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This is the overview page

Long-term effects of combined B-cell immunomodulation with Rituximab and Belimumab in severe, refractory SLE: two-year results

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date December 14th, 2019
subject Rebuttal letter

Dear Professor Fouque,

Thank you for providing us the opportunity to submit a revised manuscript entitled "*Long-term effects of combined B-cell immunomodulation with Rituximab and Belimumab in severe, refractory SLE: two year results*" for review.

We would like to thank the reviewers for their valuable comments and suggestions. We highly appreciate it, and it helped to improve our manuscript significantly. We have included a detailed point-by-point response to the reviewers with our replies in bold. Additionally, two versions of our revised manuscript are added, one with the corrections underlined in red and deletions crossed out in blue as requested and a clean version.

We hope that our replies to the reviewers' comments have successfully addressed the issues that the reviewers raised. We are looking forward to your response as we hope you will now find our manuscript suitable for publication in *NDT – Basic and Clinical Science*.

We thank you for your kind consideration of our revised manuscript.

Sincerely, on behalf of all co-authors,

Drs. T. Kraaij, MSc
Drs. E.J. Mlejnek, MSc
Dr. Y.K.O. Teng, MD

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We thank both the reviewers for their valuable comments and suggestions. We highly appreciate your effort and we agree with your comments, they contributed to improve our manuscript markedly. Below you will find our response point-by-point .

Reviewer: 1

Comments to the Author

1. These authors present the long term effects of combining rituximab with belimumab for resistant lupus disease activity. This manuscript follows an earlier paper in which the authors present short-term results. This trial and its accompanying data are important contributions to the field. In general, the paper is well written, however, there are several instances in which it is not clear what the authors are attempting to state (ex: summary of the Gong paper, reference 9).

We agree with the reviewer's point and have simplified the key conclusions of the article that is referred to in the introduction.

2. Additionally, several sentences are either grammatically incorrect (ex: he second sentence on page 11) or are missing words.

For the revised manuscript we have now thoroughly reviewed the entire manuscript for grammatical and linguistic errors and corrected them.

3. Importantly, although the limitation that this is a single arm study is listed, this limitation (ie no comparator) is significantly more than that it "impairs the ability to place the promising observed effects into perspective to standard treatment regimes."

The reviewer's point is well-taken. We have changed several aspects in order to put more emphasis on the immunological findings and avoid over-interpretation on the clinical findings of our study. We therefore shortened the result section with respect to clinical aspects, changed the title of the final paragraph of the results to manage expectations and amended the abstract and discussion to avoid over-interpretation of the clinical results observed in our study. We are convinced that with these changes we have adhered to the reviewer's point.

4. Were there early predictors of non-response (or response) in the B cell subsets? This translational data would be important if it exists, or, if it does not exist as it could contribute to hypotheses on mechanisms of action of response and/or flare.

The reviewer's point is well-taken and is in line with a comment from reviewer 2 on BAFF levels. Both reviewers value the addition of important biomarkers that can translate to clinical relevance. As such, we have added all the biomarkers measured in this study in supplemental table S3. In the main text of the manuscript we have described only the most significant biomarkers that can potentially be of added value in future studies. These included: C3, depth of CD20 depletion, repopulation of DN B-cells and baseline BAFF levels.

For the sake of clarity, we have now added a supplemental table S4 that present biomarker data of non-responders in addition to the already reported supplemental table for responders.

5. Other:

5.1. Which version of SLEDAI was used (SELENA?, 2K?).

Thank you for noting this omission. We have now included that the SLEDAI-SELENA was used in the supplemental file describing the methods.

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5.2. The statistics state that a "non-parametric t-test was used"; by definition, the t-test is used on normal data, there are non-parametric tests comparing central tendencies (medians) such as Mann-Whitney. What test was used?

We thank the reviewer for noting this error in the text. Indeed the Mann-Whitney was used, we have corrected this in the revised manuscript.

5.3. The discussion includes response rates of other lupus nephritis trials. It would be important to note that different trials utilized different definitions of response AND that the Symbiose trial uses the most lenient definition.

The reviewer's point is indeed important for the interpretation of the clinical response data which uses 0.7g as cut-off criterium for proteinuria. In line with the previous comment of the reviewer to avoid over-interpretation of clinical response data in this study, we have removed in this section the comparison with response rates of other large lupus nephritis trials.
Because the reviewer claims that our study used a lenient definition of renal response, we listed below the renal response criteria used in landmark randomized trials for lupus nephritis (LUNAR, ACCESS, ALLURE, ALMS) in the table below. As can be deducted from these data, the reviewer's point is confirmed demonstrating that the difference is solely the proteinuria threshold: All trials report the use of a UPC ratio of 0,5 which would be equal to 0.5g/24h with the side note that a portion produces less reliable results than 24 hour collection. ALMS and ACCESS used 24 hour collection in their definition of renal response but LUNAR and ALLURE did not. Therefore, we have re-analysed our study's results: at the 104 week time-point all patients with a complete response also have proteinuria values below 0.5g/24hours. We have added this notion to the discussion section of the manuscript.

Complete renal response	Proteinuria	Kidney function	Urinary sediment	Albumin	Prednisolone
SYNBIOSE	≤0.7g in 24 hour collection	≤125% compared to baseline creatinine	Normal	Normal	
LUNAR	UPC ratio 0.5	≤115% compared to baseline creatinine	Normal	Normal	
ACCESS	UPC ratio 0.5 in 24 hour collection	≤125% compared to baseline creatinine			Adherence to taper to 10mg/day at 12 weeks
ALURE	UPC ratio ≤ 0.5	≤125% compared	No cellular casts		Dosage of 10mg/day

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		to baseline creatinine			
ALMS *(renal response, no complete defined)*	24 hour collection *UPC ratio ≤0.5 (if baseline is ≤3.0) *UPC ratio <3.0 (if baseline is >3.0)	≤125% compared to baseline creatinine			

5.4. It should be noted that the comment that "We found that less profound depletion of CD20+B-cells and early repopulation of DN B-cell was associated with a poor response." confirms findings in a post-hoc analysis of responders in LUNAR.
We thank the reviewer for this valuable suggestion. This comment has been added to the discussion.

Reviewer: 2.
This is a follow up report of a small study that combined rituximab and belimumab with MMF for the treatment of refractory lupus patients, most of whom also had lupus nephritis. The patients were followed for two years but unfortunately only 8 patients completed the study.

The value in this study is understanding what happens to lymphocyte populations over time in patients treated with rituximab and belimumab. Given the drop out rate and small sample size it is imperative to be clear that no clinical predictors of response can be assessed. Therefore the paper's discussion, which emphasizes possible predictors of clinical response should be reframed to talk about the responses of B cell populations to these two B cell therapies. One could make *the argument that the last part of the results, discussion clinical correlations with response outcomes is so underpowered that it should be removed. I would suggest that section be reframed as differences between responders and non-responders that may be worth exploring in future studies.*
The reviewer's point is well-taken. As mentioned to reviewer 1, we acknowledge that over-interpretation of the clinical outcome in the study should be avoided. Therefore we have, according to the reviewer's suggestion, rephrased the manuscript in order to shift the emphasis from the clinical outcome to immunological differences between responders and non-responders. We further refer to our response to reviewer 1, point 3, who raised identical concerns.

The definition for complete renal response is very weak and not comparable to most studies of lupus nephritis. Response rate should be estimated using a conventional endpoint as in other recent trials of b cell therapies.
Th reviewer raises a similar issue as reviewer 1, point 5.3 and we would like to refer to our answer above. We furthermore acknowledge that the response rate should have been estimated with a conventional endpoint. The endpoint was 104 weeks, therefor the response rates for this timepoint have been added to the results.

Can the authors add a panel C to figure 2 that provides the kinetics of t cells in these patients and if possible T cell subsets

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We agree with the reviewer that it would be of great value to add more immune cell kinetics to this figure and therefore we thank the reviewer for this excellent suggestion. In the paragraph "Long-term immune reconstituting effects" we have added the kinetics of CD4+T-cells, CD8+Tcells and NK cells, which demonstrate the increase of T-cell counts known to be relevant in the context of (opportunistic) infections. We have added this data to a panel C in figure 2.

Given all the studies done, did the authors measure serum BAFF levels? If so please provide the results, especially over time

We thank the reviewer for this excellent suggestion. We have indeed measured serum BAFF levels up until week 48 and found a non-significant difference. We had initially chosen to describe only the significant findings, but we agree that it might be relevant information to add to the manuscript. Therefore, in the final paragraph of the result section describing the associations of immunological effects with clinical response, we have expanded the results section. Importantly it should be noted that our finding are no more than a potential discriminator for response to be further studied in larger clinical trials. We also added this notion to the first part of the discussion.

Although the Calibrate trial is not out in a manuscript yet, the data are out in abstract form, for ACR last year. The findings were not different between patients receiving belimumab or placebo. Please discuss these results in the context of the current investigation."

The reviewers point to discuss the Calibrate trial in more depth than we had already done is well taken and adjusted. Indeed more information is now available since the authors presented additional data at the recent ACR congress 2019.

Long-term effects of combined B-cell immunomodulation with Rituximab and Belimumab in severe, refractory SLE: two--year results

Rituximab and Belimumab combination for severe SLE

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Abstract

Background: Anti-CD20 B-cell depletion has not shown superior efficacy to standard immunosuppression in patients with systemic lupus erythematosus(SLE). Besides trial design, potential explanations are incomplete B-cell depletion in relation to substantial surges in B-cell activating factor(BAFF). To improve B-cell targeting strategies, we conducted the first study in SLE patients aimed at investigating immunological effects and feasibility of combining rituximab(anti-CD20) and belimumab(anti-BAFF).

Methods: Reported is the long-term follow-up of a phase 2 proof-of-concept study in 15 patients with SLE including 12(80%) with lupus nephritis(LN).

Results: In 10/15(67%) patients a clinical response was observed by achievement of lupus low disease activity state(LLDAS) of which 8(53%) continued treatment(belimumab+≤7,5mg prednisolone) during the complete 2 years of follow-up. Five patients(33%) were referred to as 'non-responders' due to persistent LN, major flare or ~~repeat~~repetitive~~ed~~ minor flare~~s~~. Out of 12 LN patients 9(75%) showed a renal response including 8(67%) complete renal responders. All anti-dsDNA^{+} patients converted to negative and both anti-C1q and extractable nuclear antigen autoantibodies(ENAs) showed significant reductions. CD19^{+}B-cells showed a median decrease from baseline of 97% at 24 weeks, with a persistent reduction of 84% up to 104 weeks. When comparing responders to non-responders, CD20^{+}B-cells were depleted significantly less in non-responders and double negative(DN) B-cells repopulated significantly earlier.

Conclusions: Combined B-cell targeted therapy with rituximab(RTX) and belimumab(BLM) prevented full B-cell repopulation including DN B-cells, with concomitant specific reduction of SLE-relevant autoantibodies. The observed ~~clinical and~~ immunological and clinical benefits in a therapy-refractory SLE population prompt further studies on RTX+BLM.

Keywords

Systemic lupus erythematosus, lupus nephritis, immune complex mediated membranoproliferative glomerulonephritis~~, systemic autoimmune disease~~, Rituximab/Belimumab, autoimmune glomerulonephritis, autoantibodies

Introduction

Systemic lupus erythematosus(SLE) is a systemic autoimmune disease in which loss of tolerance to nucleic acids and their binding proteins results in generation of autoantibodies(e.g. anti-DNA, anti-chromatin or anti-histone autoantibodies), leading to inflammation potentially involving almost every organ system, including the kidney[1]. Lupus nephritis(LN) is seen in 29-82% of patients[2] and remains difficult to treat, with short term complete renal response(CRR) rates around 10-40% at 12 months[3] and occurrence of end stage renal disease(ESRD) in 10% of LN patients[4]. Together with the fact that patients with refractory SLE ~~often~~ receive high cumulative dosage of toxic immunosuppressive medication, exploration of new therapeutic options is important.

Since autoantibodies contribute to renal pathology in SLE, targeting autoreactive B-cells has continued interest as a possible strategy for treating SLE patients. Targeting B-cells with anti-CD20 monoclonal antibody(mAb) rituximab(RTX) has been unsuccessful in randomized trials in both patients with extra-renal[5] and renal SLE[6]. Belimumab(BLM), an anti-BAFF(B-cell-activating factor) ~~monoclonal antibody~~mAb, was approved for the treatment of active SLE. Approval of BLM included a special warning on its use with concomitant B-cell targeted therapy, however RTX+BLM provides an opportunity to target the surge in circulating BAFF levels after B-cell depletion and thereby minimizing the survival of autoreactive B-cells[7,8].

The concept of combining anti-CD20 B-cell depletion with anti-BAFF cytokine inhibition is supported by mice studies showing the importance of the microenvironment and cellular competition in anti-CD20 mAb mediated killing of B-cells where ~~has previously been studied in animals. In chimeric mice expressing humanCD20 on 50% of B-cells, anti-humanCD20 therapy more effectively depleted CD20$^+$B-cells than in mice expressing humanCD20 on 100% of B-cells[9], indicating that less~~ cellular competition for survival factors(e.g. availability of ~~higher~~ BAFF) ~~levels available)~~ can underpin resistance to anti-CD20 therapy[9]. The importance of BAFF levels in anti-CD20 therapy is further illustrated in a study using an in vitro model of mature B-cells, where BAFF was able to inhibit CD20-mediated apoptosis[10]. Additionally, in

different lupus mouse models a combination of anti-CD20 and anti-BAFF therapy led to improved disease control compared to each treatment separately or cyclophosphamide[11]. We have previously reported on the effects of combination treatment with CD20 and BAFF targeting in SLE patients[12], however the long-term effects on B-cell repopulation and B-cell composition has not been reported yet.

'Synergetic B-cell immunomodulation in SLE'(Synbiose) was designed as the first translational, single-arm, proof-of-concept study in SLE patients aimed at investigating the underpinning, immunological hypothesis of combining RTX+BLM in severe, refractory SLE patients. We previously reported the early effects of RTX+BLM demonstrating a reduction in anti-nuclear antibodies(ANAs) and regression of excessive neutrophil extracellular trap(NET) formation[12]. We now report long-term effects of RTX+BLM on depletion of ANAs, B-cell repopulation and clinical response during 2 years of follow-up.

Materials and Methods

Study design

The Synbiose study is a phase 2, single-arm, open-label proof-of-concept study in which 'severe SLE' patients were included defined as a SLE disease activity index(SLEDAI-SELENA) score of ≥12 points or new, worse or persistent SLE-related activity in major organs. Patients were treated with intravenous methylprednisolone pulse therapy at baseline, 1000mg intravenous RTX at weeks 0+2 and with intravenous 10mg/kg BLM at weeks 4+6+8 and then every 4 weeks until 104 weeks. Mycophenolate mofetil was started but quickly tapered to avoid cumulative over-immunosuppression. Oral prednisolone was started at 1mg/kg/day(maximum 60mg/day) and tapered towards maintenance dose of ≤7.5mg/day. The study was approved by the Dutch LUMC medical ethics committee and all patients provided written informed consent. The study was registered at ClinicalTrials.gov(NCT02284984).

A fully detailed methods section with description of the clinical parameters, methods and materials used for experiments and statistical analysis is available as online supplemental file S1.

Results

Summarized patient characteristics

Baseline characteristics from all included patients have been reported previously[12]. Briefly, sixteen patients(88% female) were included, with median age of 31 years[19;51]. All patients had refractory disease, of which 12(80%) had active LN at baseline. One patient experienced severe hypogammaglobulinemia at week 8 after completion of ~~intravenous pulse~~ methylprednisolone and RTX, therefore BLM treatment was not initiated. This patient was excluded from the long-term follow-up study. Fifteen patients reached the primary endpoint at week 24. ~~Two patients with clinical response at week 24 withdrew from the study because of a pregnancy wish necessitating the termination of BLM. Five patients dropped out before 104 weeks due to clinical relapse or non-response necessitating alternative induction treatment, while eight patients(53%) finished the complete follow-up of 104 weeks.~~

Clinical response

During the study period, 10 out of 15(67%) patients had a clinical response. At week 104 this response is 8 out of 13(62%). Eight patients(53%) finished the complete follow-up of 104 weeks. Two patients with a clinical response stopped BLM treatment at week 24, based on a pregnancy wish(patient#14 and #15 in Figure 1). Clinical response is illustrated in Figure 1A defined by the time for patients to achieve and remain in lupus low disease activity state(LLDAS) and by attaining a renal response in patients with active LN at baseline(Figure 1B). ~~The ten patients with a clinical response at end of follow-up were classified as responders of which two stopped BLM treatment at week 24, as prespecified in the study's protocol, due to a pregnancy wish(patient#14 and #15 in Figure 1). As such, eight responders were available for analysis over the complete 2 years of follow-up.~~ In the eight responders available for analysis over the two-year follow-up~~se patients~~, the median time to the first achievement of LLDAS was 24 weeks[12;36] and the median time on LLDAS was 76 weeks[56;92]. One patient had a minor flare with pericarditis ~~at~~

week 36 and received 0.5mg/kg prednisone and colchicine(patient#3), followed by which led to quick resolution of disease activity. At week 104, 7 out of 8 patients received maintenance therapy with glucocorticoids with median dose of 7.5mg/day[2.5;7.5], and all patients continuously used hydroxychloroquine and BLM(Figure 1C).

In patients with active LN at baseline, 9 out of 12(75%) had a renal response during the trial period with CRR at week 104 in 6 out of 10(60%), all hadin which 8(67%) had a complete renal response at the proteinuria below 0.5grams/dayend of follow up. In renal responders that finished the complete study period(n=7) proteinuria decreased from a median of 4.6 gram/day[1.3;11.2] to 0.3[0.1;1],(p=0.02 at week 104), representing a median decrease of 96%.

Despite rapid decline upon treatment, one patient#4 did not reach complete renal responseCRR due to persistent proteinuria above 0.7grams/day, which clinically correlatedconsistent with histologically proven chronic renal damage warranting the continuation in the study. In renal responders that finished the complete study period(n=7) proteinuria decreased from a median of 4.6 gram/day[1.3;11.2] to 0.3[0.1;1], p=0.02 at week 104, representing a median decrease of 96%.

Five patients were classified as 'non-responders' and dropped out due to clinical relapse or non-response necessitating alternative induction treatmentof the study: Two patients had a major flare, including patient#10 experienceding a renal flare at week 46 requiring cyclophosphamide treatment and patient#11 experienceding a recurrence of transverse myelitis at week 44 upon which induction treatment with RTX+steroids was given. Two pPatients(#12 and #13) had persistent features of active LN and were excluded at week 24, as described in more detail previously[12], one was treated with cyclophosphamide, the other was given an experimental induction treatment within another study. One pPatient(#9) was excluded at week 74 due to a recurrent minor flare(complement consumption, anti-

dsDNA positivity and arthritis) and was switched to leflunomide with high dose steroids(0.5mg/kg/day). Baseline characteristics of the responders and non-responders are depicted in Table 1.

Long-term safety

Treatment-emergent adverse events(TEAE) during the complete study period are summarized in Table 2. In all patients adverse events(AE) were reported with 5 serious adverse events(SAE) in 4 patients(27%) due to hospitalization for the suspicion of infection(n=3) and for aor laparoscopic cholecystectomy(n=1) because of cholelithiasis. In all casesAll suspected infections were gastro-intestinal infections without detectable pathogen, requiring a one-night hospital admission without the need for antibiotic treatment in all cases. In 9 patients(60%) a minor infection was observed, of which upper respiratory tract infections were most prevalent. A detailed description of all infectious AEs is provided in supplemental file S2. Two patients suffered from mood disorders;: 1 patient had glucocorticoid-induced mood disorder and psychosis after methylprednisolone infusions followed by high dose oral prednisolone and another patient experienced depressive symptoms started at week 95, leading to study treatment interruption in order to exclude progressive multifocal leukoencephalopathy(PML). Once PML and neuropsychiatric SLE were ruled out, a mild depressive disorder was diagnosed and BLM treatment reinstituted.

Long-term effects of RTX+BLM on B-cell immunology

By employing high sensitivity flow cytometry, we observed prolonged inhibition of B-cell repopulation: $CD19^{+}$B-cells declined from a median of $100*10^{6}$cells/L[20.5;$248*10^{6}$] at baseline to $3.75*10^{6}$ cells/L[0.53;$64.7*10^{6}$], (p=0.005) at week 24, representing a median decrease of 97% from baseline. At week 104, the median number of $CD19^{+}$B-cells was $13.6*10^{6}$ cells/L[10.7;$47.3*10^{6}$], representing a median decrease of 84%[-92;+22] from baseline(Figure 2A) illustrating that B-cells did not repopulate to baseline values during continued BLM treatment. The low-level repopulation of B-cells was dominated by an early recurrence of plasmablasts at week 24 up to a median decrease of 17% decrease compared

to baseline(Figure 2B) and in lesser extent repopulation of switched memory B-cells up to a median decrease of 71% decrease compared to baseline values. Only from 48 weeks onwards, the resurge of immature B-cells occurred with return of transitional B-cells(+52% compared to baseline) and non-switched memory B-cells(-19% compared to baseline) at week 104. Interestingly, continuous BLM treatment prevented repopulation of naive B-cells(median decrease of 81%) as well as double negative(DN) B-cells(median decrease of 82%) at 104 weeks.

Long-term immune reconstituting effects

In the RTX+BLM treatment strategy, patients were able to taper steroids and stop MMF treatment before or at 24 weeks(Figure 1C). As a consequence, we observed significant reconstitution of circulating $CD4^+$T-cells,(from 234*10^6 cells/L[116;530*10^6] at baseline to 658*10^6 cells/L[285;1270*10^6], (p=0.02 at week 104)), $CD8^+$T-cells, (from 276*10^6 cells/L[121;418*10^6] at baseline to 493*10^6 cells/L[237;1700*10^6],(p=0.04)) and in NK-cells (from 18*10^6 cells/L[0.4;133*10^6] to 97*10^6[38;221*10^6], (p=0.08)) (Figure 2C).

Long-term effects of RTX+BLM on humoral auto-immunity

With respect to the effects of RTX+BLM on immunoglobulin levels, total IgG levels in comparison to baseline levels(median 11.3g/L[5;23.6]) initially decreased at 12 weeks(7.8g/L[2.6;14.4], p=0.05) and stabilized from 24 weeks onwards(9.7g/L[3.4;16.4], supplemental file S3). At week 104, IgG levels increased with 6.4%[-44;+30] compared to baseline levels(Figure 3A). IgA levels remained stable over the complete follow-up period while IgM levels gradually declined from 0.72g/L[0.26;1.06] at baseline to 0.27g/L[0.2;0.63], p=0.008 at week 72 and increased to 0.37g/L[0.2;0.73], p=0.02 at week 104(supplemental file S3). With regard to (auto)antigen specific IgG, anti-tetanus and anti-rubella IgG remained stable during complete follow-up(Figure 3B+C) while anti-varicella zoster virus IgG(anti-VZV IgG) showed a significant decrease(Figure 3D) from 3435mIU/mL[442;4000] at baseline to

2436[404;3625], p=0.02 at week 104. Of note, all measured anti-VZV IgG levels were within protective ranges.

Anti-dsDNA levels of 268 AU/mL[50;827] at baseline decreased at week 24 to 29.6[0;104.5], (p=0.02) equal to a median decrease of 87%[-100;+3](Figure 3E). By week 48 up to 104, all anti-dsDNA positive patients converted to negative on immunofluorescence(CLIFT) with a median titer of 52[23;132], (p=0.04) at week 104 equal to a median decrease of 81%[-91;+95] from baseline.

Similar reductions in anti-RNP70, anti-U1RNP, anti-Sm and anti-C1q autoantibodies levels were observed as illustrated in Figure 3F-I. Briefly, at 104 weeks, anti-RNP70 antibody levels were reduced with a median of 88%[-94;-48], (p=0.25), anti-U1RNP with 41%[-79;-31], (p=0.13), anti-Sm with 30%[-97;-13] and anti-C1q antibodies with 60%[-86;+2], (p=0.03). The relative reductions of *auto*-antibody compared to *allo*-antibody levels over total IgG is illustrated in Figure 3J demonstrating that RTX+BLM preferentially targeted humoral autoimmunity.

With respect to complement levels, normalization of C3 levels was seen at 104 weeks in 7 out of 8 patients with median C3 levels of 1.0g/L[0.8;1.3] compared to baseline C3 levels of 0.6g/L[0.3;0.8](, p=0.008). Also, C4 levels increased from 54[35;80] to 147mg/L[74;279](, p=0.25)((supplemental file S3).

Associations of ~~clinical and~~ immunological effects with clinical ~~in~~ response to RTX+BLM

We investigated ~~clinical and~~ immunological parameters that could potentially discriminate long-term responders(n=8) from non-responders(n=5) depicted in supplemental file S3 and S4. We observed that, not unexpectedly, after 4, 12 and 24 weeks, a significantly larger increase in C3 levels was seen in responders versus non-responders(respectively 27% versus 0%, p=0.03, 42% versus 8% , p=0.01 and 79% versus 8%, p=0.008). With high sensitivity flowcytometry, we observed two noteworthy findings: first, the total number of $CD20^{+}$B-cells at week 24 was significantly lower in

responders(1.83*10^6[0.10;17.2*10^6]) compared to the non-responders(15.8*10^6[3.01;22.1*10^6], p=0.045). Second, repopulation of DN B-cells occurred earlier in the non-responder group, at week 24[12;24](nadir levels of 0.48*10^6 cells/L[0.17;1.02*10^6]), while in responders repopulation of DN B-cells occurred at week 72[48;104], p=0.0008(nadir levels of 0.32*10^6[0.11;2.34*10^6]). Finally a trend for higher baseline BAFF levels was found in non-responders vs responders(respectively 0.97ng/ml[0.48-1.4] vs 0.44ng/ml[0.26;0.91]p=0.06) while the decrease at 24 weeks was similar between responders(0.11ng/ml[0.09-0.19]) and non-responders(0.15ng/ml[0.08-0.35]p=0.12).

Discussion

In this proof-of-concept study long-term ~~clinical and~~ immunological and clinical effects of RTX+BLM in patients with severe, refractory SLE(including LN) are described. Long-lasting, specific reduction of anti-dsDNA, anti-C1q and even ENAs were observed and full B-cell repopulation was prevented throughout two-~~-~~years ~~of~~ follow-up. ~~Importantly, response to treatment was associated with more profound depletion of CD20$^+$B-cells and prolonged suppression of DN B-cells.~~ Clinical response persisted ~~upon RTX+BLM was observed~~ in two-thirds of the patients during follow-up with maintenance treatment consisting of BLM and low dose prednisolone and allowed discontinuation of MMF associated with significant immune reconstitution. Profound depletion of CD20$^+$B-cells, prolonged suppression of DN B-cells and higher serum BAFF levels potentially discriminate~~s~~d responders from non-responders and should be validated in larger clinical trials.

~~Throughout the two year~~~~two-year~~ ~~follow-up no major safety issues were raised. The frequency of TEAEs was registered in 100% of patients containing 27% SAEs and 60% infections and was comparable to the LUNAR(99%, 27% and 85% respectively) and BLISS studies(93%, 42% and 75% respectively) even though the latter had shorter follow-up periods. Also, preliminary results of the CALIBRATE study(NCT02260934), in which 43 LN patients were randomized to receive RTX, cyclophosphamide and prednisone with or~~

~~without additional BLM treatment, showed a non-significant difference on grade 3 or higher infectious adverse events(9% vs 23% respectively, p=0.25) confirming that RTX+BLM is generally well-tolerated. Additionally, the study reported 52% renal responders in the BLM group versus 41% in the placebo group(p=ns). Our study has observed a renal response of 75%, as a reference, renal response rates in most recent RCTs for LN were 57% in LUNAR(RTX+/-MMF)[6], 59% in ACCESS(Euro-Lupus cyclophosphamide followed by azathioprine+/-abatacept)[13], 48% in ALLURE(MMF+/-abatacept)[14] and 56% in ALMS(MMF-arm)[15]. Based on LLDAS, clinical response to RTX+BLM was observed in 67% of patients in the present study. We demonstrated that responders to RTX+BLM had lasting LLDAS which is associated with reduced damage accrual[16], better quality of life[17] and can be used as an endpoint for clinical trials[18]. With RTX+BLM, clinical benefit was achieved and persisted despite tapering of steroids and discontinuation of MMF. Although unconventional, tapering of MMF was added in the study design because at that time combined B-cell targeting with RTX+BLM had not been given to patients structurally(besides case reports[19-23]) and intended to avoid over-immunosuppression which was the fundament of the previously mentioned label warning of BLM. Indeed, MMF tapering allowed for significant reconstitution of circulating CD4+T-cells and is a unique achievement for LN patients. Altogether, these data are reassuring for further studies to study clinical efficacy of RTX+BLM for active SLE including LN in a randomized setting.~~

The study encompassed refractory SLE patients ~~due to~~in which we were unable to continue immunomonitoring in non-responders who required ~~repeating~~ different conventional induction therapies nor in responders with a pregnancy wish~~y~~. Within this limitation, we investigated potential predicters of ~~long term~~ non-response to RTX+BLM predominantly in the first 6 months. ~~were all patients were still part of the study. We found that less profound depletion of CD20+B-cells and early repopulation of DN B-cell was associated with a poor response~~ It is known that the B-cell depleting potential of RTX has an inter-person variation and that the association of clinical outcome with the depth

of B-cell depletion has been made[24,25]. We found that less profound depletion of CD20$^+$B-cells was associated with a poor response, in line with findings of a post-hoc analysis of the LUNAR trial[26] where rapidness and duration of complete peripheral B-cell depletion were associated with complete response. Our observations in B-cell subsets are also in line with a recent study investigating B-cell subsets with Cytof in SLE patients during BLM therapy[27], where long-term depletion of CD20$^+$B-cells and naive B-cells was seen. ~~In vivo it is known that BAFF inhibition decreases naive B-cells in mice[28] and is important for survival of naïve B-cells in humans[29].~~ The loss of naive B-cells during BLM therapy has ~~also~~ been shown before[27,30,31]. Besides the decrease in naïve B-cells we also observed that early repopulation of DN B-cell associated with poor response. ~~Our observations regarding the DN B-cell population are relevant because~~ DN~~, activated~~ B-cells in SLE are shown to be a major source of auto-antibody secreting cells(ASCs)[32] and the number DN B-cells are associated with disease activity and the presence of ~~increased in~~ LN ~~patients~~[33]. Moreover, further characterization of the DN B-cell population elucidated that these cells were hardly found in healthy or disease controls and ~~that these cells~~ were highly responsive to TLR7 stimulation inducing their differentiation to ASCs[33]. Unfortunately, we were limited in the depth of phenotyping DN B-cells in this study partly because this subpopulation had not been described at the time of study design and initiation. Notwithstanding, taken together with our observation that memory B-cells and plasmablasts fully repopulated after RTX+BLM while long-lasting reductions of autoantibodies persisted, suggested that a prolonged suppression of autoreactive DN B-cells can be beneficial to SLE patients. Therefore, DN B-cells are highly interesting biomarker to further study in the context of RTX+BLM treatment for SLE and LN patients.

Throughout the two--year follow-up no major safety issues were raised. The frequency of TEAEs was registered in 100% of patients containing 27% SAEs and 60% infections and was comparable to the LUNAR(99%, 27% and 85% respectively) and BLISS studies(93%, 42% and 75% respectively) ~~even though the latter had shorter follow-up periods~~. Also, preliminary results of the CALIBRATE study(NCT02260934),

in which 43 LN patients were randomized to receive RTX, cyclophosphamide and prednisone with or without additional BLM treatment, showed a non-significant difference on grade 3 or higher infectious adverse events(9% with BLM vs 23% without BLM~~respectively~~, p=0.25) confirming that RTX+BLM is ~~generally~~ well-tolerated. In a~~A~~dditional~~ally~~, the CALIBRATE ~~study~~ reported 52% renal responders in the BLM group versus 41% in the placebo group~~(p=ns)~~. This non-significant difference could possibly be explained by the use of cyclophosphamide for induction treatment, in contrast to mycophenolate in the present study and the relative high dose of prednisolone maintenance(10mg/day) continued throughout two years. It is of interest that preliminary reports from the CALIBRATE study showed impaired B-cell repopulation during BLM treatment as well as specific decrease in the naïve B-cell compartment upon BLM.

Our study observed 60% CRR-rate at 104 weeks using the pre-defined CRR-criteria containing proteinuria levels of ≤0.7g/24hours, this in comparison to ≤0.5g/24hours used by landmark LN trials (LUNAR[6], ACCESS[13], ALLURE[14], ALMS[15]). Re-analyzation of the results showed that patients with a CRR at 104 weeks all have proteinuria levels below 0.5g/24hours. ~~Our study has observed a renal response of 75%, as a reference, renal response rates in most recent RCTs for LN were 57% in LUNAR(RTX+/-MMF)[6], 59% in ACCESS(Euro-Lupus cyclophosphamide followed by azathioprine+/-abatacept)[13], 48% in ALLURE(MMF+/-abatacept)[14] and 56% in ALMS(MMF-arm)[15].~~ Based on LLDAS, clinical response to RTX+BLM was observed in 62~~67~~% of patients in the present study. We demonstrated that responders to RTX+BLM had lasting LLDAS which is associated with reduced damage accrual[16], better quality of life[17] and can be used as an endpoint for clinical trials[18]. ~~With RTX+BLM~~ In this small trial clinical benefit was achieved with RTX+BLM and persisted despite tapering of steroids to a dosage ≤7,5mg and discontinuation of MMF. Although unconventional, tapering of MMF was added in the study design because at that time combined B-cell targeting with RTX+BLM had not been given to patients structurally(besides case-reports[19-23]) and intended to avoid over-immunosuppression which was the fundament of the previously-mentioned label warning of BLM. Indeed, MMF tapering allowed for significant reconstitution of circulating $CD4^+$T-cells

and is a unique achievement for LN patients. Altogether, these data are reassuring for further studies to study clinical efficacy of RTX+BLM for active SLE including LN in a randomized setting.

It is noteworthy to establish that the primary null-hypothesis to study the combination of RTX+BLM, i.e. to induce long-term B-cell depletion and indirectly(autoreactive) plasmablast depletion, was wrong. The null-hypothesis was based on dual B-cell therapy in murine studies[11] but the contrary was observed: plasmablasts repopulated fastest among the studied B-cell subsets. Importantly, this was not associated with(recurrence of) disease activity nor with autoantibodies. ~~Also, I~~it was remarkable that RTX+BLM preferentially targeted humoral autoimmunity without affecting protective ranges of anti-viral antibody levels. It can be speculated that autoreactive B-cells have an increased BAFF-dependence due to the continuous presence of antigens compared to *allo*-reactive B-cells. This might also explain the significant drop, although not below protective levels, of antibodies against the varicella-zoster virus that remains inactive in the body for many years.

The most ~~wo~~ important limitations of this study are the small size of treated patients ~~that limited us to firmly establish predictors of long-term response~~ and the single arm design. The latter ~~that~~ impairs the ability to place the ~~promising~~ observed effects into perspective to standard treatment ~~regimes~~regimens and i~~.~~ ~~I~~t could be argued that the observed effects are solely due to RTX treatment combined with concomitant immunosuppressants. However profound B-cell depletion by RTX has shown to be highly variable in SLE patients with a median time to repopulation around 32 weeks[34] and only 0-11% of patients with sustained low B-cell counts for 1-2 years without re-treatment[24 34-36]. In a comparable cohort of 7 severe, refractory SLE patients re-treated with RTX a median duration of clinical response of 13 months and B-cell depletion of 6 months were reported[37]. Together suggesting ~~an~~a synergistic role of BLM in RTX-treated SLE patients. ~~Also, these limitations have to be brought into the perspective that this~~

~~study was the first to pioneer the combination of RTX+BLM in patients with severe, refractory SLE aiming to establish its feasibility and better understand its immunological effects.~~

In conclusion, this study was the first to pioneer the combination of RTX+BLM in patients with severe, refractory SLE aiming to establish its feasibility and better understand its immunological effects.

~~we described long-term feasibility of RTX+BLM in severe SLE and LN patients together with its clinical and immunological effects.~~ RTX+BLM treatment appears to be a promising strategy to target pathological autoimmunity mechanisms in SLE with ~~strong~~ suggestions towards beneficial clinical effects. We are, therefore, reassured that RTX+BLM can be safely studied in further clinical trials to assess its added value in the treatment of SLE patients with and without renal involvement.

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Conflict of Interest Statement

YKOT received consultancy fees from GSK and Aurinia Pharmaceuticals.

Authors' Contributions

TK en EJA contributed equally as first authors. TK and YKOT contributed to the design of the study, acquisition of data, analysis and interpretation of data and manuscript preparation. EJA contributed to the acquisition of data, analysis and interpretation of data and manuscript preparation. LSvD contributed to the acquisition of data, interpretation of data and manuscript preparation. PLAvD, OWB and AR contributed significantly to the recruitment and follow-up of patients and acquisition of data. SWAK, JAB, HUS contributed to the acquisition of data. TJWH, TJR and CvK contributed to the analysis and interpretation of data and manuscript preparation. All authors discussed and agreed on the content of the manuscript before submission.

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Tables

Table 1. Baseline and historic disease characteristics of responders (n=8) and non-responders (n=5)

	Responders (n=8)	Non-responders (n=5)
Demographics		
Age, median (range)	31 (21-47)	30 (19-51)
Female sex, n (%)	6 (75)	5 (100)
Race, n (%)		
White/Caucasian	2 (25)	2 (40)
Black/African American	6 (75)	2 (40)
Asian/Oriental	0 (0)	1 (20)
Smoker (%)	2 (25)	0 (0)
Baseline disease characteristics		
SLEDAI, median (range)	19 (12-26)	18 (6-29)
Disease flare characteristics, n (%)		
Renal flare	9 (90)	3 (60)
Transverse myelitis	0 (0)	1 (20)
Persistent disease activity despite treatment	1 (10)	1 (20)
LN disease characteristics		
Histopathology, n (%)		
Class II (±V)	1 (14)	0 (0)
Class III (±V)	1 (14)	2 (67)
Class IV (±V)	4 (57)	1 (33)
Class V	1 (14)	0 (0)
Proteinuria (g/24h), median (range)	4.6 (1.3-11.2)	1.9 (1.0-8.4)
Treatment at disease flare		
Glucocorticoids[a], n (%)	8 (100)	4 (80)
Dose mg/day, median (range)	15 (5-60)	15 (5-60)
Mycophenolate mofetil, n (%)	5 (63)	3 (60)
Dose mg/day, median (range)	2000 (1500-4000)	1500 (1000-3000)
Azathioprine, n (%)	1 (13)	1 (20)
Dose mg/day, median (range)	200	100
Hydroxychloroquinine, n (%)	8 (100)	1 (20)
Biomarkers		
ANA positivity	8 (100)	5 (100)
Anti-dsDNA titer[b] (AU/ml), median (range)	268 (50-827)	479 (33-1123)
Complement consumption[c] (%)	100	100
C3[d] (g/l), median (range)	0.6 (0.3-0.8)	0.6 (0.5-1.3)
C4[e] (mg/l), median (range)	96 (35-236)	68 (21-260)
IgG (g/l), median (range)	11.5 (5-23.6)	12.9 (4.9-16.6)
IgA (g/l), median (range)	3.0 (1.2-4.5)	2.9 (1.6-6.3)
IgM (g/l), median (range)	0.7 (0.3-1.1)	0.8 (0.4-1.1)

CD19⁺B-cells (*10^6 cells/l), median (range)	90	(21-279)	65	(37-300)
Historic disease characteristics				
Disease duration in years, median (range)	7	(3-18)	10	(2-24)
No. of previous relapses, median (range)	3	(2-6)	5	(1-5)
No. of renal relapses, median (range)	2	(1-5)	1	(0-3)
SLICC damage index, median (range)	1	(0-3)	1	(0-4)
Organ involvement, n (%)				
Constitutional	8	(100)	5	(100)
Mucocutaneous	7	(88)	3	(60)
Neuropsychiatric	1	(13)	2	(40)
Musculoskeletal	5	(63)	4	(80)
Cardiorespiratory	7	(88)	4	(80)
Gastrointestinal	0	(0)	0	(0)
Ophtalmic	0	(0)	2	(40)
Renal	8	(100)	4	(80)
Hematology	4	(50)	4	(80)
Treatment history				
Steroids, n (%)	8	(100)	5	(100)
Mycophenolate mofetil, n (%)	8	(100)	5	(100)
Cyclophosphamide, n (%)	3	(38)	3	(60)
Azathioprine, n (%)	4	(50)	3	(60)
Tacrolimus, n (%)	1	(13)	0	(0)
Rituximab, n (%)	2	(25)	1	(20)
Hydroxychloroquinine, n (%)	8	(100)	5	(100)

[a]Patients were treated with the glucocorticoid equivalent prednisolone. [b]Normal anti-dsDNA IgG <10 IU/ml. [c]Complement consumption is defined as decreased CP (classical pathway) activation, decreased C3 or decreased C4. [d]Normal C3: 0.9-2 g/l. [e]Normal C4: 95-415 mg/l.

Table 2 Adverse events during 104 weeks of study

Treatment-emergent adverse events*	n=15	
All adverse events	15	(100)
Severe adverse events (hospitalization)	4	(26.7)
Major infection	3	(20.0)
Cholelithiasis	1	(6.7)
Minor infection	8	(53.3)
Upper respiratory tract	9	(60.0)
Lower respiratory tract	3	(20.0)
Urinary tract	4	(26.7)
Urogenital infection	2	(13.3)
Sinusitis	1	(6.7)
Influenza	1	(6.7)
Herpes simplex	1	(6.7)
Skin	1	(6.7)
HACA formation	4	(26.7)
Symptomatic	1	(6.7)

Asymptomatic	3 (20.0)
Hypogammaglobulinemia (<4.0 g/l)[a]	2 (13.3)
Infusion-related reaction	1 (6.7)
Myalgia	7 (46.7)
Diarrhoea	4 (26.7)
Headache	2 (13.5)
Pyrexia	2 (13.5)
Nausea	2 (13.3)
Mood disorder[b]	2 (13.3)
Fatigue	2 (13.3)
Other	10 (66.7)

*Depicted values are number of patients with percentage of patients that experienced ≥1 TEAE over 104 weeks of study. [a]Study treatment was interrupted in 1 patient. [b]Study treatment was interrupted in 1 patient, in the other patient symptoms were related to high dose steroids.

Legends to figures

Figure 1

Overview of the clinical responses, renal responses and concomitant immunosuppression upon RTX+BLM treatment. (A) Achievement of lupus low disease activity state(LLDAS) over time.(B) Achievement of a renal response in patients included with active lupus nephritis(n=12). Complete renal response was achieved when proteinuria ≤0.7 grams/day, normal serum albumin, stable kidney function, normal urinary sediment; partial response: >0.7–2.9 g/24 h with a decrease in proteinuria of ≥50% from baseline, serum albumin >30 g/L and stable kidney function. When patients did not meet any of these criteria they were considered to have persistent active lupus nephritis.(C) Overview of concomitant treatment with belimumab, mycophenolate mofetil and prednisolone throughout the study's follow-up. Patient numbers mentioned on the y-axis correspond between the 3 figures.

LLDAS, low disease activity state; SLEDAI, SLE disease activity index; SACQ, serologically active(positive antibody and or low complement) clinically quiescent; BLM, belimumab.

Figure 2

Longitudinal kinetics of circulating immune~~B~~ cells over 2 years of follow-up after RTX+BLM treatment(n=8 responders).

(A) RTX+BLM prevents the complete repopulation of circulating B-cells. Depicted is the median change from baseline in the number of $CD19^+$B-cells. (B) Repopulation of B-cell subsets upon RTX+BLM. Depicted are the median change from baseline of the following B-cell subsets: plasmablasts(CD3-CD38brightCD27brightCD19+), non-switched memory B-cells($CD3^-CD19^+CD27^+IgD^+$), switched memory B-cells($CD3^-CD19^+CD27^+IgD^-$), naive B-cells($CD3^-CD19^+CD27^-IgD^+$), double negative B-cells($CD3^-CD19^+CD27^-IgD^-$) and transitional B-cells($CD3^-CD19^+$ $CD38^{bright}CD24^{bright}$). (C) Significant reconstitution of circulating $CD4^+$ T-cells (CD3+CD4+), $CD8^+$ T-cells (CD3+CD8+) and NK-cells (CD16+CD56+). Depicted are the median changes from baseline.

Figure 3

RTX+BLM resulted in prolonged, specific reduction of autoantibody levels over 2 years follow-up(n=8 responders).

(A-D) Percentage change of physiological ~~auto~~antibody levels are depicted, i.e. total IgG, anti-tetanus toxoid, anti-rubella and anti-varicella zoster antibodies .(E-G) Percentage change of SLE-relevant autoantibodies are depicted ,i.e. anti-dsDNA(n=8), anti-U1RNP(n=4), anti-RNP70(n=3), anti-Sm antibodies(n=3) and anti-C1q antibodies(n=7).(J) To illustrate specific reductions in physiological antibody(anti-TT, anti-rubella and anti-VZV) and autoantibody levels(anti-dsDNA, anti-RNP70, anti-U1RNP, anti-Sm, anti-C1q), normalized ratio over total IgG was calculated and compared to baseline.

Long-term effects of combined B-cell immunomodulation with Rituximab and Belimumab in severe, refractory SLE: two-year results

Rituximab and Belimumab combination for severe SLE

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Abstract

Background: Anti-CD20 B-cell depletion has not shown superior efficacy to standard immunosuppression in patients with systemic lupus erythematosus(SLE). Besides trial design, potential explanations are incomplete B-cell depletion in relation to substantial surges in B-cell activating factor(BAFF). To improve B-cell targeting strategies, we conducted the first study in SLE patients aimed at investigating immunological effects and feasibility of combining rituximab(anti-CD20) and belimumab(anti-BAFF).

Methods: Reported is the long-term follow-up of a phase 2 proof-of-concept study in 15 patients with SLE including 12(80%) with lupus nephritis(LN).

Results: In 10/15(67%) patients a clinical response was observed by achievement of lupus low disease activity state(LLDAS) of which 8(53%) continued treatment(belimumab+≤7,5mg prednisolone) during the complete 2 years of follow-up. Five patients(33%) were referred to as 'non-responders' due to persistent LN, major flare or repetitive minor flares. Out of 12 LN patients 9(75%) showed a renal response including 8(67%) complete renal responders. All anti-dsDNA^{+} patients converted to negative and both anti-C1q and extractable nuclear antigen autoantibodies(ENAs) showed significant reductions. CD19^{+}B-cells showed a median decrease from baseline of 97% at 24 weeks, with a persistent reduction of 84% up to 104 weeks. When comparing responders to non-responders, CD20^{+}B-cells were depleted significantly less in non-responders and double negative(DN) B-cells repopulated significantly earlier.

Conclusions: Combined B-cell targeted therapy with rituximab(RTX) and belimumab(BLM) prevented full B-cell repopulation including DN B-cells, with concomitant specific reduction of SLE-relevant autoantibodies. The observed immunological and clinical benefits in a therapy-refractory SLE population prompt further studies on RTX+BLM.

Keywords

Systemic lupus erythematosus, lupus nephritis, immune complex mediated membranoproliferative glomerulonephritis, Rituximab/Belimumab, autoimmune glomerulonephritis, autoantibodies

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease in which loss of tolerance to nucleic acids and their binding proteins results in generation of autoantibodies(e.g. anti-DNA, anti-chromatin or anti-histone autoantibodies), leading to inflammation potentially involving almost every organ system, including the kidney[1]. Lupus nephritis (LN) is seen in 29-82% of patients[2] and remains difficult to treat, with short term complete renal response (CRR) rates around 10-40% at 12 months[3] and occurrence of end stage renal disease (ESRD) in 10% of LN patients[4]. Together with the fact that patients with refractory SLE receive high cumulative dosage of toxic immunosuppressive medication, exploration of new therapeutic options is important.

Since autoantibodies contribute to renal pathology in SLE, targeting autoreactive B-cells has continued interest as a possible strategy for treating SLE patients. Targeting B-cells with anti-CD20 monoclonal antibody (mAb) rituximab (RTX) has been unsuccessful in randomized trials in both patients with extra-renal[5] and renal SLE[6]. Belimumab (BLM), an anti-BAFF (B-cell-activating factor) mAb, was approved for the treatment of active SLE. Approval of BLM included a special warning on its use with concomitant B-cell targeted therapy, however RTX+BLM provides an opportunity to target the surge in circulating BAFF levels after B-cell depletion and thereby minimizing the survival of autoreactive B-cells[7,8].

The concept of combining anti-CD20 B-cell depletion with anti-BAFF cytokine inhibition is supported by mice studies showing the importance of the microenvironment and cellular competition in anti-CD20 mAb mediated killing of B-cells where cellular competition for survival factors(e.g. availability of BAFF) can underpin resistance to anti-CD20 therapy[9]. The importance of BAFF levels in anti-CD20 therapy is further illustrated in a study using an in vitro model of mature B-cells, where BAFF was able to inhibit CD20-mediated apoptosis[10]. Additionally, in different lupus mouse models a combination of anti-CD20 and anti-BAFF therapy led to improved disease control compared to each treatment separately or cyclophosphamide[11]. We have previously reported on the effects of combination treatment with CD20

and BAFF targeting in SLE patients[12], however the long-term effects on B-cell repopulation and B-cell composition has not been reported yet.

'Synergetic B-cell immunomodulation in SLE'(Synbiose) was designed as the first translational, single-arm, proof-of-concept study in SLE patients aimed at investigating the underpinning, immunological hypothesis of combining RTX+BLM in severe, refractory SLE patients. We previously reported the early effects of RTX+BLM demonstrating a reduction in anti-nuclear antibodies (ANAs) and regression of excessive neutrophil extracellular trap (NET) formation[12]. We now report long-term effects of RTX+BLM on depletion of ANAs, B-cell repopulation and clinical response during 2 years of follow-up.

Materials and Methods

Study design

The Synbiose study is a phase 2, single-arm, open-label proof-of-concept study in which 'severe SLE' patients were included defined as a SLE disease activity index (SLEDAI-SELENA) score of ≥12 points or new, worse or persistent SLE-related activity in major organs. Patients were treated with intravenous methylprednisolone pulse therapy at baseline, 1000mg intravenous RTX at weeks 0+2 and with intravenous 10mg/kg BLM at weeks 4+6+8 and then every 4 weeks until 104 weeks. Mycophenolate mofetil was started but quickly tapered to avoid cumulative over-immunosuppression. Oral prednisolone was started at 1mg/kg/day(maximum 60mg/day) and tapered towards maintenance dose of ≤7.5mg/day. The study was approved by the Dutch LUMC medical ethics committee and all patients provided written informed consent. The study was registered at ClinicalTrials.gov(NCT02284984).

A fully detailed methods section with description of the clinical parameters, methods and materials used for experiments and statistical analysis is available as online supplemental file S1.

Results

Summarized patient characteristics

Baseline characteristics from all included patients have been reported previously[12]. Briefly, sixteen patients(88% female) were included, with median age of 31 years[19;51]. All patients had refractory disease, of which 12(80%) had active LN at baseline. One patient experienced severe hypogammaglobulinemia at week 8 after completion of methylprednisolone and RTX, therefore BLM treatment was not initiated. This patient was excluded from the long-term follow-up study. Fifteen patients reached the primary endpoint at week 24.

Clinical response

During the study period, 10 out of 15(67%) patients had a clinical response. At week 104 this response is 8 out of 13(62%). Eight patients(53%) finished the complete follow-up of 104 weeks. Two patients with a clinical response stopped BLM treatment at week 24, based on a pregnancy wish (patient#14 and #15 in Figure 1). Clinical response is illustrated in Figure 1A defined by the time for patients to achieve and remain in lupus low disease activity state (LLDAS) and by attaining a renal response in patients with active LN at baseline (Figure 1B). In the eight responders available for analysis over the two-year follow-up, the median time to the first achievement of LLDAS was 24 weeks[12;36] and the median time on LLDAS was 76 weeks[56;92]. One patient had a minor flare with pericarditis and received 0.5mg/kg prednisone and colchicine(patient#3) followed by quick resolution of disease activity. At week 104, 7 out of 8 patients received maintenance therapy with glucocorticoids with median dose of 7.5mg/day[2.5;7.5], all patients continuously used hydroxychloroquine and BLM (Figure 1C).

In patients with active LN at baseline, 9 out of 12(75%) had a renal response during the trial period with CRR at week 104 in 6 out of 10(60%), all had proteinuria below 0.5grams/day. In renal responders that finished the study period(n=7) proteinuria decreased from a median of 4.6 gram/day[1.3;11.2] to 0.3[0.1;1](p=0.02 at week 104) representing a median decrease of 96%.Despite rapid decline upon

treatment, patient#4 did not reach CRR due to persistent proteinuria above 0.7grams/day, which clinically correlated with histologically proven chronic renal damage warranting the continuation in the study.

Five patients were classified as 'non-responders' and dropped out due to clinical relapse or non-response necessitating alternative induction treatment: Two patients had a major flare, patient#10 experienced a renal flare at week 46 requiring cyclophosphamide treatment and patient#11 experienced recurrence of transverse myelitis at week 44 upon which induction treatment with RTX+steroids was given. Patients #12 and #13 had persistent features of active LN and were excluded at week 24, as described in more detail previously[12], one was treated with cyclophosphamide, the other was given an experimental induction treatment within another study. Patient#9 was excluded at week 74 due to a recurrent minor flare(complement consumption, anti-dsDNA positivity and arthritis) and was switched to leflunomide with high dose steroids(0.5mg/kg/day). Baseline characteristics of the responders and non-responders are depicted in Table 1.

Long-term safety

Treatment-emergent adverse events (TEAE) during the study period are summarized in Table 2. In all patients adverse events (AE) were reported with 5 serious adverse events (SAE) in 4 patients(27%) due to hospitalization for the suspicion of infection(n=3) or laparoscopic cholecystectomy(n=1) because of cholelithiasis. In all cases suspected infections were gastro-intestinal without detectable pathogen, requiring a one-night hospital admission without the need for antibiotic treatment. In 9 patients(60%) a minor infection was observed, of which upper respiratory tract infections were most prevalent. A detailed description of all infectious AEs is provided in supplemental file S2. Two patients suffered from mood disorders; 1 patient had glucocorticoid-induced mood disorder and psychosis after methylprednisolone infusions and another patient experienced depressive symptoms started at week 95,

leading to study treatment interruption in order to exclude progressive multifocal leukoencephalopathy (PML). Once PML and neuropsychiatric SLE were ruled out, a mild depressive disorder was diagnosed and BLM treatment reinstituted.

Long-term effects of RTX+BLM on B-cell immunology

By employing high sensitivity flow cytometry, we observed prolonged inhibition of B-cell repopulation: $CD19^+$B-cells declined from a median of $100*10^6$cells/L[20.5;$248*10^6$] at baseline to $3.75*10^6$ cells/L[0.53;$64.7*10^6$](p=0.005) at week 24, representing a median decrease of 97% from baseline. At week 104, the median number of $CD19^+$B-cells was $13.6*10^6$ cells/L[10.7;$47.3*10^6$], representing a median decrease of 84%[-92;+22] from baseline (Figure 2A) illustrating that B-cells did not repopulate to baseline values during continued BLM treatment. The low-level repopulation of B-cells was dominated by an early recurrence of plasmablasts at week 24 up to a median decrease of 17% (Figure 2B) and in lesser extent repopulation of switched memory B-cells up to a median decrease of 71% compared to baseline values. Only from 48 weeks onwards, the resurge of immature B-cells occurred with return of transitional B-cells(+52%) and non-switched memory B-cells(-19%) at week 104. Interestingly, continuous BLM treatment prevented repopulation of naive B-cells(-81%) as well as double negative (DN) B-cells(-82%) at 104 weeks.

Long-term immune reconstituting effects

In the RTX+BLM treatment strategy, patients were able to taper steroids and stop MMF treatment before or at 24 weeks (Figure 1C). As a consequence, we observed significant reconstitution of circulating $CD4^+$T-cells,from $234*10^6$ cells/L[116;$530*10^6$] at baseline to $658*10^6$ cells/L[285;$1270*10^6$](p=0.02 at week 104), $CD8^+$T-cells, from $276*10^6$ cells/L[121;$418*10^6$] at baseline to $493*10^6$ cells/L[237;$1700*10^6$](p=0.04) and in NK-cells from $18*10^6$ cells/L[0.4;$133*10^6$] to $97*10^6$[38;$221*10^6$](p=0.08) (Figure 2C).

Long-term effects of RTX+BLM on humoral auto-immunity

With respect to the effects of RTX+BLM on immunoglobulin levels, total IgG levels in comparison to baseline levels(median 11.3g/L[5;23.6]) initially decreased at 12 weeks(7.8g/L[2.6;14.4], p=0.05) and stabilized from 24 weeks onwards(9.7g/L[3.4;16.4], supplemental file S3). At week 104, IgG levels increased with 6.4%[-44;+30] compared to baseline levels (Figure 3A). IgA levels remained stable over the follow-up period while IgM levels gradually declined from 0.72g/L[0.26;1.06] at baseline to 0.27g/L[0.2;0.63], p=0.008 at week 72 and increased to 0.37g/L[0.2;0.73], p=0.02 at week 104 (supplemental file S3). With regard to (auto)antigen specific IgG, anti-tetanus and anti-rubella IgG remained stable during follow-up (Figure 3B+C) while anti-varicella zoster virus IgG (anti-VZV IgG) showed a significant decrease (Figure 3D) from 3435mIU/mL[442;4000] at baseline to 2436[404;3625], p=0.02 at week 104. Of note, all measured anti-VZV IgG levels were within protective ranges.

Anti-dsDNA levels of 268AU/mL[50;827] at baseline decreased at week 24 to 29.6[0;104.5](p=0.02) equal to a median decrease of 87%[-100;+3] (Figure 3E). By week 48 up to 104, all anti-dsDNA positive patients converted to negative on immunofluorescence (CLIFT) with a median titer of 52[23;132](p=0.04) at week 104 equal to a median decrease of 81%[-91;+95] from baseline. Similar reductions in anti-RNP70, anti-U1RNP, anti-Sm and anti-C1q autoantibodies levels were observed as illustrated in Figure 3F-I. Briefly, at 104 weeks, anti-RNP70 antibody levels were reduced with a median of 88%[-94;-48](p=0.25), anti-U1RNP with 41%[-79;-31](p=0.13), anti-Sm with 30%[-97;-13] and anti-C1q antibodies with 60%[-86;+2](p=0.03). The relative reductions of *auto*-antibody compared to *allo*-antibody levels over total IgG is illustrated in Figure 3J demonstrating that RTX+BLM preferentially targeted humoral autoimmunity.

With respect to complement levels, normalization of C3 levels was seen at 104 weeks in 7 out of 8 patients with median C3 levels of 1.0g/L[0.8;1.3] compared to baseline C3 levels of

0.6g/L[0.3;0.8](p=0.008). Also, C4 levels increased from 54[35;80] to 147mg/L[74;279](p=0.25) (supplemental file S3).

Associations of immunological effects with clinical response to RTX+BLM

We investigated immunological parameters that could potentially discriminate long-term responders(n=8) from non-responders(n=5) depicted in supplemental file S3 and S4. We observed that, not unexpectedly, after 4, 12 and 24 weeks, a significantly larger increase in C3 levels was seen in responders versus non-responders(respectively 27% versus 0%, p=0.03, 42% versus 8% , p=0.01 and 79% versus 8%, p=0.008). With high sensitivity flowcytometry, we observed two noteworthy findings: first, the total number of $CD20^{+}$B-cells at week 24 was significantly lower in responders($1.83*10^6$[0.10;$17.2*10^6$]) compared to the non-responders($15.8*10^6$[3.01;$22.1*10^6$], p=0.045). Second, repopulation of DN B-cells occurred earlier in the non-responder group, at week 24[12;24](nadir levels of $0.48*10^6$ cells/L[0.17;$1.02*10^6$]), while in responders repopulation of DN B-cells occurred at week 72[48;104], p=0.0008(nadir levels of $0.32*10^6$[0.11;$2.34*10^6$]). Finally a trend for higher baseline BAFF levels was found in non-responders vs responders(respectively 0.97ng/ml[0.48-1.4] vs 0.44ng/ml[0.26;0.91]p=0.06) while the decrease at 24 weeks was similar between responders(0.11ng/ml[0.09-0.19]) and non-responders(0.15ng/ml[0.08-0.35]p=0.12).

Discussion

In this proof-of-concept study long-term immunological and clinical effects of RTX+BLM in patients with severe, refractory SLE(including LN) are described. Long-lasting, specific reduction of anti-dsDNA, anti-C1q and even ENAs were observed and full B-cell repopulation was prevented throughout two-year follow-up. Clinical response persisted in two-thirds of the patients during follow-up with maintenance treatment consisting of BLM and low dose prednisolone and allowed discontinuation of MMF associated with significant immune reconstitution. Profound depletion of $CD20^{+}$B-cells, prolonged suppression of

DN B-cells and higher serum BAFF levels potentially discriminated responders from non-responders and should be validated in larger clinical trials.

The study encompassed refractory SLE patients in which we were unable to continue immunomonitoring in non-responders who required different conventional induction therapies nor in responders with a pregnancy wish. Within this limitation, we investigated potential predicters of non-response to RTX+BLM predominantly in the first 6 months. It is known that the B-cell depleting potential of RTX has an inter-person variation and that the association of clinical outcome with the depth of B-cell depletion has been made[13 14]. We found that less profound depletion of $CD20^+$B-cells was associated with a poor response, in line with findings of a post-hoc analysis of the LUNAR trial[15] where rapidness and duration of complete peripheral B-cell depletion were associated with complete response. Our observations in B-cell subsets are also in line with a recent study investigating B-cell subsets with Cytof in SLE patients during BLM therapy[16], where long-term depletion of $CD20^+$B-cells and naive B-cells was seen. The loss of naive B-cells during BLM therapy has been shown before[16,17,18]. Besides the decrease in naïve B-cells we also observed that early repopulation of DN B-cell associated with poor response. DN B-cells in SLE are shown to be a major source of auto-antibody secreting cells (ASCs)[19] and the number DN B-cells are associated with disease activity and the presence of LN[20]. Moreover, further characterization of the DN B-cell population elucidated that these cells were hardly found in healthy or disease controls and were highly responsive to TLR7 stimulation inducing their differentiation to ASCs[20]. Unfortunately, we were limited in the depth of phenotyping DN B-cells in this study partly because this subpopulation had not been described at the time of study design and initiation. Notwithstanding, taken together with our observation that memory B-cells and plasmablasts fully repopulated after RTX+BLM while long-lasting reductions of autoantibodies persisted, suggested that a prolonged suppression of autoreactive DN B-cells can be beneficial to SLE patients. Therefore, DN B-cells are highly interesting biomarker to further study in the context of RTX+BLM treatment for SLE and LN patients.

Throughout the two-year follow-up no major safety issues were raised. The frequency of TEAEs was registered in 100% of patients containing 27% SAEs and 60% infections and was comparable to the LUNAR(99%, 27% and 85% respectively) and BLISS studies(93%, 42% and 75% respectively). Also, preliminary results of the CALIBRATE study(NCT02260934), in which 43 LN patients were randomized to receive RTX, cyclophosphamide and prednisone with or without additional BLM treatment, showed a non-significant difference on grade 3 or higher infectious adverse events(9% with BLM vs 23% without BLM, p=0.25) confirming that RTX+BLM is well-tolerated. In addition, the CALIBRATE reported 52% renal responders in the BLM group versus 41% in the placebo group. This non-significant difference could possibly be explained by the use of cyclophosphamide for induction treatment, in contrast to mycophenolate in the present study and the relative high dose of prednisolone maintenance(10mg/day) continued throughout two years. It is of interest that preliminary reports from the CALIBRATE study showed impaired B-cell repopulation during BLM treatment as well as specific decrease in the naïve B-cell compartment upon BLM.

Our study observed 60% CRR-rate at 104 weeks using the pre-defined CRR-criteria containing proteinuria levels of ≤0.7g/24hours, this in comparison to ≤0.5g/24hours used by landmark LN trials(LUNAR[6], ACCESS[21], ALLURE[22], ALMS[23]). Re-analyzation of the results showed that patients with a CRR at 104 weeks all have proteinuria levels below 0.5g/24hours. Based on LLDAS, clinical response to RTX+BLM was observed in 62% of patients in the present study. We demonstrated that responders to RTX+BLM had lasting LLDAS which is associated with reduced damage accrual[24], better quality of life[25] and can be used as an endpoint for clinical trials[26]. In this small trial clinical benefit was achieved with RTX+BLM and persisted despite tapering of steroids to a dosage ≤7,5mg and discontinuation of MMF. Although unconventional, tapering of MMF was added in the study design because at that time combined B-cell targeting with RTX+BLM had not been given to patients structurally(besides case-reports[27-31]) and intended to avoid over-immunosuppression which was the fundament of the previously-mentioned label

warning of BLM. Indeed, MMF tapering allowed for significant reconstitution of circulating $CD4^+$T-cells and is a unique achievement for LN patients. Altogether, these data are reassuring for further studies to study clinical efficacy of RTX+BLM for active SLE including LN in a randomized setting.

It is noteworthy to establish that the primary null-hypothesis to study the combination of RTX+BLM, i.e. to induce long-term B-cell depletion and indirectly (autoreactive) plasmablast depletion, was wrong. The null-hypothesis was based on dual B-cell therapy in murine studies[11] but the contrary was observed: plasmablasts repopulated fastest among the studied B-cell subsets. Importantly, this was not associated with (recurrence of) disease activity nor with autoantibodies. It was remarkable that RTX+BLM preferentially targeted humoral autoimmunity without affecting protective ranges of anti-viral antibody levels. It can be speculated that autoreactive B-cells have an increased BAFF-dependence due to the continuous presence of antigens compared to *allo*-reactive B-cells. This might also explain the significant drop, although not below protective levels, of antibodies against the varicella-zoster virus that remains inactive in the body for many years.

The most important limitations of this study are the small size of treated patients and the single arm design. The latter impairs the ability to place the observed effects into perspective to standard treatment regimens and it could be argued that the observed effects are solely due to RTX treatment combined with concomitant immunosuppressants. However profound B-cell depletion by RTX has shown to be highly variable in SLE patients with a median time to repopulation around 32 weeks[32] and only 0-11% of patients with sustained low B-cell counts for 1-2 years without re-treatment[13 32-34]. In a comparable cohort of 7 severe, refractory SLE patients re-treated with RTX a median duration of clinical response of 13 months and B-cell depletion of 6 months were reported[35]. Together suggesting a synergistic role of BLM in RTX-treated SLE patients.

In conclusion, this study was the first to pioneer the combination of RTX+BLM in patients with severe, refractory SLE aiming to establish its feasibility and better understand its immunological effects. RTX+BLM treatment appears to be a promising strategy to target pathological autoimmunity mechanisms in SLE with suggestions towards beneficial clinical effects. We are, therefore, reassured that RTX+BLM can be safely studied in further clinical trials to assess its added value in the treatment of SLE patients with and without renal involvement.

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Conflict of Interest Statement

YKOT received consultancy fees from GSK and Aurinia Pharmaceuticals.

Authors' Contributions

TK en EJA contributed equally as first authors. TK and YKOT contributed to the design of the study, acquisition of data, analysis and interpretation of data and manuscript preparation. EJA contributed to the acquisition of data, analysis and interpretation of data and manuscript preparation. LSvD contributed to the acquisition of data, interpretation of data and manuscript preparation. PLAvD, OWB and AR contributed significantly to the recruitment and follow-up of patients and acquisition of data. SWAK, JAB, HUS contributed to the acquisition of data. TJWH, TJR and CvK contributed to the analysis and interpretation of data and manuscript preparation. All authors discussed and agreed on the content of the manuscript before submission.

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Tables

Table 1. Baseline and historic disease characteristics of responders (n=8) and non-responders (n=5)

	Responders (n=8)		Non-responders (n=5)	
Demographics				
Age, median (range)	31	(21-47)	30	(19-51)
Female sex, n (%)	6	(75)	5	(100)
Race, n (%)				
White/Caucasian	2	(25)	2	(40)
Black/African American	6	(75)	2	(40)
Asian/Oriental	0	(0)	1	(20)
Smoker (%)	2	(25)	0	(0)
Baseline disease characteristics				
SLEDAI, median (range)	19	(12-26)	18	(6-29)
Disease flare characteristics, n (%)				
Renal flare	9	(90)	3	(60)
Transverse myelitis	0	(0)	1	(20)
Persistent disease activity despite treatment	1	(10)	1	(20)
LN disease characteristics				
Histopathology, n (%)				
Class II (±V)	1	(14)	0	(0)
Class III (±V)	1	(14)	2	(67)
Class IV (±V)	4	(57)	1	(33)
Class V	1	(14)	0	(0)
Proteinuria (g/24h), median (range)	4.6	(1.3-11.2)	1.9	(1.0-8.4)
Treatment at disease flare				
Glucocorticoids[a], n (%)	8	(100)	4	(80)
Dose mg/day, median (range)	15	(5-60)	15	(5-60)
Mycophenolate mofetil, n (%)	5	(63)	3	(60)
Dose mg/day, median (range)	2000	(1500-4000)	1500	(1000-3000)
Azathioprine, n (%)	1	(13)	1	(20)
Dose mg/day, median (range)	200		100	
Hydroxychloroquinine, n (%)	8	(100)	1	(20)
Biomarkers				
ANA positivity	8	(100)	5	(100)
Anti-dsDNA titer[b] (AU/ml), median (range)	268	(50-827)	479	(33-1123)
Complement consumption[c] (%)	100		100	
C3[d] (g/l), median (range)	0.6	(0.3-0.8)	0.6	(0.5-1.3)
C4[e] (mg/l), median (range)	96	(35-236)	68	(21-260)
IgG (g/l), median (range)	11.5	(5-23.6)	12.9	(4.9-16.6)
IgA (g/l), median (range)	3.0	(1.2-4.5)	2.9	(1.6-6.3)
IgM (g/l), median (range)	0.7	(0.3-1.1)	0.8	(0.4-1.1)

CD19⁺B-cells (*10^6 cells/l), median (range)	90	(21-279)	65	(37-300)
Historic disease characteristics				
Disease duration in years, median (range)	7	(3-18)	10	(2-24)
No. of previous relapses, median (range)	3	(2-6)	5	(1-5)
No. of renal relapses, median (range)	2	(1-5)	1	(0-3)
SLICC damage index, median (range)	1	(0-3)	1	(0-4)
Organ involvement, n (%)				
Constitutional	8	(100)	5	(100)
Mucocutaneous	7	(88)	3	(60)
Neuropsychiatric	1	(13)	2	(40)
Musculoskeletal	5	(63)	4	(80)
Cardiorespiratory	7	(88)	4	(80)
Gastrointestinal	0	(0)	0	(0)
Ophtalmic	0	(0)	2	(40)
Renal	8	(100)	4	(80)
Hematology	4	(50)	4	(80)
Treatment history				
Steroids, n (%)	8	(100)	5	(100)
Mycophenolate mofetil, n (%)	8	(100)	5	(100)
Cyclophosphamide, n (%)	3	(38)	3	(60)
Azathioprine, n (%)	4	(50)	3	(60)
Tacrolimus, n (%)	1	(13)	0	(0)
Rituximab, n (%)	2	(25)	1	(20)
Hydroxychloroquinine, n (%)	8	(100)	5	(100)

[a]Patients were treated with the glucocorticoid equivalent prednisolone. [b]Normal anti-dsDNA IgG <10 IU/ml. [c]Complement consumption is defined as decreased CP (classical pathway) activation, decreased C3 or decreased C4. [d]Normal C3: 0.9-2 g/l. [e]Normal C4: 95-415 mg/l.

Table 2 Adverse events during 104 weeks of study

Treatment-emergent adverse events*	n=15	
All adverse events	15	(100)
Severe adverse events (hospitalization)	4	(26.7)
Major infection	3	(20.0)
Cholelithiasis	1	(6.7)
Minor infection	8	(53.3)
Upper respiratory tract	9	(60.0)
Lower respiratory tract	3	(20.0)
Urinary tract	4	(26.7)

Urogenital infection	2	(13.3)
Sinusitis	1	(6.7)
Influenza	1	(6.7)
Herpes simplex	1	(6.7)
Skin	1	(6.7)
HACA formation	4	(26.7)
Symptomatic	1	(6.7)
Asymptomatic	3	(20.0)
Hypogammaglobulinemia (<4.0 g/l)[a]	2	(13.3)
Infusion-related reaction	1	(6.7)
Myalgia	7	(46.7)
Diarrhoea	4	(26.7)
Headache	2	(13.5)
Pyrexia	2	(13.5)
Nausea	2	(13.3)
Mood disorder[b]	2	(13.3)
Fatigue	2	(13.3)
Other	10	(66.7)

*Depicted values are number of patients with percentage of patients that experienced ≥1 TEAE over 104 weeks of study. [a]Study treatment was interrupted in 1 patient. [b]Study treatment was interrupted in 1 patient, in the other patient symptoms were related to high dose steroids.

Legends to figures

Figure 1

Overview of the clinical responses, renal responses and concomitant immunosuppression upon RTX+BLM treatment. (A) Achievement of lupus low disease activity state (LLDAS) over time. (B) Achievement of a renal response in patients included with active lupus nephritis (n=12). Complete renal response was achieved when proteinuria ≤0.7 grams/day, normal serum albumin, stable kidney function, normal urinary sediment; partial response: >0.7–2.9 g/24 h with a decrease in proteinuria of ≥50% from baseline,

serum albumin >30 g/L and stable kidney function. When patients did not meet any of these criteria they were considered to have persistent active lupus nephritis. (C) Overview of concomitant treatment with belimumab, mycophenolate mofetil and prednisolone throughout the study's follow-up. Patient numbers mentioned on the y-axis correspond between the 3 figures.

LLDAS, low disease activity state; SLEDAI, SLE disease activity index; SACQ, serologically active (positive antibody and or low complement) clinically quiescent; BLM, belimumab.

Figure 2

Longitudinal kinetics of circulating immune cells over 2 years of follow-up after RTX+BLM treatment (n=8 responders).

(A) RTX+BLM prevents the complete repopulation of circulating B-cells. Depicted is the median change from baseline in the number of $CD19^+$B-cells. (B) Repopulation of B-cell subsets upon RTX+BLM. Depicted are the median change from baseline of the following B-cell subsets: plasmablasts (CD3-CD38brightCD27brightCD19+), non-switched memory B-cells ($CD3^-CD19^+CD27^+IgD^+$), switched memory B-cells ($CD3^-CD19^+CD27^+IgD^-$), naive B-cells ($CD3^-CD19^+CD27^-IgD^+$), double negative B-cells ($CD3^-CD19^+CD27^-IgD^-$) and transitional B-cells ($CD3^-CD19^+$ $CD38^{bright}CD24^{bright}$). (C) Significant reconstitution of circulating $CD4^+$ T-cells (CD3+CD4+), $CD8^+$ T-cells (CD3+CD8+) and NK-cells (CD16+CD56+). Depicted are the median changes from baseline.

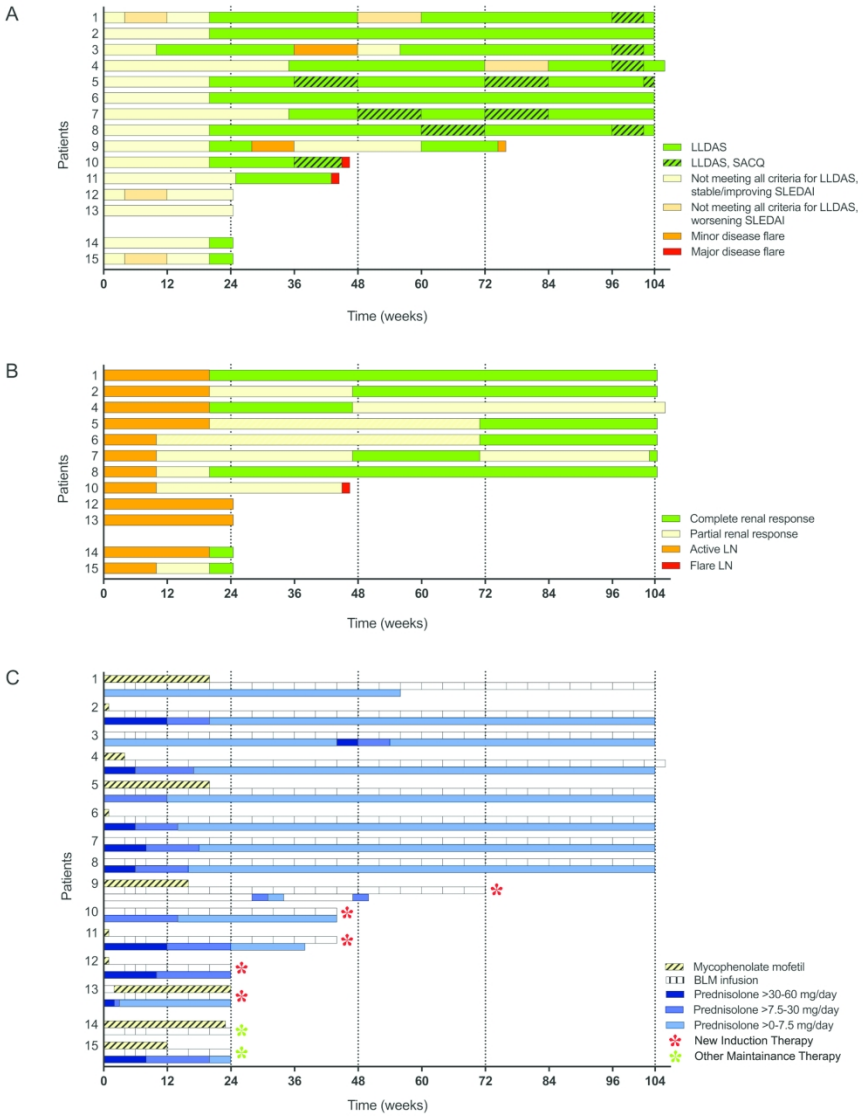
Figure 3

RTX+BLM resulted in prolonged, specific reduction of autoantibody levels over 2 years follow-up (n=8 responders).

(A-D) Percentage change of physiological antibody levels are depicted, i.e. total IgG, anti-tetanus toxoid, anti-rubella and anti-varicella zoster antibodies . (E-G) Percentage change of SLE-relevant autoantibodies are depicted ,i.e. anti-dsDNA (n=8), anti-U1RNP (n=4), anti-RNP70 (n=3), anti-Sm antibodies (n=3) and

anti-C1q antibodies (n=7). (J) To illustrate specific reductions in physiological antibody (anti-TT, anti-rubella and anti-VZV) and autoantibody levels (anti-dsDNA, anti-RNP70, anti-U1RNP, anti-Sm, anti-C1q), normalized ratio over total IgG was calculated and compared to baseline.

Figure 1



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LLDAS, low disease activity state; SLEDAI, SLE disease activity index; SACQ, serologically active(positive antibody and or low complement) clinically quiescent; BLM, belimumab.

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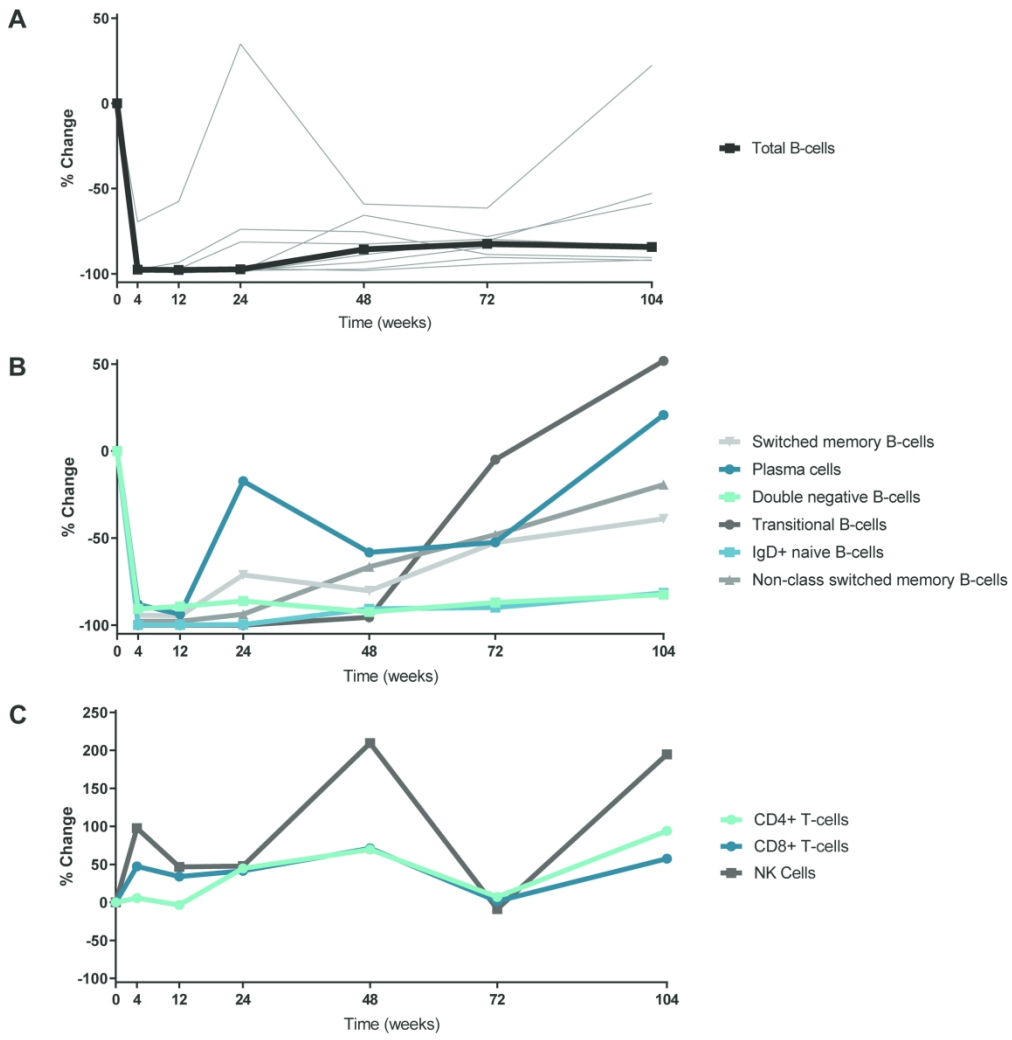
Figure 2



Longitudinal kinetics of circulating immune cells over 2 years of follow-up after RTX+BLM treatment(n=8 responders).
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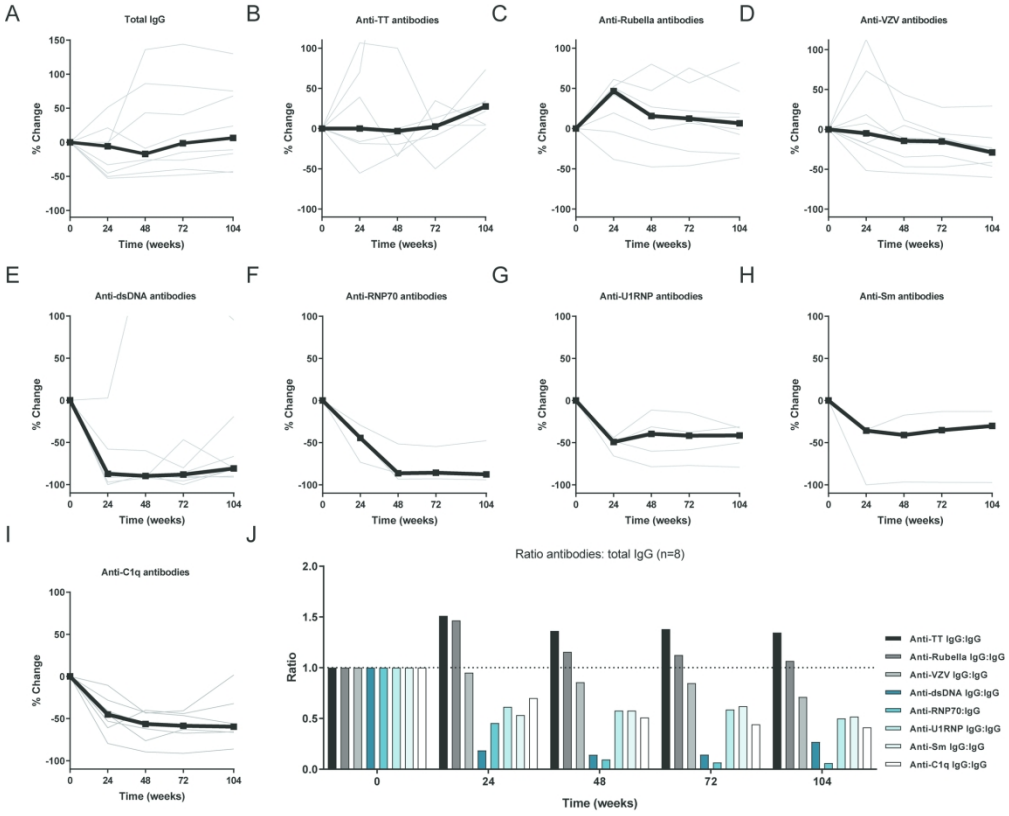
Figure 3



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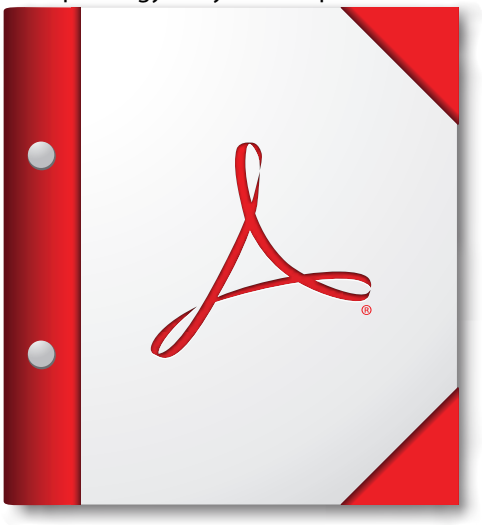

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		Reporting Item	Page Number
Title and abstract			
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	n/a
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	#3	State specific objectives, including any prespecified hypotheses	4/5, 10
Methods			

Study design	#4	Present key elements of study design early in the paper	3, 4/5, 11
Setting	#5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	11
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up.	table 1, 5, 11
Eligibility criteria	#6b	For matched studies, give matching criteria and number of exposed and unexposed	n/a
Variables	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5-8, 11-13
Data sources / measurement	#8	For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group. Give information separately for for exposed and unexposed groups if applicable.	11-13
Bias	#9	Describe any efforts to address potential sources of bias	11-13
Study size	#10	Explain how the study size was arrived at	5
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	5-8, 11-13
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	13
Statistical methods	#12b	Describe any methods used to examine subgroups and interactions	13
Statistical methods	#12c	Explain how missing data were addressed	n/a
Statistical	#12d	If applicable, explain how loss to follow-up was	n/a

methods		addressed	
Statistical methods	#12e	Describe any sensitivity analyses	n/a
Results			
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	3,6,8,14,15
Participants	#13b	Give reasons for non-participation at each stage	5
Participants	#13c	Consider use of a flow diagram	It has been made and used for presentation's, adding to the manuscript has been considered
Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	table 1
Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest	n/a
Descriptive data	#14c	Summarise follow-up time (eg, average and total amount)	Figure 1 (title, 3, 5, 9,11, 14)
Outcome data	#15	Report numbers of outcome events or summary measures over time. Give information separately for exposed and unexposed groups if applicable.	11
Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	5-8,13
Main results	#16b	Report category boundaries when continuous	n/a

		variables were categorized	
Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a
Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	8
Discussion			
Key results	#18	Summarise key results with reference to study objectives	9
Limitations	#19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	10
Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	9,11
Generalisability	#21	Discuss the generalisability (external validity) of the study results	10-11
Other Information			
Funding	#22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13