5 ml/kg/h over 6 h (8). The research protocol was approved by the Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

Generally, patients with ANCA-associated vasculitis were treated with corticosteroids (0.6–0.8mg/kg/d) with intravenous cyclophosphamide (CTX) 15 mg/kg every 2–4 weeks until the cumulative dose reached 6–8 g; or corticosteroids with mycophenolate mofetil (MMF) 1.0–1.5 g/d (9–11). Patients with pulmonary hemorrhage, biopsy-proven cellular crescents, or fibrinoid necrosis of small vessels were treated with reinforcement therapy of 500 mg pulse methylprednisolone for 3 days before CTX or MMF therapy. Renal replacement therapy was adopted as supportive treatment when patients were with severe hyperkalemia or metabolic acidosis, signs of uremia, or refractory fluid overload.

Data Collection

Clinical data including age, gender, blood pressure, smoke, medical history, organs involved, urine output, urine protein, urine red blood cell, the level of C-reactive protein (CRP), myeloperoxidase (MPO)-ANCA or proteinase 3 (PR3)-ANCA, therapies used, and renal biopsy report were retrieved through medical records. Patients were staged according to the KDIGO clinical practice guidelines for AKI (8). And if there were two or more recorded AKI episodes, only the first episode was analyzed. All patients were evaluated with the Birmingham Vasculitis Activity Score (BVAS) version 3 according to their status at admission. Vascular damage index (VDI) at admission is also evaluated (12). Survival data were acquired from digital records

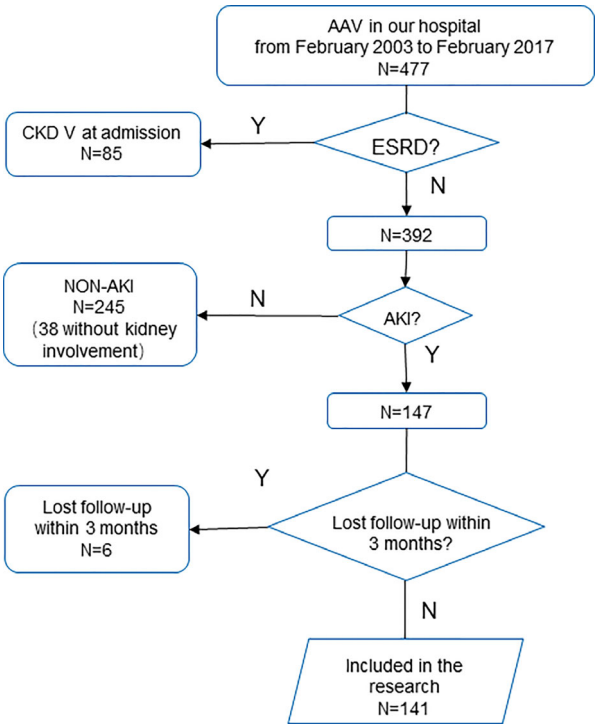

FIGURE 1 | Flowchart.

of regular follow-up visits, telephone follow-ups, and the dialysis database of Zhejiang Province.

The kidney functions of patients were evaluated through Scr levels. Scr level on the day of admission, peak level within 7 days (in the AKI episode), the level at the third month's follow-up, and the level at last follow-up were recorded. Baseline Scr was acquired through previous records or the level at admission (pre-episode). For patients who do not have previous records, the baseline Scr was back-calculated through the MDRD equation assuming an estimated glomerular filtration rate (eGFR) of 75 ml/min/1.73m^2 (6, 13).

According to the change of Scr level, patients with AKI were further divided into two groups. Patients whose Scr level at the third month decreased by 30% or more from the peak Scr level was classified into subgroup 1 (G1), and others were classified into subgroup 2 (G2). The cut-off point is defined by the approximation of the lower tertile.

Outcome Definition

As chronic kidney diseases are defined as loss of kidney function or markers of kidney damage for more than 3 months (14), the endpoint of many AKI studies was 60- or 90-day mortality (15). In this study, short-term outcomes were evaluated by the status of patients 3 months after the episode. The renal endpoint was ESRD, and the survival endpoint was defined as death from any cause. The composite endpoint was defined as reaching renal endpoint or survival endpoint. ESRD was defined as kidney failure that reaches an estimated glomerular filtration rate (eGFR) lower than 15 ml/min/1.72 m^2 or needs maintaining renal replacement therapy for more than 3 months.

Statistical Analysis

For continuous data, normally distributed data were expressed as average ± deviation, while non-normally distributed data were reported with median (interquartile range). Counting data was expressed with frequency, rate, or proportion. Student-t test, one-way ANOVA, Student–Newman–Keuls, Wilcoxon, and Kruskal–Wallis tests were used to compare continuous data. Counting data were compared with cross-table or Fisher's exact test.

Survival analysis was conducted with the Kaplan–Meier survival analysis, log-rank test, logistic regression, and Cox proportional hazards model. Factors in the univariate Cox model with P-value <0.1 were further included in multivariate analysis, and the forward LR model was used in the multivariate model. P <0.05 was considered significant.

The multivariate Cox regression model was used to build the final nomogram prognostic model, the accuracy of the model was assessed with discrimination and calibration (16, 17). The concordance index (c-index, a generalization of the area under the ROC curve) was used to assess the discrimination power of the model, and graphical plots showing observed outcomes and predicted probabilities are used for calibration.

The statistical analysis was done by SPSS 23.0 (IBM, Chicago, IL, USA). The prognostic model and its assessment were conducted in the R environment, version 3.5.3, and R packages "survival", "rms" were used.

RESULTS

Demographic and Clinical Features

In 477 patients retrieved, 85 patients diagnosed with ESRD at admission were excluded. Approximately 147 patients reached the AKI criteria, while 245 did not.

In 147 patients with AKI, six patients lost to follow up within 90 days were further excluded. Finally, 141 patients were included. In these patients, 65 (46.1%) were male, and the median age was 59.6 (51.4, 67.5) years old. The lung is the most commonly involved extrarenal organ, followed by skin and joints (**Table S1**, in **Supplementary Material**).

Patients were staged according to the KDIGO guidelines. There are 43 patients in AKI stage 1, 44 patients in AKI stage 2, and 54 patients in AKI stage 3 (Additionally, the number and percentage of patients defined by urine output or serum creatinine were listed in **Table S2**). As shown in **Table 1**, patients in AKI stages 1 to 3 had different BVAS at admission (P = 0.038). The median and interquartile ranges were 16.1 ± 3.8, 15.3 ± 3.5, and 17.3 ± 3.9 respectively. Also, the levels of CRP, serum albumin, and Scr are statistically different (P <0.001). There are no significant differences in other clinical and laboratory characteristics.

Renal biopsy was performed in 96 (68.1%) patients, and the pathological characteristics are presented in **Table 2**. The proportion of glomeruli with crescents was 11.9% (5.7%, 34.1%) in stage 1 patients, 16.1% (10.9%, 33.4%) in stage 2 patients, and 21.2% (14.3%, 51.7%) in stage 3 patients (P = 0.043), showing an upward tendency. Differences in other pathological parameters did not reach statistical significance (P >0.05).

Short-Term Renal Recovery and Outcomes

After 3 months, patients were divided into subgroups (G1 and G2) according to the variation of Scr level. There are 90 patients in the G1 subgroup and 51 patients in the G2 subgroup.

In the cohort, 84 (59.6%) patients received i.v. pulse methylprednisolone therapy, 68 (48.2%) patients used cyclophosphamide, and 58 (41.1%) patients used mycophenolate mofetil. There are no significant differences in treatment between the two groups (**Table 3**).

At the time of discharge, 14 patients in the G1 subgroup were still with renal replacement therapy, and all of them successfully withdrew dialysis at the 3rd month; 23 patients in the G2 subgroup were with renal replacement therapy at the time of discharge, and five (21.7%) of them withdrew dialysis at the third month. Two patients in the G2 subgroup died within 3 months without recovery of kidney function. There were no significant differences in clinical or laboratory characteristics between the two subgroups except for urine red blood cells [406.1 (134.3, 1302.9)/ul *vs* 139.3 (77.9, 631.2))/ul, P = 0.006] (**Table 3**). As for pathological characteristics, 64 (71.1%) patients in G1 and 32 (62.7%) patients in G2 had received renal biopsy (P = 0.496), and pathological parameters of the two groups showed no significant difference (**Table S2** in the **Supplementary Material**). During long-term follow up, 16 (17.8%) patients in the G1 subgroup and 28 (55.0%) patients in the G2 subgroup reached composite endpoint (p <0.001) (**Table 3**).

TABLE 1 | Clinical and laboratory data of AAV patients in different AKI stages.

	Stage 1 (n = 43)	Stage 2 (n = 44)	Stage 3 (n = 54)	P-value
Age, Median (IQR)	59.7(53.4,64.9)	58.2(42.0,66.9)	60.3(52.6,69.8)	0.155
Male, n (%)	22(51.2)	21(47.7)	22(40.7)	0.573
Smoke, n (%)	11(25.6)	14(31.8)	12(22.2)	0.842
Hypertension, n (%)	21(48.8)	22(50.0)	24(44.4)	0.558
Scr at admission, μmol/l, Median (IQR)	171.0(121.0,270.0)	224.5(161.0,309.8)	308.0(232.3,509.5) **##	**<0.001**
Baseline Scr, μmol/l, Median (IQR)	116(90,188)	125(82.5,156.75)	132.0 (83.3,184.0)	0.484
Urine red blood cell /ul, Median (IQR)	188.4(86.8,505.5)	429.9(93.0,1331.7)	406.1(80.3,1225.0)	0.162
White blood cell $\times10^9$/L, Median (IQR)	7.0(5.1,10.2)	7.7(5.6,10.0)	8.6(6.5,10.3)	0.130
Hemoglobin g/L, Median (IQR)	87.0(77.0,100.0)	87.0(79.0,99.0)	80.0(71.0,93.5)	0.079
Platelet$\times10^9$/L, Median (IQR)	265.0(187.0,338.0)	202.5(158.0,285.5)	226.0(166.75,289.5)	0.074
24h urine protein g, Median (IQR)	1.56(0.72,3.02)	1.75(1.13,3.47)	1.95(0.79,2.75)	0.434
Albumin g/L, Median (IQR)	33.9(29.9,37.8)	34.1(31.3,37.8)	28.6(25.6,31.1) **##	**<0.001**
Globulin g/L, Median (IQR)	29.4(26.3,34.3)	30.7(24.5,34.3)	30.0(26.8,32.9)	0.973
CRP mg/L, Median (IQR)	14.6 (4.2, 58.9)	10.5 (3.3, 58.1)	40.4 (18.8, 85.8) **##	**<0.001**
MPO-ANCA positive, n (%)	29(67.4%)	33(75.0%)	34(62.9%)	0.443
MPO, Median (IQR)	50.0(22.7,78.4)	66.0(19.4,100.0)	40.5(12.9,150.0)	0.928
PR3-ANCA positive, n (%)	4 (9.3%)	7 (15.9%)	5 (9.3%)	0.516
PR3, Median (IQR)	1.4(1.1,2.0)	1.5(1.13,2.68)	1.4(1.1,2.9)	0.823
BVAS, Mean ±std.	16.1±3.8	15.3±3.5	17.3±3.9#	**0.038**
VDI at admission, Median (IQR)	0 (0,1)	0 (0, 1)	0 (0,1)	0.699
Treatment				
I.V. Pulse Methylprednisolone	26(60.5%)	24(54.3%)	34(63.0%)	0.752
Immunosuppressants, n (%)				
CTX	19 (48.8%)	21 (47.7%)	28 (51.9%)	0.752
MMF	21 (48.8%)	18 (40.9%)	19 (35.2%)	0.309

*Scr, serum creatinine; MPO, myeloperoxidase; BVAS, Birmingham Vasculitis Activity Score; VDI, vascular damage index. **P < 0.01, compared with AKI stage1; #P < 0.05, compared with AKI stage 2; ##P < 0.01, compared with AKI stage 2.*
Bolded value: P-value < 0.05.

TABLE 2 | Pathological characteristics of AAV patients in different AKI stages.

	Stage 1 (n = 32)	Stage 2 (n = 31)	Stage 3 (n = 33)	P-value
Global sclerosis[1], Median (IQR)	20.3 (10.0, 38.5)	21.1 (12.5, 36.4)	15.4 (7.1, 20.8)	0.510
Crescent[1], Median (IQR)	11.9 (5.7, 34.1)	16.1 (10.9, 33.4)	21.2 (14.3, 51.7) **	**0.043**
Cellular crescent[1], Median (IQR)	5.8 (0.0, 21.4)	8.0 (2.0, 13.4)	15.2 (5.7, 26.7)	0.075
Capillary Necrosis, n (%)	12 (37.5)	11 (35.4)	19 (57.5)	0.140
Mesangial hypercellularity, n (%)	32 (100.0)	31 (100.0)	32 (96.970)	0.381
Endocapillary hypercellularity, n (%)	9 (28.1)	13 (41.9)	16 (48.5)	0.232
Interstitial fibrosis, n (%)				0.399
0–50%	26 (81.2)	22 (71.0)	22 (66.7)	
>50%	6 (18.8)	9 (29.0)	11 (33.3)	
Interstitial infiltration, n (%)				0.269
0–25%	11 (34.4)	4 (12.9)	8 (24.2)	
25–50%	11 (34.4)	11 (35.5)	9 (27.3)	
>50%	10 (31.2)	16 (51.6)	16 (48.5)	

[1]Proportion of glomeruli per biopsy with the lesion, data was expressed as median and interquartile range (IQR).
***P < 0.01, compared with AKI stage 1.*
Bolded value: P-value < 0.05.

Long-Term Outcomes and Predictive Model

During a median follow-up of 64.0 (34.8, 85.4) months, 36 (25.5%) patients reached renal endpoint and 22 (15.6%) patients died. The median renal survival time was 35.9 (21.3, 72.6) months, and the median overall survival was 48.4 (26.8, 82.8) months.

In the multivariate model (**Table 4**), after adjusted for age and gender, AKI stage 3 (P = 0.012 RR = 3.116, 95%CI 1.278–7.598), high BVAS score (P = 0.014, RR = 1.110, 95% CI1.021–1.207) and G2 subgroup (P <0.001, RR = 9.150, 95%CI 4.163–20.113) were independent risk factors for poor long-term kidney outcome in AAV patients with AKI. These factors (AKI stage, subgroup, BVAS) were used to construct the nomogram for predicting the renal outcome (**Figure 2A**) (calibration plot in the **Supplementary Material**), by internal validation the c-index was 0.830.

As for long-term survival outcome, age (P = 0.017, RR = 1.046, 95%CI 1.008–1.086), BVAS (P = 0.010, RR = 1.171, 95%CI 1.038–1.320), and G2 subgroup (P = 0.010, RR = 3.264,

TABLE 3 | Clinical and laboratory data of AAV patients in G1 and G2 subgroups.

	G1 (n = 90)	G2 (n = 51)	P-value
Age, Median (IQR)	60.3 (52.7, 66.7)	58.6 (51.0, 70.2)	0.597
Male, n (%)	37 (41.1)	28 (54.9)	0.114
Smoke, n (%)	23 (25.6)	14 (27.5)	0.806
Hypertension, n (%)	42 (46.7)	25 (49.0)	0.340
Scr at admission, μmol/l, Median (IQR)	257.0 (162.0, 384.5)	209.0 (157.5, 287.0)	0.075
Baseline Scr, μmol/l, Median (IQR)	117.0 (81.2, 155.5)	134.0 (99.0, 190.0)	0.070
Urine red blood cell/ul, Median (IQR)	406.1 (134.3, 1302.9)	139.3 (77.9, 631.2)	**0.006**
White blood cell × 10^9/L, Median (IQR)	7.6 (5.4, 10.0)	8.5 (6.6, 10.4)	0.063
Hemoglobin g/L, Median (IQR)	83.0 (76.2, 95.0)	82.0 (71.5, 101.5)	0.555
Platelet×10^9/L, Median (IQR)	217.0 (167.8, 291.8)	244.0 (182.0, 303.5)	0.298
24 h urine protein g/L, Median (IQR)	1.7 (1.0, 2.7)	1.9 (0.7, 3.3)	0.324
Albumin g/L, Median (IQR)	32.6 (28.1, 36.7)	31.0 (27.1, 35.0)	0.284
Globulin g/L, Median (IQR)	30.5 (27.0, 33.2)	29.5 (25.9, 35.0)	0.804
CRP mg/L, Median (IQR)	21.5 (6.6, 58.4)	33.1 (4.6, 101.7)	0.109
MPO-ANCA positive, n (%)	67(74.4)	37 (68.5)	0.806
MPO, Median (IQR)	53.1 (20.3, 116.0)	42.7 (17.9, 94.8)	0.138
PR3-ANCA positive, n (%)	10 (11.1%)	6 (11.8%)	0.906
PR3, Median (IQR)	1.5 (1.1, 2.8)	1.3 (1.1, 2.0)	0.382
BVAS, Mean ± std.	16.6 ± 3.7	15.8 ± 4.2	0.175
VDI at admission, Median (IQR)	0 (0, 1)	0 (0, 1)	0.117
AKI stage, n (%)			0.154
Stage 1	24 (26.7)	19 (37.3)	
Stage 2	33 (36.7)	11 (21.6)	
Stage 3	33 (36.7)	21 (41.2)	
Treatments, n (%)			
I.V. Pulse Methylprednisolone	58 (64.4)	26 (51.0)	0.117
Plasma exchange	3 (3.3)	2 (3.9)	0.856
Immunosuppressants, n (%)			
CTX	42 (46.7)	26 (51.0)	0.622
MMF	42 (40.0)	16 (27.5)	0.093
Renal biopsy, n (%)	64 (71.1)	32 (62.7)	0.496
Status at 3-month, n (%)			
Withdrew dialysis	14 (15.6)	5 (9.8)	**<0.001**
Dialysis	0 (0)	18 (35.3)	
Death	0 (0)	2 (2.1)	
Long-term outcome			
Death, n (%)	8 (8.9)	14 (27.5)	**0.007**
Survival time, month, Median (IQR)	56.5 (27.5, 82.0)	41.0 (24.5, 86.0)	0.480
Renal endpoint, n (%)	12 (13.3)	24 (47.1)	**<0.001**
Renal survival time, month, Median (IQR)	48.0 (26.2, 80.8)	24.0 (3.0, 39.0)	**<0.001**
Composite endpoint, n (%)	16 (17.8)	28 (55.0)	**<0.001**

Scr, serum creatinine; MPO, myeloperoxidase; BVAS, Birmingham Vasculitis Activity Score; CTX, cyclophosphamide; MMF, mycophenolate mofetil; VDI, vascular damage index. Bolded value: P-value < 0.05.

95%CI 1.326–8.035) were found to be the risk factors (**Table 4**). The risk factors (age, subgroup, BVAS) were used to construct the nomogram for predicting the renal outcome (**Figure 2B**, calibration plot in the **Supplementary Material**), by internal validation the c-index was 0.763.

DISCUSSION

Kidney involvement is common in patients with AAV. Although treatments for AAV have improved in the past decades, about 20–25% of patients progress into ESRD several years after diagnosis and need persistent dialysis or kidney transplantation (18, 19). Our study suggests that in AAV patients with AKI, both BVAS at admission and the level of kidney function recovery in three months can be independent predictors for ESRD and mortality, and the KDIGO AKI stage is a predictor for the poor renal outcome. The result provided potential indicators for both early identification of high-risk patients and prediction of long-term prognosis.

AKI stage is defined as the proportional rise of Scr and is classified according to the severity of kidney injury, and the severity of the renal injury is suggested to be related to long-term outcomes (20). In AAV patients, the KDIGO AKI stage also shows renal outcome predictive value. Of note, in the research, the AKI stage is not a predictor of long-term survival outcomes. One hypothesis is that AKI does not directly impact the mortality in AAV patients. Some researchers suggested that the intervention of AKI may not have a significant impact on mortality. For example, in situations including post-operation with low mortality risk or sepsis, though AKI is associated with

TABLE 4 | Cox model for long-term outcome.

Factors	Univariate model for renal survival			Multivariate model for renal survival			Univariate model for overall survival			Multivariate model for overall survival		
	RR	95%CI	P-value	RR	95%CI	P-value	RR	95%CI	P-value	RR	95%CI	P-value
G(G2)	6.156	2.920-12.977	**<0.001**	9.150	4.163-20.113	**<0.001**	3.286	1.378-7.835	0.007	3.264	1.326-8.035	**0.010**
BVAS	1.094	1.004-1.192	0.041	1.110	1.021-1.207	**0.014**	1.152	1.029-1.289	0.015	1.171	1.038-1.320	**0.010**
AKI1			0.057	/	/	0.038	/	/	0.101			
AKI2	0.909	0.347-2.380	0.846	1.711	0.639-4.582	0.286	0.932	0.249-3.489	0.916			
AKI3	2.116	0.923-4.951	0.077	3.116	1.278-7.598	**0.012**	2.434	0.791-7.490	0.121			
Age	1.007	0.980-1.035	0.604				1.062	1.021-1.104	0.003	1.046	1.008-1.086	**0.017**
Male	1.510	0.779-2.928	0.222				1.307	0.563-3.035	0.533			
24h urine protein	1.188	0.984-1.433	0.073				1.209	0.962-1.520	0.104			
Urine red blood cell	1.116	0.573-2.173	0.747				1.324	0.554-3.165	0.528			

BVAS, Birmingham Vasculitis Activity Score; AKI, acute kidney injury. Forward LR method was adapted in multivariate models.
Bolded value: P-value < 0.05.

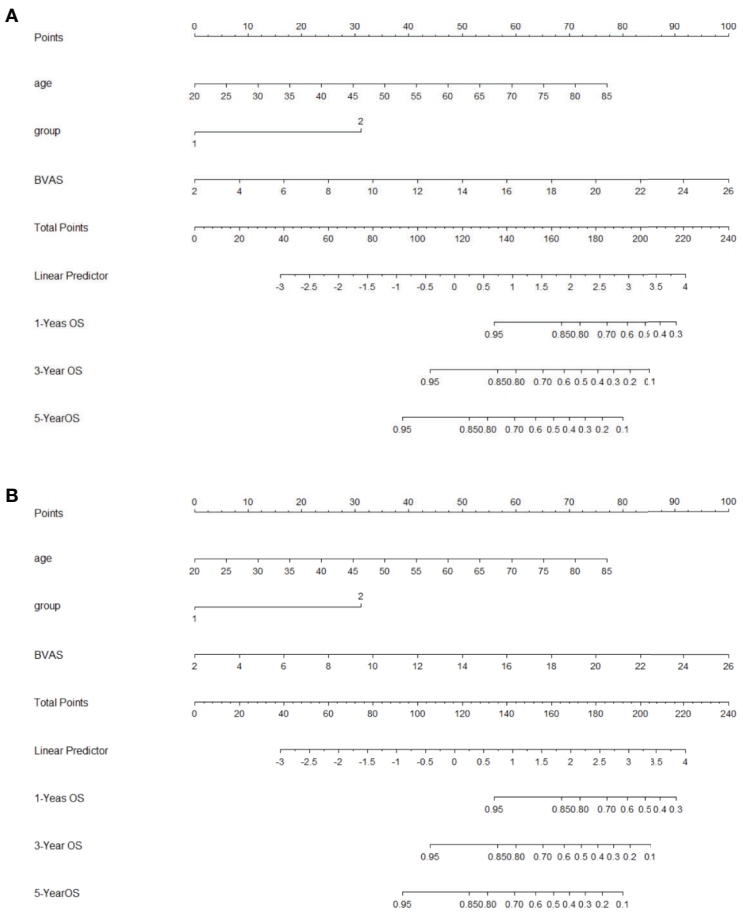

FIGURE 2 | Nomograms for outcomes **(A)**, nomogram for renal outcome; **(B)**, nomogram for survival outcome; To use the nomogram, based on patient's value, draw upward lines from each variable axis to the points, and calculate the sum of these points. And draw a downward line from total point axis to the survival axis to get the likelihood of 1-, 3-, 5-year survival).

higher mortality, it is not sure to what degree of AKI works as a marker for unrecognized risk of mortality (15).

In this study, several features showed significant differences among different AKI groups. BVAS score and CRP level were significantly higher in AKI stage 3 patients, suggesting that acute exacerbation was related to disease activity and inflammation. Though without statistically significant difference in pairwise comparison, the mean BVAS score of AKI stage 1 was higher

than stage 2. The BVAS score shows systemic active injury, however, the activity of injury may vary in different tissues or organs. Further analysis found that pulmonary hemorrhage was more frequently seen in stage 1 patients (supporting material). So, the activity in organs outside the kidney may contribute to the relatively high BVAS score. Decreased albumin level was also found in AKI stage 3. Generally, hypoalbuminemia can be caused by increased urine loss or increased consumption during inflammation or chronic disease. We showed that there was no difference in urine protein level among groups, while a higher CRP level was found in group AKI stage 3. We found that in these patients, serum albumin level was correlated with CRP level (**Supplementary Material**). So, the hyperinflammatory response may contribute to hypoalbuminemia. Previous studies have also found that high inflammation marker levels were related to lower serum albumin, and it is suggested that inflammation suppresses albumin synthesis (21). The presentation of crescent formation and tubular infiltration increased with the AKI stage. The proportion of cellular crescents showed a trend of increase but was not of statistical significance, and we infer that the significance of differences is limited by sample size. Previously, some studies have found correlations between histological features and patient outcomes. Former histopathologic studies classified ANCA-associated glomerulonephritis into four types: focal, crescentic, mixed, and sclerotic (22), the sclerotic type has the poorest outcome, followed by the crescentic type (23). Brix et al. developed a renal risk score system based on tubular atrophy, interstitial fibrosis, the proportion of normal glomeruli, and eGFR at diagnosis (24). Another study found that the renal risk score which combines histologic and laboratory features performs better in predicting the renal outcome (25).

BVAS at admission were independent risk factors of ESRD or mortality. It is used to evaluate disease activity while the Vasculitis Damage Index (VDI) evaluates long-term organ damage (12). Our finding consists of previous studies that disease activity is associated with outcomes (26).

Besides the AKI stage and BVAS at diagnosis, the recovery of kidney function at 3 months showed the potential of predicting renal and survival outcomes. This indicates assessment at 3 months may also provide important information about prognosis. In clinical studies of AKI, kidney recovery is an important short-term (60–90 d) endpoint, and if kidney damage lasts for 3 months, it and can be classified into CKD. In AAV, Gopaluni et al. indicated that disease activity 3 months after diagnosis may predict long-term outcomes (26). Our study showed that after acute exacerbation of kidney function, early follow-ups are also helpful for clinical management. Comparing to a single measurement of serum creatinine, the fold change of creatine level may better reflect the dynamic process of post-AKI recovery or damage. A single measurement of Scr cannot reflect the real-time kidney function, and the level of serum creatinine can be influenced by acute diseases, muscle mass. Some researchers presumed that there is a new baseline in patients recovered from AKI that lower than the original one, and recovery within a certain range may suggest a sustaining renal injury (15). Though how short or medium-term outcomes can be translated into long-term outcomes is not fully understood, our research indicated that the recovery of serum creatinine at 3 months can be a predictive factor of long-term outcomes.

Interestingly, the level of urine red blood cells at admission is significantly higher in G1 than in G2. We performed univariate and multivariate models, but the level of urine red blood cells did not show association with renal (P = 0.747, RR = 1.116, 95%CI 0.573–2.173) or survival outcome ((P = 0.528, RR = 1.324, 95%CI 0.554–3.165). The reason for this phenomenon is not clear. Furthermore, in the cohort, the level of urine red blood cell is not associated with long-term outcomes. Knowledge about the role of hematuria in ANCA-associated vasculitis is limited and more studies are needed (27).

There some limitations to this study. This study is a single-center retrospective study, and the number of patients with GPA or EGPA is limited. Also, in this retrospective cohort, BVAS score and VDI score ex-renal activity and damages at follow-ups of more than 50% of patients were irretrievable, so only renal outcomes and survival outcomes of patients are analyzed. We also acknowledge that due to the limitation of retrospective study, treatment differences between individuals exist. This may mask the relationship between some clinical features and long-term outcomes. However, on the whole, there were no significant differences in therapies used among different AKI stages or between different groups (**Tables 1**, **3**). We hope our conclusion can be validated in prospective multi-center cohorts.

CONCLUSION

KDIGO AKI stage3 is the risk factor for ESRD in AAV patients with AKI, while the BVAS score and level of kidney function recovery at 90 days are the independent risk factors for ESRD and all-cause mortality, and is of predictive value.

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 Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XH contributed to the conception of the study, analysis, and manuscript preparation, performed the data analyses, and wrote the manuscript. LC contributed to resource provision, maintenance of data, and performed the data analyses. LL and PR contributed to resource provision and maintenance of data. AN, YM, YW, and YZ helped perform the analysis with constructive discussions. JC and

FH contributed to commentary and revision of the manuscript. FH also contributed to funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.641655/full#supplementary-material

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Emerging Cellular Therapies for Anti-myeloperoxidase Vasculitis and Other Autoimmune Diseases

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Anti-myeloperoxidase vasculitis (MPO-AAV) is a life-threatening autoimmune disease which causes severe inflammation of small blood vessels, mainly in the kidney. As for many other autoimmune diseases, current treatments, which consist of general immunosuppressants, are partially effective, toxic and broadly immunosuppressive, causing significant and serious adverse effects in many patients. Therefore, there is an urgent need for more targeted and less harmful therapies. Tolerogenic dendritic cells, regulatory T cells and stem cells have emerged as attractive, new and safer options for the treatment for various autoimmune diseases due to their unique and selective immunosuppressive capacity. In this review, we will discuss how these cellular therapies offer potential to become novel and safer treatments for MPO-AAV.

Keywords: vasculitis, glomerulonephritis, myeloperoxidase, tolerogenic dendritic cells, regulatory T cells, stem cells

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INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a severe condition which causes inflammation and damage of small blood vessels. It is caused by autoimmunity against neutrophil proteins, mainly myeloperoxidase (MPO) and proteinase-3 (PR3). AAV consists of microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA), and it has an annual incidence of 20/million (1, 2). MPO-ANCA are found in the majority of MPA and a smaller proportion of GPA and EGPA patients, while PR3-ANCA are found predominantly in GPA (3).

Although MPO-AAV and PR3-AAV have many similarities, they are now considered to be different diseases based on their epidemiology, genetics, etiology, immunopathology and clinical features. MPO-AAV predominates in southern Europe and Asia-Pacific, is genetically weakly associated with HLA-DQ, mainly occurs as a single event, and vasculitis is mostly limited to the kidney (1, 4). In contrast, PR3-AAV is more commonly found in the northern hemisphere, is associated with HLA-DP, has a higher rate of relapse, and organs other than the kidneys such as the lungs are also affected (1, 4).

As in many other human autoimmune diseases, the aim of conventional therapies in AAV has been to damage the immune system in general to attenuate the autoimmunity-induced inflammatory injury to organs expressing the target autoantigens. The development of biological

therapies, mainly monoclonal antibodies, has allowed more accurate selection and targeting of key components of pathways which mediate auto immunopathogenesis. These are more "precise" than conventional therapies in having fewer off target injurious effects on the immune system.

However, the ultimate goal for many autoimmune diseases, including AAV, is to restore tolerance (unresponsiveness) towards the disease-causing autoantigen in a way that would turn off only the injurious autoimmune response, without adversely affecting host immune defense and causing other major side effects. Although we now have tools which can restore tolerance in an antigen-specific manner, antigen-specific immunosuppression causing disease reversal is nearly impossible to achieve in many human autoimmune diseases, but is likely to be realistic in AAV, particularly MPO-AAV, due to the reasons explained below. For a biological treatment strategy to deliver successful antigen-specific restoration of tolerance, there are several essential components:

i. The diagnosis of a particular disease must be possible before the immune injury has induced irreversible damage to the target organ. In many human autoimmune diseases such as autoimmune thyroiditis and type 1 diabetes (T1D), this is not possible.
ii. The major human disease-causing autoantigen needs to be known. This is not the case in many diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and type 1 diabetes (T1D).
iii. In diseases such as Multiple Sclerosis (MS), epitope spreading makes antigen-specific inhibition complex and difficult to achieve.
iv. There must be thorough and precise knowledge of the key pathways and the essential components of the immune pathogenesis of the disease.
v. Animal models with similar autoimmune target, immune pathogenic pathways and clinical outcomes need to exist or can be developed so that pre-clinical studies can be used to provide proof-of-concept that safe precise biological therapies can be taken on to clinical trials.

MPO-AAV is an autoimmune disease where all these criteria are met. Although patients often present with significant target organ (kidney) damage, at the time of diagnosis, the majority have sufficient kidney reserve to live a long life without the need for transplantation or dialysis once effective therapy is introduced. The major disease-causing autoantigen (MPO) is known, there is no evidence of epitope spreading occurring, and relevant animal models have been developed that have helped define the immunopathogenesis of the human disease and identify new therapeutic targets. On the other hand, although humanized mice have emerged as a promising tool to develop successful *in vivo* models of PR3-AAV (5), murine models of this disease have been difficult to induce (6). This is most likely because PR3 is not detected on mouse neutrophils and is therefore not accessible to anti-PR3 antibodies (1). Hence, this review will focus mainly on MPO-AAV. However, the potential cellular therapies discussed below may be also applied to PR3-AAV and later tested in relevant animal models.

Autologous *ex vivo*-generated regulatory T cells (Tregs) and autoantigen-loaded tolerogenic dendritic cells (DCs) are an appealing tool for the treatment of autoimmune diseases, including MPO-AAV, because they can provide safe, antigen-specific immunosuppression, without posing any risk of rejection. Alternative to inducing MPO-specific immunosuppression, other attractive cell therapies such as stem cells would desirably suppress anti-MPO autoimmunity and vasculitis without being rejected or producing major adverse effects. This review will discuss emerging evidence which suggests that such cellular therapies may offer a safer, effective therapeutic option for MPO-AAV. **Figure 1** illustrates how these therapies could be used in this disease, with their main advantages and disadvantages summarized in **Table 1**.

MPO-AAV

MPO-AAV is caused by autoimmunity to MPO, an abundant protein found inside our most common immune cell, neutrophil (3, 7). This disease causes severe inflammation and destruction of small blood vessels, leading to significant morbidity and mortality. It equally affects men and women, mainly over the age of 50, but it can also affect young adults and children (1). MPO-AAV is associated with the presence of serum anti-MPO antibodies, known as anti-neutrophil cytoplasmic antibodies or MPO-ANCA. Although the kidney bares the brunt of autoimmune injury, the major autoantigen, MPO, is not normally expressed in the kidney. MPO is, however, the major protein present in the granules of neutrophils (8). Evidence from relevant human and mouse studies shows that circulating MPO-ANCA target activated, MPO-exposing neutrophils, which subsequently lodge in glomeruli and deposit the autoantigen there (9–16). It also shows that both MPO-ANCA and MPO-specific CD4 T cells vitally contribute to the development of glomerular injury in MPO-AAV (9–16).

Animal models have provided a great deal of knowledge about the immunobiology of MPO-AAV. A few different animal models of MPO-AAV exist, including an antibody transfer mouse model in which injury is mediated by passively-transferred MPO-ANCA, transplanting irradiated, MPO-immunized MPO-deficient mice with wildtype bone marrow and immunizing WKY rats with MPO. They all closely resemble human disease, immunologically and pathologically, and have been thoroughly reviewed elsewhere (17). In one model, wildtype mice are immunized with MPO to induce active MPO-specific autoimmunity, including MPO-ANCA and MPO-specific T cells (16, 18–21). They are then administered either MPO-ANCA or low dose anti-glomerular basement membrane (GBM) globulin which cause neutrophils to deposit MPO in glomeruli for subsequent recognition by infiltrating MPO-specific T cells. In line with data from MPO-AAV patients, CD4 (13, 22) and CD8 T cells (22, 23), neutrophils

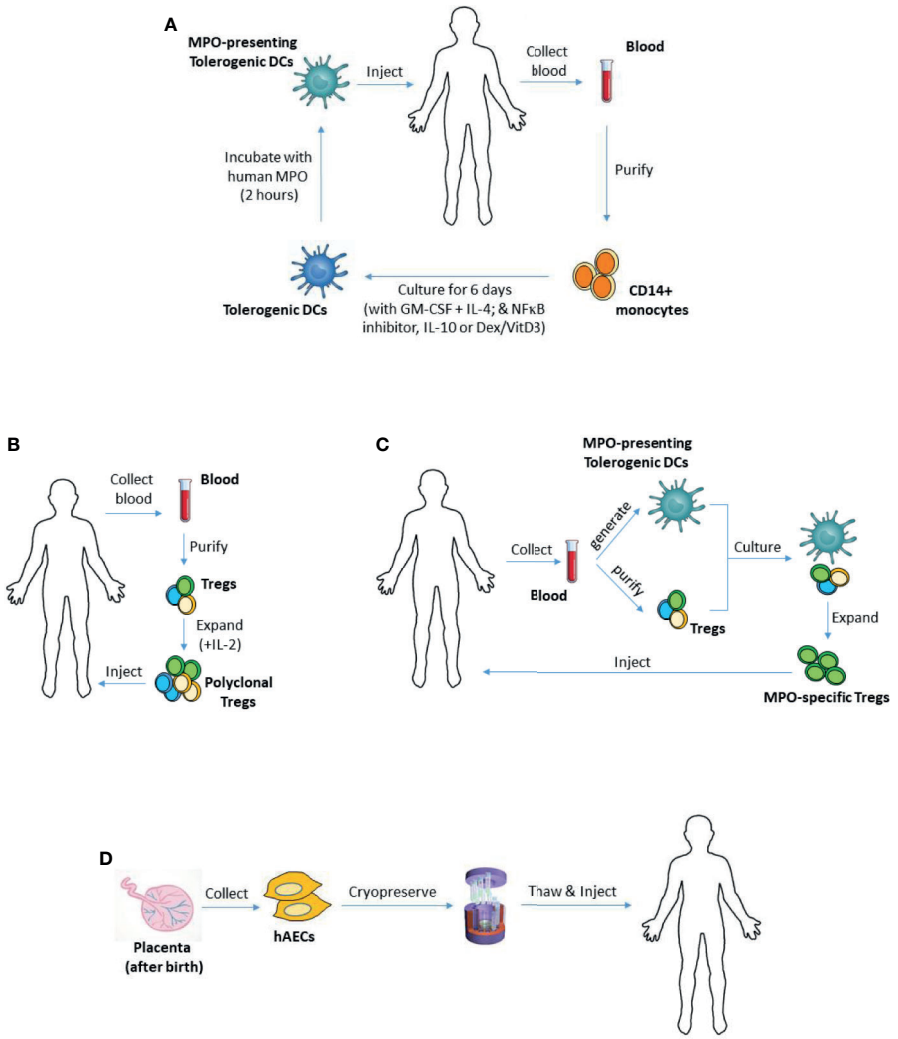

FIGURE 1 | Using tolerogenic DCs, Tregs or hAECs as cellular therapies in MPO-AAV. **(A)** To generate monocyte-derived DCs, CD14+ monocytes can be purified from patient's blood and cultured in the presence of GM-CSF and IL-4. Different types of tolerogenic DCs can be generated by adding various anti-inflammatory mediators to the culture including IL-10, Dex/VitD3 or inhibitors of NFκB. To make the DCs present MPO, they are then pulsed with purified human MPO, and injected back into the patient. **(B)** To expand polyclonal Tregs *ex vivo*, patient's Tregs ($CD4^+CD25^+CD127^{-/low}$) can be isolated from their blood, then cultured for several weeks in the presence of IL-2. **(C)** Patient's MPO-presenting tolerogenic DCs and Tregs, generated and purified as in **(A, B)** respectively, could be co-cultured to expand MPO-specific Tregs. Such antigen-specific Tregs are expected to have superior suppressive capacity compared with polyclonal Tregs. **(D)** hAECs are isolated from the amniotic membrane of the placenta after birth and cryopreserved as primary (non-cultured) cells. They can be given to MPO-AAV patients when needed. DCs, dendritic cells; Tregs, regulatory T cells; hAECs, human amniotic epithelial cells; MPO, myeloperoxidase; AAV, ANCA-associated vasculitis; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin 4; Dex, dexamethasone; VitD3, vitamin D3; NFκB, nuclear factor kappa B.

and macrophages (13, 22, 24), IL-17A (19, 25) and IFNγ (26, 27) promote injury in this model, while CD4+foxp3+ regulatory T cells (Tregs) are inhibitory (28–30). Here, our group has also defined the disease-causing immunodominant MPO T cell peptide (16), which is strikingly similar to the immunodominant human MPO peptide (16, 20). Hence, studies of MPO-specific immunomodulation in this model are very relevant to human disease.

As for many other autoimmune diseases, current treatments for MPO-AAV are only partially effective, but are harmful and non-specific, thus causing significant serious side effects in many patients which lead to considerable complications and death. Decades after their introduction, the first-line therapy for induction of remission still consists of high-dose corticosteroids and cyclophosphamide (1). These treatments induce remission in 70-90% of patients, but the incidence of dialysis or death at 5 years is still high (30%) (3, 31, 32). 1 in 3 patients also relapse while being treated (1, 31). The main problem with these therapies is that they are highly toxic and broadly immunosuppressive. Cyclophosphamide increases the risk of infection, cancer and infertility, while corticosteroids cause cardiovascular problems, diabetes, depression, anxiety,

TABLE 1 | Advantages and disadvantages of different cellular therapies for human MPO-AAV.

Cellular therapy	Advantages	Disadvantages
Antigen-loaded tolerogenic DCs	- antigen-specific - no risk of rejection (using patients' own *ex vivo*-modified cells) - abundant DCs can be generated from patients' PBMCs - expensive genetic engineering methods not required	- cost related to cell isolation and *in-vitro* culture - long-term stability and survival after transfer needs to be optimized - migratory capacity can be impaired with certain tolerogenic treatments
Polyclonal Tregs	- expensive genetic engineering methods not required - no risk of rejection (using patients' own *ex vivo*-expanded cells)	- not antigen-specific - less suppressive than antigen-specific Tregs - reduced stability during *in vitro* expansion due to the loss of foxp3 - cost related to cell isolation and *in vitro* culture
Antigen-specific Tregs	- antigen-specific - no risk of rejection (using patients' own *ex vivo*-modified cells) - more suppressive than polyclonal Tregs	- some approaches use expensive genetic engineering methods (e.g. specific TCR-Tregs and CAR-Tregs) - difficulty to generate specific TCR-Tregs due to the immunodominant peptide of human MPO being unknown - CAR-Tregs may become systemically hyper-activated due to widespread expression of MPO - cost related to cell isolation and *in vitro* culture
hAECs	- low immunogenicity and risk of rejection - low risk of causing tumors - offer protection against infection, cancer and cardiovascular disease - unique immunosuppressive capacity - isolation from the amnion after birth is non-invasive, easy, fast, relatively cheap and ethical - plentiful cells are isolated from an abundant source (placenta)	- not antigen-specific, thus pose a risk of more broadly suppressing immunity - low long-term survival after administration

insomnia, bone loss and gastric ulcers, as well as an increased risk of infection (32, 33). The rate of infection can be potentially reduced by lowering cumulative doses of corticosteroids or replacing them with avacopan (complement C5a receptor inhibitor) (34, 35). Rituximab (B cell-depleting monoclonal antibody) has been approved for use instead of cyclophosphamide, but it induces similar rates of infections, mainly due to reduced numbers of B cells, hypogammaglobulinemia and late-onset neutropenia (32, 36). Of all patient deaths in AAV, an alarming 70% are caused by treatment-related side effects, mostly infections (31). Another common downside of these therapies, including past and currently-ongoing clinical trials in ANCA vasculitis (1), is that none of them specifically target anti-MPO autoimmunity to avoid complications due to their off-target, non-antigen-specific effects. There is an urgent need for safer, more-targeted effective therapies.

TOLEROGENIC DCs

DCs are specialized immune cells and most-potent antigen-presenting cells which vitally control adaptive immunity. After activation, they upregulate MHC-II, costimulatory molecules (e.g. CD40, CD80/86) and pro-inflammatory cytokines (e.g. IL-12, TNF). In this context, they present antigen to T cells *via* MHC-II to induce protective antigen-specific T cell immunity against pathogens. In contrast, DCs are also critical to the maintenance of peripheral tolerance by presenting self-antigens in an immature or semi-mature state, thus causing T cell hyporesponsiveness, as shown by studies in which DC depletion during steady-state resulted in fatal autoimmunity (37, 38). Tolerogenic DCs are found throughout the body including mucosal surfaces where they promote airway and oral tolerance and unresponsiveness towards commensal microbiota (37). They generally express high levels of anti-inflammatory (e.g. PD-L1, IL-10, TGFβ) and low levels of pro-inflammatory mediators (e.g. CD40, CD80/86, IL-12) (37).

Tolerogenic DCs can be also made *ex vivo* by modification with various anti-inflammatory agents including IL-10, Dexamethasone (Dex; glucocorticoid), VitaminD3 (VitD), inhibitors of NFκB (one of their major pro-inflammatory pathways) and tools such as anti-sense oligonucleotides (oligos) which can inhibit gene expression of molecules critical for T cell activation like CD80, CD86 and CD40 (39–46). These DCs use various molecules to inhibit pathogenic T cells (e.g. PD-L1, IL-10, TGFβ), and can turn them off by promoting apoptosis, and by inducing endogenous inhibitory cells such as CD4+foxp3+ Tregs, type 1 regulatory cells (Tr1; inhibitory CD4+foxp3-) and regulatory B cells (Bregs) (39, 40, 47). However, mechanisms of immunosuppression are context and disease-dependent and vary between differently-modified DCs which exhibit different phenotypes.

Various antigen-loaded tolerogenic DCs, including CD40-deficient DCs, DCs treated with Dexamethasone (Dex)/Vitamin D (VitD) or NFκB inhibitors (e.g. BAY-11-7082), have rapidly, in 2-3 days, induced potent antigen-specific immunosuppression and attenuated organ damage in models of allergy, transplantation and autoimmune diseases (e.g. RA, MS, T1D), while generally remaining very stable after transfer (41, 48–51). These DCs have induced long-lasting immunosuppression, although they themselves have persisted for only a few weeks in recipients (52). Due to their stability and capacity to provide antigen-specific immunosuppression, as shown in rodents and non-human primates (41, 48–51), autologous *ex vivo*-derived

tolerogenic DCs have emerged as an excellent therapeutic candidate for the treatment of autoimmune diseases. Their clinical use in autoimmunity is now a reality, with 5 phase I trials completed in RA, T1D, MS, neuromyelitis optica (NMO) and Crohn's disease (53–57). In these clinical studies, patients' own *ex vivo*-modified tolerogenic DCs (including BAY, Dex/VitD and CD40/80/86 anti-sense oligo-treated DCs), which were biologically active, were shown to be safe and well-tolerated, without producing any major side effects for 12 months. Furthermore, it was shown that tolerogenic DC therapy produced anti-inflammatory and immunomodulatory effects in these trials, with some positive clinical outcomes. For example, in RA, the DCs decreased effector T cells and their ability to produce IL-6, increased the ratio of Tregs: effector T cells, reduced serum levels of pro-inflammatory cytokines and chemokines and diminished the disease activity score (53). In T1D, tolerogenic DCs increased the frequency of peripheral Bregs (54), while in MS/NMO, they upregulated IL-10 production by PBMCs and reduced memory CD8 T cells (57). In Crohn's disease, tolerogenic DCs reduced the disease activity index, with 1 patient reaching clinical remission and 2 a positive clinical response, and disease lesions markedly improving in 3 out of 9 patients (55). These studies demonstrate the feasibility and safety of this therapy in humans, with promising clinical outcomes which need to be further investigated in future trials. The optimum and most effective treatment regimen still needs to be determined for each autoimmune condition including DC dose, route and frequency of administration, long-term stability and survival, and the type of tolerogenic DC to be used. It also needs to be ensured that these cells can migrate to lymphoid organs to be able to exert their effects since many anti-inflammatory agents used to make tolerogenic DCs can inhibit their migratory capacity.

Recently, we demonstrated that administration of *ex vivo*-generated antigen-presenting tolerogenic DCs can induce selective, MPO-specific immunosuppression and attenuate vasculitis in mice (58). Bone marrow-derived tolerogenic DCs were generated by treatment with an NFκB inhibitor (BAY-11-7082) and pulsed with mouse MPO. These MPO-presenting inhibitory DCs were then given to mice with established anti-MPO autoimmunity. The DCs significantly decreased vasculitis and MPO-specific immunity, including effector CD4 T cell activation, proliferation, survival and pro-inflammatory cytokine production, as well as CD8 T cell and B cell responses. In line with suppressing anti-MPO immunity, MPO/BAY DCs upregulated Treg expression of inhibitory mediators including foxp3, CTLA-4, TNFR2 and IL-10, without affecting Tr1 or Bregs. Studies in Treg-depleted mice showed that the inhibitory effects of MPO/BAY DCs on anti-MPO autoimmunity were dependent on Tregs. Subsequent adoptive transfer/antibody blockade experiments showed that MPO/BAY DC-induced Tregs suppressed anti-MPO immunity and vasculitis *via* IL-10. Further *in vitro* DC : Treg co-culture experiments, supported by *in vivo* antibody blockade studies, showed that MPO/BAY DCs induced IL-10+ Tregs *via* the ICOS/ICOS-ligand pathway.

Importantly, the above-described inhibitory effects of MPO/BAY DCs on anti-MPO immunity were MPO-specific, since the same DCs did not induce Tregs or suppress immunity against an irrelevant antigen. However, in line with augmenting Th2 responses, MPO/BAY DCs did increase circulating IgE levels in recipient mice, indicating that they may adversely exacerbate allergy, but further studies are needed to test that assertion.

Overall, these pre-clinical, proof-of-concept studies demonstrated that MPO-presenting tolerogenic DCs may be a potential MPO-specific therapy for MPO-AAV which deserve further exploration. Future studies will be needed to test other types of MPO-pulsed tolerogenic DCs in pre-clinical models of MPO-AAV because the effectiveness and precise mechanism of immunosuppression varies between different types of tolerogenic DCs which exhibit different phenotypes. This will allow the best DC candidate to be identified for further studies and progression to clinical trials in MPO-AAV. In addition, before such a cellular therapy could be clinically tested, it would be important to determine if patients' own MPO-presenting tolerogenic DCs could selectively turn off their anti-MPO T cell responses *ex vivo*.

TREGS

Tregs are a specialized inhibitory subset of CD4+ T cells characterized by expression of CD25 and foxp3, a transcription factor essential for their development, stability and suppressive capacity (59). In humans, including MPO-AAV patients, they are also CD127-low (60, 61). Tregs play a vital role as regulators of pathogenic immunity in various immune-mediated conditions such as allergy, transplantation and inflammation. They are well known to critically maintain peripheral tolerance by inhibiting pathogenic autoreactive T cells and enhancing the tolerogenic capacity of DCs in an antigen-specific manner (62, 63). Tregs can also inhibit immunity and subsequent inflammation leading to organ damage by suppressing other types of injurious immune cells such as neutrophils, macrophages and B cells (62). They provide immunosuppression by expressing various inhibitory mediators including IL-10, TGFβ, IL-35, CTLA-4 and TNFR2 (58, 64–66).

Dysregulation of Treg number and/or function has been associated with the development of several autoimmune diseases including systemic lupus erythematosus, RA, T1D and MS. Similarly, several studies have shown that the frequency and suppressive capacity of Tregs is significantly reduced and negatively correlates with disease in MPO-AAV patients (28, 30). The critical importance of endogenous Tregs as negative regulators of anti-MPO autoimmunity and vasculitis has been confirmed in experimental MPO-AAV. We have shown that Tregs not only inhibit the generation of anti-MPO autoimmunity (29), but that they also suppress established responses of MPO-specific CD4 T cells, CD8 T cells and B cells (58).

Due to their ability to provide antigen-specific immunosuppression, there has been a great deal of interest to

develop and use autologous *ex-vivo*-derived Tregs as a potential therapy for autoimmune diseases. Several studies have shown that such Tregs can attenuate pathogenic immunity and thus organ damage in models of transplantation and autoimmunity (67–69). Phase I trials in autoimmune conditions have demonstrated their feasibility and safety in humans (70, 71). However, many optimizations are still required before Tregs can be clinically used such as enhancing their stability and survival post transfer and determining optimum dose and frequency of administration.

Ex vivo-derived Tregs, which have been tested as cellular therapies in animal models and human trials, can be broadly categorized as either polyclonal or antigen-specific Tregs.

Polyclonal Tregs

Many protocols expand Tregs for several weeks with Treg growth factors such as IL-2, without antigen-stimulation (64). This generates polyclonal Tregs of broad specificities. Such Tregs have attenuated immune-mediated organ damage in models of autoimmune conditions and shown relative safety and some efficacy in human trials (e.g. T1D) (70, 71). However, polyclonal Tregs are much less suppressive than their antigen-specific counterparts (72) and they become less stable and suppressive even before administration due to their progressive loss of foxp3 expression during *in vitro* expansion (73). Therefore, although not ideal, autologous *ex vivo*-expanded polyclonal Tregs could be trialled as a treatment in MPO-AAV patients, but their therapeutic efficacy needs to be first tested in pre-clinical models of the disease.

Antigen-Specific Tregs

Antigen-specific Tregs are a lot more attractive and promising due to their specificity and superior suppressive capacity. They can be generated in different ways to produce Tregs with an antigen-specific T cell receptor (TCR), chimeric antigen receptor (CAR) Tregs or DC-induced Tregs.

Antigen-Specific TCR Tregs

Tregs can be transduced in order to express a high-affinity TCR specific for the autoantigen of interest. Such genetically-engineered Tregs have been effective in models of autoimmune diseases such as T1D (68). Some of the biggest challenges in generating autoantigen-specific TCR Tregs for the treatment of autoimmunity have been the lack of knowledge of the disease-causing autoantigen and its immunodominant epitope(s) in most conditions including SLE and RA, as well as antigen and epitope shifting in others such as T1D and MS.

In MPO-AAV, the autoantigen (MPO) is known. Our group has also defined the T cell specific MPO immunodominant epitope in a murine model of the disease, which interestingly shows striking homology with the human MPO dominant peptide recognized by patients' pathogenic MPO-ANCA (16, 20). Hence, it may be possible to generate murine MPO-specific TCR and test them in pre-clinical models of MPO-AAV in the near future. The human T cell-specific MPO dominant epitope has not yet been identified, however recent studies suggest that there may be several such epitopes present within human MPO (74), thus making the generation of human MPO-specific TCR Tregs for the treatment of MPO-AAV patients more challenging.

CAR Tregs

There has also been a great deal of interest in developing CAR Tregs for the treatment of autoimmune conditions. CAR Tregs are genetically-engineered Tregs which contain an extracellular CAR molecule (autoantigen-binding antibody domain), a transmembrane region and an intracellular T cell signaling domain. CAR Tregs are able to migrate to the site of auto-inflammation where they get activated by their specific autoantigen (69). However, for this therapy to be effective, the autoantigen needs to be expressed only at the diseased site. If the autoantigen is also expressed elsewhere in the body, this could cause systemic over-activation of the Tregs, potentially leading to side effects associated with broader immunosuppression. The potential therapeutic efficacy and safety of CAR Tregs in MPO-AAV may be questionable. This is mainly due to the fact that MPO is not only expressed in the target organ (kidney), but that neutrophils, the major source of MPO, and to a lesser extent monocytes/macrophages, also release it into the extracellular space following cell activation in response to bacteria and other pathogens. MPO is also released from neutrophil precursors during its synthesis in the bone-marrow, so this autoantigen is always present in the circulation (8, 75).

DC-Induced Tregs

Antigen-specific Tregs can be expanded *ex vivo* from the polyclonal repertoire without genetic engineering by using antigen-presenting DCs. This approach has been largely employed in transplantation due to the knowledge of alloantigens (76, 77). However, it could be also applied in autoimmune conditions in which the autoantigen is known, including MPO-AAV. For example, *ex vivo* DC-induced/expanded Tregs were effective at reducing immunity and organ damage in a model of RA (78). DC-induced Tregs have increased suppressive capacity and stability due to the long-lasting, enhancing effects of DCs on Treg foxp3 expression and stability (76, 78).

We have recently shown that Tregs induced by MPO-presenting tolerogenic DCs can be used to inhibit established anti-MPO immunity and vasculitis in a pre-clinical model of MPO-AAV (58). In these studies, administration of DC-induced CD4+foxp3+ Tregs suppressed MPO-specific autoimmunity including CD4 T cells, CD8 T cells and B cells, and attenuated vasculitis. These proof-of-concept studies suggested that MPO-specific Tregs induced/expanded by MPO-presenting DCs may be a potential and feasible therapy for MPO-AAV. However, the long-term stability and effect of such Tregs needs to be explored in further studies.

Therefore, although many challenges still remain and the therapeutic efficacy of various types of Tregs needs to be tested in pre-clinical models of MPO-AAV, cell therapy utilizing patients' own *ex vivo*-modified Tregs, particularly antigen-specific ones, remains a promising potential treatment option for vasculitis patients worth further exploring.

STEM CELLS

Stem cells have also emerged as a promising therapeutic approach for the treatment of various inflammatory and autoimmune diseases due to their immunomodulatory ability. Several types of stem cells, including embryonic and mesenchymal stem cells (MSC), have attenuated organ damage in models of immune-mediated diseases and their safety and efficacy have been evaluated in clinical trials (79). One particular stem cell type, human amniotic epithelial cells (hAECs), have gained much attention in recent years as a treatment choice due to their safety and clinical applicability.

hAECs, which have pluripotent stem cell properties (80, 81), represent a novel, safe and affordable therapeutic option for MPO-AAV, for several reasons. They are isolated after birth from the amniotic which is attached to the placenta (82), therefore bypassing ethical barriers that normally occur with other (e.g. embryonic) stem cells. hAEC isolation involves non-invasive, easy, fast and low-cost procedures, resulting in an abundance of readily-available cells which are infused as primary, non-passaged cells (82). In contrast, other types of stem cells (e.g. mesenchymal and embryonic) have ethical issues regarding their isolation or have to be cultured for several weeks to generate enough infusible cells, thus significantly increasing the cost and potential for *in vitro* mal-transformation. hAECs have low immunogenicity because they do not express class IA antigens (HLA-A, HLA-B, HLA-C) or HLA-DR (class II), and are therefore not rejected upon transfer, nor do they form teratomas due to lacking telomerase, as shown in animals and humans (81, 83, 84).

Importantly, consistent with their role to protect the fetus from mother's immune system, hAECs, like other stem cells, are immunosuppressive. In fact, similar to MSC (85, 86), the protective effects of hAECs are largely due to their paracrine action and immunosuppressive capacity, rather than multilineage differentiation potential. They have suppressed pathogenic immunity by inhibiting effector T cells, altering macrophage polarisation toward the anti-inflammatory M2 phenotype, inhibiting neutrophils (87, 88) or by inducing other immunosuppressive cells such as Tregs and Bregs and as such they have attenuated organ damage in models of various inflammatory and autoimmune diseases including MS, autoimmune thyroiditis and uveitis, lung injury, liver fibrosis (89–94) and stroke (95). Similar inhibitory effects of hAECs on human T cells have been reported *in vitro*. For example, in co-culture experiments with human PBMCs or purified CD4 T cells, hAECs significantly decreased their proliferation and Th1/Th17 cytokine production (96, 97).

hAECs express a range of immunosuppressive mediators, including transforming growth factor beta (TGFβ), prostaglandin-E_2 (PGE_2) and the immunosuppressive HLA-G (81, 89), which they use to suppress immunity. However, which anti-inflammatory mediators are utilized and which immune cells are targeted by the hAECs depends on the model used. For example, in bleomycin-induced lung injury, hAECs attenuated disease by inducing Tregs *via* TGFβ (91). In a model of MS, hAECs suppressed lymphocyte proliferation *via* TGFβ and PGE_2 (89), but in those studies, hAEC-mediated suppression of disease was not associated with Treg induction. This shows that Tregs are required for hAEC-exerted effects in some, but not all, inflammatory models. Interestingly, but similar to MSC, IFNγ/TNF stimulation of hAECs enhances their production of suppressive molecules such as TGFβ (86, 91), suggesting that exposure to pro-inflammatory mediators found in many autoimmune and inflammatory conditions may further augment the inhibitory function of hAECs. Term hAECs are also more suppressive than those from pre-term donors (< 36 weeks gestation), possibly due to their increased expression of HLA-G (98), which is known to suppress T cell and neutrophil responses and induce Tregs (99, 100).

Recently, it was shown that hAECs can also mediate immunosuppression by producing exosomes (88, 101). Exosomes are released microvesicles (~50-100nm in diameter) which contain proteins, lipids and DNA/RNA with important roles in intercellular communication (102). They are released by many cell types and have been successfully used as a cell-free therapy in inflammatory conditions (103, 104). hAEC-derived exosomes inhibit various immune cells including T cells, macrophages and neutrophils *in vitro* and show protection in inflammatory models of acute lung injury and liver fibrosis (88, 101). Therefore, hAEC exosomes may represent a potential cell-derived, but cell-free, therapy in MPO-AAV.

Unlike the current AAV treatments, hAECs are unlikely to cause major side effects because they have anti-infection and anti-cancer properties. They produce various anti-microbial mediators including β-defensins and type I interferons in response to bacteria and viruses *in vitro* (105, 106). They inhibit cancers directly by inducing apoptosis of malignant cells and indirectly by enhancing anti-tumor immunity *in vivo* (107, 108). hAECs also protect against cardiovascular conditions, including stroke and myocardial infarction (95, 109).

hAECs have already entered the clinic. Amniotic membrane or hAECs have been safely used to treat eye injuries and promote wound healing for decades (84, 110). hAECs are also being tested in clinical trials as a therapy for other conditions. A phase I trial in babies with bronchopulmonary dysplasia has demonstrated their short and long-term feasibility and safety (111, 112), while another two phase I trials are underway in liver fibrosis (113) and stroke (114).

Therefore, stem cells such as hAECs represent a potentially safer, effective therapy for MPO-AAV due to their relatively harmless and immunomodulatory profile. Stem cells have never been tested as a treatment in this disease, but we are currently exploring the therapeutic efficacy of hAECs and their exosomes in pre-clinical models of MPO-AAV, which will pave the way for this therapy to be clinically tested in vasculitis patients.

CONCLUSIONS

Overall, although many obstacles still need to be overcome before tolerogenic cell therapy becomes a reality for vasculitis patients, autologous Tregs and *ex vivo*-derived MPO-loaded

tolerogenic DCs offer promise to be a feasible and successful antigen-specific treatment for MPO-AAV. This is because (i) Tregs and tolerogenic DCs have been generally shown to be stable, safe and well-tolerated in patients, and they can uniquely induce antigen-specific immunosuppression in various autoimmune conditions including experimental MPO-AAV, and (ii) MPO-AAV itself does not hold any caveats for effective antigen-specific restoration of tolerance. hAECs, if proven to be effective in pre-clinical models of MPO-AAV, may be even closer to clinical testing in MPO-AAV patients since they have already been extensively characterized, provide an off-the-shelf, abundant and relatively cheap therapeutic option, do not get rejected after transfer and clinical trials in other conditions have demonstrated their safety in humans. If successful, these cell therapies have the potential to change clinical practice in MPO-AAV and provide immense benefit to patients by decreasing their risk of death and complications from major adverse and non-antigen-specific effects which currently occur with the existing treatments.

AUTHOR CONTRIBUTIONS

DO searched the literature and wrote the manuscript. SH reviewed and edited the paper. All authors contributed to the article and approved the submitted version.

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