CLINICAL, MOLECULAR AND ADVERSE RESPONSES TO B CELL THERAPIES IN AUTOIMMUNE DISEASE

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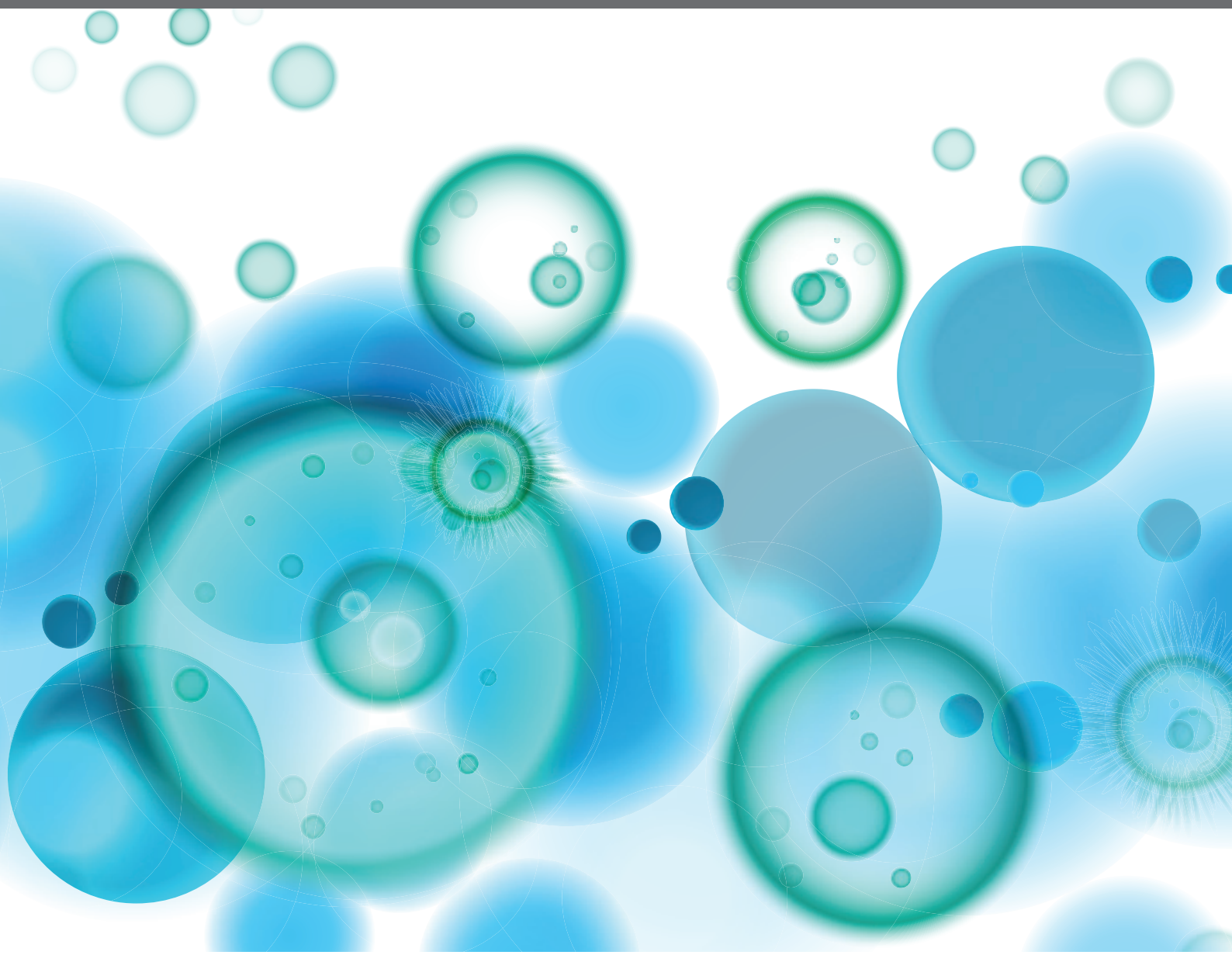

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CLINICAL, MOLECULAR AND ADVERSE RESPONSES TO B CELL THERAPIES IN AUTOIMMUNE DISEASE

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EDITORIAL
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Editorial: Clinical, Molecular and Adverse Responses to B-Cell Therapies in Autoimmune Disease

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Keywords: B-cell, rituximab, belimumab, hypogammaglobulinemia, autoimmunity, allergy, adverse, neutropenia

Editorial on the Research Topic

Clinical, Molecular and Adverse Responses to B Cell Therapies in Autoimmune Disease

In this Research Topic, we highlighted advances and addressed knowledge gaps in prediction, mechanisms and management of adverse events and efficacy of B-cell targeted therapies (BCTT) in autoimmune disease. BCTT were introduced in 1997 for treatment of lymphoma, and subsequently have become an important treatment option for a wide range of autoimmune diseases, particularly autoimmune rheumatic diseases (AIRD), including for the management of severe patients. BCTT include B-cell depleting drugs (BCDT) targeting CD20 (e.g. rituximab-RTX), CD22 (e.g. epratuzumab), and CD19 (e.g. MEDI-551); and drugs interfering with B-cell survival factors, such as belimumab. Indeed, the latter is one of only three new therapies for patients with systemic lupus erythematosus (SLE) or lupus nephritis (LN) to have received a license from the US Food and Drug Administration over the last 60 years. Several studies have tested the combination of different BCTT, e.g. RTX and belimumab. The BLISS-BELIEVE and CALIBRATE clinical trials reported negative efficacy results for add-on RTX compared with belimumab alone (SLE) or add-on belimumab compared with RTX and cyclophosphamide alone (LN), respectively. However, the BEATLupus study showed that add-on belimumab was superior over RTX alone in prolonging the time to severe SLE flare and in reducing anti-dsDNA antibody levels (1–3). Besides, as incomplete peripheral blood B-cell depletion might be associated with the inability to reduce tubulointerstitial lymphoid aggregates in the kidney and be responsible for inadequate response to treatment (4) a short-term intensified BCTT (5, 6) consisting of a combination therapy of RTX and cyclophosphamide given at sub-immunosuppressive doses aimed at potentiating the B cell depleting effects of RTX was developed and showed effective results even in the long term (without immunosuppressive maintenance therapies) (5, 6).

Clinical use of BCTT is expanding: the BCDT agent RTX is now approved in ANCA-associated vasculitis (AAV), rheumatoid arthritis (RA), and pemphigus, while ocrelizumab (relapsing-remitting and primary progressive) and ofatumumab (relapsing-remitting) are approved in multiple sclerosis. BCTT are used off-label in lupus, membranous nephropathy, Sjögren's syndrome and certain autoimmune neurological disorders. In this Research Topic, Wang et al.

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reported clinical benefit of lower dose RTX in 19/26 (73.1%) patients with severe autoimmune encephalitis, with add-on bortezomib (proteasome inhibitor targeting plasma cells) in the remaining refractory 7/26 patients. Walhelm et al. observed favourable results using bortezomib in a nationwide Swedish study of 12 patients with refractory SLE and/or lupus nephritis. Cui et al. described a case report of belimumab treatment for anti-SRP-associated immune-mediated necrotizing myopathy.

While evidence supporting BCDT efficacy in several autoimmune conditions is increasing, current monitoring of BCTT remains rudimentary, and there are major opportunities to develop predictive biomarkers and immunological monitoring for both efficacy and adverse events. From a *post-hoc* analysis of the major phase III belimumab SLE trials, Parodis et al. noted that early patterns in particular B-cell subsets following standard therapy with or without add-on belimumab might predict future SLE flares. Rapid memory B-cell (MBC) expansion may predict sustained treatment response when followed by a subsequent reduction, while no return or delayed MBC increase may predict disease flare. Arnold et al. proposed a personalized retreatment approach in AAV patients based on clinical assessment using the Birmingham Vasculitis Activity score or B-cell markers. They suggested that all BCTT-treated patients should receive concomitant oral immunosuppression, with further BCTT at 6 months in patients with incomplete clinical response or absent naïve B-cells. In pemphigus treated with RTX, Hebert et al. noted an increase in BAFF levels and BAFF-R on B-cells, in contrast to patients receiving corticosteroids alone, in whom BAFF-R was unchanged. Li et al. undertook a cluster analysis of B-cell subsets in IgG4-related disease, stratifying the patients into 3 subgroups: subgroup 1 with low MBC and normal Breg, subgroup 2 with high MBC and low Breg, and subgroup 3 with high plasmablasts and low naive B-cells. This has potential treatment implications as subgroup 2 and 3 patients were overall more treatment-resistant.

Initially, certain adverse effects of BCTT, such as hypogammaglobulinaemia appear to have been underestimated (7). This may have related to various factors, including the short duration and limited number of treatment cycles in early reports. Conversely, progressive multifocal leukoencephalopathy was perhaps over-estimated due to a number of early cases, and the severity of this condition. Studying adverse events cannot be approached in isolation. As we treat patients holistically, we recognize the need to study toxicity in the context of efficacy. Profound and prolonged B-cell depletion may induce clinical remission, but result in sustained hypogammaglobulinaemia in a proportion of patients (8).

Other important adverse effects include neutropenia, hepatitis B reactivation, allergy/infusion reactions, serum sickness, human anti-chimeric antibody responses, and primary or secondary non-response (9–11). There is a clinical need to improve selection of patients being prescribed BCTT based on their likelihood to respond or experience specific adverse events. We need to understand the role of early intervention should such adverse events occur. Tieu et al. reported on a large prospective BCTT cohort of over 400 autoimmune disease patients (Jayne D, personal communication) with long-term follow-up in Cambridge, UK. Of 142 patients (101 AAV, 18 SLE, 23 other) developing persistent hypogammaglobulinemia, 29 (20.4%) required immunoglobulin replacement therapy (IGRT), with consequent reduction in infection risk. In contrast, an Austrian study of 144 autoimmune renal disease patients by Odler et al. reported hypogammaglobulinemia in 58.5% of the patients, but this was not associated with serious infections (SI). Impaired renal function, lower BMI, nephritic glomerular disease treated with corticosteroids, were factors associated with SI. These contrasting conclusions with respect to clinical significance and infection risk may relate to several factors: underlying disease (risk appears higher in AAV); duration of follow-up; definition of hypogammaglobulinemia; cumulative dose of BCTT; cumulative dose and concomitant use of other immunosuppressive agents. This also illustrates the need to recognize that most BCTT-related hypogammaglobulinemia is minor/transient, but that in a significant minority, recurrent/severe infections and persistent hypogammaglobulinemia may justify IGRT (12).

Although RTX is the anti-CD20 agent for which most experience exists, there are several second and third-generation anti-CD20 agents which have been studied in autoimmune disease. Here, Kaegi et al. reported a systematic review of the efficacy and safety of these drugs, including obinutuzumab, ocrelizumab, ofatumumab, ublituximab, and veltuzumab. In a case series of phospholipase A2 receptor (PLA2-R)-associated membranous nephropathy, obinutuzumab showed promising results. Ofatumumab showed promising results in AAV, SLE, and RA, but mixed results in PLA2-R-associated membranous nephropathy.

Patients may be unable to tolerate BCTT due to infusion reactions, development of major allergic responses/anaphylaxis, induction of human anti-chimeric antibodies (HACA). This can lead to a clinical management quandary, for example if the patient's disease is responding well to the particular BCTT. Aun et al. reported successful desensitization of a multiple sclerosis patient who experienced an allergic reaction during the first infusion of ocrelizumab.

During the COVID-19 pandemic, it has emerged that autoimmune disease patients on immunosuppression may not respond optimally to COVID-19 vaccination – most clearly demonstrated for RTX treatment (13). Here, Stefanski et al. assessed COVID-19 vaccine responses in 15 AIRD patients treated with RTX. In vaccine responders, most B-cells were naïve and transitional, while the B-cell profile in non-responders included mainly plasmablasts and $CD27^{-}IgD^{-}$ double negative B-cells. The authors suggested that a significant repopulation of the naive B-cell compartment was positively associated while B-cell exhaustion markers (upregulation of CD95 and loss of CD21) were inversely associated with vaccine response (Stefanski et al.).

From the publications in this Research Topic, we thank the contributing authors for demonstrating the progress of BCTT use in autoimmune disease, with expansion regarding the range of diseases, choice of agents, and studies aiming at optimizing efficacy and safety. Future work will build on this progress, in order to attain multiple ambitions:

personalization of BCTT in autoimmune disease; identification of appropriate biomarkers; minimization of infectious complications; and prediction of patients at highest risk of specific side-effects.

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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Rituximab Associated Hypogammaglobulinemia in Autoimmune Disease

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Objective: To evaluate the characteristics of patients with autoimmune disease with hypogammaglobulinemia following rituximab (RTX) and describe their long-term outcomes, including those who commenced immunoglobulin replacement therapy.

Methods: Patients received RTX for autoimmune disease between 2003 and 2012 with immunoglobulin G (IgG) <7g/L were included in this retrospective series. Hypogammaglobulinemia was classified by nadir IgG subgroups of 5 to <7g/L (mild), 3 to <5g/L (moderate) and <3g/L (severe). Characteristics of patients were compared across subgroups and examined for factors associated with greater likelihood of long term hypogammaglobulinemia or immunoglobulin replacement.

Results: 142 patients were included; 101 (71%) had anti-neutrophil cytoplasm antibody (ANCA) associated vasculitis (AAV), 18 (13%) systemic lupus erythematosus (SLE) and 23 (16%) other conditions. Mean follow-up was 97.2 months from first RTX. Hypogammaglobulinemia continued to be identified during long-term follow-up. Median time to IgG <5g/L was 22.5 months. Greater likelihood of moderate hypogammaglobulinemia (IgG <5g/L) and/or use of immunoglobulin replacement therapy at 60 months was observed in patients with prior cyclophosphamide exposure (odds ratio (OR) 3.60 [95% confidence interval (CI) 1.03 – 12.53], glucocorticoid use at 12 months [OR 7.48 (95% CI 1.28 – 43.55], lower nadir IgG within 12 months of RTX commencement [OR 0.68 (95% CI 0.51 – 0.90)] and female sex [OR 8.57 (95% CI 2.07 – 35.43)]. Immunoglobulin replacement was commenced in 29/142 (20%) and associated with reduction in infection rates, but not severe infection rates.

Conclusion: Hypogammaglobulinemia continues to occur in long-term follow-up post-RTX. In patients with recurrent infections, immunoglobulin replacement reduced rates of non-severe infections.

Keywords: rituximab, hypogammaglobulinemia, autoimmune disease, immunoglobulin replacement therapy, B-cell

INTRODUCTION

B cell depletion plays a key role in the management of many autoimmune diseases. Rituximab (RTX) is licensed for use in rheumatoid arthritis (RA) and AAV, and clinical trials have evaluated RTX in other autoimmune conditions including SLE. Despite limited evidence of hypogammaglobulinemia in patients receiving RTX in these studies, it has been consistently identified in observational studies of patients with autoimmune disease (1–7).

Lower baseline immunoglobulin G (IgG) levels, including levels within population norms, have been associated with subsequent hypogammaglobulinemia (2, 4, 6, 7). An association between cumulative RTX exposure and hypogammaglobulinemia has not been demonstrated (2–4, 7).

Although there is no universal IgG threshold for hypogammaglobulinemia, the clinical significance of hypogammaglobulinemia lies in the resultant susceptibility to infection. Extrapolated from the treatment of patients with common variable immunodeficiency (CVID), prophylactic antibiotics and immunoglobulin replacement therapy are considered where there is a combination of hypogammaglobulinemia, poor vaccination responses, and recurrent and/or severe infection.

Although a proportion of patients with hypogammaglobulinemia have been identified following RTX therapy for autoimmune disease in several studies, their longer-term outcomes, including the effects of immunoglobulin replacement therapy, remain unclear.

In a previous study, from which this study cohort derives, 135/243 (56%) patients with systemic autoimmune disease treated with RTX developed hypogammaglobulinemia (4). This was classified as mild (5 to <7 g/L) in 72 (53%), moderate (3 to <5 g/L) in 53 (39%) and severe (<3 g/L) in 10 (7%). In this study, we sought to evaluate the long-term outcomes of patients with previously identified hypogammaglobulinemia.

OBJECTIVES

1. To explore the characteristics of patients with autoimmune disease who develop RTX associated hypogammaglobulinemia and their long-term outcomes.
2. To examine the outcomes of patients with autoimmune disease which develop RTX associated hypogammaglobulinemia requiring immunoglobulin replacement therapy.

METHODS

Patients with multi-system autoimmune disease who had received RTX between February 2003 and November 2012 and had an IgG <7 g/L on at least two occasions were included in this single center, retrospective cohort form the Vasculitis and Lupus Clinic, Addenbrooke's Hospital, Cambridge, United Kingdom. Data were collected until August 2017 or last recorded follow-up. A previous report from this cohort described immunoglobulin outcomes in 243 patients who had received RTX for the treatment of multi-system autoimmune disease up to November 2012 (4). This report includes extended follow-up of 142 patients who met the above inclusion criteria.

Patients received a standard departmental dose of 2x1g a fortnight apart followed by 1g every 6 months for 2 years. Extension of RTX course and shortened treatment regimens occurred when clinically appropriate. At the time of treatment for these patients, biosimilar products were not available. Clinical assessments and laboratory data were typically obtained 6-monthly, prior to each dose of RTX. Interval data, where available, were also collected.

Patients were excluded if paraproteinemia was detected at any time during follow-up. All immunoglobulin results during periods of nephrotic range proteinuria and for 3 months following plasma exchange were excluded from analyses. Patients were categorized by absolute nadir IgG levels, as mild (5 to <7 g/L), moderate (3 to <5 g/L), and severe (<3 g/L). Infection was defined as any presumed or confirmed infection warranting the use of an oral antimicrobial agent. Severe infection was defined as a presumed or confirmed infection requiring an intravenously administered antimicrobial and/or hospital admission.

Data collected on each patient included age at diagnosis, gender, disease diagnosis and manifestations, age, date, and indication for first RTX prescription, cumulative RTX dose, use of immunosuppressive agent(s) pre-RTX, concurrently and post-RTX, prednisolone use at RTX commencement, and at 6 monthly intervals until 24 months post-RTX, infections, mortality, antibiotic prophylaxis and use and duration of immunoglobulin replacement therapy (intravenous or subcutaneous). Prednisolone was the standard oral glucocorticoid prescribed, with equivalent efficacy to prednisone. Laboratory data were collected for each patient from 1 month prior to rituximab to last follow-up, including IgG, IgM and IgA levels, lymphocyte, and neutrophil counts, and CD19, CD4 and CD8 counts. Flow cytometry for lymphocyte subsets were not routine prior to every RTX infusion. Where available, B cell subsets and antibody titers to pneumococcal, haemophilus, varicella, measles, mumps, rubella, and tetanus were collected.

Concurrent immunosuppression was defined as the use of an immunosuppressive agent for at least 6 weeks from RTX commencement, except for cyclophosphamide where any use within the first 6 weeks was included. Post-RTX immunosuppression was defined as use of an immunosuppressive agent at least 6 weeks after RTX commencement, for at least 3 months.

In the setting of hypogammaglobulinemia, immunoglobulin replacement therapy was typically commenced in patients with recurrent and/or severe infections following specialist clinical immunology evaluation. This generally included the assessment of infection rates, and laboratory parameters including lymphocyte subsets and vaccine responses to *Streptococcus pneumoniae* and *Haemophilus influenzae*, and a trial of prophylactic antibiotics. Prophylactic antibiotic choice was individualized where possible; azithromycin was typically used if not available microbiological or antibiotic sensitivity data was available. Intravenous immunoglobulin replacement therapy was commenced, and patients transitioned to self-administered subcutaneous administration where appropriate.

Intravenous immunoglobulin was not used for treatment of underlying autoimmune disease in these patients.

In accordance with the UK National Health Service Research Ethics Committee guidelines, ethics approval was not required as this work comprises anonymous retrospective data and all treatment decisions were made prior to our evaluation.

Dichotomous outcomes are summarized as proportions. Continuous outcomes are summarized as mean and standard deviation if normally distributed, otherwise as median and interquartile range. Comparisons of categorical variables across the immunoglobulin categories were analyzed using Somers' D to assess for the trend across nadir IgG subgroups. Nominal categorical variables were compared using Chi squared tests or Fisher's exact test as appropriate. Continuous variables have been compared using Kruskall-Wallis tests. In patients receiving immunoglobulin replacement therapy, infection and severe infection rates were compared by Wilcoxon sign ranked tests. Nadir IgG in the first 12 months were used to examine outcome at 60 and 100 months following the first dose of RTX. A multivariable logistic regression model was used to model outcome (IgG <5g/L or on immunoglobulin replacement therapy) at 60 months. Prespecified explanatory variables were included using a step-wise approach. Model fit was assessed using -2log likelihood, Cox & Snell R square and Nagelkerke R square values. Statistical analyses were performed in SPSS version 24 and figures were produced using Graphpad prism version 7 and R (ggalluvial package).

RESULTS

Long-term clinical and immunoglobulin data were available for 142 patients with hypogammaglobulinemia. Mild hypogammaglobulinemia was recorded in 40/142 (28.2%), moderate in 66/142 (46.5%) and severe in 36/142 (25.4%) patients. Mean follow-up was 97.2 months; and was longer in lower nadir IgG subgroups (**Table 1**). Patients with more severe hypogammaglobulinemia were younger at diagnosis and first RTX (**Table 1**). AAV was the most common indication for RTX (71%) and most patients received RTX for the management of relapsing (25%) or refractory (69%) disease (**Table 1**). There was no difference in indication for RTX (new, relapsing, or refractory disease), or by diagnosis ($p = 0.27$, data not shown) by subgroup. Seventy one percent were female, with a greater proportion in patients with moderate and severe hypogammaglobulinemia (**Table 1**).

Immunosuppression and Development of Hypogammaglobulinemia

Exposure to mycophenolate mofetil prior to RTX was more common in patients with moderate or severe hypogammaglobulinemia (**Table 2**). Prednisolone use at 12 and 24 months following RTX commencement were associated with lower nadir IgG (**Table 2**). Cumulative RTX dose and prior exposure to other immunosuppressive agents were not associated with a lower nadir IgG (**Table 2**).

Immunoglobulin Levels Over Long-Term Follow-Up

Baseline values were often collected after commencement of glucocorticoids; mean IgG at baseline was 7.45 (standard deviation (SD) 3.1), mean baseline IgM was 0.8 (SD0.5) and mean baseline IgA was 1.6 (0.8).

Moderate (IgG <5 g/L) and severe (IgG <3 g/L) hypogammaglobulinemia and use of immunoglobulin replacement therapy was increasingly observed with longer follow-up (**Figure 1**). Median time to moderate hypogammaglobulinemia was 22.5 months [IQR 3.0 to 61.5] and to severe hypogammaglobulinemia was 24.5 months [IQR 4.0 to 80.8].

Of the patients who were followed up to 60 months post-RTX (n=124), substantial change was observed in IgG levels over

TABLE 1 | Patient characteristics.

	All (n = 142)	Mild (n = 40)	Moderate (n = 66)	Severe (n = 36)
Total follow-up (months)	97.2 ± 36.4	87.5 ± 33.7	95.7 ± 34.1	110.6 ± 40.1
Age (years)	45.2 ± 17.6	47.9 ± 17.7	47.6 ± 16.7	37.4 ± 17.2
Age at first RTX (years)	51.4 ± 16.5	55.8 ± 15.8	52.4 ± 15.2	44.2 ± 17.7
Disease duration (months)	43.1 [13.2 – 101.7]	63.2 [10.8 – 159.2]	31.7 [11.7 – 76.8]	56.0 [19.4 – 97.7]
Female	101/142 (71)	21/40 (53)	50/66 (76)	30/36 (83)
Diagnosis				
AAV	101/142 (71)	30/40 (75)	48/66 (73)	23/36 (64)
GPA	69/101 (68)	21/30 (70)	34/48 (71)	14/23 (61)
MPA	15/101 (15)	4/30 (13)	6/48 (13)	5/23 (22)
EGPA	17/101 (17)	5/30 (17)	8/48 (17)	4/23 (17)
SLE	18/142 (13)	5/40 (13)	6/66 (9)	7/36 (19)
Other*	23/142 (16)	5/40 (13)	12/66 (18)	6/36 (17)
Disease state				
New	8/140 (6)	1/39 (3)	5/66 (8)	2/35 (6)
Relapse	35/140 (25)	10/39 (26)	16/66 (24)	9/35 (26)
Refractory	97/140 (69)	28/39 (72)	45/66 (68)	24/35 (69)

Mild: nadir IgG 5 to < 7 g/L, Moderate: nadir IgG 3 to < 5 g/L, Severe: nadir IgG < 3 g/L.

**other: Undifferentiated connective tissue disorder (4), Neuromyelitis optica (3), Undifferentiated vasculitis (2), Behcet's syndrome (2), polychondritis (2), mixed connective tissue disease (2), IgA vasculitis (1), cryoglobulinemic vasculitis (1), polyartertitis nodosa (1), Cogan's syndrome (1), Takayasu arteritis (1), myasthenia gravis (1), cryoglobulinemic vasculitis (1).*

AAV, ANCA-associated vasculitis; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; SLE, systemic lupus erythematosus.

Mean ± standard deviation, median [interquartile range].

TABLE 2 | Use of immunosuppressive agents in patients with hypogammaglobulinemia.

	All (n = 142)	Mild (n = 40)	Moderate (n = 66)	Severe (n = 36)	p
Cumulative RTX (g)	9.0 ± 5.1	8.5 ± 4.7	9.8 ± 5.6	8.1 ± 4.4	0.23
Pre-RTX immunosuppression					
Cyclophosphamide	107/142 (75)	29/40 (73)	49/65 (75)	28/36 (78)	0.79
Cumulative cyclophosphamide dose (g)	12.0 [6.0 – 26.0]	12.0 [5.8 – 27.8]	11.5 [6.0 – 17.3]	11.0 [5.7 – 27.0]	0.91
Azathioprine	88/141 (62)	27/40 (68)	39/65 (60)	22/36 (61)	0.54
Mycophenolate mofetil	94/141 (67)	25/40 (63)	39/65 (60)	30/36 (83)	0.05
Methotrexate	36/141 (26)	10/40 (25)	20/65 (31)	6/36 (17)	0.42
Intravenous immunoglobulin	22/141 (16)	7/40 (18)	8/65 (12)	7/36 (19)	0.86
Plasma exchange	16/141 (11)	4/40 (10)	5/65 (8)	7/36 (19)	0.27
No. immunosuppressive medications	3.0 [2.0 – 4.0]	3.0 [2.0 – 3.0]	3.0 [2.0 – 3.0]	3.0 [2.0 – 4.0]	0.49
Concurrent immunosuppression					
Cyclophosphamide	25/141 (18)	6/40 (15)	13/66 (20)	6/35 (17)	0.77
Mycophenolate mofetil	21/141 (15)	5/40 (13)	9/66 (14)	7/35 (20)	0.39
Plasma exchange	10/141 (7)	4/40 (10)	3/66 (5)	3/35 (9)	0.79
Post-RTX immunosuppression					
Cyclophosphamide	20/142 (14)	6/40 (15)	8/66 (12)	6/36 (17)	0.87
Mycophenolate mofetil	27/142 (19)	5/40 (13)	14/66 (21)	8/36 (22)	0.25
No. immunosuppressive medications	0.0 [0.0 – 1.0]	0.5 [0.0 – 1.0]	0.0 [0.0 – 1.0]	1.0 [0.0 – 1.8]	0.44
Prednisolone					
Baseline	115/121 (95)	36/38 (95)	53/55 (96)	26/28 (93)	0.82
6 months	120/133 (90)	31/39 (79)	61/63 (97)	28/31 (90)	0.15
12 months	113/137 (82)	27/39 (69)	56/64 (88)	30/34 (88)	0.04
24 months	98/133 (74)	22/37 (59)	48/62 (77)	28/34 (82)	0.03

Mild: nadir IgG 5 to < 7 g/L, Moderate: nadir IgG 3 to < 5 g/L, Severe: nadir IgG < 3 g/L.
RTX, rituximab. Proportion (%), median [interquartile range].

time (**Figure 2**). In patients with moderate hypogammaglobulinemia within the first 12 months of RTX administration, 17/37 (45%) patients recovered to an IgG ≥5 g/L without the need for immunoglobulin replacement therapy at 60 months. A further 8/37 (22%) had commenced immunoglobulin replacement therapy, and the remaining 12/37 (32%) remained hypogammaglobulinemia with an IgG <5 g/L at 60 months.

In a multivariable logistic regression model, cyclophosphamide use prior to RTX, lower nadir IgG in the first 12 months, prednisolone use at 12 months following RTX, and female sex were associated with an increased likelihood of moderate hypogammaglobulinemia and/or requiring immunoglobulin replacement 60 months after RTX commencement. This model was additionally adjusted for age at RTX commencement, mycophenolate use prior to RTX and total cumulative RTX (**Table 3**).

Cumulative RTX dose was not associated with a greater likelihood of moderate/severe hypogammaglobulinemia or requiring immunoglobulin replacement therapy 60 months after RTX commencement. The inclusion of disease duration prior to RTX and number of immunosuppressive agents used post-RTX did not improve model fit or alter overall interpretation. A model inclusive of nadir IgM values within the first 12 months improved model fit, with no change in interpretation (**Table 3**).

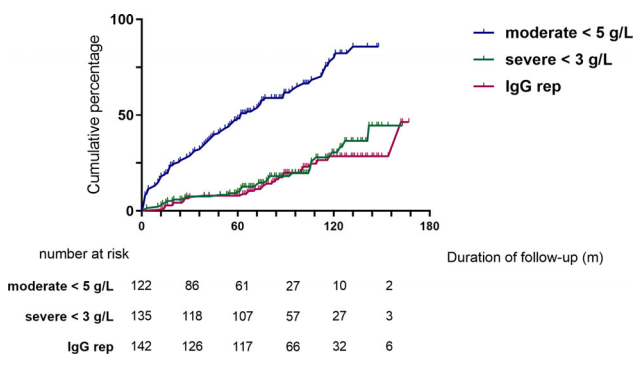

FIGURE 1 | Cumulative incidence of hypogammaglobulinemia and immunoglobulin replacement therapy commencement during follow-up.

Hypogammaglobulinemia and Infection

Overall, infection rates were low. Severe and non-severe infections predominantly involved the respiratory tract (65% and 58% respectively). There were no differences in infection rates between patients with mild, moderate, and severe hypogammaglobulinemia (**Figure 3A**). A subset of patients, however, were referred for further assessment and/or commenced prophylactic therapy due to recurrent infections.

Peripheral blood immunophenotyping was available in 30 patients at the time of Clinical Immunology assessment; CD19+ lymphocytes were detectable in 11 (37%). Where sufficient B cells were identified in 8 of these patients (7 with AAV and 1 with SLE), further B cell subset analyses were performed. This revealed a pattern of high naïve ($IgM^+IgD^+CD27^-$) and low switched memory ($IgM^-IgD^-CD27^+$) B cells in all patients (**Supplementary Table 1**).

Pneumococcal antibody titers were available in 28 patients with recurrent infection, with only 9 having protective antibody titers to at least 7 of the 13 serotypes tested. In those who went on

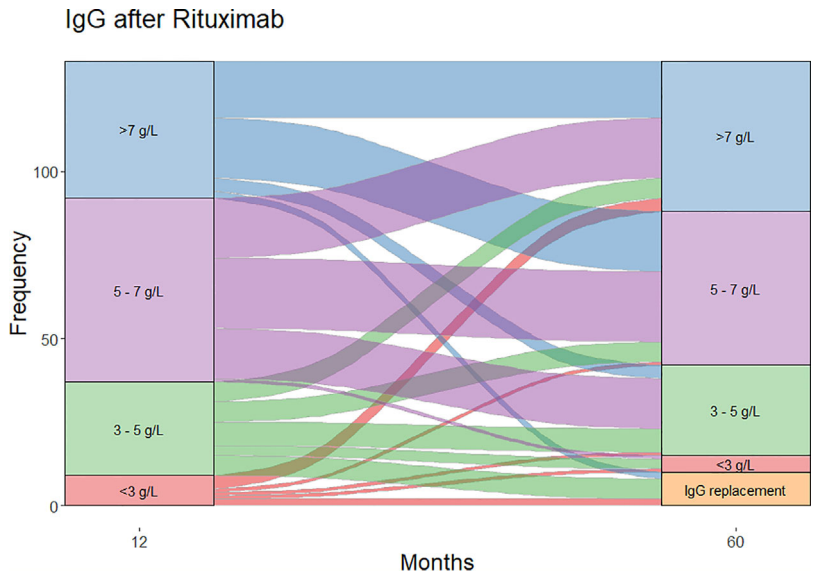

FIGURE 2 | Change in IgG strata between month 12 and month 60 of follow-up.

TABLE 3 | IgG < 5 g/L or immunoglobulin replacement at 60 months.

	Model 1 OR (95% CI)	p- value	Model 2 OR (95% CI)	p-value
Age at RTX commencement	0.98 (0.95 – 1.01)	0.21	0.97 (0.94 – 1.01)	0.10
Female	7.56 (1.88 – 30.48)	0.004	8.57 (2.07 – 35.43)	0.008
Pre-RTX cyclophosphamide	3.31 (1.00 – 10.96)	0.05	3.60 (1.03 – 12.53)	0.04
Pre-RTX mycophenolate	2.16 (0.75 – 6.26)	0.16	2.04 (0.70 – 5.95)	0.20
Nadir IgG (0 – 12 m)	0.67 (0.50 – 0.90)	0.008	0.68 (0.51 – 0.90)	0.008
Prednisolone use at 12 m	6.19 (1.12 – 33.31)	0.03	7.48 (1.28 – 43.55)	0.03
Total cumulative RTX	0.91 (0.81 – 1.02)	0.09	0.91 (0.81 – 1.02)	0.11
Nadir IgM (0 – 12 m)	–		0.12 (0.01 – 1.05)	0.06

RTX, rituximab; Ig immunoglobulin; m, month, OR, odds ratio, CI, confidence interval.

to receive immunoglobulin replacement therapy, only 4 of 18 patients tested (22%) had protective pneumococcal antibody levels, and a post-vaccination response was demonstrated in only 1/9 (11%) recorded.

Antibiotic Prophylaxis and Immunoglobulin Replacement Therapy

Antibiotic prophylaxis was initiated in 53 (37%) of patients; greater antibiotic prophylaxis use was observed in patients with moderate and severe hypogammaglobulinemia (**Figure 3B**). Of the patients who commenced antibiotic prophylaxis, 42 (79%) were AAV patients, 6 (11%) had SLE and 5 (9%) other autoimmune conditions. Immunoglobulin replacement therapy was initiated in 27/53 (51%) patients who had commenced antibiotic prophylaxis.

Immunoglobulin replacement therapy was commenced in 29 patients; with mild hypogammaglobulinemia in 1 (3%) patient, moderate hypogammaglobulinemia in 9 patients (31%) and severe hypogammaglobulinemia in 19 patients (66%). Of the patients commencing immunoglobulin replacement therapy, 21 (72%) had a diagnosis of AAV, 4 (14%) SLE and 4 (14%) other autoimmune diseases. Immunoglobulin replacement therapy was commenced a median of 71 months after first RTX. In patients commencing immunoglobulin replacement therapy, infections reduced (median [IQR] 1.02 infections/year [0.54 – 1.88] to 0.13 infections/year [0.00 – 0.35], p <0.001, **Figure 3C**). Annual severe infection rates were not reduced during immunoglobulin replacement therapy in these patients. After removal of two outliers with recurrent respiratory tract infections requiring antibiotics, there remained no difference in severe infection rates.

At the time of data collection or last recorded follow-up, 20 of 29 patients were continuing to receive immunoglobulin replacement therapy, 4 had died and 5 had ceased immunoglobulin replacement therapy. Of the four who died, the causes of death were respiratory sepsis in a patient with AAV, decompensated liver disease and pneumonia in a patient with IgA vasculitis, refractory vasculitis in a patient with AAV and was unknown in a patient with AAV. Of the five who had ceased immunoglobulin replacement therapy, 2 were intolerant and 3 were weaned off immunoglobulin replacement therapy without recurrent infection; 1 subsequently recommenced immunoglobulin replacement therapy owing to recurrent infection,

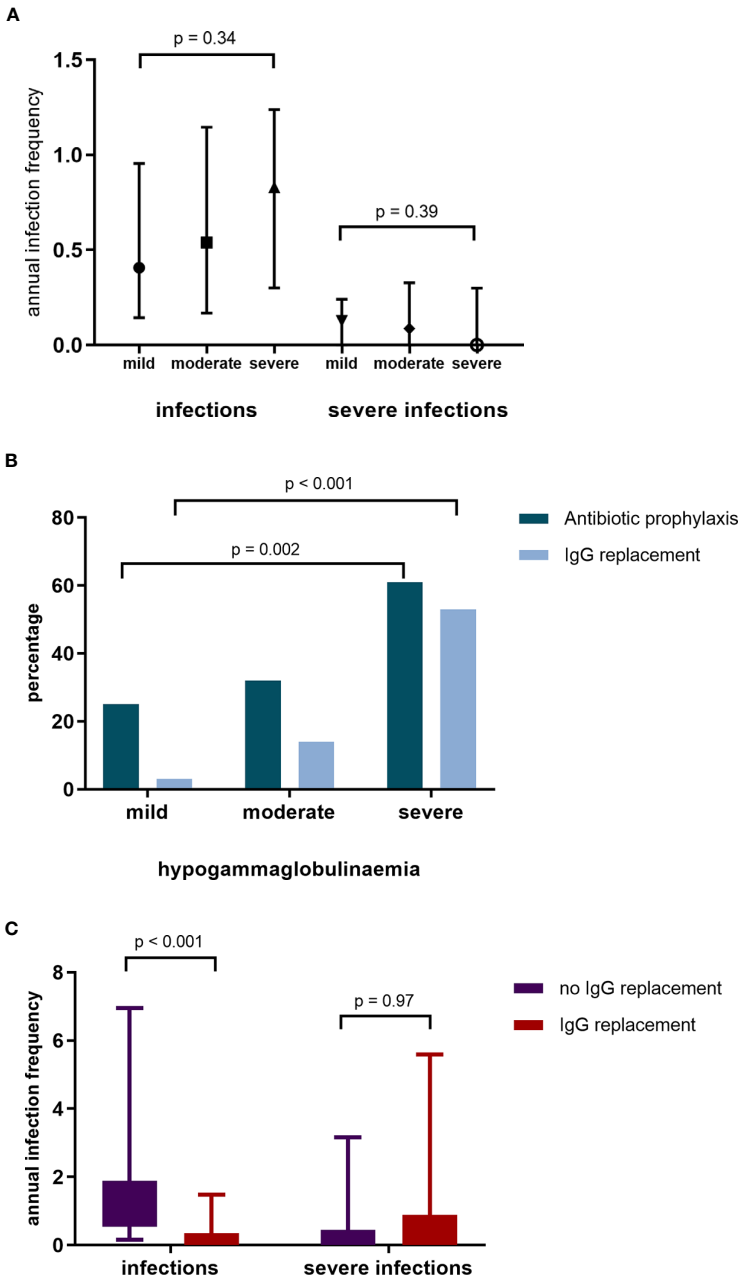

FIGURE 3 | **(A)** Annual infection and severe infection rate by IgG subgroup. **(B)** Commencement of antibiotic (Abx) prophylaxis and IgG replacement by IgG group. **(C)** Infection and severe infection rates in patients without IgG replacement and during IgG replacement.

1 has had IgG recovery to normal levels (>7 g/L), and 3 have remained off immunoglobulin replacement therapy with stable IgG levels <5 g/L.

DISCUSSION

We report on 142 patients with multi-system autoimmune disease with RTX associated hypogammaglobulinemia, their long-term outcomes and response to immunoglobulin replacement therapy. Overall, 102/142 (72%) had moderate hypogammaglobulinemia and 36/142 patients (25%) severe hypogammaglobulinemia. Factors associated with lower nadir IgG levels were prior mycophenolate use and prednisolone use 12 and 24 months after RTX initiation. Prior cyclophosphamide, prednisolone at 12 months after RTX initiation, nadir IgG in the first 12 months of RTX commencement and female sex were associated with an increased likelihood of moderate/severe hypogammaglobulinemia

and/or immunoglobulin replacement therapy use 60 months post-RTX commencement. Antibiotic prophylaxis was used in 53/142 (37%) patients and immunoglobulin replacement therapy commenced in 29/142 (20%) in whom infection rates but not severe infection rates were reduced.

The majority of patients included in this study had refractory SLE and AAV. There is substantial consistent evidence that RTX is beneficial in patients with AAV in both induction and maintenance of remission (8–10). Although data for RTX in SLE has been mixed, observational studies have demonstrated benefit (11, 12). Although hypogammaglobulinemia has been identified in multiple observational studies, the occurrence of hypogammaglobulinemia in this cohort is higher than previous estimates (7, 13–16). This cohort had a longer duration of follow-up, with nadir IgG levels occurring many months or years after commencing RTX therapy. Mean follow-up was 8 years, compared with up to an average follow-up of 4 years in other studies (2, 7, 13–18).

The rate of hypogammaglobulinemia may also be influenced by diagnosis. Although most patients in this study had AAV, other studies of hypogammaglobulinemia have included greater proportions of patients with RA (not included in this study) and SLE (13% of this cohort) (1, 16, 18). Thiel and colleagues have demonstrated delayed B cell recovery following RTX in patients with AAV compared with RA and SLE, suggesting a distinct underlying or acquired B cell dysfunction in these patients (19).

Notably, cumulative RTX doses are higher in this study than other reports (6, 7, 15, 17). This is likely influenced by multiple factors including the duration of follow-up and high proportion of patients with longstanding relapsing or refractory disease in this cohort. An association between cumulative RTX and hypogammaglobulinemia has previously been postulated (6), but not identified in other studies (2, 4, 7). In this study, there was no difference in cumulative RTX dose across the subgroups and was not associated with greater likelihood of moderate/severe hypogammaglobulinemia or requiring immunoglobulin replacement therapy at 60 months in an adjusted logistic regression model.

The impact of other immunosuppressive agents used prior to, in conjunction with or after RTX in the development of hypogammaglobulinemia has been difficult to delineate. Of note, mean baseline immunoglobulin levels were low-normal at baseline. In this study, mycophenolate and cyclophosphamide were the most common non-glucocorticoid immunosuppressive agents used. In the multivariable logistic regression model accounting for age, sex and prednisolone use post-RTX, prior cyclophosphamide, but not mycophenolate use increased the likelihood of moderate or severe hypogammaglobulinemia 60 months after RTX initiation. Venhoff and colleagues observed prolonged B cell depletion in patients who received RTX after previous cyclophosphamide use compared with RTX alone (20). In this study, 54% of patients who had received prior cyclophosphamide developed hypogammaglobulinemia, compared with 21% who received RTX alone.

Glucocorticoids alone have also been implicated in the development of hypogammaglobulinemia, and the impact of prolonged or greater glucocorticoid use in conjunction with RTX or other immunosuppressive agents on immunoglobulin levels requires further study (21). In this cohort, prednisolone use at 12 and 24 months were associated with lower nadir immunoglobulin levels. In the multivariable model examining outcomes at 5 years, prednisolone use at 12 months was associated with increased likelihood of moderate or severe hypogammaglobulinemia and/or immunoglobulin replacement. The use of prednisolone at 12 months was observed in 82.5% of patients, reflective of clinical practice in patients with historically more difficult to control, longstanding disease. Ongoing efforts to minimize glucocorticoid exposure remain important to the chronic management of these patients.

Of interest, there were more female patients were more likely to have more likely to have moderate/severe hypogammaglobulinemia and/or have commenced immunoglobulin replacement therapy at 60 months. Cross sectional studies suggest that immunoglobulin levels decline with age, with limited differences between males and females in adult age ranges (22–24). In post-hoc analyses of a trial evaluating induction therapy in AAV, female patients receiving RTX had higher serum RTX levels compared with males despite using body surface area dosing (25). Importantly, however, although higher serum levels of RTX were associated with a longer time to B cell repopulation, this was not associated with fewer relapses up to 18 months of follow-up. This association requires further assessment in larger cohorts and could have implications for dosing based on sex if confirmed.

Infections remain the key concern in patients with hypogammaglobulinemia. In a mixed cohort of patients receiving RTX for cancer (77.7%) and rheumatologic conditions (27.7%), severe infection rates were greater in patients with hypogammaglobulinemia (26). This was observed in early follow-up 12 months after RTX by MD Yusof and colleagues who in a mixed cohort of patients with autoimmune rheumatic diseases, identified an increased likelihood of severe infections in patients with hypogammaglobulinemia (16).

The use of immunoglobulin replacement therapy in patients with hypogammaglobulinemia associated with immunosuppression is extrapolated from experience in the management of the heterogenous group of patients with CVID. Both groups share a predisposition to infection, hypogammaglobulinemia, and impaired vaccination responses. In CVID, a reduction in respiratory tract infections has been demonstrated in small cohorts after commencement of immunoglobulin replacement therapy (27–29). The efficacy of immunoglobulin replacement therapy in patients with hypogammaglobulinemia and hematological malignancies has also been demonstrated in small cohorts (30). We observed a reduction in infection rates after initiation of immunoglobulin replacement therapy, supporting the efficacy of immunoglobulin replacement therapy in this population of patients with systemic autoimmune disease.

Importantly, despite the reduction in infections requiring antimicrobial therapy, the same benefit was not observed for severe infections. The majority of severe infections in these patients were respiratory tract infections; in this patient

population, disease related airways damage and colonization of the respiratory tract commonly contribute to chronic and recurrent infections, which may not be mitigated by immunoglobulin replacement. Age and other comorbidities may additionally influence infections in this cohort of patients with refractory and relapsing disease.

Given the patient and health care burdens of ongoing immunoglobulin replacement therapy, and increasing concerns regarding supply of this limited resource, trials of immunoglobulin replacement therapy cessation are considered. However, the most appropriate approach to this remains unknown. Recovery of immunoglobulin levels was observed in several individuals in longer term follow-up. In this single center study, of the 29 patients who commenced immunoglobulin replacement therapy, it was successfully ceased in 4 of the 5 patients in whom this was attempted. Although a very limited experience is presented in this study, it highlights the possibility of cessation of immunoglobulin replacement.

Again, albeit in small numbers, the pattern of high naïve and low switched memory B cells observed in a subset of these patients with hypogammaglobulinemia despite B cell repopulation warrants further investigation. Although a possible treatment effect, this could be representative of an associated underlying B cell dysfunction, which has been suggested in the associations between CVID and autoimmunity (31, 32).

Limitations of this study include the retrospective design, introducing selection bias in choice of treatments and total doses. Long-term follow-up in patients who have difficult to control rare autoimmune disease has inherent challenges. Though missing data, particularly for infection and severe infections, which were often not culture proven, is an important limitation, this group of patients typically have close clinical review focusing on infections, an important contributor to morbidity in this group of patients. The lack of control group for comparisons of infection and severe infection rates is a limitation to this study. Some studies have drawn comparisons between cyclophosphamide and rituximab treated patients. As refractory disease or disease relapse are common in long term follow-up, overlap of medications are common, and limit comparisons between groups.

In this study evaluating long-term outcomes of patients with RTX associated hypogammaglobulinemia, we have observed clinically significant hypogammaglobulinemia in a high proportion of patients, and an increasing incidence of hypogammaglobulinemia over time. The rates observed highlight the need for ongoing immunoglobulin monitoring in patients who have previously or continue to receive RTX. The use of prior immunosuppressive therapies, prolonged glucocorticoid use and female gender were associated with hypogammaglobulinemia long-term. Additionally, a reduction in infection in those receiving immunoglobulin replacement therapy for recurrent infection, provides evidence of its efficacy in this population of immunodeficient individuals. The risks and consequences of hypogammaglobulinemia should be considered with RTX therapy in multi-system autoimmune disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

In accordance with the UK National Health Service Research Ethics Committee guidelines, ethics approval was not required as this work comprises anonymous retrospective data and all treatment decisions were made prior to our evaluation.

AUTHOR CONTRIBUTIONS

JT, RS, DK, and DJ contributed to conception and design of the study. JT and SG extracted data. JT performed the statistical analysis. All authors contributed to interpretation of results. JT wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.671503/full#supplementary-material

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Modifications of the BAFF/BAFF-Receptor Axis in Patients With Pemphigus Treated With Rituximab Versus Standard Corticosteroid Regimen

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The efficacy of the B-cell-depleting agent rituximab has been reported in immune diseases but relapses are frequent, suggesting the need for repeated infusions. The B-cell activating factor (BAFF) is an important factor for B cell survival, class switch recombination and selection of autoreactive B cells, as well as maintaining long-lived plasma cells. It has been hypothesized that relapses after rituximab might be due to the increase of serum BAFF levels. From the Ritux3 trial, we showed that baseline serum BAFF levels were higher in pemphigus patients than in healthy donors (308 ± 13 pg/mL versus 252 ± 28 pg/mL, p=0.037) and in patients with early relapse compared who didn't (368 ± 92 vs 297 ± 118 pg/mL, p=0.036). Rituximab and high doses of CS alone have different effects on the BAFF/BAFF-R axis. Rituximab led to an increase of BAFF levels associated to a decreased mRNA (Day 0: 12.3 ± 7.6 AU vs Month 36: 3.3 ± 4.3 AU, p=0.01) and mean fluorescence intensity of BAFF-R in non-autoreactive (Day 0: 3232 vs Month 36: 1527, mean difference: 1705, 95%CI: 624 to 2786; p=0.002) as well as on reappearing autoreactive DSG-specific B cells (Day 0: 3873 vs Month 36: 2688, mean difference: 1185, 95%CI: -380 to 2750; p=0.20). Starting high doses of corticosteroids allowed a transitory decrease of serum BAFF levels that re-increased after doses tapering whereas it did not modify BAFF-R expression in autoreactive and non-autoreactive B cells. Our results suggest that the activation of autoreactive B cells at the onset of pemphigus is likely to be related to the presence of high BAFF serum levels and that the decreased BAFF-R expression after rituximab might be responsible for the delayed generation of memory B cells, resulting in a rather long period of mild pemphigus activity after rituximab therapy. Conversely, the incomplete B cell depletion and persistent BAFF-R expression associated with high BAFF serum levels might explain the high number of relapses in patients treated with CS alone.

Keywords: Pemphigus, BAFF - B-cell activating factor, BAFF-receptor, rituximab, Corticosteroïd

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INTRODUCTION

Pemphigus is, in most cases, mediated by anti-desmoglein (DSG) 1 and anti-DSG3 autoantibodies (1, 2). The B-cell activating factor (BAFF) is an important factor for B cell survival, class switch recombination, selection of autoreactive B cells and maintaining long-lived plasma cells (3, 4). The BAFF-receptor (BAFF-R), expressed on the majority of B cells, is the key receptor involved in promoting B cell survival (5). In mice, overexpression of BAFF results in the development of autoimmune manifestations (6). In human, a correlation between BAFF levels and disease severity has been shown in several auto-immune diseases (7–9), leading to belimumab (an anti-BAFF-R monoclonal antibody) approval in systemic lupus erythematosus (SLE) by the Food and Drug Administration. Anti-BAFF-R monoclonal antibodies (mAb) are currently being tested in pemphigus.

The Ritux3 trial showed that first-line treatment with rituximab (RTX), an anti-CD20 mAb, combined with a short-term regimen of corticosteroids (CS) was more effective and safer than a standard regimen of long-term CS (10). Despite a rather low (12 of 46) relapse rate in the RTX group compared to the CS group (20/44), most of relapses (9/12) occurred quite early in the RTX group during the first 12 months after the start of treatment, whereas relapses in the CS group occurred more regularly during patients' follow-up (7 during the 1st year and 13 during the 2nd year after the start of treatment). A high relapse rate has been consistently reported in the literature in patients with pemphigus who received only one cycle of rituximab (11–14), as well as in other auto-immune diseases. It has been hypothesized that some relapses after rituximab might be due to the increase of serum BAFF levels, which could promote the recovery of auto-reactive B cells (4, 15).

We used samples collected from patients treated with RTX or oral CS alone in the Ritux 3 trial to longitudinally assess the BAFF/BAFF-R axis. For this, we studied: i) serum BAFF levels, ii) BAFF-R mRNA in bulked total B cells and one-cell sorted autoreactive B cells, and iii) BAFF-R phenotypic expression in both treatment groups.

METHODS

Clinical Trial

Ninety newly-diagnosed pemphigus patients were randomly assigned to receive a standard regimen of CS versus RTX associated with a short-term regimen of CS. Patients in the RTX group were treated with the autoimmune regimen (two infusions of 1000 mg of RTX at Day 0 and Day 15) and a maintenance treatment corresponding to two infusions of RTX of 500 mg at Month 12 and Month 18. They also received an initial dose of prednisone of 0.5 to 1 mg/kg/day, depending on initial pemphigus severity (moderate versus severe), which was rapidly tapered over 3 to 6 months. Patients assigned to the standard oral CS group were given an initial dose of prednisone of 1 to 1.5 mg/kg/day, with a progressive tapering over 12 to 18 months, depending on initial pemphigus severity.

Patients

Blood samples from RTX-treated patients were analyzed before (Day 0) and 36 months after the initial rituximab infusion (Month 36), after recovery of B lymphocytes. Blood samples from CS-treated patients were analyzed before (Day 0), 12 months (Month 12) and 24 months (Month 24) after the start of CS therapy in order to perform biological analyses in patients still receiving CS treatment. The pemphigus disease area index (PDAI) score was used to assess pemphigus severity.

We performed a sequential analysis of serum BAFF levels by enzyme-linked immunosorbent assay (ELISA) in 88 patients (45 treated with RTX, 43 treated with CS), and 37 healthy donors (HD). We analyzed the transcriptomic and cytometric profiles of BAFF-R expression in cell sorted autoreactive DSG+ B lymphocytes, and in DSG- whole B lymphocytes from 10 patients before and after treatment with RTX or CS, as well as in 7 HD.

Desmoglein-Specific B-Cell Staining

In order to analyse DSG1 and DSG3 specific B cells, peripheral blood mononuclear cells (PBMCs) were extracted from venous blood using Ficoll-Hypaque (Lymphoprep™, Oslo, Norway). Then, B cells were isolated using Dynabeads Untouched Human B-cells kit (Invitrogen™, Carlsbad, USA) according to manufacturer's instructions. Then, purified B cells were incubated for 30 minutes at 4°C with histidine-tagged recombinant DSG1 or DSG3 (30 ng/µl).

Flow Cytometry Analysis

First, cells were incubated with Fc Blocking Reagent (eBioscience) prior to staining. For live cell analyses, dead cells were excluded by staining with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies). Then, the phenotype of B cells was determined by flow cytometry with anti-human antibodies directed against IgG (BD Biosciences), CD19, CD27, IgM, BAFF-R and TACI (BD Biosciences). Anti-histidine coupled with phycoerythrin (R&D Systems) was used to identify DSG-specific B cells. Data were collected by FACS ARIA III (BD Biosciences) and analyzed with FlowJo software 10 (TreeStar).

One-Cell Sorting and Pre-Amplification

DSG-specific single B cells were sorted by FACS ARIA III into 96-well plates containing 10 µL Platinum Taq polymerase and SuperScript III reverse transcriptase (Invitrogen), a mixture of Taqman primer-probes at 0.2×concentration specific for the transcripts of interest and CellsDirect qRT-PCR buffer (Invitrogen). Immediately following cell sorting, samples were centrifuged, incubated at 55°C for 10 minutes, and subjected to 20 cycles of Polymerase Chain Reaction (PCR) (50°C for 15 minutes then 95°C for 15 seconds for the reverse transcription, followed by 20 cycles of 95°C for 15 seconds and 60°C for 4 minutes for amplification). Subsequent pre-amplified single-cell cDNA was stored at −20°C until analysis.

Real-Time Quantitative Polymerase Chain Reaction

After ¼ dilution in TE buffer, each cDNA sample was then separated into 48 separate reactions for further quantitative Polymerase Chain Reaction (qPCR) using the BioMark 48.48 dynamic array nanofluidic chip (Fluidigm, Inc.). Briefly, following hydraulic chip priming, 48 pre-amplified cDNA samples were mixed with a mild detergent loading solution to allow capillary flow, and the samples were added

to a 48.48 nanofluidic chip (Fluidigm, Inc.) along with 38 individual Taqman primer-probe mixtures listed in along with 38 other individual Taqman primer-probe mixtures (Applied Biosystems) specific for individual transcripts of interest. The chip was then thermocycled through 40 cycles and fluorescence in the FAM channel was detected using a CCD camera placed above the chip, normalized by ROX (6-carboxy-X-rhodamine) intensity. One hundred CD19+ cells and no-cell wells were used as positive and negative controls respectively. To limit potentially biased measurement, cells with less than 2 expressed genes among the 5 control genes (HPRT1, B2M, GUSB, TUBB and GAPDH) were excluded from the analysis. Data were analyzed using Real Time PCR Analysis software with or without normalization of the Ct value for each gene using GAPDH as calibrator gene. We considered that the cell expressed the gene if the Ct value was < 40 and if the expression curve was a sigmoid. However, positive control wells containing 100 CD19+ cells showed detectable expression levels of all tested cytokine genes. The amount of RNA contained in one cell was therefore possibly too low. We analyzed qPCR results in frequency of cytokine gene expressing B cells between different groups.

BAFF Enzyme-Linked Immunosorbent Assay

The concentration of BAFF in patients' serum (42 RTX-treated and 26 CS-treated patients) was determined using ELISA: DuoSet kit from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions. BAFF levels were measured at Days 0, 90, 180, 365, 730 and Month 36.

Statistical Analysis

Prism software was used for statistical analysis. Fisher exact test was used to compare the frequencies, the T-test to compare patients before and after treatment and one-way Anova for multiple comparisons. A p value of less than 0.05 was considered significant for all analyses.

RESULTS

BAFF Levels in Pemphigus Patients

Using serum samples from 88 patients and 37 healthy donors (HD), we first showed that serum BAFF levels (mean ± SD) measured at baseline were significantly higher in pemphigus patients than in HD (308 ± 13 pg/mL vs 252 ± 28 pg/mL, p=0.037) (**Figure 1A**). Moreover, we showed that patients who further relapsed during the 12 months after the start of treatment had significantly higher serum BAFF levels than patients who did not relapse (368 ± 92 vs 297 ± 118 pg/mL, p=0.036) (**Figure 1B**). A ROC curve showed that a BAFF level ≥300 pg/mL provided a sensitivity of 54.8%, a specificity of 92.9%, and a 98% negative and a 28% positive predictive value for the occurrence of relapse during the first year after the start of treatment (AUC=0.70; 95% CI: 0.57-0.83; p=0.019), regardless of the treatment group (RTX or CS alone).

Evolution of the BAFF/BAFF-R Axis After Rituximab

Serum BAFF levels evolved inversely to the percentage of CD19+ blood B cells in patients from the RTX group. Serum BAFF levels started to decrease from Day 180 to Day 360, and from Day 730 to Day 1096, corresponding to periods of recovery of blood B cells (**Figure 2**).

BAFF-R mRNA relative expression and BAFF-R protein expression were first studied in total B cells from 11 pemphigus patients. BAFF-R mRNA expression decreased after RTX treatment from 12.3 ± 7.6 AU at Day 0 to 3.3 ± 4.3 AU at Month 36, p=0.01, which corresponded to values close to those measured in HD (2.2 ± 2.1 AU) data not shown. The mean fluorescence intensity (MFI) of the BAFF-R protein in total B cells also decreased significantly after RTX from 3232 at Day 0 to 1527 at Month 36 (mean difference: 1705, 95% CI: 624 to 2786; p=0.002), the values measured at Month 36 were even lower than those measured in HD (**Figure 3A**).

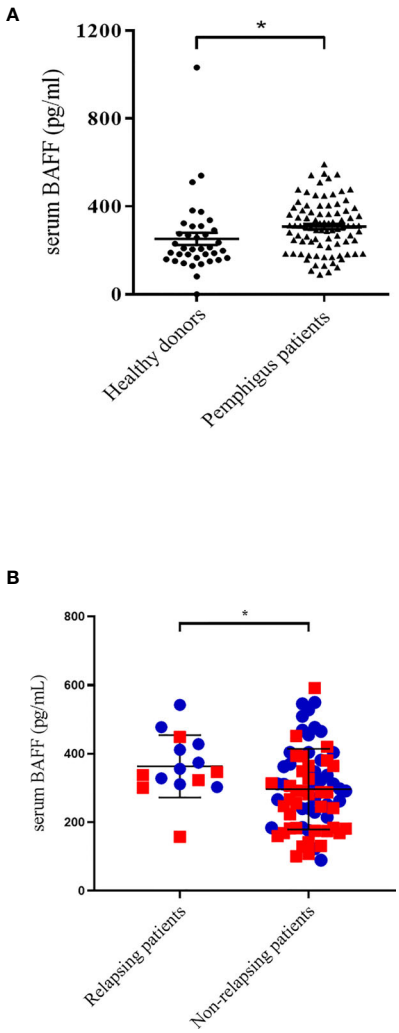

FIGURE 1 | **(A)** Comparison of serum BAFF levels between healthy donors and pemphigus patients at baseline. **(B)** Comparison of baseline serum BAFF levels between short-term relapsing patients and non-relapsing patients. Blue circles represent rituximab-treated patients. Red squares represent corticosteroids-treated patients. *p < 0.05.

We then assessed BAFF-R mRNA expression in one-cell sorted autoreactive B cells collected at baseline (n=191 cells) or after RTX (n=115 cells). MFI was measured in bulked autoreactive B cells from the same 11 patients. While we did not evidence significant modifications of BAFF-R mRNA expression after treatment (Day 0: 12/191 (6.3%) vs Month 36: 12/115, (10.5%); p =0.20), we observed a non-statistically significant decrease of BAFF-R phenotypic expression on reappearing autoreactive DSG-specific B cells relative to baseline samples (from 3873 at baseline to 2688 at Month 36, mean difference: 1185, 95% CI: -380 to 2750; p=0.20) (data not shown), which was reminiscent to that observed in the whole B cell population.

Evolution of the BAFF/BAFF-R Axis After Corticosteroids Alone

Treatment with high doses of CS alone led to a transient but significant decrease of serum BAFF levels during the first three months after the start of treatment (273.2 pg/mL vs 194.2 pg/mL, p=0.0002), when patients received the highest doses of prednisone (**Figure 2**). Serum BAFF levels then re-increased from Day 90 to the end of the study, when prednisone doses were tapered under 20 mg/day.

mRNA and cytometric MFI expression of BAFF-R in total B cells did not show significant variations after CS treatment (mRNA expression: 2.2 ± 2.1 AU vs 10.1 ± 5.4; p=0.32; phenotypic expression: 3232 vs 4100, mean difference: 898, 95% CI: -2463 to 667; p=0.43). Longitudinal transcriptomic and cytometric analyses were then performed in one-cell sorted autoreactive B cells collected at baseline (n=191 cells) and after CS (n=120 cells), and in bulked autoreactive B cells, respectively. No significant modification of BAFF-R mRNA expression (12/191 (6.3%) vs 3/120, (2.5%); p=0.18), or phenotypic expression (3873 vs 4771, mean difference: -868, 95% CI: -2086 to 650; p=0.24) (**Figure 3B**) was evidenced between autoreactive cells collected at baseline and those collected after CS treatment.

DISCUSSION

In this study, we showed for the first time, in a significantly larger cohort than in previous studies, that pemphigus patients had higher BAFF serum levels than healthy donors (16, 17). Moreover, early-relapsing patients had higher baseline serum BAFF levels than patients who did not relapse during the first year of follow-up, regardless of the treatment used. Interestingly, we calculated that patients who had a baseline serum BAFF level <300 pg/mL had a 98% chance of remaining in clinical remission during the first year of treatment. Both groups of patients treated with RTX or corticosteroids who further short-term relapsed had a higher baseline mean serum BAFF level than patients who did not further relapse, although these differences did not reach statistical significance and may due to a weak number of relapses (data not shown).

We showed that RTX and high doses of CS alone had different effects on the BAFF/BAFF-R axis. As expected, the evolution of serum BAFF levels was negatively correlated with blood B cell count in the RTX group and serum BAFF levels started to decrease when B cells started to return. Interestingly, most relapses observed in the RTX group occurred precisely during that time when B cells were in presence of the highest BAFF serum levels (10, 18). On the contrary, CS alone led to a transient decrease of serum BAFF levels, followed by a re-increase after CS doses were tapered under 20 mg/day, corresponding to the time period during which most patients relapsed.

Our findings suggest that a combined therapy associating anti-CD20 and anti-BAFF monoclonal antibodies might be of interest in pemphigus. These biologics, which work through complementary mechanisms, might result in an enhanced depletion of circulating and tissue-resident autoreactive B lymphocytes when administered together. In particular, it would make sense to use anti-BAFF therapy when B cells start to return, when serum BAFF levels are still very high, i.e. around the sixth month after the initial RTX infusion. The therapeutic regimen currently proposed to prevent short-term relapses after the initial cycle of RTX in pemphigus, is to perform additional RTX infusions whose exact time interval after the initial cycle, dosage and number are not clearly determined. Another strategy to prevent these short-term relapses might be to combine RTX maintenance infusion and anti-BAFF therapy, or to use anti-BAFF therapy alone as maintenance therapy. A phase-3 study is currently conducted to evaluate and compare the efficacy and tolerance of subcutaneous injections of belimumab in association to 2 cycles of RTX or a placebo in patients with SLE, after promising results in refractory SLE (19, 20).

Second, we showed that RTX led to decreased mRNA and phenotypic expression of BAFF-R in non-autoreactive B cells. Such a modification has already been reported in total B cells from patients with rheumatoid arthritis and idiopathic thrombocytopenic purpura (21, 22). We also observed a decrease of BAFF-R phenotypic expression on reappearing autoreactive DSG-specific B cells, which likely did not reach statistical significance due to the limited number of sorted DSG-specific B cells. Nevertheless, one can hypothesize that the decreased BAFF-R expression after RTX might be responsible for the delayed generation of memory B cells, resulting in a rather long period of mild pemphigus activity after RTX therapy. Interestingly, these modifications of the BAFF/BAFF-R axis seem likely related to a specific effect of RTX, since we did not observe these modifications in patients treated with CS alone, in whom we did not observe any modification in BAFF-R expression.

The main strength of this work is the high number of sera in which BAFF dosages were longitudinally performed in particular in patients treated with RTX, which is currently the mainstay of treatment for moderate to severe types of pemphigus. Our principal limitation was the low number of autoreactive B cells that could be analyzed due to the rarity of this cell population in patients' blood, and the quite long time after which some frozen samples were analyzed, which further lowered the number of auto-reactive cells that we were able to study. In particular, a higher number of sorted autoreactive B cells would have allowed us to reach a statistically significant difference, notably regarding the under-expression of the BAFF-receptor by recovering autoreactive B-cells.

Overall, these findings suggest that the activation of autoreactive B cells at the onset of pemphigus, is likely related to the presence of high BAFF serum levels. Furthermore, the decrease of BAFF-R expression observed in recovering B cells after RTX might prevent the binding of BAFF to these cells, resulting in a decreased activation of B cells despite the presence

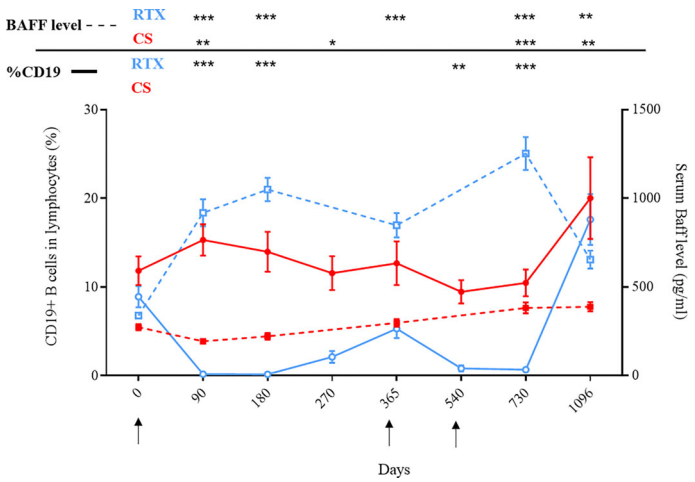

FIGURE 2 | Evolution of serum BAFF levels and CD19+ B cells according to treatments: rituximab (blue curves) or corticosteroids (red curves). Mean percentages of CD19+ B cells among peripheral blood lymphocytes were measured by flow cytometry and are indicated in full lines. Mean serum BAFF levels were measured by ELISA and are indicated in dashed lines. Arrows correspond to rituximab infusions. *p < 0.05; **p < 0.01; ***p < 0.001.

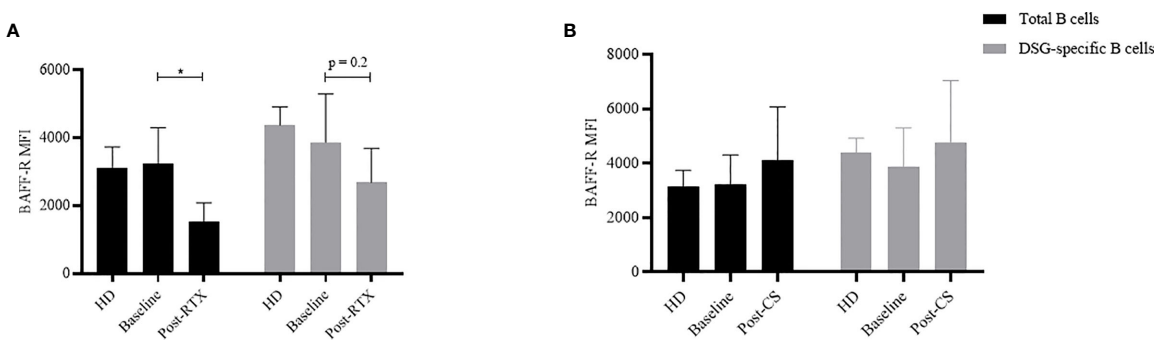

FIGURE 3 | Evolution of B-cells BAFF-R expression according to treatment by rituximab **(A)** or corticosteroids **(B)**. **(A)** Evolution of the mean cytometric BAFF-R MFI expression in total B cells (black) and autoreactive DSG-specific B cells (grey) before (n = 22) and after rituximab (n = 11) and comparison to healthy donors (n = 4). **(B)** Evolution of the mean cytometric BAFF-R MFI expression in total B cells (black) and autoreactive DSG-specific B cells (grey) before (n = 22) and after corticosteroids (n = 11) and comparison to healthy donors (n = 4). *p < 0.05.

of high BAFF serum levels. Conversely, the incomplete B cell depletion and persistent BAFF-R expression associated with high BAFF serum levels might explain the high number of relapses in patients treated with CS alone.

DATA AVAILABILITY STATEMENT

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

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 patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VH: Conceptualization, Investigation, Writing – original draft. MM-V: Investigation. M-LG: Investigation. MP: Investigation. GR: Software. OB: Resources. PM: Methodology. SC: Methodology. PJ: Writing – review & editing. All authors contributed to the article and approved the submitted version.

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Peripheral B-Cell Immunophenotyping Identifies Heterogeneity in IgG4-Related Disease

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Objectives: To elucidate heterogeneity of IgG4-related disease (IgG4-RD) based on B cell immunophenotyping.

Methods: Immunophenotyping of 4 B-cell subsets in peripheral blood from patients with active IgG4-RD (aIgG4-RD, n=105) was performed using flow cytometry to get preliminary B-cell heterogeneity spectrum. Then 10 B-cell subsets were characterized in aIgG4-RD (n = 49), remissive IgG4-RD (rIgG4-RD, n = 49), and healthy controls (HCs, n = 47), followed by principal components analysis (PCA) and cluster analysis to distinguish B-cell immunophenotypes and classify IgG4-RD patients into subgroups.

Results: Cluster analysis identified two endotypes in 105 aIgG4-RD patients based on 4 B-cell subsets: Group1 with higher Breg and naive B cells (n = 48), and Group2 with higher plasmablasts and memory B cells (MBCs) (n = 57). PCA indicated that aIgG4-RD consisted of plasmablast-naive B cell and MBCs-Breg axes abnormalities. There was a negative relationship between naive B cells and disease activity. Both plasmablasts and MBCs were positively associated with serological biomarkers. Cluster analysis stratified aIgG4-RD patients into 3 subgroups based on 10 B-cell subsets: subgroup1 with low MBCs and normal Breg, subgroup2 with high MBCs and low Breg, and subgroup3 with high plasmablasts and low naive B cells. Patients in subroup2 and subgroup3 were more likely to be resistant to treatment.

Conclusion: Patients with aIgG4-RD can be divided into 3 subgroups based on B cell heterogeneity. The B cell immunophenotyping could help elucidate the pathogenesis of IgG4-RD, identify patients with potential refractory IgG4-RD, and provide important information for the development of new therapies.

Keywords: B-cell subsets, immunophenotyping, cluster analysis, heterogeneity, IgG4-RD

INTRODUCTION

IgG4-related disease (IgG4-RD) is an immune-mediated fibrotic disease characterized by elevated serum IgG4 concentration, tissue infiltration by IgG4+ plasma cells, and a marked responsiveness to both glucocorticoids and B cell depletion with rituximab (RTX) (1). Various immunologic abnormalities contribute to generating the inflammatory masses in IgG4-RD, including M2 macrophages (2), activated B cells (3), CD4+ CTLs (4) and other immune-related cells.

The pathogenic role of B cell subsets has been given increasing attention since IgG4-RD was first recognized. In particular, plasmablasts are highly expanded and infiltrate tissue with extensive somatic hypermutation (5). Circulating plasmablasts are a useful biomarker, and correlate with other clinical and serological biomarkers of IgG4-RD activity (6, 7). B cell depletion with RTX has been proved effective for the treatment of IgG4-RD, which validates the suggested pathogenicity of B cells in this disease (8). Glucocorticoids (GCs), on the other hand, are not supposed to affect the total number of circulating B cells in IgG4-RD, but reduce naïve B cell, increase memory B cells (MBCs), and deplete circulating plasmablasts (9). The increase of circulating memory B cells after 6 months of GCs treatment might predict IgG4-RD relapse (10).

Taken together, the above findings suggest B-cell compartment in IgG4-RD is phenotypically heterogeneous. Although RTX is effective for both induction therapy and treatment of relapses in IgG4-RD, the high rate of infections and the temporary effect of RTX might be hindrances to such strategy (11), for it depletes B cell crudely, and B cell reconstitution is inevitable. Previous study has identified four homogenous clinical phenotypes based on typical patterns of organ involvement (12): Pancreato-Hepato-Biliary disease, Retroperitoneal Fibrosis and/or Aortitis, Head and Neck-Limited disease, and classic Mikulicz syndrome with systemic involvement. But patterns of B-cell subsets remain poorly defined. Moreover, the full characterization of circulating B-cell subsets in IgG4-RD patients at different stages of disease activity compared with healthy controls (HCs) was not carried out, which could offer a better understanding of their involvement in the pathogenesis of IgG4-RD.

Based on these considerations, it is worth raising three clinical questions: 1) What are the differences in B-cell immunophenotypes between IgG4-RD patients and healthy individuals, before and after treatment? 2) How do the B-cell immunophenotypes interact? 3) Can patients be divided into subgroups by immunophenotyping? 4) What's the association of B-cell subsets abnormalities and clinical phenotypes (12)? To address these questions, we initially characterized 10 B-cell subsets in IgG4-RD, and tried to obtain a broader perspective on the B-cell heterogeneity in IgG4-RD by immunophenotyping.

METHODS

Study Subjects

Peripheral blood (PB) was obtained from patients with active IgG4-RD (aIgG4-RD, n=105), remissive IgG4-RD (rIgG4-RD, n=49), and HC (n=47). IgG4-RD was diagnosed according to the 2011 comprehensive IgG4-RD diagnostic criteria (13) and the 2019 American College of Rheumatology/European League Against Rheumatism classification criteria for IgG4-RD (14). Patients with infectious diseases, other rheumatic diseases, malignancies, or conditions that could mimic IgG4-RD were excluded. HCs were matched for gender and age. All subjects were enrolled in accordance with ethics regulations, approved by the Ethics Committee of Peking Union Medical College Hospital, following written informed consent.

Laboratory Analysis and Flow Cytometry

Laboratory analyses of IgG4-RD patients before and after treatments included percentage of eosinophil (EOS%), absolute eosinophil count (AEC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), complement (C3 and C4), immunoglobin (IgG, IgA, IgM, and T-IgE), IgG1, IgG2, IgG3, and IgG4 subclasses. Peripheral blood mononuclear cells (PBMCs) were isolated from PB using Ficoll-Hypaque density gradient centrifugation. $1X10^6$ PBMCs were stained for B-cell subsets for 30 minutes at 4°C after washing and re-suspend in cell staining buffer. Flow cytometric analysis was performed immediately after sample preparation (see **Supplementary Figure 1** for gating strategy). All samples were analyzed using a BD FACSAria II system (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Treatments and Clinical Assessment

The treatments for IgG4-RD patients were classified into four categories: watchful waiting, GCs monotherapy, immunosuppressive agents (IM) monotherapy, and GCs + IM combination. IM applied in our study was graded as strong potency IM including cyclophosphamide (CTX) and mycophenolate mofetil (MMF), and weak potency IM including methotrexate (MTX) and leflunomide (LEF). The average follow-up period was 20 months. Remissive IgG4-RD included complete remission (CR) and partial remission (PR): CR was defined as IgG4-RD RI (2018) =0; PR was defined as IgG4-RD RI (2018) declining by ≥ 50%. Relapse was defined as a recurrence of symptoms and signs and/or worsening of imaging studies, with or without re-elevation of the serum IgG4 level. Potential refractory IgG4-RD was defined as no significant improvement on serological biomarkers especially reported risk factors (15, 16), although remission was achieved.

Study Design

We preliminarily evaluated the differences in B-cell immunophenotype in 105 aIgG4-RD patients. PBMCs were stained with CD19, CD24, and CD38 antibodies to obtain 4 B-cell subsets ($CD19^+CD24^+CD38^-$ MBCs, $CD19^+CD24^{int}CD38^{int}$ naïve B cells, $CD19^+CD24^{hi}CD38^{hi}$ regulatory B cells (Bregs), and $CD19^+CD24^-CD38^{hi}$ plasmablasts cells) (17), which was followed by cluster analyses to get a preliminary B-cell heterogeneity spectrum.

The gating strategy of B-cell subpopulations varies under the scientific research focus. Therefore, we further investigated and compared 10 published B-cell subsets (17–19) in aIgG4-RD

(n=49), rIgG4-RD (n=49), and HCs (n=47), including 3 plasmablasts cells ($CD19^+CD24^-CD38^{hi}$, $CD19^+CD27^{hi}CD38^{hi}$, and $CD19^+IgD^-CD38^{hi}$), 2 naive B cells ($CD19^+CD24^{int}CD38^{int}$, $CD19^+IgD^+CD38^{\pm}$), 4 MBCs ($CD19^+IgD^-CD27^+$, $CD19^+CD24^+CD38^-$, $CD19^+IgD^+CD27^+$, and $CD19^+IgD^-CD38^-CD27^+$), and $CD19^+CD24^{hi}CD38^{hi}$ Breg (**Supplementary Figure 1**). Principal components analysis (PCA) was performed to reduce the dimensionality of immunophenotyping data and followed by cluster analysis to classify the mixed samples (patients and HCs) into subgroups.

To elucidate the diversity among patients with aIgG4-RD, we performed a separate analysis of 49 aIgG4-RD using PCA and cluster analysis again. Disease activity associated changes after treatment were analyzed when these patients obtained clinical remission after treatment, including EOS%, ESR, IgG4, and T-IgE.

The immunophenotyping data and/or clinical features were compared among the different populations including HCs, aIgG4-RD, rIgG4-RD patients and the classified patients' groups. **Figure 1** presents information on the study design in flowchart format.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 24.0 software. Data including demographic data, immunophenotyping data, and longitudinal clinical data were stored in Microsoft Excel. Data were reported as mean ± SD. Normal distribution data between two groups were analyzed using independent-samples t tests or paired samples t tests, and one-way analysis of variance (ANOVA) was used among 3 groups. Non-normally distributed data between two groups were compared using the Mann-Whitney U-test, and

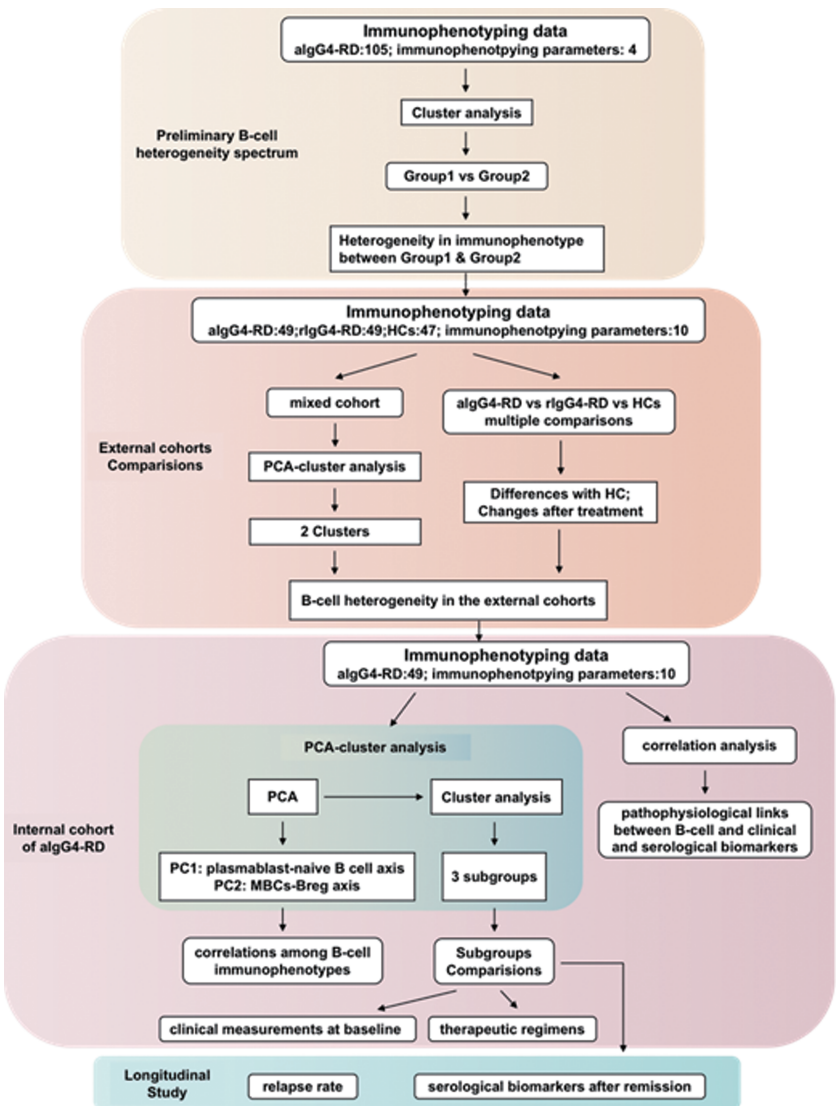


FIGURE 1 | The flow scheme of study design. HCs, (healthy controls); algG4-RD, (active IgG4-RD); rlgG4-RD, (remissive IgG4-RD); PCA, (principal components analysis).

Kruskall-Wallis was performed among 3 groups. Chi-squared tests were used to compare Categorical variables such as treatment categories, clinical phenotypes, and relapse state. The relationships between B-cell subsets and clinical features were analyzed by Pearson's or Spearman's correlation test. Paired t-tests were used to assess differences in serum biomarkers before and after treatments. P-values <0.05 were considered significant.

Principal Components Analysis and Cluster Analysis

For easy exploration and visualization of multiple variables, we used PCA to statistically aggregate 10 items of B-cell subsets, reducing the dimensionality of immunophenotyping data for subsequent cluster analysis, and exploring the correlation between these variables. According to eigenvalues ($\lambda > 1$) and cumulative contribution rate (>75%), we selected appropriate number of eigenvectors with the top highest eigenvalues as principal components (PCs). According to Component Matrix and eigenvalue, PC scores (the values for extracted PCs) were also calculated in individual samples as new variables for further cluster analysis (20, 21).

Cluster analysis was performed three times in this study using a hierarchical and agglomerative clustering algorithm with the Ward method (20–22): directly performed in 105 aIgG4-RD patients with 4 B-cell subsets data, following PCA in a mixed samples with 10 B-cell subsets data, and following PCA in aIgG4-RD patients (n=49) with 10 B-cell subsets data. We determined the number of clusters based on the scree plot (eigenvalue > 1) and tree diagram. In this study, we judged that the appropriate number of clusters was 2 in mixed samples and 3 in 49 aIgG4-RD patients.

RESULTS

General B-Cell Subsets Architecture of 105 Active IgG4-RD Patients

Baseline clinical characteristics and 4 B-cell subsets of 105 aIgG4-RD patients were shown in **Supplementary Table 1**. The mean age of diagnosis was 52.5 years, and most patients were males (64.8%). The mean duration of IgG4-RD was 3.1 years; mean diagnostic score (2019 ACR/EULAR classification criteria) and IgG4-RD RI (2018) at baseline were 32.9 and 7.6, respectively.

Cluster analysis classified aIgG4-RD patients into Group1 (n=48) and Group2 (n=57) based on 4 B-cell subsets (**Supplementary Figure 2**). Compared with patients in Group1, patients in Group2 were more likely to be male (75.4%), had shorter disease duration, but more organs involved, higher diagnostic score, higher level of IgG, IgG1, IgG4, T-IgE, and higher IgG4-RD RI. Unsurprisingly, Group2 was characterized by higher plasmablasts and memory B, but lower naive B and Breg (**Supplementary Table 1**). The proportions of B-cell subsets in individual patients in the 2 groups are plotted in three-dimensional diagrams (**Supplementary Figures 3A, B**), which confirmed that aIgG4-RD patients were clearly separated according to these 4 B-cell subsets.

Cluster Analysis Identified IgG4-RD Patients With High B-Cell Heterogeneity Spectrum Irrespective of Their Disease Activity Compared With Healthy Controls

Based on the observed heterogeneity of B-cell subsets architecture in aIgG4-RD and a variety of B-cell subsets published with different values, we hypothesized that B-cell subsets could be different among aIgG4-RD, rIgG4-RD and HCs. Therefore, we further analyzed 10 B-cell subsets in these three cohorts (aIgG4-RD, n=49; rIgG4-RD, n=49; HCs cohorts, n=47) (**Supplementary Figure 1**), and combined them into one mixed cohort (n=145) for PCA and cluster analysis.

First, we compared B-cell subsets proportions of the 3 cohorts (**Figure 2** and **Supplementary Table 2**). When compared with HCs, patients with IgG4-RD had lower proportion of $CD19^+$ total B cells, lower proportion of $CD19^+IgD^+CD38^{\pm}$ naive B cell, and higher proportion of $CD19^+IgD^-CD27^+$ MBCs no matter the disease was active or in remission. In addition, there were significant differences in 3 plasmablasts with different markers ($CD19^+CD24^-CD38^{hi}$, $CD19^+CD27^{hi}CD38^{hi}$, and $CD19^+IgD^-CD38^{hi}$) between aIgG4-RD patients and HCs, which were remarkably higher in aIgG4-RD patients and reduced drastically after treatments in rIgG4-RD patients. The proportion of $CD19^+IgD^+CD27^+$ unswitched MBCs were lower in aIgG4-RD patients than HCs, and similar to that in rIgG4-RD patients. The result was worth discussing is that $CD19^+CD24^{hi}CD38^{hi}$ Breg proportion did not differ between aIgG4-RD patients and HCs, and decreased after treatments. No significant differences in proportions of $CD19^+CD24^{int}CD38^{int}$ naive B cell and $CD19^+IgD^-CD38^-CD27^+$ switched MBCs were observed among cohorts.

We extracted 3 PCs (accumulative contribution rate = 78%) in the mixed cohort based on ten B-cell subsets ratios: PC1 (eigenvalue 4.348), PC2 (eigenvalue 2.395), and PC3 (eigenvalue 1.056). Then two distinct Clusters (based on 10 B-cell subsets profile) were identified by cluster analysis (**Supplementary Figure 4**): Cluster 1 (n=66; aIgG4-RD=32, rIgG4-RD=12, HCs=22) and Cluster 2 (n=79; aIgG4-RD=17, rIgG4-RD=37, HCs=25). Each Cluster included aIgG4-RD, rIgG4-RD and HCs. And there was striking difference in constituent ratio of cohorts between Clusters by chi-square test (P<0.001). Cluster1 more often presented with aIgG4-RD patients than Cluster2 (48.5% vs 21.5%), while Cluster2 more often presented with rIgG4-RD patients than Cluster1 (46.8% vs 18.21%). Healthy controls distributed evenly in two Clusters (33.3% vs 31.7%). As expected, the obvious heterogeneity among individual patients with IgG4-RD and significant differences in B-cell subsets profile before and after treatment were observed visually by heat map (**Supplementary Figure 4**). Together, these results suggest that IgG4-RD patients present with high B-cell heterogeneity spectrum irrespective of their disease activity compared with HCs, especially in aIgG4-RD patients.

		aIgG4 -RD (n=49)	rIgG4 -RD (n=49)	HC (n=47)	P value
Sex, n (% male)		29 (59.18%)	27 (55.10%)	29 (61.7 0%)	0.761
Lymphocyte		59.81 ± 13.56	58. 22 ± 13.07	58.37 ± 15.17	0.825
Total B cell	CD19+	7.30 ± 3.70	7.76 ± 8.37	9.26 ± 5.10	**0.011**
Plasmablast	CD24-CD38hi	9.60 ± 14.78	3.31 ± 3.41	2.18 ± 1.53	**<0.001**
	CD27hiCD38hi	8.29 ± 13.16	3.03 ± 3.85	1.88 ± 1.46	**<0.001**
	IgD-CD38hi	10.41 ± 16.31	2.51 ± 2.58	2.23 ± 1.43	**<0.001**
Naïve B	CD24intCD38int	53.16 ± 13.47	54.37 ± 12.25	55.49 ± 11.29	0.654
	IgD+CD38+/-	57.02 ± 14.10	50.57 ± 17.48	64.27 ± 9.16	**<0.001**
Memory B	IgD-CD27+	21.47 ± 14.45	22.91 ± 14.08	16.33 ± 8.77	**0.032**
	IgD-CD38-CD27+	11.54 ± 7.15	12.15 ± 6.52	11.94 ± 6.43	0.902
	IgD+CD27+	3.84 ± 3.75	5.28 ± 4.07	5.64 ± 3.65	0.054
Breg	CD24hiCD38hi	5.49 ± 4.39	2.13 ± 2.40	5.75 ± 3.11	**<0.001**

FIGURE 2 | Differences in phenotypes of B- cell subsets among patients with IgG4-RD and age- and sex-matched healthy control subjects. Values that were significantly different in the patient group compared with the healthy control group highlighted in color (blue for decreased; red for increased). aIgG4-RD, active IgG4-RD; rIgG4-RD, remission IgG4-RD.

B-Cell Subsets Ratios Correlated With Various Disease-Associated Indexes and Presented Plasmablast-Naive B Cell and MBCs-Breg Axes Abnormalities in aIgG4-RD

To assess a potential pathophysiological link between circulating B-cell subsets frequencies and aIgG4-RD, we performed correlation analysis with the following validated clinical and serological biomarkers in aIgG4-RD cohort (n=49): number of organs involved, IgG4-RD RI (2018), serum IgG, IgG_{1-4} and T-IgE levels, EOS%, AEC, ESR, CRP, and C3 (**Supplementary Table 3**). In general, circulating plasmablasts cells as well as $CD19^+IgD^-CD27^+$ MBCs ratio showed statistically significant positive correlation with disease activity and disease severity associated indexes, while naive B cells were negatively correlated with these indexes. $CD19^+IgD^+CD27^+$ unswitched MBCs and $CD19^+CD24^{hi}CD38^{hi}$ Breg proportions only showed negative correlation with T-IgE and ESR, respectively. No statistically significant correlation was found between $CD19^+IgD^-CD38^-CD27^+$ switched MBCs and disease-associated indexes. In particular, CRP had no correlation with any B-cell subsets ratio (data not shown).

To further investigate the abnormalities of B-cell subsets in aIgG4-RD, we separately used PCA in aIgG4-RD cohort to statistically aggregate these 10 immunophenotypes. Two PCs (accumulative contribution rate = 77%) were extracted based on ten B-cell subsets ratios: PC1 (eigenvalue 5.214), and PC2 (eigenvalue 2.476) (**Supplementary Figures 5A, B**). The ability of each PC to represent the corresponding B-cell subsets ratio and the correlations among ten immunophenotypes were estimated by correlation coefficient and visualized by a 2-dimensional loading plot, respectively (**Supplementary Figure 5B**, **Figure 3A**). The results showed PC1 was associated with plasmablasts cell and naive B cell phenotype. The positive side of PC1 contained 3 differently labelled plasmablasts cells ($CD19^+CD24^-CD38^{hi}$, $CD19^+CD27^{hi}CD38^{hi}$, and $CD19^+IgD^-CD38^{hi}$), while the negative side contained 2 types of naive B cells ($CD19^+CD24^{int}CD38^{int}$, $CD19^+IgD^+CD38^{\pm}$). In contrast, PC2 was mainly associated with MBCs and Breg. The positive side of PC2 contained $CD19^+CD24^+CD38^-$, $CD19^+IgD^-CD38^-CD27^+$ switched, and $CD19^+IgD^+CD27^+$ unswitched MBCs, while the negative side contained $CD19^+CD24^{hi}CD38^{hi}$ Breg. Specially, in memory B phenotype, $CD19^+IgD^-CD27^+$ MBCs seemed to play a more significant role in PC1 than in PC2.

In addition, we explored the correlations among B-cell immunophenotypes based on the distances among subsets and correlations analysis (**Figures 3A, B**). The loading plot showed clear separation among plasmablasts cells, naive B cell, MBC and Breg. On the whole, plasmablasts cells and naive B cells were located statistically opposite sides in loading plot and showed negative correlations (all pairs, P<0.001). Similarly, MBCs and Breg were locally opposite with negative correlations ($CD19^+IgD^-CD27^+$ MBCs with Breg, P=0.04; $CD19^+IgD^-CD38^-CD27^+$ switched MBCs with Breg, P=0.021). Breg also showed positive correlation with $CD19^+IgD^+CD38^{\pm}$ naive B cell (P=0.046). While both $CD19^+CD24^{int}CD38^{int}$ and $CD19^+IgD^+CD38^{\pm}$ naive B cells

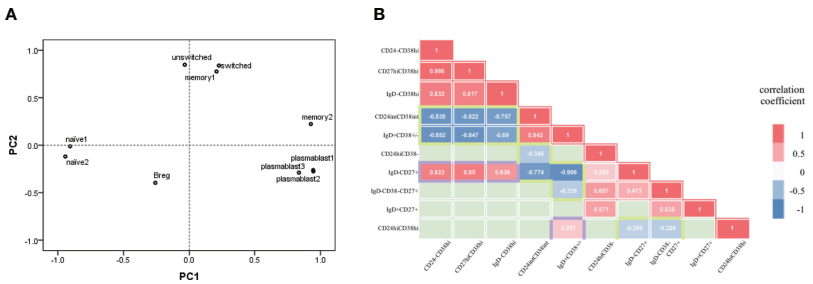


FIGURE 3 | B-cell subsets in active IgG4-RD patients presented plasmablast-naive B- cell and MBCs-Breg axes abnormalities. **(A)** Each B-cell subset immunophenotype was visualized in 2 two dimensions by principal components (PC) analysis. plasmablast1, CD19+CD24-CD38hi; plasmablast2, CD19+CD27hiCD38hi; plasmablast3, CD19+IgD-CD38hi; naïve1, CD19+CD24intCD38int; naïve2, CD19+IgD+CD38±; memory1, CD19+CD24+CD38-; memory2, CD19+IgD-CD27+; switched, CD19+IgD-CD38-CD27+ switched memory B cell; unswitched, CD19+IgD+CD27+ unswitched memory B cell; Breg, CD19+CD24hiCD38hi. **(B)** Correlation between pairs of B-cell types (10 immunological types). Correlation coefficients for each pair of cell types are represented by colour [red=positive correlation coefficient ($p<0.05$); blue=negative correlation coefficient ($p<0.05$); green=no significant correlation ($p<0.05$)].

were negatively correlated with MBCs, especially $CD19^+IgD^-CD27^+$ MBCs ($P<0.001$). Interestingly, $CD19^+IgD^-CD27^+$ MBCs and plasmablasts cells were statistically close in loading plot and showed great positive correlations (all pairs, $P<0.001$), both were negatively correlated with naive B cells, and positively correlated with disease activity and severity associated indexes as previously mentioned (**Supplementary Table 3**). The results of correlation analysis and PCA indicated that the immunophenotype of B-cell subsets in aIgG4-RD consists of plasmablast-naive B cell and MBCs-Breg axes abnormalities.

Cluster Analysis Identified Three Subgroups of Active IgG4-RD Patients

Given the heterogeneity and abnormalities of circulating B-cell subsets in patients with IgG4-RD, we next attempted to identify subgroups among 49 aIgG4-RD patients. Cluster analysis revealed that aIgG4-RD patients could be classified into 3 subgroups (subgroup1, n=36; subgroup2, n=9; subgroup3, n=4) (**Figure 4A**). The values of PC1, which was associated with plasmablast-naive B cell, and PC2, which was associated with MBCs-Breg, were calculated in individual samples and plotted in scatter plot (**Figure 4B**), which showed that aIgG4-RD patients were clearly separated and localized into 3 regions according to these 2 axes.

To assess whether the grouping also reflected distinct B-cell immunological profiles, we compared B-cell subset ratios in 3 subgroups as well as between each subgroup and HCs (**Figure 4C**). There were significant differences in all B-cell subsets except for Breg among subgroups. When compared with HCs, higher proportions of plasmablasts cells were observed in all subgroups to varying extents. Subgroup1 had lower MBCs proportions, as well as normal Breg and naive B cells proportions. Subgroup2 had the highest percentages of MBCs overall, but the lowest percentage of Breg. Subgroup3 was characterized by the highest proportions of plasmablasts cells, but the lowest percentage of naive B cells. Furthermore, we also found subgroup3 had the highest proportion of $CD19^+IgD^-CD27^+$ MBCs but lowest $CD19^+IgD^+CD27^+$ unswitched MBCs, which suggested there were inseparable relations between $CD19^+IgD^-CD27^+$ MBCs and plasmablasts again.

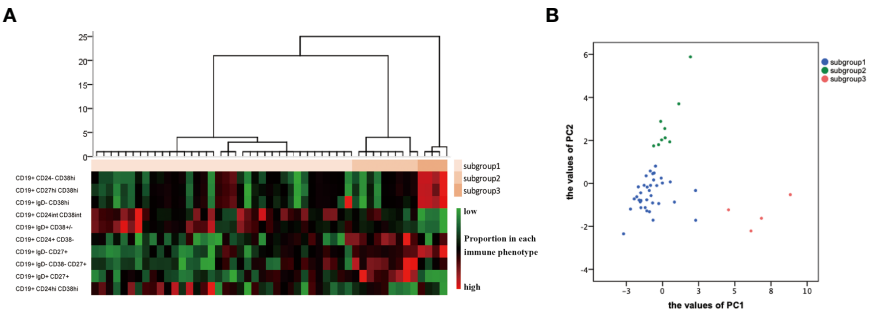
Patients With High MBCs and Plasmablast in Subgroup2&3 Were Potential Treatment-Resistant

To further investigate whether the grouping was clinically meaningful as a potential predictor of disease severity, treatment responses, and outcomes, a wide range of serological biomarker levels were compared among patients in 3 subgroups, as well as clinical measurements were collected longitudinally at subsequent encounters after treatments. Subgroup3 had significantly higher serum IgG, IgG1, IgG3, IgG4, T-IgE levels, and higher EOS%, AEC, ESR, but lower C3 than subgroup1&2 (**Supplementary Table 4**), suggesting patients in subgroup3 had higher disease severity. The number of organs involved and IgG4-RD RI did not differ. And there was no difference in therapeutic regimens ($P=0.33$), as well as intensity of treatment, including GC initial dose ($P=0.681$) and IM grades ($P=0.513$). The mean doses of initial GCs in the 3 subgroups were 41.18mg/d, 43mg/d, and 45mg/d, respectively. In GCs + IM combination therapy, the proportions of strong potency IM applyment were 64.7% in subgroup1, 80% in subgroup2, and 100% in subgroup3. Only three patients experienced relapse, all of them received IM monotherapy and belonged to subgroup1. Recurrence rate ($P=0.521$) showed no statistic difference among subgroups.

Next we compared serological biomarker levels after disease achieved remission among 3 subgroups (**Table 1**). Patients in subgroup3 still had the highest levels of EOS%, ESR, IgG4 and T-IgE. Considering subgroup3 had higher disease severity at baseline (**Supplementary Table 4**), we investigated the changes in these biomarker levels using Paired t-tests (**Figure 5**), patients in subgroup1 achieved significantly lower levels of EOS%, ESR, IgG4 and T-IgE than these at baseline, but no statistic difference in subgroup2&3. We also explored the percentages of changes to baseline levels, which didn't differ among subgroups. These results indicated patients in subgroup2&3 with potential refractory IgG4-RD.

Association of B-Cell Subsets Abnormalities and Clinical Phenotypes

We compared the distribution of clinical phenotypes in each group using Chi-squared tests (**Supplementary Figure 6**).



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		subgroup 1(n=36)	subgroup 2(n=9)	subgroup 3(n=4)	P value (Univariate)
plasmablasts	CD19+CD24-CD38hi	5.65 ± 5.90	5.19 ± 3.40	55.05 ± 7.72	<0.001
	CD19+CD27hiCD38hi	4.79 ± 4.80	4.68 ± 3.50	47.90 ± 13.89	0.004
	CD19+IgD-CD38hi	7.40 ± 11.33	3.42 ± 1.76	53.30 ± 6.43	<0.001
Naïve B cells	CD19+CD24intCD38int	57.57 ± 8.47	50.69 ± 8.83	19.00 ± 7.18	<0.001
	CD19+IgD+CD38+/-	61.99 ± 8.81	53.16 ± 6.25	21.00 ± 9.99	<0.001
Memory B cells	CD19+CD24+CD38-	6.16 ± 2.87	13.94 ± 5.15	7.22 ± 3.79	<0.001
	CD19+IgD-CD27+	15.51 ± 7.52	29.32 ± 5.68	57.45 ± 16.07	<0.001
	CD19+IgD-CD38-CD27+	8.99 ± 4.41	22.07 ± 7.96	10.83 ± 3.20	<0.001
	CD19+IgD+CD27+	2.66 ± 1.51	9.90 ± 4.73	0.84 ± 0.45	<0.001
Regulatory B cells	CD19+CD24hiCD38hi	6.20 ± 4.73	3.37 ± 2.37	3.83 ± 3.02	0.164

Colors were compared with healthy control

low 1.0 high

FIGURE 4 | Results of cluster analysis based on 10 B-cell phenotypes in patients with active IgG4-RD. **(A)** Hierarchical statistical clustering of IgG4-RD patients. **(B)** PC1 and PC2 values in individual patients in the 3 three subgroups. **(C)** B-cell subset ratios in 3 three subgroups are shown. Values are the mean ± SD, with levels that were significantly different in the patient subgroup compared with the healthy control group highlighted in color (blue for decreased; red for increased).

TABLE 1 | Serological biomarker levels of IgG4-RD patients in each subgroup after treatment.

Variables	subgroup1	subgroup2	subgroup3	P value (Univariate)
EOS%	1.77 ± 2.08	1.87 ± 1.24	10.00 ± 11.39	**0.001**
ESR (mm/h)	9.52 ± 6.85	18.57 ± 19.07	30.33 ± 27.65	**0.013**
IgG4 (mg/L)	3172.07 ± 3171.98	2359.29 ± 1347.96	22575.00 ± 36497.76	**0.008**
IgE (KU/L)	87.12 ± 94.20	85.10 ± 174.10	788.73 ± 1003.21	**0.002**

P values in the univariate analysis were determined by one-way analysis of variance (ANOVA) or Kruskall-Wallis test. P-values < 0.05 were considered statistically significant. EOS%, percentage of eosinophil; ESR, erythrocyte sedimentation rate; IgG4, immunoglobulin G4.

Variables	subgroup1		subgroup2		subgroup3		P value
	% changes	P (paired-T)	% changes	P (paired-T)	% changes	P (paired-T)	(Univariate)
EOS%	-66.67	**0.015**	7.9	0.465	-50.89	0.211	0.848
ESR (mm/h)	-64.08	**< 0.001**	-37.53	0.123	-62.74	0.074	0.885
IgG4 (mg/L)	-75.42	**< 0.001**	-41.95	0.128	-44.79	0.492	0.394
IgE (KU/L)	-67.15	**0.004**	-73.33	0.815	0	0.391	0.578

FIGURE 5 | Effects of treatment according to the the changes in serological biomarker levels using Paired paired t-test. The % changes are relative to the baseline. These changes are also shown in the chart using color (blue for decrease and red for increase) and compared among subgroups by univariate analysis (P values shown in the last column).

Between Group1 and Group2 identified by 4 B-cell subsets, Group1 showed higher proportion of clinical phenotype3, while Group2 showed higher proportion of clinical phenotype4 (P=0.001). Among subgroups identified by 10 B-cell subsets, the majority of patients in these 3 subgroups were those with clinical phenotype3&4, 2&3, and phenotype4, respectively.

We also compared the differences in phenotypes of B cell subsets among patients with different clinical phenotypes. On the whole, all clinical phenotypes had high plasmablasts. Clinical phenotype2 had the highest MBCs, but the lowest Breg. The only abnormality of B-cell subset in clinical phenotype3 was higher plasmablasts, this phenotype had relatively lower MBCs, normal naïve B cells and Breg. Clinical phenotype4 was characterized by the highest plasmablasts, the lowest naive B cells and total B cells, as well as higher MBCs and lower Breg. The features of B-cell subsets in clinical phenotype1 seemed to be intermediate between phenotype3 and phenotype4 (**Supplementary Figure 7**).

DISCUSSION

Our study represents a new classification of patients with IgG4-RD based on B-cell immunophenotyping, which confirmed the importance of plasmablast-naive B cell and memory B cell-Breg axes in IgG4-RD, established a subgroup of potential refractory IgG4-RD patients with high plasmablasts and memory B cell.

Previous immunophenotyping studies provide several landmark evidences indicating that B cells are central to the pathogenesis of IgG4-RD, including (i) $CD19^+CD24^-CD38^{hi}$ plasmablasts cells increase in active IgG4-RD (17); (ii) B cells contribute directly to tissue fibrosis in IgG4-RD, particularly of plasmablasts (3, 23); (iii) plasmablast and $CD19^+IgD^-CD27^+CD38^-$ memory B cells decreased after Iguratimod plus GCs treatment (18); (iv) GCs reduce $CD19^+CD20^+CD27^-CD38^+$ naive B cell, increase $CD19^+CD20^+CD27^+CD38^-$ memory B cells, and deplete plasmablasts (9); (v) increase of circulating $CD19^+IgD^-CD27^+CD38^-$ memory B cells after GCs treatment predicts IgG4-RD relapse (10); (vi) RTX is effective for IgG4-RD because it depletes all measurable peripheral B cells (8). We showed for the first time that patients with IgG4-RD have quite a few differences in their B-cell immunological architecture, spanning from the external comparison with healthy controls to the internal comparison among IgG4-RD subgroups.

We sketched out the general picture of B-cell heterogeneity spectrum in aIgG4-RD based on 4 immune cell subsets according to previous reports (17). Despite a predominance of plasmablasts found in IgG4-RD, patients with aIgG4-RD present heterogeneity by clustering: Group2 showed high plasmablast and MBC, low naive B and Breg, as well as a high proportion of male sex, high disease activity and severity; another cluster showed converse characterization. The results suggest clinical and B-cell heterogeneity among IgG4-RD patients as well as a potential pathophysiological link between clinical features and B-cell immunological spectrum. In addition, the results consist with the findings that male sex is associated with high serological markers and worse prognosis (24).

We further explored B-cell heterogeneity in external cohorts including IgG4-RD and HCs, and expanded 4 B-cell subsets into 10 subsets according to previous reports (18, 25, 26). The remarkable difference compared to rIgG4-RD and HCs was higher plasmablasts cells in aIgG4-RD, which has been replicated by our study as well. Compared with HCs, $CD19^+IgD^+CD38^{\pm}$ naive B cells decreased in aIgG4-RD and further declined after treatment. Another study also reported GC-induced disease remission was accompanied by a reduction of $CD19^+CD20^+CD27^-CD38^+$ naïve B cells (9). The classical function of MBCs is to retain the capability of rapidly differentiating into plasmablasts in the context of re-exposure to their cognate antigens, while different lymphocyte subsets with opposing functions are now known to be part of the MBCs (27). In our study, $CD19^+IgD^-CD27^+$ MBCs is increased in aIgG4-RD and no significant change after treatment, while the proportion of $CD19^+IgD^+CD27^+$ unswitched MBCs were lower in aIgG4-RD patients than HCs. It's reported that the percentages of $CD19^+IgD^+CD38^{\pm}$ naïve B cells decreased, while $CD19^+IgD^-CD27^+$ MBCs and plasmablasts increased following stimulation with IgG4-RD plasma exosomes (28). IgD^+CD27^+ unswitched MBCs usually present anti-inflammatory properties, for instance, are reduced in systemic lupus erythematosus (26), primary Sjögren's Syndrome (pSS) (19), and reconstitute after immunosuppressive treatment, this phenomenon also is observed in IgG4-RD in our study. IgG4-RD and pSS share several clinical and serological characteristics, such as enlargement of lacrimal and the salivary glands, and high immunoglobulin level. IgD^+CD27^+ unswitched MBCs decrease is an early feature of pSS correlated with serological autoimmunity and disease progression, and represents the loss of a MZ-equivalent endowed with protective functions such as apoptotic clearance, Interleukin-10-mediated B regulatory activity (26). But unswitched MBCs have not previously been studied in IgG4-RD pathogenesis. Our study suggests that $CD19^+IgD^-CD27^+$ MBCs and IgD^+CD27^+ unswitched MBCs may play opposite role in IgG4-RD, the former is pathogenic, the latter is protective. The role of Breg in the context of IgG4-RD is not entirely understood yet. Our data show the proportion of $CD19^+CD24^{hi}CD38^{hi}$ Breg in aIgG4-RD is similar to that in HCs, and decreases after treatment. The previous studies reported $CD19^+CD24^{hi}CD38^{hi}$ Bregs were increased in type 1 AIP patients (29), but another showed IgG4-RD patients had a lower frequency (25). There has been evidences that the profile of patients with different organs lesions differ from that of patients with other type of IgG4-RD. These conflicting results about Breg among studies may be caused by differences in the involved organs of IgG4-RD (30). Cluster analysis identified two clusters within the mixed cohort, which were characterized by differential B-cell immune signatures and had a high discriminatory capacity to identify aIgG4-RD and rIgG4-RD. In addition, both aIgG4-RD-dominant Cluster1 and rIgG4-RD-dominant Cluster2 were composed of a part of HCs. These findings highlight heterogeneous immune-pathogenic features underlying aIgG4-RD and rIgG4-RD. Patients with rIgG4-RD re-experience an

exacerbation may causally linked to the abnormal B-cell immunophenotypes.

We further explored high B cell heterogeneity in the internal cohort of aIgG4-RD. PCA and correlation analysis demonstrated relationships among B-cell immunophenotypes in aIgG4-RD. Plasmablasts cells and $CD19^+IgD^-CD27^+$ MBCs shared positive correlations with disease activity and severity, as well as the same side in PC1, indicating similar pathogenic abnormality. While naive B cells were negatively correlated with disease activity and severity, which seemed to be the anti-pathogenic factor opposite to plasmablasts in some aspects (3, 18). $CD19^+IgD^+CD27^+$ unswitched MBCs were negatively correlated with T-IgE, but $CD19^+IgD^-CD27^+$ MBCs were positively correlated. Given that unswitched MBCs are anti-inflammatory (26), we surmise T-IgE may play the opposite role in IgG4-RD, its increase may due to the unbalance between $CD19^+IgD^+CD27^+$ unswitched MBCs and $CD19^+IgD^-CD27^+$ MBCs. $CD19^+CD24^{hi}CD38^{hi}$ Breg was negatively correlated with ESR, consisting with its function of IL-10 production (31), which is known as the anti-inflammatory cytokine. Our study also first proposed that the B-cell immunophenotype of aIgG4-RD patients consisted of plasmablast-naive B cell and MBCs-Breg axes abnormalities.

Based on B cell heterogeneity, aIgG4-RD patients were clearly classified into 3 subgroups. Subgroup1: lower MBCs-near normal Breg and naive B cells proportions; subgroup2: the highest MBCs-lowest Breg proportions; subgroup3: the highest plasmablasts cells-the lowest naive B cells proportions, as well as the highest disease activity and severity. Interestingly, we found that patients in subgroup2&3 seemed to be potential treatment-resistant: (i) patients in subgroup3 received the highest doses of initial GCs and stronger potency IM, but still had the highest serological biomarker levels even if disease achieved remission; (ii) although patients in subgroup1 and subgroup2 had similar levels of serological biomarkers at baseline, subgroup2 had no significant improvement in these biomarkers after treatments. The results further indicates that huge icebergs (e.g., B-cell heterogeneity, other uncharted territories) are below the tip of the iceberg (e.g., clinical manifestations, imaging examinations, IgG4-RD RI) in IgG4-RD, which help physicians in aiming at a complete assessment of individual patients for therapeutic decision-making, relapse risk prediction, and prognosis evaluation. Therapies targeting the B-cell lineage have been applied, including RTX targeting CD20 and XmAb5871 targeting CD19 (32). Our findings provide theoretical support for B-cell depletion applying in IgG4-RD, especially in refractory cases. Considering the B-cell heterogeneity and the imperfections of B-cell depletion, such as non-selective depletion, high rate of infections, the temporary effect (11), and its inability to prevent re-emergence of pathogenic plasmablasts (33), more precision medicine based on IgG4-RD heterogeneity is expected to perfect IgG4-RD treatment.

The association of B-cell subsets abnormalities and clinical phenotypes provided support for our findings that IgG4-RD was a heterogeneous condition immunologically with plasmablast-naive B cell and memory B cell-Breg axes abnormalities, patients with higher plasmablasts and MBCs had both higher serological biomarker levels and more serious clinical condition including more potential to be systemic involved and refractory. Conversely, relatively normal naïve and Breg, or lower CD19+IgD-CD27+ MBCs may be a mild sign to patients with IgG4-RD.

Our study has limitations. First, the sample size was limited. Therefore we cannot perform stratified analysis according to different IgG4-RD types, such as types of organs involved, and different treatments. We also didn't observe clear relapse in subgroup2&3, although they had higher levels of risk factors after remission. Second, no patient received RTX in our study, we cannot comment on the impact of B-cell depletion on the B-cell immune signatures. Third, although we explored 10 B-cell immune subsets, B-cell set is complicated and continually updated. Further molecular and genetic characterization of B-cell subsets could have offered a better understanding of their involvement in the pathogenesis of IgG4-RD and will be the focus of future studies.

In conclusion, IgG4-RD is a heterogeneous condition immunologically with plasmablast-naive B cell and memory B cell-Breg axes abnormalities. Classification of patients with IgG4-RD based on B-cell immunophenotypes could help to identify potential refractory patients. A deeper understanding of these findings will improve our understanding of IgG4-RD pathogenesis, and lead to the development of more precise and effective therapies.

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 authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical ethics committee of Peking Union Medical College Hospital (Beijing, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Design of research study: WZ and JL. Acquiring data: ZL, PZ, and WL. Data analysis: JL. Recruiting patients: HL, YP, LP, JZ, MW, HC, LZ, LW, CQ, and CH. Analyzing data: WZ and JL. Writing the manuscript: JL. Review of the manuscript: XZ, YZ, YF, and WZ. 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.2021.747076/full#supplementary-material

Supplementary Figure 1 | Flow cytometry analysis of B cell subsets. **(A)** Gating strategy of B-cell subsets. **(B)** High percentage of plasmablasts and memory B cells in active IgG4-RD. SMB, CD19+IgD-CD27+ switched memory B cell, UMB, unswitched memory B cell.

Supplementary Figure 2 | Results of hierarchical statistical cluster analysis based on immune cells in 105 IgG4-RD patients.

Supplementary Figure 3 | Results of scatter gram based on B cell subsets in 105 patients with IgG4-RD. **(A)** The three-dimensional diagrams based on CD19+ CD24-CD38hi plasmablast, CD19+CD24hiCD38hi Breg, and CD19+CD24+CD38- memory B cell. **(B)** The three-dimensional diagrams based on CD19+ CD24intCD38int naive B cell, CD19+CD24+CD38- memory B cell, and CD19+ CD24hiCD38hi Breg.

Supplementary Figure 4 | Results of statistical cluster analysis based on 10 B-cell subsets in the mixed cohort.

Supplementary Figure 5 | Principal components selected based on B-cell subsets in patients with active IgG4-RD. **(A)** Scree plot for cluster analysis. **(B)** Principal component loading of two axes. Blue for negative and red for positive. PC1: principal component 1, PC2: principal component 2.

Supplementary Figure 6 | Distribution of clinical phenotypes in each group. **(A)** Distribution of clinical phenotypes compared between Group1 and Group2 identified by 4 B-cell subsets. **(B)** Distribution of clinical phenotypes compared among subgroups identified by 10 B-cell subsets. Phenotype, Clinical phenotypes of IgG4-related disease.

Supplementary Figure 7 | Differences in B cell subsets among patients with different IgG4-RD clinical phenotypes. **(A)** Four B-cell subsets compared among patients with different clinical phenotypes in initial cohort of active IgG4-RD. n=105, one patient met none of the 4 clinical phenotypes. Total B, CD19+ B cells; Plasmablasts, CD19+CD24-CD38hi; Naïve B, CD19+CD24intCD38int; Memory B cell, CD19+CD24+CD38-; Regulatory B cell, CD19+CD24hiCD38hi. **(B)** Ten B-cell subsets compared among patients with different clinical phenotypes in the internal cohort of active IgG4-RD. n=49, four patient met none of the 4 clinical phenotypes. Phenotype, Clinical phenotypes of IgG4-related disease. Values are the mean ± SD, with levels compared with the healthy control group highlighted in color (blue for decreased; red for increased).

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Clinical Experience of Proteasome Inhibitor Bortezomib Regarding Efficacy and Safety in Severe Systemic Lupus Erythematosus: A Nationwide Study

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As treatment options in advanced systematic lupus erythematosus (SLE) are limited, there is an urgent need for new and effective therapeutic alternatives for selected cases with severe disease. Bortezomib (BTZ) is a specific, reversible, inhibitor of the 20S subunit of the proteasome. Herein, we report clinical experience regarding efficacy and safety from all patients receiving BTZ as therapy for SLE in Sweden during the years 2014–2020. 8 females and 4 males were included with a mean disease duration at BTZ initiation of 8.8 years (range 0.7–20 years). Renal involvement was the main target for BTZ. Reduction of global disease activity was recorded by decreasing SLEDAI-2K scores over time and remained significantly reduced at the 6-month (p=0.007) and the 12-month (p=0.008) follow-up visits. From BTZ initiation, complement protein 3 (C3) levels increased significantly after the 2nd treatment cycle (p=0.05), the 6-month (p=0.03) and the 12-month (p=0.04) follow-up visits. The urine albumin/creatinine ratio declined over time and reached significance at the 6-month (p=0.008) and the 12-month follow-up visits (p=0.004). Seroconversion of anti-dsDNA (27%), anti-C1q (50%) and anti-Sm (67%) was observed. 6 of 12 patients experienced at least one side-effect during follow-up, whereof the most common adverse events were infections. Safety parameters (C-reactive protein, blood cell counts) mainly remained stable over time. To conclude, we report favorable therapeutic effects of BTZ used in combination with corticosteroids in a majority of patients with severe SLE manifestations irresponsive to conventional immunosuppressive agents. Reduction of proteinuria was observed over time as well as seroconversion of some autoantibody specificities. In most patients, tolerance was

acceptable but mild adverse events was not uncommon. Special attention should be paid to infections and hypogammaglobinemia.

Keywords: bortezomib (BTZ), systemic lupus - erythematosus, Lupus nephritis (LN), adverse (side) effects, antinuclear antibodies, clinical efficacy analysis, observational study

INTRODUCTION

Despite advances in treatment strategies leading to an improved prognosis, several challenges and unmet needs remain for patients living with systemic lupus erythematosus (SLE) (1, 2). In sharp contrast to other rheumatic diseases, treatment options in advanced SLE are limited. Subsequently, there is an urgent need for new and effective therapeutic alternatives for selected cases with severe disease. After many years of disappointing results from randomized clinical trials, recent outcomes of phase III trials on, *e.g.* anifrolumab and voclosporin, raise hope for clinicians and patients with SLE (3). However, many patients still experience refractory disease.

B cells have a prominent role in the pathogenesis of SLE as they mediate inflammation *via* production of a broad spectrum of autoantibodies directed against nuclear or cytoplasmic constituents and plasma proteins (4). Arguments for a pathogenic role include the fact that autoantibodies, such as anti-Smith (Sm) and anti-double stranded DNA (dsDNA), are associated with the clinical presentation of the disease, and the level of anti-dsDNA frequently correlates with SLE disease activity (5, 6). Today, different strategies are used to target the various stages of B cell development and, besides clinical disease activity, autoantibody levels are frequently used as surrogate markers of efficacy of the B cell-directed therapies (7). Most of these immunosuppressants, commonly used in combination with corticosteroids, primarily exert their therapeutic effects on B cells, plasmablasts and/or short-lived plasma cells (8, 9). However, to achieve effects also on the long-lived plasma cells, the available alternatives are autologous stem cell transplantation, atacicept [which blocks both the B cell activating factor (BAFF) and a proliferation-inducing ligand [APRIL)] and proteasome inhibition (9–11).

Bortezomib (BTZ) is a specific, reversible, and cell permeable dipeptide boronic acid inhibitor of the chymotryptic activity of the 20S subunit of the proteasome (12). Plasma cells are vulnerable to proteasome inhibition as it causes accumulation of defective immunoglobulin chains, resulting in endoplasmic reticulum stress, misfolded protein response, and subsequent apoptosis of plasma cells (13, 14). Long-lived plasma cells are significant antibody producers, they are highly sensitive to proteasome inhibition. In addition, proteasome inhibitors also effectively function as inhibitors of the production of pro-inflammatory cytokines through the regulation of NF-κB activation (7, 11).

Besides multiple myeloma and mantle cell lymphoma where BTZ is approved since the beginning of this century, advantageous effect of BTZ was demonstrated in German patients with renal and extra-renal severe SLE some years ago (15). Later, positive experience of BTZ in refractory lupus nephritis (LN) was reported from Spain and China although side-effects such as infections and neuropathy led to discontinuation in some cases (16, 17). Furthermore, the autoantibody repertoires of patients with SLE receiving BTZ or rituximab (RTX) ± belimumab (BLM) have been shown to differ illustrating the drugs' separate mechanism of action and highlight their impact on different B cell subsets (18). In a Japanese multicenter double-blind randomized controlled phase II trial including 14 patients, favorable clinical effects were observed on an individual level in some patients but also adverse events like fever, liver dysfunction and hypersensitivity reactions (19). Nevertheless, the study overall could not demonstrate efficacy of BTZ.

In 2014, the first Swedish patient with SLE was treated with BTZ. This female had life-threating disease characterized by proliferative LN, which was resistant to both cyclophosphamide (CYC) and RTX, and concomitant diffuse alveolar bleeding (20). Since then, another 11 patients with severe lupus manifestations have been started on BTZ at rheumatology practices in Sweden. Herein, we describe clinical efficacy and safety data from all Swedish patients receiving BTZ as therapy for SLE during the years 2014–2020. To our knowledge, high-quality nationwide real-life data of BTZ in SLE has previously not been communicated.

MATERIALS AND METHODS

Patients

Patient data were retrieved from all Rheumatology practices at Swedish University hospitals; 4 out of 7 tertiary referral centers offering high-specialized rheumatology health-care services (Göteborg, Linköping, Stockholm and Uppsala) and one county hospital (Jönköping) had experience of using BTZ for SLE during the years 2014–2020. All subjects eligible for BTZ treatment had been classified with SLE according to the 1997 American College of Rheumatology (ACR) criteria (21). Clinical characteristics of the included patients are detailed in **Table 1**.

Treatment Regimen

The provided dosage of BTZ was 1.3 mg/m^2 subcutaneously on day 1, 4, 8 and 11 along with dexamethasone (20–50 mg), followed by 10 days of rest before start of the next treatment cycle as illustrated in **Figure 1** (15). Two or three BTZ cycles were administrated for all but one patient. Data on disease-modifying anti-rheumatic drugs (DMARDs) and prednisolone dose used before BTZ initiation as well as concomitant immunomodulatory treatment during and following BTZ were collected.

TABLE 1 | Characteristics of the included patients (n = 12) at the initiation of BTZ treatment.

Patient characteristics	
Background variables	**Mean (range) or %**
Females	66.7
Age at SLE onset (years)	30.1 (6–71)
SLE duration (years)	8.8 (0.7–20)
SLEDAI-2K (score)	14.4 (10–20)
SDI (score)	0.8 (0–3)
Body Mass Index (kg/m^2)	30.9 (20.2–43.0)
Caucasian ethnicity	58.3
Number of fulfilled ACR-97 criteria	6.7 (4–9)
Antiphospholipid syndrome	1 (8.3)
Clinical phenotypes (ACR-97 definitions)	**n (%)**
Malar rash	7 (58.3)
Discoid lupus	2 (16.7)
Photosensitivity	5 (41.7)
Oral ulcers	4 (33.3)
Arthritis	10 (83.3)
Serositis	4 (33.3)
Renal disorder	11 (91.7)
Neurological disorder	1 (8.3)
Hematological disorder	12 (100)
Immunological disorder	12 (100)
Anti-nuclear antibody	12 (100)
Target organ system	**n (%)**
Renal	11 (91.7)
Histopathology[1]	**n (%)**
Class III	3 (27.3)
Class IV	5 (45.5)
Class V	2 (18.2)
No biopsy available	1 (9.1)
Central nervous system (transverse myelitis)	1 (8.3)
Liver (autoimmune hepatitis)[2,3]	1 (8.3)
Lung (diffuse alveolar bleeding)[2]	1 (8.3)

ACR, American College of Rheumatology; SLEDAI-2K, Systemic Lupus Erythematosus disease activity index 2000; SDI, SLICC/ACR damage index.
[1]Histopathology staged according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification for LN (22).
[2]Concomitant with active Class IV lupus nephritis.
[3]Liver biopsy showed inflammation grade 3–4 and fibrosis stage 3 according to Batts & Ludwig (23).

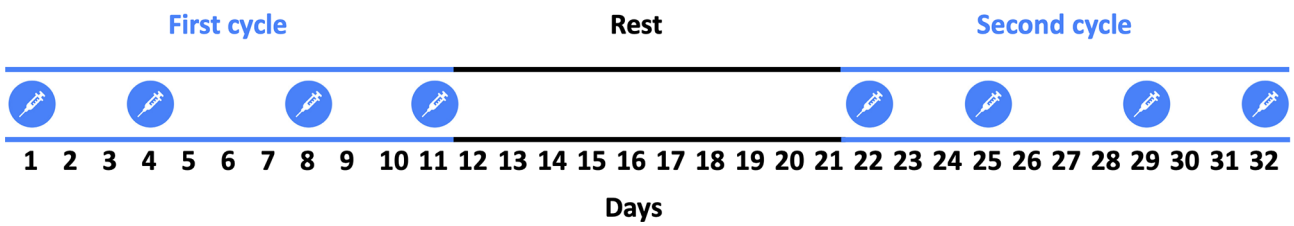

FIGURE 1 | As shown in the schematic illustration, the provided dosage of bortezomib (BTZ) was 1.3 mg/m^2 subcutaneously on day 1, 4, 8 and 11 along with dexamethasone (20–50 mg), followed by 10 days of rest before start of the next treatment cycle.

Clinical Evaluation

SLE disease activity was assessed using the Physician's Global Assessment (PGA, graded 0–100) and the Systemic Lupus Erythematosus disease activity index 2000 (SLEDAI-2K) (24, 25). Acquired organ damage, required to have been persistent for at least 6 months, was recorded by the Systemic Lupus International Collaborating Clinics (SLICC)/ACR damage index (SDI) encompassing damage in 12 defined organ systems (26).

Laboratory Measurements

Safety was continuously monitored by blood cell counts (erythrocytes, leukocytes, lymphocytes, neutrophils, and platelets) and C-reactive protein (CRP). Inflammatory and serological disease activity were followed by the autoantibodies (anti-dsDNA, anti-Sm, anti-C1q), erythrocyte sedimentation rate (ESR), and plasma analyses of albumin, immunoglobulin G (IgG), complement protein 3 (C3) and 4 (C4) according to clinical routine at the treating hospitals. Renal function was

monitored by plasma creatinine, estimated glomerular filtration rate (eGFR) based on plasma creatinine, according to the MDRD 4-Variable Equation (27), and the urine albumin/creatinine ratio.

Histopathology

Renal (n=10) and liver (n=1) biopsies were performed by percutaneous ultrasonography-guided puncture in accordance with a standard protocol. The renal tissue obtained was staged according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification for LN (22). The liver biopsy was assessed according to the standardized semi-quantitative histologic scoring system for hepatitis developed by Batts and Ludwig (23).

Statistics

Wilcoxon's matched-pairs test was used for comparing paired patient data. Associations between adverse events (binary variable) and hypogammaglobulinemia (binary variable) were examined with Fisher's exact test. Statistical analyses were performed using the SPSS software version 27.0.0.0 (SPSS Inc., Chicago, IL, USA) and Prism 9 (GraphPad Software Inc., La Jolla, USA) for construction of graphs. All laboratory data graphs are showing means with standard deviation. P-values ≤0.05 were considered statistically significant.

RESULTS

Subjects Treated With BTZ

In total, 8 females and 4 males with a disease duration ranging from 8 months to 20 years (mean 8.8 years) were considered eligible for BTZ and included in the follow-up. The mean age at BTZ initiation was 38.5 years and the global disease activity assessed by SLEDAI-2K was 14.4 (mean score). The target organ system for BTZ treatment was kidney (n=11) with proliferative (Class III and IV) histopathology dominating (n=8). One of the patients with proliferative LN had concomitant active autoimmune hepatitis requiring immunosuppression. In addition, one female had active central nervous system (CNS) involvement manifested by transverse myelitis without LN. 10 of 12 patients had an inadequate response to CYC and/or RTX ahead of BTZ initiation (**Table 2**).

BTZ Cycles and Maintenance Therapy

Of the included 12 subjects, 9 patients received two cycles of BTZ and two individuals three cycles. In the last patient, BTZ was discontinued before the 1st cycle was completed due to adverse effects (see below). One patient (Nr 4, see **Table 2**) received BTZ therapy at two occasions, with two cycles given both times.

Data of concomitant immunomodulatory treatment ahead of BTZ and following immunomodulatory treatment post-BTZ are shown in **Table 2**. Hydroxychloroquine (HCQ) was administrated during BTZ treatment in 9 of the 12 subjects. Four patients used combined mycophenolate mofetil (MMF) and BLM after completed BTZ cycles, whereas 10 of 12 subjects were prescribed HCQ in combination with other DMARDs. The mean daily prednisolone dosage at initiation of BTZ was 14.6 mg (5–40 mg); the prednisolone dose 30 days after ended BTZ treatment was 10.6 mg (5–20 mg).

Efficacy

Disease activity assessments are shown in **Figures 2A, B**. As compared to BTZ initiation, SLEDAI-2K scores (mean 14.4) were significantly reduced (i) at the end of BTZ treatment (mean 6.1, p=0.003), (ii) the 6-month follow-up visit (mean 4.0, p=0.007) and (iii) the 12-month follow-up visit (mean 4.0, p=0.008). By assessing disease activity with PGA, a significant reduction was observed at the end of the last treatment cycle (p=0.03) as well as the 6-month follow-up visit (p=0.04) compared to start of BTZ.

Complement proteins during follow-up are illustrated in **Figures 2C, D**. From BTZ initiation, C3 levels increased significantly after the 2nd cycle (p=0.05), the 6-month follow-up visit (p=0.03) and the 12-month follow-up visit (p=0.04). C4 levels increased significantly but only after the 2nd cycle (p=0.03) and at the 6-month follow-up visit (p=0.03) compared to BTZ start.

Plasma albumin (**Figure 2E**) increased over time at the 6-month follow-up visit (p=0.02) and at the 12-month follow-up visit (p=0.005). ESR (**Figure 2F**) decreased over time at the 6-month follow-up visit (p=0.03) and at the 12-month follow-up visit (p=0.05). The urine albumin/creatinine ratio (**Figure 2G**) declined over time, reaching statistical significance at the 6-month follow-up visit (p=0.008) as well as at the 12-month follow-up visit (p=0.004) post-BTZ treatment. eGFR (**Figure 2H**) was significantly improved at the 6-month follow-up visit (p=0.05).

Anti-dsDNA antibodies were positive in 11 of 12 subjects (91.7%) at start; 3/11 (27.3%) had seroconverted at the last follow-up. Anti-C1q antibodies were positive in 4 of 10 subjects (40%) at start; 2/4 (50%) had seroconverted at the last follow-up. Anti-Sm antibodies were positive in 3 of 11 subjects (27.3%) at start; 2/3 (66.7%) had seroconverted at the last follow-up.

Safety

As demonstrated in **Table 2**, 6 of 12 patients experienced at least one side-effect during follow-up. The most common adverse events were infections. One of four infections were severe and led to hospitalization. One individual did not complete the 1st cycle due to fever (viral infection) and emerging nephrotic syndrome with subsequent edema. The other patients fulfilled at least two cycles. Neuropathy was not reported in any subject.

Plasma IgG levels decreased during the BTZ cycles (median values: 9.1 g/L pre-BTZ *vs*. 7.8 g/L post-BTZ; p=0.008). 5/12 (42%) patients developed hypogammaglobulinemia (<6.7 g/L) during the BTZ treatment. 3 of 5 individuals who developed hypogammaglobulinemia experienced adverse events compared to 3 of 7 of those with IgG levels within the reference interval (not significant).

CRP and blood cell counts, including hemoglobin, leukocytes, neutrophils, lymphocytes and platelets, are illustrated in **Figure 3**. The hemoglobin concentration decreased after the 1st cycle (p=0.04), followed by a significant increase at the 12-month

TABLE 2 | Individual descriptions of the 12 included patients.

Patient number and gender	Age at BTZ initiation	BTZ cycles	Prednisolone, daily dose (mg)*		DMARDs ahead of BTZ initiation**	Concomitant immunomodulatory treatment	Immunomodulatory treatment following BTZ***	Adverse events
			Before	After				
1/F	26	2	5	5	HCQ, MMF, RTX	HCQ	HCQ, MMF	None reported
2/M	38	0.75	7.5	20	CYC, HCQ	HCQ	ABA, AZA, HCQ	Massive edema, viral infection
3/M	37	2	7.5	5	MMF	HCQ	BLM, HCQ, MMF	None reported
4/F	41	2	10	10	BLM, CYC, HCQ,	HCQ	BLM, HCQ, MMF	Otitis media, lower UTI
	43	2	5	5	MMF, RTX			
5/M	71	2	20	10	MMF, RTX		MMF	None reported
6/F	40	2	10	7.5	CYC, HCQ, MMF, RTX	HCQ	BLM, MMF, HCQ	None reported
7/F	29	2	10	10	CsA, HCQ, RTX	HCQ	BLM, HCQ	None reported
8/F	36	3	25	10	HCQ, MMF	HCQ	HCQ, MMF	None reported
9/M	21	2	15	15	HCQ, MMF, RTX	HCQ	HCQ, MMF, TAC	Renal anemia, diarrhea, hyperkalemia, hypernatremia, elevated liver enzymes
10/F	37	3	15	10	HCQ, RTX	HCQ	ABA, BLM, HCQ, MMF, PE	Pulmonary embolism
11/F	59	2	40	20	CYC, MMF, MTX, RTX	PE	BLM, HCQ	Cryptogenic organizing pneumonia
12/F	29	3	20	10	BLM, CsA, CYC, HCQ, MMF, RTX		MMF	Fever

**30-day average value, **12 months before the 1st BTZ cycle, ***12 months after the last BTZ cycle.*

ABA, abatacept; BLM, belimumab; BTZ, bortezomib; CsA, cyclosporine A; CYC, cyclophosphamide; DMARDs, disease-modifying anti-rheumatic drugs; F, female; HCQ, hydroxychloroquine; M, male; MMF, mycophenolate mofetil; MTX, methotrexate; RTX, Rituximab; TAC, tacrolimus; PE, plasma exchange; UTI, urinary tract infection.

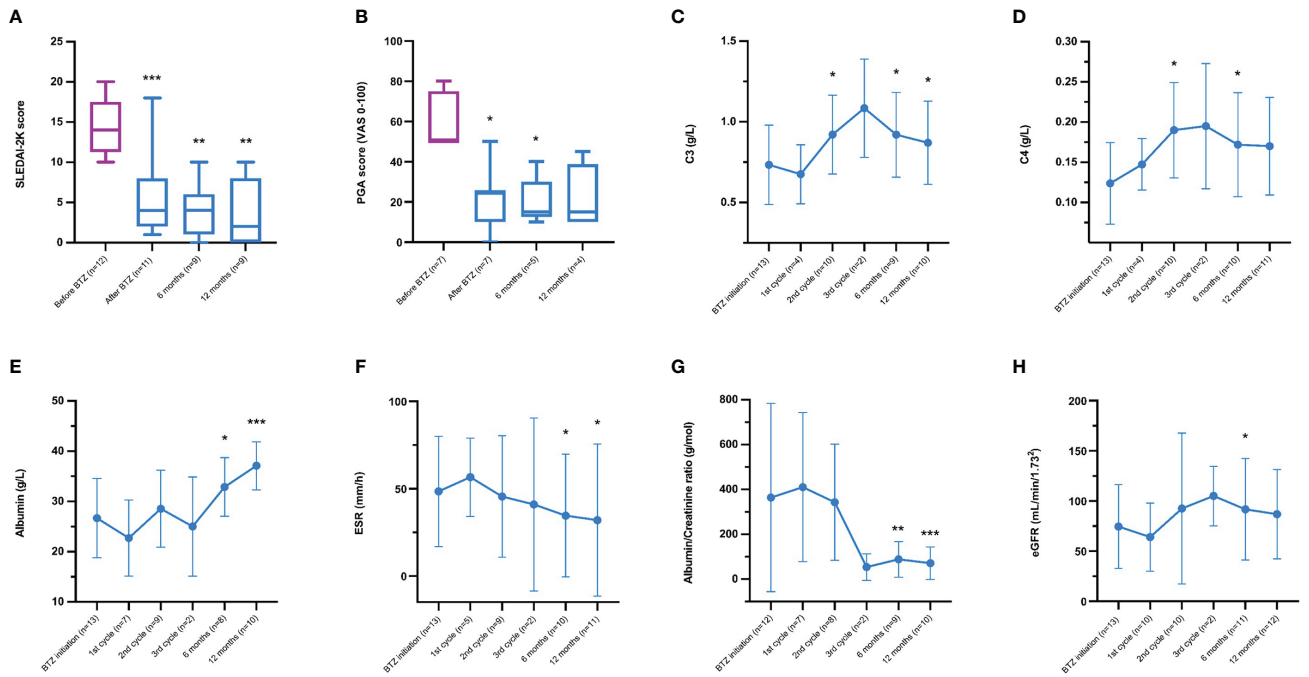

FIGURE 2 | **(A–H)** Clinical evolution and laboratory efficacy data over time from bortezomib (BTZ) initiation to the 12-month follow-up visit; **(A)** Systemic Lupus Erythematosus disease activity index 2000 (SLEDAI-2K), **(B)** Physician's Global Assessment graded (PGA) by visual analogue scale (VAS) 0–100 mm, **(C)** complement protein 3 (C3), **(D)** complement protein 4 (C4), **(E)** albumin in plasma, **(F)** erythrocyte sedimentation rate (ESR), **(G)** urine albumin/creatinine ratio, and **(H)** estimated glomerular filtration rate (eGFR) based on plasma creatinine, according to the MDRD 4-Variable Equation (27). Since one patient (without genetic deficiency of C1q, C3 or C4) received BTZ therapy at two occasions, the number of observations was 13 at some time-points. The graphs represent the mean and standard deviation; significances correspond to comparisons with baseline values. *$p < 0.05$. **$p < 0.01$ and ***$p < 0.005$.

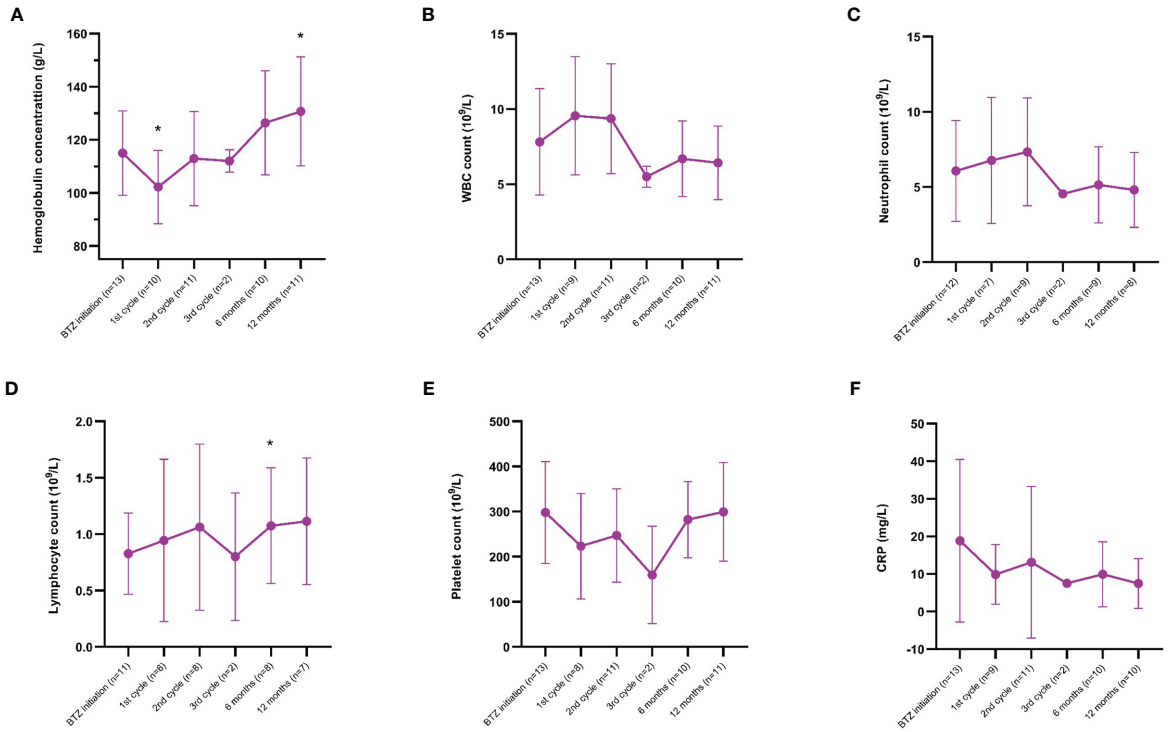

FIGURE 3 | **(A–F)** Laboratory safety data over time from bortezomib (BTZ) initiation to the 12-month follow-up visit; **(A)** hemoglobin, **(B)** white blood cell (WBC) count, **(C)** neutrophil count, **(D)** lymphocyte count, **(E)** platelet count, and **(F)** C-reactive protein (CRP). Since one patient received BTZ therapy at two occasions, the number of observations was 13 at some time-points. The graphs represent the mean and standard deviation; significances correspond to comparisons with baseline values. *$p < 0.05$.

follow-up visit (p=0.03). The lymphocyte count increased over time at the 6-month follow-up visit (p=0.04). Leukocyte, neutrophil and platelet counts, as well as CRP levels, did not change significantly over time.

DISCUSSION

The data presented originate from clinical follow-up of patients at Swedish academic practices and monitored by a limited number of SLE specialists. The efficacy of BTZ was continuously evaluated using a validated global disease activity scoring system. Overall, reduced SLEDAI-2K scores were observed early and remained reduced at the 6- and 12-month follow-up. Our findings are in line with experiences from both European and Chinese centers (15–17). Furthermore, we found that complement consumption, mirroring serological disease activity at BTZ initiation, tended to normalize over time.

Due to different immunoassays used for anti-dsDNA analysis at the four University units, we were not able to report reliable data on longitudinal autoantibody levels. However, seroconversion of anti-dsDNA (27.3%), anti-C1q (50%) and anti-Sm (66.7%) were observed. Reduction of several autoantibody specificities and antiphospholipid antibodies during BLM therapy have been reported (28–30), but it should be emphasized that anti-Sm is mainly produced by long-lived plasma cells which usually are not reached by drugs like CYC, RTX or BLM (31). The finding of seroconversion of anti-Sm in up to two-thirds of the SLE patients after BTZ could thus reflect a deeper depletion than with CYC/RTX. These observations partially contrast the findings reported by van Dam et al. who described significant decrease of anti-dsDNA during BTZ therapy whereas anti-C1q antibodies essentially remained unaffected (18). However, in the Japanese multicenter double-blind randomized controlled phase II trial of BTZ in SLE, reduction of anti-dsDNA levels was not observed (19).

In contrast to reported beneficial effects of BTZ in severe SLE herein, the only accomplished randomized controlled trial did not demonstrate clinical (or serological) efficacy although the number of included cases were very low (19). Nevertheless, refractory disease is not uncommon and BTZ could be one of several pharmaceutical alternatives to be considered in severe SLE resistant to conventional immunosuppressive agents. As patients with severe renal or CNS involvement, which have failed on drugs like CYC or RTX, are usually not suitable for randomized controlled trials clinicians often need to rely on empirical knowledge and off-label therapy may be required. Consequently, clinical guidance for these severe cases may be valuable. This was the rationale why we decided to retrospectively compile the available 7-years nationwide clinical experience of BTZ in SLE.

The majority of patients selected for BTZ had severe renal involvement. Based on this, it was crucial to follow renal function, i.e. eGFR, plasma albumin and the urine albumin/creatinine ratio. Interestingly, the significant beneficial effects on plasma albumin and albuminuria were slow and met statistical significance after the BTZ cycles were ended. Whether the decreasing proteinuria was associated with BTZ *per se* or with the DMARDs prescribed after BTZ cycles remain an open question. However, it is important to remember that, prior to the BTZ cycles, most patients had been resistant to the same DMARDs.

For all treatments, the level of efficacy must be accompanied by a reasonable safety profile. Side-effects during BTZ treatment were reported in as much as 50% of the patients, particularly infections. Prior studies have also observed infections as a frequent adverse event (15, 19). Pulmonary embolism and cryptogenic organizing pneumonia (COP) were the most severe reported side-effects, whereof the latter required therapeutic plasma exchange. To notice, the included patients were heavily immunosuppressed and had manifestations refractory to standard treatment regimens. Adverse events in this group of patients are not unexpected (32, 33). Nevertheless, neuropathy was not recorded in any of our patients in contrast to previous reports (15–17). Besides the clinical side-effects, we further investigated safety regarding blood cell counts and CRP. Hemoglobin was significantly decreased early, but elevated levels were observed over time and reached statistical significance at the 12-month follow-up visit. However, as shown in **Figure 3**, most safety parameters remained stable.

This was not a randomized controlled trial, and a comparator group was not included which must be taken into account. Thus, the observational nature of the data inevitably leads to selection bias. In addition, patients who responded to BTZ without significant side-effects have contributed with longitudinal data to a greater extent than those who experienced adverse events and discontinued treatment. The number of included patients were indeed low but represent a complete coverage of Swedish cases with SLE receiving BTZ during 2014–2020 and the sample size is comparable, or even larger, than previous reports (15–17). Short-time follow-up of the first two Swedish cases treated with BTZ has previously been reported but substantially longer follow-up was reported herein (20). The Swedish healthcare system's universal access and the use of large SLE cohorts with well-characterized patients at each University unit with close follow-up by a limited number of experienced rheumatologists constitute major strengths of the study (34). Patients' ethnicity is known to affect treatment effects in SLE. Although both Asian, African and Hispanic subjects were included herein, 7 of 12 were Caucasians. Extrapolation of therapeutic effects to different ethnicities should be done with caution.

To summarize, in a majority of patients with severe SLE manifestations irresponsive to conventional immunosuppressive agents, we observed favorable therapeutic effects of BTZ used in combination with corticosteroids. Reduction of proteinuria was seen over time as well as seroconversion of several autoantibodies. The tolerance was good in most patients, but mild adverse events was not uncommon. As BTZ may cause hypogammaglobinemia, special attention should be paid to infections.

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

Ethical approval was not required for this study, in accordance with the local legislation and institutional requirements, because this was an observational study evaluating treatment effects of a drug approved for another diagnosis. The study complied with the ethical principles of the Declaration of Helsinki. In Sweden, drugs are allowed to be used off label. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

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The Risk of Severe Infections Following Rituximab Administration in Patients With Autoimmune Kidney Diseases: Austrian ABCDE Registry Analysis

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Objective: To characterize the incidence, type, and risk factors of severe infections (SI) in patients with autoimmune kidney diseases treated with rituximab (RTX).

Methods: We conducted a multicenter retrospective cohort study of adult patients with immune-related kidney diseases treated with at least one course of RTX between 2015 and 2019. As a part of the ABCDE Registry, detailed data on RTX application and SI were collected. SI were defined by Common Terminology Criteria for Adverse Events v5.0 as infectious complications grade 3 and above. Patients were dichotomized between "nephrotic" and "nephritic" indications. The primary outcome was the incidence of SI within 12 months after the first RTX application.

Results: A total of 144 patients were included. Twenty-five patients (17.4%) presented with SI, mostly within the first 3 months after RTX administration. Most patients in the nephritic group had ANCA-associated vasculitis, while membranous nephropathy was

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the leading entity in the nephrotic group. Respiratory infections were the leading SI (n= 10, 40%), followed by urinary tract (n=3, 12%) and gastrointestinal infections (n=2, 8%). On multivariable analysis, body mass index (BMI, 24.6 kg/m^2 *versus* 26.9 kg/m^2, HR: 0.88; 95%CI: 0.79-0.99; p=0.039) and baseline creatinine (HR: 1.25; 95%CI: 1.04-1.49; p=0.017) were significantly associated with SI. All patients in the nephritic group (n=19; 100%) who experienced a SI received oral glucocorticoid (GC) treatment at the time of infection. Hypogammaglobulinemia was frequent (58.5%) but not associated with SI.

Conclusions: After RTX administration, impaired kidney function and lower BMI are independent risk factors for SI. Patients with nephritic glomerular diseases having concomitant GC treatment might be at higher risk of developing SI.

Keywords: rituximab, infections, glomerular disease, vasculitis, lupus, nephrotic, nephritic

INTRODUCTION

Severe infections (SI) are a major cause of morbidity and mortality in patients with kidney disease. In particular, glomerulopathies, either primary forms or secondary to systemic disorders, exhibit a heightened risk for such complications, which is determined by the underlying disease but to a major extent a direct consequence of immunosuppressive therapy.

Rituximab (RTX), a chimeric monoclonal antibody directed against the B cell CD20 antigen, was initially approved for the treatment of hematologic malignancies and subsequently for rheumatoid arthritis. In 2010, its label was expanded for the treatment of ANCA-associated vasculitis (AAV). In parallel, it has become an important off-label agent in the treatment of various forms of autoimmune kidney diseases, such as primary membranous nephropathy (MN), minimal change disease (MCD), and immune-mediated forms of focal segmental glomerulosclerosis (FSGS). While efficacy data of RTX in most glomerular pathologies are still limited to observational studies, several randomized controlled trials (RCT) investigating RTX in MN have recently been published (1–3).

Although the safety profile of RTX is considered favorable, SI can occur during and after anti-CD20 therapy. While few infectious complications were reported in recent key trials, a French study, involving 98 individuals with various types of glomerulopathies treated with RTX, noted infectious episodes in a quarter of patients; cumulative RTX dose and kidney failure were identified as independent risk factors (4). Generally, infectious complications appear to be mainly determined by the indication of RTX, i.e. the underlying disease, individual patient characteristics, and concurrent therapy.

In a large, contemporary multicenter cohort, we assessed incidence, type and risk factors for SI in patients treated with rituximab. Moreover, we wanted to investigate whether SI differ between "nephrotic "(e.g. MN, MCD, FSGS) and "nephritic" (AAV, lupus nephritis (LN)) indications. As the classification of autoimmune kidney disease is becoming increasingly granular and treatment approaches are being constantly refined, such data are important to allow for more individualized recommendations regarding prophylaxis strategies.

MATERIALS AND METHODS

Patient Population and Data Collection

The Austrian B-Cell Depletion Evaluation (ABCDE) study is based on a multicenter retrospective data collection of adult patients with immune-related kidney diseases, treated with at least one course of RTX. Austrian tertiary centers for the management of autoimmune kidney diseases were invited to participate in this National Registry. From January 2015 until December 2019 data on 144 patients from the participating centers were collected.

The registry contains data from patients with any of the following disorders: AAV, FSGS, immunoglobulin G4-related disease (IgG4RD), LN, MCD, MN and membranoproliferative glomerulonephritis (MPGN). Additional inclusion criteria consisted of age ≥ 18 years at the start of RTX therapy and a minimum follow-up time of 3 months after the first RTX application.

Data on clinical characteristics, comorbidities, prior immunosuppressives [i.e., calcineurin inhibitors (CNI), cyclophosphamide (CYC), and mycophenolate mofetil (MMF)], and baseline laboratory data of all patients were collected. Additionally, detailed data on RTX application including dose, treatment line, concomitant glucocorticoid treatment, and further maintenance therapies, were gathered. Patients were treated with RTX in accordance with their physician's standard practice. Dialysis was defined as newly started kidney replacement therapy (KRT) after first RTX administration.

Serial measurements on immunoglobulin G (IgG) and absolute neutrophil count (ANC) were conducted during the observation period. Hypogammaglobulinemia and neutropenia were classified by nadir IgG levels (IgG levels < 7 g/L) (5) and ANC (neutrophil count <1.5 x 10^9/L) (defined by the local laboratory) during the observation period. In addition, data on occurrence of malignancies (solid tumors and malignant hematological disorders) during RTX therapy were also recorded.

All data, if available, were derived retrospectively from the electronic medical records of the attending centers. The date of the first RTX application (index date) was registered to calculate the time to outcome event. The duration of RTX therapy was

measured from the time of drug initiation to discontinuation of the drug or censored at the date of the last follow-up for patients remaining on the drug at the time of data analysis. Data were collected until patient death, loss of follow-up, or end of the follow-up on 31 December 2019.

Definition of Infections and Outcomes

The NCI Common Terminology Criteria for Adverse Events Version 5.0 (NCI-CTCAE v5.0) was used to define and grade infections. Clinical and hospitalization reports were reviewed during RTX treatment to identify severe (grade 3-5) infections. Notably, mild infections (grade 1-2) were not considered. The site of SI was defined as respiratory, gastrointestinal, urinary tract, and other infections. Additionally, the daily corticosteroid dose (prednisolone equivalent) at the time of infection was collected.

The primary outcome of this study was the incidence of SI within 12 months after the first RTX application. In addition, incidence rates of hypogammaglobulinemia and neutropenia, as well as new onset of malignancies, were also calculated.

Ethical Statement

The study protocol was approved by the institutional ethics committee of the Medical Faculty of the Johannes Kepler University, Linz, Austria (Nr. 1117/2018) and subsequently by the local ethics committee of all participating centers. Informed consent was not obtained from the participants, as it was a noninterventional retrospective data analysis of real-life data collected on patients' regular visits. The study was conducted under the principles of the Declaration of Helsinki.

Statistical Analysis

Continuous parameters are summarized as the median and range (minimum, maximum) and categorical parameters are presented as absolute and relative frequencies. Baseline characteristics and investigated possible risk factors for SI at start of RTX therapy included age, BMI, sex, creatinine level, comorbidities, prior IS and RTX induction protocol are presented for all patients, and for nephrotic (including MN, MCD, FSGS) and nephritic (including AAV, LN, MPGN, IgG4RD) patients separately. Missing specific data were not imputed. Characteristics between both groups are compared by Mann–Whitney U and Fisher's exact test. To compare incidences of SI between the groups and accounting for the different baseline characteristics, inverse probability treatment weighting (IPTW) was performed. The propensity score was calculated by using logistic regression with group as outcome and including age, BMI, sex, creatinine level, comorbidities (yes/no) and prior IS (yes/no) as covariates. Additionally, Kaplan Meier curves for 12-month SI are presented for nephrotic and nephritic patients. To evaluate risk factors for SI within 12 months after RTX therapy start, univariable Cox proportional hazard regression analyses were performed. Time to event is defined as time from start of RTX to SI or death, lost to follow up or month 12, whatever occurs first. In a multivariable Cox regression model, all parameters with a p-value < 0.2 were included. Results are presented as Hazard ratios (HR) and their corresponding 95% confidence intervals (CIs). A p-value < 0.05 was considered statistically significant, and all analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA).

RESULTS

Patient Characteristics

One hundred forty-four patients with autoimmune kidney diseases were included. Detailed baseline demographic and clinical characteristics are shown in **Table 1**. Eighty-three patients had a nephritic glomerular disease, while 61 patients belonged to the nephrotic group. The majority of patients had AAV in the nephritic, while MN was the leading diagnosis in the nephrotic group (54% and 33% of all patients, respectively). The comorbidities were comparable in both groups. A significantly higher baseline median creatinine was observed in the nephritic group as compared to the nephrotic patients (1.7 [range: 0.7-15.4] *vs*. 1.2 [range: 0.6-2.8] mg/dL, p<0.001) at the time of RTX initiation. Nearly 40% of the patients in both groups received an induction therapy protocol using 2 x 1000 milligram (mg) RTX a fortnight apart or 4 x 375 mg/m^2 in weekly intervals, respectively, while fifty percent of all patients received RTX as maintenance therapy.

Incidence Rate and Type of Severe Infections

Twenty-five out of 144 patients (17.4%) presented with SI during a median follow-up time of 2.2 (0-4.9) years. Respiratory infections were the most frequent ones, but the majority of patients (58% and 67% nephritic and nephrotic groups) had other sites affected. The distribution of all SI according to the infection sites is shown in **Figure 1**. Two patients had a SI of grade 4 and 5 (n=1, respectively, both in nephritic group) according to the CTCAE v5.0 criteria.

In the long term, patients in the nephritic group tended to have more SI compared to nephrotic group patients (n=19, 22.9% *vs*. n=6, 10.2%; HR=1.82, 95% CI: 0.73-4.54, p=0.198). The median time to the first SI was 139 (range: 17-1345) days. Patients in the nephrotic group had a clearly shorter time until experiencing their first infection as compared to those in the nephritic group (median: 35 [range: 17-274] *vs*. 259 [range: 20-1345] days, respectively; p=0.024).

Within 3 and 12 months after first RTX administration n=10 (7.0%) and n=17 (12.0%) SI were observed. For nephritic and nephrotic patients, the infection rates were 6% *versus* 8.5% within 3 months (HR=0.39, 95% CI: 0.10-1.51, p=0.172) and 13.3% *versus* 10.2% within 12 months (HR=0.91, 95% CI: 0.33-2.51. p=0.852). Kaplan Meier estimates for 12-month SI rates are presented in **Figure 2**.

Predictors of Severe Infections

Univariable Cox regression analysis assessed predictors of SI within 12 months after first RTX administration

TABLE 1 | Baseline patient characteristics in the whole study population and stratified according to the classification on nephritic or nephrotic groups.

	Whole study population N = 144	Patients with nephritic diseases N = 83	Patients with nephrotic diseases N = 61	p-value
Age (years)	61.2 (20.4, 83.8)	65.1 (21.1, 83.8)	57.6 (20.4, 78.7)	**0.012**
BMI (kg/m^2)	26.2 (17.7, 39.9)	25.6 (17.7, 37.6)	27.3 (19.9, 39.9)	**0.036**
Female sex, n (%)	51 (35.4)	39 (47.0)	12 (19.7)	**<0.001**
Diagnosis, n (%)				
AAV	78 (54.2)	78 (94.0)	–	–
LN	3 (2.1)	3 (3.6)	–	–
MPGN	1 (0.7)	1 (1.2)	–	–
IgG4RD	1 (0.7)	1 (1.2)	–	–
MN	48 (33.3)	–	48 (78.7)	–
MCD	8 (5.6)	–	8 (13.1)	–
FSGS	5 (3.5)	–	5 (8.2)	–
Comorbidities, n (%)				
Pulmonary disease	12 (8.3)	7 (8.4)	5 (8.2)	1
Cardiovascular disease	30 (20.8)	15 (18.1)	15 (24.6)	0.408
Diabetes mellitus	17 (11.8)	9 (10.8)	8 (13.1)	0.795
Arterial hypertension	93 (64.6)	53 (63.9)	40 (65.6)	0.862
Dialysis (any time), n (%)	25 (17.4)	25 (30.1)	0 (0)	**<0.001**
Creatinine (mg/dL)*	1.3 (0.6, 15.4)	1.7 (0.7, 15.4)	1.2 (0.6, 2.8)	**<0.001**
Prior IS, n (%)				
MMF	17 (11.8)	11 (13.3)	6 (9.8)	0.608
CNI	29 (20.1)	0 (0)	29 (47.5)	**<0.001**
CYC	53 (36.8)	49 (59.0)	4 (6.6)	**<0.001**
RTX induction protocol, n (%)				
1000 mg (2x/2-weeks apart)	62 (43.1)	34 (41.0)	28 (45.9)	**0.001**
375 mg/m^2 (4x/weekly)	64 (44.4)	32 (38.6)	32 (52.5)	
Other	18 (12.5)	17 (20.5)	1 (1.6)	
RTX maintenance, n (%)	72 (50.0)	49 (59.0)	23 (37.7)	**0.018**

*Statistically significant p-values appear in boldface type (p < 0.05). Continuous variables are expressed as median (minimum and maximum). Categorical variables are n (%). *Non-dialysis dependent patients. AAV, anti-neutrophil cytoplasmatic antibody (ANCA), associated vasculitis; BMI, body mass index; CNI, calcineurin inhibitor; CYC, cyclophosphamide; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; IgG4RD, immunoglobulin G4-related disease; IS, immunosuppression; LN, lupus nephritis; MCD, minimal change disease; MN, membranous nephropathy; MMF, mycophenolate-mofetil; MPGN, membranoproliferative glomerulonephritis; RTX, rituximab.*

(**Supplementary Table 1**). In multivariable analysis, BMI (hazard ratio [HR]: 0.88; 95% confidence interval [CI]: 0.79-0.99; p=0.039) and baseline creatinine (HR: 1.25; 95% CI: 1.04-1.49; p=0.017) significantly affected SI within 12 months after the first RTX administration (**Table 2**).

All the patients in the nephritic group (n=19; 100%) who experienced a SI after the first RTX administration received oral corticosteroid (CS) treatment at the time of the infection. The median dose of CS used at the time of infection was 7.5 (range: 2.5-50.0) mg. On the other hand, only two out of six (33%) patients were treated with oral CS (median dose: 26.3 [range: 15.0-37.5] mg) at the time of SI in the nephrotic group.

Incidence Rate of Hypogammaglobulinemia, Neutropenia, and Malignancies

Hypogammaglobulinemia was observed in 72 out of 123 (58.5%) patients with serum IgG measurements. Most of these periods (40 out 72 [55.5%]) occurred within 12 months after the first RTX administration. Nineteen patients had a SI and hypogammaglobulinemia; however, no association between hypogammaglobulinemia and SI was observed (p=0.067). Interestingly, most of the infections (n=17) occurred before hypogammaglobulinemia was observed.

In total, 16 out of 140 (11.4%) patients with measurements experienced neutropenia, and it was significantly associated with SI during the whole observation period (p=0.030). Specifically, nine episodes of neutropenia (n=5 and n=4 in the nephritic and nephrotic groups, respectively) occurred during the first 12 months after the first RTX administration, which showed, however, no association with SI neither in the nephritic nor in the nephrotic group (p>0.05). Notably, no neutropenia was observed at the time of the first RTX administration, while only two patients were neutropenic at the time of the infection.

In addition, five patients (3.4%) experienced malignancy during the observation period. Two patients had a skin tumor, while one prostatic cancer, one breast cancer, and one malignant testicular tumor were observed in the other three patients.

DISCUSSION

The ABCDE Registry, an Austrian registry focusing on the use of RTX in kidney disease indications, included 144 patients, with the majority of patients having a diagnosis of AAV (54.2%) and MN (33.3%). During the observational period, 25 patients presented with a SI defined as CTCAE grade 3 and higher. Among these, there was no significant difference between

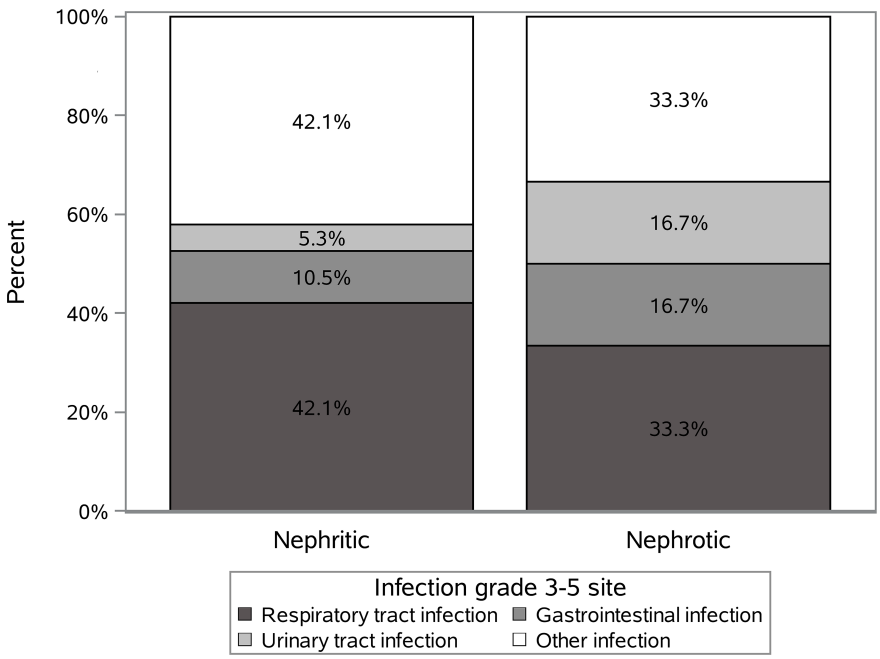

FIGURE 1 | The distribution of all severe infections according to the infection sites in the nephritic and nephrotic groups.

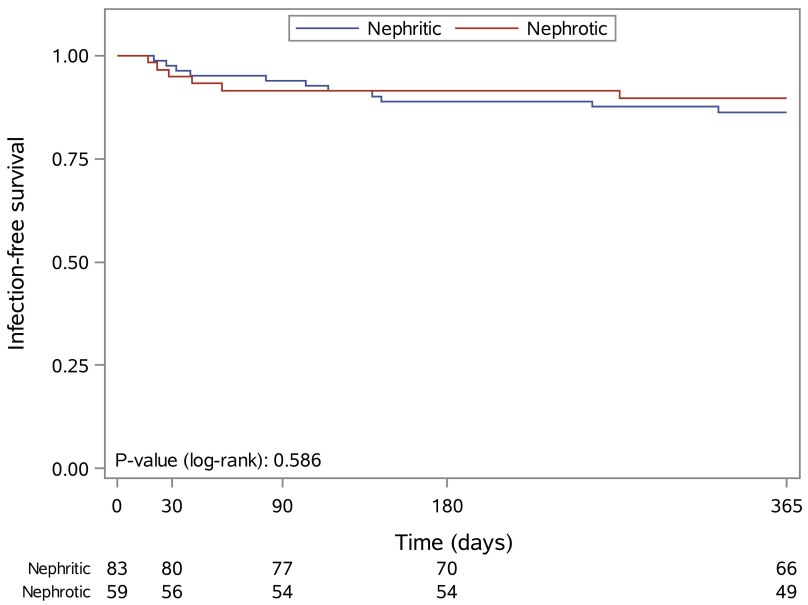

FIGURE 2 | Kaplan-Meier curves for infection-free survival within the first 12 months after the first rituximab administration in patients with nephritic (blue line) and nephrotic (red line) syndrome.

nephritic and nephrotic glomerular diseases (22.9% *versus* 10.2%). Most infections occurred within the first 3 months of RTX use, and this was most prominent for nephrotic diseases (5 infections out of 6 in total).

This study found that for nephritic glomerular diseases the risk of SI might be high when glucocorticoids are concomitantly prescribed. All SI were recorded on background glucocorticoid use. Reduced glucocorticoid regimens showed comparable

TABLE 2 | Multivariable Cox regression analysis on the predictors of severe infections within 12 months after the first rituximab administration.

Covariate	Hazard ratio	95% confidence interval	p-value
BMI (kg/m^2)	**0.88**	**0.79-0.99**	**0.039**
Creatinine (mg/dL)	**1.25**	**1.05-1.49**	**0.017**
Dialysis	0.90	0.22-3.76	0.887
Arterial hypertension	2.40	0.65 – 8.86	0.189

Statistically significant p-values appear in boldface type (p < 0.05). BMI, body mass index.

efficacy estimates in AAV (6, 7). In the PEXIVAS trial, the reduced-dose glucocorticoid regimen reached the primary end point, a combination of end-stage kidney disease and death from any cause, less frequently at one year of follow-up, although the differences were not significant. However, a significant reduction in SI at one year was observed with a reduction by over 30% in comparison to a standard-dose glucocorticoid regimen (6). More informative data on the use of a low-dose glucocorticoid regimen in combination with RTX was recently provided by the LOVAS trial, randomizing patients with AAV to either a reduced-dose or high-dose glucocorticoid regimen. The cumulative glucocorticoid dose was 1.3 g in the reduced-dose in comparison to 4.2 g in the high-dose arm, corresponding to a reduction of 68%. A similar proportion of patients achieved remission, while serious adverse events were reduced in the reduced-dose arm. Notably, only 7 SI in 5 patients were recorded in the reduced-dose arm in comparison to 20 events in 13 patients in the high-dose glucocorticoid arm (7). In the ABCDE Registry, fourteen of the 19 recorded SI in the nephritic group occurred beyond 3 months of follow-up, and 8 after 12 months. Studies such as LOVAS or the RAVE trial with a prescribed glucocorticoid withdrawal before month 6 after induction therapy highlighted both the safety and efficacy of such an approach (7, 8). A retrospective study from Germany subdivided patients by the use of a glucocorticoid dose of either below 7.5 mg or greater/equal to 7.5 mg by month 6. Patients with a higher glucocorticoid dose had more infectious episodes (1.7 *versus* 0.6), while the glucocorticoid dose had no impact on patient survival, kidney function, or relapse rate (9). The presented data of the ABCDE Registry also support a paradigm change towards a reduced time of glucocorticoid use. Notably, only 2 patients in the nephrotic group had concomitant glucocorticoid therapy, which argues against a significant impact of glucocorticoids on SI risk in this population.

Lower BMI (24.6 kg/m^2 *versus* 26.9 kg/m^2) was associated with higher risk of SI. A recent study from Japan involving 93 patients with a diagnosis of microscopic polyangiitis (MPA) subdivided patients into three groups, one group with low BMI (<18.5 kg/m^2, n=22), one group with normal BMI (18.5-23.0 kg/m^2, n=53) and one with high BMI (>23 kg/m^2, n=18). SI were recorded in 63.6%, 24.5% and 11.1% of patients in the respective groups. Patients in the low and normal BMI group were more likely to suffer from a body weight loss > 10% within six months before diagnosis of MPA was made. Patients in the low and normal BMI group also exhibited higher mortality rates in comparison to patients in the high BMI group (10). Sub-analysis of the RAVE trial indicated that newly diagnosed patients have an increase in total and low-density lipoprotein cholesterol after achieving remission. Reduced lipid levels at baseline correlated with erythrocyte sedimentation rate (11), indicating that inflammatory processes may play a critical role explaining the initial weight loss and altered lipid levels observed in patients with AAV. This persistent inflammatory process may in part explain the higher risk of SI observed in these patients. Lower BMI might also indicate a higher disease severity and these patients might have received higher glucocorticoid doses at baseline, again explaining the higher risk of SI. Despite non-significant, patients with nephrotic syndrome had a numerically lower frequency of SI, and water retention/edema development leading to a higher BMI might also influence our findings.

Most SI in our analysis occurred within the first months of RTX administration, which is in line with several investigations. A single-center study including 221 patients with autoimmune indications found SI in 42 patients. The prevalence of infectious complications was most pronounced within the first three months of follow-up (7.2%) and increased to 15.5% at one and 17.8% at two years. Most patients presented with pneumonia (45%) and/or bacteremia (21%) (12). RTX use for several indications was associated with an increase in SI from 17.2% pre-rituximab to 21.7% after administration among 8633 patients. Again, most infections occurred within the first six months of RTX administration. Pre-existing hypogammaglobulinemia was a strong risk factor for SI before RTX initiation and a third of these patients experienced SI following RTX (13). Hypogammaglobulinemia was frequently observed in our cohort (72 out of 123 with measurements, 58.5%), but was not associated with SI in our study. Notably, the sample size might have impacted this finding. Another complication of RTX is late-onset neutropenia (14). Neutropenia was associated with SI in our analysis of the ABCDE Registry, as 8 out of 16 patients with neutropenia had a SI during follow-up. A large single-center study found at least one episode of late-onset neutropenia in 71/738 adult patients receiving RTX. Its occurrence is more frequent within the first year of RTX administration and is not observed in patients with minimal change disease or focal segmental glomerulosclerosis. A majority of patients was asymptomatic during the neutropenic episode, while 31.3% and 8.5% presented with fever and septicemia (15).

Further analyses revealed an association of baseline creatinine with the risk of SI, which remained significant after adjustment for dialysis-dependency. Similar results were reported from a French study focusing on a combined end point of infection and/or death. Twenty-six out of 98 patients reached the end point during follow-up and baseline creatinine showed a borderline significance (4). Baseline creatinine was comparable to our cohort. Most analyses focusing on patients with AAV found a similar tendency towards more SI in patients with impaired kidney function (16–20), which is in line with our results.

Infections are one of the leading complications of nephrotic syndrome. Our analysis indicated that patients with nephrotic syndrome are prone to infections particularly within the first three months after RTX administration. In MN, there is an increased risk to develop invasive pneumococcal infection and there is a recommendation that patients receive pneumococcal and annual influenza vaccination (21). Vaccination might mitigate the risk to develop severe courses of these infections, while the humoral response to vaccines is severely impaired by

rituximab within the first six months (22) Antibiotic prophylaxis with trimethoprim/sulfamethoxazole, known to reduce SI in patients with AAV (17, 23), might be used in this vulnerable phase.

This study has several limitations. The ABCDE Registry was initiated as a retrospective survey among nephrology departments to capture complications of RTX therapy in glomerular diseases, before initiating a prospective study phase. The analyzed groups (nephritic and nephrotic) differed significantly in terms of age, BMI, sex, the history of immunosuppressive drug use and maintenance RTX administration, some of these factors potentially influencing our results. The presented study assessed data retrospectively, so limitations inherent with its retrospective character need to be considered (i.e. missing data, such as information on IgG levels). Initial data collection did not include the assessment of antibiotic prophylaxis used in these patients (i.e. *Pneumocystis jirovecii* prophylaxis). This information would be of particular interest in patients with nephrotic syndrome presenting with SI early after RTX use. Information about cumulative glucocorticoid use, the use of intravenous methylprednisolone to control initial disease activity and longitudinal follow-up of BMI values is missing, all of particular importance to understand the finding of a higher rate of SI in patients with lower BMI. The sample size of our study is small, but comparable to other investigations published, and multi-national efforts are needed to inform about frequency, severity and risk factors of SI in patients with glomerular diseases receiving rituximab.

In conclusion, analysis of the ABCDE Registry retained impaired kidney function and lower BMI as independent risk factors to develop SI after RTX administration. All infectious complications in patients with nephritic glomerular diseases occurred during concomitant GC treatment, while the risk might be independent of glucocorticoid use in nephrotic patients. Further studies are needed assessing the exact use of antibiotic prophylaxis, the capacity of antibiotics to mitigate the risk of SI, and the role of low-dose glucocorticoid protocols and influence on SI.

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 Johannes Kepler University, Linz, Austria (Nr. 1117/2018). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MWin and AK initially designed the ABCDE Registry. BO, MWin, MK, MSt, CH, MU, EZ, KL, MA, DC, PG, MWie, MSa, ARR, KE, and AK contributed to data acquisition, drafting the manuscript and approving the manuscript. RR performed statistical analysis. The first draft of the manuscript was written by BO, MWin, RR and AK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Belimumab for Immune-Mediated Necrotizing Myopathy Associated With Anti-SRP Antibodies: A Case Report and Retrospective Review of Patients Treated With Anti-B-Cell Therapy in a Single Center and Literature

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Background: Immune-mediated necrotizing myopathy (IMNM) is characterized by markedly elevated creatinine kinase and histologically scattered necrotic muscle fibers and generally associated with autoantibodies against signal recognition particle (SRP) or 3-hydroxy-3-methylglutaryl-coA-reductase (HMGCR). Poor clinical response to conventional therapies and relapses commonly occur in severe cases. Anti-B-cell therapies have been used in refractory/relapsing cases.

Methods: The characteristics of a patient with IMNM associated with anti-SRP antibodies including physical examination, laboratory tests, and disease activity assessment were evaluated. Conventional therapy, belimumab treatment schedule, and follow-up data were recorded. Medical records of IMNM patients treated in our department from September 2014 to June 2021 were reviewed to evaluate the efficacy and safety of anti-B-cell therapy for anti-SRP IMNM. A literature review of patients with anti-SRP IMNM treated with anti-B-cell therapies was performed.

Results: We describe a case of a 47-year-old woman with IMNM associated with anti-SRP antibodies who relapsed twice after conventional therapy but showed good response and tolerance to belimumab at 28 weeks follow-up. In this review, three patients from our department were treated with rituximab. Two of the three patients

rapidly improved after treatment. Twenty patients and five retrospective studies were included in the literature review. All patients were administered rituximab as an anti-B-cell drug.

Conclusion: Despite a lack of rigorous clinical trials, considerable experience demonstrated that anti-B-cell therapy might be effective for patients with IMNM associated with anti-SRP antibodies. Belimumab in association with steroids might be an encouraging option for refractory/relapsing cases.

Keywords: immune-mediated necrotizing myopathy, SRP antibody, refractory IMNM, belimumab, BAFF, rituximab

INTRODUCTION

Immune-mediated necrotizing myopathy (IMNM), also known as necrotizing autoimmune myopathy, is characterized by markedly elevated creatinine kinase and histologically scattered necrotic muscle fibers and generally associated with autoantibodies against signal recognition particle (SRP) or 3-hydroxy-3-methylglutaryl-coA-reductase (HMGCR) (1). Poor clinical response to conventional therapies and relapses commonly occur in severe cases.

In previous reports, anti-B-cell therapy, especially rituximab (RTX), an anti-monoclonal CD20 antibody, has been used in IMNM (2–12). In some cases, patients benefited from RTX, while in other cases, patients showed poor response or died from complications, such as infection (2–12).

Belimumab is a human monoclonal antibody targeting B-cell-activating factor (BAFF). Belimumab has been used in several rheumatoid diseases, including systemic lupus erythematosus, Sjogren's syndrome, systemic sclerosis, and antiphospholipid syndrome (13–16).

In this study, we report a case of a patient with anti-SRP IMNM who relapsed twice after conventional therapy but showed a good response and tolerance to belimumab. We also reviewed patients with anti-SRP IMNM who received anti-B-cell therapy in our department and in the literature.

PATIENTS AND METHODS

Case Record

Patient characteristics, including medical history, physical examination, laboratory tests, and radiological examinations, were recorded. Disease activity was assessed using the Myositis Disease Activity Assessment Visual Analogue Scale (MYOACT), Myositis Intention-to-Treat Activity Index (MITAX), 36-item Short Form Health Survey Physical Component Score (SF-36 PCS), 36-item Short Form Health Survey Mental Component Score (SF-36 MCS), and Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-F). Conventional therapy, belimumab treatment schedule, and follow-up data were recorded.

Abbreviations: IMNM, immune-mediated necrotizing myopathy; SRP, signal recognition particle; HMGCR, 3-hydroxy-3-methylglutaryl-coA-reductase; RTX, rituximab; BAFF, B-cell-activating factor; IIM, idiopathic inflammatory myopathies.

Retrospective Review of Patients With Anti-SRP IMNM Treated With Anti-B-Cell Therapies at a Single Center

We retrospectively reviewed all the medical records of patients in our institution between September 2014 and June 2021. Patients treated with anti-B-cell therapy for anti-SRP IMNM were included. All the subjects meet the 119th ENMC or 224th ENMC classification criteria for IMNM (1, 17).

Clinical characteristics, treatment schedules, and follow-up data were recorded.

Refractory was defined as disease worsening after treatment with high-dose glucocorticoids (equivalent of prednisone 1.0 mg/kg/day for at least 1 month) and at least one immunosuppressant (including methotrexate, azathioprine, and mycophenolate mofetil) or intravenous immunoglobulin.

A Literature Review of Patients With Anti-SRP IMNM Treated With Anti-B-Cell Therapy

We searched in PubMed, Web of Science, Embase, and Cochrane for all cases of anti-SRP IMNM treated with anti-B-cell therapy, until June 2021. All items of anti-B-cell agents that have been presented in Cochrane were included in the study; these include the following: RTX, rituxan, mabthera, ofatumumab, GA101, ofatumumab, inotuzumab, SM03, eprattuzumab, belimumab, LY2127399, imalumab, VAY736, tabalumab, AMBER, isatuximab, SAR650984, daratumumab, dara, or MOR202. The disease was searched with "exp Neuromuscular Disease/or (neuromuscular disease or neuromuscular disorder or muscular disease or muscular disorder or muscle disease).tw. or exp Muscular Disease/or exp Myositis/or (myotoni dystroph, myotoni disorder, muscular dystroph, myopath, myotonia congenita, or paramyotonia congenita).tw. or (periodic paralysis or central core disease or mitochondrial cytopath).mp. or glycogen storage disease, glycogen storage disorder, fatty oxidation disorder, inflammatory myopathy, polymyositis, dermatomyositis, inclusion body myositis, or endocrine myopathy).mp." and "anti-srp.mp. or anti-signal recognition particle. or signal recognition particle.mp."

Inclusion criteria are as follows: (1) adults >18 years of age, (2) following the 119th ENMC or 224th ENMC classification criteria

for IMNM, and (3) the patient tested positive for anti-SRP antibodies. Patients with other myopathy diseases were excluded from the study.

RESULTS

Belimumab Treatment in a Patient With Relapsing Anti-SRP IMNM

A 47-year-old woman presented with upper and lower extremity weakness. Elevated creatinine kinase (CK) and positive antinuclear antibodies (ANA) and anti-SRP antibodies were identified. Other autoantibodies, including anti-Sm, anti-RNP, anti-SSA/Ro, anti-SSB/La, anti-topoisomerase 1, anti-hystidyl-tRNA synthetase, anti-ribosomal P, and anti-chromatin, were negative. A muscle biopsy showed scattered necrotic muscle fibers. The patient was diagnosed with immune-mediated necrotizing myopathy and she began to receive prednisone at a dose of 50 mg/day and methotrexate at a dose of 15 mg once weekly. The patient responded well to the treatment, and the dose of prednisone was gradually tapered to 10 mg/day in 1 year.

Seventeen months later, muscle weakness recurred and creatinine kinase increased again. The patient was administered cyclosporine 75 mg twice daily, combined with methotrexate and prednisone. Creatinine kinase decreased but did not return to the normal range, and muscle weakness persisted. The patient was hospitalized for a second relapse of the disease 7 months later. Belimumab was added at a dose of 10 mg/kg once every 2 weeks for 6 weeks, followed by 10 mg/kg once a month. Meanwhile, the dose of prednisone was changed to 60 mg once a day as well as methotrexate at a dose of 12.5 mg once a week. The patient showed a good response and tolerance to this combination therapy, and no adverse effects were noted with the use of belimumab. All scores, including MYOACT, MITAX, SF-36 PCS, SF-36 MCS, and FACIT-F, improved after belimumab therapy (**Table 1**). Twenty-three weeks later, the CK level of the patient decreased to normal and was maintained while the dose of

TABLE 1 | Biochemical variables and scale scores in response to different methods of immunosuppression.

	Onset time of treatment	First flare of the disease	Second flare of the disease	Onset time of belimumab	2 Weeks after belimumab treatment	5 Weeks after belimumab treatment	13 Weeks after belimumab treatment	23 Weeks after belimumab treatment	28 Weeks after belimumab treatment
	December 12, 2017	March 18, 2019	November 28, 2019	January 04, 2020	January 21, 2020	February 10, 2020	April 06, 2020	June 15, 2020	July 20, 2020
	MTX, predinisone	MTX, predinisone, cyclosporine		MTX, predinisone, belimumab					
Biochemical variable[a]									
Creatinine kinase (IU/L)	1,529[b]	1,417[c]	4,850	1,073	1,544	1,446	545	131	119
LDH (IU/L)	395	439	613	493	509	537	494	229	213
HBDH (IU/L)	316	378	492	413	445	435	432	190	177
Count of B cells (cell/μl)				534				438	
ANA	1:10,000			1:3,200				1:1,000	
Anti-SRP	+							Negative	
Disease activity by scale scores									
MYOACT				6.2/60				2.1/60	1.9/60
MITAX				9/63				5/63	5/63
MDI-Muscle Severity				6.6/110				1.8/110	1.3/110
MDI-Muscle Extent				1/38				1/38	1/38
Health-related quality of life (mean (SD) g)									
SF36-MCS				81.1				86.6	86.6
SF36-PCS				44.8				61.8	61.8
FACIT-F				14				12	12
HAQ				0.4				0.1	0.1

MTX, methotrexate; LDH, lactate dehydrogenase; HBDH, hydroxybutyrate dehydrogenase; MYOACT, Myositis Disease Activity Assessment Visual Analog Scale; MITAX, Myositis Intention-to-Treat Activity Index; MDI, Myositis Damage Index; SF-36 PCS, 36-item Short-Form Health Survey Physical Component Score; SF-36 MCS, 36-item Short-Form Health Survey Mental Component Score; FACIT-F, The Functional Assessment of Chronic Illness Therapy-Fatigue; HAQ, Health Assessment Questionnaire.

[a]*The reference ranges for the biochemical variables are as follows: for creatinine kinase, 19 to 226 IU/L; for LDH, 120 to 250 IU/L; for HBDH, 72 to 182 IU/L; and for count of B cell, 175–332 cells/μl.*

[b]*After treatment, strength and creatine kinase returned to normalization.*

[c]*After treatment, creatinine kinase decreased but not back to normal range and muscle weakness persisted.*

prednisone was gradually tapered to 12.5 mg once a day. The anti-SRP antibody test results were negative.

Retrospective Review of Patients With Anti-SRP IMNM Treated With Anti-B-Cell Therapies in a Single Center

A total of 112 patients with anti-SRP IMNM who visited our department between September 2014 and June 2021 were reviewed. Only three patients were treated with RTX (anti-B-cell therapy). The first patient was a refractory case and received RTX six times (a dose of 500 mg/week for 2 weeks, then repeated 1 month later and 500 mg, two times, 1 year apart). The symptoms persisted. The other two patients responded to RTX; however, herpes zoster developed in the third patient after the first infusion and the treatment was discontinued. Patients' characteristics, laboratory data, treatment schedules, and outcomes are presented in **Table 2**.

A Literature Review of Patients With Anti-SRP IMNM Treated With Anti-B-Cell Therapy

A total of 124 articles were identified from the database. After excluding articles that are not written in English or patients who matched the exclusion criteria, 15 articles were finally selected. Twenty patients with anti-SRP IMNM and RTX treatment from case reports and case series were reviewed, and five retrospective studies were included. Details are summarized in **Tables 3**, **4**.

DISCUSSION

To our knowledge, this is the first case of belimumab in anti-SRP IMNM. The patient showed good response and tolerance to belimumab.

There are still no randomized trials or large enough case series to make formal recommendations for IMNM treatment. Based on the European Neuromuscular Center workshop, corticosteroids are considered the first-line treatment (1). High-dose corticosteroids should be used immediately upon diagnosis. For patients with an incomplete response to corticosteroid monotherapy or multisystem involvement, second-line treatments are warranted; these include methotrexate, azathioprine, and mycophenolate mofetil. In some cases, cyclosporine and tacrolimus may be used as adjuncts. In addition to conventional immunosuppression, IVIg is considered an effective treatment for initial therapy, especially in anti-HMGCR myopathy (1).

In our study, we reviewed all B-cell therapies for IMNM, and only RTX was identified to be effective (1–12, 18–21). B-cell depletion therapy with RTX in anti-SRP IMNM is commonly effective. As our study showed, all patients from case reports and case series showed a decline in CK, while three patients relapsed during tapering. In addition, five patients developed infections after RTX therapy, and one patient died from pneumonia and congestive heart failure. From the reported literature, it was gathered that most patients responded; in one study, however, only half of patients achieved remission (1). The author of the study supposed that a low ratio of remission might be related to the delay in RTX use. Despite a lack of rigorous clinical trials, considerable experience has demonstrated that anti-B-cell therapy might be effective for patients with IMNM.

Although belimumab has never been reported for use in IMNM treatment, the important role of BAFF or B-lymphocyte stimulator in the pathogenesis of idiopathic inflammatory myopathies (IIM) has been demonstrated in previous studies (22–24). In a study by Yuan, 10 of 29 patients with refractory anti-SRP IMNM showed positive BAFF in necrotic tissue regenerated muscle fibers and individual lymphocytes, while BAFF receptor was found in 24 of 29 patients. Moreover, refractory patients with anti-SRP IMNM had more BAFF receptors than nonrefractory patients. These findings suggest that BAFF and its receptors may participate in muscle fiber injury (22).

The efficacy and safety of belimumab in other autoimmune diseases have been evaluated in randomized clinical trials. The BLISS trial, a randomized, double-blind, placebo-controlled trial, demonstrated the efficacy of belimumab in SLE (13). The BLISS-LN study, a multicenter, randomized, double-blind trial included 448 patients with lupus nephritis. At week 104, primary responses occurred more often in the belimumab group than in the placebo group. Infection and infestation occurred in 15 of 224 patients in the belimumab group and 18 of 224 patients,

TABLE 2 | Retrospective review of patients with anti-SRP IMNM treated with anti-B-cell therapies in a single center.

Patient No./ Age/ Gender	Severe symptoms	Strength prior to RTX[a]	Strength after RTX[a]	CK prior to RTX (IU/L)	CK after RTX (IU/L)	Other outcomes	RTX treatment schedule	Cointerventions	Adverse event
1/40/M	None	2/5	2/5	1,851	1,014	None	2 doses of 500 mg/weekly, repeated 1 month later	CsA, Pred	None
2/57/M	Dysphagia, cardiomyopathy	3/5	5/5	5,811	390	Improvement of myocardial markers	2 doses of 100 mg/weekly, and 2 doses of 500/weekly 1 month later	Pred, CTX	None
3/54/M	Dysphagia, cardiomyopathy	4/5	4/5	7,238	134	Improvement of myocardial markers	1 dose of 100 mg	MMF, IVIG	Herpes zoster infection

MTX, methotrexate; IVIG, intravenous immunoglobulin; CTX, cyclophosphamide; MMF, mycophenolate mofetil; CsA, cyclosporine A; Pred, prednisone; RTX, rituximab.
[a]*Strength was evaluated with MRC score.*

TABLE 3 | Case reports and case series of anti-SRP IMNM patients treated with anti-B-cell therapies.

Study (year)	Age/ Gender	Severe symptoms	Prior treatment	RTX treatment schedule	Cointervention	Outcome	Adverse effects
Mazeda et al. (2021) (2)	76/F	Dysphagia	Pred, IVIG	One treatment A		Rapid symptomatic improvement	N/A
Ying et al. (2020) (3)	34/F	EN, dysphagia	MP	100 mg on Day 0, then 500 mg on Day1	MP	Decline in CK and improvement in strength	N/A
Mehta et al. (2019) (4)	30/F	15-week gestation	MP, IVIG,AZA, RTX repeated every 6 months until pregnancy	One infusion	Pred	Decline in CK and improvement in strength	N/A
Novoa Medina et al. (2018) (5)	30/F		CS, IVIG, AZA	1 treatment, repeated 6 months later	MTX	Clinical remission, but relapsed with MTX tapering	N/A
Komiya et al. (2018) (6)	71/M	Dysphagia, lymphoma	CS, IVIG, tacrolimus	R-CHOP therapy every 3 weeks for 6 cycles and an additional 2 cycles	Pred, CTX, DEX, VCR	Complete remission	N/A
Mamarabadi et al. (2018) (7)	28/F	Dysphagia	CS, IVIG	One treatment B for 5 times, repeated every 6 months	CS, IVIG	Decline in CK and improvement in strength	N/A
Valiyil et al. (2010) (8)	20/F		CS, AZA, MTX	1 treatment A	MTX and Pred	Decline in CK and improvement in strength	N/A
	34/F	Dysphagia	Pred, MTX, AZA, IVIG	1 treatment A	PE 5 times	Decline in CK and improvement in strength	N/A
	44/F		CS, MTX, MMF	1 treatment A, repeated 6 months later and 1 infusion 8 months later	CS	Decline in CK and improvement in strength	Facial abscess 1 month after initial dosing
	72/M	Dysphagia	CS, IVIG, PE	1 infusion		Decline in CK	Pneumonia and congestive heart failure, died 1 month later
	21/F		CS, MTX	One treatment A		Decline in CK and improvement in strength	Herpes zoster infection 3 months later
	26/F	Dysphagia	CS, IVIG, MTX, and MMF	1 treatment A		Decline in CK and improvement in strength	N/A
	51/M		Pred, MTX, MMF	1 treatment A		Decline in CK	N/A
	32/F		Pred, AZA, MTX, IVIG	1 treatment A		Decline in CK and improvement in strength	N/A
Fernandes das Neves et al. (2015) (9)	50/F	Dyspnea	MTX, CTX, IVIG, Pred.	1 treatment A, repeated every 6 months	CTX, Pred	Clinical remission	N/A
Curtin (2016) (10)	54/M		CS, IVIG	1 treatment A	CTX, Pred	Decline in CK and improvement in strength	N/A
Whelan and Isenberg (2009) (11)	44/F		CS, AZA, MTX	1 treatment A	MP and CTX	Decline in CK, relapsed 3 months later	Herpes zoster infection
	41/F		AZA, MTX, IVIG, MMF	1 treatment A		Decline in CK and improvement in strength	N/A
Arlet (2006) (12)	20/M		CS, IVIG, CS,PE, CsA, CTX, MMF	1 treatment B for 4 times, repeated every 4 months for 3 times	Pred, PE	Symptomatic improvement, but then relapsed 6 months after second infusion	A flare of hepatitis B with delta coinfection after the 2nd single additional infusion
	24/F		Pred, IVIG, MTX, AZA, PE, CTX	1 treatment B for 4 times and every 4 months	Pred	Decline in CK and improvement in strength	N/A

EN, erythema nodosum; AZA, azathioprine; CK, creatinine kinase; CTX, cyclophosphamide; IMNM, immune-mediated necrotizing myopathy; IVIG, intravenous immunoglobulin; MTX, methotrexate; N/A, information not available; PE, plasma exchange; RTX, rituximab; Pred, prednisone; CsA, cyclosporine A; CS, glucocorticoid; DEX, dexamethasone; MMF, mycophenolate mofetil.

Treatment A protocol: two doses of 1,000 mg, 2 weeks apart. Treatment B protocol: one dose of 375 mg/m^2 weekly. One infusion: one dose of 1,000 mg.

while the number of infection-associated deaths were equal to the two groups (three patients in each group) (25). In a bicentric prospective 1-year open-label trial on Sjogren's syndrome, patients achieved improvement in several aspects, including disease activity index, dryness, fatigue, and VAS scores. Only one of 30 patients suffered from a severe adverse event (pneumococcus meningitis) (14). A multicenter, double-blind, placebo-controlled trial on the efficacy and safety of belimumab in IIMs is ongoing by Northwell Health (NCT02347891).

Consistent with previous reports, not only improvement of disease activity but also a decline in anti-SRP antibodies was observed in our case. Some evidence has demonstrated that

TABLE 4 | Literature review of studies on anti-SRP IMNM patients treated with anti-B-cell therapies.

Study (year)	Population	No. of anti-SRP IMNM patients treated with RTX	Study design	RTX schedule	Outcome
Benveniste et al. (2011) (18)	8 anti-SRP IMNM/ PM	4/8	R	Not specified	3 patients significantly improved in strength, 1 patient slightly improved in strength.
Pinal-Fernandez (2017) (19)	37 anti-SRP IMNM	21/37	R	Not specified	13 patients responded[b]; 4 patients could not be evaluated.
Needham (2016) (20)	20 IMNM (2 anti-SRP IMNM)	1/2	R	Not specified	The patient responded very well but relapsed with prednisone weaning.
Allenbach et al. (2018) (1)	18 IMNM	18 IMNM[a]	R	1 g D1 and D14 followed by a median of 4 infusions (1 g each, ranging from 1 to 10)	Remission was obtained in 9 patients.
De Visser (2019) (21)	64 IMNM (15 with anti-SRP antibodies)	3 IMNM[a]	R	Not specified	N/A

R, retrospective study.

[a]*Not specified for anti-SRP IMNM.*

[b]*Response is defined as strength increased 2 points or CK levels declined by 10-fold within 6 months.*

anti-SRP antibodies may participate in the pathogenesis of IMNM by triggering an immune reaction, resulting in the release of myotoxic cytokines (8, 22, 26). *In vitro*, positive SRP was found on the plasma membrane of cultured myoblast cells stained with anti-SRP serum (27). In animal models, muscle weakness was observed in C57/Bl6 or Rag2-deficient or complement 3-deficient mice after passive IgG transfer from patients with anti-SRP IMNM (28). Moreover, SRP protein was identified in the muscle of anti-SRP IMNM patients *via* colabeling with the transsarcolemmal protein dysferlin and sarcoplasmic neural cell adhesion molecule, respectively, and further cellular experiments demonstrated exposed SRP protein localized at the surface of myotubes (29).

There are some limitations to this case: since this is the first case describing belimumab in IMNM, more cases and studies are needed to confirm the effects. Based on this case, we observed that belimumab was effective in IMNM associated with anti-SRP antibodies and suggest that belimumab might be option for severe cases.

CONCLUSION

Belimumab improved the clinical condition of our patient without any severe adverse events. In the review of the records of our center and literature, B-cell therapy with RTX benefited some patients with anti-SRP IMNM, but at the same time, increased the risk of infection. In conclusion, the present study demonstrates that belimumab in association with steroids might be an encouraging option for refractory/relapsing cases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors contributed to one or more of the following aspects of the manuscript: conception, acquisition of data, drafting, and revising the article. All authors contributed to the article and approved the submitted version.

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A Personalized Rituximab Retreatment Approach Based on Clinical and B-Cell Biomarkers in ANCA-Associated Vasculitis

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Background: Time to relapse after rituximab for the treatment of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is variable, and optimal retreatment strategy has remained unclear. In AAV following rituximab induction, the study objective was to evaluate clinical and B-cell predictors of relapse in order to develop a retreatment algorithm.

Methods: A retrospective observational study was conducted in 70 rituximab-treated ANCA-associated vasculitis patients followed up for over 10 years. Complete response (CR) was defined as Birmingham Vasculitis Activity Score v3.0 = 0. Retreatment was given on clinical relapse, defined as new features or worsening of persistent disease (not by biomarker status). Peripheral B-cell subsets were measured using highly sensitive flow cytometry. Predictors were tested using multivariable Cox regression.

Results: Median time to retreatment for cycles 1–5 were 84, 73, 67, 60, and 73 weeks. Over 467 patient-years follow-up, 158 relapses occurred in 60 patients; 16 (in 15 patients) were major (renal = 7, neurological = 4, ENT = 3, and respiratory = 2). The major-relapse rate was 3.4/100 patient-years. In multivariable analysis, concomitant immunosuppressant [HR, 0.48 (95% CI, 0.24–0.94)], achieving CR [0.24 (0.12–0.50)], and naïve B-cell repopulation at 6 months [0.43 (0.22–0.84)] were associated with longer time to relapse. Personalized retreatment using these three predictors in this cohort would have avoided an unnecessary fixed retreatment in 24% of patients. Area under the receiver operating characteristic for prediction of time to relapse was greater if guided by naïve B-cell repopulation than if previously evaluated ANCA and/or $CD19^+$ cells return at 6 months had been used, 0.82 and 0.53, respectively.

Conclusion: Our findings suggest that all patients should be coprescribed oral immunosuppressant. Those with incomplete response or with absent naïve B cells should be retreated at 6 months. Patients with complete response and naïve

repopulation should not receive fixed retreatment. This algorithm could reduce unnecessary retreatment and warrant investigation in clinical trials.

Keywords: B cell, rituximab, cyclophosphamide, immunoglobulin, vasculitis

INTRODUCTION

Rituximab, a chimeric anti-CD20 monoclonal antibody is licensed for remission induction of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). However, the majority of patients with AAV experience a clinical relapse following this initial induction and repeat cycles of rituximab are required for maintenance of remission (1–6). There is a need to establish an optimal long-term strategy that is effective and safe for rituximab-treated patients in AAV.

Three strategies have been proposed. (i) Fixed retreatment, which may vary internationally either using 500 mg × 2 infusions followed by 500 mg infusion every 6 months or 1,000 mg infusion every 4 months or 1,000 mg infusion every 6 months for 18 months (7, 8), with this regimen extended to 5 years in patients at higher risk of relapse (9). This is associated with low rates of relapse but may lead to hypogammaglobulinemia and serious infection; an effect that we showed was exacerbated if cyclophosphamide had also been previously used and which predicted severe infection (2, 10–13). (ii) Retreatment-on-clinical relapse. We have used this strategy and demonstrated low rates of hypogammaglobuliemia and a longer time to relapse of between 6 months and 4 years. However, this may permit severe disease flares and consequent glucocorticoid exposure (3). (iii) Retreatment according to biomarkers. This aims to avoid both problems by retreating according to predicted time to relapse.

Biomarker-led retreatment was investigated in the MAINRITSAN2 study using $CD19^+$ cells or ANCA to trigger repeat cycles. However, this biomarker-led protocol resulted in numerically more relapses compared with fixed retreatment, 14/81 and 8/81 patients, respectively, but this difference was described as not statistically significant (14). Surprisingly, 11/19 (58%) patients with no B-cell return experienced ≥1 relapse while only 11/142 (8%) patients with B cells detected on at least one occasion had relapsed ($p < 0.001$ in *post-hoc* analysis).

The MAINRITSAN2 biomarker-led protocol used $CD19^+$ cells as a pharmacodynamic and pharmacokinetic marker to guide an intention for perpetual absence of B cells. However, the association between $CD19^+$ return and clinical relapse in this trial was indeed counterintuitive. More recent data have given a more nuanced picture of B-cell monitoring that explains the results from that trial. Analysis of B-cell subsets reveals disease-specific signatures. Systemic lupus erythematosus (SLE), for example, is characterized by expansion of plasmablast numbers in proportion to autoantibody repertoire (15, 16), while in contrast, AAV is characterized by naïve lymphopenia in proportion to CRP (3). In both these diseases, we showed that analysis of B-cell subsets in early repopulation after rituximab using highly sensitive flow cytometry (HSFC) can identify these signatures and guide retreatment decisions. Accordingly, in SLE, early plasmablast repopulation predicts early relapse. In AAV, repopulation of naïve B cells (which are the majority of cells detected by a $CD19^+$ assay), is in fact a *good* prognostic marker for sustained response. Whereas failure to repopulate naïve B cells at 6 months is a sign of disease-specific B-cell activity and heralds early relapse (3).

Since our original publication (3), we have gathered data in more rituximab-treated patients with longer follow-up. Retreatment continued to be prescribed according to clinical relapse, enabling us to further evaluate clinical and B-cell relapse predictors. The objectives of the present study were to validate early naïve B-cell repopulation as a relapse biomarker in a second cohort and evaluate other predictors using multivariable analysis (MVA) in this larger cohort with a view to developing a proposal for a more effective personalized retreatment algorithm in AAV treated with rituximab induction.

METHODS AND MATERIALS

Patients and Design

A retrospective observational cohort study was conducted of the first 1,000 consecutive rituximab-treated patients with any rheumatological diagnosis in a single center between January 2006 and July 2020. Inclusion criteria were adults (≥18 years old) and fulfilling the Chapel Hill Consensus Conference definitions of systemic vasculitides (17). Exclusion criteria were no clinical and/or B-cell data in cycle 1 (C1) rituximab and receiving repeat cycles in C2 based on fixed-retreatment strategy.

Leeds (West) Research Ethics Committee (REC) confirmed that ethical approval was not required because all treatment decisions were made before evaluation of data, in accordance with the National Health Service (NHS) REC guidelines. The use of off-label rituximab prior to its licensing was approved by Leeds Teaching Hospitals NHS Trust Drug and Therapeutic Committee. To compare baseline B-cell data, results were compared with pre-existing disease controls in rheumatoid arthritis (RA) ($N = 62$) (18) and SLE ($N = 89$) (16) as previously published.

Treatment

All patients received a first cycle of therapy consisting of 100 mg of methylprednisolone and 1,000 mg of MabThera® on days 1 and 14. Further cycles consisted of the same regimen repeated on clinical relapse (defined below). Continuation of a stable dose or reduction of concomitant immunosuppressant, including oral prednisolone was left to clinicians' discretion, aiming to stop glucocorticoid if remission was achieved at 6 months. Concomitant cyclophosphamide was used in 5/60 (8.3%) patients with severe organ-threatening AAV.

Clinical Data and Outcomes

Disease activity was assessed at baseline and every 3 months postrituximab using Birmingham Vasculitis Activity Score (BVAS) version 3.0 (19) without knowledge of B-cell results. Complete response (CR) was defined as BVAS = 0 while partial response (PR) was defined as clinically significant improvement of disease activity without fulfilling the criteria for CR. Relapse was defined as new, reappearance, or worsening of persistent disease (i.e., BVAS increasing by ≥1).

Laboratory Measures

ANCA staining pattern was determined by indirect immunofluorescence, its antigen specificity for myeloperoxidase (MPO) or proteinase-3 (PR3) by Bioplex 2200 Immunoassay and immunoglobulin titers were measured by nephelometry at baseline and every 6 months posttherapy at routine NHS laboratory.

Peripheral blood B-cell subsets (naïve, memory, and plasmablast cells) were quantified using HSFC as a part of routine clinical practice in our department at an accredited Leeds Haematological Malignancy Diagnostic Service clinical laboratory as previously described (20) at weeks 0, 6, 26, and 52 and at clinical relapse without knowledge of clinical status other than time since rituximab. Naïve B cells were defined as $CD19^+CD27^-CD14^-CD3^-$ mononuclear cells. Memory B cells were defined as $CD19^+CD27^+CD38^-CD14^-CD3^-$ mononuclear cells (excluding cells gated as plasmablasts). Plasmablasts were defined as $CD19^{+/-}CD27^+CD38^{++}CD14^-CD3^-$ mononuclear cells. CD45 was used to calculate absolute cell count. Complete B-cell depletion was defined as a sum of all three subsets below the limit of detection ($<0.0001 \times 10^9$ cells/L for a white cell count of 5.0×10^9/L) and repopulation as counts above this level.

To compare these HSFC data with a conventional CD19 flow cytometry protocol, $CD19^+$ cell count was calculated as the sum of naïve and memory B cells. Detectable CD19 was defined as counts ≥16 cells/μl, a limit of detection typically reported in conventional flow cytometry studies (21).

Statistical Analyses

At rituximab baseline, peripheral B-cell subsets were compared between patients with AAV, RA, and SLE using Kruskal-Wallis for multiple comparison followed by Mann-Whitney *U* test. For the prediction of clinical relapse in cycle 1 rituximab, multiple imputation by chained equations was used to estimate missing data, and twenty multiple imputation sets were used to provide stability of results. In MVA, only variables with $p < 0.20$ in UVA and two other variables of interest (i.e., concomitant immunosuppressant and BVAS score at baseline) were analysed. The proportional hazard assumption was tested by examining the Kaplan-Meier curves and the Schoenfeld residuals plots. Cox proportional hazards regression was performed using backward elimination, with $p < 0.20$ associated with the deviance used for exclusion from the model. Survival analyses for the categorically distributed biomarkers were calculated using Kaplan-Meier plot and log-rank test.

Receiver operator characteristic (ROC) curves were used to compare the predictive strength of time to relapse using biomarkers between naïve B-cell repopulation and the protocol used in MAINRITSAN2, new or reappearance of ANCA as measured using indirect immunofluorescence or increased titer by at least doubling of either anti-PR3 or anti-MPO antibody and/or $CD19^+$ cells return (14) at 6 and 12 months post-therapy. All statistical analysis was performed using Stata MP version 16 and Graph Pad Prism version 8 for Windows.

RESULTS

Patient and Treatment Characteristics

The flow chart of participant is illustrated in **Figure 1**. A total of 80/1,000 patients had a diagnosis of AAV. Of these, 70 were included in the analysis (published discovery cohort = 35; validation cohort = 35). Four patients were excluded as they were retreated using 6 monthly retreatment following remisison induction due to organ-threatening manifestations while another 6 had no complete baseline data since their care was transferred to our unit later on during rituximab therapy.

Baseline characteristics of the 70 patients with AAV are described in **Table 1**. There was no difference in salient baseline clinical characteristics and laboratory measures apart from slight predominant Caucasians in the validation cohort compared with the published discovery cohort (94.3% vs. 80%, respectively).

A total of 282 rituximab cycles were administered during a total follow-up of 535.3 patient-years (PYs). Median (IQR) duration of follow-up per patient was 7.1 years (4.5–11.1).

Clinical Response

A high rate of clinical response (PR or CR) at 6 months were observed; rates for cycles C1–5 were 68/70 (97.1%), 55/57 (96.5%), 36/41 (87.8%), 24/27 (88.9%), and 18/20 (90%), respectively.

The duration of response in rituximab responders was considerably longer than 26 weeks; median (range) time-to-rituximab retreatment for C1–5 were 84 weeks (39–402), 73 weeks (39–246), 67 weeks (38–156), 60 weeks (40–196), and 73 weeks (42–263), respectively, thus indicating that a 6-month interval for fixed-schedule dosing is unnecessarily short for the majority of patients.

Details about long-term efficacy and safety of retreatment on clinical relapse strategy are described in the **Online Supplementary File**, **Figure S1** and **Table S2**.

Relapse

In C1, 59/70 (84.3%) patients had experienced a clinical relapse. We next analyzed relapse episodes in the first five rituximab cycles since these would roughly equate to the number of courses given in the fixed-schedule dosing group in the MAINRITSAN protocol.

In C1–5 with a follow-up of 467 PYs, there were 158 relapse episodes in 60 patients. Of these, 16 were major relapses in 15 patients (renal = 7; neurology = 4; ears, nose, and throat (ENT) = 3; respiratory = 2) (**Online Supplementary Table S1**). The rate of major relapse was 3.4/100 PY. The majority of major relapses were retreated with rituximab and glucocorticoids apart from two

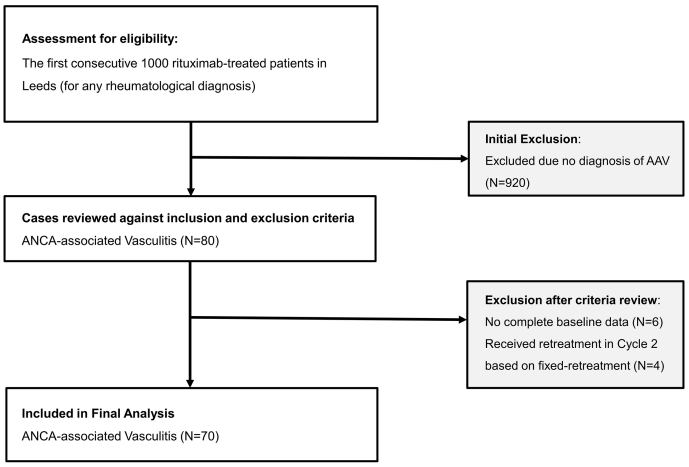

FIGURE 1 | Flow chart of participant into the study.

patients who were treated with intravenous cyclophosphamide and one with plasma exchange.

Comparison of B-Cell Signatures Across Three Diseases at Rituximab Initiation

Prior to first rituximab infusion, naïve and plasmablast cells differed between AAV, RA, and SLE groups ($p < 0.001$ and $p = 0.018$, respectively) using Kruskal-Wallis test. The data from this larger AAV cohort reconfirmed our previous finding, that active AAV is associated with naïve lymphopenia, and this effect is stronger if CRP was raised (i.e., ≥10 mg/L; $p = 0.031$). Naïve B cells were also lower in active AAV compared with RA and SLE **(Figure 2A)**. While there was no difference in memory B cell between the three diseases ($p = 0.172$) **(Figure 2B)**, plasmablasts were higher in SLE than active AAV ($p = 0.006$) **(Figure 2C)**.

Validation of Naïve B-Cell Repopulation as a Biomarker of Longer Time to Relapse

The published discovery cohort included 32/35 AAV patients with complete B-cell data (3). In this validation cohort, 25/35 subsequent and consecutive patients with B-cell data available were analyzed. Similar to the discovery cohort **(Figure 2D)**, the Kaplan-Meier survival analysis showed a significant association between repopulation of naïve B cells at 6 months and longer time to relapse ($p = 0.003$) in this validation cohort **(Figure 2E)**.

Predictors of Time to Relapse to First-Cycle Rituximab

Baseline and 6-month variables were analyzed as predictors of relapse in patients who responded to rituximab. Complete B-cell data were available in 57/70 patients. In imputed MVA, concomitant immunosuppressant HR [0.48 (95% CI, 0.24–0.94)], achieving CR at 6 months [0.24 (0.12–0.50)], and naïve repopulation at 6 months [0.43 (0.22–0.84)] were associated with longer time to relapse. Higher baseline memory B cells were associated with shorter time to relapse [1.01 (1.00–1.02)] **(Table 2)**.

Comparison of Relapse Prediction Based on Naïve Repopulation Versus ANCA and/or CD19+ Cell Return at 6 Months

In order to estimate the likelihood effectiveness of different personalized treatment strategies, we compared the accuracy of relapse prediction based on ANCA and/or total $CD19^+$ cell return (according to a conventional flow cytometry protocol) as per MAINRITSAN2 versus prediction based on absent naïve B cells using HSFC. At 6 months postrituximab, the proportion of patients with anti-PR3/anti-MPO positivity had reduced from 50/70 (71.4%) to 24/70 (34.3%) ($p < 0.001$). No patient had new or worsening of ANCA titers. Only 3/57 (5.3%) patients had detectable $CD19^+$ cells based on conventional flow cytometry whereas $CD19^+$ cells were detected in 31/57 (54.4%) if enumerated using HSFC.

Using HSFC, patients with naïve B-cell repopulation at 6 months had longer time to relapse compared with those without naïve repopulation ($p < 0.001$) **(Figure 3A)**. Relapse rates at 12 and 18 months were 2/24 (8%) and 4/24 (17%) with naïve repopulation at 6 months and 13/33 (39%) and 20/33 (61%) without naïve repopulation. In contrast, there was no difference in time to relapse between those with or without ANCA and/or $CD19^+$ return at 6 months ($p = 0.534$), although the analysis was limited by only 3/48 patients in the former **(Figure 3B)**.

The area under the ROC (AUROC) curve for time to relapse was greater for naïve B-cell repopulation using HSFC compared with absence of ANCA and/or $CD19^+$ return using conventional flow cytometry at 6 months, 0.82 (95% CI, 0.71–0.93) and 0.53 (0.26–0.80), respectively **(Figure 3C)**.

Comparison of Relapse Prediction Based on Naïve Repopulation Versus ANCA and/or $CD19^+$ Cells Return at 12 Months

Of 59/70 patients who had a clinical relapse in cycle 1 rituximab, 44/59 had not yet relapsed at 12 months. Data for B cells and ANCA were available in 37/44 patients for analysis.

TABLE 1 | Characteristics/measures of 70 AAV patients at first rituximab infusion.

Characteristics or measures	Discovery cohort (*N* = 35)	Validation cohort (*N* = 35)	Total cohort (*N* = 70)
Age [mean (SD) years]	51 (16.9)	53 (20.2)	52 (18.5)
Male [*N* (%)]	19 (54.3)	19 (54.3)	38 (54.3)
Ethnicity [*N* (%)]			
Caucasian	28 (80.0)	33 (94.3)	61 (87.1)
South Asian	5 (14.2)	2 (5.7)	7 (10.1)
Chinese/South East Asian	1 (2.9)	0	1 (1.4)
Mixed race	1 (2.9)	0	1 (1.4)
Disease duration [median (IQR) years]	2.2 (0.9–5.3)	1.9 (0.4–3.5)	2 (0.6–4.4)
Disease type [*N* (%)]			
Granulomatosis with polyangiitis (GPA)	29 (82.9)	22 (62.9)	51 (72.9)
Microscopic polyangiitis (MPA)	6 (17.1)	10 (28.6)	16 (22.9)
Eosinophilic granulomatosis with polyangiitis (EGPA)	0	3 (8.6)	3 (4.3)
Positive ANCA at diagnosis [No. (%)]	34 (97.1)	30 (85.7)	64 (91.4)
Anti-PR3 antibody	25 (71.4)	19 (54.3)	44 (62.9)
Anti-MPO antibody	5 (14.3)	10 (28.6)	15 (21.4)
Immunofluoresence only	4 (11.4)	1 (2.9)	5 (7.1)
Negative but with a positive histology of GPA/EGPA	1 (2.9)	5 (14.3)	6 (8.6)
Positive anti-PR3/anti-MPO at cycle 1 rituximab infusion [*N* (%)]	26 (74.3)	25 (71.4)	51 (72.9)
Prior/concomitant therapy with cyclophosphamide [*N* (%)]	32 (91.4)	30 (85.7)	62 (88.6)
No. of prior immunosuppressant failure (including Cyclophosphamide and plasma exchange but excluding steroid) [median (range)]	2 (0–5)	2 (0–4)	2 (0–5)
Concomitant immunosuppressant/started within 3 months of cycle 1 rituximab infusion [*N* (%)]	23 (65.7)	23 (65.7)	46 (65.7)
Methotrexate	6 (17.1)	4 (11.4)	10 (14.3)
Azathioprine	8 (22.9)	11 (31.4)	19 (27.1)
Mycophenolate mofetil	9 (25.7)	6 (17.1)	15 (21.4)
Cyclophosphamide[a]	2 (5.7)	3 (8.6)	5 (7.1)
Tacrolimus	0	1 (2.9)	1 (1.4)
Concomitant oral prednisolone [*N* (%)]	30 (85.7)	32 (91.4)	62 (88.6)
Oral prednisolone dose [mean (SD), mg/day]	13 (9.6)	23 (13.3)	18 (12.6)
Organ system involvement [*N* (%)]			
Ear, nose, and throat (ENT)	25 (71.4)	23 (65.7)	48 (68.6)
Musculoskeletal and general	20 (57.1)	22 (62.9)	21 (58.3)
Chest	16 (45.7)	17 (48.6)	33 (47.1)
Renal	12 (34.3)	13 (37.1)	25 (35.7)
Mucocutaneous	8 (22.9)	6 (17.1)	14 (20)
Nervous system	3 (8.6)	6 (17.1)	9 (12.9)
Eyes	6 (17.1)	3 (8.6)	9 (12.9)
Abdominal	1 (2.9)	1 (2.9)	2 (2.9)
BVAS 3.0 score [mean (SD)]	10.5 (5.9)	11.5 (5.5)	11 (5.7)
VDI score (median (range)]	0 (0–5)	1 (0–5)	1 (0–5)
Immunoglobulin level [mean (SD), g/dl]			
IgM (normal range, 0.5–2.0 g/L)	0.95 (0.67)	0.91 (0.85)	0.93 (0.76)
IgA (normal range, 0.8–4.0 g/L)	2.22 (1.35)	1.73 (0.79)	1.97 (1.13)
IgG (normal range, 6.0–16.0 g/L)	10.03 (4.92)	8.86 (3.86)	9.44 (4.43)
Lymphocyte count [mean (SD), $\times 10^9$/L] (normal range 1.00–4.50)	1.35 (0.65)	1.10 (0.63)	1.2 (0.6)
Total B cells [median (IQR), $\times 10^9$ cells/L]	0.0402 (0.0181–0.0835)	0.0512 (0.0144–0.1741)	0.0410 (0.0160–0.1200)
Naïve B cells [median (IQR), $\times 10^9$ cells/L]	0.0259 (0.0086–0.0540)	0.0275 (0.0060–0.1021)	0.0259 (0.0075–0.0782)
Memory B cells [median (IQR), $\times 10^9$ cells/L]	0.0148 (0.0057–0.0331)	0.0129 (0.0045–0.0358)	0.0132 (0.0055–0.0344)
Plasmablasts [median (IQR), $\times 10^9$ cells/L]	0.0021 (0.0011–0.0032)	0.0014 (0–0.0033)	0.0018 (0.0007–0.0032)
CRP [mean (SD), mg/L]	29.1 (37.4)	27.1 (37.5)	28.1 (37.2)
Total B-cell counts [median (interquartile range), $\times 10^9$ cells/L]			
Group 1: Patients without concomitant oral immunosuppressant	0.0519 (0.0713)	0.0584 (0.2244)	0.0551 (0.1115)
Group 2: Patients with concomitant oral immunosuppressant	0.0370 (0.0641)	0.0362 (0.1582)	0.0369 (0.0789)
Difference between groups	$p = 0.899$	$p = 0.232$	$p = 0.509$
Total B-cell counts [median (interquartile range), $\times 10^9$ cells/L]			
Group 1: Patients without concomitant oral prednisolone	0.0445 (0.0399)	0.1708 (0.1923)	0.0583 (0.1338)
Group 2: Patients with concomitant oral prednisolone	0.0402 (0.0804)	0.0362 (0.1511)	0.0399 (0.1070)
Difference between groups	$p = 0.659$	$p = 0.226$	$p = 0.171$

BVAS, Birmingham Vasculitis Activity Score version 3.0; IS, immunosuppressant; rituximab, rituximab; VDI, Vasculitis Damage Index.

[a]*Combination of rituximab and 2–4 pulses of intravenous cyclophosphamide were administered for remission induction of severe AAV to 5 patients with critical subglottic stenosis (N = 3), renal involvement with rapidly rising serum creatinine (N = 1), and probable cardiac involvement (N = 1).*

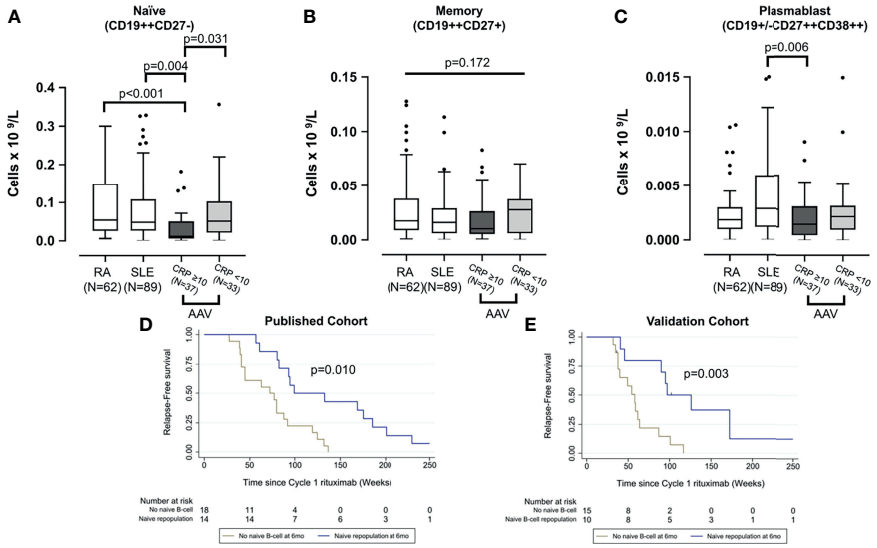

FIGURE 2 | Comparison of peripheral B-cell subsets across three diseases and validation of B-cell biomarkers of relapse. B-cell subsets including naïve **(A)**, memory **(B)**, and plasmablast **(C)** were compared between patients with rheumatoid arthritis, systemic lupus erythematosus, and AAV at rituximab initiation. The latter was divided into those with and without severe systemic inflammation; raised CRP (i.e., >10 mg/L). The box plots denote median, and the error bars represent Tukeys. Analyses were performed using Kruskal-Wallis followed by Mann-Whitney *U* test. Naïve B-cell repopulation at 6 months as a biomarker of later relapse was analyzed using Kaplan-Meier survival analysis in both the published discovery cohort **(D)** and the validation cohort **(E)**.

At 12 months postrituximab, only 11/37 (30%) patients had either reappearance of ANCA or increased titer by at least doubling of either anti-PR3 or anti-MPO antibody. A total of 12/37 (32%) patients had $CD19^+$ cells detectable as defined by a conventional cytometry protocol. The total number of patients with ANCA and/or $CD19^+$ cell return was 20/37. Using HSFC, 28/37 patients had naïve B-cell repopulation and 9/37 lacked naïve repopulation at 12 months. Of 11/37 patients with reappearance of ANCA or increased antibody titer, 9/11 had naïve repopulation at 12 months.

TABLE 2 | Factors associated with time to relapse to first cycle rituximab.

Risk factors	Univariable analysis HR (95% CI); *p*-values (with multiple imputation)	Multivariable analysis (MVA) HR (95% CI); *p*-values (with multiple imputation)
Baseline clinical/serological characteristics		
Age at rituximab initiation (per 10 years)	1.01 (0.86–1.17); $p = 0.954$	Not included in MVA
Female	1.15 (0.65–2.02); $p = 0.629$	Not included in MVA
Disease duration at rituximab initiation (years)	1.06 (0.98–1.15); $p = 0.160$	Included in MVA but removed from final model as $p < 0.20$
Concomitant immunosuppressant	0.69 (0.39–1.22); $p = 0.205$	**0.48 (0.24–0.94); $p = 0.034$**
Positive ANCA immunofluorescence	0.89 (0.46–1.71); $p = 0.725$	Not included in MVA
Positive anti-PR3/anti-MPO at rituximab initiation	0.57 (0.31–1.06); $p = 0.077$	Included in MVA but removed from final model as $p < 0.20$
CRP at ri initiation (mg/L)	1.00 (0.99–1.01); $p = 0.456$	Not included in MVA
BVAS 3.0 per point score	0.99 (0.94–1.05); $p = 0.763$	Included in MVA but removed from final model as $p < 0.20$
VDI per point score	1.14 (0.87–1.50); $p = 0.353$	Not included in MVA
Clinical and serological characteristics at 26 weeks		
Complete response	**0.34 (0.19–0.61); p<0.001**	**0.24 (0.12–0.50); p<0.001**
Positive ANCA immunofluorescence	0.99 (0.56–1.75); $p = 0.962$	Not included in MVA
Positive anti-PR3/anti-MPO	0.79 (0.44–1.42); $p = 0.426$	Not included in MVA
CRP (mg/L)	0.99 (0.97–1.02); $p = 0.618$	Not included in MVA
B-cell subsets, depletion, and repopulation		
Naïve B cells at rituximab initiation ($\times 10^9$/L)[a]	1.00 (1.00–1.01); $p = 0.797$	Not included in MVA
Memory B cells at rituximab initiation ($\times 10^9$/L)[a]	**1.01 (1.00–1.02); $p = 0.040$**	**1.01 (1.00–1.02); $p = 0.045$**
Plasmablasts at rituximab initiation ($\times 10^9$/L)[a]	1.04 (0.94–1.16); $p = 0.459$	Not included in MVA
Complete depletion at 6 weeks postrituximab	0.90 (0.50–1.61); $p = 0.721$	Not included in MVA
Naïve B-cell repopulation at 26 weeks	**0.38 (0.19–0.76); $p = 0.006$**	**0.43 (0.22–0.84); $p = 0.013$**
Memory B-cell repopulation at 26 weeks	**0.45 (0.20–0.99); $p = 0.046$**	Included in MVA but removed from final model as $p < 0.20$
Plasmablast cell repopulation at 26 weeks	1.14 (0.61–2.13); $p = 0.675$	Not included in MVA

[a]*(Count $\times 10^9$ cells/L) for each subset multiplied by 1,000 prior to analysis.*

The bold values denote variables which are statistically significant in the analyses.

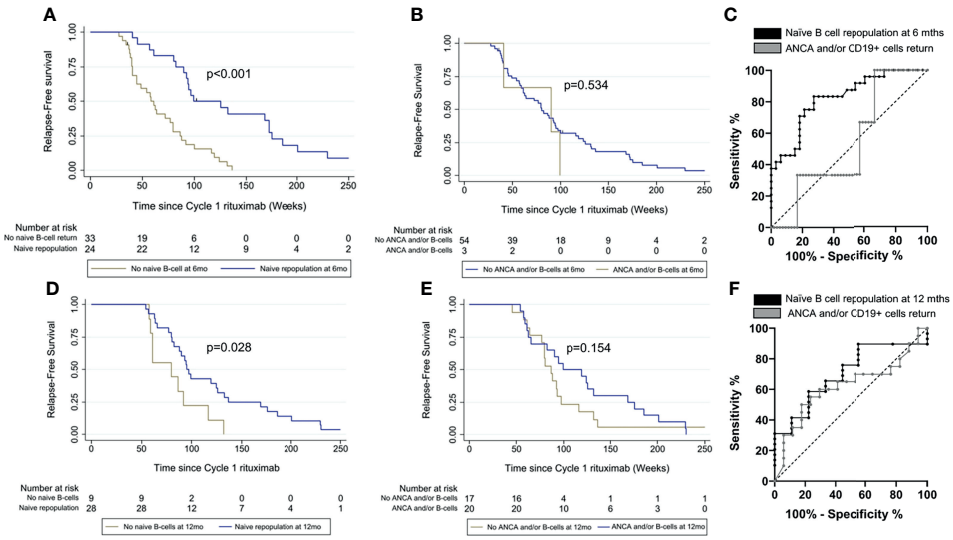

FIGURE 3 | Comparison of relapse prediction based on naïve B cells and ANCA and/or CD19+ cell return. Time to relapse was compared between patients with and without naïve repopulation in **(A)** at 6 months and **(D)** at 12 months and between patients with and without ANCA and/or CD19+ cells return in **(B)** at 6 months and **(E)** at 12 months respectively using Kaplan-Meier survival analyses. Areas under the receiver operating characteristic (AUROC) were compared between the two biomarker-led retreatment strategies **(C)** at 6 months and **(F)** at 12 months postrituximab.

Using HSFC, patients with naïve B-cell repopulation at 12 months had longer time to relapse compared with those without naïve repopulation ($p = 0.028$) **(Figure 3D)**. Relapse rates at 18 and 24 months were 6/28 (21%) and 16/28 (57%) with naïve repopulation and 4/9 (44%) and 7/9 (78%) without repopulation at 12 months. There was no association between ANCA and/or $CD19^+$ cells return and longer time to relapse ($p = 0.154$) **(Figure 3E)**.

The AUROC for time to relapse was greater for naïve B-cell repopulation using HSFC compared with absence of ANCA and/or $CD19^+$ return using conventional flow protocol at 12 months, 0.70 (95% CI, 0.52–0.88) and 0.62 (0.43–0.80), respectively **(Figure 3F).**

Proposed Algorithm for Personalized Rituximab Retreatment Based on Clinical and B-Cell Biomarkers

Based on the results above, we propose an algorithm for personalized rituximab retreatment as illustrated in **Figure 4**. Our data suggest that the key decisions for sustained response at 6 months are as follows: (i) use of concomitant immunosuppressants; (ii) retreatment if clinical response is incomplete; and (iii) retreatment if naïve B-cell repopulation is not detected. In our cohort, this would have led to retreatment at 6 months in 47/62 (76%) of patients, with the remainder not requiring fixed

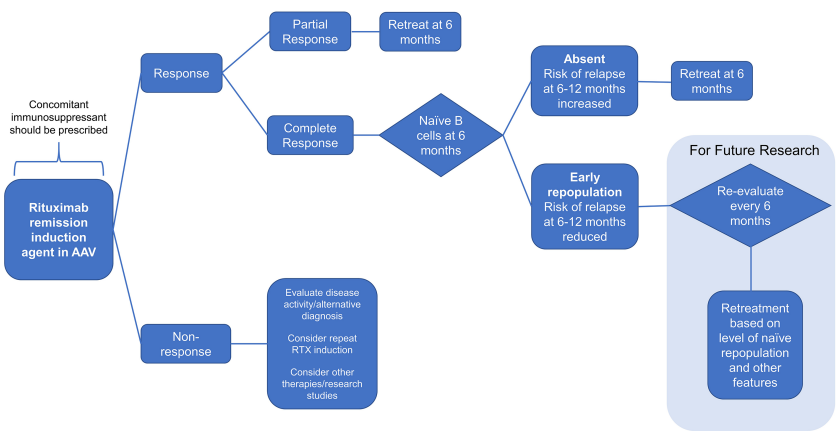

FIGURE 4 | Flow diagram. A proposal for personalized rituximab retreatment algorithm based on clinical predictors and early naïve B-cell return in ANCA-associated vasculitis.

retreatment. Further research is needed to characterize the use of clinical and B-cell biomarkers beyond the 6-month time-point as our sample size is currently insufficient to address this question.

DISCUSSION

In this study, we further characterized the use of naïve B cell (as enumerated by HSFC) as a disease-specific biomarker to guide rituximab retreatment decisions in AAV alongside new data on clinical predictors of relapse.

Our key finding, that B-cell return was associated with sustained response, may initially appear counterintuitive. However, repopulation of nonautoimmune B cells is desirable and would lead to the observed repopulation with naïve cells (22). Naïve B cells are produced by the bone marrow constantly and will become detectable as soon as the serum concentration of rituximab becomes too low to kill them. Repopulation of naïve B cells is an expected and healthy outcome of rituximab therapy and does not indicate recrudescence of autoimmunity (23, 24). Other factors in B-cell homeostasis and function may also be considered in understanding our results. We only monitor B cells in peripheral blood, but in fact these cells traffic between bone marrow, inflamed tissues, and secondary lymphoid tissues. The numbers measured in blood may not correlate with these other sites, which are perhaps more clinically relevant (25). Next, some investigators have proposed that IL-10-producing regulatory B cells may be important in autoimmunity. $CD19^{+}CD24^{hi}CD38^{hi}27^{-}$ cells are regulatory B cells which have previously been identified within the transitional B-cell subsets. This is part of the naïve B-cell gate that we associate with maintenance of remission in AAV (26).

The predictive value of naïve B-cell repopulation in relapse prediction may not only be useful at 6 months but also predict outcomes at 12 months postrituximab if retreatment was not already given. Despite having only a small number of patients without naïve repopulation at 12 months available for comparison (i.e., the majority of those without naïve repopulation at 6 months had been retreated within 12 months of rituximab), our results still showed that repopulation and higher naïve B-cell numbers were associated with longer time to relapse. This finding requires further work to confirm, which will be done in future analyses of our cohort. Although higher baseline memory B cells were predictive of shorter time to relapse in MVA, its effect size was the smallest compared with the other three significant predictors in the model.

A few studies have reported predictors of relapse to rituximab, but these data were analyzed using cohort treated with mixed retreatment strategies (27, 28). Using retreatment-on-relapse strategy, our cohort is unique and valuable for discovery of novel biomarkers and other clinical predictors of relapse. First, patients who were coprescribed immunosuppressant had longer time to relapse. A previous randomized controlled trial supported the continuation of immunosuppressant for long-term remission maintenance and improved renal survival in AAV (29). Consistent with this, concomitant immunosuppressant has been shown to prolong duration of response in randomized studies in other B-cell-mediated diseases like RA (30, 31). Second, patients with incomplete response had earlier relapse, suggesting they should have early retreatment, both to prevent relapse and to improve their level of response. The latter point is consistent with data from RA, in which patients with incomplete or nonresponse to a first cycle of rituximab had improved response after retreatment at 6 months (18, 32). Third, in the current study, no added value of ANCA monitoring up to 12 months was found, since no patient had changes in ANCA at 6 months while the majority of patients with ANCA changes at 12 months (i.e., 9/11) also had naïve repopulation. Our data are therefore consistent with previous reports on the limited value of ANCA in guiding retreatment (33, 34).

Herein, we therefore propose a personalized rituximab retreatment regimen; that all patients should be coprescribed an oral immunosuppressant with rituximab therapy; patients with PR at 6 months should be retreated pre-emptively with rituximab at 6 months; and patients with CR at 6 months and no repopulation of naïve B cell at 6 months receive retreatment at 6 months. Patients with CR and naïve B-cell return at 6 months should not receive fixed retreatment and should be monitored for a further 6 months. Applying this algorithm to our own cohort would have avoided an unnecessary fixed retreatment in 24% of patients without allowing those patients to relapse in the subsequent 6 months.

This study has some limitations. First, B-cell data were missing for a small number of patients due to their nonattendance for review at the 6-month time-point. As these were deemed missing at random, multiple imputation was used to reduce potential bias in parameter estimation as well as enhancing generalizability of the results. Second, concomitant immunosuppressant was used in about two-thirds of the patients, thus efficacy could not be attributed to rituximab alone. Concomitant immunosuppressants showed an association with time to relapse but were not prescribed in a randomized fashion. Importantly, there was no difference in either lymphocyte or B-cell numbers between those with and without concomitant immunosuppressant at rituximab baseline. Third, 73% of our patients had granulomatosis with polyangiitis (GPA), hence our proposed algorithm may not be generalized to those with microscopic polyangiitis or eosinophilic GPA predominant. Fourth, the remission induction agent used in this study was rituximab. Our results therefore cannot be generalized to patients who received cyclophosphamide induction followed by rituximab maintenance. Lastly, in terms of clinical applicability, we acknowledge that B cells are not routinely measured in every department. If only complete remission at 6 months was used for predicting relapse, this algorithm would avoid retreatment at 6 months in 38/70 (54%) patients but with 7/38 (18%) relapse rate at 12 months postrituximab. Therefore, our results showed the added value of naïve B-cell monitoring in reducing frequency of retreatment without allowing those patients to relapse within 12 months of rituximab therapy. Future health economic studies will ascertain the cost-effectiveness of B-cell monitoring in AAV patients treated with rituximab.

In conclusion, this observational study has led to a proposal for a rituximab retreatment algorithm that should be evaluated in interventional trials.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JA, EV, PE, and MYMY: substantial contributions to the conception and design of the work, the acquisition, analysis, and interpretation of data, drafting the work or revising it critically for important intellectual content, and final approval of the version published. SD, AA, AR, and SS: substantial contributions to the acquisition and interpretation of data, drafting the work or revising it critically for important intellectual content, and final approval of the version published. All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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.2021.803175/full#supplementary-material

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

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SYSTEMATIC REVIEW
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Systematic Review of Safety and Efficacy of Second- and Third-Generation CD20-Targeting Biologics in Treating Immune-Mediated Disorders

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Background: B cells can contribute to immune-mediated disorders. Targeting CD20 has proved to be efficacious in several B cell-mediated immunopathologies, as illustrated by the use of rituximab, the first anti-CD20 monoclonal antibody (mAb). Following rituximab, second- and third-generation anti-CD20 mAbs have been developed and tried in immune-mediated diseases, including obinutuzumab, ocrelizumab, ofatumumab, ublituximab, and veltuzumab. However, their safety and efficacy has not been systematically reviewed.

Objective: To evaluate safety and efficacy of obinutuzumab, ocrelizumab, ofatumumab, ublituximab, and veltuzumab for the treatment of immune-mediated disorders compared to placebo, conventional treatment or other biologics.

Methods: The PRISMA checklist guided the reporting of the data. We searched the PubMed database between 4 October 2016 and 22 July 2021 concentrating on immune-mediated disorders.

Results: The literature search identified 2220 articles. After screening titles and abstracts against the inclusion and exclusion criteria and assessing full texts, 27 articles were finally included in a narrative synthesis.

Conclusions: Obinutuzumab has shown promising results in a case series of patients with phospholipase A_2 receptor-associated membranous nephropathy and mixed results in systemic lupus erythematosus. Ocrelizumab has been approved for the use in patients with relapsing-remitting multiple sclerosis and primary progressive multiple sclerosis. Ocrelizumab was also tested in patients with rheumatoid arthritis, demonstrating promising results, and in systemic lupus erythematosus, revealing mixed results; however, in these conditions, its use was associated with increased risk of serious

infections. Ofatumumab received approval for treating patients with relapsing-remitting multiple sclerosis. Moreover, ofatumumab showed promising results in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis, rheumatoid arthritis, and systemic lupus erythematosus, as well as mixed results in phospholipase A_2 receptor-associated membranous nephropathy. Ublituximab was assessed in relapsing-remitting multiple sclerosis and neuromyelitis optica spectrum disorder, with promising results, however, the included number of patients was too small to conclude. Veltuzumab was tested in patients with immune thrombocytopenia resulting in improved platelet counts.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/, identifier CRD4201913421.

Keywords: obinutuzumab, ocrelizumab, ofatumumab, ublituximab, veltzumab, immune-mediated diseases, systemic lupus erythematosus, multiple sclerosis

INTRODUCTION

Most polygenic immune-mediated disorders, including autoimmune and chronic-inflammatory diseases, result from an imbalance of activating versus regulatory immune effector pathways (1). In certain autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE), such immune dysregulation is characterized by activated B cell responses. Dysregulated B cell responses can result in the production of autoantibodies, as typically seen in SLE and RA, or they can contribute to activation of autoreactive T cells without evidence of autoantibody production, as observed in MS (2). Traditional therapies of immune-mediated disorders, including B cell-mediated autoimmune diseases, consisted in the use of corticosteroids (also termed glucocorticoids) and immunosuppressive drugs. However, the long-term application of these treatments is hampered by an increased risk of severe infections and cutaneous malignancies as well as by corticosteroid-mediated side effects (3, 4). Starting in the 1990s, the introduction of biological agents (also called biologics or biologicals) has revolutionized the treatment of allergic, autoimmune and chronic-inflammatory disorders (5, 6). The advantage of biologics stems from their precise targeting of specific molecules, which in turn minimizes unwanted damage to off-target tissues and cells.

Abbreviations: AAV, ANCA-associated vasculitis; ACR, American College of Rheumatology; AE, adverse event; ANCA, anti-neutrophil cytoplasmic antibody; anti-PLA_2R, anti-phospholipase A_2 receptor; CD, cluster of differentiation; CDC, complement-dependent cytotoxicity; DAS28, 28-joint disease activity score; DAS28-ESR, DAS28–erythrocyte sedimentation rate; DFPP, double-filtration plasmapheresis; DMARD, disease-modifying antirheumatic drug; EDSS, expanded disability status scale; eGFR, estimated glomerular filtration rate; EMA, European Medicines Agency; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; FDA, U.S. Food and Drug Administration; Fc, fragment crystallizable; GdE, gadolinium-enhancing; IRR, infusion-related reaction; ITP, immune thrombocytopenia; IV, intravenous(ly); mAb, monoclonal antibody; MMF, mycophenolate mofetil; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; OBI, obinutuzumab; OCR, ocrelizumab; OFA, ofatumumab; PGA, patient global assessment; PLA_2R, phospholipase A_2 receptor; PPMS, primary progressive multiple sclerosis; QoL, quality of life; RA, rheumatoid arthritis; RCT, randomized controlled trial; RRMS, relapsing-remitting multiple sclerosis; RTX, rituximab; SC, subcutaneous(ly); SAE, serious adverse event; SLE, systemic lupus erythematosus; TADAI, total adjusted disease activity index; UBL, ublituximab; VEL, veltuzumab.

Also B cell-mediated immunopathologies have greatly benefitted from the advent of biologics, including monoclonal antibodies (mAbs) targeting different B cell surface molecules or survival factors of B cells (7–9). B cells can contribute to immune-mediated diseases by secreting autoantibodies, acting as antigen-presenting cells, producing cytokines, and forming ectopic lymphoid tissues (2, 10, 11). Targeting the antigen cluster of differentiation 20 (CD20) has proved to be efficacious in several B cell-mediated pathologies, as illustrated by the use of rituximab (RTX), the first anti-CD20 mAb (7, 12). Following RTX, second- and third-generation anti-CD20 mAbs have been developed, including ibritumomab tiuxetan, obinutuzumab (OBI), ocaratuzumab, ocrelizumab (OCR), ofatumumab (OFA), tositumomab, ublituximab (UBL), and veltuzumab (VEL). Notably, most of these anti-CD20 mAbs have initially been generated for the treatment of B cell malignancies (12).

CD20 is a cell surface molecule present as homodimers or homotetramers, which is expressed on B cells starting at the pre-B cell stage, whereas its expression is lost during B cell differentiation into plasmablasts and plasma cells (12–14). CD20 is thought to regulate calcium (Ca^{2+}) influx into B cells downstream of the B cell receptor. CD20-targeting mAbs act by depleting all $CD20^+$ B cell subsets, while sparing pro-B cells, plasmablasts and plasma cells (14). Thus, administration of RTX rapidly reduces the counts of circulating B cells (15), whereas tissular B cells and antibody-producing B cells are affected to a lesser extent by RTX treatment (16). Repeated use of RTX can result in hypogammaglobulinemia by decreasing serum concentrations of immunoglobulin G (IgG), particularly, when it is used in combination with other immunosuppressive agents, such as high doses of corticosteroids and mycophenolate mofetil (MMF) (17).

B cell depletion by CD20-targeting mAbs is thought to be the result of several mechanisms, such as direct apoptosis of the targeted B cells, complement-dependent cytotoxicity (CDC) of B cells, and fragment crystallizable (Fc) receptor-mediated effector functions, including antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis of B cells (12). Different anti-CD20 mAbs preferentially employ different

mechanisms of B cell depletion and modulation of CD20 molecules, with type I mAbs resulting in the redistribution of CD20 into lipid rafts and internalization, whereas type II mAbs do not appear to cause clustering of CD20 with CD20 remaining on the cell surface. Thus, the type I mAbs RTX, ocaratuzumab, OCR, OFA, UBL and VEL lead to compartmentalization of CD20 into lipid rafts and high CDC activity (12). Conversely, the type II mAbs OBI, ibritumomab tiuxetan, and tositumomab show no or little CD20 clustering and CDC activity, but instead they cause very efficient apoptosis of targeted B cells as well as antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis (18–20). In addition to its type II modality, OBI was glycoengineered to abrogate a fucose sugar residue in the Fc region, which limits its binding to complement and enhances its affinity for activating Fc γ receptors on natural killer cells and neutrophils, thus causing more efficient antibody-dependent cellular cytotoxicity of both malignant B cells and B cells from RA and SLE patients, compared to RTX (21, 22). Notably, $CD20^+$ B cells bind twice as many type I anti-CD20 mAb molecules per cell compared to type II mAbs, which is likely due to different binding modes of these mAbs (13, 23). When bound to CD20, type I mAbs form "seeding" complexes that allow the recruitment of further IgG or CD20 molecules, thus favoring efficient complement activation, whereas type II mAbs interacting with CD20 result in "terminal" complexes that prevent the association of additional type II mAbs and complement components (23).

In a previous publication, we systematically reviewed the safety and efficacy of RTX (7). The anti-CD20 mAbs OBI, OCR, OFA, UBL and VEL have been tried in immune-mediated diseases. Conversely, the murine mAbs ibritumomab tiuxetan and tositumomab, which are conjugated to radioactive yttrium-90 and iodine-131, respectively, have so far only been assessed in patients with B cell malignancies. Similarly, ocaratuzumab has been solely tested in patients with B cell malignancies. In the present article, we provide a systematic review of the current available studies assessing the safety and efficacy of the second- and third-generation anti-CD20 mAbs OBI, OCR, OFA, UBL and VEL in immune-mediated diseases.

METHODS

Study Design and Protocol Registration

The PRISMA checklist (**Table 1**) guided the reporting of this systematic review (24). We initially registered OCR and VEL on PROSPERO, and subsequently updated our protocol to also include OBI, ocaratuzumab, OFA, and UBL; PROSPERO number CRD42019134321.

Search Strategy

We searched the PubMed database and reference lists of included studies for suitable clinical trials. The search was conducted between 4 October 2016 and 22 July 2021 for OCR, VEL, OBI, and OFA. Ocaratuzumab and UBL were added during the revision process of this paper and the search was carried out on the 28th of November. Our full search strategy and research terms were defined in advance (**Table 2**). We also used filters for randomized controlled trials (RCTs). If publications were not available via institutional access or open access, study authors were contacted to receive the article or missing trial information.

Eligibility Criteria

We included RCTs, their extension trials and their substudies with predefined endpoints investigating the use of OBI, ocaratuzumab, OCR, OFA, tositumomab, UBL, and VEL in immune-mediated diseases. If RCTs were not available, we included non-randomized clinical studies with at least five patients per intervention group and case series including at least three patients, with the exception of case series stating to be retrospective. We excluded retrospective trials, posthoc-analyses, substudies without predefined endpoints, meta-analyses, reviews, and studies from registries as well as studies carried out on animal models or where the primary endpoint was non-clinical. Trials had to be available in either English or German.

We included primary immune-mediated conditions, including rare diseases. We excluded studies in hematological malignancies and allergic disorders, as they were not within the scope of this article.

Study Selection, Data Collection Process and Analysis

Three authors (CK, BW, and OB) developed and tested a data extraction sheet, whereupon two authors independently (CK and BW) searched PubMed according to the predefined search terms, checked titles and abstracts, carried out a full-text review of the selected studies, and extracted the relevant data. Any disagreements about study inclusion were resolved by consensus.

Risk of Bias Assessment

CK used a modified version of the Downs and Black tool (see **Table S1**) to assess the retrieved studies for bias (25). The studies were scored out of a maximum of 28 points for the following categories: (i) reporting, (ii) external validity, (iii) internal validity, and (iv) power, and the scores were summed and ranked high (23-28 points), medium (15-22 points) and low (0-14) quality. Any discrepancies were resolved by consensus.

As we limited our research strategy to the PubMed database, the reference list of these studies, and the expertise of the authors involved, we did not conduct a risk of bias assessment across the studies, as we believed the risk of publication bias was high.

Principal Summary Measures and Synthesis of Results

The aim of this systematic review was to provide a structured and complete overview of the current available studies assessing the safety and efficacy of OBI, OCR, OFA, tositumomab, UBL, and VEL as well as their influence on quality of life (QoL) when used in immune-mediated diseases. Since we wanted to give an overview we did not specify in more detail these endpoints in order not to exclude potentially important studies.

TABLE 1 | The preferred reporting of systematic reviews and meta-analyses (PRISMA) checklist.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2-3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	2-3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	3
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	3
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	3
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	3
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	3
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	3
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	3
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	3
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	3
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	3
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	NA
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	4
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	4-11
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see Item 12).	11
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	4-11
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	NA
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	NA
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	11-12
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	12-14
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	NA

RESULTS

Study Selection and Characteristics

The PubMed search resulted in 2220 articles. We screened 192 of them for title and abstract and, finally, 27 publications were included in the systematic review (**Figure 1**). The main characteristics are available in **Table S2**.

Synthesized Findings

Obinutuzumab

Membranous Nephropathy

Our systematic literature search revealed one prospective case series using OBI in three patients with phospholipase A_2 receptor (PLA_2R)-mediated membranous nephropathy who had previously been refractory to treatment with RTX (26). The duration of the study

TABLE 2 | Search terms.

01.	obinutuzumab	633
02.	obinutuzumab AND ITP	1
03.	obinutuzumab AND immune	82
04.	obinutuzumab AND thrombocytopenia	26
05.	obinutzumab AND vasculitis	3
06.	ocrelizumab	531
07.	ocrelizumab AND ITP	0
08.	ocrelizumab AND immune	118
09.	ocrelizumab AND thrombocytopenia	1
10.	ocrelizumab AND rheumatoid arthritis	33
11.	ocrelizumab AND rheumatoid arthritis; Filters: Randomized Controlled Trial	5
12.	ocrelizumab AND multiple sclerosis	435
13.	ocrelizumab AND multiple sclerosis; Filters: Randomized Controlled Trial	14
14.	ocrelizumab AND lupus	24
15.	ocrelizumab AND lupus; Filters: Randomized Controlled Trial	2
16.	ofatumumab	627
17.	ofatumumab AND lupus	20
18.	ofatumumab AND nephritis	16
19.	ofatumumab AND SLE	8
20.	ofatumumab AND multiple sclerosis	78
21.	ofatumumab AND multiple sclerosis; Filters: Randomized Controlled Trial	3
22.	ofatumumab AND rheumatoid arthritis	27
23.	ofatumumab AND rheumatoid arthritis; Filters: Randomized Controlled Trial	3
24.	ofatumumab AND vasculitis	7
25.	ofatumumab AND ANCA	4
26.	tositumomab	348
27.	veltuzumab	51
28.	veltuzumab AND pemphigus vulgaris	5
29.	veltuzumab AND pemphigus	5
30.	veltuzumab AND immune thrombocytopenia	5
31.	veltuzumab AND thrombocytopenia	5
32.	veltuzumab AND multiple sclerosis	1
33.	veltuzumab AND rheumatoid arthritis	3
34.	veltuzumab AND arthritis	3
35.	veltuzumab AND SLE	1
36.	veltuzumab AND lupus	4
37.	ublituximab	33
38.	ocaratuzumab	11

was 27 months for the first two cases and 30 months for the third case. The article does not mention the source of funding of the study.

The first patient (case 1, 54-year old white woman) presented with nephrotic syndrome, diagnosed as PLA_2R-associated membranous nephropathy based on a kidney biopsy. She showed persistently elevated anti-PLA_2R antibody titers and severe proteinuria despite a treatment with two courses (six months apart) of twice 1 g RTX. Thus, the patient was premedicated with 40 mg intravenous (IV) methylprednisone plus 25 mg oral diphenhydramine and 650 mg oral acetaminophen, followed by treatment with 1 g OBI, given 100 mg IV the first day and 900 mg IV the second day, to reduce possible infusion reactions. 12 and 18 months after treatment with OBI, the patient's anti-PLA_2R antibody titers and proteinuria became low and kept on decreasing, respectively, along with an improvement of serum albumin and serum creatinine concentrations.

The second patient (case 2, 61-year old white man) had also nephrotic syndrome, diagnosed as PLA_2R-associated membranous nephropathy based on a kidney biopsy. He was treated with cyclosporine and prednisone, which resulted in a transient improvement of anti-PLA_2R antibody titers and proteinuria. Because proteinuria continued to be severe, he was given prednisone and cyclophosphamide, which did not improve the patient's situation, followed by discontinuation of cyclophosphamide after nine months of treatment and administration of RTX. Despite these treatments, the patient showed an increase in anti-PLA_2R antibody titers and very severe proteinuria, which motivated a treatment with OBI, given 100 mg IV on day 1, 900 mg IV on day 2, and 1 g IV on day 8, along with a premedication similar to case 1. Seven and nine months after treatment with OBI, the patient's anti-PLA_2R antibody titers and proteinuria became low and kept on decreasing, respectively, along with an improvement of serum albumin and serum creatinine concentrations.

The third patient (case 3, 54-year old white man) also presented with nephrotic syndrome, diagnosed as PLA_2R-associated membranous nephropathy based on a kidney biopsy. He received a treatment with two courses (three months apart) of twice 1 g RTX, following which his anti-PLA_2R antibody titers decreased, however, his severe proteinuria remained unchanged. Thus, a treatment with OBI was initiated, given 100 mg IV on day 1, 900 mg IV on day 2, and 1 g IV on day 15. Six, 18 and 24 months after receiving OBI, the patient's anti-PLA_2R antibody titers remained undetectable and his proteinuria decreased and kept on decreasing, along with an improvement of serum albumin concentrations.

Only one adverse event was noted during treatment with OBI. Patient 3 experienced localized herpes zoster reactivation, which was managed conservatively. There were no other adverse events (AEs) or serious adverse event (SAEs).

The health-related QoL was not assessed.

Synopsis: Based on a case series of three patients with PLA_2R-associated membranous nephropathy whose disease was refractory to treatment with RTX, OBI was more efficacious than RTX in reducing proteinuria and improving serum albumine concentrations. RCTs are needed to confirm these promising results.

Systemic Lupus Erythematosus

We found one multicenter double-blind RCT comparing OBI to placebo treatment in 125 patients with SLE and proliferative lupus nephritis (27). All patients received maintenance treatment with MMF and corticosteroids. Furthermore, concomitant treatment with an antimalarial drug, angiotension-converting enzyme inhibitor, angiotensin II receptor blocker, calcium and vitamin D was allowed.

62 patients received placebo and 63 patients OBI. OBI was administered at a dose of 1000 mg on day 1, week 2, week 24, and week 26. The primary endpoint, proportion of patients with complete renal response – measured by urine protein-to-creatinine ratio of less than 0.5, normal serum creatinine and inactive urinary sediment – at 52 weeks was met more often in patients treated with OBI, but the difference was not statistically significant between OBI and placebo ($p = 0.115$). However, significantly more patients in the OBI group reached an overall renal response ($p = 0.025$). Although clinical endpoints did not differ markedly between OBI and placebo, patients receiving OBI significantly increased complement factors C3 and C4

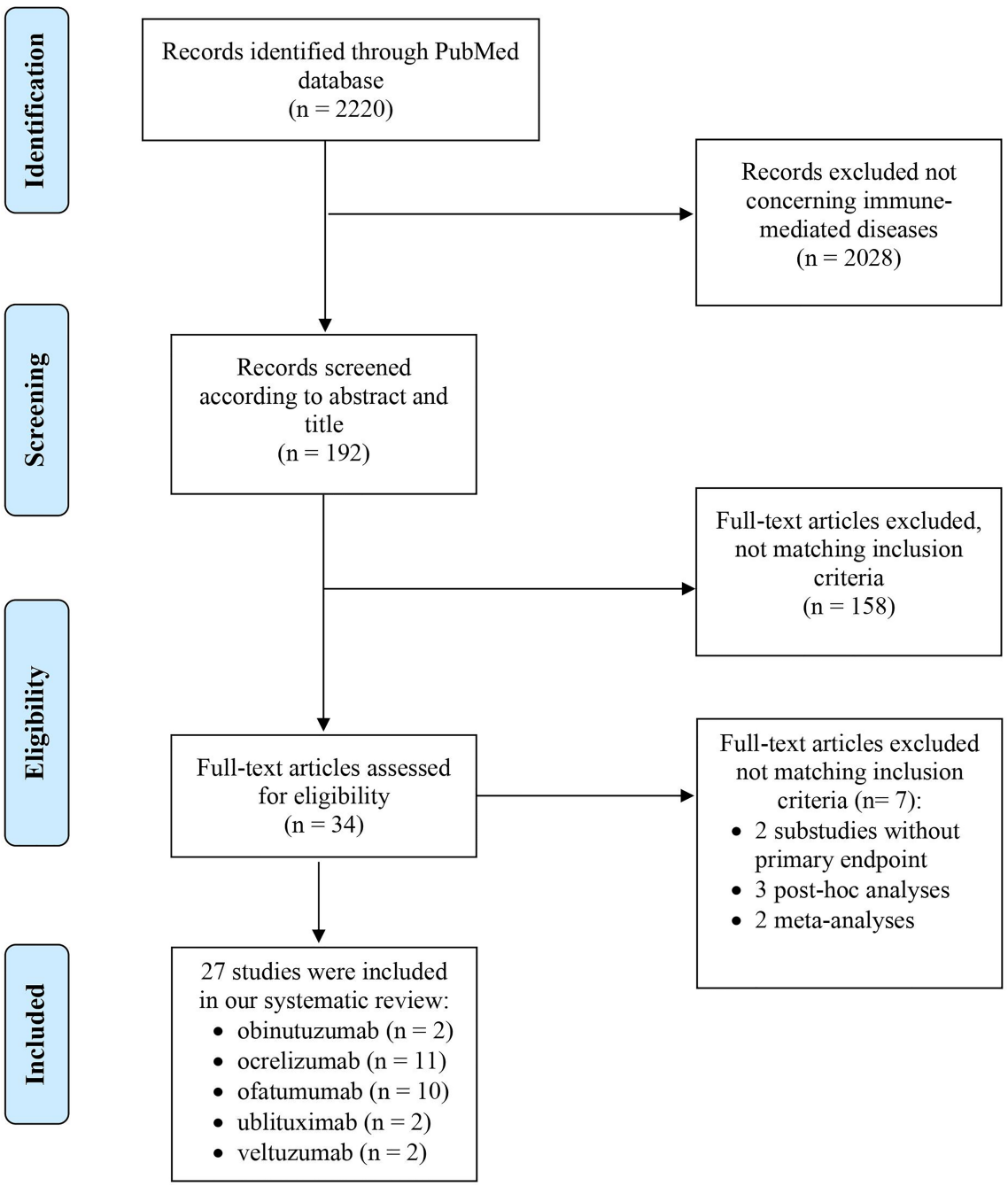

FIGURE 1 | PRISMA diagram of the literature search.

and significantly decreased titers of anti-double stranded DNA antibodies.

91% of patients receiving OBI experienced at least one AE and 25% had a SAE. There was one death in the OBI group caused by a gastrointestinal perforation. Urinary tract infection and bronchitis were the most common AEs.

Synopsis: This RCT in SLE with active lupus nephritis showed little efficacy of OBI on disease progression when compared to placebo. Further studies with different dosing regimens of OBI are needed to draw a conclusion.

Ocrelizumab

Multiple Sclerosis

We identified four double-blind placebo-controlled RCTs, one open-label extension study, and one substudy with predefined endpoints using OCR in patients suffering from MS (28–32).

Study duration varied from 24 to 192 weeks. All studies were funded by the industry.

In total 2621 patients with relapsing-remitting multiple sclerosis (RRMS) (28, 29) and 732 patients with primary progressive multiple sclerosis (PPMS) (29) were treated with either OCR or a control medication. In three of the studies diagnosis was made based on the McDonald criteria (29–31). Predefined expanded disability status scale (EDSS) had to be between 0 and 6.5 and all patients had to be at least 18 years of age.

In the first study, published in 2011, patients in the active treatment arms received IV OCR (300 mg or 1000 mg) on days 1 and 15 and again on day 1 (600 mg or 1000 mg, respectively) of the second, third, and fourth cycle (weeks 24, 48 and 72) (28). The control group was treated with matching placebo. A fourth treatment arm received open-label interferon-β-1a weekly until week 24. The placebo and interferon-β-1a arms were both offered two doses of OCR (300 mg) on days 1 and 15 of the second, third, and fourth cycle. The OPERA I and II trials used a similar treatment regimen administering 600 mg of IV OCR every 24 weeks compared to interferon β-1a (29). The only study available in patients with PPMS used the same dosing regimen for OCR compared to placebo (29). 100 mg IV methylprednisolone was given as premedication in all four studies. The primary endpoint was either the total number of gadolinium-enhancing (GdE) T1 lesions at weeks 12, 16, 20, and 24, the annualized relapse rate at week 96, the percentage of patients with a disability progression at week 12, or the proportion of infusion-related reactions (IRR). Secondary endpoints comprised the relapse rate, disability progression, proportion of relapse-free patients, safety, as well as various assessments concerning MRI lesions.

The mean number of GdE T1 lesions, the primary endpoint of the study by Kappos et al., decreased significantly as compared to placebo (28). There was an 89% reduction in the 300 mg group ($p<0.0001$) and a 96% reduction in the 1000 mg group ($p<0.0001$). Furthermore, the annualized relapse rate was significantly reduced and the total number of new and persisting GdE lesions was significantly lower in both OCR groups.

In the OPERA I and II trials, also conducted in patients with RRMS, there was also a significant reduction (46% and 47%, respectively) in the annualized relapse rate as compared to interferon-β-1a (29). Thus, the primary endpoint was achieved. Furthermore, OCR led to a significant decrease in GdE lesion on T1 MRI and a reduced number of new or newly enlarged T2 lesions. After completion of the double-blind phase, patients could enter an open-label extension trial, where OCR was administered at a dose of 600 mg every 24 weeks (32). The trial was planned for a duration of eight years, with results of the three-year follow-up available currently. Annualized relapse rates remained low in the group previously receiving OCR and continuing to receive OCR during the open-label extension phase. Moreover, there was a significant reduction in annualized relapse rates in the group receiving interferon-β-1a during the double-blind period, followed by OCR during the open-label extension phase. A significant difference between these two groups in terms of mean change in EDSS, brain atrophy, and clinical disease progression remained in the open-label extension phase. No significant differences were noted concerning the number of MRI lesions. Safety data during the extension phase were consistent with the double-blind phase.

Remarkably, results in patients with PPMS were similar to those seen in patients with RRMS. There was a significant reduction in disease progression as early as week 12 (29). The results remained significant until at least week 24. Furthermore, patients in the OCR group had a significantly smaller volume of hyperintense T2 lesions and a significantly smaller change in brain volume.

The ENSEMBLE PLUS substudy in patients with RRMS investigated the occurrence of IRRs in patients receiving OCR at a conventional infusion rate amounting to an infusion time of 3.5 hours versus a shorter infusion time of 2 hours (31). Primary endpoint was the proportion of patients with IRRs following the first dose of OCR. Although there was a slight increase in IRRs in patients receiving the shorter infusion rate, this difference was not significant and there were no serious IRRs in either group. Thus, a shorter infusion rate was considered safe.

AEs and SAEs occurred at a similar frequency in patients treated with OCR, interferon-β-1a, and placebo. Nine patients died during the studies, including two cases of suicide (29), one road-traffic accident (30), one mechanical ileus (29), one pulmonary embolism (30), one pancreatic carcinoma (30), one systemic inflammatory response syndrome of unknown cause (28), one case of pneumonia (30), and one case of aspiration (30). The number of deaths during the open-label extension study was unavailable.

The health-related QoL was assessed in neither of the studies.

Synopsis: Above-mentioned trials demonstrated a superiority of OCR above placebo and interferon-β-1a leading to the approval of the drug by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) for the use in patients with RRMS and PPMS.

Rheumatoid Arthritis

Five placebo-controlled, double-blind RCTs using OCR in patients with RA met our inclusion criteria (33–37). The study durations ranged from 48 to 104 weeks, including two trials, which were terminated early (34, 36).

2835 patients participated in either of the trials. Main inclusion criteria were diagnosis of RA according to the 1987 revised American College of Rheumatology (ACR) criteria and active disease. In most studies a minimum disease duration of three months was required for inclusion. Inflammatory joint disease other than RA and systemic involvement secondary to RA were the most common exclusion criteria (35–37).

OCR was usually given two weeks apart at doses ranging from 10 mg to 1000 mg with either concomitant methotrexate or leflunomide. In all but one trial (37), other disease-modifying antirheumatic drugs (DMARDs) had to be discontinued four weeks prior to enrollment (33–36). Premedication consisted of 100 mg IV methylprednisolone with the exception of the ACTION trial (33). All patients were allowed to use acetaminophen and an antihistamine as premedication (34–37).

Three studies assessed the ACR20 response rate as primary endpoint (34, 35, 37). In contrast, the FILM trial was planned to investigate the change in the van der Heijde-modified total Sharp score at week 104, but due to early termination this endpoint was analyzed earlier at week 52. The ACTION trial (33) analyzed safety events as primary outcome measure. Secondary endpoints comprised ACR50/70 response rates, change in the health assessment questionnaire–disability index, remission rate according to the 28-joint disease activity score (DAS28), and European League Against Rheumatism (EULAR) responses.

Rigby et al. (35), Stohl et al. (36), and Tak et al. (37), reported significant results concerning ACR20, ACR50, and ACR70 response rates as well as DAS28-erythrocyte sedimentation rate (ESR) remission rates. One of the two remaining trials reported significant ACR20 response rates in all OCR-treated patients while ACR50 response rates were only significant in two OCR arms (50 mg and 200 mg) (34). The last trial did not report any *p*-values concerning those endpoints (33).

There was no statistically significant difference in the occurrence of AEs between patients treated with OCR and patients receiving placebo. Although the STAGE and the SCRIPT studies reported comparable frequencies of SAEs and infections, the number of serious infections was elevated in patients receiving OCR leading to the early termination of two other trials (34, 36). In total 8/1951 patients receiving OCR and 5/1007 placebo-treated patients died.

Three studies assessed change in health assessment questionnaire–disability index as a marker for QoL (35–37). All three studies showed a significant improvement.

Synopsis: Although OCR led to significantly better results when assessing the ACR response rates as well as the DAS28-ESR remission rates, two studies reported increased rates in serious infections.

Systemic Lupus Erythematosus

Only one double-blind, placebo-controlled RCT assessed the efficacy of OCR in patients with SLE (38). The study lasted 96 weeks. Patients with an adequate response at week 48 continued blinded treatment, whereas patients with an inadequate response had the option of open-label treatment.

378 patients were initially enrolled. Diagnosis of SLE according to the ACR criteria with active lupus nephritis class III/IV were the main inclusion criteria. Patients with an eGFR <25ml/min were excluded. Minimum age for inclusion was 16 years.

OCR (400 mg or 1000 mg) was given on days 1 and 15 followed by a single infusion at week 16 and every 16 weeks thereafter. The control group received matching placebo. A premedication consisting of methylprednisolone, acetaminophen, and an antihistamine was given. Furthermore, all patients received concomitant treatment with MMF (3 mg/d) or cyclophosphamide (500 mg IV every 2 weeks for 6 times) followed by a maintenance therapy with azathioprine.

The proportion of patients with a renal response at week 48 was the primary endpoint and was higher in patients receiving OCR. However, the difference was not statistically significant.

83.4% of the patients receiving OCR had at least one AE, which was comparable with the 88% in the placebo group. The percentage of patients with at least one SAE was also comparable, amounting to 28.85% vs 27.2% in the OCR and placebo group, respectively. Remarkably as in patients with RA, the rate of serious infections was increased at 18.2% in patients receiving OCR vs 14.4% in placebo-treated patients, leading to early termination of the study.

Influence on QoL was not assessed.

Synopsis: OCR improved the renal response rate, however, this change was not significant when compared to placebo. OCR led to an increased rate of serious infections.

Ofatumumab

ANCA-Associated Vasculitis

There was only one case series eligible for our review. It tested the efficacy and safety of OFA in patients with ANCA-associated vasculitis (AAV) over a period of 2 years (39). Eight patients with a mean age of 52 years matched the only reported inclusion criteria being a diagnosis of AAV.

IV OFA was given at a dose of 700 mg on days 0 and 14. Concomitant treatment comprised 1 mg/kg oral prednisolone and 10 mg/kg cyclophosphamide, the latter given IV on days 0 and 14 and every 14 days thereafter. After three months, maintenance therapy with azathioprine or MMF was introduced. All patients received prophylactic co-trimoxazole for 3 months, a proton pump inhibitor, and calcium and vitamin D3 supplementation.

There were no predefined endpoints set. All patients achieved clinical remission by month 3. This was accompanied by the ability to taper corticosteroids and by a reduction in acute phase reactants. No relapse occurred during the first year of the study.

Five patients experienced an AE. None of them were considered severe AEs or SAEs.

QoL was not analyzed.

Synopsis: Currently available results seem promising, although OFA was only used in eight patients suffering from AAV. Further trials with a randomized-controlled design involving more patients are needed to confirm theses findings.

Membranous Nephropathy

We found one prospective case series publication on treatment with OFA in three patients with PLA_2R-mediated membranous nephropathy (40).

Patient 1 was a 74-year-old man suffering from nephrotic syndrome positive for anti-PLA_2R antibodies. After ineffective treatment with RTX, he was assigned to receive three cycles of double-filtration plasmapheresis (DFPP) followed by OFA. Despite the depletion of B cells, anti-PLA_2R levels remained high and the nephrotic syndrome persisted leading to end-stage renal disease.

The second patient, a 69-year-old man, experienced an anaphylactic reaction after a single RTX infusion, which was associated with a transient reduction of anti-PLA_2R antibodies. Thus, he was offered a rescue therapy with OFA and DFPP. Six days after a 100 mg OFA infusion he received three cycles of DFPP. Anti-PLA_2R titers remained low for three months but increased again to pretreatment levels after six months. Accordingly, proteinuria persisted.

The third patient, a 80-year-old man, had very high anti-PLA_2R titers and was treated with 100 mg OFA followed by 4 cycles of DFPP. During follow-up, anti-PLA_2R antibody titers decreased and were undetectable at six months. Partial remission of nephrotic syndrome was observed.

The study did not report safety data or effects on QoL.

Synopsis: The available case series included only three patients with rather negative results. Only one of three treated patients achieved partial remission of kidney disease.

Multiple Sclerosis

We identified four placebo-controlled RCTs using OFA in 1136 patients with RRMS (41–43) or secondary progressive MS (43) according to the McDonald criteria. The treatment period lasted 24, 48 weeks, and 30 months, respectively. Patients were aged between 18-55 years old and had an EDSS of 0 to 5 (41) or 5.5 (42, 43).

In the study of Sorensen et al. (41), patients received two doses OFA (100 mg, 300 mg, or 700 mg) or placebo IV two weeks apart. After 24 weeks, treatment was switched and another two infusions were administered in a blinded manner. The primary endpoint was safety. There were significant reductions noted in the number of new GdE T1 lesions, total number of GdE T1 lesions, and new and/or enlarging T2 lesions (41). However, there were no significant changes found in the EDSS score.

In the MIRROR study (42), patients received OFA 3 mg, 30 mg, or 60 mg every 12 weeks subcutaneously (SC). A fourth treatment arm received OFA 60 mg SC every four weeks. The cumulative number of new GdE lesions at week 12 was the primary endpoint and was found to be reduced by 65% in patients receiving OFA ($p<0.001$). However, there was no significant difference concerning EDSS and relapse rates (42).

The ASCLEPIOS I and II trials were multicenter RCTs conducted concurrently and following the same study design (43). 20 mg OFA were administered SC every four weeks with loading doses on days one, seven, and 14. After one month of treatment, patients were allowed to apply the medication at home. The control group received daily teriflunomide orally. Both groups received matching placebo in order to blind the study. The primary endpoint, reduction in annualized relapse rate, was achieved in both trials. For the secondary endpoints a pooled analysis of both trials was performed, which showed a significant reduction in disability worsening at three and six months, whereas there was no significant disability improvement noted. While there was a significant reduction in GdE T1 and T2 lesions in the OFA groups, the annually brain volume loss was comparable in the teriflunomide and OFA groups.

The frequency of AEs and SAEs was comparable between OFA and placebo in all studies. There was one death in the teriflunomide group of the ACLEPIOS II trial.

QoL was not analyzed.

Synopsis: Based on the available data, the FDA and EMA approved subcutaneous OFA for the treatment of patients with RRMS or secondary progressive MS.

Rheumatoid Arthritis

Four placebo-controlled RCTs (44–47) including 852 patients investigating OFA in RA patients were included in our systematic review. The main inclusion criteria were diagnosis of RA according to the ACR criteria with a minimum disease duration of six months and a patient age of at least 18 years. In all but one trial (47), disease needed to be active. Concomitant treatment with DMARDs, another autoimmune disease, and significant comorbidity were the most important exclusion criteria.

Except in the trial by Kurrasch et al. (44) where patients received a single SC dose, IV OFA was given with a dosing interval of two weeks. IV doses ranged from 300 mg to 1000 mg. All patients were allowed to receive concomitant methotrexate and oral corticosteroids at stable dosages. In all, except the SC trial (44), non-steroidal anti-inflammatory drugs, analgesics and one inter-articular injection of corticosteroids were permitted. Premedication consisted of acetaminophen, an antihistamine, and corticosteroids. Only Kurrasch et al. did not administer corticosteroids as premedication (44).

Kurrasch et al. (44) and part A of Ostergaard et al. (45) investigated safety as primary endpoint. Part B of Ostergaard et al. (45) assessed the proportion of patients with an ACR20 improvement as primary endpoint, while the extension trial explored time to treatment withdrawal. The third RCT assessed the ACR20 response rate at week 24 (47). Secondary endpoints comprised pharmacokinetics, anti-drug antibodies, EULAR responses, DAS28 response, and B cell depletion.

Both studies with available results demonstrated significantly better outcomes for OFA-treated patients ($p<0.001$ for both studies) when ACR20 was assessed. Ostergaard et al. also proved superiority in ACR50 response rates and proportion of patients with EULAR good or moderate response (45). Similarly, Taylor et al. (47) reported significantly better results concerning ACR50/70 response rates, proportion of patients with good or moderate EULAR response, and change in DAS28-ESR or DAS28–C-reactive protein. The open-label study of Quattrocchi et al. was terminated early due to the study sponsor's refocus on the investigation of SC administration and no efficacy results were available at study termination (46). Kurrasch et al. did not report markers of disease activity (44).

All four studies determined the occurrence of AEs. The incidence of AEs in OFA-treated patients ranged from 85% to 89% and in the placebo group from 55% to 62.5%. Thus, AEs occurred with a numerically but not significantly higher frequency in patients treated with OFA. SAEs occurred in 3.7%, 5%, 9.4%, 9.5%, 13%, and 20% of the OFA-treated patients compared with 0%, 0%, 3%, 5%, and 7% of the placebo treated-patients. Only one death was reported (interstitial lung disease) occurring in a patient that received 700 mg OFA.

Health-related QoL was assessed in the study of Taylor et al. (47) using scoring by FACIT-F and version 2 of the 36-Item Short Form Health Survey. For both scores significant improvements were seen in OFA-treated patients (47).

Synopsis: Available results show that OFA in combination with methotrexate is more effective than placebo treatment. There were no safety concerns. However, results from SC administered OFA are sparse and need further investigation.

Systemic Lupus Erythematosus

One study matched our inclusion criteria assessing OFA in SLE patients with refractory lupus nephritis (48). It was a case series

including four patients with initial response to RTX, however, during the course of RTX treatment, patients had developed infusion reactions and were thus treated with OFA.

IV OFA was administered at different dosing regimens. All patients received prednisolone as concomitant treatment. One patient was additionally treated with cyclosporine A and another with antimalarial drugs. No primary nor secondary endpoints were defined.

The efficacy of OFA treatment was assessed using the urine albumin-to-creatinine ratio. Although it decreased in all four patients, only one reached normalization.

The only observed AE occurring in one patient one day after OFA infusion was widespread urticaria, which caused discontinuation of OFA in that patient.

The influence on their QoL was not assessed.

Synopsis: Available results are sparse but indicate a treatment effect in SLE patients with lupus nephritis. However, RCTs involving more patients are needed to confirm these initial findings.

Ublituximab

Multiple Sclerosis

We found one study assessing UBL in 48 patients with relapsing-remitting MS, as defined by the 2010 McDonald criteria (49). Patients were randomized to receive either placebo (12 patients) or UBL (36 patients), within six cohorts treated with different doses (450 mg or 600 mg) given over 1–4 hours of infusion. The study was unblinded on day 28 and patients in the placebo group could cross over to the corresponding treatment group.

The primary endpoint, $CD19^+$ B cell depletion of at least 95%, was achieved in all patients receiving UBL. In most patients $CD19^+$ B cell depletion was achieved within 24 hours after the first dose of UBL and was maintained for up to 48 weeks. No new or persisting GdE T1 lesions were observed, however, 8 patients developed one or more new GdE T2 lesions. 93% of all patients remained relapse-free, and, overall, 74% had no evidence of disease activity.

UBL was well tolerated, with infusion-related reactions representing the most common AEs. There was only one SAE observed. No deaths were reported.

Synopsis: UBL was well tolerated and resulted in a significant reduction of circulating $CD19^+$ B cells and a reduced annualized relapse rate of MS. However, the included number of patients was too small to conclude. Moreover, future studies should compare UBL to established treatments of MS.

Neuromyelitis Optica Spectrum Disorder

One phase I open-label study tested UBL in patients with neuromyelitis optica spectrum disorder (NMOSD) (50). 5 patients with NMOSD and new neurological symptoms received an IV infusion of 450 mg UBL in addition to standard treatment with IV methylprednisolone.

The primary endpoint was safety. Secondary endpoints included efficacy and assessment of B cell counts. Efficacy was assessed by measuring the EDSS score at baseline, during relapse, at discharge, and at a 90-day follow-up visit. Overall, EDSS increased from 4.0 at baseline to 6.5 during relapse and remained high until discharge. However, it returned to 4.0 at the 90-day follow-up visit.

Only one patient experienced a SAE, which was leukopenia without corresponding symptoms or complications.

Synopsis: This small phase I study using UBL in NMOSD patients showed promising safety results. However, the currently available data on the efficacy of UBL in NMOSD are sparse and need further assessment in RCTs.

Veltuzumab

Immune Thrombocytopenia

We identified two clinical trials conducted as open-label studies without control group matching our inclusion criteria (51, 52). The study durations were 48 weeks (51) and five years (52).

91 patients were treated with VEL during either of the two trials. Patients needed to have a diagnosis of primary immune thrombocytopenia (ITP) according to the American Society of Hematology guidelines with a platelet count $<30 \times 10^9$ g/L on two separate occasions to enter the study. Marked or major bleeding were exclusion criteria.

VEL was either given IV (51) or SC (51, 52). All but one treatment arm, which received weekly VEL, was treated with two doses given two weeks apart. Single doses ranged from 80 to 320 mg. One study permitted the concomitant use of prednisone and danazol if given at stable doses (51), whereas a second trial only allowed concomitant prednisone (52). Before IV administration, antipyretics and antihistamines were given as premedication.

Both studies had no predefined primary endpoint. However, studies were planned to determine safety, efficacy, pharmacodynamics, pharmacokinetics, and immunogenicity.

Efficacy was assessed through objective response, corresponding to a platelet count of $\geq 30 \times 10^9$ g/L measured twice at least one week apart with at least two-fold increase from baseline count, and complete response, corresponding to a platelet count of $\geq 100 \times 10^9$ g/L. Of the IV treated patients 67% achieved an objective response with 33% complete responders. SC administration led to 53% and 49% objective responses and 28% and 32% complete responses in the two studies, respectively. Median time to relapse was eight months (51) and 1.3 years (52), respectively. One study also reported a bleeding reduction in all treatment groups (52).

71.4% of the IV VEL-treated patients had at least one treatment-related AE, whereas 73.5% and 78% of the SC groups had at least one AE. A total of two SAEs occurred, one in a SC treated patient (grade 3 viral gastroenteritis) and one in a patient receiving IV VEL (grade 3 hypersensitivity reaction).

Neither of the studies assessed QoL.

Synopsis: Available efficacy results of VEL treatment of 91 patients suffering from primary ITP seemed promising, with no unexpected safety events. However, both studies were conducted as open-label uncontrolled trials making the available data rather unreliable. Thus, blinded RCTs need to verify the results reported above.

Risk of Bias Assessment

We assessed the quality and risk of bias of the included studies using a modified Downs and Black checklist (**Table 3**).

TABLE 3 | Risk of bias.

	Reporting										External validity			Internal validity								Source of patients included					Power	Summary
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
OBINUTUZUMAB																												
Membranous nephropathy																												
Klomjit et al., 2020 (26)	x	o	x	x	o	x	o	o	x	–	o	o	o	o	o	o	–	o	x	x	o	o	–	–	–	o	–	7
Furie et al., 2021 (27)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	o	x	o	x	x	x	o	x	x	21
OCRELIZUMAB																												
Multiple sclerosis																												
Kappos et al., 2011 (28)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	x	x	o	o	x	x	–	x	x	22
Hauser et al., 2017 (OPERA I trial) (29)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	x	x	o	x	x	x	x	x	x	24
Montalban et al., 2017 (30)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	x	x	o	o	x	x	x	x	x	23
Rheumatoid arthritits																												
Genovese et al., 2008 (ACTION trial) (33)	x	x	x	x	x	x	–	x	o	–	o	o	o	x	x	x	x	x	o	x	o	o	x	x	–	x	–	17
Harigai et al., 2012 (34)	x	x	x	x	x	o	–	x	x	x	o	o	o	x	o	x	x	x	x	x	o	o	x	o	–	x	–	17
Rigby et al., 2012 (STAGE trial) (35)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	x	x	o	o	x	x	x	x	x	23
Stohl et al., 2012 (FILM trial) (36)	x	x	x	x	x	o	x	x	x	x	o	o	o	x	x	x	x	x	x	x	o	x	x	o	x	x	x	22
Tak et al., 2012 (SCRIPT trial) (37)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	x	x	o	o	o	o	x	x	x	21
Systemic lupus erythematosus																												
Mysler et al., 2013 (38)	x	x	x	x	x	o	x	x	x	x	o	o	o	x	o	x	x	x	x	x	o	o	o	o	x	–	–	17
OFATUMUMAB																												
ANCA-associated vasculitis																												
McAdoo et al., 2016 (39)	x	–	–	x	x	x	–	x	–	–	o	x	o	–	–	x	o	o	o	x	o	o	–	–	–	o	–	9
Multiple sclerosis																												
Sorensen et al., 2014 (41)	x	x	x	x	x	x	–	x	x	x	o	o	o	x	o	x	x	x	x	x	o	o	x	o	x	x	–	19
Bar-Or et al., 2018 (MIRROR trial) (42)	x	x	x	x	x	x	x	x	o	x	o	o	o	x	x	x	x	x	o	x	o	o	x	x	x	x	x	21
Hauser et al., 2020 (43)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	o	x	o	o	x	x	x	x	x	22
Rheumatoid arthritis																												
Ostergaard et al., 2010 (45)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	o	x	x	x	x	x	o	o	x	o	x	x	x	21
Taylor et al., 2011 (47)	x	x	x	x	x	x	x	x	x	x	o	o	o	x	x	x	x	x	x	x	o	o	x	x	x	x	x	23
Kurrasch et al., 2013 (44)	x	x	x	x	x	o	–	x	x	–	o	o	o	x	–	x	x	o	x	x	o	o	x	–	–	x	–	15
Quattrocchi et al., 2016 (46)	x	x	x	x	x	o	–	x	x	–	o	o	o	x	x	o	o	–	o	o	o	o	x	x	x	o	–	13
Quattrocchi et al., 2016 (Extension trial) (46)	x	x	x	x	x	o	–	x	x	–	o	o	o	–	–	o	o	–	o	o	o	o	–	–	x	o	–	9
Systemic lupus erythematosus																												
Haarhaus et al., 2016 (48)	x	–	–	x	–	o	–	o	–	–	o	o	o	–	–	o	o	o	o	–	o	o	–	–	–	o	–	2
UBLITUXIMAB																												
Multiple sclerosis																												
Fox et al., 2021 (49)	x	x	x	x	–	x	x	–	–	x	o	o	o	o	–	o	–	o	o	x	o	o	o	o	o	o	–	8
Neuromyelitis optica spectrum disorder																												
Mealy et al., 2019 (50)	x	–	x	–	–	–	–	–	–	–	–	–	–	–	–	o	–	o	o	–	–	o	–	–	–	o	–	2
VELTUZUMAB																												
Immune thrombocytopenia																												
Liebman et al., 2013 (51)	x	x	x	x	x	x	–	x	–	–	o	o	o	–	–	x	o	o	o	x	o	o	–	–	x	o	–	11
Liebman et al., 2016 (52)	x	x	x	x	x	x	–	x	–	–	o	o	o	–	–	x	o	o	o	x	o	o	–	–	x	o	–	11

DISCUSSION

To provide a prompt synopsis we created a table summarizing the current state of research and clinical efficacy of OBI, OCR, OFA, UBL and VEL (**Table 4**). To address safety we also created a table highlighting the AEs reported in the studies included in this systematic review (**Table 5**). However for most of the included biologics only short-term safety data were available. Long-term safety data should be obtained in future studies testing these CD20-targeting biologics and should also assess their combination with other immunosuppressive drugs. As mentioned in the introduction, the repeated use of RTX in combination with high doses of corticosteroids and MMF has been found to increase the risk of persistent hypogammaglobulinemia (17).

OBI allowed an improvement of nephrotic syndrome-grade proteinuria and of serum albumin concentrations in three patients with PLA_2R-associated membranous nephropathy refractory to treatment with RTX. Based on these promising results, RCTs using OBI in PLA_2R-associated membranous nephropathy are warranted. Moreover, OBI was tested in SLE patients with active lupus nephritis and

TABLE 4 | Summary of the evidence.

Biologic	Obinutuzumab		Ocrelizumab			Ofatumumab					Ublituximab		Veltuzumab
Disease	**PLA_2R-MN**	**SLE**	**MS**	**RA**	**SLE**	**AAV**	**MS**	**PLA_2R-MN**	**RA**	**SLE**	**MS**	**NMOSD**	**ITP**
Level I													
Level IIa													
Level IIb				*	*								
Level IIIa													
Level IIIb													
Level IV													
Too little information													

Level I Approved by the EMA and/or FDA.
Level IIa Multicentric double-blind RCTs proving a significant superiority over standard-of-care treatment.
Level IIb Multicentric double-blind RCTs proving a significant superiority over placebo.
Level IIIa Clinical study, not fulfilling the above-mentioned criteria, but proving a superiority over standard-of-care treatment.
Level IIIb Clinical study, not fulfilling the above-mentioned criteria, but proving a superiority over placebo.
Level IV Case series or open-label trials without control group with positive results.

Achieved
Failed
Mixed result

*Increased risk of serious infections.

AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration; ITP, immune thrombocytopenia; MS, multiple sclerosis; PLA_2R-MN, phospholipase A_2 receptor-associated membranous nephropathy; RA, rheumatoid arthritis; RCT, randomized controlled trial; SLE, systemic lupus erythematosus.

led to a significantly improved overall response in comparison to placebo.

OCR achieved a significant reduction of the annualized relapse rate in patients with RRMS as well as a significantly lower disease progression in PPMS patients, thus the EMA and FDA approved its administration in patients with PPMS or RRMS. OCR was further used in patients with RA leading to significant improvement of ACR rates. However, rates of serious infections were elevated with use of OCR. These safety concerns were also raised in SLE patients treated with OCR, leading to early termination of the only available RCT. Thus, a close post-marketing monitoring of MS patients treated with OCR is warranted.

For the use of OFA in patients with AAV, PLA_2R-associated membranous nephropathy, and SLE, there were only case series available. The studies in AAV and SLE showed promising results, whereas the data in patients with membranous nephropathy were rather negative. Furthermore, eight RCTs assessed the use of OFA in patients with either RRMS, secondary progressive MS, or RA where treatment with OFA resulted in a significant clinical improvement with no increased safety concerns. Thus, OFA was approved by the FDA and EMA for use in RRMS and in secondary progressive MS.

UBL was tested in a placebo-controlled RCT with MS patients and showed an improvement in frequency of T1 lesions and volume of T2 lesions. A phase I trial in patients with NMOSD showed promising safety data, whereas the trial was too small to conclude on efficacy.

VEL has only been tested in patients with ITP and showed a positive influence on platelet counts and bleeding complication in the available open-label trials.

Limitations

This is the first systematic review on the safety and efficacy of OBI, OCR, OFA, UBL and VEL in a number of immune-mediated diseases. We have used standardized systematic overview techniques, which have helped to minimize the risk of bias. Furthermore, we assessed the quality and bias of each study using a modified version of the Downs and Black checklist.

Nonetheless, our systematic review has several limitations. Firstly, we included studies with different outcome measures, inclusion criteria, concomitant treatment, premedication, control groups, and study duration, making a direct comparison difficult. Since we also considered certain case series and open-label trials, the reported results may be influenced by chance and may in consequence not be as reliable as those found by a double-blind RCTs involving more patients. Furthermore, we did not assess for risk of bias across the studies. However, we aimed to minimize the risk by double-checking the presented data as well as the inclusion of trials.

Conclusions

OBI appeared to be beneficial in three patients with PLA_2R-associated membranous nephropathy who were refractory to treatment with RTX. OBI was also tested in SLE patients with active lupus nephritis with mixed results. OCR was approved by the EMA and FDA for treatment of patients with RRMS or PPMS. Furthermore, OCR showed promising or mixed results in patients with RA or SLE, respectively, however, in these trials, OCR was associated with an increased rate of serious infections. OFA was approved by the EMA and FDA for its use in RRMS.

TABLE 5 | Adverse events.

Obinutuzumab

Organ systems affected	Adverse event(s)		Refs.
Systemic	**a) Immediate-type adverse reactions**	Infusion reaction	(27)
	b) Infection	Urinary tract infection, bronchitis, herpes zoster, upper respiratory tract infection, influenza, gastroenteritis	(26, 27)
	c) Neoplasm	None reported. Further studies in patients with immune-mediated diseases are needed to determine the frequency.	
Cardiovascular	Hypertension, peripheral edema		(27)
Gastrointestinal and hepatic	Abdominal pain, nausea, diarrhea		(27)
Hematologic events	Anemia, neutropenia		(27)
Musculoskeletal	Arthralgia		(27)
Nervous system (including eyes)	Headache, conjunctivitis, insomnia		(29, 30, 33–36, 38)
Renal	None reported. Further studies in patients with immune-mediated diseases are needed to determine the frequency.		
Upper and lower airways	Nasopharyngitis, pharyngitis, sinusitis, cough		(27)
Urogenital	Frequent urination		(27)
Skin	None reported. Further studies in patients with immune-mediated diseases are needed to determine the frequency.		

Ocrelizumab

Organ systems affected	Adverse event(s)		Refs.
Systemic	**d) Immediate-type adverse reactions**	Infusion reaction	(28–38)
	e) Infection	Upper respiratory tract infection, oral herpes, typhoid fever, urinary tract infection, urosepsis, bacterial arthritis, sepsis, septic shock	(28–38)
	f) Neoplasm	Breast cancer, cervix cancer, endometrial cancer, ovarian cancer, bladder cancer, renal-cell carcinoma, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, laryngeal cancer, lung cancer, adenocarcinoma of colon, esophageal adenocarcinoma, pancreatic carcinoma, lymphoma, malignant fibrous histiocytoma, papillary thyroid cancer	(29, 30, 32–37)
Cardiovascular	Chest pain, hypertension, hypotension, pallor, bradycardia, tachycardia, palpitations, ventricular extrasystole, myocardial infarction		(31, 35–38)
Gastrointestinal and hepatic	Nausea, dysphagia, dyspepsia, odynophagia, oral pain, diarrhea, constipation, esophagitis, elevated liver enzyme values, appendicitis		(31, 33, 35–38)
Hematologic events	Neutropenia, disseminated intravascular coagulopathy, hypogammaglobulinemia		(28, 31–34, 36–38)
Musculoskeletal	Back pain, pain in extremity, arthralgia, myalgia		(30–32, 35–37)
Nervous system (including eyes)	Headache, migraine, conjunctivitis, fatigue, sensory disturbance, tremor, somnolence, vertigo, depression, stroke, cerebral hemorrhage, suicide		(29–36, 38)
Renal	Acute renal failure		(38)
Upper and lower airways	Nasopharyngitis, nasal congestion, throat irritation, dyspnea, pharyngeal swelling, oropharyngeal edema, bronchitis, bronchospasm, pneumonia, pulmonary embolism		(29–32, 34–38)
Urogenital	Epididymitis, cystitis		(31, 34)
Skin	Pruritus, rash, flushing, urticaria, angioedema, erythema, cellulitis		(29, 31, 34–38)

Ofatumumab

Organ systems affected	Adverse event(s)		Refs.
Systemic	**a) Immediate-type adverse reactions**	Infusion reaction	(39, 41–48)
	b) Infection	Upper respiratory tract infection, urinary tract infection, genital infections, tooth infection, skin infections, sepsis	(39, 41–47)
	c) Neoplasm	Breast cancer, ovarian cancer, malignant melanoma, basal cell carcinoma, lymphoma, gingival carcinoma	(42–46)
Cardiovascular	Tachycardia, bradycardia, palpitations, hypertension, hypotension, atrial fibrillation, atrioventricular block, cardiac ischemia, pericardial effusion, left ventricular hypertrophy		(43–45, 47)
Gastrointestinal and hepatic	Nausea, vomiting, abdominal pain, dysphagia, dyspepsia, stomatitis, duodenal ulcer, diarrhea, constipation, gastroenteritis, cholelithiasis, diverticulitis, pancreatic necrosis, elevated liver enzyme values, appendicitis		(42–47)
Hematologic events	Anemia, leukopenia, neutropenia, lymphopenia, thrombocytosis, eosinophilia		(39, 43, 46)
Musculoskeletal	Back pain, pain in extremity, synovitis, bursitis, arthritis, myalgia		(41, 42, 45, 47)
Nervous system (including eyes)	Headache, fatigue, periorbital edema, vertigo, tinnitus, ear pain, hypoacusis, deafness, hypothyroidism, eye disorder (blurred vision, eye pain, diplopia, dry eye, blepharospasm, conjunctivitis, cataract, chalazion), paresthesia, migraine, syncope, tremor, somnolence, restless legs syndrome, amnesia, myasthenia gravis, depression, anxiety, insomnia, suicide attempt		(41–47)
Renal	Nephrolithiasis, pollakiuria, hematuria, leukocyturia, proteinuria		(43)

(Continued)

TABLE 5 | Continued

Obinutuzumab		
Organ systems affected	**Adverse event(s)**	**Refs.**
Upper and lower airways	Nasopharyngitis, throat irritation, laryngitis, sinusitis, bronchitis, cough, bronchospasm, pneumonia, interstitial lung disease, pulmonary embolism	(39, 41–47)
Urogenital	Endometritis, urinary incontinence, menorrhagia, dysmenorrhea, cervical dysplasia, erectile dysfunction, balanoposthitis	(46)
Skin	Pruritus, rash, flushing, erythema, urticaria, angioedema, alopecia	(41, 42, 45–47)
Ublituximab		
Organ systems affected	**Adverse event(s)**	**Refs.**
Systemic	**a) Immediate-type adverse reactions** Infusion reaction	(49)
	b) Infection Upper respiratory tract infection, influenza, fungal infection	(49)
	c) Neoplasm None reported. Further studies in patients with immune-mediated diseases are needed to determine the frequency.	
Cardiovascular	None reported. Further studies in patients with immune-mediated diseases are needed to determine the frequency.	
Gastrointestinal and hepatic	Nausea, diarrhea, constipation, upper abdominal pain, vomiting	(49)
Hematologic events	Leukopenia	(50)
Musculoskeletal	Arthralgia, back pain	
Nervous system (including eyes)	Dizziness, fatigue, headache, contusion, depression, blurred vision	(49, 50)
Renal	None reported. Further studies in patients with immune-mediated diseases are needed to determine the frequency.	
Upper and lower airways	Cough, nasopharyngitis, sinusitis	(49)
Urogenital	None reported. Further studies in patients with immune-mediated diseases are needed to determine the frequency.	
Skin	Rash	(49)
Veltuzumab		
Organ systems affected	**Adverse event(s)**	**Refs.**
Systemic	**d) Immediate-type adverse reactions** Infusion reaction	(51, 52)
	e) Infection Upper respiratory tract infection, urinary tract infection	(51, 52)
	f) Neoplasm None reported	
Cardiovascular	Atrial fibrillation, palpitations	(51, 52)
Gastrointestinal and hepatic	Nausea, vomiting, abdominal pain, abdominal bloating, gastroenteritis, dyspepsia, elevated liver enzyme values	(51, 52)
Hematologic events	Bleeding, neutropenia, lymphopenia, thrombocytopenia	(51, 52)
Musculoskeletal	Pain in extremity, myalgia, back pain, chest pain	(51, 52)
Nervous system (including eyes)	Headache, fatigue, peripheral neuropathy	(51, 52)
Renal	Elevated creatinine values, chronic renal failure	(51)
Upper and lower airways	Nasopharyngitis, sinusitis, throat irritation	(51, 52)
Urogenital	Increased thirst and urination	(52)
Skin	Pruritus, burning, erythema, swelling, edema, bruising, cellulitis	(51, 52)

Moreover, OFA was tested in patients with AAV, RA, and SLE and resulted in disease improvement. Conversely, OFA showed mixed results in patients with PLA_2R-associated membranous nephropathy. UBL was tested in MS and in NMOSD, revealing promising results, although the numbers of treated patients were small. VEL was tried in patients with ITP in open-label designed studies and appeared to be effective.

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.

AUTHOR CONTRIBUTIONS

Conception and design of the work: CK and OB. Data collection: CK and BW. Data analysis and interpretation: CK and BW. Drafting the article: CK, CC, and OB. Critical revision of the article: CK, BW, CC, and OB. Final approval of the version to be published: CK, BW, CC, and OB.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.788830/full#supplementary-material

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Case Report: Rapid Desensitization to Ocrelizumab for Multiple Sclerosis Is Effective and Safe

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Monoclonal antibodies have become a mainstay of treatment for many inflammatory diseases and malignancies. Multiple sclerosis is a chronic inflammatory, demyelinating, and neurodegenerative disease of the central nervous system and a common cause of disability in young adults. Ocrelizumab is a recombinant humanized monoclonal antibody that targets CD20-positive B cells and has been approved in the treatment of multiple sclerosis. Although considered safe, more than 30% of patients treated with Ocrelizumab developed infusion-related reactions, mostly regarded as mild. When severe, they can lead to a definite suspension of that drug. We present a case report of Ocrelizumab desensitization in a female patient who presented an immediate hypersensitivity reaction (urticaria and angioedema) during the first Ocrelizumab infusion. Although mechanisms involved in the response were not elucidated, the procedure occurred uneventfully and permitted first-line multiple sclerosis treatment maintenances. Desensitization should be considered a safe therapeutic option in patients with immediate hypersensitivity reactions to Ocrelizumab.

Keywords: hypersensitivity, allergy, multiple sclerosis, monoclonal antibodies, Ocrelizumab, desensitization

INTRODUCTION

Monoclonal antibodies (mAbs) have become a mainstay for many inflammatory diseases and malignancies. There are four different types of mAbs used in the treatment of human disease, listed in decreasing order of immunogenicity: chimeric (suffix "ximab"; e.g., infliximab); humanized (suffix "zumab"; e.g., omalizumab); fully human (suffix "umab"; e.g., adalimumab); and receptor fusion (suffix "cept"; e.g., etanercept) (1, 2). As they are "non-self" proteins, the immune system can recognize these biological products and trigger an immune response (1). Initial biologics included more significant parts of non-human proteins (as chimeric, for example) and more immunogenic. As they became more similar to human proteins, their immunogenicity diminished progressively (1).

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating, and neurodegenerative disease of the central nervous system and a common cause of disability in young adults. MS can be categorized as relapsing (RMS) or primary progressive (PPMS) but is primarily considered a progressive disease in most patients (3).

MS was long thought to be a T-cell-mediated autoimmune disorder, causing inflammatory demyelination and neuronal damage, which slows or prevents nerve signaling (4). More recently, B cells have been shown to play an essential role in the pathogenesis of MS *via* many mechanisms, such as the presentation of autoantigens and costimulatory signals to activate T cells and the secretion of pro-inflammatory cytokines (5).

Ocrelizumab is a recombinant humanized mAb that targets CD20-positive B cells and has been approved for the treatment of RMS and PPMS (1, 4). The precise mechanisms by which Ocrelizumab exerts its therapeutic clinical effects in MS are not fully elucidated. Still, it is believed that it eliminates B cells from the peripheral blood, primarily through antibody-dependent cellular cytotoxicity and to a lesser extent by antibody-dependent cellular phagocytosis, complement-dependent cytotoxicity, and the direct apoptosis of B cells (6).

Ocrelizumab is the first CD20+ B-cell-selective monoclonal antibody for treating MS, at a dose of 600 mg IV twice yearly, with significant benefit on disability progression and with sustained efficacy with continuous efficacy therapy up to 6.5 years in the open-label extensions of the phase III studies (3).

Although considered safe, more than 30% of patients treated with Ocrelizumab in phase III trials developed infusion-related reactions (IRRs), mostly regarded as mild. When IRRs are moderate to severe, they can lead to a definitive suspension of that drug and to a scheme modification, which has been previously described in clinical trials (7).

Rapid drug desensitization (RDD) is a cornerstone in the management of immediate hypersensitivity reactions (IHRs) and can be applied to allergic (IgE-mediated) and non-allergic reactions. It is indicated when there is no alternative drug to replace the one that elicits the initial reaction (8).

We describe a female patient with MS who presented an IHR to Ocrelizumab at the first infusion and was successfully and safely desensitized to that drug.

CASE DESCRIPTION

A 39-year-old woman with a history of progressive left spasticity since the age of 24, extensively investigated by an orthopedist, with no detailed record of fluctuations in the motor condition, was evaluated in a neurological consultation that showed a left pyramidal syndrome with no changes in superficial and deep sensitivity.

She was investigated with serological tests of autoimmunity, including serum anti-AQP4 and anti-MOG, with negative results, and a brain, cervical, and thoracic magnetic resonance was performed that showed a bulbar lesion with a demyelinating pattern (**Figure 1**). Given the characteristics of the lesion and the clinical history, the investigation was complemented with a study of the cerebrospinal fluid (average cell count, normal protein levels, and absence of oligoclonal bands) and genetic panel for genetically determined leukoencephalopathies including the *GFAP* gene, to rule out the adult form of Alexander disease. After these last exams, the diagnosis of PPMS was defined, followed by infusion of Ocrelizumab as the only approved disease-modifying therapy for this condition.

As defined by clinical trials, the first dose should be 600 mg divided into two 300-mg doses, separated by 14 days, with a premedication scheme including a 100-mg dose of IV methylprednisolone. When about 290 mg of Ocrelizumab had been administered, the patient started to present pruritus and flushing (**Figure 2A**), followed by generalized urticaria and facial angioedema (**Figure 2B**). She did not develop dyspnea, tachycardia, or hypotension. The infusion was stopped, and the reaction was successfully treated with an extra dose of 100 mg methylprednisolone succinate and diphenhydramine 50 mg IV. The patient had a previous history of allergic rhinitis and nonsteroidal anti-inflammatory drugs (NSAIDs)-induced urticaria and angioedema. However, she had never presented any reactions to injectable medications. She had presented three urticaria or angioedema attacks after taking aspirin, ibuprofen, and dipyrone orally.

To maintain MS first-line treatment, she was evaluated by the Allergy Unit, and an RDD was indicated until a therapeutic 300-mg dose as previously described (9). The woman underwent risk stratification and was classified as low risk (Brown Classification grade I IHR with no respiratory or cardiovascular comorbidities) (10). Although it would help define the mechanisms involved and completely stratify the patient's risk, we could not perform skin tests.

Then, the patient was submitted to the 3-bag, 12-step RDD protocol (9), which is summarized in **Table 1**. She was premedicated with 100 mg methylprednisolone and diphenhydramine 50 mg IV, and the protocol was successfully performed in the Day Hospital Unit, with no breakthrough reactions.

Six months later, the patient received the next infusion, with a total 600 mg Ocrelizumab dose, under an RDD 12-step protocol, with no adverse reactions. However, the regular protocol for the injection of 600 mg indicates that the drug must be diluted in a 500-ml bag of saline solution. Thus, we adapted the RDD protocol so that the third bag (steps 9–12) included 500 ml. Still, infusion rates administered at each stage were doubled compared to the first desensitization (**Supplementary File**).

The patient signed the consent form, and the Ethics Committee from the University of São Paulo Medical School approved the study (CAAE 38855420.0.0000.0068, Plataforma Brasil).

DISCUSSION

We presented a female patient successfully desensitized to Ocrelizumab after an initial IHR. Although the reaction had

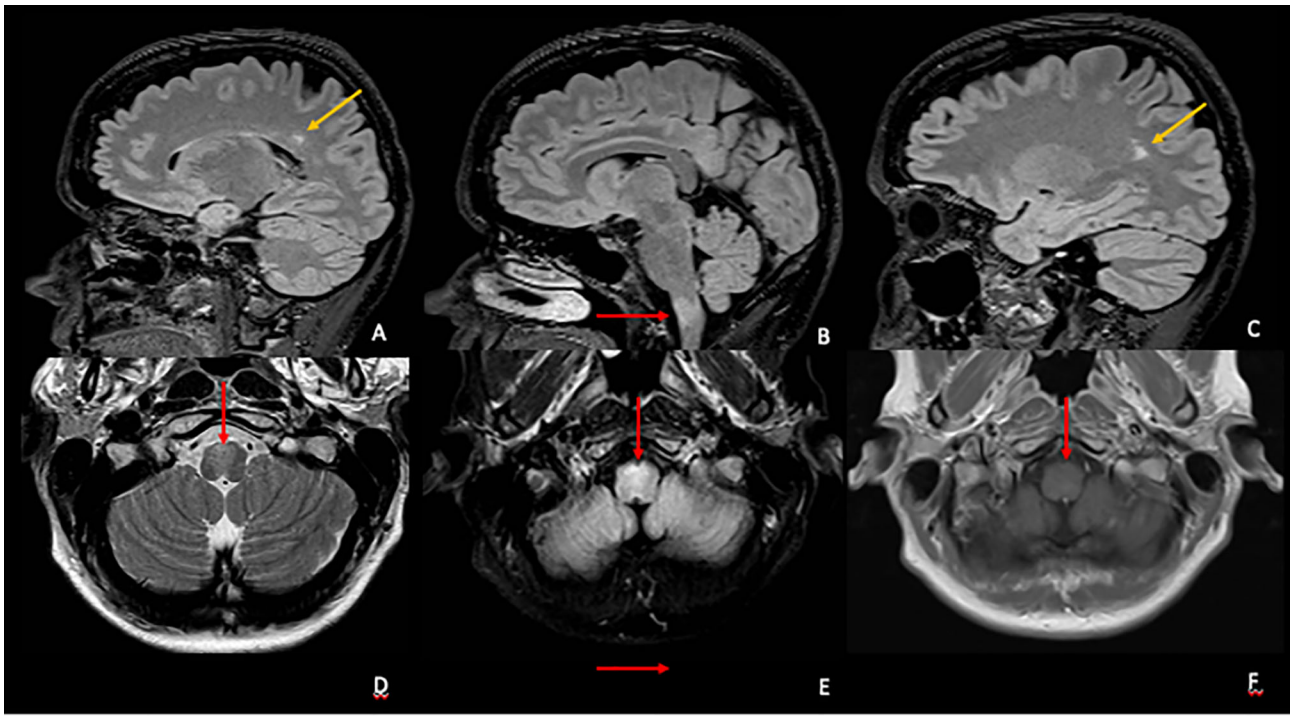

FIGURE 1 | Top row **(A–C)** Sagittal volumetric fluid-attenuated inversion recovery (FLAIR) images showing at least two periventricular hyperintense lesions (yellow arrows) with perivenular distribution and one ventral medula oblongata lesion (red arrow). Bottom row—Axial T2 **(D)**, FLAIR **(E)**, and T1 pos gadolinium **(F)** images showing the ventral medulla oblongata hyperintense lesion compromising pyramidal decussation **(D, E)** without gadolinium enhancement **(F)**.

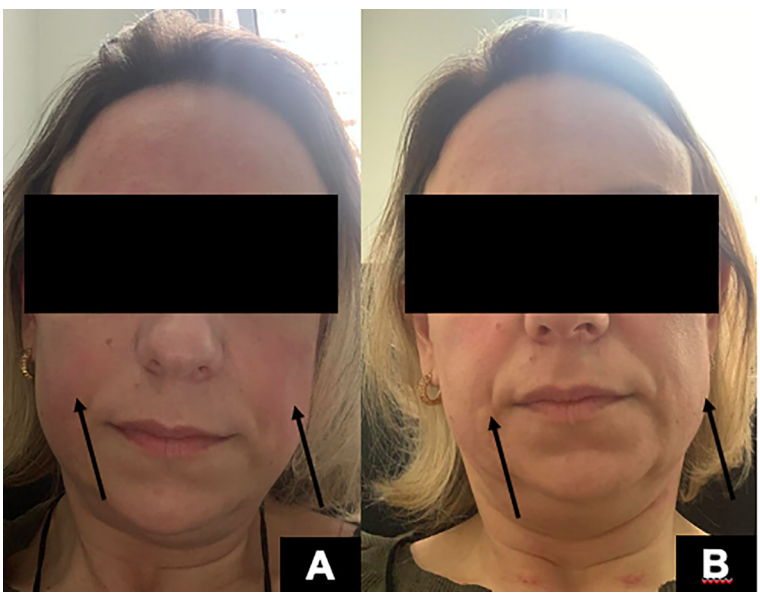

FIGURE 2 | **(A)** Facial erythema and flushing (arrows) during Ocrelizumab infusion. **(B)** Facial angioedema (arrows) minutes after the initial facial erythema.

TABLE 1 | First rapid desensitization with Ocrelizumab 300 mg using the 3-bag, 12-step protocol published elsewhere (9).

					Total mg per bag	Amount of bag infused (ml)	
Solution 1	250	ml of	0.012	mg/ml	3.000	9.25	
Solution 2	250	ml of	0.120	mg/ml	30.000	18.75	
Solution 3	250	ml of	1.191	mg/ml	297.639	250.00	
Step	**Solution**	**Rate (ml/h)**	**Time (min)**	**Volume infused per step (ml)**	**Dose administered with this step (mg)**	**Cumulative dose (mg)**	**Fold increase per step**
1	1	2.0	15	0.50	0.0060	0.0060	–
2	1	5.0	15	1.25	0.0150	0.0210	2.5
3	1	10.0	15	2.50	0.0300	0.0510	2
4	1	20.0	15	5.00	0.0600	0.1110	2
5	2	5.0	15	1.25	0.1500	0.2610	2.5
6	2	10.0	15	2.50	0.3000	0.5610	2
7	2	20.0	15	5.00	0.6000	1.1610	2
8	2	40.0	15	10.00	1.2000	2.3610	2
9	3	10.0	15	2.50	2.9764	5.3374	2.48032
10	3	20.0	15	5.00	5.9528	11.2902	2
11	3	40.0	15	10.00	11.9056	23.1957	2
12	3	80.0	174.375	232.50	276.8043	300.0000	2
				Total time (minutes) = 339.375 = 5.66 h			

The total volume and dose dispensed are more than the final dose given to the patient because the initial solutions are not entirely infused.

not been severe, as the patient developed urticaria and angioedema, which is highly suggestive of mast cell activation despite the mechanisms involved, future infusions could induce anaphylaxis and be life threatening. On the other hand, the replacement of the MS therapy could lead to disease exacerbation. As far as we know, this is the first case published as a complete article in a journal. In 2019, two case reports were presented as abstracts in a scientific meeting (11).

It has been recently demonstrated that clinically significant IRRs include four major phenotypes: type-I-like hypersensitivity (IgE mediated or non-IgE mediated), cytokine-release, mixed reactions, and delayed type IV (12). A minority of individuals also present a type-III hypersensitivity reaction after biologic administration. It can include systemic serum-sickness disease or only a local Arthus reaction because of IgM and IgG deposition (12).

IRRs and cytokine-release reactions to mAbs can occur at first infusion and may typically present with mild to severe symptoms, including flushing, chills, fever, tachycardia, hypertension, dyspnea, nausea, vomiting, and syncope. The difference between IRRs and cytokine-release reactions is the self-limiting nature of IRRs on repeat exposure and the response to premedication (13).

Type I-like reactions to biologics can manifest with flushing, pruritus, urticaria, shortness of breath, hypotension, and life-threatening anaphylaxis that typically initiate during the infusion. These symptoms are associated with releasing mast cells/basophils mediators, including tryptase, histamine, leukotrienes, and prostaglandins, whose actions affect cutaneous, respiratory, gastrointestinal, and cardiovascular organ systems (12). They have delayed reactions that usually occur more than 12 h after the infusion and may range from mild maculopapular rash to severe cutaneous adverse reactions, such as Stevens–Johnson syndrome or drug rash with eosinophilia and systemic symptoms (12). Type-IV hypersensitivity includes <5% of mAb-induced responses.

Our female patient presented an immediate reaction characterized by flushing, followed by urticaria and facial angioedema, without respiratory or cardiovascular compromise. According to this classification, its phenotype could be considered a type-I-like hypersensitivity reaction based on clinical features. Type-III (14) and type-IV reactions (15, 16) have been previously associated with Ocrelizumab infusion, but type-I-like reactions have only been cited during phase III clinical trials (7).

Using a different classification of IRR severity that combined all kinds of adverse reactions, Mayer et al. described that more than 30% of Ocrelizumab-exposed individuals presented any IRR during clinical trials. It is essential to point out that all patients received a 100-mg dose of IV methylprednisolone before Ocrelizumab infusion (7). Our patient was also premedicated with methylprednisolone, which did not prevent the reaction. Considering the three trials altogether, more than 2,000 individuals were evaluated. Only one patient presented a severe Common Terminology Criteria for Adverse Events (CTCAE) grade IV IRR, namely, severe bronchospasm, during their first infusion in one of the OPERA studies. This treatment was then withdrawn (7). That severe reaction could have been a type-I-like hypersensitivity reaction. On the other hand, in ORATORIO, two patients presented severe IRRs, but the authors described clinical pictures as compatible with cytokine-release or mixed reactions, not a type-I-like reaction (7).

If we consider the Brown classification for severity of IHRs, our patient presented a grade I reaction (10). It is impossible to know if the reaction was less severe because of corticosteroid administration before the biologic. Thus, even if it was not severe, it probably involved mast cells and/or basophils activation, which could induce a future anaphylactic reaction during subsequent exposure. The patient became afraid of a

future infusion, and the neurologist was concerned about her first-line therapy maintenance. Thus, we decided to perform an RDD with Ocrelizumab using the 12-step protocol published by Prof. Mariana Castells et al. (9, 17).

Although considered safe, rapid desensitization to Ocrelizumab has not been previously described. In the most extensive case series already published, including patients submitted to chemotherapeutic agents and biologics rapid desensitization, <30% of patients present any breakthrough reaction during the procedure. Moreover, <10% present severe breakthrough reactions, confirming that RDD is safe and effective (18, 19).

Mechanisms involved in Ocrelizumab hypersensitivity are unknown. Despite being clinically compatible with a type-I allergic reaction, we could not prove the involvement of IgE. IHRs induced by rituximab, another CD20-targeted mAb largely used in clinical practice to manage autoimmune diseases and hematological malignancies, can be IgE or non-IgE mediated (12). Our patient presented the IHR during the first infusion, but as we did not perform a skin test, it is impossible to postulate whether the reaction should be considered allergic or non-allergic. However, independently of the pathophysiology, RDD can be successfully performed in all type-I-like reactions, allowing maintenance of first-line therapy (12).

Our case description has a few limitations. First, we did not repeat standard infusion with a different premedication scheme, including an antihistamine. Nevertheless, it is also unlikely to be safe, since a 100 mg dose of IV methylprednisolone before infusion could not prevent the IHR. Furthermore, as cited above, we did not perform skin tests, making it impossible to define the mechanisms involved in the initial reaction. Future studies involving more individuals will permit a conclusion about the pathophysiology of hypersensitivity to Ocrelizumab.

In summary, we described that rapid desensitization with Ocrelizumab using the 3-bag, 12-step protocol is safe. It allowed the patient to be treated with the only approved disease-modifying therapy for PPMS and then prevent the progression of this severe neurological condition. Some other drugs, such as Ofatumumab and Rituximab, have been used off-label in selected cases. However, as they are still not approved for PPMS management, desensitization to Ocrelizumab can be safely considered to keep patients with this approved and efficacious treatment.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was reviewed and approved by University of São Paulo Medical School (CAAE 38855420.0.0000.0068, Plataforma Brasil). The patient provided her written informed consent to participate in this study. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MA, FF, and VM assisted the patient and collected the data. MA, FF, and PG-B coordinated and performed the desensitization. MA and FF reviewed literature 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.2022.840238/full#supplementary-material

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Early B Cell and Plasma Cell Kinetics Upon Treatment Initiation Portend Flares in Systemic Lupus Erythematosus: A *Post-Hoc* Analysis of Three Phase III Clinical Trials of Belimumab

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Objective: To investigate changes in B cell subsets in relation to disease flares upon initiation of standard therapy (ST) plus belimumab or placebo in patients with systemic lupus erythematosus (SLE).

Patients and Methods: Using data from the BLISS-76, BLISS-SC and BLISS Northeast Asia trials, we investigated associations of relative to baseline rapid (through week 8) and early (through week 24) changes in peripheral B cell subsets, anti-dsDNA and complement levels with the occurrence of disease flares from week 24 through week 52 (Mann-Whitney *U* tests) or the entire study follow-up (Cox regression analysis), assessed using the SELENA-SLEDAI Flare Index.

Results: Patients on ST alone who flared displayed less prominent early decreases in $CD19^+CD20^-CD138^+$ long-lived plasma cells (-16.1% versus -35.1%; P=0.012). In all arms combined, patients who developed severe flares showed less prominent early decreases in $CD19^+CD20^-CD138^+$ long-lived plasma cells (-23.5% versus -39.4%; P=0.028) and $CD19^+CD27^{bright}CD38^{bright}$ SLE-associated plasma cells (-19.0% versus -27.8%; P=0.045). After adjustment for rapid changes, early increases in overall $CD19^+CD20^+$ B cells (HR: 1.81; 95% CI: 1.08–3.05; P=0.024) and early increases or no return after a rapid expansion in $CD19^+CD20^+CD27^+$ memory B cells (HR: 1.58; 95% CI: 1.18–2.11; P=0.002) portended subsequent severe flares. Patients who developed flares of any severity showed no or less prominent rapid (0.0% versus -12.5%; P<0.001) or early (-1.9% versus -21.7%; P<0.001) decreases in anti-dsDNA levels, and patients who developed severe flares showed no or less prominent early decreases in anti-dsDNA levels (0.0% versus -13.3%; P=0.020). Changes in complement levels exhibited no ability to distinguish flaring from non-flaring patients.

Conclusions: Increase or lack of decrease in certain circulating B cell subsets or anti-dsDNA levels upon treatment initiation for active SLE heralded subsequent severe disease flares. A rapid expansion of memory B cells may signify sustained response to therapy when followed by a subsequent drop, while no return or delayed increases in memory B cells may portend flaring. Peripheral B cell and serological marker kinetics may help identify patients in whom therapeutic modifications could protect against flare development, and may hence prove a useful complement to traditional surveillance and early treatment evaluation in SLE.

Keywords: systemic lupus erythematosus, biomarkers, flares, plasma cells, B cells, belimumab, biologics

1 INTRODUCTION

Although the prognosis of patients with systemic lupus erythematosus (SLE) has improved during the last decades, occurrence of disease flares still endangers organ function and long-term outcomes (1–5), contributing to the burden of direct and indirect disease- and treatment-related morbidity and costs (6). Multiple definitions of flares have been proposed in SLE (7–9). Usually, flares are classified into mild/moderate or severe, based on the degree of therapeutic modification that is required and the impact on patient performance and eventually survival (10). To date, the risk of disease flares in patients with SLE is mainly determined based on short-term fluctuations of serological markers, which may show inconsistent results owing to different assays, time of sample collection, and the prominent heterogeneity in disease manifestations (11–13).

Belimumab blocks the soluble counterpart of B cell activating factor (BAFF; also known as B lymphocyte stimulator, BLyS) and has been used for the treatment of SLE for longer than a decade (14). Belimumab has shown ability to induce durable disease control and reduce the risk of flares in multiple clinical trials and real-life observational studies (15–21). However, early identification of patients at risk for subsequent flares upon commencement of belimumab treatment remains a challenge, leaving an area of uncertainty during the critical stages of early follow-up. This need was recently exemplified in a report of *de novo* lupus nephritis cases after initiation of belimumab therapy (22).

In this regard, biological changes occurring soon after treatment initiation might provide measurable tools that could be used to improve patient monitoring and stratification according to the risk for relapses. In this study, we aimed at investigating early changes in B cell and plasma cell subsets in relation to the development of disease flares during non-biological standard therapy (ST) plus belimumab or placebo within the frame of three phase III clinical trials of belimumab in SLE.

2 PATIENTS AND METHODS

2.1 Study Population

We analysed longitudinal data from patients with active SLE who participated in three multicentre, randomised, double-blind, placebo-controlled trials comparing belimumab (administered intravenously or subcutaneously) with placebo, i.e., BLISS-76 (NCT00410384; N=797) (21), BLISS-SC (NCT01484496; N=822) (23), and BLISS Northeast Asia (NEA; NCT01345253; N=60) (24). The study population (N=1679) was selected based on availability of data on B cell subset counts and clinical data needed to determine flares. In the BLISS programmes, belimumab or placebo was administered on top of non-biological ST, including antimalarial agents, glucocorticoids, immunosuppressive agents, or combinations thereof.

In terms of design, the three trials were similar. Briefly, all patients were required to have a Safety of Estrogens in Lupus Erythematosus National Assessment - Systemic Lupus Erythematosus Disease Activity Index (SELENA-SLEDAI) (25) score ≥6 (BLISS-76) or ≥8 (BLISS-SC and BLISS-NEA) and had to be autoantibody positive (antinuclear antibody titres ≥1:80 and/or anti-double stranded (ds)DNA levels ≥30 IU/mL) at the screening. All patients had received stable dosages of ST for at least 30 days prior to baseline. For BLISS-76 and BLISS-NEA, belimumab or placebo were administered intravenously on days 0, 14, and 28, and every 4th week thereafter through week 48 (BLISS-NEA) or week 72 (BLISS-76). The actual number of patients enrolled in BLISS-NEA was 702, and the selection of the 60 patients that were included in the present study was based on availability of B cell data from the initial trials. In BLISS-SC, belimumab 200 mg or placebo was administered subcutaneously weekly through week 52, on top of non-biological ST. Progressive restrictions were imposed during the trial periods on concurrent immunosuppressive and antimalarial medications, as well as glucocorticoid intake. The primary endpoint in all trials was the proportion of responders at week 52, with response being determined using the composite SLE Responder Index (SRI)-4 (26). The similar trial design and endpoints allowed pooling of the data to increase power during statistical analyses.

Occurrence of flares graded into mild/moderate or severe according to the SELENA-SLEDAI Flare Index (SFI) (10) was determined every fourth week.

2.2 Determination of B Cell Subsets and Serological Markers

Peripheral B cell and plasma cell subsets were determined by flow cytometry within the frame of the BLISS study programmes (21, 23, 24), and classified into total peripheral $CD19^+CD20^+$ B cells,

$CD19^+CD20^+CD69^+$ activated B cells, $CD19^+CD20^+CD27^-$ naïve B cells, $CD19^+CD20^+CD27^+$ memory B cells, $CD19^+CD20^-CD27^{bright}$ plasmablasts, $CD19^+CD20^+CD138^+$ short-lived plasma cells, $CD19^+CD20^-CD138^+$ long-lived plasma cells, and $CD19^+CD38^{bright}CD27^{bright}$ SLE-associated plasma cells (27–29). Levels of anti-dsDNA, C3 and C4 were determined within the frame of the BLISS programmes (21, 23, 24).

We analysed relative to baseline (i.e., treatment initiation) changes in B cell subsets and serum levels of anti-dsDNA, C3 and C4 that occurred through week 8, 24 and 52. Changes occurring through week 8 were deemed rapid and changes occurring through week 24 were deemed early. We next investigated associations between rapid or early changes in B cell or plasma cell subsets or changes in serological markers and flares occurring from week 24 through week 52 (Mann-Whitney *U* tests) or through the last observation (week 52 for BLISS-SC and BLISS-NEA, and week 76 for BLISS-76; Cox proportional hazards regression analysis).

2.3 Ethics

Data from the BLISS trials were made available by GlaxoSmithKline (Uxbridge, UK) through the Clinical Study Data Request (CSDR) consortium. The trial protocols were approved by regional ethics review boards for all participating centres and complied with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all study participants prior to enrolment. The present study was approved by the Swedish Ethical Review Authority (2019-05498).

2.4 Statistical analysis

Descriptive statistics are reported as means and standard deviations or medians and interquartile ranges for continuous variables, while frequencies and percentages are reported for categorical variables. Values (relative to baseline percentage change) above the 97.5^{th} percentile were treated as extreme values and set to the same max value (equal to the 97.5^{th} percentile) for each cell variable.

Comparisons of distributions of the relative to baseline changes between groups (e.g., flaring versus non-flaring patients, or patients receiving belimumab versus placebo) were conducted using the non-parametric Mann-Whitney *U* test. For determination of time-dependent associations between rapid or early biological changes and flare occurrence, we used Cox proportional hazards regression models. All models were adjusted for age, sex, ethnicity, SLE disease duration, belimumab use (any dose), use of methotrexate, use of azathioprine, use of mycophenolate mofetil, use of immunosuppressants other than those mentioned before, and the BLISS study to account for batch variations in cell analyses. The potential interaction between cell alterations and belimumab use was accounted for. One set of models investigating associations between early B cell changes and flares occurring from week 24 through week 76 or the last available follow-up visit was also adjusted for the relative to baseline cell alterations from baseline through week 8 to account for alterations in opposing directions in the two follow-up phases.

P values below 0.05 were deemed significant. All analyses were performed using the R version 4.01 software (R Foundation for Statistical Computing, Vienna, Austria).

3 RESULTS

3.1 Patient Characteristics

Demographics, clinical and serological data of the patients including comparisons between patients who developed and patients who did not develop flares (any grade or severe) are reported in **Table 1**. Baseline B cell and plasma cell data, including comparisons between patients who developed and patients who did not develop flares (any grade or severe) are reported in **Table 2**, where results are stratified by study to account for batch variations in cell analyses across studies.

3.2 Associations With Flares Occurring From Week 24 Through Week 52

3.2.1 Flares of Any Severity (Mild/Moderate or Severe)

In the pooled datasets, 892/1533 patients (58.2%) developed at least one SFI flare of any degree of severity from week 24 through week 52. Among patients who flared, the first flare occurred after a mean time of 244.8 ± 61.0 days from baseline.

3.2.1.1 B Cell Changes

In the entire cohort (all treatment arms) and among patients who received add-on belimumab, no difference in rapid or early changes in any B cell subset was observed between patients who developed and patients who did not develop SFI flares of any severity from week 24 onwards (**Figure 1** and **Supplementary Tables S1**, **S2**). Among patients who received ST alone, patients who flared showed a slight decrease in $CD19^+CD20^+CD27^+$ memory B cells through week 8 (-2.1%) while patients who did not flare showed an increase (+4.2%; P=0.037). Additionally, patients who flared exhibited less prominent decreases in $CD19^+CD20^-CD138^+$ long-lived plasma cells from baseline through week 24 compared with patients who did not flare (-16.1% versus -35.1%; P=0.012). No difference was observed between flaring and non-flaring patients regarding rapid or early changes in $CD19^+CD20^+$ B cells (P=0.630 and P=0.082, respectively), $CD19^+CD20^+CD69^+$ activated B cells (P=0.439 and P=0.681, respectively), $CD19^+CD20^-CD27^{bright}$ plasmablasts (P=0.967 and P=0.772, respectively), or $CD19^+CD27^{bright}CD38^{bright}$ SLE-associated plasma cells (P=0.681 and P=0.366, respectively).

3.2.1.2 Serological Markers

In the entire cohort (all treatment arms), patients who developed flares of any severity from week 24 onwards showed no rapid change in anti-dsDNA levels (0.0%) while patients who did not flare showed rapid (-12.5%; P<0.001) and persistent decreases, which were consistently greater compared with those observed in flaring patients (baseline through week 24: -21.7% versus -1.9%; P<0.001), as well as in a subgroup analysis of patients with positive anti-dsDNA levels at baseline, both regarding rapid (through week 8; -22.2% versus -15.8%; P<0.001) and early changes (through

TABLE 1 | Characteristics of patients who developed versus patients who did not develop flares from week 24 through week 76 in the pooled BLISS study population.

	Any flare from week 24 through week 76				Severe flare from week 24 through week 76			
	All patients N=1533	Yes N=959	No N=574	P value	All patients N=1533	Yes N=187	No N=1346	P value
Patient characteristics								
Age at baseline (years)	39.3 ± 11.8	39.3 ± 11.4	39.3 ± 12.5	0.703	39.3 ± 11.8	38.8 ± 12.6	39.3 ± 11.7	0.461
Female sex	1439 (93.9%)	898 (93.6%)	541 (94.3%)	0.629	1439 (93.9%)	173 (92.5%)	1266 (94.1%)	0.410
Ancestry								
Asian	250 (16.3%)	133 (13.9%)	117 (20.4%)	**0.001**	250 (16.3%)	29 (15.5%)	221 (16.4%)	0.752
Black/African American	172 (11.2%)	125 (13.0%)	47 (8.2%)	**0.004**	172 (11.2%)	32 (17.1%)	140 (10.4%)	**0.006**
Indigenous American*	153 (10.0%)	108 (11.3%)	45 (7.8%)	**0.031**	153 (10.0%)	21 (11.2%)	132 (9.8%)	0.543
White/Caucasian	958 (62.5%)	593 (61.8%)	365 (63.6%)	0.492	958 (62.5%)	105 (56.1%)	853 (63.4%)	0.056
Clinical data								
SLE duration at baseline (years)	5.1 (1.7–10.6)	5.2 (1.7–10.6)	4.9 (1.5–10.8)	0.551	5.1 (1.7–10.6)	5.6 (2.3–11.1)	5.1 (1.6–10.5)	0.129
Treatment at baseline								
Glucocorticoids	1263 (82.4%)	747 (77.9%)	516 (89.9%)	**<0.001**	1263 (82.4%)	151 (80.7%)	1112 (82.6%)	0.530
AMA†	984 (64.2%)	626 (65.3%)	358 (62.4%)	0.251	984 (64.2%)	115 (61.5%)	869 (64.6%)	0.413
Immunosuppressants‡	787 (51.3%)	538 (56.1%)	249 (43.4%)	**<0.001**	787 (51.3%)	109 (58.3%)	678 (50.4%)	**0.042**
Azathioprine	301 (19.6%)	194 (20.2%)	107 (18.6%)	0.449	301 (19.6%)	43 (23.0%)	258 (19.2%)	0.217
Methotrexate	218 (14.2%)	159 (16.6%)	59 (10.3%)	**0.001**	218 (14.2%)	30 (16.0%)	188 (14.0%)	0.446
Mycophenolate mofetil or sodium	214 (14.0%)	156 (16.3%)	58 (10.1%)	**0.001**	214 (14.0%)	32 (17.1%)	182 (13.5%)	0.184
Trial intervention								
Placebo	505 (32.9%)	339 (35.3%)	166 (28.9%)	**0.010**	505 (32.9%)	82 (43.9%)	423 (31.4%)	**0.001**
Belimumab	1028 (67.1%)	620 (64.7%)	408 (71.1%)	**0.010**	1028 (67.1%)	105 (56.1%)	923 (68.6%)	**0.001**
i.v. 1 mg/kg	245 (16.0%)	186 (19.4%)	59 (10.3%)	**<0.001**	245 (16.0%)	31 (16.6%)	214 (15.9%)	0.812
i.v. 10 mg/kg	274 (17.9%)	193 (20.1%)	81 (14.1%)	**0.003**	274 (17.9%)	39 (20.9%)	235 (17.5%)	0.256
s.c. 200 mg	509 (33.2%)	241 (25.1%)	268 (46.7%)	**<0.001**	509 (33.2%)	35 (18.7%)	474 (35.2%)	**<0.001**
Serological markers at baseline								
C3; mg/dL	96.0 (75.0 –118.5)	95.0 (73.0 –119.0)	96.0 (77.0 –117.0)	0.524	96.0 (75.0 –118.5)	89.0 (64.0 –110.0)	97.0 (76.0 –119.0)	**<0.001**
C4; mg/dL	15.0 (9.0–22.0)	15.0 (9.0–22.0)	15.0 (9.0–21.0)	0.862	15.0 (9.0–22.0)	12.0 (7.0–19.0)	15.0 (9.0–22.0)	**0.001**
anti-dsDNA; IU/mL (all patients)	92.0 (29.0 –275.0)	89.0 (29.0 –285.0)	100.0 (29.0 –268.3)	0.582	92.0 (29.0 –275.0)	127.0 (29.0 –429.0)	89.0 (29.0 –254.3)	**0.002**
anti-dsDNA; IU/mL (patients positive at baseline)	162.0 (88.0 –477.0); N=1045	167.0 (88.0 –498.0); N=643	149.5 (86.0 –426.0); N=402	0.443	162.0 (88.0 –477.0); N=1045	245.0 (101.5 –652.5); N=136	151.0 (86.0 –450.5); N=909	**0.013**

Data are presented as number (percentage), mean ± standard deviation, or median (interquartile range), as appropriate. In case of missing values, the total number of patients with available data is indicated. Statistically significant P values are in bold.

**Alaska Native or American Indian from North, South or Central America.*

†Hydroxychloroquine, chloroquine, mepacrine, mepacrine hydrochloride or quinine sulfate.

‡Azathioprine, cyclosporine, oral cyclophosphamide, leflunomide, methotrexate, mizoribine, mycophenolate mofetil, mycophenolate sodium or thalidomide.

AMA, antimalarial agents; C3, complement component 3; C4, complement component 4; i.v., intravenous; s.c., subcutaneous; SLE, systemic lupus erythematosus; SRI-4; SLE, Responder Index 4.

week 24; -33.0% versus -24.0%; P<0.001). Changes in complement levels exhibited no ability to distinguish flaring from non-flaring patients. The results are illustrated in **Figure 2** and detailed in **Supplementary Tables S1**–**S3**.

Among patients who received add-on belimumab, patients who developed flares of any severity showed less prominent rapid (-4.6% versus -17.7%; P<0.001) and early (-10.8% versus -26.4%; P<0.001) relative to baseline decreases in anti-dsDNA levels compared with patients who did not flare, which was also the case in a subgroup analysis of patients with positive anti-dsDNA levels at baseline, both regarding rapid (-20.2% versus -27.4%; P=0.012) and early (-31.5% versus -39.0%; P=0.008) changes. No differences were observed regarding rapid or early changes in C3 or C4 levels (**Figure 2**).

Among patients who received ST alone, no differences were found between patients who flared and patients who did not flare from week 24 onwards regarding rapid or early changes in anti-dsDNA or complement levels.

3.2.2 Severe Flares

In the pooled datasets, 163/1533 patients (10.6%) developed at least one severe flare from week 24 through week 52. Among patients who developed severe flares, the first severe flare occurred after a mean time of 253.6 ± 64.8 days from baseline.

3.2.2.1 B Cell Changes

In the entire cohort (all treatment arms), patients who developed at least one severe flare from week 24 onwards showed less prominent rapid increases through week 8 in $CD19^+CD20^+CD27^+$ memory B cells compared with patients who did not develop severe flares (+50.0% versus +83.5%; P=0.037), as shown in **Figure 3**. Furthermore, patients who developed severe flares displayed less

TABLE 2 | B cell subset counts at baseline in patients who developed versus patient who did not develop flares from week 24 through week 76 in the BLISS-76, BLISS-SC and BLISS-NEA study population.

B cell subsets	All patients	Yes	No	P value
		BLISS-76		
		Any flare from week 24 through week 76		
	N=720	**N=553**	**N=167**	
$CD19^+CD20^+$ ($x10^3$/mL)	91.5 (42.0–175.0); N=662	95.0 (42.3–175.0); N=504	81.0 (40.0–163.0); N=158	0.270
$CD19^+CD20^+CD27^+$ ($x10^3$/mL)	14.0 (6.0–27.0); N=662	14.5 (6.0–27.0); N=504	13.0 (7.0–25.0); N=158	0.464
$CD19^+CD20^+CD69^+$ (/mL)	2096.5 (939.3–4357.5); N=650	2141.0 (867.5–4422.5); N=493	1958.0 (1010.0–4221.5); N=157	0.886
$CD19^+CD20^+CD27^-$ ($x10^3$/mL)	75.5 (32.8–141.3); N=662	79.0 (33.0–144.0); N=504	67.5 (30.5–127.0); N=158	0.209
$CD19^+CD20^+CD138^+$ (/mL)	791.5 (329.3–1768.0); N=656	832.0 (357.0–1848.0); N=499	549.0 (263.5–1544.5); N=157	**0.014**
$CD19^+CD20^-CD138^+$ (/mL)	474.0 (212.0–1059.0); N=655	485.0 (212.0–1083.0); N=499	449.0 (211.5–1040.0); N=156	0.931
$CD19^+CD20^-CD27^{brt}$ (/mL)	312.0 (117.0–714.5); N=653	275.5 (107.0–668.3); N=496	456.0 (162.5–880.0); N=157	**0.004**
$CD19^+CD27^{brt}CD38^{brt}$ (/mL)	320.0 (115.3–722.3); N=660	292.0 (109.8–675.5); N=502	438.0 (153.5–865.3); N=158	**0.008**
		Severe flare from week 24 through week 76		
	N=720	**N=120**	**N=600**	
$CD19^+CD20^+$ ($x10^3$/mL)	91.5 (42.0–175.0); N=662	91.0 (37.0–161.0); N=113	92.0 (43.0–175.5); N=549	0.463
$CD19^+CD20^+CD27^+$ ($x10^3$/mL)	14.0 (6.0–27.0); N=662	12.0 (5.0–26.5); N=113	15.0 (7.0–27.0); N=549	0.183
$CD19^+CD20^+CD69^+$ (/mL)	2096.5 (939.3–4357.5); N=650	2385.0 (1063.3–5261.8); N=110	2046.5 (864.3–4296.3); N=540	0.196
$CD19^+CD20^+CD27^-$ ($x10^3$/mL)	75.5 (32.8–141.3); N=662	70.0 (30.0–136.5); N=113	76.0 (33.0–142.0); N=549	0.575
$CD19^+CD20^+CD138^+$ (/mL)	791.5 (329.3–1768.0); N=656	756.0 (258.0–1961.0); N=113	795.0 (342.0–1696.0); N=543	0.942
$CD19^+CD20^-CD138^+$ (/mL)	474.0 (212.0–1059.0); N=655	498.0 (209.0–1100.0); N=113	469.5 (211.8–1061.0); N=542	0.813
$CD19^+CD20^-CD27^{brt}$ (/mL)	312.0 (117.0–714.5); N=653	274.5 (113.5–609.3); N=112	320.0 (119.0–743.5); N=541	0.480
$CD19^+CD27^{brt}CD38^{brt}$ (/mL)	320.0 (115.3–722.3); N=660	285.0 (105.0–649.0); N=113	334.0 (120.0–732.0); N=547	0.274
		BLISS-SC		
		Any flare from week 24 through week 76		
	N=757	**N=377**	**N=380**	
$CD19^+CD20^+$ ($x10^3$/mL)	107.0 (58.0–197.5); N=736	102.0 (53.0–189.0); N=363	108.0 (59.5–205.5); N=373	0.161
$CD19^+CD20^+CD27^+$ ($x10^3$/mL)	14.0 (7.0–29.0); N=736	12.0 (6.0–25.0); N=363	17.0 (7.0–32.0); N=373	**0.001**
$CD19^+CD20^+CD69^+$ (/mL)	79.0 (32.0–198.8); N=736	74.0 (29.0–171.0); N=363	85.0 (35.0–230.0); N=373	**0.045**
$CD19^+CD20^+CD27^-$ ($x10^3$/mL)	89.0 (44.0–167.0); N=736	90.0 (43.0–158.0); N=363	89.0 (44.5–177.0); N=373	0.414
$CD19^+CD20^+CD138^+$ (/mL)	53.0 (20.0–131.8); N=736	55.0 (22.0–130.0); N=363	52.0 (19.0–133.5); N=373	0.735
$CD19^+CD20^-CD138^+$ (/mL)	198.0 (67.0–501.8); N=736	224.0 (69.0–566.0); N=363	176.0 (62.5–449.5); N=373	0.168
$CD19^+CD20^-CD27^{brt}$ (/mL)	2000.0 (1000.0–4000.0); N=736	2000.0 (1000.0–4000.0); N=363	2000.0 (1000.0–4000.0); N=373	0.132
$CD19^+CD27^{brt}CD38^{brt}$ (/mL)	1723.5 (728.3–3887.3); N=736	1594.0 (630.0–3733.0); N=363	1795.0 (763.0–4046.0); N=373	0.184
		Severe flare from week 24 through week 76		
	N=757	**N=63**	**N=694**	
$CD19^+CD20^+$ ($x10^3$/mL)	107.0 (58.0–197.5); N=736	70.0 (29.5–165.3); N=62	108.5 (60.8–200.0); N=674	**0.002**
$CD19^+CD20^+CD27^+$ ($x10^3$/mL)	14.0 (7.0–29.0); N=736	8.5 (5.0–21.3); N=62	15.0 (7.0–30.0); N=674	**0.001**
$CD19^+CD20^+CD69^+$ (/mL)	79.0 (32.0–198.8); N=736	55.0 (26.0–111.0); N=62	82.0 (33.0–205.0); N=674	**0.007**
$CD19^+CD20^+CD27^-$ ($x10^3$/mL)	89.0 (44.0–167.0); N=736	61.0 (23.8–146.3); N=62	92.0 (46.0–170.3); N=674	**0.007**
$CD19^+CD20^+CD138^+$ (/mL)	53.0 (20.0–131.8); N=736	44.0 (16.0–100.5); N=62	54.5 (20.0–135.0); N=674	0.155
$CD19^+CD20^-CD138^+$ (/mL)	198.0 (67.0–501.8); N=736	248.0 (65.0–611.5); N=62	194.5 (67.0–496.5); N=674	0.460
$CD19^+CD20^-CD27^{brt}$ (/mL)	2000.0 (1000.0–4000.0); N=736	1500.0 (750.0–3000.0); N=62	2000.0 (1000.0–4000.0); N=674	0.421
$CD19^+CD27^{brt}CD38^{brt}$ (/mL)	1723.5 (728.3–3887.3); N=736	1698.5 (649.8–3620.0); N=62	1723.5 (728.8–3909.3); N=674	0.912
		BLISS NEA		
		Any flare from week 24 through week 76		
	N=60	**N=40**	**N=20**	
$CD19^+CD20^+$ ($x10^3$/mL)	54.0 (22.0–102.0); N=51	54.0 (28.0–121.0); N=27	53.5 (17.3–90.5); N=24	0.503
$CD19^+CD20^+CD27^+$ ($x10^3$/mL)	7.4 (3.5–10.7); N=52	7.2 (3.2–11.7); N=28	7.4 (4.4–10.7); N=24	0.673
$CD19^+CD20^+CD69^+$ (/mL)	106.6 (45.5–182.8); N=52	114.4 (46.8–182.8); N=28	106.6 (45.0–182.4); N=24	0.883
$CD19^+CD20^+CD27^-$ ($x10^3$/mL)	40.5 (18.7–94.5); N=52	43.1 (25.1–99.2); N=28	38.9 (15.0–77.8); N=24	0.533
$CD19^+CD20^+CD138^+$ (/mL)	100.1 (58.3–247.3); N=52	84.9 (50.3–457.2); N=28	114.1 (64.1–201.3); N=24	0.783
$CD19^+CD20^-CD138^+$ (/mL)	301.2 (175.6–685.7); N=52	390.5 (179.3–708.7); N=28	257.2 (128.1–596.8); N=24	0.322
$CD19^+CD20^-CD27^{brt}$ (/mL)	970.6 (229.7–2204.8); N=52	1053.1 (290.2–2204.8); N=28	935.7 (213.4–2537.5); N=24	0.646
$CD19^+CD27^{brt}CD38^{brt}$ (/mL)	954.4 (263.2–2218.4); N=52	998.5 (269.7–2218.4); N=28	919.8 (210.4–2274.6); N=24	0.633
		Severe flare from week 24 through week 76		
	N=56	**N=4**	**N=52**	
$CD19^+CD20^+$ ($x10^3$/mL)	54.0 (22.0–102.0); N=51	61.5 (14.3–158.3)	54.0 (28.0–95.0); N=47	0.879
$CD19^+CD20^+CD27^+$ ($x10^3$/mL)	7.4 (3.5–10.7); N=52	5.1 (3.6–59.6)	7.5 (3.5–10.7); N=48	0.882
$CD19^+CD20^+CD69^+$ (/mL)	106.6 (45.5–182.8); N=52	100.6 (50.0–139.4)	106.6 (45.0–186.6); N=48	0.778
$CD19^+CD20^+CD27^-$ ($x10^3$/mL)	40.5 (18.7–94.5); N=52	57.2 (9.3–99.2)	40.5 (19.5–85.5); N=48	0.728
$CD19^+CD20^+CD138^+$ (/mL)	100.1 (58.3–247.3); N=52	143.8 (40.8–442.3)	89.7 (58.3–247.3); N=48	0.753
$CD19^+CD20^-CD138^+$ (/mL)	301.2 (175.6–685.7); N=52	173.4 (80.2–357.4)	309.6 (185.6–701.8); N=48	0.121

(Continued)

TABLE 2 | Continued

B cell subsets	All patients	Yes	No	P value
$CD19^+CD20^-CD27^{brt}$ (/mL)	970.6 (229.7–2204.8); N=52	1059.9 (280.8–2322.3)	970.6 (229.7–2204.8); N=48	0.960
$CD19^+CD27^{brt}CD38^{brt}$ (/mL)	954.4 (263.2–2218.4); N=52	1096.7 (296.8–2048.0)	954.4 (263.2–2308.9); N=48	1.000

Data are presented as medians (interquartile range) of absolute counts. In case of missing values, the total number of patients with available data is indicated. P values are derived from non-parametrical Mann-Whitney U tests. Statistically significant P values are in bold.
NEA, Northeast Asia; SC, subcutaneous.

prominent relative to baseline decreases through week 24 in $CD19^+CD20^-CD138^+$ long-lived plasma cells (-23.5% versus -39.4%; P=0.028), $CD19^+CD20^+CD138^+$ short-lived plasma cells (21.5% versus -41.1%; P=0.024) and $CD19^+CD27^{bright}CD38^{bright}$ SLE-associated plasma cells (-19.0% versus -27.8%; P=0.045) compared with patients who did not develop severe flares. No differences were observed between patients who developed severe flares compared with patients who did not regarding rapid or early changes in $CD19^+CD20^+$ B cells (P=0.967 and P=0.323, respectively), $CD19^+CD20^+CD69^+$ activated B cells (P=0.378 and P=0.431, respectively) or $CD19^+CD20^+CD27^-$ naïve B cells (P=0.273 and P=0.313, respectively), or rapid changes in $CD19^+CD20^+CD138^+$ short-lived plasma cells (P=0.599). The results are delineated in **Figure 3** and detailed in **Supplementary Tables S1**–**S3**.

Among patients who received add-on belimumab, no differences in rapid or early changes across any B cell subset were observed between patients who developed severe flares and patients who did not.

Among patients who received non-biological ST alone, patients who developed severe flares showed an increase while patients who did not develop severe flares showed a decrease from baseline through week 24 in $CD19^+CD20^+CD27^+$ memory B cells (+14.3% versus -7.7%; P=0.023), $CD19^+CD20^-CD138^+$ long-lived plasma cells (+6.7% versus -27.2%; P=0.002) and $CD19^+CD27^{bright}CD38^{bright}$ SLE-associated plasma cells (+41.2% versus -6.1%; P=0.038), resulting in a significant difference in all cases. No difference was observed between patients who developed severe flares and patients who did not regarding rapid or early changes in the overall $CD19^+CD20^+$ B cell pool (P=0.972 and P=0.062, respectively), $CD19^+CD20^+CD69^+$ activated B cells (P=0.653 and P=0.159, respectively), $CD19^+CD20^+CD27^-$ naïve B cells (P=0.761 and P=0.101, respectively), $CD19^+CD20^-CD27^{bright}$ plasmablasts (P=0.272 and P=0.184, respectively), or $CD19^+CD20^+CD138^+$ short-lived plasma cells (P=0.755 and P=0.106, respectively; **Figure 3**).

3.2.2.2 Serological Markers

In the entire cohort (all treatment arms), no differences between patients who developed severe flares and patients who did not were documented regarding rapid changes in anti-dsDNA or complement levels. Patients who developed at least one severe flare from week 24 onwards showed no early change (0.0%) while patients who did not develop severe flares exhibited early decreases in anti-dsDNA levels (-13.3%; P=0.020). In a subgroup analysis of patients with positive anti-dsDNA levels at baseline, the relative to baseline decrease in anti-dsDNA levels through week 24 was less prominent in patients who developed severe flares from week 24 onwards compared with patients who did not (-11.2% versus -29.8%; P=0.003), as shown in **Figure 4**. No differences between patients who developed severe flares and patients who did not were seen regarding early changes in C3 or C4 levels (**Figure 4**).

A similar pattern was seen among patients who received add-on belimumab. Patients who developed at least one severe flare from week 24 onwards showed a trend towards less prominent decreases in anti-dsDNA levels through week 24 compared with patients who did not develop severe flares (-10.5 versus -20.5%), which however did not reach statistical significance (P=0.071). Nevertheless, in the subgroup analysis of patients with positive anti-dsDNA levels at baseline, the decreases in anti-dsDNA levels through week 24 were less prominent in patients who developed severe flares from week 24 onwards compared with patients who did not (-19.6% versus -35.9%; P=0.022). No differences between patients who developed severe flares and patients who did not were seen regarding rapid or early changes in C3 or C4 levels (**Figure 4**).

Among patients who received non-biological ST alone, no differences between patients who developed severe flares and patients who did not were seen regarding rapid or early relative to baseline changes in anti-dsDNA, C3 or C4 levels (**Figure 4**).

3.3 Associations With Disease Flares in Time-Dependent Cox Regression Models

3.3.1 Flares of Any Severity (Mild/Moderate or Severe)

In the pooled datasets, 959/1533 patients (62.6%) developed at least one SFI flare of any degree of severity from week 24 through the end of the study period (week 52 in BLISS-SC and BLISS-NEA; week 76 in BLISS-76). Among patients who flared, the first flare occurred after a mean time of 254.4 ± 76.7 days from baseline.

Proportional hazards (Cox) regression models showed no ability of alterations in B cell or plasma cell subsets to portend flares of any severity occurring from week 24 onwards in the entire study population, being the case for both rapid changes through week 8 and early changes through week 24, the latter also in models adjusted for the rapid phase B cell changes (**Figure 5A**). By contrast, use of belimumab was shown to be overall protective against disease flares. The results are detailed in **Supplementary Table S4**, including the interaction term between belimumab use and relative to baseline B cell changes. Thus, the hazard ratio (HR) of flare development in belimumab-treated patients is derived by multiplication of the HR for the interaction term with the HR for B cell changes in the respective

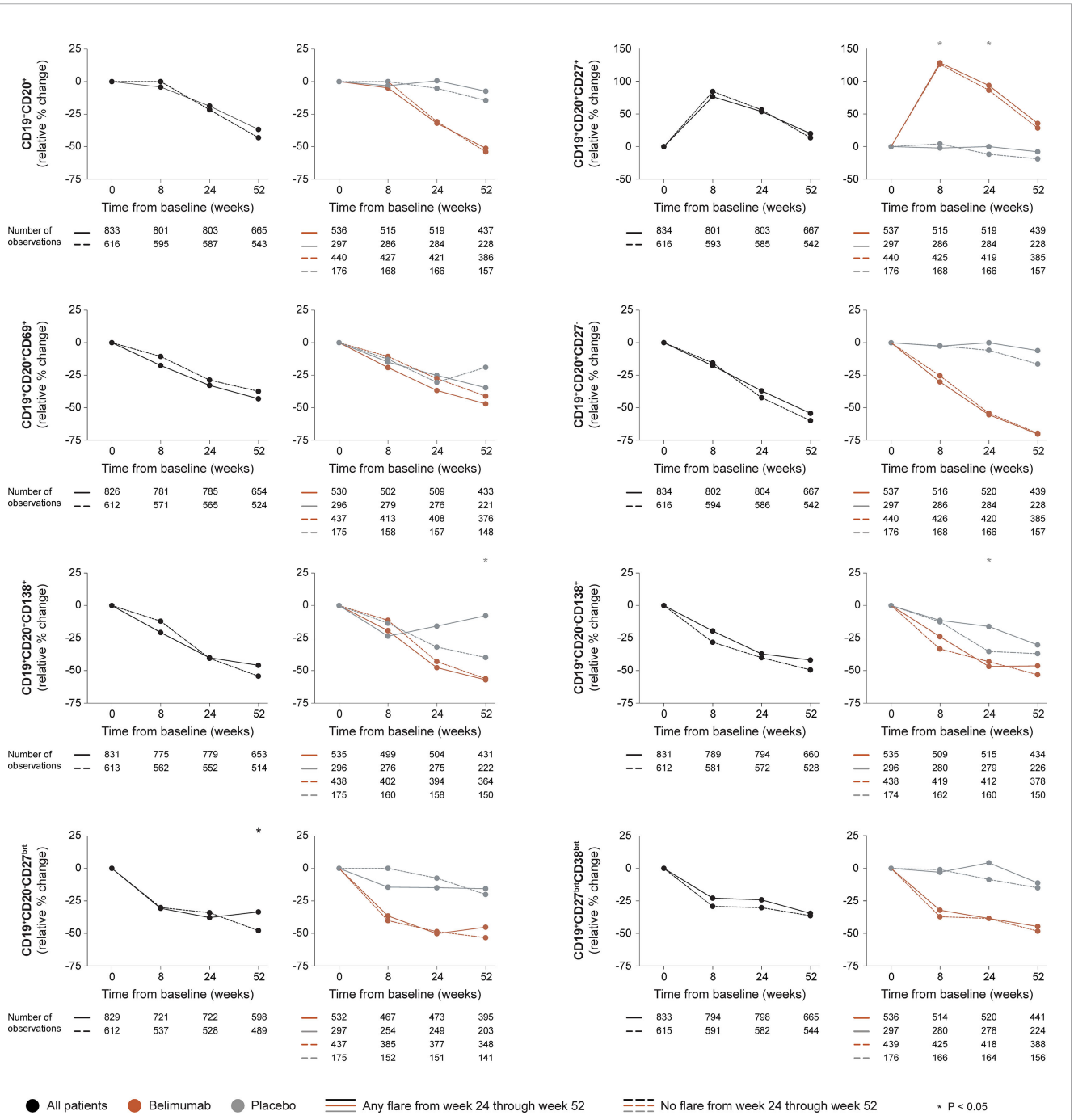

FIGURE 1 | B cell alterations in relation to flares occurring from week 24 through week 52. The graphs delineate relative to baseline percentage changes in selected B cell and plasma cell subsets from baseline through different time points in patients who developed at least one SFI flare (mild/moderate or severe) from week 24 through week 52 (continuous lines) and patients who did not (dashed lines). Comparisons between patients who flared and patients who did not were conducted for the entire population with available data (black lines), and after stratification into patients who received standard therapy plus belimumab (terracotta lines) and patients who received standard therapy alone (grey lines). P values derived from non-parametric Mann-Whitney *U* tests. The number of patients with available data at each time point is indicated for each patient subgroup. SFI, Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) - Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) Flare Index.

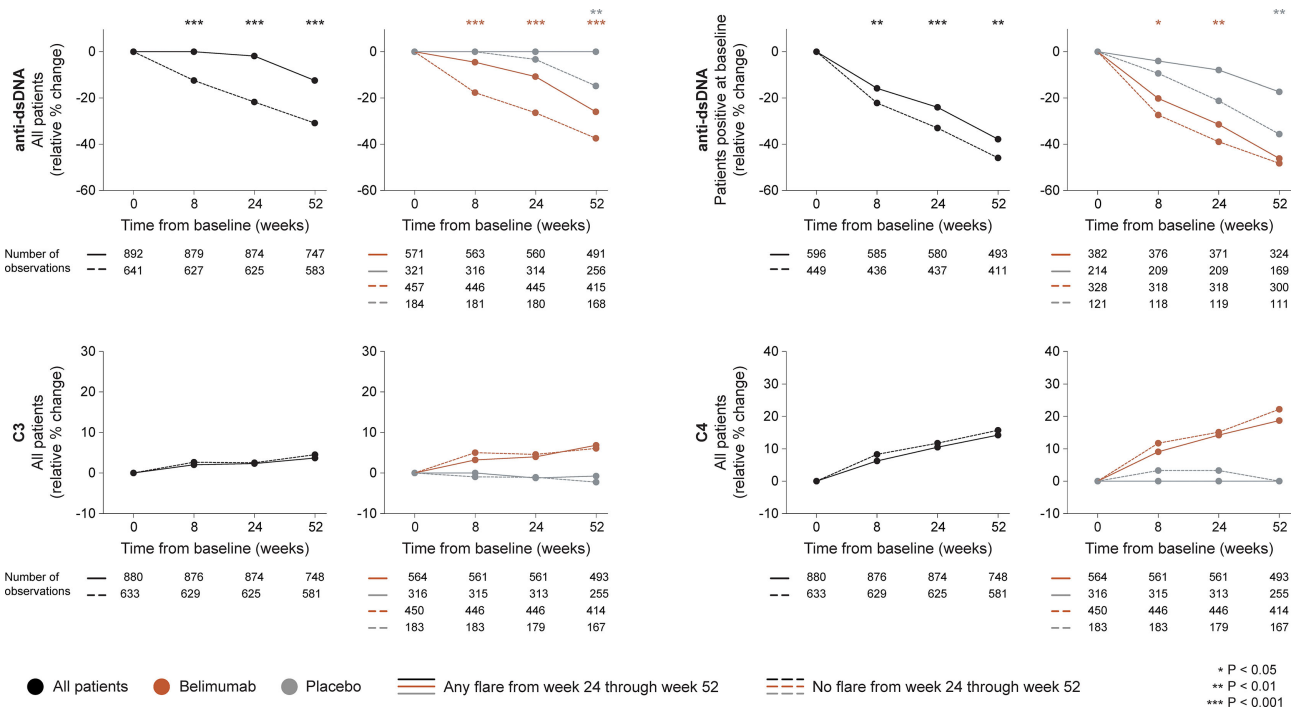

FIGURE 2 | Changes in serological markers in relation to flares occurring from week 24 through week 52. The graphs delineate relative to baseline percentage changes in anti-dsDNA, C3 and C4 levels from baseline through different time points in patients who developed at least one SFI flare (mild/moderate or severe) from week 24 through week 52 (continuous lines) and patients who did not (dashed lines). Comparisons between patients who flared and patients who did not were conducted for the entire population with available data (black lines), and after stratification into patients who received standard therapy plus belimumab (terracotta lines) and patients who received standard therapy alone (grey lines). For anti-dsDNA levels, a separate analysis for patients with positive anti-dsDNA levels (≥30 IU/mL) at baseline is also demonstrated. P values derived from non-parametric Mann-Whitney *U* tests. The number of patients with available data at each time point is indicated for each patient subgroup. SFI, Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) - Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) Flare Index; anti-dsDNA, anti-double stranded DNA antibodies; C3, complement component 3; C4, complement component 4.

model. Regarding flares of any severity, the interaction term did not reach statistical significance in any model.

3.3.2 Severe Flares

In the pooled datasets, 187/1533 patients (12.2%) developed at least one severe flare from week 24 through the end of the study period. Among patients who developed severe flares, the first severe flare occurred after a mean time of 274.3 ± 88.4 days from baseline.

Rapid increases in $CD19^+CD20^-CD138^+$ long-lived plasma cells from baseline through week 8 were associated with a higher likelihood and/or shorter time to the first severe flare from week 24 onwards (HR: 1.11; 95% CI: 1.01–1.22; P=0.024), while changes in the other B cell or plasma cell subsets during the rapid phase exhibited no significant association with development of severe flares. Add-on belimumab was shown to exert an overall protective effect, which however did not reach significance in the models of $CD19^+CD20^+CD27^+$ memory B cells, $CD19^+CD20^-CD138^+$ long-lived plasma cells and $CD19^+CD27^{bright}CD38^{bright}$ SLE-associated plasma cells (**Figure 5B**; **Supplementary Table S4**).

Notably, early increases in $CD19^+CD20^+CD27^+$ memory B cells from baseline through week 24 were associated with a higher likelihood and/or shorter time to the first severe flare from week 24 onwards, both before (HR: 1.39; 95% CI: 1.05–1.84; P=0.022) and after (HR: 1.58; 95% CI: 1.18–2.11; P=0.002) adjustment for changes in $CD19^+CD20^+CD27^+$ memory B cells during the rapid phase (from baseline through week 8), while add-on belimumab showed no protective effect in these models (**Figure 5B**). The interaction term between belimumab use and relative to baseline changes in $CD19^+CD20^+CD27^+$ memory B cells through week 24 was statistically significant (HR:0.72; 95% CI: 0.52–0.99; P=0.044) in the unadjusted model for the changes through week 8. Thus, relative to baseline changes in $CD19^+CD20^+CD27^+$ memory B cells through week 24 were associated with a 39% increased hazard of subsequent severe flare development when the patient was on placebo, while for belimumab-treated patients this hazard was minimal (1.39 x 0.72 = 1.0008), in line with the unadjusted analysis presented in **Figure 3**. Changes in the other B cell or plasma cell subsets from baseline through week 24 exhibited no significant association with development of severe flares before adjustment for the rapid phase. Following adjustment for the rapid phase, early increases in the total $CD19^+CD20^+$ B cell pool were associated with a higher likelihood and/or shorter time to the first severe flare (HR: 1.81; 95% CI: 1.08–3.05; P=0.024; **Figure 5B**). The results are detailed in **Supplementary Table S4**.

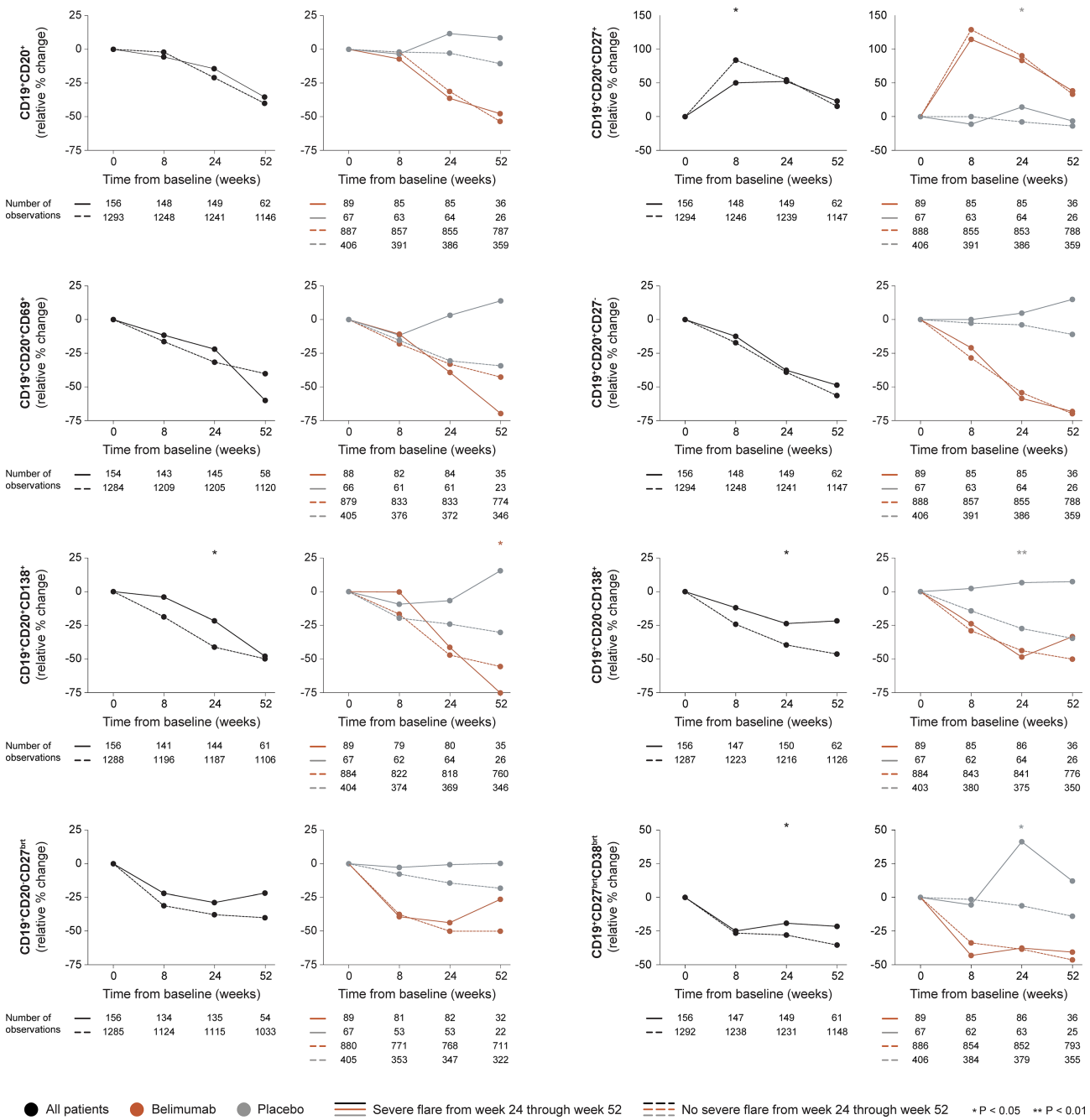

FIGURE 3 | B cell alterations in relation to severe flares occurring from week 24 through week 52. The graphs delineate relative to baseline percentage changes in selected B cell and plasma cell subsets from baseline through different time points in patients who developed at least one severe SFI flare from week 24 through week 52 (continuous lines) and patients who did not (dashed lines). Comparisons between patients who flared and patients who did not were conducted for the entire population with available data (black lines), and after stratification into patients who received standard therapy plus belimumab (terracotta lines) and patients who received standard therapy alone (grey lines). P values derived from non-parametric Mann-Whitney *U* tests. The number of patients with available data at each time point is indicated for each patient subgroup. SFI, Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) - Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) Flare Index.

4 DISCUSSION

In this paper, we analysed data from three phase III clinical trials of SLE. We demonstrated that increasing trends in long-lived plasma cells during an initial rapid phase and in memory B cells during a later intermediate phase upon commencement of therapy with belimumab or placebo on top of non-biological ST were associated with subsequent severe flares. Our study

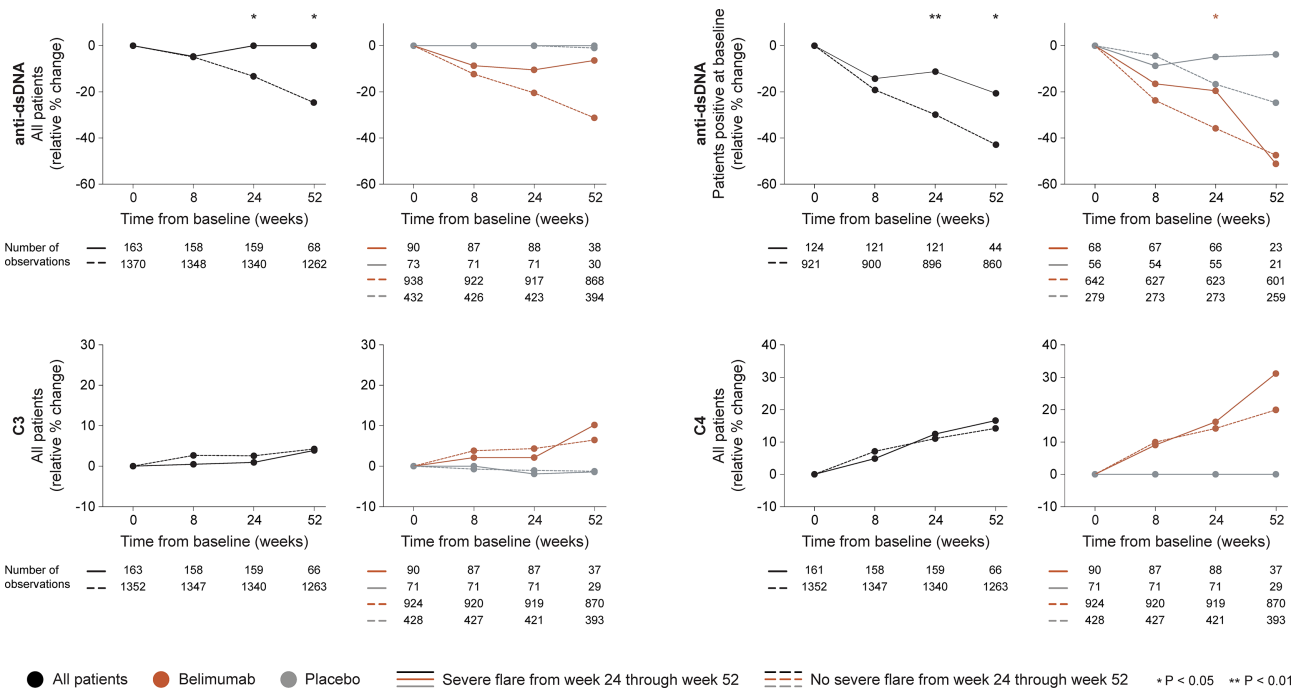

FIGURE 4 | Changes in serological markers in relation to severe flares occurring from week 24 through week 52. The graphs delineate relative to baseline percentage changes in anti-dsDNA, C3 and C4 levels from baseline through different time points in patients who developed at least one severe SFI flare from week 24 through week 52 (continuous lines) and patients who did not (dashed lines). Comparisons between patients who flared and patients who did not were conducted for the entire population with available data (black lines), and after stratification into patients who received standard therapy plus belimumab (terracotta lines) and patients who received standard therapy alone (grey lines). For anti-dsDNA levels, a separate analysis for patients with positive anti-dsDNA levels (≥30 IU/mL) at baseline is also demonstrated. P values derived from non-parametric Mann-Whitney *U* tests. The number of patients with available data at each time point is indicated for each patient subgroup. SFI, Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) - Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) Flare Index; anti-dsDNA, anti-double stranded DNA antibodies; C3, complement component 3; C4, complement component 4.

introduces dynamics in peripheral B cell and plasma cell subsets as a potential complemental tool in the surveillance of lupus patients. It is worth noting that among patients treated with add-on belimumab, patients who developed flares exhibited more modest decreases in anti-dsDNA levels compared with patients who did not flare, providing important implications about the potential usefulness of anti-dsDNA dynamics in early evaluation of belimumab therapy. To the best of our knowledge, this is the first documentation of the relationship between rapid and early changes in circulating B lymphocyte subsets and subsequent disease flares in a large SLE population, with potential implications regarding surveillance strategies and early treatment evaluation in patients with SLE.

Prevention of flares is included among SLE treatment goals, since they may heavily influence the patients' prognosis, e.g., by contributing to organ damage accrual and morbidity (30, 31). Unfortunately, despite advanced therapeutics during the last decades (14), implementation of efficient preventive strategies remains an unmet need, and flares are not rare even upon treatment initiation (32, 33), the latter *per se* thus not constituting a guarantee for disease quiescence. Moreover, early determination of the risk for disease flares in patients commencing treatment for active SLE is still not feasible in clinical practice. Considering the important role of B cells in SLE (30), exploration of the relationship between their kinetics upon treatment initiation and disease flaring is intriguing.

Following the advent of the anti-BAFF biological agent belimumab, several studies have highlighted that this drug reduces the burden of flares in patients with SLE (2, 16–18). Considering its mode of action, belimumab is expected to hamper the survival of B cells, especially immature B cells, which has been corroborated in previous research (28, 34–36). Thus, declining B cell subsets, especially B cell subsets of early developmental stages, could be expected to signify better responses to belimumab therapy, in a similar manner as successful B cell depletion has been shown to be coupled with good responses to treatment with rituximab (37, 38).

Our hypothesis was that prominent biological changes towards abatement of B cell activity upon therapy initiation would be associated with a protection against flares, and since biological changes have been shown to precede the measurable clinical improvement induced by belimumab (34), one could expect that alterations in B cell subsets in patients who are protected from flares occur early after treatment initiation. The concept of monitoring early biological changes to portend therapeutic outcome should not be regarded as contradicting that of baseline predictors, but rather complemental towards optimised surveillance, early and efficient decision-making, and

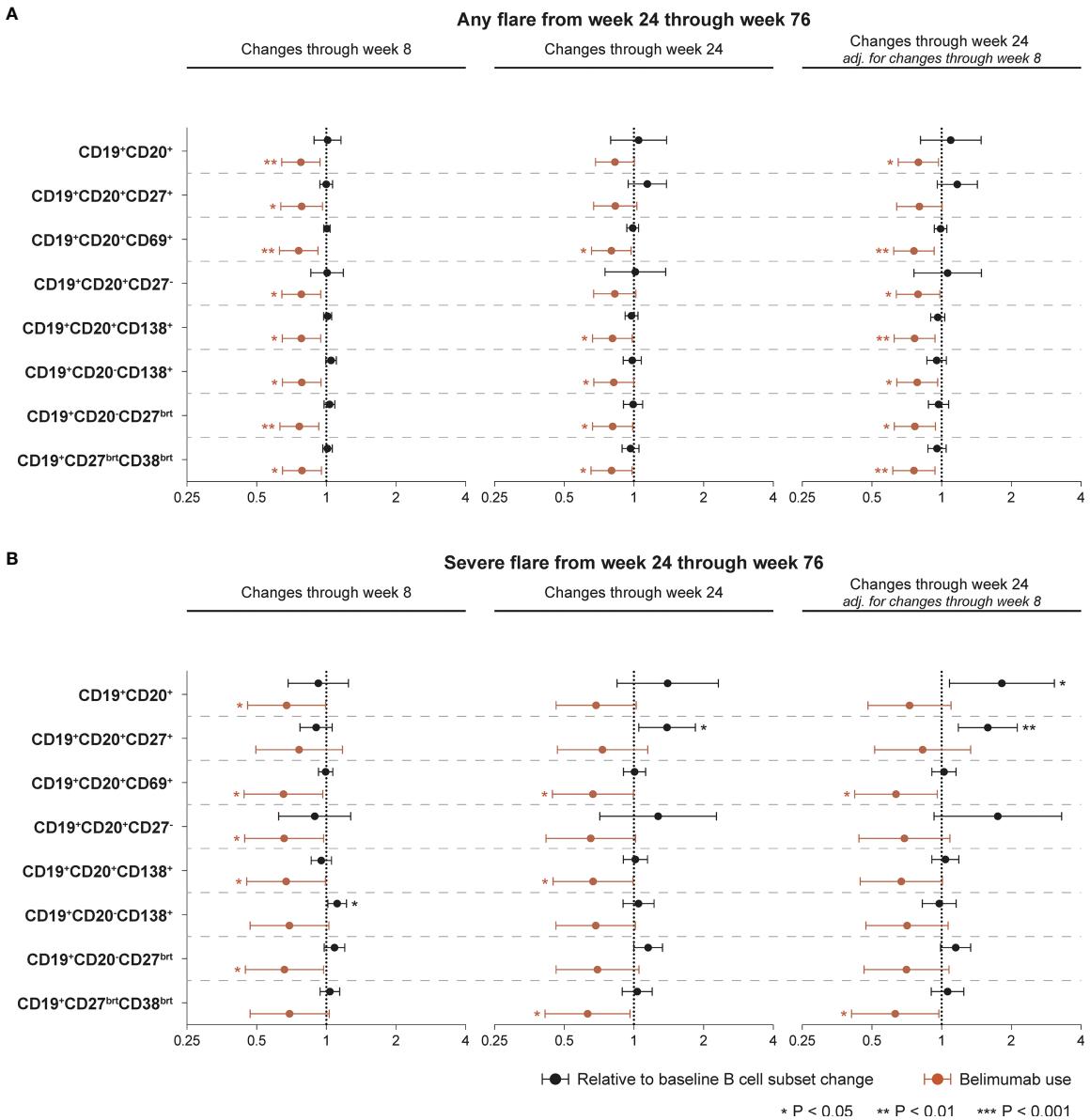

FIGURE 5 | Associations between B cell alterations and flare development. The forest plots illustrate results from proportional hazards (Cox) regression analysis, investigating associations between rapid or early relative to baseline percentage changes in selected B cell and plasma cell subsets and development of the first SFI flare of any severity (mild/moderate or severe; **(A)** or the first severe SFI flare **(B)** occurring from week 24 through week 76 or the last available follow-up visit. All models included belimumab use (any dose) as a covariate, and the result for the respective model is plotted in terracotta colour. The potential interaction between cell alterations and belimumab use were accounted for. Additionally, all models were adjusted for age, sex, ethnicity, SLE disease duration, use of methotrexate, use of azathioprine, use of mycophenolate mofetil, use of immunosuppressants other than those mentioned before, and the BLISS study to account for batch variations in cell analyses. One set of models investigating associations between early B cell changes and flare development was also adjusted for the relative to baseline cell alterations from baseline through week 8 to account for alterations in opposing directions in the rapid and early follow-up phase. Circles denote hazard ratios and whiskers denote 95% confidence intervals. Statistically significant associations are indicated with asterisks. SFI, Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) - Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) Flare Index.

better outcomes. For instance, serological status at baseline has been shown to be informative regarding the outcome of belimumab therapy (39, 40), as have early decreases in levels of interleukin (IL)-6 (41).

Flares may occur at any time during patient follow-up and have been reported both as an early and a delayed event upon treatment initiation (3, 5, 17). In the present study, we assessed changes in peripheral B cell and plasma cell subsets preceding disease flares which occurred from week 24 from baseline and throughout a follow-up of up to 76 weeks. We showed that more prominent rapid and early decreases in long-lived plasma cells were inversely associated with subsequent flares, particularly

severe flares. Stratification of patients by treatment arm (ST plus belimumab and ST alone) revealed that the inverse association between early decreases in long-lived plasma cells and subsequent flaring was significant in patients who received non-biological ST alone in unadjusted analysis but not in patients who received add-on belimumab. Thus, early and profound decreases in long-lived plasma cells may signify greater expected drug efficacy and a protective effect against flares when broad immunosuppression is commenced, whereas belimumab may rather be expected to induce decreases irrespective of the treatment outcome. While this observation should be interpreted with caution since it was not replicated in the Cox regression analysis for the early treatment phase, it has some interest in light of inconsistent results in previous research regarding the impact of belimumab therapy on plasma cell subsets (28, 34–36). In this respect, the large study population and the investigation of several distinct plasma cell subsets carried out in the present study may have facilitated the detection of subsets within the plasma cell pool, the kinetics of which may have particular prognostic value.

By contrast, a rapid increase in memory B cells was found to be inversely associated with subsequent occurrence of severe flares. Interestingly, however, a later relative to baseline increase in memory B cells through week 24 was also shown to portend severe flares in time-dependent Cox regression analysis. This seemingly conflicting finding becomes interesting in light of knowledge that belimumab therapy induces an early expansion of memory B cells, with a subsequent return towards baseline values (35, 36), which however has not been put in relation to a longer-term treatment outcome. The findings herein imply that while this initial expansion may be associated with belimumab efficacy and a lower likelihood to develop severe flares, the lack of return or a continued increase in memory B cells may be associated with abatement of the drug efficacy and flare development. Following stratification by treatment arms, the rapid expansion of memory B cells was evidently driven by belimumab, although the numbers were not sufficient to demonstrate significant differences between flaring and non-flaring patients within treatment groups. Notably, it was also evident that among patients who received non-biological ST alone, those who developed severe flares from week 24 onwards displayed an increase in memory B cells through week 24 following an initial drop, whereas belimumab-treated patients displayed a rapid increase in circulating memory B cells followed by a subsequent return regardless of flare occurrence.

Importantly, relative increases in the overall B cell pool through week 24 were also found to herald subsequent severe flares in Cox regression analysis, however only after adjustment for B cell changes during the rapid treatment phase, which complicates the interpretation of this finding. The link between changes in the circulating B cell pool and clinical response has been investigated in response to anti-CD20 treatment in SLE and lupus nephritis, with overall depletion of B cells being associated with better responses (38, 42), whereas a quick repopulation of memory B cells and plasmablasts heralded lupus flares (37). Our findings yield further merit to the concept of B cell monitoring as a relevant tool for patient follow-up upon therapy, especially B cell modulatory therapy, and provide novel implications of a connection between changes in distinct B cell and plasma cell subsets in the periphery following anti-BAFF treatment and occurrence of lupus flares.

We also investigated changes in anti-dsDNA and complement levels. In this analysis, anti-dsDNA antibody levels decreased more prominently through week 24 in belimumab-treated patients who did not develop subsequent severe flares compared with belimumab-treated patients who developed severe flares, whereas in placebo-treated patients this difference reached significance only at week 52. This corroborates the known usefulness of anti-dsDNA antibodies in surveillance of patients with SLE (43, 44), here also in the context of treatment evaluation (45), especially early evaluation of treatment with belimumab. While add-on belimumab overall induced increases in C3 and C4 levels, those could not distinguish patients who flared from patients who did not.

Among the limitations of the present study, one should mention the selected clinical trial population, which was enriched with patients with active musculoskeletal and mucocutaneous SLE, raising concerns about the generalisability of our findings. On the other hand, this is the first study to assess early changes in B cell subsets upon treatment initiation in relation to the development of SLE flares in a large study population. Importantly, when interpreting the results, one should bear in mind that we investigated relative and not absolute changes in cell subsets, which on the one hand may pose hurdles in interpretation and direct clinical implementation, whereas on the other hand normalised the values and circumvented batch effects from the varying methods at different laboratories. Lastly, in this investigation we stratified flares according to their severity, which forms a rather generalised concept for flaring. However, even severe articular or mucocutaneous flares may be less likely to result in life-threatening complications and irreversible organ damage compared with renal or neuropsychiatric flares. While it was beyond the scope of this study, flare stratification by organ involvement would have merit in a future analysis, as would stratification by background immunosuppressive therapy.

In summary, we showed that a rapid increase in long-lived plasma cells, an early increase in the total pool of circulating B cells, and an early or intermediate increase in memory B cells upon treatment initiation for active SLE heralded subsequent severe disease flares. Moreover, no or less prominent rapid or early decreases in anti-dsDNA antibody levels were also associated with the development of flares of any severity and severe flares, especially in patients treated with add-on belimumab. An initial expansion of memory B cells may signify sustained response to therapy when followed by a subsequent drop, while intermediate increases in memory B cells may portend flaring. Therapeutic adjustments in patients showing no dynamics in peripheral plasma cell subsets or anti-dsDNA levels might help prevent flares and disease progression. Overall, anti-dsDNA may be an important marker in the monitoring of patients treated with belimumab, and peripheral B cell and plasma cell subsets may prove a useful complement to traditional surveillance and early treatment evaluation in patients with SLE.

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 trial protocols were approved by regional ethics review boards for all participating centres and complied with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all study participants prior to enrolment. The present study was reviewed and approved by the Swedish Ethical Review Authority (2019-05498).

AUTHOR CONTRIBUTIONS

Study conception and design, IP and MG. Acquisition of data, IP, AG, JC, AB, and JL. Analysis and interpretation of data, IP, AG, and MG. All authors were involved in the drafting of the manuscript or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.796508/full#supplementary-material

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PERSPECTIVE
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B Cell Characteristics at Baseline Predict Vaccination Response in RTX Treated Patients

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Background: Vaccination is considered as most efficient strategy in controlling SARS-CoV-2 pandemic spread. Nevertheless, patients with autoimmune inflammatory rheumatic diseases receiving rituximab (RTX) are at increased risk to fail humoral and cellular responses upon vaccination. The ability to predict vaccination responses is essential to guide adequate safety and optimal protection in these patients.

Methods: B- and T- cell data before vaccination were evaluated for characteristics predicting vaccine responses in altogether 15 patients with autoimmune inflammatory rheumatic diseases receiving RTX. Eleven patients with rheumatoid arthritis (RA) on other therapies, 11 kidney transplant recipients (KTR) on regular immunosuppression and 15 healthy controls (HC) served as controls. A multidimensional analysis of B cell subsets *via* UMAP algorithm and a correlation matrix were performed in order to identify predictive markers of response in patients under RTX therapy.

Results: Significant differences regarding absolute B cell counts and specific subset distribution pattern between the groups were identified at baseline. In this context, the majority of B cells from vaccination responders of the RTX group (RTX IgG+) were naïve and transitional B cells, whereas vaccination non-responders (RTX IgG-) carried preferentially plasmablasts and double negative (CD27-IgD-) B cells. Moreover, there was a positive correlation between neutralizing antibodies and B cells expressing HLA-DR and CXCR5 as well as an inverse correlation with CD95 expression and CD21low expression by B cells among vaccination responders.

Summary: Substantial repopulation of the naïve B cell compartment after RTX therapy appeared to be essential for an adequate vaccination response, which seem to require the additional capability of antigen presentation and germinal center formation. Moreover, expression of exhaustion markers represent negative predictors of vaccination responses.

Keywords: RTX (rituximab), B-cells, vaccination, SARS – CoV – 2, prediction

INTRODUCTION

Patients with autoimmune inflammatory rheumatic diseases (AIIRD) are at increased risk for infections, attributed to the underlying autoimmune disease, immunosuppressive therapy and comorbidities (1). Thus, COVID-19, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requires particular considerations in AIIRD patients by rheumatologists. Rituximab (RTX), a first generation anti-CD20 monoclonal antibody leading to B cell depletion and largely used in rheumatologic diseases, has been found as risk factor for poor COVID-19 associated outcomes regarding hospitalization and death (2, 3). Severe COVID-19 can be prevented by vaccination in healthy individuals (4–6), however, B cell depleting therapy with rituximab has been reported to result in substantially diminished vaccination responses following SARS-CoV-2 vaccination (7–9).

The ability to predict vaccination responses is crucial to ensure safety and optimal protection in this patient group. We have previously described that a minimum level of B cell repopulation (at least 10 cells/µl, 0.4% of lymphocytes accordingly) is necessary for RTX treated patients to develop humoral and adequate T cellular responses upon SARS-CoV-2 vaccination (9). In this study, we described predictive markers of vaccination response by assessing qualitative characteristics of the B cell compartment before vaccination (d0) predicting IgG responses by analyzing B cell subsets and molecular B cell markers at baseline.

MATERIALS AND METHODS

Study Participants

Outpatient rheumatic patients treated with RTX, who received SARS-CoV-2 vaccination according to federal and Berlin state recommendations between February and May 2021 and participated at our initial study (9), were screened for the availability of baseline data before vaccination (d0). From the previously studied 19 RTX treated patients, we included 13 rheumatoid arthritis (RA) patients [according 2010 ACR Rheumatoid Arthritis Classification Criteria (10)] and 2 ANCA associated vasculitis [AAV patients, defined as (11)] under RTX treatment. Eleven RA patients receiving other therapies (RA group), 11 kidney transplant recipients (KTR) on regular immunosuppression (KTR group) and 15 healthy controls (HC group) served as control groups. All participants gave written informed consent according to the approval of the ethics committee at the Charité University Hospital Berlin (EA2/010/21, EA4/188/20). Peripheral blood samples (EDTA anti-coagulated or serum-tubes, BD Vacutainersystem, BD Diagnostics, Franklin Lakes, NJ, USA) were collected at baseline and 3 weeks after vaccination with either 2x SARS-CoV-2 BNT162b2, 2x ChAdOx1 nCoV-19 or 1x ChAdOx1 nCoV-19 followed by 1x SARS-CoV-2 BNT162b2. Serologic data (**Figure 1A**) and antigen specific (RBD+) B cells (**Figure 1B**) of HC, RA, RTX patients and KTR 3 weeks after 2nd vaccination have been partially previously published (9, 12). Regarding the absolute numbers of CD19+, CD4+ and CD8+ lymphocytes, there was no difference between baseline and after 2nd vaccination (data not shown). Donor information is summarized in **Table 1**.

Enzyme-Linked Immunosorbent Assay

The Euroimmun anti-SARS-CoV-2 assay is a classical enzyme-linked immunosorbent assay (ELISA) for the detection of IgG and IgA to the S1 domain of the SARS-CoV-2 spike (S) protein, and IgG to the SARS-CoV-2 NCP protein. The assay was performed according to the manufacturer´s instructions, as described (12). Briefly, serum samples were diluted at 1:100 in sample buffer and pipetted onto strips of 8 single wells of a 96-well microtiter plate, precoated with recombinant SARS-CoV-2 spike or nucleocapsid proteins. Calibrators, a positive and a negative control were carried out on each plate. After incubation for 60 minutes at 37°C, wells were washed 3 times and the peroxidase-labelled anti-IgG or anti-IgA antibody solution was added, followed by a second incubation step for 30 min. After three additional washing steps, substrate solution was added and the samples incubated for 15 - 30 minutes in the dark. OD values were measured on a POLARstar Omega plate reader (BMG Labtech, Ortenberg, Germany) at 450 nm and at 620 nm. Finally, OD ratios were calculated based on the sample and calibrator OD values. To identify previously SARS-CoV-2 infected individuals we measured antibodies against the nucleocapsid protein (NCP, not a vaccine component) 6 ± 3 days after 2nd vaccination (indicated in red in **Figure 1**).

Surrogate SARS-CoV-2 Neutralization Test (GenScript)

The assay was performed according to the manufacturer´s instructions, as described (12). This blocking ELISA qualitatively detects anti-SARS-CoV-2 antibodies suppressing the interaction between the receptor binding domain (RBD) of the viral spike glycoprotein (S) and the angiotensin-converting enzyme 2 (ACE2) protein on the surface of cells. After pre-incubation of samples and controls, which allows antibodies in the serum to bind to a horseradish peroxidase (HRP)-conjugated RBD fragment (HRP-RBD), the mixture is added to a capture plate coated with human ACE2 protein. Any unbound HRP-RBD or HRP-RBD bound to non-neutralizing antibodies is captured on the plate. Complexes of neutralizing antibodies

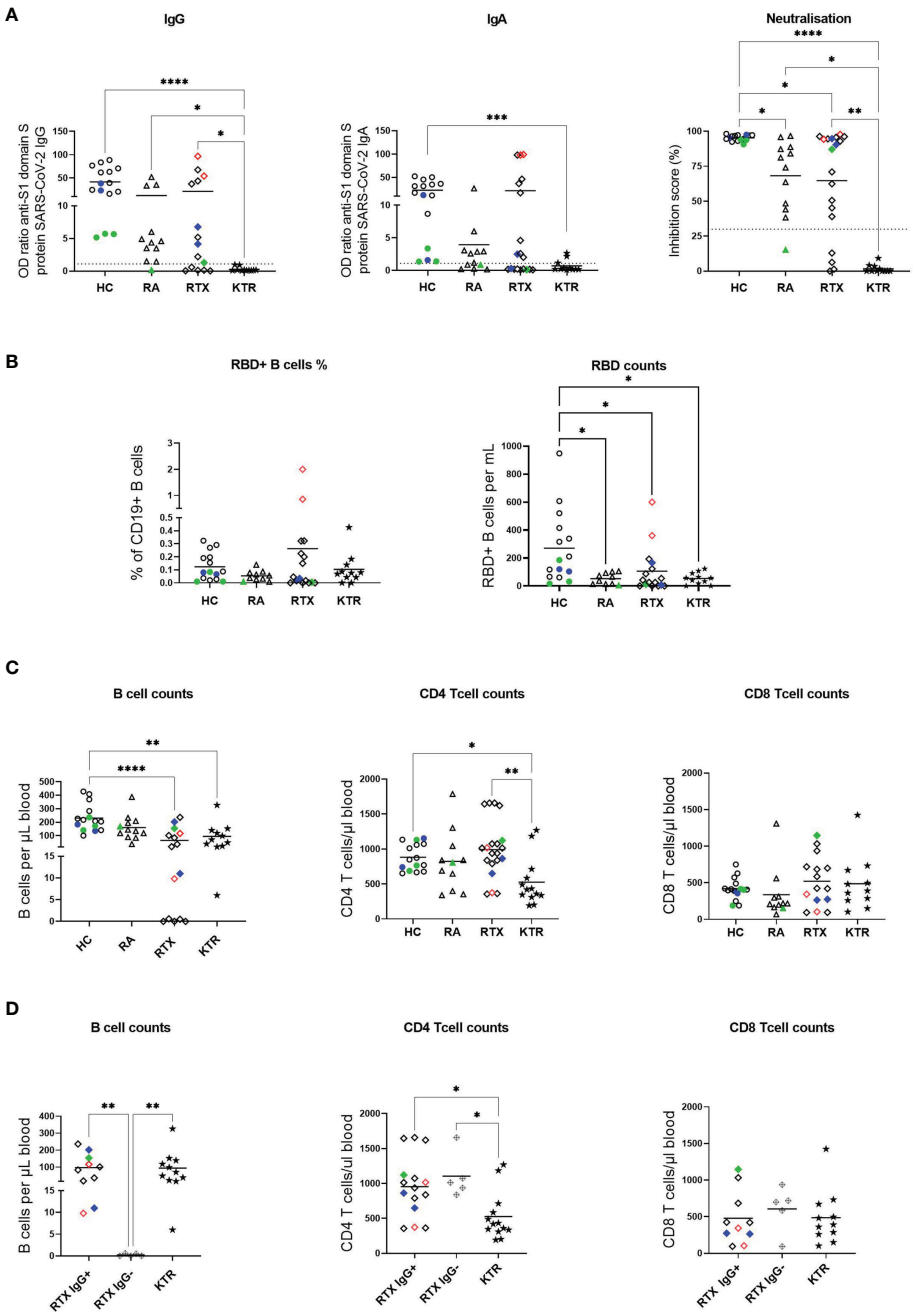

FIGURE 1 | Impaired humoral and cellular anti-SARS-CoV-2 vaccination response in RTX treated patients. **(A)** Humoral immune response against SARS-CoV-2 was assessed by ELISA for spike protein S1 IgG, spike protein S1 IgA and virus neutralization by a blocking ELISA 3 weeks after 2nd SARS-CoV-2 vaccination. Threshold of upper limit of normal is indicated by dotted lines. **(B)** Frequencies and absolute numbers of RBD+ cells among total CD19+ B cells measured 6 ± 3 days after 2nd vaccination. **(C)** Absolute cell counts of CD19+ B cells, CD4+ and CD8+ T cells among the groups at baseline (d0) before vaccination. **(D)** Absolute numbers of CD19+ B cells, CD4+ and CD8+ T cells among the groups at baseline (d0) before vaccination in KTR, RTX IgG+ and RTX IgG- patients. Color code: previously infected individuals are indicated as red quadrats; 2x vaccinated with ChAdOx1 indicated in green; 2x heterologous vaccinated 1x ChAdOx1 followed by 1x BNT162b2, indicated in blue. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

and HRP-RBD do not bind on the plate and are removed after three washing steps. Then, TMB is added as a substrate, allowing HRP to catalyze a colour reaction. The colour of the solution changes from blue to yellow after addition of the stop reagent, and can be read by a mictotiter plate reader at 450nm (OD450). The absorbance of the sample is inversely correlated with the amount of SARS-CoV-2 neutralizing antibodies. Positive and negative controls serve as internal controls, the test is considered

TABLE 1 | Patient characteristics.

	HC N=15	RA N=11	RTX N=15 (13 RA, 2 AAV)	KTR N=11
Age				
Median [IQR]	54 [41.5 – 70]	65 [62.5 – 79.5]	59 [57.5 – 64,5]	59 [51.7 – 63]
Under 50	7	2	3	2
Between 50-69	5	4	9	9
> 70	3	5	3	0
Gender				
Female	8	8	12	2
Male	7	3	3	9
Vaccines (n)				
2x BNT162b2	10	10	12	11
2x ChAdOx1	3	1	1	0
1x ChAdOx1 + 1x BNT162b2	2	0	2	0
Immunosuppression (n)				
MTX		6	4	
Leflunomid		1	0	
Sulfasalazin		0	1	
AZA		0	1	
JAKI		4	2	
TNFI		1	0	
Abatacept		1	1	
MMF				11
CNI				10
Prednisolone		2 (max 4mg/d)	6 (max 7.5mg/d)	11 (max. 5mg/d)
DAS 28				
Median [IQR]		3.1 [2.4 – 3.5]	2.58 [1,7 – 3.1]	
Months since last RTX				
Median [IQR]			8.5 [5.5 – 15]	
Years on RTX				
Median [IQR]			3 [1.5 – 6.5]	

IQR, interquartile range; MTX, methotrexate; AZA, azathioprine; JAKI, janus kinase inhibitor; TNFI, tumor necrosis factor alpha inhibitor; MMF, mycophenolate mofetil; CNI, calcineurin inhibitor.

valid only if the OD450 for each control falls within the respective range (OD450negative control > 1.0, OD450positive control < 0.3). For final interpretation, the inhibition rates were determined using the following formula: Inhibition score (%) = (1 - [OD valuesample/OD valuenegative control] x 100%). Unless stated otherwise, scores < 30% were considered negative, scores ≥ 30% were considered positive.

Isolation of Peripheral Blood Mononuclear Cells and Staining

PBMCs were prepared by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Chicago, IL, USA). For surface staining 1 x 10^6 cells were suspended in 50 µl of PBS/0.5% BSA/EDTA and 10 µl Brilliant Buffer (BD Horizon, San Jose, CA, USA). Cells were stained for 15 min on ice and washed afterwards with PBS/0.5% BSA/EDTA (810 xg, 8 min, 4°C).

Staining of Antigen-Specific B Cells

To identify RBD-specific B cells, recombinant purified RBD (DAGC149, Creative Diagnostics, New York, USA) was labeled with either AF647 or AF488 as reported (9, 12). Double positive cells were considered as antigen-specific. A blocking experiment using unlabeled RBD in 100-fold concentration was used to ensure specificity of detection.

Flow Cytometry Analysis

All flow cytometric analyses were performed using a BD FACS Fortessa (BD Biosciences, Franklin Lakes, NJ, USA). To ensure comparable mean fluorescence intensities (MFIs) over time of the analyses, Cytometer Setup and Tracking beads (CST beads, BD Biosciences, Franklin Lakes, NJ, USA) and Rainbow Calibration Particles (BD Biosciences, Franklin Lakes, NJ, USA) were used. For flow cytometric analysis, the following fluorochrome-labeled antibodies were used: BUV737 anti-CD11c (BD, clone B-ly6), BUV395 anti-CD14 (BD, clone M5E2), BUV395 anti-CD3 (BD, clone UCHT1), BV786 anti-CD27 (BD, clone L128), BV711 anti-CD19 (BD, clone SJ25C1), BV605 anti-CD24 (BD, clone ML5), BV510 anti-CD10 (BD, clone HI10A), BV421 anti-CXCR5 (BD, clone RF8B2), PE-Cy7 anti-CD95 (ThermoFischer, Waltham, MA, USA clone APO-1/Fas), PE-CF594 anti-IgD (Biolegend, San Diego, CA, USA, clone IA6-2), APC-Cy7 anti-CD38 (Biolegend, clone HIT2), PE-Cy7 anti-IgG (BD, clone G18-145), anti-IgA-Biotin (BD, clone G20-359), BV650 anti-IgM (BD, clone MHM-88), FITC anti-HLA-DR (Biolegend, clone L234), PE anti-CD21 (BD, clone B-ly4), APC anti-CD22 (BD, clone S-HCL-1). The absolute number of B cells, CD4+ and CD8+ T cells was measured with Trucount (BD) and samples were processed according to the manufacturer's instruction (B cells were defined as CD19+CD45+ CD3-CD14-CD16-CD56- lymphocytes, CD4+ T cells as CD45+CD3+CD4+CD8-CD19- CD14-CD16-CD56- lymphocytes, CD8+ T cells as CD45+CD3+CD8+CD4-CD19- CD14-CD16-CD56- lymphocytes).

Data Analysis

All samples included in the final analyses had at least 1×10^6 events with a minimum threshold for CD19+ cells of 2,000 events apart from RTX patients: minimal recorded CD19+ events in the RTX group were 13 and 17 events respectively, out of > 1 Mio total recorded events. Flow cytometric data were analyzed by FlowJo software 10.7.1 (TreeStar, Ashland, OR, USA). For UMAP analysis of CD19+ B cells flow cytometry data of all study participants was pre-gated on alive CD19+ B cells, concatenated, down sampled to 350 cells per cohort (total CD19 B cells in the RTX IgG- cohort; DownSmapleV3; FlowJo plugin) and clustered by CD27, IgD, CD38, CD10, CD24. As settings we selected the Euclidean distance function, nearest neighbor value of 15 and a minimum distance of 0.5.

Statistics

GraphPad Prism Version 5 (GraphPad software, San Diego, CA, USA) was used for statistical analysis. For group comparison Kruskal-Wallis with Dunn´s post-test was used. P-values < 0.05 were considered significant. Correlation matrix was calculated using base R and corrplot package (R Foundation for Statistical Computing) using the Spearman method (n=13 due to limited B cell numbers in 2 RTX patients).

RESULTS

Cohorts and Patient Characteristics

For the current vaccination study, we included 15 patients receiving rituximab (13 with rheumatoid arthritis, RA, and 2 with ANCA associated vasculitis, AAV; RTX group), 15 healthy controls (HC group), 11 RA patients on other therapies (RA group) and 11 kidney transplant recipients (KTR group) as additional control groups. The majority of study participants were vaccinated twice with the mRNA vaccine BNT162b2. There were three HC, one RA and one RTX vaccinated twice with the viral vector vaccine ChAdOx1 (indicated in green throughout the figures). Two RTX patients and two HC, respectively received 1x ChAdOx1 followed by a heterologous vaccination with 1x BNT162b2, according to national recommendations (indicated in blue throughout the figures). Demographics and co-medication of all study participants are summarized in **Table 1**. HC were younger than RA patients, but had a comparable age as RTX and KTR patients. The majority of RA and RTX patients were female, while the majority of KTR were male, as characteristic of these patients. At the time of vaccination, RTX patients had received B cell depleting therapy on average for 3 years and median time since the last RTX treatment was 8.5 months. The majority of the KTR patients received triple immunosuppression with mycophenolate mofetil (MMF), a calcineurin-inhibitor (CNI) and low-dose prednisolone.

Impaired Humoral Response and Induction of RBD+ B Cells Upon SARS-CoV-2 Vaccination in All Patient Groups

Antibody responses to SARS-CoV-2 vaccines were assessed in all individuals, 3 weeks after 2nd vaccination. All HC became positive for anti-S1 IgG and IgA and showed very high (> 90%) SARS-CoV-2 neutralisation. As previously reported (9, 12), KTRs failed to develop IgA and IgG anti-vaccine including neutralizing titres, while in the RA and the RTX group, the titre of neutralizing antibodies were significantly diminished upon vaccination (**Figure 1A**). 10/15 (66.7%) of RTX treated patients compared to 15/15 (100%) of HC, 10/11 (90.9%) of patients in the RA control and 0/11 (0%) of patients from the KTR group mounted anti-spike-IgG SARS-CoV-2 antibodies 3 weeks upon 2nd vaccination. Two RTX patients with unknown prior infection (identified as anti-nucleocapsid protein positive, indicated in red), developed high titers of anti-S1 IgG, IgA and neutralizing antibodies comparable with HC.

Next, we studied SARS-CoV-2 specific B cell responses using flow cytometry to quantify receptor-binding domain (RBD) specific B cells in peripheral blood [as previously described (9, 12), gating strategy shown in **Supplementary Figures 1A, B**]. While percentages of RBD+ B cells were comparable among the groups, there were significantly diminished absolute B cell numbers in all patient groups compared with HC (**Figure 1B**).

RTX Patients Show Diminished CD19+ B Cell Counts but Normal Range of CD4+/ CD8+ T Cell Counts Before Vaccination

To identify predictive factors regarding vaccination response in RTX treated patients, we analyzed cellular data at baseline (d0) before vaccination. First, absolute CD19+, CD4+ and CD8+ cell counts were measured for all groups (**Figure 1C**). While absolute B cell counts were significantly reduced in the RTX and KTR group compared with HC, only KTR showed significantly diminished CD4+ T cell numbers when compared with HC and RTX patients. There were no significant differences regarding CD8+ T cell counts between the groups. A deeper insight into the differences between IgG seroconverted (RTX IgG+), non-seroconverted (RTX IgG-) RTX patients and the KTR group revealed significantly lower B cell counts for the RTX IgG- patients (**Figure 1D**). Furthermore, absolute numbers of CD4+ T cells were significantly diminished in the KTR group compared with RTX IgG+ and RTX IgG- groups.

Therapy-Related B Cell Subset Distribution Is Characteristic Among the Groups

Next, we implemented a high-dimensional flow cytometry analysis of circulating B cell populations before vaccination using Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction (**Figure 2**). After down sampling to comparable B cell numbers across all groups, clusters corresponding to distinct subsets of CD19+ B cells were defined as: transitional (CD24+CD38+CD10+), naïve (CD27-IgD+), pre-switch-memory (CD27+IgD+), switched memory (CD27+IgD-) and double negative B cells (CD27-IgD-) as well as plasmablasts (CD27+CD38+, **Figure 2A**, distribution of key markers shown in **Supplementary Figure 2**). Clusters gated in each donor group are shown in **Figures 2B, C**. While the majority of B cells in RTX IgG+ group consisted of naïve and transitional B cells, the predominant subsets in non-responders

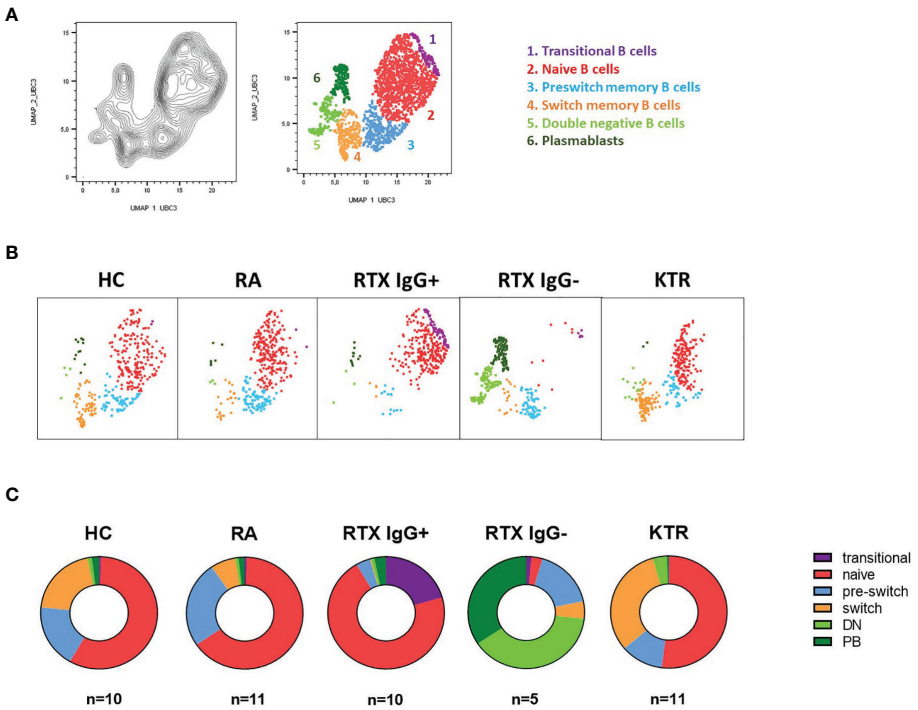

FIGURE 2 | Distinct B cell subsets characterize HC and patient groups. UMAP clustering was performed on a concatenated file of pre-gated CD19+ B cells composed of 350 events in each group. **(A)** Cluster overlay of 1750 B cells of all groups for subset identification. **(B)** Corresponding clusters gated in each donor group. **(C)** Distribution of certain B lineage cell subsets as shown in A showed characteristic differences between the HD, RA, RTX IgG+, RTX IgG- and KTR groups.

(RTX IgG-) were plasmablasts and double negative B cells. RA patients on other therapies than RTX and KTR revealed no substantial differences compared to HC, suggesting a specific signal for patients treated with B cell targeted therapy.

Qualitative B Cell Alterations Before Vaccination Predict Vaccination Response in RTX Treated Patients

Next, we screened B cells for the expression of several molecules related to their activation status and functions. Notably, there was a positive correlation between neutralizing antibodies and B cell numbers, HLA-DR and CXCR5 expression on B cells as well as an inverse correlation with CD95 expression and the percentage of CD21low B cells (**Figures 3A, B**). There was no correlation with the expression of PD-1 and PD-L1 on B cells. RBD+ B cell counts correlated with total B cell counts only. There was no significant correlation between CD4+ and CD8+ T cell counts, vaccination response and B cell markers.

DISCUSSION

Protection through immunization is achieved by an orchestrated immune response between different cellular subsets of innate (APCs) and adaptive immunity, such as B and T cells. Understanding vaccine responsiveness in the context of B cell depleting therapies is essential for preventing infectious diseases in this at-risk patient group. Furthermore, it offers unique insides into B cell biology in general and the function of a B cell impaired immune system in humans.

In the current study, we analyzed the B cell compartment before vaccination in RTX treated patients (13 with RA and 2 with AAV), to identify qualitative predictive markers of an anti-SARS-CoV-2 vaccination response. The RTX patients presented with a wide range of total circulating B cell counts (0.5 - 484/µl blood), providing the opportunity to analyze the B cell compartment at different stages of repopulation. 10/15 patients of the RTX group were able to develop IgG anti-S1-SARS-CoV-2 antibodies upon vaccination, while 5/15 were non-responders. The comparison with the other patient control groups, RA on other therapies (10/11 vaccination responders) and KTR (the majority on triple immunosuppression with MMF/CNI/low-dose-PDN; 11/11 non-responders), addressed the question about the impact of different immunosuppressive therapies.

High-dimensional flow cytometry analysis of B cell subsets before vaccination revealed specific patterns of vaccine prediction between the groups. As known for healthy controls (13), the majority of B cells showed a naïve B-cell phenotype followed by memory compartment (pre-switch, switched memory subsets). Additionally, low numbers of transitional/immature B cells (recent bone marrow migrants), plasma cells and antigen-experienced, double negative B cells were found in peripheral blood.

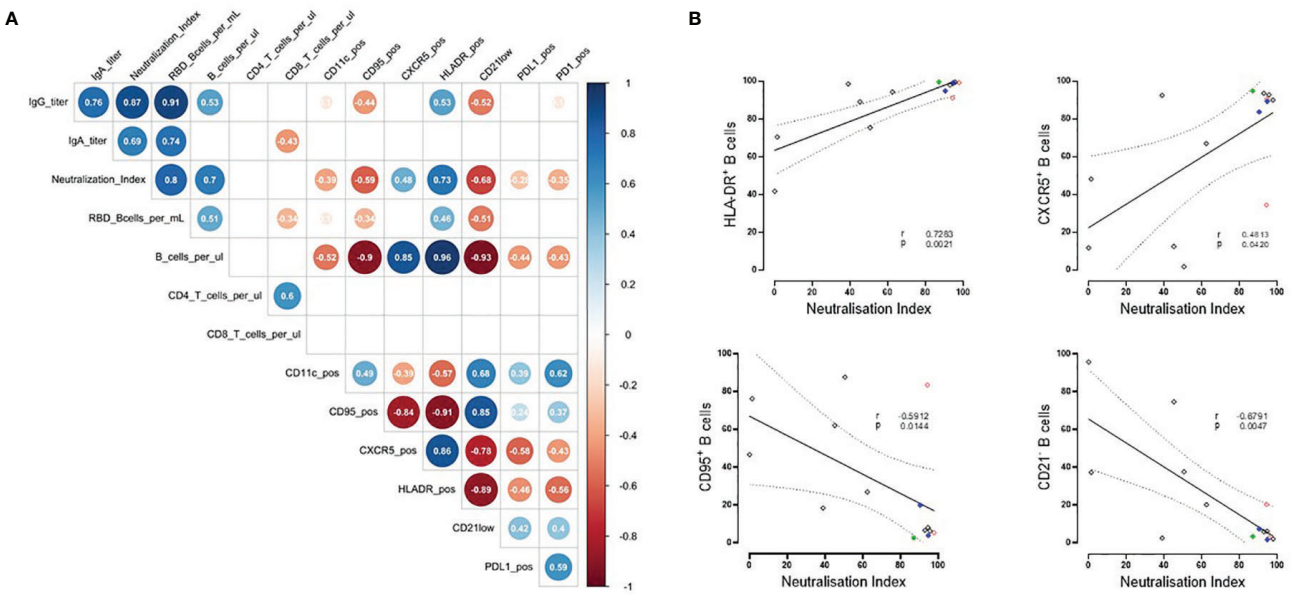

FIGURE 3 | Correlation of humoral vaccine responses, absolute lymphocyte counts and molecule expression in RTX treated patients. **(A)** Spearman´s correlation matrix showing relation between humoral responses, absolute cell counts and expression of CXCR5, HLA-DR, CD95, CD21low, PD-1, PD-L1. Corresponding correlations are represented by red (negative) or blue (positive) circles; size and intensity of color refer to the strength of correlation (RTX n=13 due to limited B cell numbers in 2 RTX patients). Only correlations with $p \leq 0.01$ are indicated. Values in circles indicates r value of correlation. **(B)** Significant correlations between neutralizing antibodies and expression of HLA-DR, CXCR5, CD95 and CD21low by B cells from RTX patients.

Other than in HC, the majority of B cells in RTX IgG+ group consisted of naïve and transitional B cells, while the memory compartment counted for less than 10%. More strikingly, the predominant subsets in vaccination non-responding RTX patients were plasmablasts and double negative B cells. Absolute B cell counts and subset distribution suggest that there is still a relevant B cell depletion upon RTX therapy in the RTX IgG- group. Plasmablasts and DN B cells show a lower CD20 expression, and these cells may rather escape CD20 depletion. After rituximab treatment, numerical reconstitution of the B cell compartment is highly variable, but typically begins in 6–9 months. As seen in our cohort, during initial reconstitution, transitional B cells followed by naive B cells predominate in the peripheral blood B cell pool (14). For the immunologic response to neoantigens, like SARS-CoV-2, the diversity within the naive B-cell repertoire appears to be crucial. As evidenced by our study, an adequate vaccination response after RTX therapy required substantial repopulation of naïve B cells with their capacity to differentiate into B lineage memory.

Interestingly, in the KTR cohort, where all patients were vaccination non-responders, we saw significantly reduced total B cell counts, comparable with the RTX IgG+ cohort. While in RTX patients, the repopulation of naïve B cells decides about vaccination response, KTR show also significantly reduced CD4 T cells, which emphasize the more comprehensive effects of the triple immunosuppression and severely limitations in the ability of T cell dependent antibody responses. This analysis reveals the impact of different immunosuppressive therapies (RTX versus MMF/CNI/low-dose-PDN) upon B and T cell compartments critically involved in successful vaccination.

Screening for the expression of several molecules related to B cell activation and functional properties, revealed additional qualitative characteristics, predictive for an adequate vaccination response. The expression level of MHC class II molecules, such as HLA-DR involved in presentation of peptide antigens to T cells, correlated with neutralizing antibodies and B cell counts. Another positive correlation was found for the expression of the chemokine receptor CXCR5 on B cells, which directs B cells into germinal centers and lymphoid tissues. On the other hand, the presence of CD95+ on B cells or CD21low marking exhausted B cells were predictive at baseline for an insufficient vaccination responses in patients who received RTX.

Adding to the known relationship between B cell counts and vaccination response in RTX treated patients, the current study identified qualitative B cell alterations at baseline indicative of their impaired function. The molecules herein identified can serve as predictive biomarkers regarding a successful vaccination in this patient group. Furthermore, we were able to dissect the impact of different immunosuppressive therapies on certain B cell characteristics and their subset distribution.

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 Charité University Hospital Berlin (EA2/010/21, EA4/188/20). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The concept of the study was developed by A-LS, AL and TD. Patient's samples were collected by KK, A-LS, and JR. Data were obtained by A-LS, HR-A, FS, JR, YC, BJ, HS, CL. Data were analyzed by A-LS and H R-A. The theoretical framework was developed by TD, A-LS and AL. The work was supervised by AL, and TD. All authors developed, read, and approved the current manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.822885/full#supplementary-material

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Clinical Features, Treatment, and Prognostic Factors in Neuronal Surface Antibody-Mediated Severe Autoimmune Encephalitis

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Objective: This study aimed to determine the clinical characteristics and evaluate the efficacy of immunotherapy and the long-term prognosis of severe autoimmune encephalitis (AE) in China.

Methods: Clinical features, laboratory or radiological findings, and treatment outcomes of 60 severe patients with AE from January 1, 2014, to December 31, 2020, were collected. Continuous variables were compared using the *t*-test and the nonparametric Mann–Whitney *U* test, as appropriate. Univariate and multivariable logistic regression analyses were performed to assess the correlations between factors, treatment responses, and prognosis of severe AE.

Results: The median age of symptom onset was 35 years. Tumors were identified in 23.3% of patients, and 36/60 (60%) patients responded to first-line immunotherapy. Second-line immunotherapy was implemented in 26/60 (43.3%) patients. A significant clinical benefit was observed in 19/26 (73.1%) patients treated with lower dosage rituximab; seven patients were still refractory and received bortezomib as an add-on therapy. During the last follow-up, 48/60 (80%) patients achieved good outcomes (mRS, 0–2), and 10 died. Seventeen patients experienced relapses. A high $CD19^+$ B-cell count (OR, 1.197; 95% CI [1.043–1.496]; $p = 0.041$) and a lower neutrophil-to-lymphocyte ratio (NLR; OR, 0.686; 95% CI [0.472–0.884]; $p = 0.015$) predict the response to first-line treatment and good prognosis, respectively.

Conclusions: Patients with severe AE were in critical condition at baseline but could be salvaged after effective rescue immunotherapy. A lower dosage of rituximab could be an optimal option for severe AE. $CD19^+$ B-cell count and NLR may provide prognostic information for predicting treatment response and outcome of severe AE.

Keywords: severe, autoimmune encephalitis, rituximab, bortezomib, treatment and prognosis

INTRODUCTION

Autoimmune encephalitis (AE) constitutes a group of diseases with autoantibodies against neurosurface and synaptic antigens, characterized by abnormal psychiatric behavior or cognitive dysfunction, speech dysfunction, seizures, movement disorder, decreased levels of consciousness, autonomic dysfunction, and central hypoventilation (1). Following infectious encephalitis, AE is the second most common cause of encephalitis, with an estimated incidence of approximately 6.5/10,000 (2, 3). Approximately 80%–85% of patients with AE respond favorably to timely immunosuppressive therapies (4); however, a significant portion of patients with AE progress to critical conditions and often require long-term hospitalization.

The pathogenic mechanisms of severe AE remain poorly understood. Previous studies have shown that innate immunity plays a role in AE pathogenesis (5). Neutrophils, monocyte infiltration, and several proinflammatory cytokines produced by neutrophils during neuroinflammatory conditions are known to affect the function of the blood–brain barrier (BBB), leading to increased permeability of immune cells and inflammatory mediators (6). Lymphocytes can permeate through the damaged BBB and differentiate into plasma cells. Dysfunction of the BBB and intrathecal immunopathogenesis by the infiltration of B cells and $CD138^+$ antibody-secreting cells are considered responsible for disease severity (7–9). In addition, tumors express a wide variety of nontissue-specific surface proteins, including neuronal antigens that can be presented to T cells, generating an immune response against the central nervous system. Moreover, genetic analysis of paraneoplastic syndrome (PNS)-associated tumors has revealed specific molecular signatures and mutations in genes encoding onconeural proteins, leading to the production of highly immunogenic neoantigens, which may also contribute to disease pathogenesis (10).

Patients may suffer from the poor consequences of severe AE with functional and psychosocial sequelae due to delayed diagnosis and therapy. Hence, the emphasis on timely and effective interventions for severe AE has increased, which may salvage this critical zone and consequently prevent disease progression and relapse, facilitating neurological function recovery. The development of monoclonal antibody treatment and protease inhibitors has made significant progress since the characterization of the targeted depletion of B cells and long-lived plasma cells (9, 11–13).

Current knowledge regarding severe AE is limited, and more detailed information about its epidemiologic and clinical characteristics, the potential mechanisms of severe AE, and more effective regimens for severe AE are needed. In this study, we performed a retrospective cohort analysis of patients with severe AE. The main challenges confronted in clinical practice are discussed, which will contribute to innovations in the exploration of severe AE.

METHODS

Study Design and Patients

This study was approved by the institutional review board of Shandong Provincial Hospital (SWYX : No.2022-160). All procedures performed on human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki Declaration. Informed consent was obtained from all participants or their legal representatives.

Patients with severe AE admitted to Shandong Provincial Hospital between January 1, 2014, and December 31, 2020, were enrolled in this retrospective study (**Figure 1**). The inclusion criteria were as follows: (1) the presence of one or more of the following six major groups of symptoms: abnormal psychiatric behavior or cognitive dysfunction; speech dysfunction (pressured speech, verbal reduction, mutism); seizures, movement disorder, dyskinesias, or rigidity/abnormal postures;

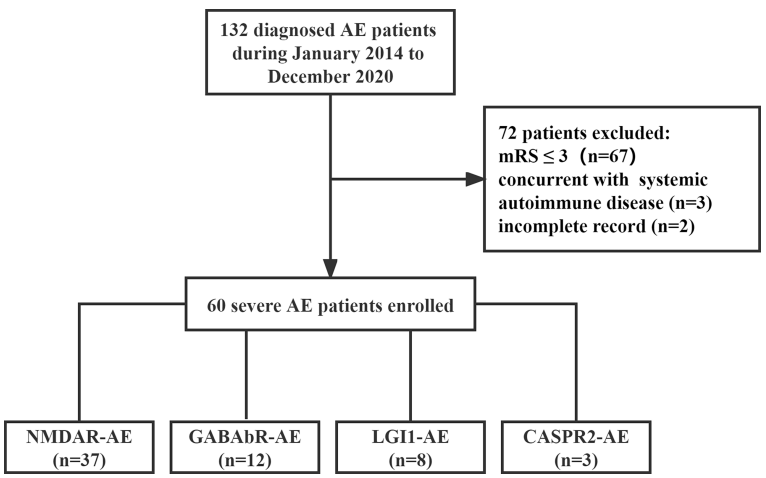

FIGURE 1 | The flowchart of included study.

decreased level of consciousness; autonomic dysfunction; or central hypoventilation. (2) The presence of serum or cerebrospinal fluid (CSF) autoantibodies to neuronal cell surface antigens, including *N*-methyl-D-aspartate receptor (NMDAR), leucine-rich glioma-inactivated 1 (LGI1), contactin-associated protein-like 2 (CASPR2), gamma-aminobutyric acid-b receptor (GABAbR), and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid 1/2 (AMPA1/2) receptor, were analyzed using cell-based assays. (3) Severe neurological dysfunction at the onset of disease with a modified Rankin scale (mRS) score of 4–5. (4) Respiratory failure leading to ventilator support. (5) Status epilepticus or decreased consciousness requiring care in the intensive care unit (ICU). Patients with concurrent systemic autoimmune disease and neurological dysfunction at the onset of the disease with a mRS score of 0–3 and incomplete records were also excluded from this study.

Demographic data included sex, age at AE onset, antibody profile, clinical features, and neuroimaging findings. To screen for an associated neoplasm, all patients underwent a CT scan of the thorax/abdomen/pelvis, an ultrasound of the abdomen and the pelvic region, and a transvaginal ultrasound was performed in married women. Peripheral B-cell levels ($CD19^+$ B-cell count) and routine blood examinations were performed on freshly acquired blood samples within 12 h of admission before any immunosuppressive treatment. The neutrophil-to-lymphocyte ratio (NLR) is defined as the number of neutrophils divided by the number of lymphocytes and is used to assess the state of inflammation in the body. Patients with infectious diseases were excluded, which may have had a potential impact on white blood cell counts. Lumbar punctures were performed on the second day after admission, and CSF protein and white cell counts were analyzed.

The immunotherapy treatment forms and application time points were reviewed. First-line immunotherapy was defined as corticosteroid therapy at a dosage of 500–1,000 mg for 3–5 days and 0.4 g/kg intravenous immunoglobulins (IVIG) for 5 days. Second-line immunotherapies include rituximab and cyclophosphamide, alone or in combination. Long-term immunotherapy was mycophenolate mofetil (MMF) of >1 year; bortezomib was used as an add-on immunotherapy. Rituximab infusion was administered when there was no meaningful clinical response (improvement in the mRS, <1 point) after 2–4 weeks of optimized first-line therapy or when patients relapsed despite long-term immunotherapy. Bortezomib was administered to rituximab-resistant patients who showed no substantial improvement after the last dose of rituximab for at least 1 month.

Good outcomes or functional independence were defined as mRS 0 to 2; relapse was defined as the appearance of new-onset symptoms or the worsening of preexisting symptoms after improvement or stabilization of the disorder for at least 2 months, not explainable by other causes. Early diagnosis was defined as the median duration from the disease to diagnosis.

Statistical Analysis

Data were analyzed using R software. Continuous variables are expressed as means ± standard deviations; otherwise, numerical variables are described as medians and ranges. Continuous variables with >2 subgroups were compared using the Kruskal–Wallis test, and two subgroups were compared using the Mann–Whitney *U* test or *t*-test. Factors affecting outcomes were assessed using univariate logistic regression analysis. Clinically or statistically relevant variables from the univariate analyses were used in the multivariate logistic regression analysis. Receiver operating characteristic (ROC) curve analysis was performed to assess the predictive performance for outcomes based on the NLR values and $CD19^+$ B-cell count obtained at admission. The cutoff values were estimated using the ROC curve, and the corresponding sensitivities and specificities were calculated based on the area under the curve (AUC). Statistical significance was set at $p < 0.05$.

RESULTS

Epidemiological Characteristics

We investigated the clinical course of 60 severe patients with AE associated with antibodies against neuronal cell surface antigens. The ratio of female to male patients was 8:7. Among all patients, 37 (61.7%) were positive for anti-NMDAR antibodies (23 female and 14 male patients), 12 (20%) for anti-GABAbR antibodies (6 female and 6 male patients), 8 (13.3%) for anti-LGI1 antibodies (1 female and 7 male patients), and 3 (5%) for anti-CASPR2 antibodies (2 female and 1 male patient) (**Figure 2A**). The median age of 60 severe AE was 35 years (range, 14 to

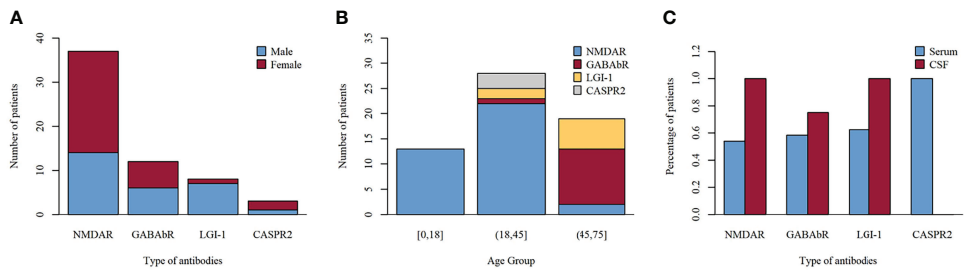

FIGURE 2 | Frequency of distributions of sex **(A)**, age **(B)**, and antibodies **(C)** among subtypes of autoimmune encephalitis.

72 years). The percentage of patients under 18 years old, in the age range between 18 and 45 years, and older than 45 years was 13 (21.7%), 30 (50%), and 17 (28.3%), respectively (**Figure 2B**). The sensitivity of antibody testing in the serum and CSF of all the patients with severe AE was 57.6% and 91.5%, respectively. All 37 patients with NMDAR-AE were CSF positive, and 20 (54%) were seropositive. Among the GABAbR-AE cases, seven (58.3%) had detectable antibodies in the serum, and nine (75%) were positive for antibodies in the CSF. For LGI1-AE, antibodies were found in the serum and CSF in five (62.5%) and eight (100%) cases, respectively. All three CASPR2-positive patients had anti-CASPR2 antibodies in serum (**Figure 2C**).

Clinical Characteristics

The median time lag from symptom onset to diagnosis of severe AE was 19 days (ranging from 3 to 180 days), and the clinical manifestation of severe AE displayed a distinct phenotype. In the initial description, the most common clinical symptoms were seizures (43 patients, 73%), psychosis (23 patients, 39%), and decreased level of consciousness (21 patients, 36%). In our study, we noted that psychosis (23 patients, 62.2%) was most frequent in the NMDAR subgroup. Seizures occurred in the 12 GABAbR-positive patients; eight patients with LGI1 antibodies presented with seizures or cognitive impairment, which was in line with previously reported studies. The median mRS at the onset of the disease was 5 (range, 4 to 5), and 49 patients (81.7%) had an mRS score of 5. Twenty-four patients (40.6%) were admitted to the ICU because of status epilepticus, central hypoventilation requiring respiratory support, and serious complications.

Associated tumors were detected in 14 patients (23.3%). Five ovarian teratomas (13.5%) were identified in patients with NMDAR encephalitis (median age, 22 years), and complete tumor resection was performed at a median time of 16.5 days after disease onset. Two patients showed neurologic improvement, and the other three did not respond. In the GABAbR-AE group, 9 (75%) patients were diagnosed with small-cell lung cancer (median age of 67 years). Seven of the 9 patients with a tumor were treated with surgery or chemotherapy; however, only 3 patients showed a partial response. Compared to the NMDAR-AE and GABAbR-AE groups, patients with LGI1-AE and CASPR2-AE did not show the presence of underlying cancer on tumor screening.

Auxiliary Examinations

T2 fluid-attenuated inversion recovery showed high signal in the bilateral temporal lobes (6 patients, 10%), hippocampus (7 patients, 11.7%), parietal lobe (3 patients, 5%), and cortex (2 patients, 33.3%). The CSF analysis revealed lymphocytic pleocytosis (1–286 cells/L; median, 10 cells/L) in 25 (41.7%) patients, while 11 (18.3%) patients had increased protein concentration (0.1–1.39 g/L; median, 0.3 g/L). Inflammatory changes in CSF most frequently occur in the NMDAR-AE and GABAbR-AE groups. The distribution of CSF white cell counts and protein concentrations in the different AE subtypes is shown in **Table 1**.

The $CD19^+$ B-cell count was similar between those patients who had reached functional independence at discharge from the hospital (mRS, ≤2) and those with nonfunctional independence (mRS, >2) (22.84 ± 8.61 vs 22.16 ± 7.98; $p = 0.809$) (**Figure 3A**). NLR was higher in patients without functional independence (range, 2.07–23.7; median, 4.87) than in patients with functional independence (range, 0.51–16.07; median, 3.67) ($p = 0.045$) (**Figure 3B**).

Treatment Outcomes

No randomized controlled trials have yet been conducted to investigate standard immunotherapy protocols for AE. In our cohort, 60/60 (100%) patients received high-dose corticosteroids (500–1,000 mg) for 5 days, and IVIG was administered to 52/60 (86.7%) patients. Overall, 36/60 (60%) patients responded to first-line immunotherapy, and the median change in mRS score was 1 (range, 0–3). Compared to the other subgroups, the LGI1-AE subgroup exhibited greater mRS improvement, but the difference was not significant (**Table 2**) ($p = 0.262$). The median mRS after first-line therapy in the entire cohort was 4 (range, 1–6).

Second-line immunotherapy was initiated 25 (range, 5–300) days after the definitive diagnosis. Rituximab,

TABLE 1 | Characterization of the whole cohort.

	Total cases	NMDAR	GABAbR	LGI1	CASPR2
N	60	37	12	8	3
Female/male (*n*)	32/28	23/14	6/6	1/7	2/1
Age at disease onset (median, range)	32 (14–72)	28 (14–62)	65 (35–72)	54.5 (43–64)	32 (26–40)
Time of diagnosis (median, range)	19 (3–540)	20 (9–540)	17 (3–370)	40 (5–120)	10 (7–40)
mRS at the peak of disease (median, range)	5 (4–5)	5 (4–5)	5 (4–5)	4 (4–5)	5 (5–5)
ICU admission (*n*, %)	24 (40)	20 (54)	3 (37.5)	0	1 (33)
Tumor (*n*, %)	14 (23.3)	5 (13.5)	9 (75)	0	0
Tumor type (*n*)		Ovarian teratoma (5)	SCLC(9)		
Abnormal MRI (*n*, %)	20 (33.3)	13 (35)	4 (33.3)	3 (37.5)	0
Abnormal CSF (*n*, %)	30 (50)	22 (59.4)	7 (58.3)	1 (12.5)	0
CSF protein [g/L (median, range)]	0.3 (0.1–1.39)	0.29 (0.1–1.39)	0.36 (0.21–0.59)	0.295 (0.21–0.63)	0.3 (0.2–0.45)
Elevated CSF protein (*n*, %)	11 (18.3)	8 (21.6)	2 (16.7)	1 (12.5)	0
CSF WCC [cells/L(median, range)]	10 (1–286)	16 (1–286)	13 (3–118)	3 (1–8)	6 (2–8)
Elevated CSF WCC (*n*, %)	25 (41.7)	19 (51.3)	6 (50)	0	0

mRS, modified Rankin scale; ICU, intensive care unit; MRI, magnetic resonance imaging; CSF, cerebrospinal fluid; WCC, white cell count; SCLC, small-cell lung cancer; NMDAR, N-methyl-D-aspartate receptor; CASPR2, contactin-associated protein-like 2; GABAbR, g-aminobutyric acid receptor B; LGI1, leucine-rich glioma-inactivated protein 1.

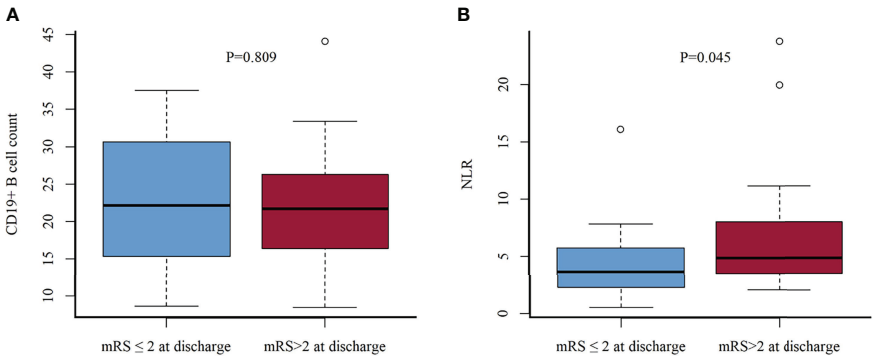

FIGURE 3 | CD19$^+$ B-cell count **(A)** and NLR **(B)** in functional independence group (mRS scores, ≤2) vs. nonindependence group (mRS scores, >2) at discharge. NLR, neutrophil-to-lymphocyte ratio.

cyclophosphamide, or a combination of the two were implemented in 26/60 (43.3%) patients who showed no significant improvement to first-line therapy or experienced a definite clinical relapse. Rituximab (26 patients, 43.3%) was the most frequently applied second-line immunotherapy with two regimens (100 mg IV infusion once per week for 4 consecutive weeks or 600 mg IV infusion in 1 day). In total, 19/26 (73.1%) patients treated with rituximab showed significant improvement, and 7/26 (26.9%) patients pretreated with rituximab were still refractory and received further immunosuppressant drugs with bortezomib as an add-on therapy at a median time of 32 (range, 29–45) days after the last dose of rituximab. A total dose of 1.3 mg/m^2 was administered subcutaneously on days 1, 4, 8, and 11 of the 21-day cycle. Each patient received a median of 1 (range, 1–3) cycle. Although 36/60 (60%) patients showed improvement after first-line immunotherapy, based on the severity of the initial attack and the risk of relapse, long-term immunosuppression (MMF) was administered in 24/60 (40%) patients. At discharge, the median mRS score was 3 (range 2–6), which was significantly lower than the score of 5 (range 4–5) at the peak of the disease ($P < 0.001$). Surprisingly, anti-LGI1 encephalitis patients typically showed substantial recovery, with none having a moderate or severe deficit at discharge.

The mean duration of follow-up was 40 (range, 1–84) months. The distribution of mRS scores at the peak of the disease and the last follow-up improved significantly in patients with NMDAR-AE, LGI1-AE, and CASPR2-AE; no significant improvement was observed in patients in the GABAbR-AE group (**Figure 4**). Although patients with severe AE were severely affected at baseline (**Table 1**), at the final follow-up, 48/60 (80%) patients had achieved independent living (mRS score, ≤2) with a median mRS of 1 (0–6) ($p < 0.01$) (**Table 2**). Ten (16.7%) patients (three NMDAR-AE, seven GABAbR-AE) died of consequences associated with lung tumors, symptoms, and severe bacteremia.

TABLE 2 | Immunotherapy and follow-up of patients.

	Total cases	NMDAR	GABAbR	LGI1	CASPR2
First-line therapy					
Steroids (*n*, %)	60 (100)	37 (100)	12 (100)	8 (100)	3 (100)
IVIG (*n*, %)	52 (86.7)	36 (97)	9 (75)	4 (50)	3 (100)
Response to first-line therapy (*n*, %)	36 (60)	19 (51.3)	8 (66.7)	7 (87.5)	2 (66.7)
Δ mRS scores post-first-line therapy (median, range)	1 (0–3)	1 (0–2)	1 (0–3)	2 (0–3)	1 (0–2)
mRS score post-first-line therapy (median, range)	4 (1–6)	4 (2–6)	4 (2–5)	3 (1–4)	4 (3–5)
Second-line therapy (*n*, %)	26 (43.3)	20 (54.1)	2 (16.7)	3 (37.5)	1 (33.3)
Rituximab (*n*, %)	26 (43.3)	20 (54.1)	2 (16.7)	3 (37.5)	1 (33.3)
Cyclophosphamide (*n*, %)	1 (1.7)	1 (2.9)	0	0	0
Add on immunotherapy					
Bortezomib (*n*, %)	7 (11.7)	7 (18.9)	0	0	0
Long-term immunotherapy					
Mycophenolate mofetil [MMF (*n*, %)]	24 (40)	20 (54.1)	1 (8.3)	2 (25)	1 (33.3)
mRS score at discharge (median, range)	3 (2–6)	3 (2–6)	3 (2–5)	2 (2–2)	3 (2–3)
mRS score ≤2 at discharge (*n*, %)	28 (46.7)	16 (43.2)	3 (25)	8 (100)	1 (33.3)
mRS score at final follow-up (median, range)	1 (0–6)	1 (0–6)	6 (0–6)	1 (0–2)	2 (0–2)
mRS score ≤2 at final follow-up (*n*, %)	48 (80)	32 (86.5)	5 (41.7)	8 (100)	3 (100)
Relapse (*n*, %)	17 (28.3)	10 (27)	6 (50)	1 (12.5)	0
Mortality (*n*, %)	10 (16.7)	3 (8.1)	7 (58.3)	0	0

IVIG, intravenous immunoglobulins; mRS, modified Rankin scale; Δ mRS, changes in the mRS; NMDAR, N-methyl-D-aspartate receptor; CASPR2, contactin-associated protein-like 2; GABABR, g-aminobutyric acid receptor B; LGI1, leucine-rich glioma-inactivated protein 1.

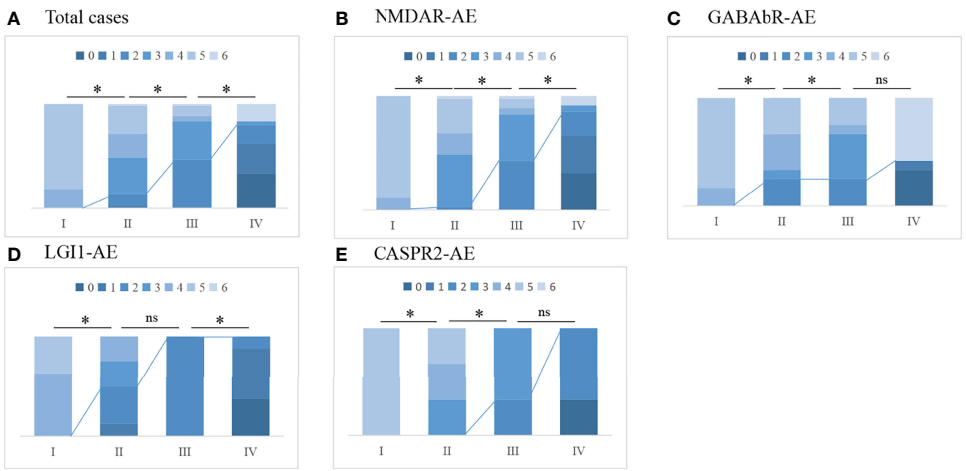

FIGURE 4 | The change in mRS scores and the outcome of total cases **(A)** and different subtypes of AE **(B–E)**. I, maximal mRS at symptom onset; II, mRS post-first-line immunotherapy; III, mRS at discharge from hospital; IV, mRS at last follow-up. The line represents the change in mRS scores dividing favorable mRS scores (0–2) and unfavorable mRS scores (≥3); *p < 0.05. ns, not significant.

Relapses occurred in 17/60 (28.3%) patients in our study at a median time of 10 months (2–36 months) after the initial episode (10 anti-NMDAR encephalitis cases, 6 GABAbR encephalitis cases, and 1 LGI1 encephalitis). Of the relapsed cases, 9/17 (52.9%) were treated with second-line immunotherapy or long-term immunotherapy after the initial attack. One patient with NMDAR-AE relapse had an ovarian teratoma at disease onset, and six patients with GABAbR-AE relapse had small-cell lung cancer. Three of 17 (17.6%) patients experienced further relapses (range, 2–7). All relapsed patients underwent reinitiation of first-line immunotherapy, six patients subsequently received long-term MMF, and 11 patients were treated with rituximab.

Predictors of Treatment Efficiency and Prognosis

Univariate logistic regression analysis indicated that younger age (p = 0.045), nontumor status (p = 0.003), nonpulmonary infection complications (p = 0.007), lower NLR levels (p = 0.022), and response to first-line treatment (p = 0.005) were associated with good outcomes at the final follow-up. A high $CD19^+$ B-cell count corresponded with failure of first-line treatment (OR, 1.109; 95% CI [1.013–1.24]; p = 0.04). Sex, CSF protein, and early diagnosis were not related to any of our outcomes (**Table 3**). Multivariable logistic regression analysis confirmed that patients with a high $CD19^+$ B-cell count exhibited an OR of 1.197 (95% CI [1.043–1.496]) for predicting failure of first-line treatment at a statistically significant level (p = 0.041). Lower NLR levels were more likely to have good functional outcomes at final follow-up for severe AE (OR, 0.686; 95% CI [0.472–0.884]; p = 0.015). Tumors corresponded with increased odds of relapse (OR, 29.506; 95% CI [2.79–757.342]; p = 0.014) and mortality (OR, 8.034, 95% CI [1.388–58.033]; p = 0.024) (**Table 4**).

The ROC curve analysis was performed to evaluate the predictive value for a good outcome at the final follow-up using the full multivariate model (final model) and univariate model (NLR model, $CD19^+$ B-cell count model). As shown in **Figure 5**, the full multivariate model demonstrated a good predictive value [AUC = 0.925; 95% CI (0.847–1)] compared to the univariate model. Based on the ROC curve, the optimal cutoff values of NLR and $CD19^+$ B-cell count to predict good outcomes were 10.19 (sensitivity, 0.977; specificity, 0.384) and 22.33 (sensitivity, 0.515; specificity, 1), respectively (**Figure 5**).

DISCUSSION

In this retrospective analysis, we identified several novel findings. First, this is the most detailed description of clinical features in patients with severe AE to date, and long-term outcomes in the overall cohort were favorable. Second, we showed that a lower dosage of rituximab is the most frequently applied second-line immunotherapy used in 43.3% of all patients with severe AE. We also observed a clinical benefit and provided preliminary evidence that a lower dosage of rituximab may be as effective as standard doses for treating severe AE with good tolerance and less financial burden. Finally, we found that $CD19^+$ B-cell count and NLR can help predict the response to treatment and prognosis, respectively, and could thus be valuable in guiding clinicians to offer aggressive rescue immunotherapy.

In our cohort, severe AE cases mostly comprised anti-NMDAR encephalitis. The sex ratio and age distribution among those with anti-NMDAR encephalitis were similar to those of previous studies, showing a higher frequency in women (14–16). However, the sex disparity in the rest of the subtypes was not in concordance with the results of prior studies, which reported male predominance in LGI1, GABAbR, and CASPR2 encephalitis (17). In our cohort, cerebellar ataxia and brainstem encephalitis were uncommon manifestations that occurred in patients with NMDAR-AE and GABAbR-AE. When altered

TABLE 3 | Univariate logistic regression analysis for all severe AE patients.

Variables [OR ([95% CI); *p*-value]	mRS ≤2 at discharge	mRS ≤2 at final follow-up	ICU admission	Failure of first-line treatment	Mortality	Relapse
Age	0.996 [0.968–1.025]; $p = 0.802$	**0.964 [0.928–0.998]; $p = 0.045$**	0.975 [0.944–1.004]; $p = 0.104$	0.976 [0.946–1.005]; $p = 0.12$	**1.054 [1.014–1.102]; $p = 0.012$**	0.991 [0.958–1.023]; $p = 0.588$
Gender	1.686 [0.609–4.776]; $p = 0.317$	1.533 [0.445–5.724]; $p = 0.504$	0.946 [0.332–2.672]; $p = 0.916$	0.946 [0.332–2.672]; $p = 0.916$	0.595 [0.141–2.233]; $p = 0.451$	0.6 [0.177–1.904]; $p = 0.393$
Tumor	**0.147 [0.021–0.623]; $p = 0.02$**	**0.125 [0.029–0.49]; $p = 0.003$**	2.059 [0.592–7.384]; $p = 0.255$	0.921 [0.246–3.199]; $p = 0.898$	**12.542 [2.958–62.048]; $p = 0.001$**	3.171 [0.854–11.819]; $p = 0.081$
Pulmonary infection complications	**0.087 [0.021–0.291]; $p < 0.01$**	**0.141 [0.028–0.535]; $p = 0.007$**	**391 [49.681–9387.565]; $p < 0.01$**	**8.500 [2.736–29.527]; $p < 0.01$**	**5.02 [1.27–25.275]; $p = 0.029$**	0.365 [0.091–1.232]; $p = 0.122$
Early diagnosis	0.765 [0.274–2.113]; $p = 0.605$	0.821 [0.232–2.834]; $p = 0.754$	1.750 [0.621–5.080]; $p = 0.294$	2.333 [0.821–6.933]; $p = 0.117$	1.25 [0.334–4.86]; $p = 0.739$	1.408 [0.446–4.588]; $p = 0.56$
CSF WCC	0.986 [0.966–1.001]; $p = 0.115$	0.993 [0.979–1.008]; $p = 0.343$	**1.018 [1.003–1.038]; $p = 0.038$**	1.003 [0.989–1.016]; $p = 0.668$	1.007 [0.992–1.022]; $p = 0.315$	0.994 [0.971–1.009]; $p = 0.488$
CSF protein	2.851 [0.077–124.592]; $p = 0.566$	0.838 [0.014–88.474]; $p = 0.935$	1.201 [0.029–46.085]; $p = 0.92$	0.167 [0.002–6.715]; $p = 0.367$	0.772 [0.004–58.939]; $p = 0.913$	0.289 [0.002–18.328]; $p = 0.588$
$CD19^+$ B-cell count	1.011 [0.930–1.100]; $p = 0.800$	1.128 [0.954–1.421]; $p = 0.219$	0.953 [0.867–1.037]; $p = 0.283$	**1.109 [1.013–1.24]; $p = 0.04$**	0.814 [0.556–1.026]; $p = 0.162$	0.947 [0.857–1.034]; $p = 0.248$
NLR	0.856 [0.693–0.997]; $p = 0.089$	**0.823 [0.677–0.953]; $p = 0.022$**	1.063 [0.937–1.225]; $p = 0.346$	1.108 [0.974–1.297]; $p = 0.146$	1.105 [0.960–1.280]; $p = 0.150$	0.970 [0.813–1.110]; $p = 0.686$
Failure of first-line treatment	**0.149 [0.041–0.465]; $p = 0.002$**	**0.127 [0.026–0.487]; $p = 0.005$**	**7.00[2.290–23.562]; $p = 0.001$**	–	**5.5 [1.387–27.784]; $p = 0.022$**	0.598 [0.165–1.947]; $p = 0.407$

Univariate logistic regression analyses was performed to determine correlations between covariates (including NLR, $CD19^+$ B-cell count) and the outcomes(mRS, ICU admission, failure of first-line treatment, mortality, relapse). OR, 95% CI, and their respective p-values are shown for all correlations. Significant values ($p < 0.05$) are highlighted (in bold). Lower NLR level was associated with good outcome at final follow-up [OR, 0.823; 95% CI (0.677–0.953); $p = 0.022$]. High $CD19^+$ B-cell count corresponded with failure of first-line treatment [OR, 1.109; 95% CI (1.013–1.24); $p = 0.04$]. mRS, modified Rankin scale; CSF, cerebrospinal fluid; WCC, white cell count; NLR, neutrophil-to-lymphocyte ratio; ICU, intensive care unit; OR, odds ratios; CI, confidence intervals.

consciousness is accompanied by these atypical symptoms, Bickerstaff encephalitis, which is characterized by a typical picture of cranial nerve involvement and consciousness alterations, should be excluded (18). Data regarding the prevalence of tumor association was also confirmed in our study; the occurrence of an underlying teratoma in female patients was lower (13.5%) compared to those reported in previous publications (19.5%–58%) (16, 17, 19). The reasons for heterogeneity may be explained by the inclusion criteria, sample sizes, or other factors, including genetic background and epidemiology. In GABAbR encephalitis, the tumor type was small-cell lung cancer, occurring in 75% of anti-GABABR-positive patients, which is in accordance with the findings of Hayden et al. (20). In tumor-associated AE, surgical treatment or

TABLE 4 | Multivariable logistic regression analysis for all severe AE patients.

Variables [OR (95% CI); *p*-value]	mRS ≤2 at discharge	mRS ≤2 at final follow-up	ICU admission	Failure of first-line treatment	Mortality	Relapse
Age	0.984 [0.936–1.031]; $p = 0.51$	0.895 [0.801–0.963]; $p = 0.013$	0.895 [0.726–0.982]; $p = 0.094$	1.003 [0.949–1.065]; $p = 0.914$	**1.083 [1.021–1.174]; $p = 0.02$**	0.956 [0.905–1.003]; $p = 0.082$
Gender	2.575 [0.565–13.698]; $p = 0.236$	2.797 [0.277–47.809]; $p = 0.405$	–	–	–	1.905 [0.346–12.874]; $p = 0.473$
Tumor	0.243 [0.01–2.53]; $p = 0.281$	0.426 [0.048–3.443]; $p = 0.42$	–	1.517 [0.083–32.847]; $p = 0.772$	**8.034 [1.388–58.033]; $p = 0.024$**	**29.506 [2.79–757.342]; $p = 0.014$**
Pulmonary infection complications	**0.082 [0.01–0.437]; $p = 0.007$**	**0.014 [0–0.196]; $p = 0.008$**	**6895.308 [100.529–470,891,290.905]; $p = 0.008$**	**20.15 [2.054–522.743]; $p = 0.028$**	**16.376 [1.68–387.747]; $p = 0.035$**	**0.069 [0.004–0.553]; $p = 0.03$**
CSF WCC	1.003 [0.981–1.022]; $p = 0.755$	1.015 [0.994–1.045]; $p = 0.217$	–	0.995 [0.97–1.021]; $p = 0.692$	1.001 [0.981–1.018]; $p = 0.912$	0.996 [0.966–1.021]; $p = 0.772$
$CD19^+$ B-cell count	–	–	–	**1.197 [1.043–1.496]; $p = 0.041$**	–	–
NLR	0.835 [0.624–1.03]; $p = 0.146$	**0.686 [0.472–0.884]; $p = 0.015$**	0.851 [0.548–1.279]; $p = 0.357$	–	–	0.846 [0.611–1.082]; $p = 0.252$
Failure of first-line treatment	–	–	1.14 [0.044–17.424]; $p = 0.925$	–	–	–

Variables with statistical significance in the univariate logistic regression analysis and clinically relevant variables were included in multivariable logistic regression models. OR, 95% CI, and their respective p-values are shown for all correlations. Significant values ($p < 0.05$) are highlighted (in bold). High $CD19^+$ B-cell count has exhibited an OR of 1.197 (95% CI = 1.043–1.496) for predicting failure of first-line treatment at a statistically significant level ($p = 0.041$). Lower NLR levels were more likely to have good functional outcome at final follow-up of severe AE [OR, 0.686; 95% CI (0.472–0.884); $p = 0.015$]. mRS, modified Rankin scale; CSF, cerebrospinal fluid; WCC, white cell count; NLR, neutrophil-to-lymphocyte ratio; ICU, intensive care unit; OR, odds ratios; CI, confidence intervals.

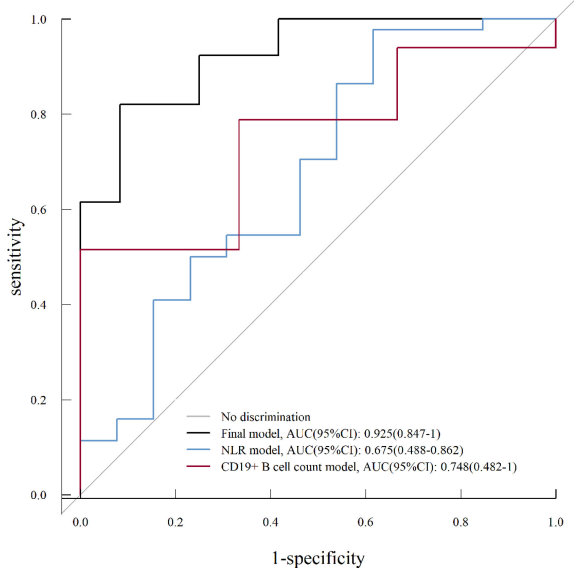

FIGURE 5 | Receiver operating characteristic (ROC) curve analysis for a univariate analysis (NLR model, $CD19^+$ B-cell count model) and multivariate analysis (final model) on good prognosis (mRS score, ≤2) of severe AE. NLR, neutrophil-to-lymphocyte ratio; AUC, area under the curve; CI, confidence interval.

radiation/chemotherapy should be initiated as soon as possible to relieve the symptoms and allow a more favorable long-term outcome. In the study by Lee et al. (19), nine teratomas were not detected in the initial workup but by a follow-up pelvic MRI, resulting in delayed removal of the teratoma, which suggests that the extent of tumor screening and regular tumor screening should be taken into consideration.

Inflammatory changes in the CSF were noted in 30 (50%) of the patients. We found that patients in the NMDAR-AE and GABAbR-AE groups were more likely to develop CSF pleocytosis than the other subtypes of the cohort; this result is similar to those of other studies (20, 21). Previous studies have found an association between CSF changes and worse outcomes (22). In the current cohort, we also confirmed that abnormal CSF white cell counts increased the odds of ICU admission.

The NLR has previously been proposed as an indicator of systemic inflammation. A high NLR implies overwhelmed inflammation or imbalanced innate and adaptive immunity, which are frequently used to predict outcomes (23). Our results showed that NLR was higher in patients without functional independence at discharge, while those with lower NLR levels were more likely to have good functional outcomes at final follow-up (OR, 0.686; 95% CI [0.472–0.884]; $p = 0.015$). This was in line with earlier reports that noted that the percentage of patients who exhibited severe disease increased significantly in the higher NLR subgroup, and a high NLR was associated with higher odds of first-line treatment failure in AE (24, 25). In addition to NLR, evaluation of the peripheral $CD19^+$ B-cell count revealed that a high $CD19^+$ B-cell count is a predictor of first-line treatment failure. B cells are the major effector cells in AE through antibody production and proinflammatory cytokine production. However, the effects of first-line immunotherapy, such as corticosteroids, on B-lymphocytes are limited (26). This indicates that drugs targeting B lymphocytes are required.

Decisions regarding immunotherapy initiation were based on clinical symptoms. Our data confirmed that 36/60 (60%) patients responded to first-line immunotherapy, and anti-LGI1 encephalitis was associated with faster recovery, possibly due to low-affinity IgG4 antibodies (27). Recently, a study of the largest Chinese anti-NMDAR encephalitis cohort concluded that repeated first-line immunotherapy, involving mostly a combination of steroids and IVIG, can achieve favorable clinical outcomes (16). Notably, Zhang et al. reported that therapeutic plasma exchange might be an effective rescue therapy associated with rapid functional improvement in patients with severe steroid/IVIG-refractory antibody-associated AE (28).

In addition, early initiation of second-line immunotherapy with rituximab has been shown to result in a more favorable prognosis (11, 12). In the meta-analysis of Nepal et al. (11), good outcomes at last follow-up were noted in 71.8% of patients following rituximab therapy, with a mean mRS score decrease of 2.67, and relapse occurred in only 17.5% of patients with an acceptable toxicity profile. Similarly, in a study by Thaler et al. (12), early and short-term rituximab therapy was shown to be an effective and safe treatment option in most patients with NMDAR-, LGI1-, and CASPR2-AE. These study outcomes were consistent with the therapeutic outcomes for rituximab in AE. In our study, in terms of rituximab dose, we used reduced-dose rituximab, considering rituximab's off-label use for AE in China and the cost of hospitalization. The median time lag from definite diagnosis of the disease to rituximab administration in our study was 25 (range, 5–300) days. As rituximab is used as a second-line drug, the delay in initiation of rituximab therapy may affect the outcomes. We found that 19/26 (73.1%) patients treated with rituximab showed a significant improvement, and 7/26 (26.9%) patients were still refractory, consistent with Titulaer et al. (4, 11), while the mechanism of rituximab-resistant AE remains undefined. In a novel immune-mediated model of anti-NMDAR encephalitis provided by Wagnon et al. (8), the differentiation of B cells into plasma cells coincided with an increase in protein concentration and the detection of anti-NMDAR IgG in the CSF. In anti-NMDAR encephalitis brains, antibody-secreting cells (ASCs) reside in perivascular, interstitial, and Virchow–Robin spaces, which may be the main source of continuously synthesized Ig (20). This suggests that these plasma cells are responsible for the production of anti-NMDAR autoantibodies that may contribute to disease progression. Previous studies have demonstrated the rescuing effects of proteasome inhibitor bortezomib in patients unresponsive to rituximab, depleting extra-CNS ASCs in a targeted manner (9, 13). In our cohort, seven patients received bortezomib treatment, six showed clinical improvement, and one patient died due to serious complications.

Therapeutic recommendations related to the long-term management of AE are influenced by multiple factors: (1) the presence and type of neuronal autoantibodies and their relevance to the patient's presentation, (2) relapse rates in different AE subtypes, and (3) severity of the initial attack and individual risks related to immunosuppression. Of note, overlapping with oral corticosteroids is needed for 3–6 months when using MMF owing to its delayed onset of action (29). Moreover, vigilant management of airway complications, especially pulmonary infection and monoclonal antibody infusion-related reactions, is required.

In our cohort, the relapse rate was 28.3%, compared with rates of 10%–35% calculated in previous studies (4, 17, 30). Tumors corresponded with increased odds of relapse (OR, 29.506; 95% CI [2.79–757.342]; $p = 0.014$). The high relapse rate may be due to the severity of the disease, the detection of tumors, and the fact that some patients were not treated with second-line immunotherapies or long-term immunotherapy after the initial attack. Patients who experience a definite relapse should be treated with the same first-line treatment scheme as at the first clinical presentation; for long-term immunosuppression, rituximab is the most popular choice, chosen by 46% of responders for relapsing AE (29).

Our treatment regimens showed promising outcomes as more than 46.7% of patients had an mRS of ≤2 at discharge, and 48/60 (80%) patients had achieved independent living (mRS score, ≤2) at final follow-up, which may be due to the early and high-frequency (43.3%) application of rituximab and aggressive administration of bortezomib. This study also provides preliminary evidence that lower doses of rituximab may be as effective as the standard doses to treat severe AE.

This study had some limitations. First, the retrospective collection and analysis of clinical information and the lack of a control cohort resulted in the heterogeneity of rituximab treatment regimens. Prolonged monitoring is required to assess the long-term efficacy of rituximab in treating severe AE. The second constraint is the relatively small sample size of the cohort made up of different subtypes of severe AE, which may introduce bias in this study. Furthermore, the mRS is a scale developed to measure global disability, and a novel clinical scale, such as the clinical assessment scale in autoimmune encephalitis (CASE), should be applied to evaluate the severity in patients with diverse AE syndromes (31). Prospective multicenter studies are required to address this question.

In summary, we showed the clinical characteristics of severe AE and the predictive value of peripheral immune cells for treatment response and prognosis. We present evidence of the efficacy of early lower dosage of rituximab treatment in severe AE and suggest that short-term therapy could be a viable treatment option; bortezomib can be used as rescue immunotherapy in rituximab-resistant patients. Future studies are needed to investigate new therapeutic strategies, such as IL-6 receptor blockers, which may interfere with the pathologic activation of B cells (tocilizumab) and anti-CD19 agents (inebilizumab). More *in vitro* and *in vivo* studies are needed to improve our understanding of the molecular mechanism of severe AE.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics institutional review board of Shandong Provincial Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BW collected the data and drafted the manuscript. CW and JF analyzed the data. MH rechecked the data and revised the manuscript. SG designed and conceptualized the study, interpreted the data, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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