Targeting autoimmunity in renal diseases: focus on neutrophil extracellular traps and autoreactive B-cells

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A reverse translational study on the effect of rituximab, rituximab plus belimumab, or bortezomib on the humoral autoimmune response in SLE

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ABSTRACT

Objectives: SLE is a severe autoimmune disease characterized by autoreactive B-cells and immune-complex (ICx) formation, which causes systemic inflammation. B-cell targeted therapy could be a promising treatment strategy in SLE patients, nevertheless randomized clinical trials have not always been successful. However, some groups have demonstrated beneficial effects in severe SLE patients with off-label rituximab (RTX) with belimumab (BLM), or bortezomib (BTZ), which targeted different B-cell subsets. This study assembled sera from SLE cohorts treated with RTX+BLM (n=15), BTZ (n=11) and RTX (n=16), to get an in-depth insight in the immunological effects of these therapies on autoantibodies and ICx-formation.

Methods: Autoantibodies relevant for ICx-formation and the avidity of anti-dsDNA were determined by ELISA. ICx-mediated inflammation was studied by complement levels and *ex vivo* serum-induced neutrophil extracellular trap (NET) formation.

Results: Reductions in autoantibodies were observed after all approaches, but the spectrum differed depending upon the treatment. Specifically, only RTX+BLM significantly decreased anti-C1q. Achieving seronegativity of ≥1 autoantibody, specifically anti-C1q, was associated with lower disease activity. In all SLE patients, the majority of anti-dsDNA autoantibodies had low-avidity. RTX+BLM significantly reduced low-, medium- and high-avidity anti-dsDNA, while RTX and BTZ only significantly reduced medium-avidity. ICx-mediated inflammation, measured by C3 levels and neutrophil extracellular trap formation, improved after RTX+BLM and RTX but less after BTZ.

Conclusion: This study demonstrates the impact of different B-cell targeted strategies on autoantibodies and ICx formation and their potential clinical relevance in SLE.

KEY MESSAGES

- RTX, RTX+BLM and BTZ resulted in different targeting of anti-C1q, high-avidity anti-dsDNA and the autoantibody repertoire.
- Reduction of anti-C1q and high-avidity anti-dsDNA by RTX+BLM led to reduced immune-complex mediated inflammation.
- Achievement of autoantibody negativity, specifically anti-C1q, after B-cell targeted strategies, was beneficial for SLE patients.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder characterized by a break of tolerance leading to the development of autoreactive B-cells that produce anti-nuclear autoantibodies (ANAs)[1,2]. These autoantibodies can form immune-complexes (ICx) that deposit in affected tissues, leading to complement activation and a systemic inflammatory cascade. A severe manifestation of ICx-mediated inflammation in SLE patients is lupus nephritis (LN), which is histologically represented by a "full-house" pattern of ICx-deposition in glomeruli[3,4]. Typically, activation of the classical pathway of the complement system is an indirect biomarker of ICx-mediated inflammation in SLE. Additionally, SLE-specific-ICx can trigger excessive neutrophil extracellular trap (NET) formation[5], an immunogenic and toxic release of DNA by neutrophils[6-8]. In addition, NETs themselves are an important source of nuclear autoantigens, creating a pathological vicious cycle of perpetuating inflammation in SLE[5-9].

A reduction of autoantibodies, or even reversal to negativity, upon immunosuppressive treatment has been found to be associated with a beneficial clinical outcome in SLE[10-14]. These autoantibodies can be secreted by two distinct antibody-secreting cell (ASC) populations: short-lived proliferating plasma blasts (PBs) and non-dividing long-lived plasma cells (PCs)[15-17]. Typically, autoreactive PBs, originating from an activated naïve B-cell population[16,18] or the autoreactive memory compartment[15], are associated with increasing autoantibody (anti-dsDNA, anti-C1q) levels and diseases flares. Autoreactive PCs are thought to maintain the persistence of circulating autoantibodies and specifically those against extractable nuclear antigens (ENAs) that are refractory to immunosuppressive treatment[15,19]. Additionally, B-cells and PCs also have antibody-independent functions, such as antigen-presentation, T-cell activation, and cytokine production, which makes them an even more valuable therapeutic target in SLE[20].

Currently, a variety of therapeutic strategies can be used to target B-cells, PBs and PCs[20], but the most optimal approach for SLE has not yet been elucidated. Strategies that could be beneficial include: direct targeting of the ASC population by proteasome inhibition or by TACI-Ig (atacicept)[20], which blocks both proliferation inducing ligand (APRIL) and B-cell activating factor (BAFF) by binding to the TACI receptor, or by targeting of precursors of ASCs, including PBs, by B-cell depleting monoclonal antibodies (mAbs) (e.g. directed against CD20, CD21 or CD19) or neutralization of B-cell survival factors with anti-cytokine mAbs against APRIL or BAFF. Currently, anti-BAFF mAb Belimumab (BLM) is the only officially approved B-cell targeted therapy for SLE patients without renal and/or neurological manifestations, and it is currently being tested in the BLISS-LN trial for LN (NCT01639339). BLM decreased naïve and transitional B-cells, but not switched

memory B-cells, whereas PCs did not decrease until after 2 years of BLM[21]. Rituximab (RTX), an anti-CD20 mAb, was not effective in two large randomized controlled trials in SLE patients, however off-label use of RTX was promising, with strong effects on autoantibodies and the complement system[10,22,23]. The exact subpopulation of B-cells targeted and eliminated by RTX remains uncertain[24]. Some B-cell populations can also escape RTX, such as switched memory B-cells, despite CD20 expression[24] and tissue-resident $CD20^-$ PCs[25]. In general, it is thought that RTX does not decrease $CD20^-$ PCs, however, presumably by targeting of their precursors, all $CD27^{br}CD38^{br}$PBs/PCs ($CD20^{-/+}$) were significantly depleted after RTX in SLE[26]. Importantly, B-cell depletion with RTX results in an increase in serum BAFF levels[9,27], which can effectively be decreased with sequential BLM treatment after RTX[9]. This approach of combining RTX and BLM led to clinical responses in refractory SLE patients and significantly decreased memory B-cells, transitional B-cells and $CD27^{br}CD38^{br}$ PBs/PCs[9]. During monthly BLM, PCs started to repopulate from week 24 onwards, while anti-dsDNA, anti-C1q and even ENAs remained suppressed. On the other hand, bortezomib (BTZ), a proteasome inhibitor targeting PCs, was effective in refractory SLE[28,29]. BTZ decreased $CD20^-$ PCs, including both $HLA\text{-}DR^{+/-}$ in the peripheral blood and bone marrow, but did not target their precursors.

Altogether, B-cell-targeted therapies may be effective in the treatment of SLE and LN, because different off-label approaches have demonstrated potential clinical benefit, but it is unclear which approach in SLE patients would be most optimal from an immunological perspective. To address this issue, the present study assembled previously published cohorts of SLE patients treated with experimental B-cell-targeted strategies: RTX+BLM[9], BTZ[28,29] and (as a comparator) RTX[22] and investigated the immunological effects of these B-cell targeted strategies on autoantibodies and ICx-mediated NET inflammation to increase the understanding for B-cell-targeting in SLE.

METHODS

Study population

Serum samples were assembled from 42 severe, refractory SLE patients (each of whom met the 1997 revised ACR or 2012 SLICC criteria) from previously published cohorts treated with RTX[22] (n=16), RTX+BLM[9] (n=15) and BTZ[28,29] (n=11). All patients provided informed consent locally, and the study was approved by the local ethical committee at each center. Demographics, clinical parameters, disease activity scores, and current medications were recorded locally by the treating physicians. Local routine laboratory assessments were collected at each center separately including immunoglobulin (Ig)-G, IgM and IgA, absolute count of $CD19^+$ B-cells, and C3 serum titers. Low C3 was defined as below normal cut-off values as defined by each local laboratory; RTX <0.79 g/L, RTX+BLM <0.9 g/L, BTZ <0.9 g/L. Patients' characteristics are summarized in Supplementary Table S1, available at *Rheumatology* online, and detailed treatment schedules in Supplementary Table S2, available at *Rheumatology* online.

Autoantibody measurements

To compare autoantibody titers of the SLE patients in different cohorts, serum levels of anti-dsDNA, -histones, -nucleosomes and -C1q IgG autoantibodies were measured centrally in the Leiden University Medical Center (LUMC) in Leiden, the Netherlands. Details of the ELISAs are described in Supplementary Materials 1, section Methods, available at *Rheumatology* online, and Supplementary Figure S1, available at *Rheumatology* online.

Preparation of neutrophils and quantification of NETs

Paul Karl Horan (PKH)-labeled (Sigma-Aldrich, USA) neutrophils from a healthy donor (HC) were stimulated with 10% SLE serum for 4 hours to induce NET formation. After 3.75 hours of stimulation, 1 µM SYTOXgreen (ThermoFisher, USA) was added for 15 minutes, after which neutrophils were fixed with 4% paraformaldehyde (PFA) (Added Pharma, Netherlands). Hereafter, the neutrophil extracellular traps (NETs) were visualized and quantified by 3D confocal microscopy using the automated BD Pathway 855 (BD Biosciences, USA), or the Image Xpress Micro Confocal (Molecular Devices, USA) as described previously[30,31].

Statistics

All clinical data are expressed as median ± [interquartile ranges (IQR)] for numerical data or given as percentage for nominal data. NET formation data are expressed as medians [IQR] NET area per imaged neutrophil. NET formation ratios are expressed as median [IQR]. To determine statistical differences between three independent groups the Kruskal-Wallis test for numerical data and the Chi-squared test for nominal variables was used. Statistical difference between two groups was determined with Mann-Whitney U test, and Wilcoxon's matched-pairs test was used for paired samples. Statistical analyses were performed with GraphPad software (La Jolla, USA) and SPSS version 32 (IBM, USA).

RESULTS

Study population

The characteristics of SLE patients (n=42) who were treated with RTX[22] (n=16), a combination of RTX+BLM[9] (n=15) or BTZ[28,29] (n=11) are summarized in Table 1. Briefly, most patients were females (86%), with a median age of ~35 [IQR: 30-41] years and a median disease duration of ~9 [6-16] years. SLE organ involvement included renal (69%), cardiorespiratory (64%) or neurologic (31%) disease. All SLE patients had comparable high disease activity scores (DAS) at baseline of ~14 [10-19], which decreased significantly for all approaches (Table 1, Supplementary Figure S2A, available at *Rheumatology* online). The refractory nature of the SLE was illustrated by previously used immunosuppressants (Supplementary Table S1, available at *Rheumatology* online). Both RTX+BLM-treated and BTZ-treated cohorts included a comparable number of patients that were refractory to RTX therapy (27% and 55% resp.). 47% of RTX-treated patients had complement consumption, which was less than 91% of BTZ-treated (p=0.03) and less than 87% of RTX+BLM-treated SLE patients (p=0.01). 69% of RTX-treated SLE patients were positive for anti-dsDNA autoantibodies, compared with 100% in both BTZ and RTX+BLM cohorts (p=0.01). The measured autoantibody repertoire included autoantibodies against dsDNA in 88%, histones in 88%, nucleosomes in 95% and C1q in 81% of all SLE patients. Importantly, the serum levels of anti-dsDNA, anti-histone, anti-nucleosome and anti-C1q autoantibodies, our primary objective in this study, and the number of patients per cohort positive for these autoantibodies, other than anti-dsDNA, were comparable between the cohorts at baseline.

B-cell depletion was associated with reduced autoantibody levels

To assess the effects of the different treatment strategies on humoral autoimmunity, the depleting effects on circulating $CD19^+$ B-cells were evaluated (Table 2, Figure 1A). After RTX, circulating $CD19^+$ B-cells were ~23 x 10^6 cells/litre [2-109], corresponding to a median of -76% [-7; -93] change from baseline (p=0.08). After RTX+BLM, circulating $CD19^+$ B-cells were ~7 x 10^6 cells/litre [3-24], corresponding to a median of -95% [-79; -97] change from baseline (p=0.0001). After BTZ, circulating $CD19^+$ B-cells were ~13x10^6 cells/litre [3-30], corresponding to a median -68% [-81; +11] change as compared with baseline (p=0.16). More detailed impact on B-cell subsets by each therapy has been published separately for RTX[10,22,23,26], RTX+BLM[9] and BTZ[28,29]. Of interest, RTX+BLM was previously demonstrated to result in a more persistent reduction of B-cells as compared with RTX alone in a comparative study[32].

Table 1. Patient characteristics.

	Total (n=42)	RTX (n=16)	RTX+BLM (n=15)	BTZ (n=11)
Demographics				
Age (years)	35 (30-41)	39 (33-47)	31 (25-42)	34 (30-36)
Females (%)	86%	88%	87%	82%
Ethnicity				
Caucasian (%)	46%	27%	33%	91%
Black (%)	44%	53%	60%	9%
Asian (%)	10%	20%	7%	0%
SLE parameters				
Disease duration (years)	9 (6-16)	9 (7-17)	10 (6-16)	9 (5-15)
Disease activity score	14 (10-18)	13 (10-16)	18 (12-21)	13 (10-18)
Disease activity score after therapy	4 (1-6)	2 (2-6)	2 (0-4)	7 (5-7)
Low C3 (%)	73%	47%	87%	91%
CD19+ (10x10^6/L)	110 (50-206)	157 (72-221)	117 (80-228)	41 (15-53)
Follow-up (weeks)	24 (10-26)	30 (24-34)	24 (24-24)	6 (3-7)
Autoantibody profile				
anti-dsDNA positivity (%)	88%	69%	100%	100%
anti-dsDNA AU/mL	230 (86-585)	123 (99-230)	255 (67-455)	438 (168-1116)
anti-histones positivity (%)	88%	75%	93%	100%
anti-histone AU/mL	227 (71-535)	167 (39-428)	190 (67-467)	227 (85-981)
anti-nucleosomes positivity (%)	95%	100%	93%	82%
anti-nucleosome AU/mL	47 (15-207)	47 (13-127)	29 (15-163)	208 (21-617)
anti-C1q positivity (%)	81%	69%	87%	91%
anti-C1q IU/mL	61 (34-117)	55 (28-115)	85 (47-107)	60 (36-125)

BLM: belimumab; BTZ: bortezomib; RTX: rituximab; Low C3 was defined as below normal values according to local laboratory.

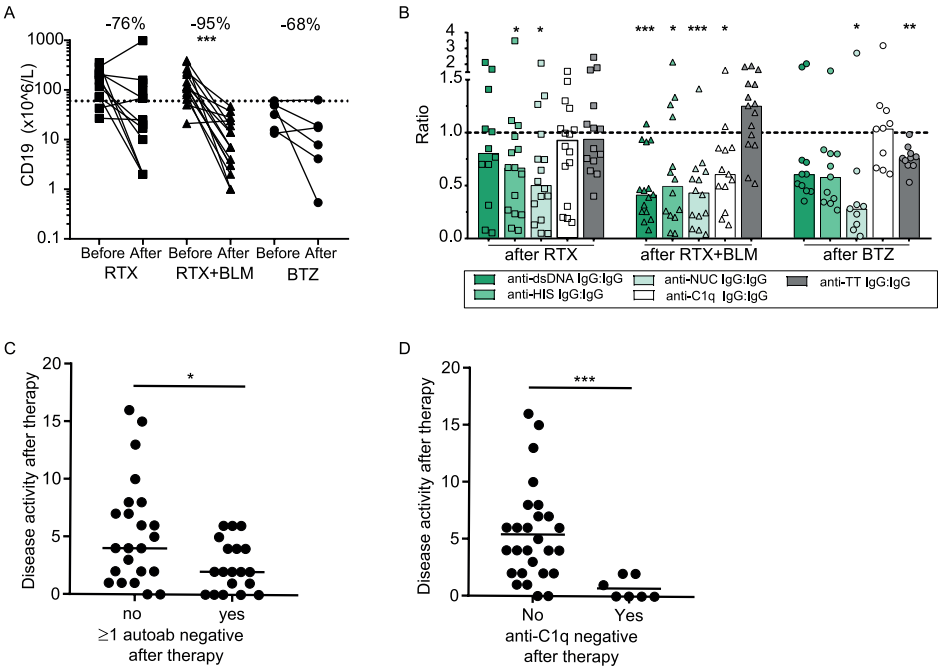

Figure 1. B-cell depletion associated with reduced autoantibody levels. (A) The absolute values of CD19+ B-cells are shown for each individual patient per cohort before and after treatment. Percentages indicate the median change per cohort. **(B)** The change in (auto) antibodies for each individual patient per cohort is expressed as a ratio of the normalized ratio of anti-TT IgG (grey bars), anti-dsDNA (dark green), anti-histones (middle green), anti-nucleosomes (light green) and anti-C1q (white) to total IgG after therapy and compared with the ratio at the baseline of these (auto) antibodies to total IgG. Bars indicate the median per cohort. **(C)** SLE disease activity was assessed in SLE patients who did not achieve seronegativity of any of the autoantibodies compared with SLE patients who did achieve seronegativity of at least one autoantibody. **(D)** SLE disease activity was assessed in anti-C1q positive SLE patients who achieved seronegativity of these anti-C1q autoantibodies and compared with the patients who did not achieve anti-C1q seronegativity. The Wilcoxon matched-pairs signed rank test was used to test statistical differences between baseline and after different targeted therapies in paired patient serum samples. The Mann Whitney U test was used to test statistical difference between two groups. * p<0.05, ** p< 0.01, ***p<0.001. BLM: belimumab; BTZ: bortezomib; HIS: histones; NUC: nucleosomes; RTX: rituximab.

No significant change in total serum IgG was observed after any of the treatment strategies, whereas anti-TT-IgG serum levels decreased significantly upon BTZ treatment to a median of 18% less [-4;-31] (p=0.03), but not after RTX or RTX+BLM (Table 2). In contrast, total serum IgM levels decreased significantly after RTX and RTX+BLM, but not after BTZ (Table 2). Only BTZ decreased total serum IgA significantly, which was not decreased by RTX nor by RTX+BLM. These data confirmed the premise that BTZ targeted long-lived PCs, while RTX with or without BLM targeted short-lived PBs without directly affecting long-lived PCs.

Next, we investigated the effects of the different B-cell targeted strategies on SLE-specific autoantibodies, as summarized in Table 2 and Supplementary Figure S3, available at *Rheumatology* online. Anti-dsDNA antibodies decreased significantly after RTX+BLM to a median of -68% [-48;-84] (p=0.0003) and upon BTZ to a median of -48% [-29; -57] (p=0.03), but not following RTX to a median of -22% [-10;-62] (p=0.20) (Table 2). Anti-histone autoantibody titers decreased significantly following RTX to a median -52% [-23; -62] (p=0.03), following RTX+BLM to a median of -57%[-17;-85] (p=0.003), and following BTZ to a median of -51% [-19; -63] (p=0.005). Anti-nucleosomes autoantibodies titers decreased significantly after RTX+BLM to a median of -62% [-48; -86] (p=0.0004) and after BTZ to a median of -77% [-42; -91] (p=0.02), but non-significantly after RTX to a median of -39% [-77; +8] (p=0.06). Anti-C1q antibodies decreased only significantly following RTX+BLM to median -45% [-69; -27] (p=0.0005), but not after RTX to a median of -9% [-47; +19] (p=0.28), nor after BTZ to median -14% [-26; +14] (p=0.32). Specific targeting of autoantibody levels was assessed by comparing relative changes of IgG autoantibodies to total IgG antibodies per individual patient (Figure 1B). Following RTX, anti-TT IgG remained stable, whereas significant reductions in anti-histone and anti-nucleosome autoantibodies were observed. Also, after RTX+BLM, anti-TT IgG remained unaffected, while preferential reductions of anti-dsDNA, anti-histone, anti-nucleosome and anti-C1q autoantibodies were observed. After BTZ, both anti-histone autoantibodies and anti-TT IgG were significantly reduced, while anti-C1q antibodies were unaffected.

ICx-formation is influenced by the extent of the autoantibody repertoire in SLE patients[33,34]. SLE patients in all cohorts had a similar median number of positive autoantibodies, ~4 [3-4] out of 4 measured specificities (Table 2, Supplemental figure S4A, available at *Rheumatology* online). Overall, we observed that 16 out of 42 SLE patients achieved seronegativity of ≥1 autoantibody specificity after treatment, RTX: 6/16, RTX+BLM: 8/15 and BTZ: 2/11 (Supplemental Figure S4B, available at *Rheumatology* online). This resulted in a significantly smaller autoantibody repertoire for RTX and RTX+BLM patients (Table 2). Interestingly, SLE patients who achieved negativity of one or more autoantibodies after therapy had a significantly lower disease

activity score of ~2 [0-4] compared with those who did not (4 [2-8]; p=0.02) (Figure 1C). This was predominantly reflected in anti-C1q-positive SLE patients who achieved anti-C1q-negativity after RTX (2/11), RTX+BLM (5/13), and BTZ (0/10), who had significantly lower disease activity scores of ~0 [0-2] compared with SLE patients who remained anti-C1q-positive (~4 [2-7]; p=0.0005) (Figure 1D).

RTX+BLM targeted low-, medium- and high-avidity anti-dsDNA, whereas RTX and BTZ targeted only medium-avidity anti-dsDNA

The avidity of anti-dsDNA antibodies is thought to be an important contributor to their pathogenic potential[35], and especially high-avidity anti-dsDNA antibodies closely associated with SLE disease activity and complement activation, thereby contributing to LN[36,37]. To further dissect the effects of each treatment strategy on specific ASC populations, the avidity of anti-dsDNA autoantibodies, with anti-TT-IgG as control, was determined (Figure 2), as previously described[38]. An example of avidity curves for anti-dsDNA and anti-TT IgG and the definition of low-, medium- and high-avidity anti-dsDNA are displayed in Supplementary Figure S1, available at *Rheumatology* online. In summary, in 37 anti-dsDNA positive SLE patients, the majority (59-74%) of anti-dsDNA autoantibodies were of low-avidity (eluted from antigen with 0.25M NaSCN or less) (Figure 2A). Medium-avidity autoantibodies (eluted with ≥0.25M and <1M NaSCN) constituted 16-23% of anti-dsDNA in the cohorts, whereas high-avidity autoantibodies (eluted with ≥1M NaSCN) encompassed 11-19%. The distribution of low-, medium- and high-avidity anti-dsDNA within total anti-dsDNA antibodies did not differ significantly between the cohorts before treatment (Figure 2A). Anti-TT-IgG consisted entirely of high-avidity antibodies (eluted with ≥1M NaSCN) (data not shown). Next, we analyzed the absolute serum titers of low-, medium- and high-avidity anti-dsDNA autoantibodies (Supplementary Figure S5, available at *Rheumatology* online) and observed that medium-avidity anti-dsDNA decreased significantly after all three treatment strategies, whereas RTX+BLM significantly also decreased the low- and high-avidity anti-dsDNA (Figure 2B, Supplementary Figure S5).

Table 2. Immunological parameters.

	RTX		
	Before	**After**	**p**
Circulating B-cells			
CD19+ B-cells (x10^6/L)	157 [72-221]	23 [2-109]	0.08
Immunoglobulins			
IgG (g/L)	9.5 [8.3-13.1]	10.8 [9.5-11.8]	0.2
IgM (g/L)	0.5 [0.4-1.0]	0.4 [0.3-0.8]	0.0002
IgA (g/L)	3.2 [2.5-3.9]	2.9 [2.6-3.8]	0.5
Anti-TT IgG (AU/mL)	12 [6-16]	13 [6-16]	0.95
Autoantibody profile			
anti-dsDNA	69%	63%	
anti-Histones	75%	56%	
anti-Nucleosomes	100%	88%	
anti-C1q	69%	56%	
Repertoire	4 [2-4]	3 [2-4]	0.03
Autoantibody titers			
anti-dsDNA (AU/mL)	123 [99-230]	129 [49-180]	0.20
anti-Histones (AU/mL)	167 [39-428]	86 [27-321]	0.03
anti-Nucleosomes (AU/mL)	47 [13-127]	17 [8-129]	0.06
anti-C1q (IU/mL)	55 [28-115]	50 [24-117]	0.28

	RTX+BLM			BTZ		
	Before	**After**	**p**	**Before**	**After**	**p**
	117 [80-228]	7 [3-24]	0.0001	41 [15-53]	13 [3-30]	0.16
	11.6 [10.4-13.4]	9.6 [7.0-12.5]	0.07	12.6 [10.9-13.3]	11.0 [8.4-12.8]	0.41
	0.7 [0.5-0.9]	0.4 [0.3-0.6]	0.0004	0.8 [0.6-1.1]	0.7 [0.5-1.0]	0.27
	2.8 [1.9-4.3]	2.7 [1.7-3.4]	0.09	2.9 [2.4-4.0]	2.2 [1.6-3.1]	0.03
	14 [5-28]	13 [7-22]	0.76	17 [6-47]	14 [5-50]	0.03
	100%	87%		100%	100%	
	94%	80%		100%	100%	
	87%	60%		82%	73%	
	87%	53%		91%	91%	
	4 [3-4]	3 [2-4]	0.03	4 [3-4]	4 [3-4]	0.99
	255 [67-455]	68 [32-118]	0.0003	438 [168-1116]	355 [104-798]	0.03
	190 [67-467]	51 [39-198]	0.003	227 [85-981]	108 [66-371]	0.005
	29 [15-163]	13 [6-24]	0.0004	208 [21-617]	45 [15-113]	0.02
	85 [47-107]	32 [19-57]	0.0005	60 [36-125]	68 [26-106]	0.32

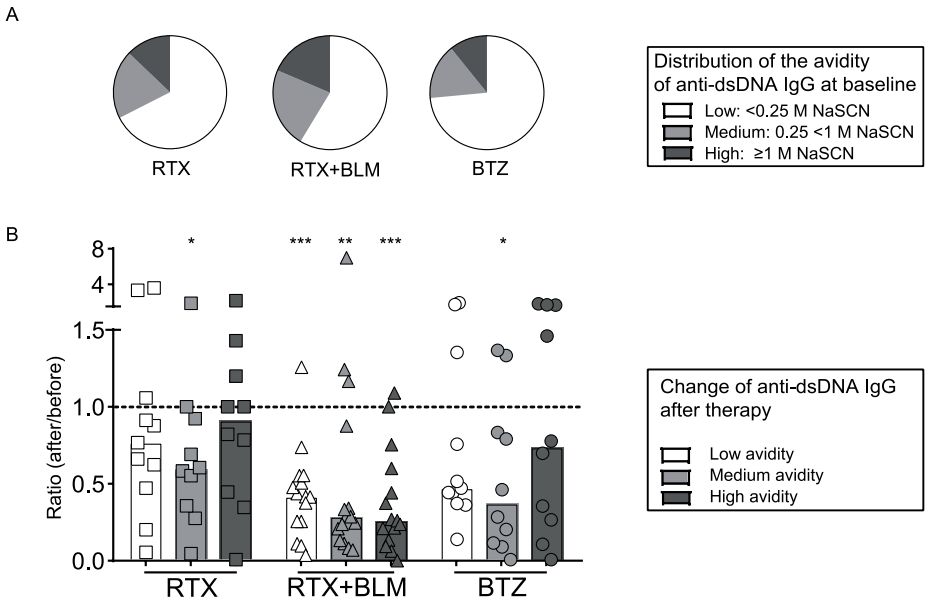

Figure 2. RTX+BLM targets low-, medium- and high-avidity anti-dsDNA autoantibodies, whereas RTX and BTZ target only medium-avidity anti-dsDNA. (A) Distribution of low-, medium- and high-avidity anti-dsDNA autoantibodies is displayed as mean percentage within the total of anti-dsDNA autoantibodies per cohort at baseline. **(B)** The ratio of the absolute serum titers of low-, medium- and high-avidity anti-dsDNA autoantibodies as compared with baseline are shown for each patient. Bars indicate the median per cohort. * p<0.05, ** p< 0.01, ***p<0.001. BLM: belimumab; BTZ: bortezomib; RTX: rituximab.

ICx-mediated inflammation was targeted by RTX and RTX+BLM, but not by BTZ

Finally, we investigated the treatment effects on functional parameters of ICx formation, i.e. complement consumption and *ex vivo* serum-induced NET formation. After RTX, C3 levels normalized in 5/7 SLE patients (Figure 3A, Supplementary Figure S2B, available at *Rheumatology* online) in whom C3 serum levels increased by ~27% [19-116%] (p=0.02) (Figure 3B). After RTX+BLM, C3 levels normalized in 6/13, in whom C3 serum levels increased by ~38% [21-93] (p=0.001). After BTZ, 0/10 SLE patients normalized C3 serum levels, however absolute C3 levels increased significantly by ~20% [4-42] (p=0.04).

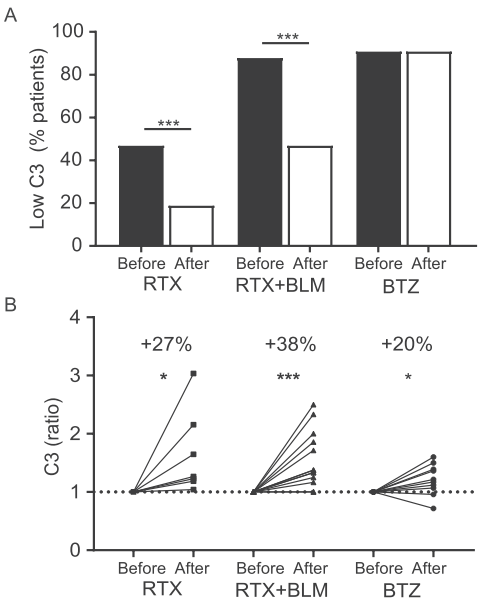

Figure 3. Complement consumption is normalized after RTX and RTX+BLM, but not after BTZ. (A) The percentage of SLE patients that had reduced C3 levels (Supplementary Figure S2B) in sera before (black bars) and after (white bars) rituximab (RTX), rituximab with belimumab (RTX+BLM) or bortezomib (BTZ) are displayed. **(B)** The change of C3 serum titers after RTX, RTX+BLM and BTZ as ratios compared with baseline are displayed. The Wilcoxon matched-pairs signed rank test was used to test statistical differences between baseline and after different targeted therapies in paired patients. *p<0.05, ***p<0.001, RTX: rituximab; BLM: belimumab; BTZ: bortezomib.

SLE-specific-ICx can induce excessive neutrophil extracellular trap (NET) formation[5-7]. Therefore, NET formation induced by SLE sera as a quantitative measurement of ICx formation was quantified. Representative illustrations of the 3D NET quantification assay before and after RTX (A), RTX+BLM (B) and BTZ (C) are shown in Figure 4. Typical SLE-induced clusters of NET-ting neutrophils are observed at baseline[5] (Figure 4A-C). NET formation significantly decreased to a median of -75%[-88; -45] (p=0.008) after RTX+BLM and to a median of -42%[-54; 10] (p=0.03) after RTX, but not after BTZ, when it increased to a median of +31% [-36; 104] (p=0.15) (Figure 4D).

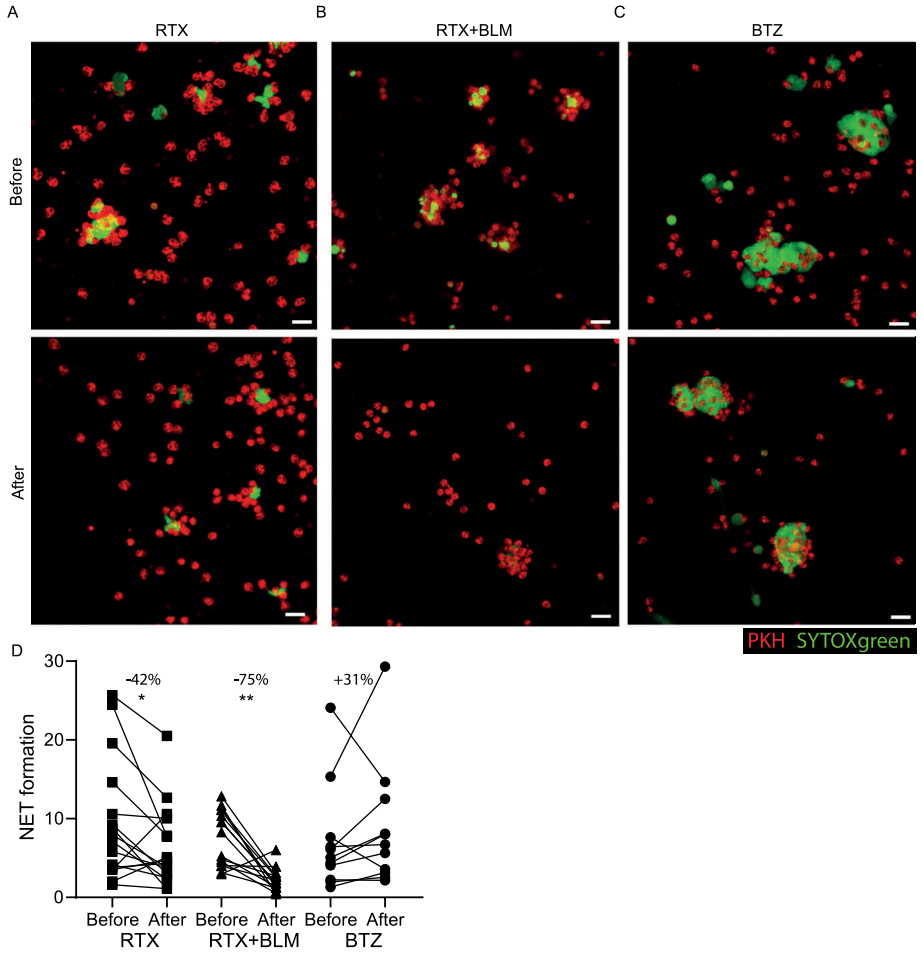

Figure 4. Serum-induced NET formation is significantly decreased after RTX and RTX+BLM, but is not affected by BTZ. Healthy PKH-labelled neutrophils were stimulated with sera of SLE patients at baseline and after treatment with B-cell targeted therapies to induce NET formation. NET formation was quantified three-dimensionally with immuno-fluorescence microscopy through analysing the cumulative extracellular DNA area (SYTOX green) over Z-stacks per imaged neutrophils (red). Representative pictures of the quantitative assay of SLE serum-induced NET formation at baseline and after **(A)** RTX, **(B)** RTX+BLM and **(C)** BTZ. White scale bar = 20uM. **(D)** NET formation is expressed as absolute NET area per imaged neutrophil before and after B-cell targeted therapy for each individual SLE patient (individual lines) per cohort. Percentages indicate the median change per cohort. Wilcoxon matched-pairs signed rank test was used to test statistical differences between baseline and after different targeted therapies in paired patient serum samples. *p<0.05, ** p<0.01. RTX: rituximab; BLM: belimumab; BTZ: bortezomib.

DISCUSSION

RTX, RTX+BLM and BTZ are novel B-cell targeted strategies that differentially target B-cell and plasma cell subsets, the sources of autoantibodies in SLE. In this reverse translational study, we demonstrated that autoantibody levels decreased upon each treatment strategy, but the extent of targeted autoantibodies was most significant for RTX+BLM in a quantitative manner (reduced autoantibody repertoire) as well as a qualitative manner (reduced low, medium and high-avidity anti-dsDNA autoantibodies). These effects were less pronounced for RTX only and not observed in BTZ-treated patients. Especially the reversal of anti-C1q to seronegative was associated with reduced ICx-mediated inflammation and clinical disease activity, which happened most frequent after RTX+BLM, less after RTX and not after BTZ treatment. These observations collectively demonstrated the relevance of in-depth monitoring of the immunological effects of B-cell targeted strategies that have potential implications for the clinic.

Immunomonitoring of the humoral autoimmune response in SLE is challenging because a wide variety of autoantibodies mediate organ damage directly or indirectly through the formation of ICx[2], which subsequently trigger the complement system and NET formation[6,9]. Anti-dsDNA[39], -histones[40], -nucleosomes[41] and -C1q[42] autoantibodies were shown to be involved in ICx formation, correlated with disease activity and thereby are closely linked to the pathogenesis of SLE. Specifically, anti-C1q antibodies have a pathogenic role in LN because they activated the classical and lectin pathway of the complement system[43] and C1q-containing immune complexes are found in the glomeruli[44]. Increased anti-C1q titers predicted renal flares[42,45] and after successful treatment titers decreased[46]. Of interest, immunoadsorption of anti-C1q on C1q columns led to depletion of ICx and anti-C1q autoantibodies and was shown to be beneficial in SLE patients[47].

Overall, a reduction of pathogenic autoantibody levels after any treatment was associated with beneficial clinical outcome in SLE patients[11-13]. Therefore, achieving a reduction of autoantibody load, and ultimately achieving negativity of autoantibodies, could be a key treatment target in SLE patients. In this study, some B-cell targeted strategies were found to not always effectively eradicate pathogenic ICx formation in SLE patients.

An important premise within this retrospective study of three uniquely treated cohorts of SLE patients is that RTX and RTX+BLM do not directly target long-lived PCs, but cause depletion of their precursors (i.e. B-cells and short-lived PBs)[9,22], whereas BTZ does predominantly target long-lived PCs[28,29]. Additionally, B-cell depletion was

sustained longer after RTX+BLM as compared to RTX alone[32]. Different groups have demonstrated that both RTX and RTX+BLM reduced $CD27^{br}CD38^{br}$ PBs[9,26], although their maturation stage remained unclear. On the other hand, BTZ was shown to cause a significant depletion of $CD20^{-}$ PCs in peripheral blood (PB) and bone marrow (BM) in SLE patients[29], whereas their pre-cursor B-cells and T cells remained largely unaffected[28]. After BTZ withdrawal, a rapid repopulation of short-lived HLA-DR+PCs, but not long-lived HLA-DR^{-}PC occurred, accompanied by increasing autoantibody levels[28]. Indeed, our study further corroborated these previously published data by demonstrating a significant effect of BTZ on anti-TT-antibodies typically derived from long-lived PCs, which remained unaffected by RTX+BLM or RTX only. It is therefore of great interest that BTZ did not affect anti-C1q autoantibody levels in our study, whereas RTX+BLM did, strongly suggesting anti-C1q autoantibody production is predominantly derived from short-lived PBs susceptible to BLM treatment. Moreover, RTX+BLM also effectively targeted anti-dsDNA autoantibodies with low to high avidity. It is reasonable to assume that these phenomena underpinned the reduction in ICx-mediated inflammation and the associated amelioration of clinical disease activity in SLE patients treated with RTX+BLM. This study showed similar effects in SLE patients treated with RTX only, but to a lesser extent, whereas ICx-mediated inflammation was not affected in BTZ-treated SLE patients. Taken together, our study suggests that the pathogenic, ICx-forming autoantibodies are derived from short-lived PBs. As such, these autoreactive PBs could be relevant immunomonitoring markers for the evaluation of novel, experimental therapies in SLE and LN patients.

We also demonstrated that most anti-dsDNA autoantibodies were of low avidity. The role of low-avidity anti-dsDNA in the pathophysiology of SLE is uncertain; however, high-avidity anti-dsDNA antibodies detected by the Farr assay and the *Crithidia luciliae* immunofluorescence test (CLIFT) were demonstrated to associate closely with disease activity and complement consumption, and to contribute to LN[36]. Therefore, it is of interest that RTX+BLM targeted significantly high-avidity anti-dsDNA autoantibodies, in contrast to RTX and BTZ, which did not affect the levels of high-avidity anti-dsDNA antibodies. This was in line with a previous publication of RTX+BLM treatment in SLE[9], which demonstrated that 7/12 patients had reversal of anti-dsDNA autoantibodies, measured by CLIFT, from positive to negative.

Several limitations inherent in the design and analysis of this study need to be mentioned. First, the study was limited by the relatively small number of patients treated worldwide with off-label RTX+BLM, BTZ, or RTX. Despite in-depth studies in these small patient cohorts, the serendipity of some of the observations should be considered. Second, because this study assembled three independent cohorts

(which were therefore without randomization), baseline differences were inevitable (Table 1, Supplementary Table S1, available at *Rheumatology* online), and this might have indirectly influenced our results. Although the cohorts were different with respect to the distribution of patients; ethnicities[48] (fewer black patients included in the BTZ-cohort), renal involvement (more patients in the RTX+BLM cohort), reduced C3 levels at baseline (less patients in RTX cohort) and anti-dsDNA positivity at baseline (less patients in RTX cohort), all baseline autoantibody serum titers and the number of patients per cohort positive for the autoantibodies, except anti-dsDNA were comparable between the cohorts (Supplementary Figure S3, available at *Rheumatology* online). As such, relevant changes in unbiased, immunological end points could be monitored in this study. Moreover, to avoid the issue of comparability, we only compared individual patients with their own baseline, and direct comparisons between cohorts were withheld. Third, we cannot exclude the possibility that some observed effects were due to concomitant immunosuppression within these separate treatment strategies. However, even though the cohorts differed significantly with respect to the use of MMF and CYC (Supplementary Table S1-S2, available at *Rheumatology* online), previous studies have shown that MMF and CYC were comparable with respect to the reduction in IgG levels or anti-dsDNA in SLE patients[49].

Additionally, we did not study anti-ENA autoantibodies in this study. But we have previously shown significant decreases after RTX+BLM for anti-Sm (-35%), anti-RNP70 (-48%) and anti-U1RNP (-58%) antibodies[9]. After BTZ, 2/5 SLE patients decreased anti-RNP or anti-Sm levels[28] while RTX did not affected these anti-ENAs[12]. Overall, these data underscored indirectly the effects of the studied B-cell targeted strategies on ENA-specific PCs, which are in line with the results of our study.

Lastly, it is important to note that reported data on BTZ-treated patients were evaluated at a significantly shorter follow-up time as compared to RTX and RTX+BLM-treated patients. This was a well-considered, deliberate part of the study's design because the immunological effects of proteasome inhibition by BTZ arise quicker and last shorter than the effects of B-cell depletion by RTX+/-BLM. For the sake of clarity, we also investigated follow-up sera after a median of 12 weeks after start of BTZ treatment, which demonstrated that autoantibody levels, B-cells, complement levels and NET formation did not further improve while some even worsened over time (Supplementary Figure S6, available at *Rheumatology* online).

In conclusion, this reverse translational study monitored the differential effects of RTX, RTX+BLM and BTZ on the humoral autoimmune response of severe SLE patients. Immunological effects of each B-cell-targeted strategy elucidated the relevance of reducing, and even eradicating, SLE-relevant autoantibodies, in particular anti-C1q. These findings were related to a reduction in ICx-mediated inflammation and translated to clinical benefit. These results support the relevance of immunomonitoring in the context of emerging B-cell targeted strategies for severe SLE patients.

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