Title: The NET-effect of combining Rituximab with Belimumab in severe systemic lupus erythematosus

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Abstract

Objective In systemic lupus erythematosus (SLE) patients, excessive formation of neutrophil extracellular traps (NETs) is observed and their degradation is impaired. In vitro, immune complexes (ICx) trigger NET formation while NET-derived DNA is a postulated autoantigen for anti-nuclear autoantibodies (ANAs), found in SLE. Based on these self-perpetuating mechanisms in SLE, this study investigates whether interfering with ICx formation using a combination of rituximab (RTX) and belimumab (BLM) could decrease NET formation and ameliorate disease.

Methods A phase 2A, open-label, single arm proof-of-concept study was performed wherein 16 SLE patients with severe, refractory disease were treated with a combination of CD20-mediated B-cell depletion with rituximab and sustained inhibition of B-cell activating factor BlyS with belimumab. Besides safety, the study's endpoints were chosen to address the concept of autoantibodies in relation to excessive NET formation.

Results We demonstrated a surge of BlyS levels upon RTX-mediated B-cell depletion which was abrogated by subsequent BLM treatment.. As such, therapeutic intervention with RTX+BLM led to specific reductions in ANAs and regression of excessive NET formation. RTX+BLM appeared to be safe and achieved clinically significant responses: low lupus disease activity state was achieved in 10 patients, renal responses in 11 patients and concomitant immunosuppressive medication was tapered in 14 out of the 16 patients.

Conclusions This study provides novel insights into clinical beneficence of reducing excessive NET formation in SLE by therapeutic targeting ANA production with RTX+BLM. Altogether putting forward a new treatment concept that specifically ameliorates underlying SLE pathophysiology.

Trial registration. ClinicalTrials.gov NCT02284984

Highlights

I. Combination treatment of rituximab with belimumab leads to significant reductions of autoantibodies and consequently regression of excessive NET formation.
II. Combined B cell targeted therapy affects neutrophil-derived autoantigens.
III. Combination therapy led to significant clinical responses in previous refractory patients while reducing other immunosuppressive drugs

Keywords

Systemic lupus erythematosus
Lupus nephritis
Refractory lupus
Rituximab
Belimumab
Neutrophil extracellular traps
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Clinical trial

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Competing interests

T.K., T.J.R., C.K. and Y.K.O.T. are inventors of the UK patent application No. 1517458.4 dealing with the assay to quantify extracellular traps. T.W.J/the department of rheumatology LUMC has received research support/lecture fees/consultancy fees from Roche and from GSK. Y.K.O.T./the department of Nephrology has received lecture fees/consultancy fees from GSK and from Aurinia Pharmaceuticals.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the loss of tolerance to nucleic acids and their binding proteins. This results in the generation of anti-nuclear autoantibodies (ANAs), including anti-dsDNA, anti-chromatin and anti-histone autoantibodies. Neutrophil extracellular traps (NETs) have been demonstrated as prominent autoantigens leading to disease-relevant autoantibody production[1–8] . Excessive NET formation [9] together with the impaired degradation of NETs [10,11] has been associated with disease severity in SLE including the presence of lupus nephritis (LN), anti-dsDNA levels and complement usage[11]. The triggers of excessive NET formation in SLE have been associated with anti-RNP[4] and anti-LL37[2] autoantibodies. Moreover, the impaired degradation of NETs is also associated with DNA-binding autoantibodies that block access for the DNAse-I complex which is functionally intact in SLE [12]. So, regression of excessive NET formation has been postulated as an important therapeutic goal in SLE [13].

Additional to NETs, which are proposed as important autoantigens for the development of ANAs, SLE patients display characteristics of B-cell hyperactivity[14] including the typical increase in circulating plasma cells[15,16]. Despite the eminent role of autoreactive B-cells in SLE, specific targeting of B-cells with rituximab (RTX) has been unsuccessful in randomized trials[17,18]. The well-described surge in circulating B-cell activating factor (BAFF) after B-cell depletion has been postulated to influence repopulation of autoreactive B-cells [19] and negatively impact the efficacy of rituximab [20,21]. The pathophysiological importance of BAFF is further supported by the increased serum BAFF levels in SLE patients compared to healthy subjects [22,23] and its association with disease activity [24], disease relapse [20] and increased numbers of circulating plasma cells [25]. The recently approved anti-BAFF monoclonal antibody, belimumab (BLM), [26], is able to specifically target these pathophysiological processes in SLE subsequent to rituximab treatment. Interestingly, a few case reports have reported the clinical use of combined anti-CD20 and anti-BAFF treatment in patients [27–30]: three of the cases had refractory lupus nephritis (LN) showing beneficial effects in response to combination therapy.

The present proof-of-concept study was designed to investigate the hypothesis that combination treatment of rituximab with belimumab (RTX+BLM) would have a synergetic reducing effect on autoantibody production and thereby diminishing NET formation in SLE patients. As a novel therapeutic approach in SLE, this proof-of-concept study was designed as a single-arm translational

study aimed at determining the immunological effects while simultaneously evaluating safety and clinical responses in patients with severe, refractory disease eligible for rescue treatment with RTX+BLM.

2. Materials and methods

2.1. Clinical study

We conducted a phase 2, single-arm, proof-of-concept study in which SLE patients were included who had severe and refractory disease. 'Severe SLE' was defined as an SLE disease activity index (SLEDAI) score of 12 or more points or new, worse, or persistent SLE-related activity in major organs. Refractory disease was defined according to national Dutch guidelines[31]: 1) the failure of initial induction treatment for which a switch to another induction therapy regimen was already carried out; 2) intolerance or contraindication for cyclophosphamide and mycophenolate mofetil (MMF); 3) a second relapse within two years after the start of initial induction therapy; or 4) a relative contraindication for high-dose oral or intravenous prednisone. A renal biopsy was performed in 2 refractory LN patients prior to inclusion where the diagnosis active LN was uncertain. Patients were excluded if pregnant, had low peripheral B-cell counts ($<60x10^6$ cells/liter), hypogammaglobulinemia (IgG<4.0 g/l), IgA deficiency (IgA<0.1 g/l), active infection or a history of primary immunodeficiency or active malignancy in the past 5 years.

Patients were treated with 1000 mg RTX at weeks 0 and 2 and with 10 mg/kg BLM at weeks 4, 6, 8 and then every 4 weeks. In accordance with international guidelines[32], any patient with active lupus nephritis or severe neurological involvement (e.g. transverse myelitis) received concomitant intravenous methylprednisolone pulse therapy (variable dose/regimens). High dose glucocorticoids were started at 1 mg/kg (maximum dose was 60 mg per day) and tapered towards a maintenance dose of 7.5 mg/day. The study was approved by the LUMC medical ethics committee and all patients provided written informed consent. The study was registered at ClinicalTrials.gov (NCT02284984).

2.2. Endpoints

Primary endpoints were decrease in autoantibodies and NET formation at 24 weeks. Secondary outcomes were seroconversion of anti-dsDNA autoantibodies, complement normalization, safety, feasibility, and clinical response. Autoantibodies were measured at screening, baseline and at 4, 12, and 24 weeks. Ex vivo NET induction was determined at screening, at week 12 and at week 24. Clinical response was investigated by determining the SLEDAI-2000 (SLEDAI-2K) [33] and the number of patients that achieved lupus low disease activity state (LLDAS) after 24 weeks. LLDAS was defined

according to recent international recommendations[34]: 1) SLEDAI-2K ≤4, with no activity in major organ systems; 2) no new lupus disease activity; 3) physician global assessment ≤1; 4) prednisolone dose ≤7.5 mg per day; and 5) well-tolerated treatment with immunosuppressive drugs and/or biological agents[34]. In patients with lupus nephritis, renal responses were defined as follows: a complete renal response was achieved when proteinuria decreased to ≤0.7 g/24h and normal serum albumin, stable kidney function and a normal urinary sediment were achieved. Partial renal response was achieved when proteinuria: >0.7-2.9 g/24h with a decrease in proteinuria of ≥50% from baseline, serum albumin >30 g/l and a stable kidney function as measured by serum creatinine. Urine sediment did not necessarily had to be normalized for achieving a partial renal response. All other patients were considered to be renal non-responders.

2.3. Preparation of neutrophils and ex vivo NET induction

Whole blood (20 ml) from healthy donors was collected into EDTA-coated tubes (BD, Franklin Lakes, NJ, USA). Neutrophils were isolated by density gradient centrifugation with a Ficoll-amidotrizoate gradient (LUMC, Leiden, The Netherlands) followed by erythrocyte lysis at 4°C. Cells were counted using trypan blue, labelled with PKH26 (2 µM, Sigma-Aldrich, Saint-Louis, MO, USA), and 37,500 neutrophils per well were seeded into a 96-well culture plate (Falcon, Tewksbury, MA, USA) in phenol red-free RPMI 1640 medium (Life Technologies, The Netherlands) supplemented with 2% heat-inactivated fetal calf serum (FCS). To induce NETosis, neutrophils were stimulated for 3.75 hours with one of the following: medium (negative control), 10% serum, 10% IgG-depleted serum, 250 or 25 µg/ml IgG, 25 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Saint-Louis, MO, USA) or IgG derived from intravenous immunoglobulin (IVIG; Sanquin, Amsterdam, the Netherlands), healthy controls and patients. When immobilized IgG was used, 10 µg/ml IgG was coated overnight at 4^{o}C in a 96-well Falcon plate, after which neutrophils were incubated in the wells. After stimulation, 1 µM of the impermeable DNA dye SYTOX green (Thermo Fisher, Waltham, MA, USA) was added for 15 minutes and then the neutrophils were fixed with 4% paraformaldehyde (PFA) (Added Pharma, Oss, the Netherlands).

2.4. NET visualization and quantification

NETs were visualized by confocal laser scanning microscopy (CLSM) using the automated BD Pathway 855 (BD Biosciences, San Jose, CA, USA), as described previously[35]. Briefly, 12 z-stacked images of 25 predefined high-power fields (HPFs) at 20x magnification were automatically captured. The HPFs were evenly spread throughout the well using a standardized 5×5 zig-zag pattern that accounts for 11.1% of the area of the well. The microscope was programmed to automatically focus

on PKH26 membrane staining. PKH26 (Cy3; named after its discoverer Paul Karl Horan) and SYTOX green (alexa488) were visualized for each image. The same exposure time was used for all images in the same experiment. Acquired images were analyzed automatically with ImageJ software (NIH, Bethesda, MD, USA) by determining the area of PKH and the area of extracellular DNA, using a pixel threshold to exclude potential intracellular DNA staining. The extracellular DNA of NETs was quantified as the cumulative area of positive SYTOX green staining. To correct for the number of neutrophils, the mean area of positive PKH26 staining was quantified. Next, the ratio of both areas is calculated, representing the NET area corrected for the amount of imaged neutrophils in each sample. Quantification of the NET induction data is expressed as the fold induction relative to the mean NHS values in each experiment. The correlation of NET induction with the SLEDAI and anti-dsDNA titers was assessed by Pearson and Spearman correlation coefficients.

2.5. Fluorescence immunocytochemistry of NETs

Neutrophils were seeded onto chambered coverslips (ibiTreat, Ibidi, Martinsried, Germany), and NETs were induced as described above. Fixed neutrophils were blocked with 1% BSA and 20% normal goat serum (NGS) in PBS with 5 mM EDTA to inhibit nuclease activity and then stained with 1 µg/ml polyclonal rabbit anti-human citrullinated histone H3 (Abcam, Cambridge, UK), 5 µg/ml polyclonal rabbit neutrophil elastase (NE) (Abcam), or a polyclonal rabbit IgG isotype control (Dako, Santa Clara, CA, USA) in PBS plus 1% NGS with 5 mM EDTA and incubated for two hours. The neutrophils were then washed and incubated with a 1:500 secondary goat anti-rabbit Alexa488 antibody (Thermo Fisher). The secondary antibodies were pre-incubated for 30 minutes with 5% normal human serum (NHS) to bind anti-human immunoglobulins. After incubation for 60 minutes with the secondary antibodies and 1:100 phalloidin (Sigma-Aldrich), neutrophils were washed and stained with 1 µg/ml Hoechst 33258 (Thermo Fisher). Images were acquired with the Leica DMI6000 inverted microscope using a 20x magnification.

2.6. High sensitivity flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood collected in EDTA-coated tubes using density gradient centrifugation with Ficoll-amidotrizoate (LUMC). At least one million events were stained with the following antibodies in 6 different panels for FACS staining: CD3 (SK7), CD8 (SK1), CD11B (ICRF44), HLA-DR (G46-6), all from BD Biosciences, and CD4 (SK3), CD14 (MϕP9), CD16 (CB16), CD19 (SJ25C1), CD20 (2H7), CD21 (HB5), CD24 (SN3 A5-2H10), CD27 (O323), CD38 (HB7), CD56 (B159), CD66B (G10F5), IgM (SADA4), and IgD (IA6-2), all from eBioscience (Thermo Fisher Scientific). Cells were read on LSRII (Becton Dickinson, San Jose, CA, USA) and

analyzed with Flowjo (Ashland, Oregon, USA). B-cells were defined as $CD3^{-}CD19^{+}$ cells, wherein memory B-cells were $CD27^{+}$cells, transitional B-cells were $CD24^{bright}CD38^{bright}$ cells, and circulating plasma cells were $CD38^{bright}CD27^{bright}$ cells. Absolute cell numbers were calculated using the absolute lymphocyte counts.

2.7. *Autoantibody measurements*

Anti-dsDNA autoantibodies were semi-quantitatively measured using the in-house, Crithidia luciliae indirect fluorescent test (CLIFT). For quantitative measurements of anti-dsDNA, anti-U1RNP, anti-RNP70 and anti-Sm (anti-ENA) autoantibody serum levels Phadia 250 (Thermo Fisher, Waltham, MA, USA) was used. This is a fully automated and high-throughput system using fluorescence enzyme immunoassay for routine laboratory testing where the fluorescence signal of measured serum samples is compared to calibrators with known concentrations. Anti-C1q levels were determined using commercially available ELISA (Inova, San Diego, CA, USA) according to the manufacturer's instructions.

2.8. Measuring serum levels of free BlyS

The assay to quantify free BLyS is a bespoke assay, developed and validated as fit for purpose at GlaxoSmithKline (GSK) with sera from patients treated with belimumab. Briefly, the capture reagent, biotinylated-belimumab-antibody fragment (Fab), is bound to a microtiter streptavidin-coated 96-well plate. BLyS, in the diluted serum sample, is captured by binding to the biotinylated-drug-Fab and detected by the addition of Alexa-Fluor labeled goat anti-BLyS polyclonal antibody. The plate is washed and Elution Buffer B is added for acidification, thereby cleaving the Alexa labelled antibody. Neutralization Buffer C is added to a new 384-well plate. The eluate is then transferred from the 96-well assay plate to a 384-well plate. The 384-well plate is then read on the Erenna immunoassay system (Singulex, Alameda, CA, USA). The concentration of free BLyS in serum samples is interpolated from a reference standard curve. One signal (DE, PE, or TP) is chosen for export and analysed within Softmax GxP (Molecular Devices, San Jose, CA, USA).

2.9. Statistical analysis

All data are reported as individual data points or as median [range]. Differences between week 0 and week 24 or between two groups were compared using a nonparametric paired t-test. Correlations were assessed using Pearson and Spearman correlation coefficient. All statistical analyses were performed with GraphPad (La Jolla, CA, USA).

3. Results

3.1. Patient characteristics

A therapeutic approach that targets B-cells with RTX+BLM was investigated to determine its immunological effects on humoral autoimmunity in SLE patients. To do so, we included 16 patients with severe refractory SLE from a total of 31 screened patients in this study. Patient characteristics are shown in Table 1. Briefly, the majority of SLE patients was female and had severe disease with major organ involvement. The median [range] SLEDAI was 18 [6–29] and 13 patients (81%) had active lupus nephritis (LN) with a median [range] proteinuria of 2.3 g/day [1–8.2]. One patient had transverse myelitis at inclusion and presented with paralysis of the lower extremities. All patients had refractory disease with a median of 3 relapses that had failed to respond to previous treatment with mycophenolate acid and at least one other immunosuppressive regimen.

Table 1. Baseline and historic disease characteristics of patients in the SynBioSe study (n=16)

Demographics		
Age, median (range)	31	(19-51)
Female sex, n (%)	14	(88)
Race, n (%)		
White/Caucasian	5	(31)
Black/African American	10	(63)
Asian/Oriental	1	(6)
Smoker, n (%)	3	(19)
Baseline disease characteristics		
SLEDAI, median (range)	18	(6-29)
Disease flare characteristics, n (%)		
Renal flare	13	(81)
Transverse myelitis	1	(6)
Persistent disease activity despite treatment	2	(13)
LN disease characteristics (n=13)		
Histopathology, n (%)		
Class II (±V)	1	(8)
Class III (±V)	4	(31)
Class IV (±V)	5	(38)
Class V	3	(23)
Proteinuria (g/24h), median (range)	2.3	(1.0-11.2)
Treatment at disease flare		
Glucocorticoids*, n (%)	13	(81)
Dose mg/day, median (range)	15	(5-60)
Mycophenolate mofetil, n (%)	10	(63)
Dose mg/day, median (range)	2000	(1000-4000)
Azathioprine, n (%)	3	(19)
Dose mg/day, median (range)	150	(100-200)
Hydroxychloroquinine, n (%)	12	(75)
Biomarkers		
ANA positivity	16	(100)

Anti-dsDNA titer (IU/ml)†, median (range)	144	(18-505)
Complement consumption‡ (%)	16	(100)
C3§ (g/l), median (range)	0.6	(0.3-1.3)
C4¥ (mg/l), median (range)	96	(21-260)
IgG (g/l), median (range)	11.5	(4.9-23.6)
IgA (g/l), median (range)	3	(1.2-6.3)
IgM (g/l), median (range)	0.7	(0.3-1.1)
CD19+ B-cells (*10^6 cells/l), median (range)	100	(21-302)
Historic disease characteristics		
Disease duration in years, median (range)	10	(2-24)
No. of previous relapses, median (range)	3	(1-6)
No. of renal relapses, median (range)	1	(0-5)
SLICC damage index, median (range)	1	(0-4)
Organ involvement, n (%)		
Constitutional	16	(100)
Mucocutaneous	13	(81)
Neuropsychiatric	2	(13)
Musculoskeletal	10	(63)
Cardiorespiratory	11	(69)
Gastrointestinal	0	(0)
Opthalmic	2	(13)
Renal	15	(94)
Hematology	10	(63)
Treatment history		
Steroids, n (%)	16	(100)
Mycophenolate mofetil, n (%)	16	(100)
Cyclophosphamide, n (%)	7	(44)
Azathioprine, n (%)	9	(56)
Tacrolimus, n (%)	1	(6)
Rituximab, n (%)	4	(25)
Hydroxychloroquinine, n (%)	14	(88)

*Patients were treated with the glucocorticoid equivalent prednisolone. †Normal anti-dsDNA IgG <10 IU/ml. ‡Complement consumption is defined as decreased CP (classical pathway) activation, decreased C3 or decreased C4. §Normal C3: 0.9-2 g/l. ¥Normal C4: 95-415 mg/l.

SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; LN, Lupus Nephritis; ANA, Anti-Nuclear antibodies; SLICC, Systemic Lupus International Collaborating Clinics.

3.2. RTX+BLM specifically reduces humoral autoimmune phenomena in refractory SLE patients

First, we confirmed the hypothesis that belimumab effectively inhibited a spike of circulating BlyS after B-cell depletion. Indeed, by quantifying free circulating BlyS levels (Figure 1A) we observed a significant increase of serum BlyS levels at 4 weeks (1.17 ng/ml [0.02-4.81]) after initiation of rituximab compared to baseline levels (1.08 ng/ml [0.14-3.22], P=0.03). Belimumab treatment thereafter effectively reduced BlyS levels to 0.15 ng/ml [0.05-0.4], P<0.01, at 24 weeks.

Second, we used high sensitivity flow cytometry to monitor the kinetics of different B-cell subsets in SLE patients treated with RTX+BLM. We observed that B-cells were significantly depleted from

100×10^6 cells/liter [21–302×10^6] at baseline to a nadir of 2.43×10^6 cells/liter [1.17–8.0×10^6] (*P*<0.0001) equal to 98% decrease from baseline. At 24 weeks, the B-cells had repopulated to a level of 11×10^6 cells/liter [0.5–64.7×10^6] (*P*=0.0002) (Supplementary Table 1), which still represented 94% decrease (Figure 1B). B-cell subset analysis demonstrated that memory B-cells were depleted from 17.5×10^6 cells/liter [6.2–142] at baseline to a nadir of 1.34×10^6 cells/liter [0.15–4.4] (*P*=0.0001) equal to 94% decrease. Transitional B-cells were completely depleted from 0.93×10^6 cells/liter [0.08–3.43] at baseline to a nadir of 0 [0–0.15×10^6] (*P*=0.0002) representing 100% decrease. Circulating plasma cells were depleted from 2.13×10^6 cells/liter [0.07–10.5×10^6] at baseline to a nadir of 0.11×10^6 cells/liter [0–11×10^6] (*P*=0.104) equal to 95% decrease. Looking more closely at B-cell subsets during repopulation at 24 weeks, memory B-cells were the dominant subset, with 4.46×10^6 cells/liter [0.31–49.5×10^6] equal to 76% decrease from baseline. Circulating plasma cells repopulated to 1.26×10^6 cells/liter [0–18.3×10^6] equal to 72% decrease from baseline. Transitional B-cells did not repopulate and remained depleted at a level of 0×10^6 cells/liter [0–6.22×10^6] at 24 weeks (Figure 1B).

Third, we assessed the effects of RTX+BLM on (auto-)antibody levels (Figure 2 and Supplementary Table S1). Total IgG levels transiently declined from baseline levels of 11.5 g/l [4.8–23.6] to 8.1 g/l [2.6–14.4] at week 12 (*P*=0.03) and returned to 8.9 g/l [3.4–16.4] at week 24 (*P*=0.12). A median decrease of 21% from baseline was observed at week 24 (Figure 2A). Of note, IgM levels steadily decreased from baseline levels of 0.7 [0.3–1.1] to 0.4 g/l [0.2–0.7] at week 24 (*P*=0.0007). IgA levels had not significantly changed at week 24. Anti-tetanus toxoid (Figure 2B), anti-rubella (Figure 2C) and anti-varicella zoster virus (Figure 2D) antibody levels did not change significantly during treatment. Based on the fluorescence enzyme immunoassay, 12 out of 16 patients were anti-dsDNA positive at baseline with a median anti-dsDNA level of 144 IU/ml [range 18–505]. Following RTX+BLM treatment, the anti-dsDNA levels steadily decreased to 57 IU/ml [10–374, *P*=0.0004] at week 24. We found a median decrease in anti-dsDNA antibody levels of 58% (Figure 2E). Seven out of 12 (58%) anti-dsDNA positive patients had converted to negative immunofluorescence at week 24. Additionally, based on immunofluorescence, 7 patients were positive for anti-Sm autoantibodies, 6 patients for anti-RNP70 autoantibodies and 9 patients for anti-U1RNP autoantibodies at baseline. Anti-Sm antibody levels decreased by 35% (Figure 2F), anti-RNP70 antibody levels by 48% (Figure 2G) and anti-U1RNP antibody levels by 58% (Figure 2H) at week 24. Fourteen of 16 patients (88%) had anti-C1q autoantibodies and anti-C1q antibody levels decreased by 45% at week 24 (Figure 2I). To assess whether RTX+BLM specifically targeted autoantibody levels, the ratios of (auto-)antigen-specific antibodies to total IgG levels were determined in individual patients (Figure 2J), demonstrating preferential reductions of autoantibodies.

Fourth, we investigated systemic complement activation: C3 and C4 levels and classical pathway (CP) activity were reduced in all patients at baseline. The reduced C3 concentrations were restored to normal levels within 24 weeks after RTX+BLM treatment in 6 out of 14 patients. Overall, the C3 levels increased from 0.6 g/l [0.3–0.8] at baseline to 0.8 g/l [0.5–1.3] at week 24, *P*=0.002 (Supplementary Table S1). Reduced C4 concentrations were restored to normal levels within 24 weeks in 5 out of 8 patients; overall, C4 levels increased from 54 [21–80] at baseline to 110 mg/l [39–292] at week 24, *P*=0.04. CP activity had normalized in 8 out of 14 patients at week 24. Overall, complement activation had normalized in 7 out of 15 (47%) patients at week 24.

Taken together, these data demonstrate that RTX+BLM treatment led to complete but transient B-cell depletion, with early repopulation of memory B-cells and circulating plasma cells. RTX+BLM specifically reduced relevant circulating SLE-specific autoantibodies and improved complement profiles, which suggests that RTX+BLM effectively targets humoral autoimmune phenomena in SLE patients.

3.3. Excessive NET formation is normalized upon RTX+BLM treatment

Based upon previous reports that SLE-specific autoantibodies were demonstrated to induce excessive NET formation *in vitro* [4] due to crosslinking of Fc-gamma receptors (FcγRs) by pathogenic immune complexes (ICx) [2], we investigated whether reducing ANAs also resulted in a regression of NET formation in our patients with severe SLE. Representative images of ex vivo NET formation before and after RTX+BLM in 2 patients are shown in Figure 3A. In our study population, excessive NET formation was observed at baseline, which was 6.8-fold [2.6–12.8] increased compared to NHS controls (*P*<0.0001) (Figure 3B). RTX+BLM treatment significantly diminished excessive NET formation at 12 weeks and at 24 weeks: 1.6 fold [0.9–13.7] (*P*=0.003) and 1.9 fold [0.4–6.1] (*P*=0.0006), respectively. These data show that RTX+BLM treatment regressed excessive NET formation in severe SLE patients.

3.4. RTX+BLM leads to beneficial clinical responses in refractory SLE patients

Finally, we investigated whether the observed immunological effects translated into clinical disease amelioration. Ten patients, 9 patients with active lupus nephritis and 1 patient with transverse myelitis, received concomitant pulse intravenous glucocorticoids prior to rituximab. All 16 patients received RTX+BLM according to the study protocol. At baseline, the median SLEDAI was 18 [6–29] and this significantly decreased to a median SLEDAI of 2 (0–13, P<0.0001) at week 24 (Figure 4A).

LLDAS was achieved in 10 patients at week 24. Three patients were classified as non-responders. One patient showed persistent disease activity of SLEDAI 13 at week 24, including persistent complement activation, positivity for anti-dsDNA antibodies, proteinuria, hematuria and pyuria. Importantly, this patient did not achieve B-cell depletion after RTX due to preformed human anti-chimeric antibodies (HACAs). A second patient showed increased complement activation, positivity for anti-dsDNA antibodies, and persistent proteinuria at week 24. This patient was treated with methylprednisolone and high-dose glucocorticoids for active LN at baseline and developed a severe mood disorder and psychosis for which the glucocorticoids had to be abruptly reduced to 10 mg. The third patient dropped out of the study at 8 weeks due to severe hypogammaglobinemia (IgG nadir of 1.8 g/l), requiring intravenous immunoglobulin treatment. Notably, this patient also received pulse intravenous glucocorticoids. In the 13 patients with LN, a renal response was observed in 11, with 5 complete responders. In these patients, the median proteinuria levels at baseline of 2.3 g/24h [range 1–11.2] significantly decreased to a median of 0.7 g/24h [0.1–1.8] at 24 weeks (*P*=0.0005) (Figure 4B).

These clinical responses were achieved during RTX+BLM treatment while tapering concomitant immunosuppressive medication. In 14 patients who were treated with glucocorticoids, the median baseline glucocorticoid dose of 60 mg/day [5–60] was successfully tapered to a median dose of 7.5 mg/day [5–12.5], *P*=0.001 (Figure 4C). Only one patient was still treated with >7.5 mg/day glucocorticoids at week 24. Additionally, 10 out of 16 patients were on MMF at baseline which was stopped successfully in all patients (Figure 4D). In 5 out of 10 patients on MMF, tapering of MMF was started after 8 weeks and in the other 5 patients, tapering was started before 8 weeks. In the patient who developed a glucocorticoid-induced severe mood disorder and psychosis, MMF treatment was started at 4 weeks and continued throughout the study. There was also a significant improvement in systemic inflammatory markers as measured by the erythrocyte sedimentation rate (ESR) and leukocyte counts during RTX+BLM treatment (Supplementary Table S1). Compared to baseline values, the median ESR decreased from 47 mm [6–129] to 19 mm [2–77] at 24 weeks (*P*=0.02).
As mentioned above, autoantibody levels (see also Supplementary Table S1), complement activation (see also Supplementary Table S1) and ex vivo NET formation all significantly improved in response to RTX+BLM treatment. Strikingly, ex vivo NET formation showed a significant correlation with SLEDAI (r=0.52, *P*=0.0003) (Figure 4E) that was stronger than the weak correlation between circulating anti-dsDNA autoantibody levels and SLEDAI (r=0.34, *P*=0.03) (Figure 4F).

The safety profile of RTX+BLM is summarized in Table 2. Briefly, 41 treatment-emergent adverse events (AEs) were observed. All patients experienced at least 1 adverse event. Hospitalization for self-limiting viral gastroenteritis classified as major infection. Twelve (38%) minor infections were observed and circulating HACAs in 4 patients. Hypogammaglobulinemia was observed in 3 patients and led to the withdrawal of one patient. In a second patient, IgG levels decreased to 2.5 g/l with symptoms of viral sinusitis responding to increasing BLM treatment interval to 8 weeks for 3 consecutive infusions. In a third patient, IgG levels dropped to 3.4 g/l reconstituting by tapering concomitant immunosuppression.

Table 2. Adverse events during 24 weeks of study

Any adverse events*, n (%)	41	
Major infection (hospitalisation) , n (%)	1	(2%)
Minor infection, n (%)	15	(37%)
Upper respiratory tract	6	
Lower respiratory tract	3	
Urinary tract	3	
Sinusitis	1	
Influenza	1	
Herpes simplex	1	
HACA formation, n (%)¶	4	(10%)
Symptomatic¥	1	
Asymptomatic¥	3	
Hypogammaglobulinemia† (<4.0 g/l), n (%)	3	(7%)
Glucocorticoid-induced mood disorder/ psychosis, n (%)	1	(2%)
Diarrhoea, n (%)	5	(12%)
Myalgia, n (%)	3	(7%)
Infusion-related reaction, n (%)	2	(5%)
Headache, n (%)	1	(2%)
Nausea, n (%)	1	(2%)
Other‡, n (%)	5	(12%)

*Treatment-emergent adverse events. †Study agent was interrupted in one patient, one patient was excluded from the study. ‡Other events include: iron deficiency anemia, rash, onycholysism, hyperkalemia, dry eyes.

¶HACA, human anti-chimeric antibodies, as quantified by using validated antigen-binding **tests**(RIA).

¥ Patients with HACA formation were classified as symptomatic when clinical signs of serum sickness were observed in conjunction with a newly positive test for HACAs. Patients were classified as asymptomatic when a newly positive test for HACAs was found without any objectified clinical symptoms.

4. Discussion

This translational proof-of-concept study is the first clinical trial to investigate the immunological and clinical responses of RTX+BLM in patients with severe, refractory SLE. Recently we found that circulating ICx contributed to excessive NET formation in SLE in line with data from others [2,4]. Here we now demonstrate that RTX+BLM effectively reduced ANAs and regressed excessive NET formation *ex vivo* while achieving significant clinical responses in patients with severe refractory SLE. As such, we describe the unique finding that B-cell targeting by RTX+BLM indirectly affected neutrophils when assessing NET formation. Even though a proper control group was lacking, this study provides novel insight into the autoimmunity of SLE with respect to NETs and ANA formation and puts forward a new therapeutic concept to target this pathophysiology.

Our study showed preferential reductions of autoantibody levels compared to physiological antibody levels suggesting that autoantibody-secreting plasma cells are more susceptible to RTX+BLM. Interestingly, after RTX+BLM led to profound B-cell depletion, early repopulation was dominated by memory B-cells and circulating plasma cells in the absence of the reconstitution of transitional B-cells. These observations suggest that early repopulation after RTX+BLM is derived from the recruitment of differentiated B-cells from lymphoid tissues rather than reconstitution from precursor B-cells from the bone marrow. These observations corroborated findings in patients treated with belimumab only where an increase in memory B-cells was observed [26,36,37]. These data support the hypothesis that neutralizing BAFF in the context of B-cell depletion can result in migration of tissue-resident mature B-cells into the circulation.

The present study was designed to investigate the feasibility of RTX+BLM in severe SLE patients while simultaneously studying the thus far unknown immunological effects. At the time of the study's design, it was considered unethical to randomize severe, refractory patients to either a RTX only arm, which failed to be superior over conventional treatment these patients already failed[18] or a BLM only arm which was not investigated in severe SLE patients. Despite this limitation, we observed significant clinical responses in previously unremitting, treatment- refractory SLE patients: on SLEDAI and LLDAS was achieved in 75% while tapering concomitant immunosuppression. Of note, LLDAS associated with lower damage accrual [34,38]. As such, this proof-of-concept study is an important stepping stone to further study RTX+BLM in SLE patients.

5. Conclusion

We demonstrated that RTX+BLM ameliorated autoimmune phenomena by reducing circulating ANAs and regressing ICx-mediated excessive NET formation in SLE. Simultaneously, RTX+BLM elicited

significant clinical responses in patients with severe, refractory SLE. RTX+BLM therapy represents a promising new treatment concept that specifically targets pathological autoimmunity in SLE patients.

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References

[1] K. Kessenbrock, M. Krumbholz, U. Schönermarck, W. Back, W.L. Gross, Z. Werb, H.-J. Gröne, V. Brinkmann, D.E. Jenne, Netting neutrophils in autoimmune small-vessel vasculitis., Nat. Med. 15 (2009) 623–625. doi:10.1038/nm.1959.

[2] R. Lande, D. Ganguly, V. Facchinetti, L. Frasca, C. Conrad, J. Gregorio, S. Meller, G. Chamilos, R. Sebasigari, V. Riccieri, R. Bassett, H. Amuro, S. Fukuhara, T. Ito, Y.-J. Liu, M. Gilliet, Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus., Sci. Transl. Med. 3 (2011) 73ra19. doi:10.1126/scitranslmed.3001180.

[3] E. Villanueva, S. Yalavarthi, C.C. Berthier, J.B. Hodgin, R. Khandpur, A.M. Lin, C.J. Rubin, W. Zhao, S.H. Olsen, M. Klinker, D. Shealy, M.F. Denny, J. Plumas, L. Chaperot, M. Kretzler, A.T. Bruce, M.J. Kaplan, Netting Neutrophils Induce Endothelial Damage, Infiltrate Tissues, and Expose Immunostimulatory Molecules in Systemic Lupus Erythematosus, J. Immunol. 187 (2011) 538–552. doi:10.4049/jimmunol.1100450.

[4] G.S. Garcia-Romo, S. Caielli, B. Vega, J. Connolly, F. Allantaz, Z. Xu, M. Punaro, J. Baisch, C. Guiducci, R.L. Coffman, F.J. Barrat, J. Banchereau, V. Pascual, Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus., Sci. Transl. Med. 3 (2011) 73ra20. doi:10.1126/scitranslmed.3001201.

[5] P.C. Grayson, C. Carmona-Rivera, L. Xu, N. Lim, Z. Gao, A.L. Asare, U. Specks, J.H. Stone, P. Seo, R.F. Spiera, C.A. Langford, G.S. Hoffman, C.G.M. Kallenberg, E.W. St. Clair, N.K. Tchao, S.R. Ytterberg, D.J. Phippard, P.A. Merkel, M.J. Kaplan, P.A. Monach, Neutrophil-related gene expression and low-density granulocytes associated with disease activity and response to treatment in antineutrophil cytoplasmic antibody-associated vasculitis, Arthritis Rheumatol. 67 (2015) 1922–1932. doi:10.1002/art.39153.

[6] C. Carmona-Rivera, M.J. Kaplan, Low-density granulocytes: A distinct class of neutrophils in systemic autoimmunity, Semin. Immunopathol. 35 (2013) 455–463. doi:10.1007/s00281-013-0375-7.

[7] C. Carmona-Rivera, M.M. Purmalek, E. Moore, M. Waldman, P.J. Walter, H.M. Garraffo, K.A. Phillips, K.L. Preston, J. Graf, M.J. Kaplan, P.C. Grayson, A role for muscarinic receptors in neutrophil extracellular trap formation and levamisole-induced autoimmunity., JCI Insight. 2 (2017) e89780. doi:10.1172/jci.insight.89780.

[8] C. Carmona-Rivera, P.M. Carlucci, E. Moore, N. Lingampalli, H. Uchtenhagen, E. James, Y. Liu, K.L. Bicker, H. Wahamaa, V. Hoffmann, A.I. Catrina, P.R. Thompson, J.H. Buckner, W.H.

Robinson, D.A. Fox, M.J. Kaplan, Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis, Sci. Immunol. 2 (2017) eaag3358. doi:10.1126/sciimmunol.aag3358.

[9] L.S. van Dam, T. Kraaij, S. Kamerling, H.U. Scherer, C.D. Pusey, T.J. Rabelink, C. van Kooten, Y.K.O. Teng, Ex Vivo Induced Neutrophil Extracellular Traps Are Intrinsically Different in ANCA-Associated Vasculitis- and Systemic Lupus Erythematosus, 2017. https://www.asn-online.org/education/kidneyweek/2017/program-abstract.aspx?controlId=2774738 (accessed January 12, 2018).

[10] A. Hakkim, B.G. Furnrohr, K. Amann, B. Laube, U.A. Abed, V. Brinkmann, M. Herrmann, R.E. Voll, A. Zychlinsky, Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis, Proc. Natl. Acad. Sci. 107 (2010) 9813–9818. doi:10.1073/pnas.0909927107.

[11] J. Leffler, M. Martin, B. Gullstrand, H. Tydén, C. Lood, L. Truedsson, A. a Bengtsson, A.M. Blom, Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease., J. Immunol. 188 (2012) 3522–31. doi:10.4049/jimmunol.1102404.

[12] J. Leffler, K. Ciacma, B. Gullstrand, A.A. Bengtsson, M. Martin, A.M. Blom, A subset of patients with systemic lupus erythematosus fails to degrade DNA from multiple clinically relevant sources, Arthritis Res. Ther. 17 (2015) 205. doi:10.1186/s13075-015-0726-y.

[13] S. Gupta, M.J. Kaplan, The role of neutrophils and NETosis in autoimmune and renal diseases., Nat. Rev. Nephrol. 12 (2016) 402–13. doi:10.1038/nrneph.2016.71.

[14] P.E. Lipsky, Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity., Nat. Immunol. 2 (2001) 764–6. doi:10.1038/ni0901-764.

[15] M. Odendahl, A. Jacobi, A. Hansen, E. Feist, F. Hiepe, G.R. Burmester, P.E. Lipsky, A. Radbruch, T. Dorner, Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus, J Immunol. 165 (2000) 5970–5979. doi:10.4049/jimmunol.165.10.5970.

[16] A. Hostmann, A.M. Jacobi, H. Mei, F. Hiepe, T. Dorner, Peripheral B cell abnormalities and disease activity in systemic lupus erythematosus, Lupus. 17 (2008) 1064–1069. doi:10.1177/0961203308095138.

[17] J.T. Merrill, C.M. Neuwelt, D.J. Wallace, J.C. Shanahan, K.M. Latinis, J.C. Oates, T.O. Utset, C. Gordon, D.A. Isenberg, H.J. Hsieh, D. Zhang, P.G. Brunetta, Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: The randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial, Arthritis Rheum. 62 (2010) 222–233. doi:10.1002/art.27233.

[18] B.H. Rovin, R. Furie, K. Latinis, R.J. Looney, F.C. Fervenza, J. Sanchez-Guerrero, R. Maciuca, D.

Zhang, J.P. Garg, P. Brunetta, G. Appel, LUNAR Investigator Group, Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: The lupus nephritis assessment with rituximab (LUNAR) study., Arthritis Rheum. 64 (2012) 1215–1226. doi:10.1002/art.34359.

[19] M.P. Cancro, D.P.D. Cruz, M. a Khamashta, The role of B lymphocyte stimulator (BLyS) in systemic lupus erythematosus., J. Clin. Invest. 119 (2009) 1066–73. doi:10.1172/JCI38010.1066.

[20] L.M. Carter, D.A. Isenberg, M.R. Ehrenstein, Elevated serum BAFF levels are associated with rising anti-double-stranded DNA antibody levels and disease flare following B cell depletion therapy in systemic lupus erythematosus, Arthritis Rheum. 65 (2013) 2672–2679. doi:10.1002/art.38074.

[21] M.R. Ehrenstein, C. Wing, The BAFFling effects of rituximab in lupus: danger ahead?, Nat. Rev. Rheumatol. 12 (2016) 367–372. doi:10.1038/nrrheum.2016.18.

[22] J. Zhang, V. Roschke, K.P. Baker, Z. Wang, G.S. Alarcón, B.J. Fessler, H. Bastian, R.P. Kimberly, T. Zhou, Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus., J. Immunol. 166 (2001) 6–10. doi:10.4049/jimmunol.166.1.6.

[23] G.S. Cheema, V. Roschke, D.M. Hilbert, W. Stohl, Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases, Arthritis Rheum. 44 (2001) 1313–1319. doi:10.1002/1529-0131(200106)44:6<1313::AID-ART223>3.0.CO;2-S.

[24] M. Petri, W. Stohl, W. Chatham, W.J. McCune, M. Chevrier, J. Ryel, V. Recta, J. Zhong, W. Freimuth, Association of plasma B lymphocyte stimulator levels and disease activity in systemic lupus erythematosus, Arthritis Rheum. 58 (2008) 2453–2459. doi:10.1002/art.23678.

[25] F. Mackay, J.L. Browning, BAFF: a fundamental survival factor for B cells., Nat. Rev. Immunol. 2 (2002) 465–475. doi:10.1038/nri844.

[26] R. Furie, M. Petri, O. Zamani, R. Cervera, D.J. Wallace, D. Tegzová, J. Sanchez-Guerrero, A. Schwarting, J.T. Merrill, W.W. Chatham, W. Stohl, E.M. Ginzler, D.R. Hough, Z.J. Zhong, W. Freimuth, R.F. van Vollenhoven, A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus, Arthritis Rheum. 63 (2011) 3918–3930. doi:10.1002/art.30613.

[27] T. Kraaij, T.W.J. Huizinga, T.J. Rabelink, Y.K.O. Teng, Belimumab after rituximab as maintenance therapy in lupus nephritis., Rheumatology (Oxford). 53 (2014) 2122–2124. doi:10.1093/rheumatology/keu369.

[28] F. Simonetta, D. Allali, P. Roux-Lombard, C. Chizzolini, Successful treatment of refractory lupus nephritis by the sequential use of rituximab and belimumab, Jt. Bone Spine. 84 (2017) 235–236. doi:10.1016/j.jbspin.2016.01.008.

[29] C. Gonzalez-Echavarri, A. Ugarte, G. Ruiz-Irastorza, Rituximab-refractory lupus nephritis successfully treated with belimumab., Clin. Exp. Rheumatol. 34 (2016) 355–6. http://www.ncbi.nlm.nih.gov/pubmed/26886714 (accessed July 27, 2017).

[30] S. de Vita, L. Quartuccio, S. Salvin, L. Picco, C.A. Scott, M. Rupolo, M. Fabris, Sequential therapy with belimumab followed by rituximab in Sjögren's syndrome associated with B-cell lymphoproliferation and overexpression of BAFF: Evidence for long-term efficacy, Clin. Exp. Rheumatol. 32 (2014) 490–494. doi:7852 [pii].

[31] A. Van Tellingen, NfN Richtlijn Lupus Nefritis februari 2014, (2014).

[32] G. Bertsias, J.P.A. Ioannidis, J. Boletis, S. Bombardieri, R. Cervera, C. Dostal, J. Font, I.M. Gilboe, F. Houssiau, T. Huizinga, D. Isenberg, C.G.M. Kallenberg, M. Khamashta, J.C. Piette, M. Schneider, J. Smolen, G. Sturfelt, A. Tincani, R. van Vollenhoven, C. Gordon, D.T. Boumpas, EULAR recommendations for the management of systemic lupus erythematosus. Report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics, Ann. Rheum. Dis. 67 (2008) 195–205. doi:10.1136/ard.2007.070367.

[33] D.D. Gladman, D. Ibañez, M.B. Urowitz, Systemic lupus erythematosus disease activity index 2000., J. Rheumatol. 29 (2002) 288–91. doi:10.3899/jrheum.110550.

[34] K. Franklyn, C.S. Lau, S. V Navarra, W. Louthrenoo, A. Lateef, L. Hamijoyo, C.S. Wahono, S. Le Chen, O. Jin, S. Morton, A. Hoi, M. Huq, M. Nikpour, E.F. Morand, Definition and initial validation of a Lupus Low Disease Activity State (LLDAS), Ann. Rheum. Dis. (2015) 1–7. doi:10.1136/annrheumdis-2015-207726.

[35] T. Kraaij, F.C. Tengström, S.W.A. Kamerling, C.D. Pusey, H.U. Scherer, R.E.M. Toes, T.J. Rabelink, C. van Kooten, Y.K.O. Teng, A novel method for high-throughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes, Autoimmun. Rev. (2016). doi:10.1016/j.autrev.2016.02.018.

[36] D.J. Wallace, W. Stohl, R.A. Furie, J.R. Lisse, J.D. McKay, J.T. Merrill, M.A. Petri, E.M. Ginzler, W.W. Chatham, W.J. McCune, V. Fernandez, M.R. Chevrier, Z.J. Zhong, W.W. Freimuth, A phase II, randomized, double-blind, placebo-controlled, dose-ranging study of belimumab in patients with active systemic lupus erythematosus., Arthritis Rheum. 61 (2009) 1168–78. doi:10.1002/art.24699.

[37] Dascalu C, Davidson A, Mackay MC, Furie R, Huang W, Aranow C, The Effect of Belimumab on Peripheral Blood Cells in Patients with Systemic Lupus Erythematosus - ACR Meeting

Abstract, Arthritis Rheumatol. 67 (2015). http://acrabstracts.org/abstract/the-effect-of-belimumab-on-peripheral-blood-cells-in-patients-with-systemic-lupus-erythematosus/ (accessed July 28, 2017).

[38] M.W.P. Tsang-A-Sjoe, I.E.M. Bultink, M. Heslinga, A.E. Voskuyl, Both prolonged remission and Lupus Low Disease Activity State are associated with reduced damage accrual in systemic lupus erythematosus, Rheumatology. 56 (2017) 121–128. doi:10.1093/rheumatology/kew377.

Figure legends

Figure 1

RTX+BLM specifically reduces humoral autoimmune phenomena in refractory SLE patients. (A) The percentage change of free serum BlyS in individual patients throughout the 24-week study. The thick black line represents the median and thin grey lines represent individual data. (B) The median change from baseline in the number of circulating B-cells (CD19+), memory B-cells (CD3-CD19+CD27+), transitional B-cells (CD3-CD19+CD38brightCD24bright) and plasma cells (CD3-CD38brightCD27brightCD19+) as measured in peripheral blood using high sensitivity flow cytometry.

Figure 2

RTX+BLM specifically reduces autoantibodies in refractory SLE patients. (A) The percentage change in the total IgG in individual patients throughout the 24-week study. (B) The percentage change in anti-tetanus toxoid (TT), (C) anti-rubella, (D) anti-VZV antibodies (n=16), (E) anti-dsDNA antibodies (n=12), (F) anti-Sm antibodies (n=7), (G) anti-RNP70 antibodies (n=6), (H) anti-U1RNP antibodies (n=9) and (I) anti-C1q antibodies in individual patients throughout the 24-week study. (J) The median normalized ratio of the reference condition anti-TT IgG to the total IgG (black bars), anti-rubella and anti-VZV to total IgG, and the normalized ratios of various autoantibodies (anti-dsDNA, anti-RNP70, anti-U1RNP, anti-Sm, anti-C1q) to total IgG. Thick black lines represent the median and thin grey lines represent individual data. *P<0.05, **P<0.01, ***P<0.001.

Figure 3

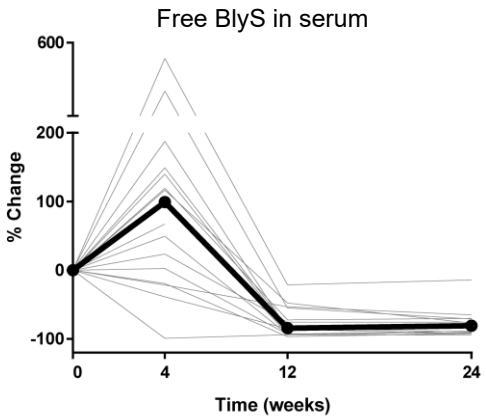
RTX+BLM treatment significantly reduces excessive *ex vivo* NET formation. (A) Representative images of ex vivo NET formation at week 0, week 12 and week 24 in 2 patients. Extracellular DNA is stained with SYTOX (green) and cells are labelled with the membrane stain PKH26 (red). Bars = 100 µm. (B) Ex vivo NET formation in SLE patients during RTX +BLM treatment. The dotted line represents the normal human serum (NHS) control. Data are expressed as fold increase compared to the NHS control. Each point represents one patient, solid lines represent the median fold induction. ***P*<0.01, ****P*<0.001.

Figure 4

Beneficial clinical responses in refractory patients treated with RTX+BLM. (A) SLE Disease Activity Score (SLEDAI) at weeks 0, 4, 12, and 24. (B) Proteinuria (g/day) at weeks 0, 12, and 24 in patients with active lupus nephritis at baseline (n=13). (C) Successful tapering of glucocorticoids in patients on glucocorticoids at baseline (n=14), in mg/day prednisolone. (D) Successful discontinuation of mycophenolate mofetil (MMF) within 24 weeks of treatment in patients treated with MMF at baseline (n=10). Correlation of SLEDAI scores with NET formation (E) and with anti-dsDNA titers (F). Thick black lines represent median values and thin grey lines represent individual data points. *P<0.05, ***P<0.001.

Figure 1

A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600
200
100
0
-100
% Change
0
4
12
24
Time (weeks)

B

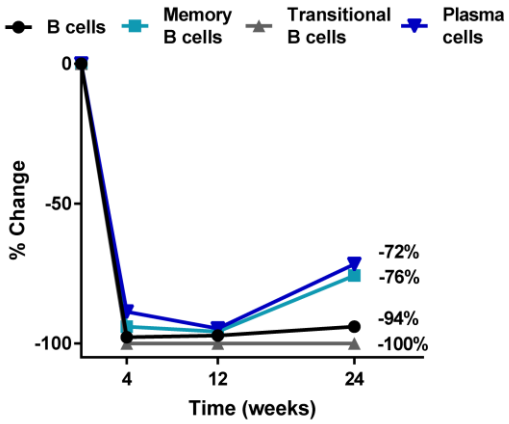

B cells
Memory B cells
Transitional B cells
Plasma cells
0
-50
-100
% Change
-72%
-76%
-94%
-100%
4
12
24
Time (weeks)

Figure 2

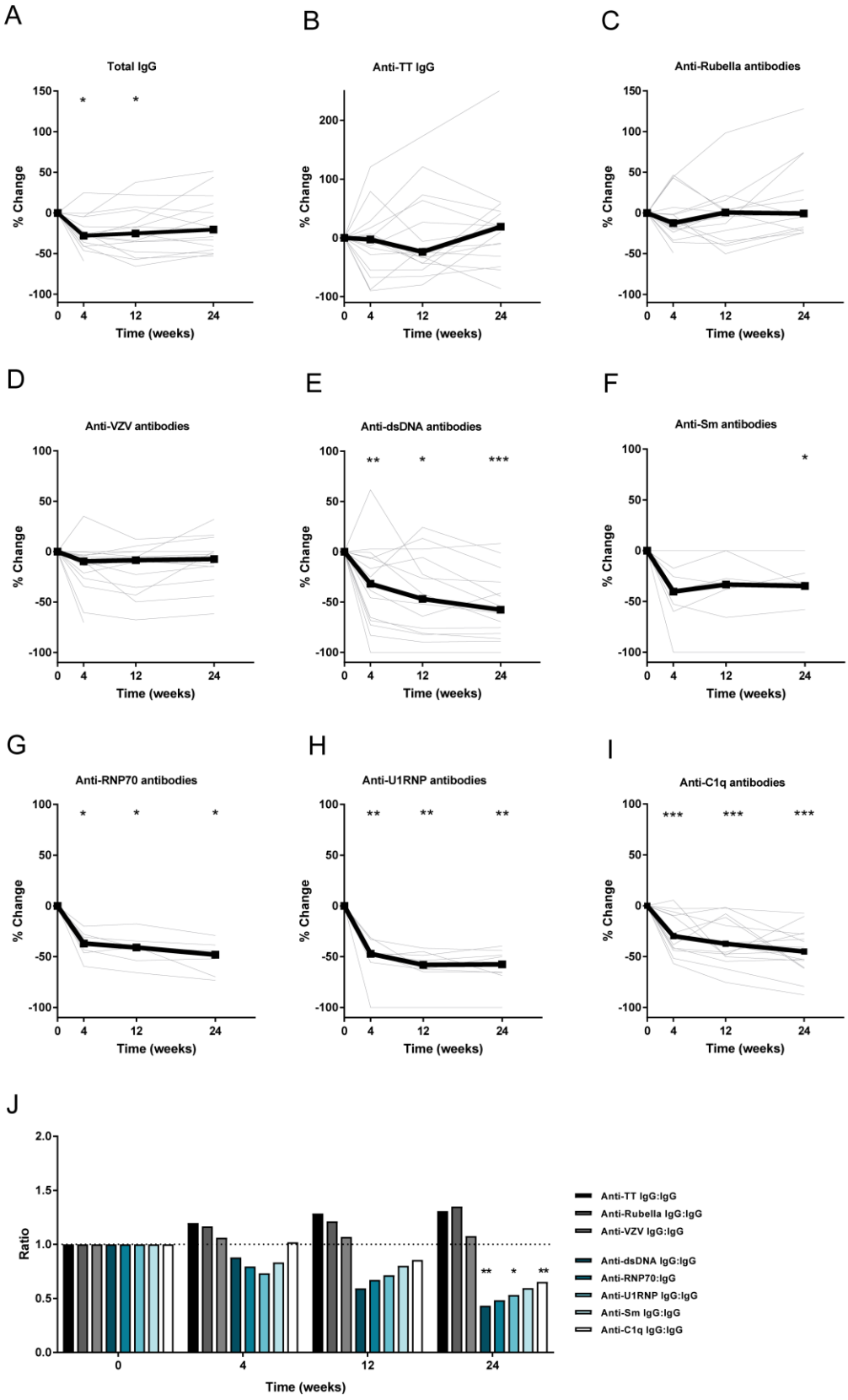

A
Total IgG
B
Anti-TT IgG
C
Anti-Rubella antibodies
D
Anti-VZV antibodies
E
Anti-dsDNA antibodies
F
Anti-Sm antibodies
G
Anti-RNP70 antibodies
H
Anti-U1RNP antibodies
I
Anti-C1q antibodies
% Change
Time (weeks)
J
Ratio
Time (weeks)
Anti-TT IgG:IgG
Anti-Rubella IgG:IgG
Anti-VZV IgG:IgG
Anti-dsDNA IgG:IgG
Anti-RNP70:IgG
Anti-U1RNP IgG:IgG
Anti-Sm IgG:IgG
Anti-C1q IgG:IgG

Figure 3

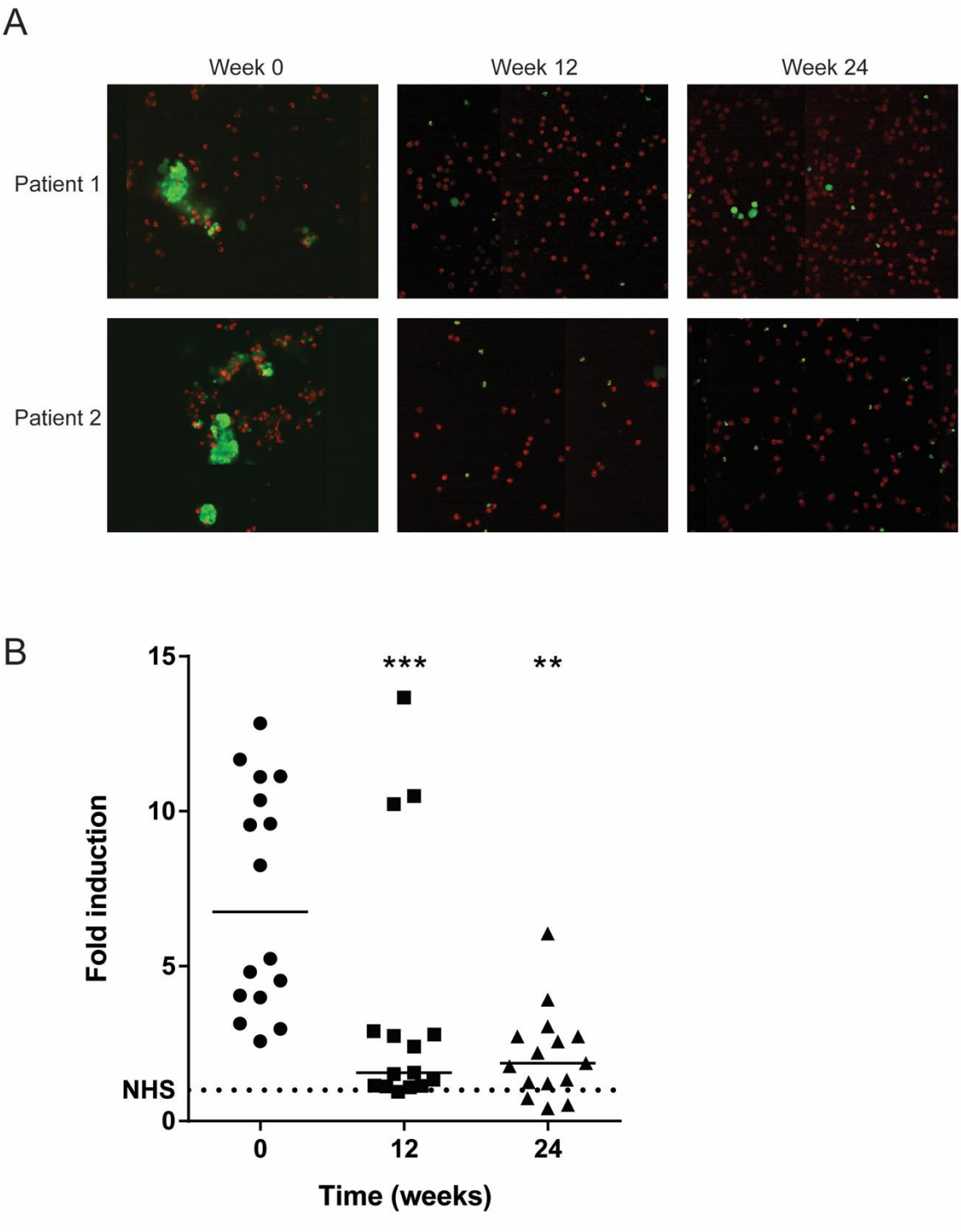
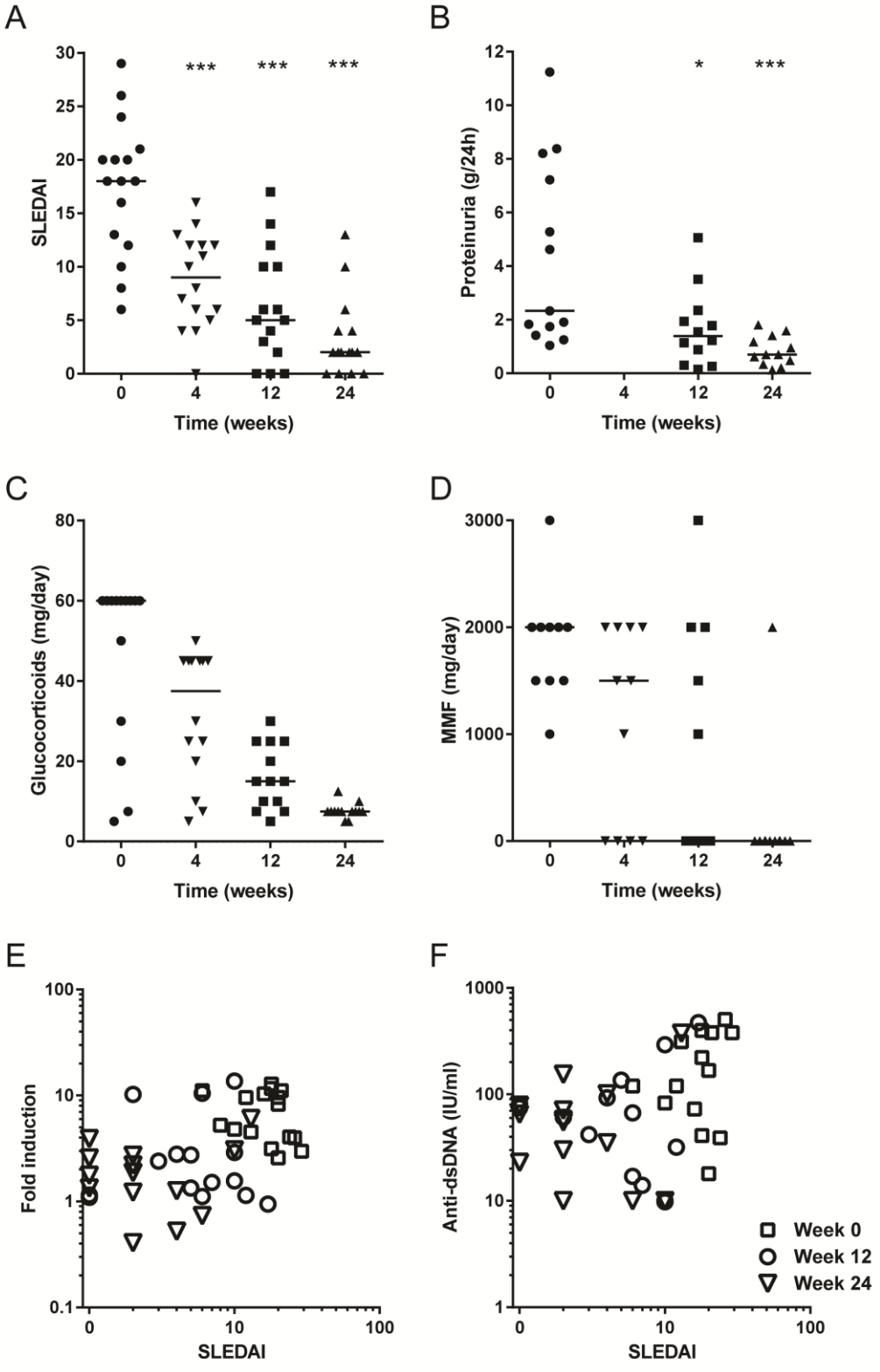

A
Week 0
Week 12
Week 24
Patient 1
Patient 2
B
15
10
5
NHS
0
Fold induction
***
**
0
12
24
Time (weeks)

Figure 4


A
SLEDAI
Time (weeks)
***
B
Proteinuria (g/24h)
Time (weeks)
*
***
C
Glucocorticoids (mg/day)
Time (weeks)
D
MMF (mg/day)
Time (weeks)
E
Fold induction
SLEDAI
F
Anti-dsDNA (IU/ml)
SLEDAI
Week 0
Week 12
Week 24