The NET effect of novel treatments in lupus nephritis

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Voor Marjan en Annelies

Contents

Chapter 1

General introduction and outline of this thesis

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease in which loss of tolerance to nucleic acids and their binding proteins results in generation of autoantibodies (e.g. anti-dsDNA), leading to inflammation in virtually every organ, including skin, kidneys, lungs, heart or brain. SLE predominantly affects young women with childbearing potential (20-40 years) and the estimated incidence of SLE in North America is 23.2 per 100 000 person years. SLE patients have an increased mortality with 1 of 8 patients dying within 8 years of follow-up, which is 2,5 times higher than the general population [1].

Lupus nephritis (LN) is seen in 29-82% of patients with SLE [2] and remains difficult to treat. The short term complete renal response ranges between 10-40% at 12 months [3] and end stage renal disease (ESRD) occurs in 10% of LN patients [4]. LN is associated with a 6-fold increase in mortality compared to the general population [5]. Current guidelines on the treatment of LN recommend corticosteroids in combination with cyclophosphamide or mofetil mycophenolate (MMF) as induction treatment and azathioprine or MMF as maintenance treatment [6,7]. Nevertheless, there is a persistent need for new therapeutic options, since the cumulative renal flare rate is 50% within 10 years following the first-choice conventional treatments [8]. For these refractory patients, guidelines are less specific in their recommendations and stress the ongoing need for novel treatment options. In order to develop novel treatment approaches, translational studies require a profound knowledge of pathophysiological mechanism that play a role in SLE patients which will be addressed below.

Brief overview of B cell development and humoral immune response

B cell development

Before addressing the pathogenesis of SLE in detail, it is necessary to briefly introduce B cell development and the humoral immune response. B cell development occurs in the bone marrow and in peripheral lymphoid tissues (e.g. lymph nodes, spleen). The pre-B cell, present in the bone marrow, expresses a pre-B cell receptor (pre-BCR). Rearrangement of the immunoglobin present on the pre-B cell leads to a mature BCR that is able to bind antigen. Immature B cells express immunoglobin M (IgM). The presence thereof initiates the release of the immature B cells from the bone marrow into the circulation. They enter the spleen as transitional B cells where they undergo final stages of development to form mature B cells. Guided by survival signals, e.g. BAFF, they develop into marginal zone B cells or follicular B cells. Further development goes on in the germinal center, a structure that forms within peripheral lymphoid organs in response to T cell-dependent antigens. Here, B cells interact with T cells which drives B cell proliferation

together with cytokines secreted by T cells. This also triggers molecular events leading to immunoglobin isotype switching. B cells further acquire high rates of mutations through somatic hypermutation leading to generation of mutant clones with a broad range of affinities, therefore imposing the necessity for a new round of selection. These processes will ultimately lead to differentiation of B cells into memory B cells or plasma cells. [9–11].

Autoreactive B cells

The random rearrangement process of immunoglobulin genes during B cell development is important for the generation of a large variation of BCRs, in order to recognize a large amount of autoantigen. In fact, 75% of immature B cells in humans are estimated to be autoreactive [12]. 20-50% of autoreactive clones are eliminated during a process termed central tolerance that takes place in the bone marrow. The immature cell that strongly recognizes autoantigens either changes its antigen receptor (receptor editing) or goes into apoptosis (clonal deletion). Additional mechanisms exist in the peripheral lymphoid organs that can further remove autoreactive cells. Mature B cells in the peripheral lymphoid organs that encounter autoantigens become anergic, they leave the lymphoid follicles and may die because of lack of survival signals [13].

Humoral immune response

The humoral immune response is defined by the production of antibodies by B cells leading to destruction of microorganisms present in extracellular space. In response to antigens derived from microorganisms, naive B cells are activated leading to clonal expansion, which is the proliferation of antigen specific B cells. This process can be T cell dependent or independent. Effector CD4+ T helper cells can interact with activated B cells in lymphoid follicles in the peripheral lymphoid organs. The naive CD4+ T cell is activated by the presentation of antigen by an antigen presenting cell (APC), usually a dendritic cell, leading to differentiation into an effector cell. The migration of the effector CD4+ T cell at the same time as antigen-stimulated B cells toward each other depends on expression of certain chemokine receptors, such as CCR7 and CXCR5. When the cells meet, the B cell presents antigen to the T cell via MHC class II, which can activate T cells to express CD40L (CD154), leading to subsequent CD40 engagement on B cells and further to clonal expansion. CD40 engagement is also important for heavy chain isotype switching, a process leading to differentiation of B cells into IgG, IgE or IgA antibody-secreting cells. The secreted antibodies recognize the antigen that initiated the immune response. The different isotypes have different effector functions, e.g. IgG opsonizes antigens for phagocytosis, leads to antibody-dependent cellular toxicity (ADCC) and activates the classical complement pathway.

Next to the uptake and presentation of antigens derived from microorganisms by APCs, autoantigen can be taken up as well, potentially leading to the production of autoantibodies.

Humoral autoimmune response in SLE

The central concept in the pathogenesis of SLE is the loss of tolerance against nuclear antigens as a result of autovaccination with these components [14,15], see also Figure 1. This results in the formation of autoantibodies, a hallmark for diagnosis of SLE. Formation of autoantibodies in SLE patients suggests that the presentation of nuclear components to APCs might be an inciting pathological event. Indeed, extracellular nucleic acids are very potent immunostimulatory agents of the nucleic acid recognition receptors toll like receptor 7 and 9. Toll like receptors (TLRs) recognize pathogen associated molecular patterns (PAMPs) and TLR7 and TLR9 specifically recognize nucleic acids derived from pathogens. They are found in endosomes or endoplasmic reticulum (ER) of mainly plasmacytoid dendritic cells (pDCs) and B cells. Activation is not exclusive for PAMPs, since also when endogenous nuclear particles become present in the extracellular space, they can be recognized by TLR7/9 [16,17].

TLRs form an important link between innate immunity and autoantibody response in SLE. In a study performed with lupus-prone mice without adequate TLR signaling, autoantibodies were not generated [18]. Another murine study showed that pDCs were activated by immune complexes containing small nuclear ribonucleoproteins (snRNPs) to produce interferon-α (IFN-α), a process dependent on TLR7 [19]. In humans, it was shown that serum from SLE patients, containing immune complexes, can activate pDCs [20], and that serum from SLE patients with active disease, and thus potentially containing immune complexes, induced TLR7 expression [21]. TLR signaling leads to pDC activation and production of large amounts of IFN-α, promoting an inflammatory response which normally occurs during viral infection. The chronic pDC activation and IFN-α production results in the so-called interferon signature in PBMCs of SLE patients [22,23].

Presentation of autoantigens by pDCs to T cells together with the production of pro-inflammatory cytokines can further activate B cells, leading to autoantibody production. Also, autoreactive B cells can be stimulated directly via their TLRs [24,25]. B cell stimulation leads to maturation towards autoantibody producing plasma cells [14,24,26]. Autoantibodies form immune complexes (ICx) with autoantigen and -as mentioned before- can deposit in any tissue leading to tissue damage via recruitment of inflammatory cells and complement activation. An example is the subendothelial deposition of immune complexes in the glomerulus of the kidney, leading to class 3 or 4 LN.

Despite the vast amount of data that the break of tolerance to nuclear particles leads to autoimmunity in SLE patients, it is still important to address how nuclear particles can be presented to the immune system in SLE. This question is important because, under normal

circumstances, endogenous nuclear particles are protected from exposition to the immune system by a nuclear envelope and cell membrane. To address this issue, it is important to expand on potential mechanisms leading to release of DNA in the extracellular space.

Extracellular DNA in SLE

High levels of extracellular DNA in SLE were already described in 1966 [27]. In SLE, several DNA clearance defects have been described, that can potentially lead to the presence of nuclear particles in extracellular compartments [28]. Notably, SLE has been referred to as a 'clearance' disease [29]. Examples are impaired phagocytosis by macrophages and granulocytes in SLE patients [30,31] and impaired clearance in early phases of apoptosis [31]. Early in vitro work studying apoptosis of human skin cells upon the exposure to UV light, shows the development of apoptotic bodies or blebs containing autoantigens at the surface [32]. They might be involved in the induction of disease flare, as in some SLE patients the disease flares up when exposed to UV light. Nucleosomes are found at the surface op apoptotic cells as well [33]. Elevated levels of such autoantigens might lead to impaired removal of apoptotic cells [34]. Furthermore, complement components have a role in the clearance of dead cells. C1q binds apoptotic cells and thereby promotes clearance. C1q deficiency almost always leads to development of SLE, although this is a rare phenomenon [35]. Other deficiencies of complement system components associated with SLE have been described, such as deficiency of C2, C4 and CR3. Mutations in complement inhibitors, such as factor H (FH), have been described as well [36]. Anti-C1q antibodies are found in about one third of SLE patients [37,38] and in almost all patients with lupus nephritis [39,40]. They are potentially pathological in SLE by binding to immune complexes and apoptotic cells containing C1q in the kidney leading to complement activation and inflammation [37]. Without the presence of C1q in the glomerular basement membrane, anti-C1q autoantibodies do not seem to initiate renal disease [41].

Besides the aberrant clearance of extracellular DNA in SLE, accelerated cell death has a role in the break of tolerance as well. The next part focuses on a relatively new described form of cell death called NETosis, by which neutrophils die upon the release of neutrophil extracellular traps.

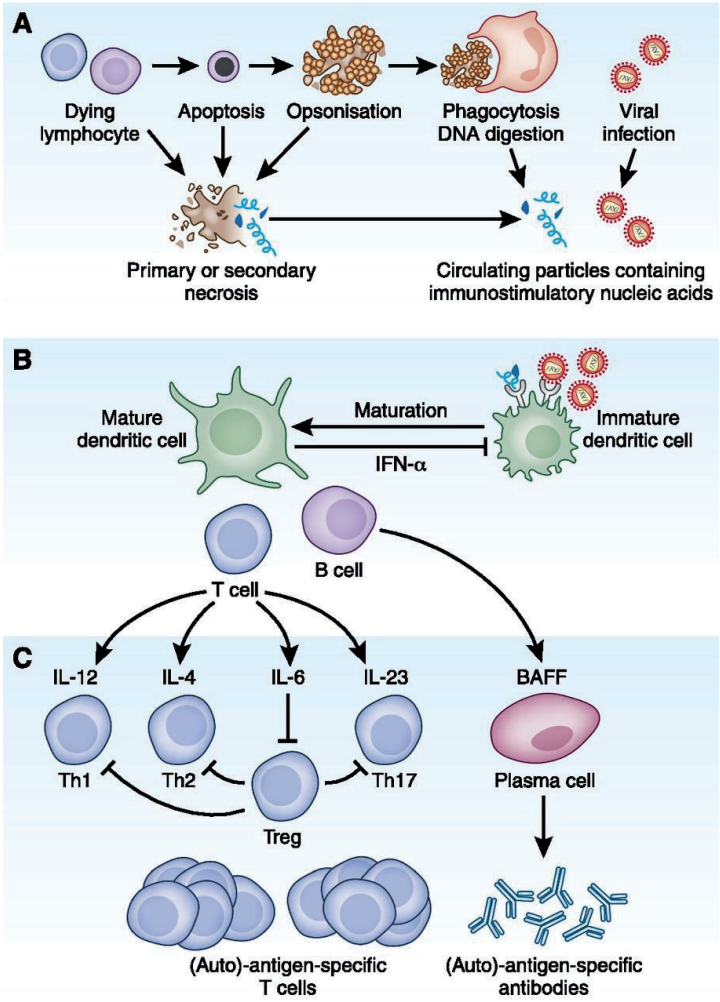

Figure 1. Autovaccination with autoantigen.
In **(A)**, cell death by apoptosis and primary or secondary necrosis leads to circulating particles containing immunostimulatory nucleic acids. In **(B)**, dendritic cells take up this autoantigenic material in a similar way as viral particles are taken up and present it to T cells after which the humoral immune response is initiated in **(C)**, leading to production of autoantibodies. Figure adapted from Lech et al [42] with permission.

NETs in autoimmune disease

Neutrophils act as a first line defense against microorganisms. Besides phagocytosis, production of ROS species and release of antimicrobial peptides from their granules, neutrophils can release neutrophil extracellular traps (NETs) that trap and kill pathogens. In 2004, NETs were described

for the first time [43]. NETs are strands of extracellular DNA covered with antimicrobial proteins, e.g. myeloperoxidase (MPO), neutrophil elastase (NE) and LL37 [44]. They are a proposed source of extracellular DNA in SLE.

Different forms of NET release have been described. The 'classic' form is the release of NETs upon which the neutrophil lyses and dies, so called 'suicidal NETosis'. This form of NET release is seen upon the stimulation of neutrophils by the chemical compound phorbol 12-myristate 13-acetate (PMA) [45], interleukin-8 (IL-8)[46] or monosodium urate (MSU) crystals [47]. However, when stimulated with other stimuli such as lipopolysaccharide (LPS) [48] or Staphylococcus aureus [49], the neutrophil releases vesicles containing NETs, without the disruption of the cell membrane, a process called vital NET release. Several important molecules are involved in NET release. The classic form is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, leading to production of reactive oxygen species (ROS) and oxidative burst [50]. In this form of NET release, chromatin decondensation is seen after about one hour of stimulation [45], a process dependent on the peptidylarginine deiminase 4 (PAD4) enzyme [51]. PAD4 targets histone arginine and mediated the conversion to histone citrulline, called citrullination [52].

It has been proposed that NETs can also contribute to the break of tolerance in several autoimmune diseases, such as SLE, ANCA associated vasculitis (AAV) and rheumatoid arthritis (RA) [21,53–61]. Not surprisingly, NETs were described for the first time as an autoimmune phenomenon in the context of SLE because they could act as a source of extracellular nuclear antigens. Indeed, it was found that the rate of NET release is increased in SLE. It was shown that low density granulocytes (LDG) from SLE patients spontaneously release NETs [22,54,62] and that SLE neutrophils release higher amounts of NETs when stimulated with anti-RNP IgG [21]. In addition, serum from SLE patients contains high levels of NET remnants [63] and self-DNA complexed with NET-related peptides [55]. Furthermore, clearance of NETs seems to be defective in SLE, which could be a result of DNase inhibitors, present in about one third of SLE patients [64]. Of note, mutations in the DNase gene that lead to development of SLE have been described as well, although they are rare [65].

In vitro studies showed that NETs are able to activate pDCs [21,55] and lead to the production of IFN-α [21,55,58,66]. Also, NETs were found in skin and in glomeruli of LN patients [54], suggesting that they could contribute to tissue damage. Furthermore, products from the complement system were found on NETs and C5a was generated in normal human serum (NHS) incubated with NETs [66], indicating complement activation by NETs and thereby increasing inflammation. Leffler et al. [66] showed that addition of C1q to NHS decreased its NET degradation ability, possibly due to the binding of C1q to NETs, thereby interfering with DNase access. Therefore,

antibodies against NET components, present during disease flare, can potentially interfere with NET degradation in a similar way. Taken together, NETs could be a source of extracellular nuclear antigens in SLE, contributing to autovaccination as shown in Figure 1, break of tolerance to self-DNA antigens and production of pathological autoantibodies.

ANCA-associated vasculitis

ANCA-associated vasculitis (AAV) leads to inflammation in small vessels and can affect any organ in the body. Anti-neutrophil cytoplasmic autoantibodies (ANCAs) are directed against PR3 or MPO and their presence is highly associated with granulomatosis with polyangiitis (GPA) or microscopic polyangiitis (MPA), respectively [67]. The incidence of all AAV in Europe is about 1.3-2 per 100 000. GPA is more common in the north, while MPA is more common in southern regions of Europe [68,69]. Prevalence of AAV is relatively high among middle-aged and elderly people, with also quite similar prevalence between men and women [69].

Renal involvement of AAV leads to rapidly progressive glomerulonephritis, showing glomerular crescent formation in the kidney biopsy in 50% of cases, which can lead to renal failure within 3 months after clinical onset when left untreated [70]. Kidney biopsies show 'pauci-immune' glomerulonephritis, meaning there is no glomerular staining for immunoglobulins, which is seen in 'full house' glomerulonephritis, e.g. in lupus nephritis. The pathological role of ANCAs is evident; e.g. anti-MPO IgG treatment in mice induces crescentic glomerulonephritis [71] and primed neutrophils activated by ANCA lead to necrotizing inflammation of the vascular endothelium [72,73]. NETs were also shown to play a pathological role in ANCA-associated vasculitis. Sangaletti et al. [74] showed that the injection of NET-loaded myeloid dendritic cells (mDCs) in mice, resulted in the production of autoantibodies, especially MPO-ANCA and PR3-ANCA, more so than injection of mDCs loaded with apoptotic neutrophils. This shows that NETs are able to transfer neutrophil cytoplasmic antigens to DCs which can lead to ANCA production in a mouse model. NETs were also found in kidney biopsies from AAV patients [75] and NETs have been shown to be able to damage the endothelium [54,76]. In vitro studies showed NET induction by ANCA [77,78], strongly suggesting a pathological role of NETs in AAV.

Targeting autoantibody production in SLE

As mentioned before, current treatment options for SLE are focused on suppressing inflammation [79] and do not specifically target the production of pathological autoantibodies. Learning more about the pathophysiology of SLE has led to the development of new therapeutic strategies [26], including antibodies directed against CD20 [80,81], CD22 [82] and BAFF [83,84] or with the proteasome inhibitor bortezomib [85]. In our studies we focused on targeting of autoreactive

plasma cells, and thereby autoantibody production, with a combination of anti-CD20 and anti-BAFF antibodies.

B cell activating factor

B cell activating factor (BAFF), also named Blys (B lymphocyte stimulator) or TNFSF13B (tumor necrosis factor ligand superfamily member 13B), is a B cell survival factor produced by myeloid cells, such as dendritic cells, neutrophils, monocytes and macrophages as well as by stromal cells. BAFF promotes survival and maturation of transitional B cells into mature B cells, supports B cell proliferation, class-switch recombination and plasma cell survival [12,86]. Peripheral tolerance mechanisms could be ineffective due to elevated levels of BAFF and T cell help of anergic B cells, enabling survival of autoreactive cells. Thus, BAFF seems to be important in the development and survival of autoreactive B cells [13]. In mice, overexpression of BAFF leads to development of SLE [87] and BAFF blockade reduces symptoms in an SLE mouse model [88]. Furthermore, high BAFF levels are found in patients with SLE [89–91] and are associated with disease flare [92,93]. These results imply that BAFF could be an interesting target in SLE treatment.

Belimumab

Belimumab (BLM) is a human IgG1 monoclonal antibody that binds soluble BAFF. Biologically active BAFF can bind to three receptors present on B cells, illustrated in Figure 2; B cell maturation antigen (BCMA), transmembrane activation and calcium modulating ligand interactor (TACI) and BAFF receptor (BAFFR) or BR3. BAFF binds strongly to BAFFR and TACI and weakly to BCMA, whereas the related survival factor APRIL (a proliferation-inducing ligand) binds strongly to BCMA and TACI [94].

Belimumab was studied in the BLISS study. The BLISS study showed a significantly better clinical response in patients with active SLE when treated with BLM in addition to standard of care [84]. In this study, BLM was compared to placebo as add on therapy in non-renal SLE patients with mildly active disease (SLE disease activity index (SLEDAI) ≥ 6), patients with active LN or CNS involvement were excluded. Besides better clinical outcome, BLM treatment led to reduction of anti-dsDNA antibodies and increasing C3 and C4 levels compared to placebo [96]. Post hoc analysis showed that renal response was higher in BLM treated patients with proteinuria $>$ 1 gram/day and renal flare rates were lower [97]. Also, patients with higher SLE disease activity (based on anti-dsDNA levels, complement levels and corticosteroids use) seem to have a more effective response to BLM [98]. Overall, BLM shows potential for SLE treatment which could especially be true for LN patients. These and other studies performed with subcutaneous BLM [99,100], led to the approval of BLM as add-on therapy in SLE in the Netherlands in serologically active SLE patients.

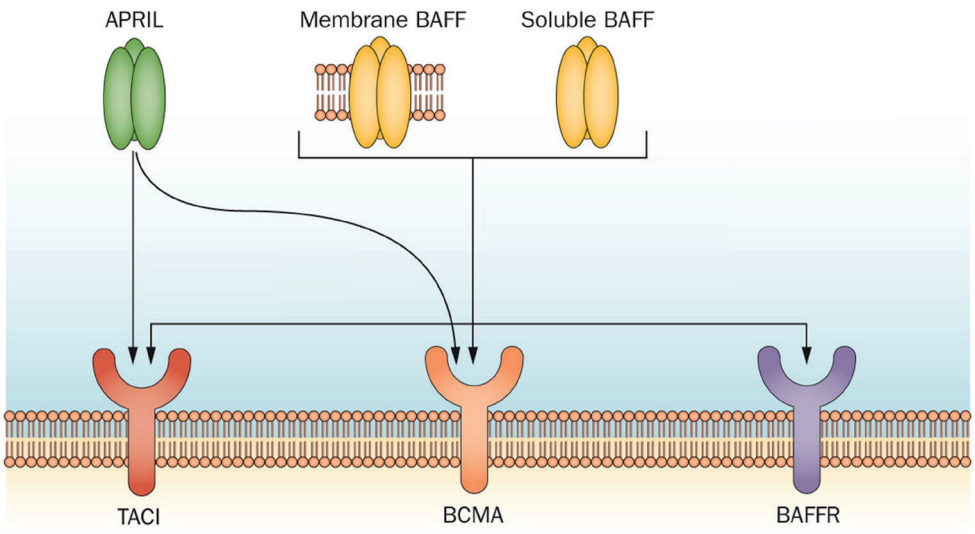

Figure 2. BAFF axis.
Figure adapted from Stohl et al. [95] with permission.

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Rituximab

B cell targeting has already been studied in 2 RCTs in SLE patients with the anti-CD20 monoclonal IgG1 antibody rituximab (RTX), where RTX was shown not to be superior compared to placebo [80,81]. The EXPLORER study included non-renal SLE patients [81] and the LUNAR study

included LN patients [80]. It has been suggested, that their failure supposedly could have to do with their trial design, i.e. the fact that patients received high dose of steroids (0.5-1 mg/kg) for several weeks and concomitant immunosuppression, which could have clouded the ability of RTX, as well as a 'strict' definition of response compared to other clinical studies performed with SLE patient [101].

Multiple meta-analysis have been published on RTX in SLE. One meta-analysis focused on refractory LN patients and found complete renal response rate (CR) and partial renal response rate (PR) of 40% and 34% after 60 weeks, respectively [102]. These results are comparable to renal response rates in various RCTs in LN [103]. Also, there is evidence from prospective trials showing efficacy of RTX and MMF in LN [104]. The Rituxilup study is an open label study using MMF and RTX without steroids, showing 52% CR and 34% PR in patients with LN [105]. Currently, RTX has a place in treatment of refractory SLE [6,106,107].

It has further been proposed that RTX failed in RCTs due to the production of BAFF upon B cell depletion [108,109], possibly leading to survival of autoreactive B cells. Due to the rise in BAFF after anti-CD20 therapy [110,111] and its important role in development of autoreactive B cells, we hypothesized that combining RTX with BLM could target autoreactive plasma cells, and thereby production of pathological antibodies.

Dual B cell therapy in mouse models

In murine studies, there are several important findings supporting the use of combined anti-CD20 B cell depletion with anti-BAFF cytokine inhibition. First, in an in vitro model using mature B cells, BAFF therapy was able to inhibit CD20-mediated apoptosis, showing the importance of BAFF levels in anti-CD20 therapy [112]. The important contribution of BAFF to anti-CD20 killing was further shown in a study with chimeric mice expressing human CD20 on 50% of B cells. Anti-human CD20 therapy more effectively depleted splenic B cells than mice expressing human CD20 on 100% of B cells [113]. This indicates that cellular competition for survival factors, such as BAFF, leads to resistance to anti-CD20 therapy. Further, combined treatment with anti-CD20 and BR3-Fc fusion protein, neutralizing BAFF, leads to depletion of all splenic B cells compared to BR3-Fc and anti-CD20 treatment alone.

Further murine studies were performed with combined B cell treatment. Two other murine studies, both using different mouse models, used anti-mCD20 antibodies instead of anti-human CD20 antibodies [114,115]. One compared B cell depletion and clinical markers after treatment with anti-CD20, anti-BR3 (a murine BLM substitute) or cyclophosphamide in three different murine SLE models. The combination treatment led to a better clinical response and more effective B cell depletion was shown in mice treated with combined therapy in both blood and spleen [114].

Another in a in vivo study using a murine anti-CD20 antibody combined with BAFF blockade with BR3-Fc, found enhanced B cell depletion in lymph nodes and spleen, but no enhanced effect in peritoneum, blood and bone marrow [115]. This study showed no benefit of combined therapy in glomerulonephritis treatment compared to anti-CD20 therapy alone.

Another interesting finding was described in a murine study with non-obese diabetic (NOD) mice, investigating anti-CD20 and anti-BAFFR treatment for the inhibition of development of type 1 diabetes (T1D) [116]. The number of non-diabetic NOD mice that developed diabetes, was similar in mice continuously treated with anti-BAFFR alone and with the combination treatment compared to mice treated with a control antibody. Effect of treatment with anti-CD20 alone was similar to the control group. The greatest depletion of B cells in the spleen and pancreatic lymph nodes (PLN) was seen in the combined therapy group. Interestingly, a short cycle of anti-BAFFR treatment led to long term T1D prevention, but the combination therapy did not. It was hypothesized anti-BAFFR treatment led to enrichment of regulatory B cells, that are depleted after subsequent anti-CD20 treatment. Overall, the authors conclude that addition of anti-CD20 therapy is not beneficial compared to anti-BAFFR treatment alone.

In conclusion, combined treatment shows more effective B cell depletion in above described models and in SLE mouse models and this also leads to better clinical response, which makes combination treatment an interesting option in SLE in humans.

Outline of this thesis

This thesis describes the translational studies performed to better understand the role of neutrophil extracellular traps in the pathogenesis of SLE and the development of a novel strategy to target autoreactive plasma cells in order to reduce pathological autoantibodies and NETs in SLE patients.

For the studies described in this thesis, a highly sensitive method to quantify ex vivo NET release, based on confocal microscopy, was developed and is described in **chapter 2**. With this novel assay, we measured NET release upon stimulation of neutrophils by sera from patients with autoimmune disease. Chapter 2 shows that the assay is applicable in a high throughput manner and allows us to study mechanisms of NET release in autoimmune diseases. **Chapter 3** employs our novel assay describing a comparative study investigating NET formation by sera from SLE patients compared to AAV patients. We demonstrate intrinsically different mechanisms of NET formation, leading to the hypothesis that features of NET formation in these diseases are different. Most importantly, we were able to demonstrate that only in sera from SLE patients

NET formation was triggered by immune complexes. **Chapter 4** characterizes in more detail the effects of AAV serum on ex vivo NET release within a large group of GPA and MPA patients and its relation to clinical disease.

In the second part of the thesis we explored potential therapeutic strategies to reduce immune complexes in SLE, with the aim to consequently improve disease activity. In **chapter 5**, we present two refractory LN patients that were successfully treated with belimumab after rituximab, supporting the rationale of combining these two antibodies for treatment of LN. We then investigated whether this novel therapeutic approach is capable to reduce immune complex-mediated inflammation in a prospective study, described in **chapter 6, 7 and 8**. In **chapter 6**, we describe the short term results of the so-called SynBioSe study (**Syn**ergetic **B** cell **i**mmunomodulation in **S**L**E**, Figure 3). This study was developed to study the safety and feasibility of the combination treatment and to assess the effect on autoantibody production and B cell subsets in SLE patients with severe and refractory disease. In **chapter 7**, we investigated whether a novel method measuring C4d in the circulation could confirm the effective reduction of circulating immune complexes by RTX+BLM. In **chapter 8**, we expand on the long-term clinical and immunological results of the Synbiose study. Lastly, in **chapter 9**, we explored the potential of tacrolimus, a calcineurin inhibitor, as a treatment option for refractory LN patients with a meta-analysis of available studies on the use of tacrolimus in LN to help guide clinical judgement.
The final chapter, **chapter 10**, provides a summary and discussion of this thesis.

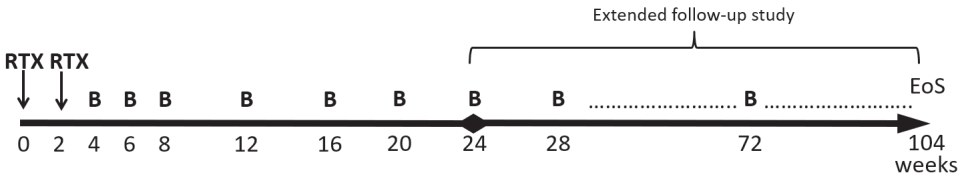


Figure 3. Flowchart Synbiose study.
RTX; Rituximab, B; Belimumab, EoS; end of study.

References

1. S. Bernatsky, J.-F. Boivin, L. Joseph, S. Manzi, E. Ginzler, D.D. Gladman, M. Urowitz, P.R. Fortin, M. Petri, S. Barr, C. Gordon, S.-C. Bae, D. Isenberg, A. Zoma, C. Aranow, M.-A. Dooley, O. Nived, G. Sturfelt, K. Steinsson, G. Alarcón, J.-L. Senécal, M. Zummer, J. Hanly, S. Ensworth, J. Pope, S. Edworthy, A. Rahman, J. Sibley, H. El-Gabalawy, T. McCarthy, Y. St Pierre, A. Clarke, R. Ramsey-Goldman, Mortality in systemic lupus erythematosus., Arthritis Rheum. 54 (2006) 2550–2557. doi:10.1002/art.21955.
2. S. Almaani, A. Meara, B.H. Rovin, Update on Lupus Nephritis., Clin. J. Am. Soc. Nephrol. 12 (2017) 825–835. doi:10.2215/CJN.05780616.
3. S. V. Parikh, B.H. Rovin, Current and Emerging Therapies for Lupus Nephritis, J. Am. Soc. Nephrol. (2016). doi:10.1681/ASN.2016040415.
4. J.G. Hanly, A.G. O'Keeffe, L. Su, M.B. Urowitz, J. Romero-Diaz, C. Gordon, S.C. Bae, S. Bernatsky, A.E. Clarke, D.J. Wallace, J.T. Merrill, D.A. Isenberg, A. Rahman, E.M. Ginzler, P. Fortin, D.D. Gladman, J. Sanchez-Guerrero, M. Petri, I.N. Bruce, M.A. Dooley, R. Ramsey-Goldman, C. Aranow, G.S. Alarcón, B.J. Fessler, K. Steinsson, O. Nived, G.K. Sturfelt, S. Manzi, M.A. Khamashta, R.F. Van Vollenhoven, A.A. Zoma, M. Ramos-Casals, G. Ruiz-Irastorza, S. Sam Lim, T. Stoll, M. Inanc, K.C. Kalunian, D.L. Kamen, P. Maddison, C.A. Peschken, S. Jacobsen, A. Askanase, C. Theriault, K. Thompson, V. Farewell, The frequency and outcome of lupus nephritis: Results from an international inception cohort study, Rheumatol. (United Kingdom). (2015). doi:10.1093/rheumatology/kev311.
5. D.Y.H. Yap, C.S.O. Tang, M.K.M. Ma, M.F. Lam, T.M. Chan, Survival analysis and causes of mortality in patients with lupus nephritis, Nephrol. Dial. Transplant. (2012). doi:10.1093/ndt/gfs073.
6. B.H. Hahn, M. a McMahon, A. Wilkinson, W.D. Wallace, D.I. Daikh, J.D. Fitzgerald, G. a Karpouzas, J.T. Merrill, D.J. Wallace, J. Yazdany, R. Ramsey-Goldman, K. Singh, M. Khalighi, S.-I. Choi, M. Gogia, S. Kafaja, M. Kamgar, C. Lau, W.J. Martin, S. Parikh, J. Peng, A. Rastogi, W. Chen, J.M. Grossman, American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis., Arthritis Care Res. (Hoboken). 64 (2012) 797–808. doi:10.1002/acr.21664.
7. G.K. Bertsias, M. Tektonidou, Z. Amoura, M. Aringer, I. Bajema, J.H.M. Berden, J. Boletis, R. Cervera, T. Dorner, a. Doria, F. Ferrario, J. Floege, F. a. Houssiau, J.P. a. Ioannidis, D. a. Isenberg, C.G.M. Kallenberg, L. Lightstone, S.D. Marks, a. Martini, G. Moroni, I. Neumann, M. Praga, M. Schneider, a. Starra, V. Tesar, C. Vasconcelos, R.F. van Vollenhoven, H. Zakharova, M. Haubitz, C. Gordon, D. Jayne, D.T. Boumpas, Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis, Ann. Rheum. Dis. 71 (2012) 1771–1782. doi:10.1136/annrheumdis-2012-201940.
8. F. Tamirou, D. D'Cruz, S. Sangle, P. Remy, C. Vasconcelos, C. Fiehn, M.D.M. Ayala Guttierez, I.-M. Gilboe, M. Tektonidou, D. Blockmans, I. Ravelingien, V. le Guern, G. Depresseux, L. Guillevin, R. Cervera, F. a. Houssiau, D. Abramowicz, F. Atzeni, M.G. Danieli, L.D. Clercq, E. de Ramon Garrido,

F.D. Keyser, M. Delahousse, G. Espinosa, M. Golstein, M. Hirsch, a. Karras, P. Lang, M. Marchal, a. Marinho, R. Max, P. Peeters, P. Petera, R. Petrovic, T. Quemeneur, F. Raeman, P. Sarzi-Puttini, L. V. de Santis, L. Verresen, L. Weiss, R. Westhovens, Long-term follow-up of the MAINTAIN Nephritis Trial, comparing azathioprine and mycophenolate mofetil as maintenance therapy of lupus nephritis, Ann. Rheum. Dis. (2015) 1–6. doi:10.1136/annrheumdis-2014-206897.

9. K. Pieper, B. Grimbacher, H. Eibel, B-cell biology and development, J. Allergy Clin. Immunol. 131 (2013) 959–971. doi:10.1016/j.jaci.2013.01.046.
10. D. Gatto, R. Brink, The germinal center reaction, J. Allergy Clin. Immunol. 126 (2010) 898–907. doi:10.1016/j.jaci.2010.09.007.
11. N.S. De Silva, U. Klein, Dynamics of B cells in germinal centres, Nat. Rev. Immunol. 15 (2015) 137–148. doi:10.1038/nri3804.
12. W. Hoffman, F.G. Lakkis, G. Chalasani, B cells, antibodies, and more, Clin. J. Am. Soc. Nephrol. (2016). doi:10.2215/CJN.09430915.
13. J.C. Cambier, S.B. Gauld, K.T. Merrell, B.J. Vilen, B-cell anergy: From transgenic models to naturally occurring anergic B cells?, Nat. Rev. Immunol. (2007). doi:10.1038/nri2133.
14. M. Lech, H.-J. Anders, The Pathogenesis of Lupus Nephritis, J Am Soc Nephrol. 24 (2013) 1357–1366. doi:10.1681/ASN.2013010026.
15. H.-J. Anders, B. Rovin, A pathophysiology-based approach to the diagnosis and treatment of lupus nephritis., Kidney Int. 90 (2016) 493–501. doi:10.1016/j.kint.2016.05.017.
16. H. Takagi, K. Arimura, T. Uto, T. Fukaya, T. Nakamura, N. Choijookhuu, Y. Hishikawa, K. Sato, Plasmacytoid dendritic cells orchestrate TLR7-mediated innate and adaptive immunity for the initiation of autoimmune inflammation, Sci. Rep. (2016). doi:10.1038/srep24477.
17. S.K. Devarapu, H.J. Anders, Toll-like receptors in lupus nephritis, J. Biomed. Sci. 25 (2018). doi:10.1186/s12929-018-0436-2.
18. S.R. Christensen, J. Shupe, K. Nickerson, M. Kashgarian, R.A.A. Flavell, M.J. Shlomchik, Toll-like Receptor 7 and TLR9 Dictate Autoantibody Specificity and Have Opposing Inflammatory and Regulatory Roles in a Murine Model of Lupus, Immunity. 25 (2006) 417–428. doi:10.1016/j.immuni.2006.07.013.
19. E. Savarese, O.W. Chae, S. Trowitzsch, G. Weber, B. Kastner, S. Akira, H. Wagner, R.M. Schmid, S. Bauer, A. Krug, U1 small nuclear ribonucleoprotein immune complexes induce type I interferon in plasmacytoid dendritic cells through TLR7, Blood. 107 (2006) 3229–3234. doi:10.1182/blood-2005-07-2650.
20. T.K. Means, E. Latz, F. Hayashi, M.R. Murali, D.T. Golenbock, A.D. Luster, Human lupus autoantibody–DNA complexes activate DCs through cooperation of CD32 and TLR9, J. Clin. Invest. 115 (2005) 407–417. doi:10.1172/JCI23025.
21. 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.

22. L. Bennett, a K. Palucka, E. Arce, V. Cantrell, J. Borvak, J. Banchereau, V. Pascual, Interferon and granulopoiesis signatures in systemic lupus erythematosus blood., J. Exp. Med. 197 (2003) 711–723. doi:10.1084/jem.20021553.
23. E.C. Baechler, F.M. Batliwalla, G. Karypis, P.M. Gaffney, W.A. Ortmann, K.J. Espe, K.B. Shark, W.J. Grande, K.M. Hughes, V. Kapur, P.K. Gregersen, T.W. Behrens, Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 2610–2615. doi:10.1073/pnas.0337679100.
24. A. Migliorini, H.-J. Anders, A novel pathogenetic concept—antiviral immunity in lupus nephritis, Nat. Rev. Nephrol. 8 (2012) 183–189. doi:10.1038/nrneph.2011.197.
25. C.J. Desmet, K.J. Ishii, Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination, Nat. Rev. Immunol. 12 (2012) 479–491. doi:10.1038/nri3247.
26. H.-J.H.J.H.-J. Anders, B.H.B. Rovin, J.G. Hanly, A.G. O'Keeffe, L. Su, E. Al., C.C. Goodnow, M. Lech, H.-J.H.J.H.-J. Anders, Y. Ghodke-Puranik, T.B. Niewold, R. Aggarwal, A.L. Sestak, A. D'Sousa, E. Al., Y.J. Crow, N. Manel, A. Rahman, D.A. Isenberg, F. Hiepe, T. Dörner, A.E. Hauser, E. Al., C. Cornaby, L. Gibbons, V. Mayhew, E. Al., H.-J.H.J.H.-J. Anders, P.S. Patole, D. Zecher, R.D. Pawar, E. Al., R.D. Pawar, A. Ramanjaneyulu, O.P. Kulkarni, E. Al., A. Migliorini, H.-J.H.J.H.-J. Anders, S.A. Chung, E.E. Brown, A.H. Williams, E. Al., H.-J.H.J.H.-J. Anders, A.B. Fogo, B.I. Freedman, C.D. Langefeld, K.K. Andringa, E. Al., A. Chang, S.G. Henderson, D. Brandt, E. Al., R. Allam, H.-J.H.J.H.-J. Anders, C. Kurts, U. Panzer, H.-J.H.J.H.-J. Anders, E. Al., J.J. Weening, V.D. D'Agati, M.M. Schwartz, E. Al., H.-J.H.J.H.-J. Anders, J.J. Weening, D. Song, L.H. Wu, F.M. Wang, E. Al., A. Vivante, F. Hildebrandt, H.-J.H.J.H.-J. Anders, D.R. Jayne, B.H.B. Rovin, A. Malvar, P. Pirruccio, B. Lococo, E. Al., A. Zickert, B. Sundelin, E. Svenungsson, I. Gunnarsson, A.S. Alvarado, A. Malvar, B. Lococo, E. Al., K. Kopetschke, J. Klocke, A.-S. Grießbach, E. Al., H.I. Brunner, M.R. Bennett, R. Mina, E. Al., A. Alsuwaida, S. Husain, M. Alghonaim, E. Al., K.D.I.G.O. (KDIGO), B.H.B. Rovin, I.E. Stillman, D. Wofsy, J.L. Hillson, B. Diamond, R. Furie, K. Nicholls, T.T. Cheng, E. Al., M. Dall'Era, M.G. Cisternas, D.E. Smilek, E. Al., F. Tamirou, B.R. Lauwerys, M. Dall'Era, E. Al., F.A. Houssiau, C. Vasconcelos, D. D'Cruz, E. Al., B.H.B. Rovin, S.V. Parikh, N. Maroz, M.S. Segal, L. Henderson, P. Masson, J.C. Craig, E. Al., E.V. Lourenco, M. Wong, B.C. Hahn, E. Al., D.R. Jayne, G.B. Appel, D.T.M. Chan, E. Al., S. Manzi, J. Sánchez-Guerrero, J.T. Merrill, E. Al., M.A. Dooley, F.A. Houssiau, C. Aranow, E. Al., G. AT, R. Furie, J.T. Merrill, V. Werth, E. Al., N. Hainz, S. Thomas, K. Neubert, E. Al., K. Neubert, S. Meister, K. Moser, E. Al., M.M. Seavey, L.D. Lu, K.L. Stump, E. Al., N.J. Jansen, W. van Oeveren, M. van Vliet, E. Al., A. Singh, R. Ghosh, P. Kaur, E. Al., H.-J.H.J.H.-J. Anders, M. Weidenbusch, B.H.B. Rovin, A pathophysiology-based approach to the diagnosis and treatment of lupus nephritis, Kidney Int. 90 (2016) 252–262. doi:10.1016/j.kint.2016.05.017.

27. E.M. Tan, P.H. Schur, R.I. Carr, H.G. Kunkel, Deoxyribonucleic Acid (DNA) and Antibodies to DNA in the Serum of Patients with Systemic Lupus Erythematosus*, 1966. https://dm5migu4zj3pb.cloudfront.net/manuscripts/105000/105479/JCI66105479.pdf (accessed July 6, 2019).
28. A. Mahajan, M. Herrmann, L.E. Muñoz, Clearance Deficiency and Cell Death Pathways: A Model for the Pathogenesis of SLE, Front. Immunol. 7 (2016) 35. doi:10.3389/fimmu.2016.00035.
29. L.E. Munoz, U.S. Gaipl, S. Franz, A. Sheriff, R.E. Voll, J.R. Kalden, M. Herrmann, SLE - A disease of clearance deficiency?, in: Rheumatology, 2005: pp. 1101–1107. doi:10.1093/rheumatology/keh693.
30. M. Herrmann, R.E. Voll, O.M. Zoller, M. Hagenhofer, B.B. Ponner, J.R. Kalden, Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus, Arthritis Rheum. 41 (1998) 1241–1250. doi:10.1002/1529-0131(199807)41:7<1241::AID-ART15>3.0.CO;2-H.
31. U.S. Gaipl, L.E. Munoz, G. Grossmayer, K. Lauber, S. Franz, K. Sarter, R.E. Voll, T. Winkler, A. Kuhn, J. Kalden, P. Kern, M. Herrmann, Clearance deficiency and systemic lupus erythematosus (SLE), J. Autoimmun. 28 (2007) 114–121. doi:10.1016/J.JAUT.2007.02.005.
32. L. a Casciola-Rosen, G. Anhalt, a Rosen, Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes., J. Exp. Med. 179 (1994) 1317–1330. doi:10.1084/jem.179.4.1317.
33. M. Radic, T. Marion, M. Monestier, Nucleosomes Are Exposed at the Cell Surface in Apoptosis, J. Immunol. (2004). doi:10.4049/jimmunol.172.11.6692.
34. J.W.C. Dieker, J. van der Vlag, J.H.M. Berden, Deranged removal of apoptotic cells: Its role in the genesis of lupus, Nephrol. Dial. Transplant. (2004). doi:10.1093/ndt/gfg485.
35. L. Schejbel, L. Skattum, S. Hagelberg, A. Åhlin, B. Schiller, S. Berg, F. Genel, L. Truedsson, P. Garred, Molecular basis of hereditary C1q deficiency—revisited: identification of several novel disease-causing mutations, Genes Immun. 12 (2011) 626–634. doi:10.1038/gene.2011.39.
36. J. Leffler, A.A. Bengtsson, A.M. Blom, The complement system in systemic lupus erythematosus: An update, Ann. Rheum. Dis. 73 (2014) 1601–1606. doi:10.1136/annrheumdis-2014-205287.
37. M.C. Pickering, M. Botto, Are anti-C1q antibodies different from other SLE autoantibodies?, Nat. Rev. Rheumatol. (2010). doi:10.1038/nrrheum.2010.56.
38. M. Trendelenburg, Antibodies against C1q in patients with systemic lupus erythematosus, Springer Semin. Immunopathol. 27 (2005) 276–285. doi:10.1007/s00281-005-0007-y.
39. M. Trendelenburg, M. Lopez-Trascasa, E. Potlukova, S. Moll, S. Regenass, V. Fremeaux-Bacchi, J. Martinez-Ara, E. Jancova, M.L. Picazo, E. Honsova, V. Tesar, S. Sadallah, J. Schifferli, High prevalence of anti-C1q antibodies in biopsy-proven active lupus nephritis, Nephrol. Dial. Transplant. 21 (2006) 3115–3121. doi:10.1093/ndt/gfl436.
40. C. Siegert, M. Daha, M.L. Westedt, E. Van der Voort, F. Breedveld, IgG autoantibodies against C1q are correlated with nephritis, hypocomplementemia, and dsDNA antibodies in systemic lupus erythematosus, J. Rheumatol. 18 (1991) 230–234.

41. L.A. Trouw, T.W.L. Groeneveld, M.A. Seelen, J.M.G.J. Duijs, I.M. Bajema, F.A. Prins, U. Kishore, D.J. Salant, J.S. Verbeek, C. van Kooten, M.R. Daha, Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes, J. Clin. Invest. 114 (2004) 679–688. doi:10.1172/jci21075.
42. M. Lech, H.-J. Anders, The Pathogenesis of Lupus Nephritis, J. Am. Soc. Nephrol. 24 (2013) 1357–1366. doi:10.1681/ASN.2013010026.
43. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria., Science (80-.). 303 (2004) 1532–1535. doi:10.1126/science.1092385.
44. J.S. Knight, C. Carmona-Rivera, M.J. Kaplan, Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases, Front. Immunol. 3 (2012). doi:10.3389/fimmu.2012.00380.
45. T.A. Fuchs, U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann, A. Zychlinsky, Novel cell death program leads to neutrophil extracellular traps., J. Cell Biol. 176 (2007) 231–41. doi:10.1083/jcb.200606027.
46. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria., Science. 303 (2004) 1532–1535. doi:10.1126/science.1092385.
47. C. Schorn, C. Janko, M. Latzko, R. Chaurio, G. Schett, M. Herrmann, Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells, Front. Immunol. 3 (2012). doi:10.3389/fimmu.2012.00277.
48. B.G. Yipp, P. Kubes, NETosis: how vital is it?, Blood. 122 (2013) 2784–94. doi:10.1182/blood-2013-04-457671.
49. F.H. Pilsczek, D. Salina, K.K.H. Poon, C. Fahey, B.G. Yipp, C.D. Sibley, S.M. Robbins, F.H.Y. Green, M.G. Surette, M. Sugai, M.G. Bowden, M. Hussain, K. Zhang, P. Kubes, A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus., J. Immunol. 185 (2010) 7413–7425. doi:10.4049/jimmunol.1000675.
50. A. Hakkim, T.A. Fuchs, N.E. Martinez, S. Hess, H. Prinz, A. Zychlinsky, H. Waldmann, Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation, Nat. Chem. Biol. 7 (2011) 75–77. doi:10.1038/nchembio.496.
51. M. Leshner, S. Wang, C. Lewis, H. Zheng, X.A. Chen, L. Santy, Y. Wang, PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures, Front. Immunol. 3 (2012). doi:10.3389/fimmu.2012.00307.
52. Y. Wang, M. Li, S. Stadler, S. Correll, P. Li, D. Wang, R. Hayama, L. Leonelli, H. Han, S.A. Grigoryev, C.D. Allis, S.A. Coonrod, Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation, J. Cell Biol. 184 (2009) 205–213. doi:10.1083/jcb.200806072.

53. 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.

54. 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.

55. 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.

56. R. Khandpur, C. Carmona-Rivera, A. Vivekanandan-Giri, A. Gizinski, S. Yalavarthi, J.S. Knight, S. Friday, S. Li, R.M. Patel, V. Subramanian, P. Thompson, P. Chen, D.A. Fox, S. Pennathur, M.J. Kaplan, NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis, Sci. Transl. Med. 5 (2013). doi:10.1126/scitranslmed.3005580.

57. 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.

58. C. Lood, L.P. Blanco, M.M. Purmalek, C. Carmona-Rivera, S.S. De Ravin, C.K. Smith, H.L. Malech, J.A. Ledbetter, K.B. Elkon, M.J. Kaplan, Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease, Nat. Med. (2016) 1–11. doi:10.1038/nm.4027.

59. 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.

60. 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.

61. D. Nakazawa, H. Shida, U. Tomaru, M. Yoshida, S. Nishio, T. Atsumi, A. Ishizu, Enhanced Formation and Disordered Regulation of NETs in Myeloperoxidase-ANCA-Associated Microscopic Polyangiitis., J. Am. Soc. Nephrol. (2014) 1–8. doi:10.1681/ASN.2013060606.

62. M.F. Denny, S. Yalavarthi, W. Zhao, S.G. Thacker, M. Anderson, A.R. Sandy, W.J. McCune, M.J. Kaplan, A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs., J. Immunol. 184 (2010) 3284–3297. doi:10.4049/jimmunol.0902199.

63. S. Zhang, X. Lu, X. Shu, X. Tian, H. Yang, W. Yang, Y. Zhang, G. Wang, Elevated Plasma cfDNA May be Associated with Active Lupus Nephritis and Partially Attributed to Abnormal Regulation of Neutrophil Extracellular Traps (NETs) in Patients with Systemic Lupus Erythematosus, Intern. Med. (2014). doi:10.2169/internalmedicine.53.2570.

64. 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.

65. A. Bodaño, J. Amarelo, A. González, J.J. Gómez-Reino, C. Conde, Novel *DNASE I* mutations related to systemic lupus erythematosus, Arthritis Rheum. 50 (2004) 4070–4071. doi:10.1002/art.20721.

66. J. Leffler, M. Martin, B. Gullstrand, H. Tyden, 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) 3522–3531. doi:10.4049/jimmunol.1102404.

67. C.G.M. Kallenberg, P. Heeringa, C.A. Stegeman, Mechanisms of disease: Pathogenesis and treatment of ANCA-associated vasculitides, Nat. Clin. Pract. Rheumatol. 2 (2006) 661–670. doi:10.1038/ncprheum0355.

68. R.A. Watts, S. Elane, D.G.I. Scott, Epidemiology of vasculitis in Europe [4], Ann. Rheum. Dis. (2001). doi:10.1136/ard.60.12.1156.

69. R.A. Watts, A. Mahr, A.J. Mohammad, P. Gatenby, N. Basu, L.F. Flores-Suárez, Classification, epidemiology and clinical subgrouping of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, Nephrol. Dial. Transplant. 30 (2015) i14–i22. doi:10.1093/ndt/gfv022.

70. G. Moroni, C. Ponticelli, Rapidly progressive crescentic glomerulonephritis: Early treatment is a must, Autoimmun. Rev. 13 (2014) 723–729. doi:10.1016/j.autrev.2014.02.007.

71. H. Xiao, P. Heeringa, P. Hu, Z. Liu, M. Zhao, Y. Aratani, N. Maeda, R.J. Falk, J. Charles Jennette, Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice, J. Clin. Invest. 110 (2002) 955–963. doi:10.1172/JCI200215918.

72. R.J. Falk, R.S. Terrell, L. a Charles, J.C. Jennette, Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro., Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4115–4119. doi:10.1073/pnas.87.11.4115.

73. D.J. Radford, C.O.S. Savage, G.B. Nash, Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion, Arthritis Rheum. 43 (2000) 1337–1345. doi:10.1002/1529-0131(200006)43:6<1337::AID-ANR16>3.0.CO;2-M.

74. S. Sangaletti, C. Tripodo, C. Chiodoni, C. Guarnotta, B. Cappetti, P. Casalini, S. Piconese, M. Parenza, C. Guiducci, C. Vitali, M.P. Colombo, Neutrophil extracellular traps mediate transfer of cytoplasmic

neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity, Blood. 120 (2012) 3007–3018. doi:10.1182/blood-2012-03-416156.

75. K.M. O'Sullivan, C.Y. Lo, S.A. Summers, K.D. Elgass, P.J. McMillan, A. Longano, S.L. Ford, P.Y. Gan, P.G. Kerr, A.R. Kitching, S.R. Holdsworth, Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis, Kidney Int. 88 (2015) 1030–1046. doi:10.1038/ki.2015.202.
76. M. Saffarzadeh, C. Juenemann, M.A. Queisser, G. Lochnit, G. Barreto, S.P. Galuska, J. Lohmeyer, K.T. Preissner, Neutrophil extracellular traps directly induce epithelial and endothelial cell death: A predominant role of histones, PLoS One. 7 (2012). doi:10.1371/journal.pone.0032366.
77. 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.
78. D. Nakazawa, H. Shida, U. Tomaru, M. Yoshida, S. Nishio, T. Atsumi, A. Ishizu, Enhanced Formation and Disordered Regulation of NETs in Myeloperoxidase-ANCA-Associated Microscopic Polyangiitis., J. Am. Soc. Nephrol. (2014) 1–8. doi:10.1681/ASN.2013060606.
79. A. Fanouriakis, M. Kostopoulou, A. Alunno, M. Aringer, I. Bajema, J.N. Boletis, R. Cervera, A. Doria, C. Gordon, M. Govoni, F. Houssiau, D. Jayne, M. Kouloumas, A. Kuhn, J.L. Larsen, K. Lerstrøm, G. Moroni, M. Mosca, M. Schneider, J.S. Smolen, E. Svenungsson, V. Tesar, A. Tincani, A. Troldborg, R. Van Vollenhoven, J. Wenzel, G. Bertsias, D.T. Boumpas, 2019 Update of the EULAR recommendations for the management of systemic lupus erythematosus, Ann. Rheum. Dis. 78 (2019) 736–745. doi:10.1136/annrheumdis-2019-215089.
80. 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.
81. 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.
82. M.E.B. Clowse, D.J. Wallace, R.A. Furie, M.A. Petri, M.C. Pike, P. Leszczyński, C.M. Neuwelt, K. Hobbs, M. Keiserman, L. Duca, K.C. Kalunian, C. Galateanu, S. Bongardt, C. Stach, C. Beaudot, B. Kilgallen, C. Gordon, EMBODY Investigator Group, Efficacy and Safety of Epratuzumab in Moderately to Severely Active Systemic Lupus Erythematosus: Results From Two Phase III Randomized, Double-Blind, Placebo-Controlled Trials., Arthritis Rheumatol. (Hoboken, N.J.). 69 (2017) 362–375. doi:10.1002/art.39856.

83. J.T. Merrill, D.J. Wallace, S. Wax, A. Kao, P.A. Fraser, P. Chang, D. Isenberg, Efficacy and Safety of Atacicept in Patients With Systemic Lupus Erythematosus, Arthritis Rheumatol. 70 (2018) 266–276. doi:10.1002/art.40360.
84. S. V Navarra, R.M. Guzman, a E. Gallacher, S. Hall, R. a Levy, R.E. Jimenez, E.K.-M. Li, M. Thomas, H.Y. Kim, M.G. Leon, C. Tanasescu, E. Nasonov, J.L. Lan, L. Pineda, Z.J. Zhong, W. Freimuth, M. a Petri, Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: A randomised, placebo-controlled, phase 3 trial2774, Lancet. 377 (2011) 721–731. doi:10.1016/S0140-6736(10)61354-2.
85. T. Alexander, R. Sarfert, J. Klotsche, A.A. Kühl, A. Rubbert-Roth, H.M. Lorenz, J. Rech, B.F. Hoyer, Q. Cheng, A. Waka, A. Taddeo, M. Wiesener, G. Schett, G.R. Burmester, A. Radbruch, F. Hiepe, R.E. Voll, The proteasome inhibitior bortezomib depletes plasma cells and ameliorates clinical manifestations of refractory systemic lupus erythematosus, Ann. Rheum. Dis. (2015). doi:10.1136/annrheumdis-2014-206016.
86. F.B. Vincent, E.F. Morand, P. Schneider, F. Mackay, The BAFF/APRIL system in SLE pathogenesis, Nat. Rev. Rheumatol. 10 (2014) 365–373. doi:10.1038/nrrheum.2014.33.
87. F. Mackay, S.A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, J.L. Browning, Mice Transgenic for Baff Develop Lymphocytic Disorders along with Autoimmune Manifestations, J. Exp. Med. 190 (1999) 1697–1710. doi:10.1084/jem.190.11.1697.
88. J. Gross, J. Johnston, S. Mudri, R. Enselman, S.D.- Nature, undefined 2000, TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease, Nature.com. (n.d.). https://www.nature.com/articles/35010115 (accessed July 7, 2019).
89. G.Ø. Eilertsen, M. Van Ghelue, H. Strand, J.C. Nossent, Increased levels of BAFF in patients with systemic lupus erythematosus are associated with acute-phase reactants, independent of BAFF genetics: a case–control study, Rheumatology. 50 (2011) 2197–2205. doi:10.1093/rheumatology/ker282.
90. 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.
91. 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.
92. 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.
93. G. Cambridge, D.A. Isenberg, J.C.W. Edwards, M.J. Leandro, T.-S. Migone, M. Teodorescu, W. Stohl, B cell depletion therapy in systemic lupus erythaematosus: relationships among serum B lymphocyte stimulator levels, autoantibody profile and clinical response, Ann. Rheum. Dis. 67 (2008) 1011–1016. doi:10.1136/ard.2007.079418.

94. F.B. Vincent, E.F. Morand, P. Schneider, F. Mackay, The BAFF/APRIL system in SLE pathogenesis, Nat. Rev. Rheumatol. 10 (2014) 365–373. doi:10.1038/nrrheum.2014.33.
95. W. Stohl, Future prospects in biologic therapy for systemic lupus erythematosus, Nat. Rev. Rheumatol. 9 (2013) 705–720. doi:10.1038/nrrheum.2013.136.
96. W. Stohl, F. Hiepe, K.M. Latinis, M. Thomas, M.A. Scheinberg, A. Clarke, C. Aranow, F.R. Wellborne, C. Abud-Mendoza, D.R. Hough, L. Pineda, T.S. Migone, Z.J. Zhong, W.W. Freimuth, W.W. Chatham, Belimumab reduces autoantibodies, normalizes low complement levels, and reduces select B cell populations in patients with systemic lupus erythematosus, Arthritis Rheum. 64 (2012) 2328–2337. doi:10.1002/art.34400.
97. M.A. Dooley, F. Houssiau, C. Aranow, D.P. D'Cruz, A. Askanase, D.A. Roth, Z.J. Zhong, S. Cooper, W.W. Freimuth, E.M. Ginzler, Effect of belimumab treatment on renal outcomes: results from the phase 3 belimumab clinical trials in patients with SLE., Lupus. 22 (2013) 63–72. doi:10.1177/0961203312465781.
98. R.F. Van Vollenhoven, M.A. Petri, R. Cervera, D.A. Roth, B.N. Ji, C.S. Kleoudis, Z.J. Zhong, W. Freimuth, Belimumab in the treatment of systemic lupus erythematosus: High disease activity predictors of response, Ann. Rheum. Dis. (2012). doi:10.1136/annrheumdis-2011-200937.
99. W. Stohl, A. Schwarting, M. Okada, M. Scheinberg, A. Doria, A.E. Hammer, C. Kleoudis, J. Groark, D. Bass, N.L. Fox, D. Roth, D. Gordon, Efficacy and Safety of Subcutaneous Belimumab in Systemic Lupus Erythematosus: A Fifty-Two–Week Randomized, Double-Blind, Placebo-Controlled Study, Arthritis Rheumatol. 69 (2017) 1016–1027. doi:10.1002/art.40049.
100. A. Doria, W. Stohl, A. Schwarting, M. Okada, M. Scheinberg, R. van Vollenhoven, A.E. Hammer, J. Groark, D. Bass, N.L. Fox, D. Roth, D. Gordon, Efficacy and Safety of Subcutaneous Belimumab in Anti–Double-Stranded DNA–Positive, Hypocomplementemic Patients With Systemic Lupus Erythematosus, Arthritis Rheumatol. 70 (2018) 1256–1264. doi:10.1002/art.40511.
101. D.A. Isenberg, Rituximab-it was the best of times, it was the worst of times, Autoimmun. Rev. (2012). doi:10.1016/j.autrev.2012.02.005.
102. M. Weidenbusch, C. Römmele, A. Schröttle, H.J. Anders, Beyond the LUNAR trial. Efficacy of rituximab in refractory lupus nephritis, Nephrol. Dial. Transplant. (2013). doi:10.1093/ndt/gfs285.
103. G.B. Appel, G. Contreras, M.A. Dooley, E.M. Ginzler, D. Isenberg, D. Jayne, L.-S. Li, E. Mysler, J. Sánchez-Guerrero, N. Solomons, D. Wofsy, Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis., J. Am. Soc. Nephrol. 20 (2009) 1103–1112. doi:10.1681/ASN.2008101028.
104. R. Pepper, M. Griffith, C. Kirwan, J. Levy, D. Taube, C. Pusey, L. Lightstone, T. Cairns, Rituximab is an effective treatment for lupus nephritis and allows a reduction in maintenance steroids, Nephrol. Dial. Transplant. (2009). doi:10.1093/ndt/gfp336.
105. M.B. Condon, D. Ashby, R.J. Pepper, H.T. Cook, J.B. Levy, M. Griffith, T.D. Cairns, L. Lightstone, Prospective observational single-centre cohort study to evaluate the effectiveness of treating lupus

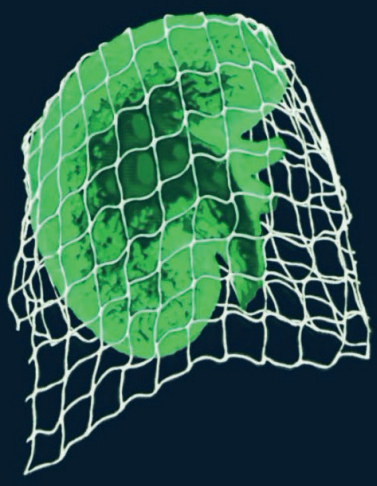
nephritis with rituximab and mycophenolate mofetil but no oral steroids, Ann. Rheum. Dis. (2013). doi:10.1136/annrheumdis-2012-202844.

106. S. Iwata, K. Saito, S. Hirata, N. Ohkubo, S. Nakayamada, K. Nakano, K. Hanami, S. Kubo, I. Miyagawa, M. Yoshikawa, Y. Miyazaki, H. Yoshinari, Y. Tanaka, Efficacy and safety of anti-CD20 antibody rituximab for patients with refractory systemic lupus erythematosus, Lupus. 27 (2018) 802–811. doi:10.1177/0961203317749047.
107. R. Aguiar, C. Araújo, G. Martins-Coelho, D. Isenberg, Use of Rituximab in Systemic Lupus Erythematosus: A Single Center Experience Over 14 Years, Arthritis Care Res. (2017). doi:10.1002/acr.22921.
108. 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.
109. F. Lavie, C. Miceli-Richard, M. Ittah, … J.S.-A. of the, undefined 2007, Increase of B cell-activating factor of the TNF family (BAFF) after rituximab treatment: insights into a new regulating system of BAFF production, Ard.bmj.com. (n.d.). https://ard.bmj.com/content/66/5/700.short (accessed July 11, 2019).
110. R. Gualtierotti, M.O. Borghi, M. Gerosa, T. Schioppo, P. Larghi, J. Geginat, P.L. Meroni, Successful sequential therapy with rituximab and belimumab in patients with active systemic lupus erythematosus: a case series., Clin. Exp. Rheumatol. 36 (n.d.) 643–647. http://www.ncbi.nlm.nih.gov/pubmed/29533753 (accessed February 22, 2019).
111. F. Lavie, C. Miceli-Richard, M. Ittah, J. Sellam, J.E. Gottenberg, X. Mariette, Increase of B cell-activating factor of the TNF family (BAFF) after rituximab treatment: Insights into a new regulating system of BAFF production, Ann. Rheum. Dis. 66 (2007) 700–703. doi:10.1136/ard.2006.060772.
112. Y. Saito, Y. Miyagawa, K. Onda, H. Nakajima, B. Sato, Y. Horiuchi, H. Okita, Y.U. Katagiri, M. Saito, T. Shimizu, J. Fujimoto, N. Kiyokawa, B-cell-activating factor inhibits CD20-mediated and B-cell receptor-mediated apoptosis in human B cells, Immunology. 125 (2008) 570–590. doi:10.1111/j.1365-2567.2008.02872.x.
113. Q. Gong, Q. Ou, S. Ye, W.P. Lee, J. Cornelius, L. Diehl, W.Y. Lin, Z. Hu, Y. Lu, Y. Chen, Y. Wu, Y.G. Meng, P. Gribling, Z. Lin, K. Nguyen, T. Tran, Y. Zhang, H. Rosen, F. Martin, A.C. Chan, Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy, J. Immunol. (Baltimore, Md 1950). 174 (2005) 817–826. doi:10.4049/jimmunol.174.2.817.
114. W. Lin, D. Seshasayee, W.P. Lee, P. Caplazi, S. McVay, E. Suto, A. Nguyen, Z. Lin, Y. Sun, L. Deforge, M. Balazs, F. Martin, A.A. Zarrin, Dual B cell immunotherapy is superior to individual Anti-CD20 depletion or baff blockade in murine models of spontaneous or accelerated lupus, Arthritis Rheumatol. 67 (2015) 215–224. doi:10.1002/art.38907.
115. K.W. Bekar, T. Owen, R. Dunn, T. Ichikawa, W. Wang, R. Wang, J. Barnard, S. Brady, S. Nevarez, B.I. Goldman, M. Kehry, J.H. Anolik, Prolonged effects of short-term anti-CD20 B cell depletion therapy in murine systemic lupus erythematosus, Arthritis Rheum. 62 (2010) 2443–2457. doi:10.1002/art.27515.

116. Q. Wang, J.J. Racine, D. V Serreze, C. Wasserfall, M.A. Atkinson, J.J. Ratiu, R. Ettinger, S. Wang, Transient BAFF Blockade Inhibits Type 1 Diabetes Development in Nonobese Diabetic Mice by Enriching Immunoregulatory B Lymphocytes Sensitive to Deletion by Anti-CD20 Cotherapy, J. Immunol. 199 (2017) 3757–3770. doi:10.4049/jimmunol.1700822.

PART 1

Neutrophil extracellular traps in autoimmune disease



Chapter 2

A novel method for high-throughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes

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Abstract

A newly-described first-line immune defense mechanism of neutrophils is the release of neutrophil extracellular traps (NETs). Immune complexes (ICxs) induce low level NET release. As such, the in vitro quantification of NETs is challenging with current methodologies. In order to investigate the role of NET release in ICx-mediated auto-immune diseases, we developed a highly sensitive and automated method for quantification of NETs. After labelling human neutrophils with PKH26 and extracellular DNA with Sytox green, cells are fixed and automatically imaged with 3-dimensional confocal laser scanning microscopy (3D-CLSM). NET release is then quantified with digital image analysis whereby the NET amount (Sytox green area) is corrected for the number of imaged neutrophils (PKH26 area). A high sensitivity of the assay is achieved by a) significantly augmenting the area of the well imaged (11%) as compared to conventional assays (0.5%) and b) using a 3D imaging technique for optimal capture of NETs, which are topologically superimposed on neutrophils. In this assay, we confirmed low levels of NET release upon human ICx stimulation which were positive for citrullinated histones and neutrophil elastase. In contrast to PMA-induced NET release, ICx-induced NET release was unchanged when co-incubated with diphenyleneiodonium (DPI). We were able to quantify NET release upon stimulation with serum from RA and SLE patients, which was not observed with normal human serum. To our knowledge, this is the first semi-automated assay capable of sensitive detection and quantification of NET release at a low threshold by using 3D CLSM. The assay is applicable in a high-throughput manner and allows the in vitro analysis of NET release in ICx-mediated autoimmune diseases.

Introduction

Neutrophil extracellular traps (NETs) are extracellular strands of DNA that are expulsed upon the interaction of neutrophils with infectious pathogens such as Staphylococcus aureus [1], Salmonella typhi, Shigella flexneri [2], Candida albicans [3] and Aspergillus fumigatus [4]. The extracellular NET fibers were demonstrated to be composed of nuclear chromatin, citrullinated histones and multiple granular antimicrobial and cytoplasmic proteins like myeloperoxidase (MPO) and neutrophil elastase (NE) [2]. The physiological function of NETs is the entrapment and subsequent elimination of these pathogens. NETs are therefore considered as part of a first line defense mechanism and, as such, the antimicrobial armamentarium of neutrophils consists of phagocytosis, degranulation of lysozymes and extrusion of NETs.

The discovery of NETs as a source of extracellular DNA has triggered several investigations to study its role in autoimmune diseases (AIDs), because extracellular DNA is an established trigger of autoimmunity [5]. NETs are postulated to have a potentially pathogenic role in ANCA-associated small-vessel vasculitis [6,7], systemic lupus erythematosus [8,9] and rheumatoid arthritis [10]. Moreover, reduced degradation of NETs was associated with more severe disease in AID patients [11,12]. Because almost any pathogen can induce NET formation, it seems unlikely that in light of the many (subclinical) microbial attacks that humans continuously face, every 'infection' also potentially induces an autoimmune response. Therefore, it is relevant to identify non-infectious triggers of NET formation which contribute to increased NETosis as underpinning pathogenic mechanisms of autoimmunity.

To address the issue of NET-inducing triggers, current studies are hampered by the lack of a reliable and sensitive quantification method of NET formation. In contrast to PMA-induced NETosis which usually leads to an overwhelming extrusion of DNA, physiological stimuli like immune complexes (ICxs)) induce more subtle and lower levels of NET formation [13]. Currently, two methods are commonly used to quantify NET release in vitro: DNA measurements in supernatant [7,8,14] or demonstration of extracellular DNA by immunocytochemistry [6,15–17]. With respect to the first method, DNA measurements are straightforward and objective but not specific for NETs. Also, several molecules, most importantly immunoglobulins, can interfere with fluorescence measurements [18]. With respect to the second method, quantification based on immunocytochemistry is heterogeneously reported by number of netting neutrophils, neutrophils with decondensed nuclei or area of extracellular DNA after image analysis. This method is prone to subjectivity of the observer and is quite labor intensive. Therefore, the aim of the present study was to develop a highly sensitive, objective and reproducible NET quantification assay applicable in a high-throughput system. Here, we describe how ICx-induced NET formation is sensitively detected and quantified by a semi-automated, confocal laser scanning microscopy (CLSM) 3D-imaging technique.

Methods

Patient samples

All serum samples were obtained after informed consent. Peripheral blood was obtained without anticoagulants and serum samples were stored at −80 until use. Twenty-seven rheumatoid arthritis (RA) and 20 systemic lupus erythematosus (SLE) serum samples were collected. Six normal human serum (NHS) samples, acquired from a healthy donor bank, were used as a control. 10% serum was used in the assay and each sample was tested in triplicate within one experiment.

Preparation of neutrophils

Twenty milliliters of whole blood was collected in EDTA-coated tubes. Neutrophils were isolated by density gradient centrifugation with Ficoll-amidotrizoaat (LUMC, Leiden, The Netherlands) followed by erythrocyte lysis. Cells were counted using trypan blue, labelled with PKH26 (2 μM, Sigma-Aldrich, Saint-Louis, MO, USA) and then 3.75 $\times$ 104 neutrophils were seeded per well into a 96-well culture plate in a phenol red-free RPMI 1640 medium (Life Technologies, The Netherlands) supplemented with 2% heat-inactivated fetal calf serum (FCS). Neutrophils were stimulated during 3:45 h with any given stimulus. Stimuli used were medium (negative control), intravenous immunoglobulins (IVIG) (Nanogam 50 mg/mL; Sanquin, Amsterdam, The Netherlands), purified IgG, heat-aggregated IgG immune complexes or phorbol myristate acetate (PMA) (Sigma-Aldrich). For NADPH oxidase inhibition, 1 μM diphenyleneiodonium (DPI) (Sigma-Aldrich) was used. Hereafter, an impermeable DNA dye, 1 μM Sytox green (Life Technologies), was added for 15 min after which neutrophils were fixed with 4% formaldehyde (Added Pharma).

Fluorescence immunocytochemistry of NETs

PKH-labelled neutrophils were seeded onto poly-D-lysine-coated coverslips (Corning, NY, USA) and NETs were induced according to the abovementioned protocol. Fixed neutrophils were blocked with 1% BSA and 5% normal goat serum in PBS and stained with polyclonal rabbit anti-human citrullinated histon3 (10 μg/ml, Abcam) or a mouse monoclonal anti-human citrullinated histon3 (10 μg/ml, Abcam) and polyclonal rabbit neutrophil elastase (NE) (10 μg/ml, Abcam) in PBS and incubated for 2 h. Then, neutrophils were washed and incubated with a 1/500 dilution secondary goat anti-rabbit Alexa488 antibody (Life Technologies) or with a 1/500 dilution secondary goat anti-mouse Alexa488 antibody (Life Technologies). The secondary antibodies were pre-incubated for 30 min with 10% normal human serum to bind anti-human immunoglobulins. After 60 min incubation, neutrophils were washed and stained with Hoechst 33258 (1 μg/ml, Life Technologies). Images were acquired with the Leica DMI6000 inverted microscope using a 20 $\times$ magnification.

Preparation of heat-aggregated IgG immune complexes

IgG was enriched from pooled healthy human sera by the use of a diethylaminoethyl (DEAE) cellulose anion exchange column (GE Healthcare) [19]. Samples were centrifuged over the DEAE column and subsequently the column was flushed with TRIS buffer. The eluate containing IgG was concentrated with a centrifugal filter unit with a cut-off at 30 kDa (Merck Millipore). IgG enrichment was confirmed with SDS-polyacrylamide gel electrophoresis (10% Mini-Protean TGX gel, Biorad). Briefly, the precast gel was run with non-reduced and reduced samples (reducing agent DTT, New England Biolabd) at 30 mA at room temperature. Bands were detected with Coomassie Brilliant Blue protein staining (Biorad). The obtained IgG was aggregated by heating in a water bath at 63° for 20 min at a concentration of 25 mg/ml. Then, the solution was centrifuged at 3000 rpm for 10 min and the supernatant, containing the aggregates, was loaded on a Sepharose 4B column (GE Healthcare) for fractionation of the IgG aggregates. A solution of monomeric IgG was used as a standard. Large aggregates, consisting of more than 40 IgG monomers, were collected to use in the confocal laser scanning microscopy (CLSM) NET assay. Monomeric IgG was used as a control. The IgG concentration was defined with a Pierce assay.

Visualization of neutrophil extracellular traps

Immediately following fixation, neutrophil extracellular traps (NETs) were visualized by confocal laser scanning microscopy (CLSM) using the automated BD Pathway 855 (BD Biosciences, San Jose, CA, USA). Briefly, 12 z-stacked images of 25 predefined high power fields (HPFs) at a 20 $\times$ magnification were captured. The HPFs were evenly spread throughout the well by a standardized 5 $\times$ 5 zig-zag pattern with 400 μm (length) and 500 μm (width) spaced between each high HPF.

The well area covered by the above-described microscopic imaging was calculated as follows: 1) the area of a HPF at a 20$\times$ magnification was calculated at 3.3 * 106 μm^2 (length 417.39 μm $\times$ width 318.01 μm); 2) the area of a HPF at a 40 $\times$ magnification was calculated at 0.8 * 106 μm^2 (length 210.49 μm $\times$ width 160.38 μm); 3) total area covered was calculated by multiplying the number of HPFs by the HPF-area at a given magnification. The microscope was programmed to automatically focus on PKH26 membrane staining. Then, for each image PKH26 (Cy3) and Sytox green (alexa488) were visualized. The CLSM exposure time for Sytox green was set on the positive control and for PKH26 on the negative control. The same exposure time was applied to all images in the same experiment.

Automatic digital image analysis for the quantification of NET formation

Acquired images were automatically analyzed by ImageJ image analysis software (NIH, Bethesda, MD, USA). Extracellular DNA of NETs was quantified as the cumulative area of positive Sytox green. To correct for the number of neutrophils, the mean area of positive PKH26 staining

was quantified. Thus, the ratio of both areas is calculated, representing the NET area corrected for the number of imaged neutrophils in each sample. A higher ratio indicates a larger NET area present.

Results

NETs are induced at lower levels by human immune complexes as compared to PMA

Immune complexes are considered as pathogenic compounds in human autoimmune diseases and capable of inducing NETs [20,21]. We first confirmed that human heat-aggregated IgG ICx indeed induced NET formation. Extracellular DNA was detected by immunofluorescence and we confirmed that the extracellular DNA was positive for citrullinated histones (citH3) and neutrophil elastase (NE) (Fig. 1A, B). Visually, ICx induced NET release at much lower levels compared to PMA (Fig. 1C). We were unable to pick up low levels of ICx-induced NET release compared to medium control, which was indeed the case for PMA-induced NET release (Fig. 1D). Of note, similar results were obtained when using Picogreen and Sytox green fluorescence measurements on supernatants of these conditions (data not shown). In-depth exploration to improve visualization of NETs, we noted that NETs were topologically superimposed on the neutrophils: Fig. 1E illustrates that by using stacked imaging, neutrophils were found attached to the bottom of a well while released NETs were predominantly observed on top of the neutrophils. Comparing NET quantification, we demonstrated a significant higher result for ICx-induced NET release in three-dimensional (3D) imaging (mean NET area $\pm$ SEM: 1.03 $\pm$ 0.11) as compared to 2D imaging (mean NET area $\pm$ SEM: 0.04 $\pm$ 0.01, $p = 0.01$) (Fig. 1F). Altogether, sensitivity to quantify low levels of NETs upon ICx stimulation was increased by visualizing NET release 3-dimensionally (3D).

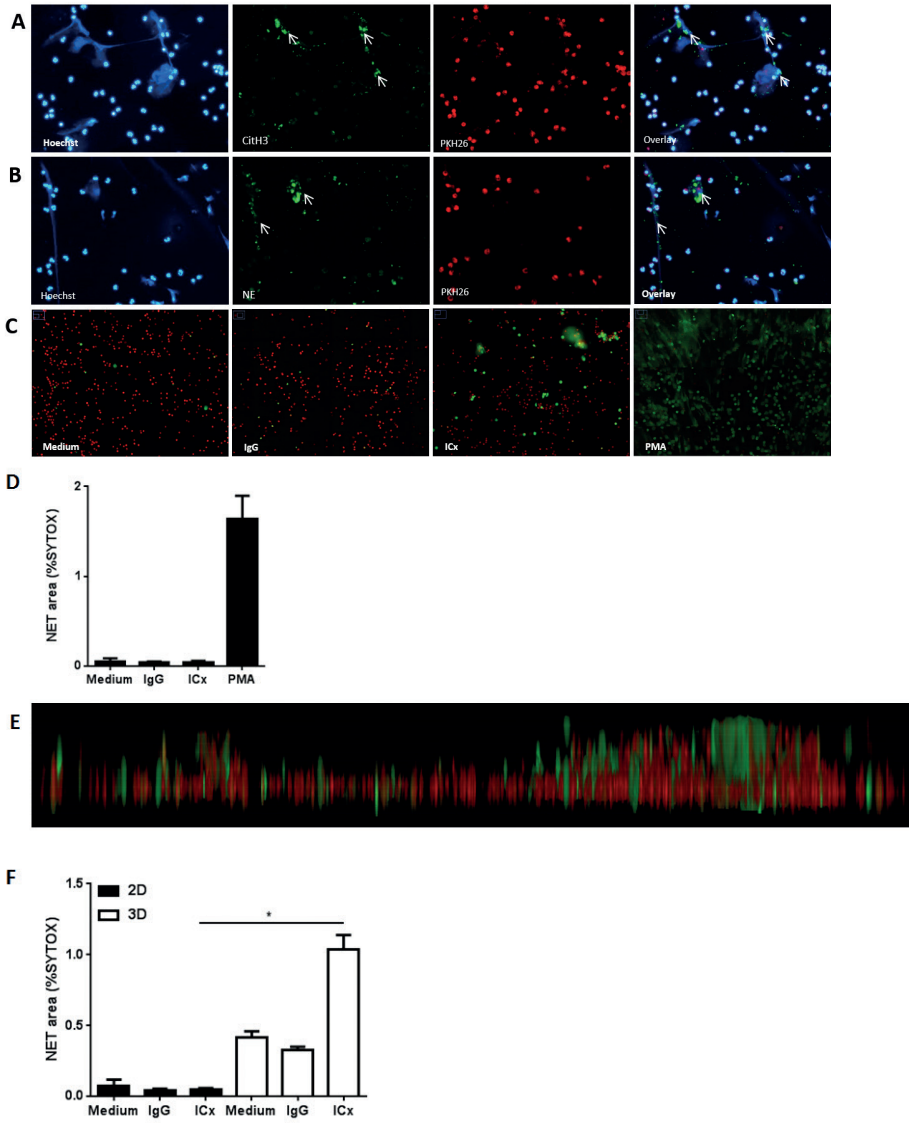

Figure 1. Human immune complexes (ICx) induce low level NET release detectable with three-dimensional CSLM. **(A)** PKH26-positive (red) neutrophils incubated with human ICx release extracellular DNA detected by Hoechst (blue) which are positive for the NET-specific marker citrullinated histon3 (citH3 — green) and, **(B)** the NET-specific marker neutrophil elastase (NE — green). Staining is representative of 3 separate experiments. **(C)** The amount of NET release is visually lower compared to PMA-stimulated NET release. PKH-labelled (red) neutrophils were stimulated for 4 h with either 12.5 μg/ml ICx or 12.5 nM PMA and NETs were stained with Sytox (green). Medium and the corresponding IgG concentration were taken along as controls. Subsequently, a montage image consisting of 25 high power fields (HPFs) was taken with confocal

microscopy of which a part is shown here. **(D)** Low level NET release induced by ICx is not detected when quantified with the % area of positive Sytox staining. **(E)** Cross-sectional overview of a 3-dimensional image using 12 z-stacks demonstrated that NETs (green) are topologically superimposed on neutrophils (red). **(F)** Quantification of NETs by percentage area of Sytox green positive DNA in a 3D z-stack image, yields significantly more NETs as compared to a single focus 2D image (p b 0.05 for ICx 12.5 μg/mL). Bars represent mean $\pm$ SEM. *$p < 0.05$. Results are representative for three experiments.

Automatization of 3D CSLM image acquisition

Because ICx induced NETs in fewer neutrophils as compared to PMA, we aimed to further augment the sensitivity of the NET quantification assay by increasing the total imaged surface to yield a higher number of neutrophils. By varying the fully automated image acquisition technique we showed that increasing number of HPFs and reducing the magnification led to larger image areas, correlating with a 10-fold increase of imaged neutrophils (Fig. 2A, B). When the 40$\times$ magnification was used, the area of the well captured is 2.8% when 25 HPFs were imaged, representing 194 $\pm$ 66 neutrophils (mean $\pm$ SEM). For optimal acquisition, 11.1% area of the well was captured when 25 HPFs were imaged with the 20 $\times$ magnification, thereby analyzing 1500 $\pm$ 247 neutrophils (mean $\pm$ SEM). We confirmed that imaging a larger well area by increasing the number of HPFs, resulted in a higher yield of neutrophils (Fig. 2C). To exclude observer subjectivity, 25 HPFs were captured in a constant pattern throughout each well, as illustrated in Fig. 2D. Captured images from 25 HPFs were stitched together to form one montage and 12 montages per well were mounted to form a 3D-image, which was used for digital image analysis (Fig. 2E and Supplemental movie). When performing image analysis, the amount of NET release is calculated by the total area of Sytox green (%), corrected for the number of imaged cells calculated by the total area of PKH26 (%). The NET area per amount of imaged neutrophil is thus calculated by the total area of NETs (Sytox positive) divided by the number of neutrophils, represented by the mean area of PKH26. Regardless of the induction of NETosis, we demonstrated that the total area of PKH26 (%) correlated significantly with neutrophil counts in unstimulated conditions ($r = 0.99$, $p < 0.0001$) and ICx-stimulated conditions ($r = 0.95$, $p < 0.0001$) (Fig. 2F, G). PKH staining is drastically decreased upon PMA stimulation (75% with 12.5 nM PMA) compared to ICx stimulation (34% with 12.5 μg/ml ICx) (Fig. 2H, I), suggesting increased neutrophil death and/or cell loss with PMA compared to ICx-induced NETosis.

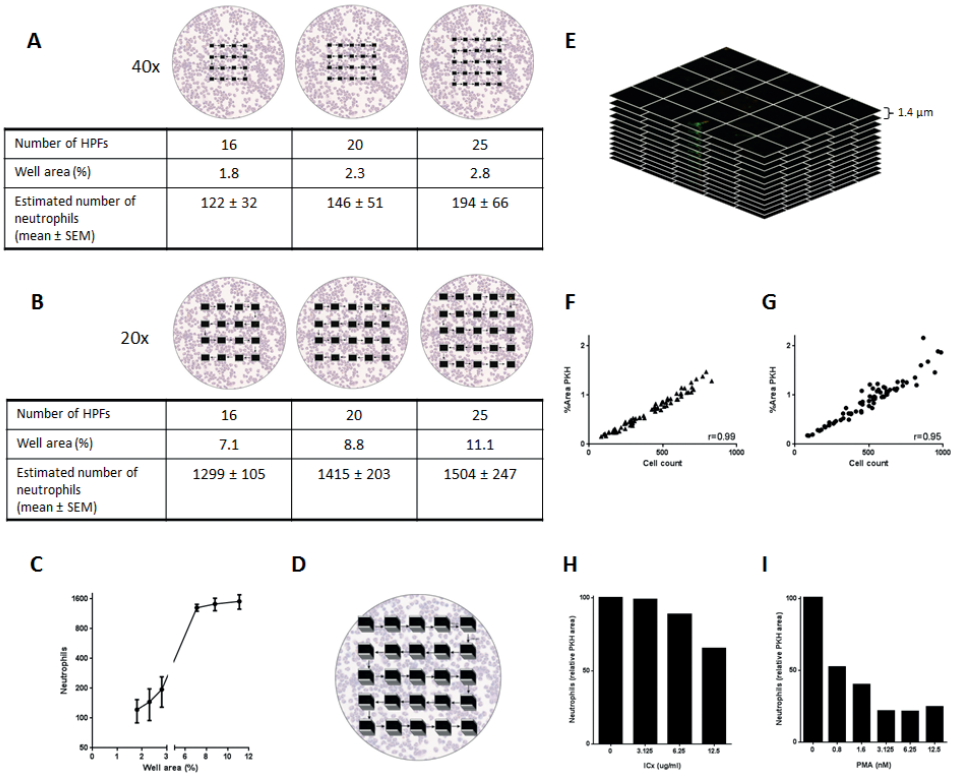

Figure 2. Automatization of 3D CSLM image acquisition. The yield of (potentially netting) neutrophils is augmented by increasing the area per well imaged. **(A)** At a 40× magnification, 3D CSLM is capable of automatically imaging up to 2.8% of each well area. **(B)** At a 20× magnification, 3D CSLM is capable of automatically imaging up to 11.1% of each well area. **(C)** Depicted is the total amount of imaged neutrophils correlated to the imaged well area. Mean ± SEM are shown. **(D)** Thus, optimal yield was achieved by automatically capturing a fixed pattern 25 high power fields (HPFs) images for each well. **(E)** For image analysis, 25 HPF images are merged to one montage resulting in a total of 12montages for each well suitable for digital image analysis. **(F)** When capturing high numbers of neutrophils, the percentage area of PKH26 correlates significantly with the number of imaged neutrophils (unstimulated). **(G)** This correlation is preserved even after ICx-induced NET release (ICx 12.5 μg/ml). Seventy-two montages were analyzed. **(H, I)** Relative in vitro neutrophil cell loss after ICx-induced NET release is lower compared to PMA-induced NETosis. The amount of neutrophil was quantified by percentage area of PKH26 positivity. Depicted is the relative reduction in percentage area.

High sensitivity quantification of ICx-induced NET release shows a ROS-independent process

Using the above-described high-sensitivity NET quantification assay, we confirmed its applicability by further investigating ICx-induced NET release (see Fig. 3). Low levels of ICx induced NET release (mean NET area per neutrophil ± SEM: 1.1 ± 0.5) were not significantly different when diphenyleneiodonium (DPI) was added (mean NET area per neutrophil ± SEM: 0.8 ± 0.2, $p > 0.05$). PMA induced large amounts of NET release (mean NET area per neutrophil ± SEM: 107 ± 5.8) which was almost completely inhibited by DPI (mean NET area per neutrophil ± SEM: 2.9 ± 0.0, $p < 0.005$). Fig. 3B confirmed these findings in cross-sectional overviews of 3-dimensional images of each condition with and without DPI. Overall, these findings indicate that human ICx induce NETs in a ROS-independent manner.

RA and SLE serum samples show NET-inducing capability compared to normal human serum

To further confirm applicability of this assay in the analysis of NET release in ICx-mediated autoimmune diseases, we next used this assay to quantify NET release induced by serum of patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). The mean NET area ± SEM per imaged neutrophil upon induction with RA serum was 0.46 ± 0.08, 0.69 ± 0.18 for SLE and 0.12 ± 0.06 for the NHS control (Fig. 4A). For RA, 18 out of 27 serum samples (67%) showed increased NET-inducing capacity compared to the NHS control and in SLE, 17 out of 20 samples (85%) showed increased NET-inducing capacity (Fig. 4B).

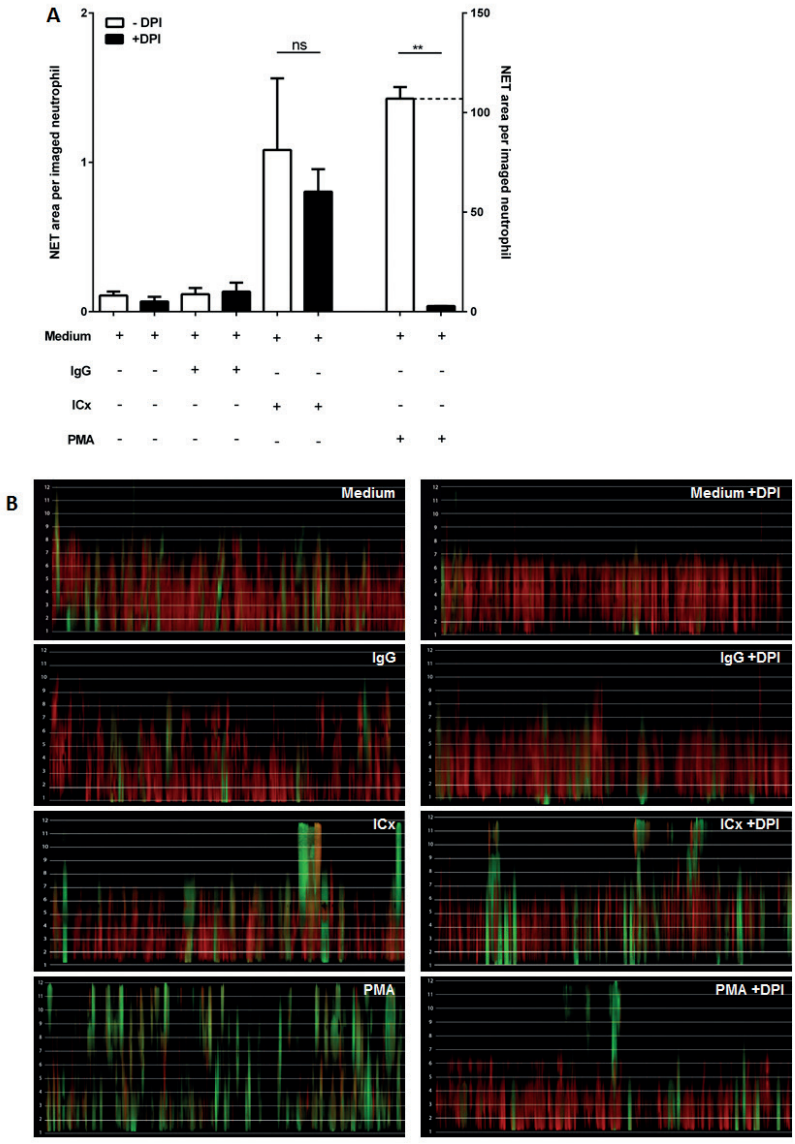

Figure 3. High sensitivity quantification of ICx-induced NET release is ROS-independent. **(A)** NADPH-oxidase was inhibited through pre-incubation of neutrophils with 1 μM diphenyleneiodonium (DPI) and subsequently NETs were induced with human IgG (6.25 μg/ml), ICx (6.25 μg/ml) and PMA (25 nM) or medium (unstimulated control). ROS inhibition did not influence ICx-induced NET release, plotted on the left y-axis, whereas PMA-induced NET release, plotted on the right y-axis, was almost completely blocked. ns = not significant, **$p < 0.005$. **(B)** Corresponding cross-sectional overview of a 3-dimensional images using 12 z-stacks illustrating ICx-induced NET release is ROS independent. Results are representative for three experiments. Bars represent mean ± SEM.

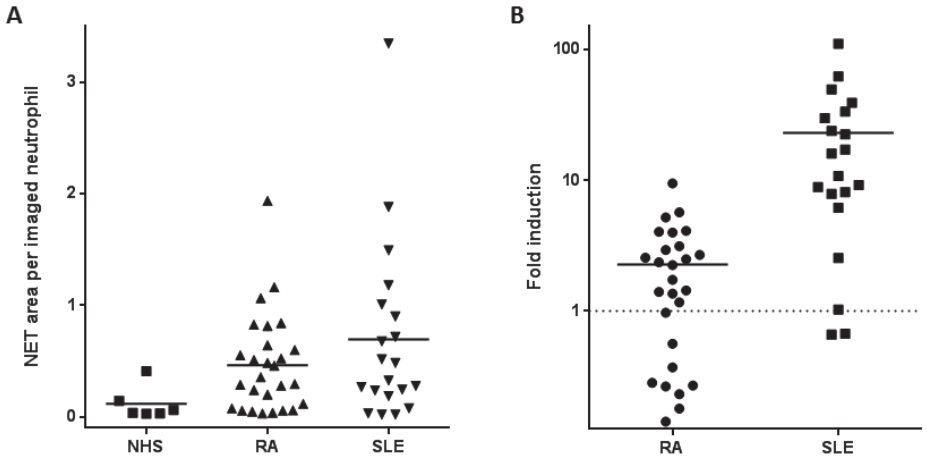

Figure 4. Quantification of NET release induced by RA and SLE serum. **(A)** NET release was measured upon induction with 10% serum from 27 RA patients, 20 SLE patients and 6 NHS controls. **(B)** The amount of NET release upon stimulation with RA and SLE serum is shown relative to the NHS control (fold induction). The NHS control value is based on three different NHS samples used per experiment. The fold induction was calculated relative to the mean NHS value within the same experiment. All samples were tested in triplicate. NET release is induced in 18 out of 27 RA samples and in 17 out of 20 SLE samples. Each sample is represented by one dot or cube.

Discussion

The present study describes a novel method for automatic and highly sensitive quantification of NET release based on CLSM. With this assay, we were able to demonstrate that the NET release upon stimulation with human heat-aggregated IgG immune complexes is independent of ROS production. Moreover, we show the applicability of this assay to quantify NET-inducing capacity in sera from RA and SLE patients.

The hallmark of the current method is its high sensitivity, which is based on two novel assay features. First, we introduced for the first time 3D imaging in a NET quantification assay. Since we observed that NETs are superimposed on neutrophils, we developed a more reliable detection of NETs by obtaining 12 z-stack images. Thus, we were able to demonstrate that low levels of NET release could be detected with 3D but not with 2D imaging. Second, the acquisition of (potentially netting) neutrophils is increased by imaging 11% of the total well area. In comparison, conventional methods based on fluorescence microscopy quantify 5–10 HPFs at 40× magnification correlating with an image area of 0.5–1% of each well [22,23]. The automated

quantification method developed by Brinkmann obtained 5% of each well area by capturing 5 images randomly spread throughout the well with a 10× magnification [16]. When increasing the imaged area, we also show that an increased number of neutrophils is captured. Because the amount of measured NET release is directly related to the number of neutrophils captured in each HPF, we validated that PKH26 matched with the large number of neutrophils imaged. Of note, it is important to realize that the current assay includes limited pipetting steps, since no antibody stainings are used. Taken together, this novel assay has a significantly increased yield of NET detection, due to an increased sensitivity as compared to the currently reported assays to quantify NET release.

Quantifying NETs by immunofluorescence microscopy is inherently associated with observer subjectivity when images are acquired. To address this issue, we automated image acquisition which made it possible to capture 300 HPFs (25 HPFs in 12 z-stacks) for each well and, additionally, eliminate observer subjectivity and inter-observer variability during image acquisition. As a consequence, to handle the large image data sets, we semi-automated the digital image analysis. Thus, in contrast to previous NET quantification methods, we were able to identify a major reduction in PKH staining upon stimulation with PMA. This is most likely a result of general neutrophil death through NETosis induced by PMA. Importantly, neutrophil cell loss by NETosis was much smaller upon incubation with ICx illustrative of a quantitatively and qualitatively different process compared to PMA. Collectively, automated image acquisition of large areas from each well increased sensitivity while reducing observer subjectivity when quantifying NET release.

An important advantage of this assay is that it is compatible with the use of human IgG immune complexes. Previous reports investigating NET induction by ICx studied non-human ICx, such as soluble BSA/ rabbit anti-BSA ICx [21] or immobilized HSA/rabbit polyclonal anti-HSA ICx [13]. Until now, it was a common problem to measure human ICx-induced NET release in a Sytox green fluorescence assay (data not shown) or with Picogreen DNA measurements due to the interference of human IgG with the detection method [18]. Another complication is the lack of sensitivity of previous conventional methods. To our knowledge, this is the first assay using a fully humanized system for NET quantification with human ICx.

There are a few limitations to this assay. First of all, inter-assay variability is present since the optimal imaging settings (e.g. exposure time) and analysis settings (e.g. intensity threshold) can vary between experiments. The use of different neutrophil donors is an uncontrollable variable that could add to inter-assay variability. Another limitation is the use of the specific dyes PKH26 and Sytox. A more specific NET marker could improve the assay's specificity for NETs. However, as yet, the current available NET-specific stainings such as citrullinated histone and neutrophil elastase have a low sensitivity and would therefore reduce the current assay's sensitivity to

detect low levels of extracellular DNA [24,25]. Future investigations will attempt to further reduce assay variability and decrease duration of image acquisition.

The applicability of this method was first established in our experiments characterizing human ICx-induced NET release. By using DPI, which inhibits NADPH oxidase and ROS production, we demonstrated that ICx induction of (low levels of) NET release was different from PMA-induced (high levels of) NET release. This observation was in accordance with a previous report showing that soluble BSA/anti-BSA ICx-induced NET release is independent on the formation of ROS [21]. Moreover, we show the ability of this assay to quantify NET release upon stimulation with sera from RA and SLE patients. Compared to the NHS control, RA and SLE sera show increased capability to induce NET release. To minimize the potential inter-assay variability, the NET-inducing capacity is shown relative to the NHS control used in the corresponding experiment as well in Fig. 4B. In summary, we could successfully quantify the NET-inducing capacity of RA and SLE sera. Further investigations will focus on the NET-inducing capacity of multiple AID samples and factors involved in NET release in AID.

In conclusion, we have described a novel method to quantify NET release in vitro by applying a semi-automated 3D CLSM image analysis. This assay has four advantages over the currently reported, conventional methods for NET quantification: 1) the assay has increased sensitivity to detect low levels of NETs; 2) the assay is semi-automated minimizing observer variability; 3) the assay is performed in a fully humanized system; 4) the assay allows high-throughput analysis. With this novel method we were able to detect NET release induced by RA and SLE serum samples with high sensitivity. Future investigations will aim to apply this assay in order to investigate the ability of circulating factors to induce NET release in autoantibody-mediated diseases, such as ANCA-associated vasculitis, SLE, rheumatoid arthritis, antiphospholipid syndrome and cryoglobulinemic vasculitis.

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References

1. Pilsczek FH, Salina D, Poon KKH, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol 2010;185:7413–25. http://dx.doi.org/10.4049/ jimmunol.1000675.
2. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303(80-):1532–5. http:// dx.doi.org/10.1126/science.1092385.
3. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog 2009;5, e1000639. http://dx.doi. org/10.1371/journal.ppat.1000639.
4. McCormick A, Heesemann L, Wagener J, Marcos V, Hartl D, Loeffler J, et al. NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. Microbes Infect 2010;12:928–36. http://dx.doi.org/10.1016/j.micinf. 2010.06.009.
5. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang Y-H, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 2007;449:564–9. http://dx.doi.org/10.1038/nature06116.
6. Kessenbrock K, Krumbholz M, Schönermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 2009;15: 623–5. http://dx.doi.org/10.1038/nm.1959.
7. Nakazawa D, Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. J Am Soc Nephrol 2014;1–8. http://dx.doi.org/10.1681/ ASN.2013060606.
8. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med 2011;3:73ra20. http://dx.doi.org/10.1126/scitranslmed. 3001201.
9. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med 2011;3:73ra19. http://dx.doi.org/10. 1126/scitranslmed.3001180.
10. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med 2013;5:178ra40. http://dx. doi.org/10.1126/scitranslmed.3005580.
11. Hakkim A, Fürnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A 2010;107:9813–8. http://dx.doi.org/10.1073/pnas.0909927107.
12. Leffler J, Martin M, Gullstrand B, Tydén H, Lood C, Truedsson L, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol 2012;188:3522–31. http://dx. doi.org/10.4049/jimmunol.1102404.

13. Behnen M, Leschczyk C, Moller S, Batel T, Klinger M, Solbach W, et al. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via Fc RIIIB and Mac-1. J Immunol 2014;193:1954–65. http://dx.doi.org/10.4049/jimmunol.1400478.
14. Guimarães-Costa AB, Nascimento MTC, Froment GS, Soares RPP, Morgado FN, Conceição-Silva F, et al. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A 2009;106:6748–53. http://dx.doi.org/10.1073/pnas.0900226106.
15. Van Avondt K, Fritsch-Stork R, Derksen RHWM, Meyaard L. Ligation of signal inhibitory receptor on leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus. PLoS One 2013;8, e78459. http://dx.doi. org/10.1371/journal.pone.0078459.
16. Brinkmann V, Goosmann C, Kühn LI, Zychlinsky A. Automatic quantification of in vitro NET formation. Front Immunol 2012;3:413. http://dx.doi.org/10.3389/ fimmu.2012.00413.
17. Coelho LP, Pato C, Friães A, Neumann A, von Köckritz-Blickwede M, Ramirez M, et al. Automatic determination of NET (neutrophil extracellular traps) coverage in fluorescent microscopy images. Bioinformatics 2015;31:2364–70. http://dx.doi.org/10. 1093/bioinformatics/btv156.
18. Invitrogen. Quant-iT™ PicoGreen® dsDNA reagent and kits; 2008 1–7.
19. Lems-Van Kan P, Verspaget HW, Peña AS. ELISA assay for quantitative measurement of human immunoglobulins IgA, IgG, and IgM in nanograms. J Immunol Methods 1983;57:51–7. http://dx.doi.org/10.1016/0022-1759(83)90064-9.
20. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. Nat Med 2016;1–11. http://dx. doi.org/10.1038/nm.4027.
21. Chen K, Nishi H, Travers R, Tsuboi N, Martinod K, Wagner DD, et al. Endocytosis of soluble immune complexes leads to their clearance by FcyRIIIB but induces neutrophil extracellular traps via FcyRIIA in vivo. Blood 2012;120:4421–31. http://dx.doi. org/10.1182/blood-2011-12-401133.
22. Tanaka K, Koike Y, Shimura T, Okigami M, Ide S, Toiyama Y, et al. In vivo characterization of neutrophil extracellular traps in various organs of a murine sepsis model. PLoS One 2014;9, e111888. http://dx.doi.org/10.1371/journal.pone.0111888.
24. Yipp BG, Petri B, Salina D, Jenne CN, Scott BNV, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med 2012;18:1386–93. http://dx.doi.org/10.1038/nm.2847.
24. Zhao W, Fogg DK, Kaplan MJ. A novel image-based quantitative method for the characterization of NETosis. J Immunol Methods 2015. http://dx.doi.org/10.1016/j.jim. 2015.04.027.
25. Ciepiela O, Ostafin M, Bystrzycka W, Sieczkowska S, Moskalik A, Demkow U, et al. Flow cytometric quantification of neutrophil extracellular traps: limitations of the methodological approach. Am J Hematol 2015. http://dx.doi.org/10.1002/ajh.24257.

Supplementary material

Supplemental movie illustrating a rotating view of the 3-dimensional visualization of NET release upon incubation with immune complexes can be found online at https://doi.org/10.1016/j.autrev.2016.02.018.

Chapter 3

Intrinsically distinct role of neutrophil extracellular trap formation in antineutrophil cytoplasmic antibody–associated vasculitis compared to systemic lupus erythematosus

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Abstract

Different studies have demonstrated that neutrophil extracellular traps (NETs) may be involved in the pathophysiology of both antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) and systemic lupus erythematosus (SLE). AAV and SLE are clinically and pathologically divergent autoimmune diseases with different autoantibodies. However, the respective autoantigens recognized in AAV and SLE have been shown to be an intricate part of NETs. This study aimed to examine whether the mechanisms of NET formation and the composition of NETs are distinct between AAV and SLE. To investigate this hypothesis, healthy neutrophils were stimulated with serum from patients with AAV ($n = 80$) and patients with SLE ($n = 59$), and the mechanisms of NET formation and NET composition were compared. Both patients with AAV and patients with SLE had excessive NET formation, which correlated with the extent of disease activity (in AAV $r = 0.5$, $P < 0.0001$; in SLE $r = 0.35$, $P < 0.01$). Lytic NET formation over several hours was observed in patients with AAV, as compared to rapid (within minutes), non-lytic NET formation coinciding with clustering of neutrophils in patients with SLE. AAV-induced NET formation was triggered independent of IgG ANCAs, whereas SLE immune complexes (ICx) induced NET formation through Fcγ receptor signaling. AAV-induced NET formation was dependent on reactive oxygen species and peptidyl arginine deaminases, and AAV-induced NETs were enriched for citrullinated histones (mean ± SEM 23 ± 2%). In contrast, SLE-induced NETs had immunogenic properties, including binding with high mobility group box chromosomal protein 1 (mean ± SEM 30 ± 3%) and enrichment for oxidized mitochondrial DNA, and were involved in ICx formation. The morphologic features, kinetics, induction pathways, and composition of excessive NET formation are all intrinsically distinct in AAV compared to SLE. Recognizing the diversity of NET formation between AAV and SLE provides a better understanding of the pathophysiologic role of NETs in these different autoimmune diseases.

Introduction

Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) and systemic lupus erythematosus (SLE) are both life-threatening systemic autoimmune diseases. These patients are distinguished by their clinical phenotypes, histopathology, and autoantibody profiles. Patients with AAV display ANCAs against myeloperoxidase (MPO) or proteinase 3 (PR3), whereas patients with SLE develop diverse autoantibodies against nuclear autoantigens (ANAs) [1,2]. Typically, renal involvement in AAV manifests as a pauci-immune, crescentic glomerulonephritis (GN), while in SLE, a "full house" proliferative GN is seen.

A growing body of evidence indicates that neutrophil extracellular traps (NETs) may have an important role in the pathogenesis of both AAV and SLE [3–13]. NETs are immunogenic [5] and toxic [13,14] extracellular DNA structures released by neutrophils that contain a pool of autoantigens relevant for both AAV and SLE [15,16]. NET-derived DNA complexed with danger-associated molecular patterns, such as LL-37 [4,5] or high mobility group box chromosomal protein 1 (HMGB-1) [4], converts the NET DNA to potent immunogenic structures [4]. Indeed, NETs were demonstrated to activate plasmacytoid dendritic cells (4) and autoreactive B cells in vitro [17], which resulted in the production of interferon-α (IFNα) and autoantibodies, respectively. Furthermore, NETs also have direct cytotoxic effects on (glomerular) endothelial cells [18], mediated by histones [13,18] and MPO [14], which, in a murine model, was found to lead to severe, crescentic GN [19]. In addition, murine plasmacytoid dendritic cells loaded with NET-derived DNA led to the production of both ANAs and ANCAs [11]. Taken together, these findings provide ample evidence to indicate that NETs have the capability of inducing autoimmunity related to both AAV and SLE.

In clinical studies, we and others have demonstrated that excessive NET formation or impaired NET degradation is present both in patients with active AAV [3,7,20,21] and in patients with severe SLE [4,5,8,12,17,22,23], and this is correlated with the severity of disease activity. Thus, preclinical and clinical studies have demonstrated an important role for NETs in the pathogenesis of both AAV and SLE. However, as both diseases are divergent clinical and histologic entities, we hypothesized that excessive NET formation should have a different pathophysiologic role in each disease. The present study addressed this hypothesis by characterizing the quantitative, qualitative, and immunologic properties of NET formation in a direct comparison of AAV and SLE patients.

Patients and methods

Study population

Serum samples were collected from 80 patients with ANCA-positive AAV who met the classification criteria for vasculitis according to the Chapel Hill Consensus Conference definitions [24], and 59 patients with ANA-positive SLE who met the American College of Rheumatology 1997 updated classification criteria for SLE [25]. The patients were followed up at the Lupus-, Vasculitis-, and Complement-mediated Systemic Autoimmune Diseases outpatient clinic (LuVaCs) at Leiden University Medical Center (LUMC). All patients consented to participate in the LUMC biobank. Clinical data were extracted from the patients' electronic records at the time of the collection of the serum sample. The control group consisted of 29 healthy subjects who consented to participate in the LUMC healthy donor biobank. Both biobanking studies were approved by the local ethics committee at LUMC. The characteristics of the patients and healthy controls are summarized in Supplementary Table 1, and an extended methods section is provided in Supplementary Patients and Methods (available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract).

Preparation of neutrophils

Ficoll-amidotrizoaat (LUMC) was used to isolate neutrophils from 20 ml of whole blood obtained from a healthy donor and collected in EDTA-coated tubes. Cell isolation was carried out using density gradient centrifugation followed by erythrocyte lysis with sterilized water at 4°C. The neutrophils were then labeled with 2 μM PKH (Sigma-Aldrich) for all NET quantification experiments or 2.5 μM MitoSox Red (Thermo-Fisher Scientific) for immunofluorescence microscopy experiments. Thereafter, the neutrophils were stimulated for 4 hours (or as otherwise indicated) with 10% serum, IgG-depleted serum, 250 μg/ ml or 25 μg/ml isolated IgG from healthy controls or patients with AAV or SLE, or IgG derived from intravenous immunoglobulin (Sanquin) in phenol red–free RPMI 1640 medium–2% fetal calf serum (FCS) (Life Technologies), to induce NET formation. Details on the methods used for IgG depletion and separation are provided in Supplementary Patients and Methods (http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract). When immobilized IgG was used, 10 μg/ml IgG was coated overnight at 4°C in a 96-well Falcon plate, after which the neutrophils were incubated in the wells.

Quantification of ex vivo NET formation

NET formation was quantified using an approach previously described by our group (26,27). Briefly, PKH-labeled neutrophils were plated in a 96-well culture plate (Falcon) at 37,500 cells per well in phenol red–free RPMI 1640 medium (Life Technologies) supplemented with 2% heat-inactivated FCS. After 3.75 hours of stimulation, 1 μM Sytox green, an impermeable DNA dye

(Thermo-Fisher Scientific), was added for 15 minutes, and the neutrophils were then fixed with 4% paraformaldehyde (Added Pharma).

NETs were then visualized and quantified by confocal laser scanning microscopy using an automated BD Biosciences Pathway 855 apparatus, as described previously by our group, or an Image Xpress Micro confocal microscopy device (Molecular Devices). Further details on the NET quantification, live-cell imaging, and inhibition experiments are described in Supplementary Patients and Methods (http://onlinelibrary.wiley.com/doi/10.1002/ art.41047/abstract). Per well, the total amount of NET formation was quantified as the cumulative area of Sytox green staining corrected for the number of neutrophils (as reflected by the mean area of PKH26-labeled cells). In addition, the mean NET size per image and mean size of the PKH-labeled cell area, as a reflection of clustering of neutrophils, were each analyzed and quantified. For comparison of values between different experiments, the fold increase in NET area as compared to the mean NET area per imaged neutrophil from 3 normal human serum control samples was calculated for each sample in each experiment.

Immunofluorescence staining of NETs

Healthy neutrophils were seeded onto chambered coverslips (ibiTreat; Ibidi), and NET formation was induced as described previously. For each staining, at least 3 different SLE and AAV serum samples were used. Slides were stained with antibodies to detect NET-related proteins and immunoglobulins. Details are described in Supplementary Patients and Methods (http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract). The area of colocalization of NET-related proteins and DNA was quantified with ImageJ software, through a colocalization macro in which the colocalization area of the NET-related protein (stained with Alexa Fluor 488) and DNA (stained with Hoechst dye) was measured. The percentage colocalization per NET marker was calculated as the colocalization area divided by the total area of DNA.

Statistical analysis

Data on NET formation are expressed as the median (interquartile range [IQR]) area of NET formation per imaged neutrophil or as the fold induction relative to the mean value from 3 normal human serum control samples per experiment. NET formation ratios are expressed as the mean ± SEM. Colocalization data are expressed as the mean ± SEM. Statistically significant differences between 2 groups were determined with the Mann-Whitney U test, and Wilcoxon's matched pairs test was used for paired serum samples. Pearson's r was used for statistical correlation tests. All statistical analyses were performed with GraphPad software.

Results

Lytic NET formation in AAV versus non-lytic NET formation in SLE

We have previously demonstrated that serum from both AAV patients and SLE patients can induce excessive NET formation in healthy neutrophils [20,22,26]. We therefore performed a side-by-side quantitative comparison of 2 large cohorts of patients with ANCA-positive AAV ($n = 80$) and patients with ANA-positive SLE ($n = 59$). We observed that both AAV and SLE patient sera induced excessive NET formation as compared to that in the serum from healthy controls (each $P < 0.0001$) (Figure 1A). Furthermore, excessive NET formation was significantly higher in patients with AAV compared to patients with SLE (median NET area per neutrophil 1.29 [IQR 0.58–3.38] versus 0.77 [IQR 0.33–1.50]; $P = 0.02$).

The quantitative difference between AAV-induced NET formation and SLE-induced NET formation was associated with a differing morphologic appearance. In AAV, large areas of DNA with interconnected DNA stretches were observed, whereas in SLE, small clusters of "NET-ting" neutrophils were typically seen around extracellular DNA (Figure 1B).

To further investigate the observed morphologic differences between AAV-induced and SLE-induced NETs, we performed live-cell imaging to assess the morphologic features over time (Figure 1C and Supplementary Movies 1–3, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract). We observed that AAV-induced NETs were characterized by a lytic expulsion of extracellular DNA strands, which occurred from 1–2 hours onward (see Movie 1). In contrast, SLE-induced NET formation was characterized by clustering of neutrophils within minutes, and the neutrophils released NETs without visible neutrophil lysis and with maintenance of their motility (see Movie 2).

To quantify the different features of AAV-induced NET formation compared to SLE-induced NET formation, NETs were quantified at various time points. The results confirmed the delayed kinetics of AAV-induced NET formation as compared to the fast kinetics of SLE-induced NET formation (see Supplementary Figure 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract).

In addition, the morphologic appearance of AAV-induced NET formation was compared to that of SLE-induced NET formation in a subset of patient's sera. The size of individual AAV-induced NETs (median 1.1×106 pixels [IQR 0.8×106–1.2×106]) was significantly larger than that of individual SLE-induced NETs (median 0.7×106 pixels [IQR 0.5×106–0.9×106]; $P < 0.0001$) (see Supplementary Figure 2A, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract). Furthermore, the size of the neutrophil

(PKH) area was significantly larger for SLE-induced NETs (see Supplementary Figure 2B [http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract]), reflecting the typical phenomenon of neutrophil clustering in SLE.

In patients with AAV, NET formation correlated moderately with the extent of disease activity measured by the Birmingham Vasculitis Activity Score (BVAS) ($r = 0.5$, $P < 0.0001$) (Figure 1D). Of interest, NET formation in AAV patients with active disease (BVAS >0) was significantly higher compared to AAV patients without active disease (BVAS 0) (median NET area per neutrophil 2.5 [IQR 0.9–8.4] versus 1.2 [IQR 0.5–3.2]; $P = 0.02$) (Figure 1E).

In patients with SLE, NET formation correlated weakly with the extent of disease activity measured by the SLE Disease Activity Index (SLEDAI) ($r = 0.35$, $P < 0.01$) (Figure 1F). Of interest, NET formation was significantly higher in SLE patients with high disease activity (SLEDAI score >4) compared to SLE patients with low disease activity (SLEDAI score ≤4) (median NET area per neutrophil 1.2 [IQR 0.5–1.9] versus 0.5 [IQR 0.3–1.0]; $P = 0.02$) (Figure 1G).

Triggering of NET formation by SLE immune complexes (ICx) in an Fcγ receptor (FcγR)–dependent manner, but lack of effect of IgG ANCAs in AAV serum

Recently, our group demonstrated that AAV-induced NET formation occurred in a manner independent of IgG ANCAs (20), whereas SLE-specific autoantibodies were reported to induce NET formation (4). We therefore further corroborated the involvement of autoantibodies in a direct comparison of AAV-induced and SLE-induced NET formation. Detailed information on the selected patient serum samples are included in Supplementary Patients and Methods (http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract). Complete depletion of IgG did not lead to a reduction in NET formation in cultures with AAV patient serum (mean ± SEM ratio 1.29 ± 0.33; $P = 0.74$). In contrast, upon IgG depletion, a significant reduction in SLE-induced NET formation was observed (mean ± SEM ratio 0.53 ± 0.19; $P = 0.049$) (Figures 2A and B).

Surprisingly, NET formation could not be restored with the addition of soluble IgG isolated from either AAV or SLE patient serum (Figure 2C). However, immobilized IgG, mimicking ICx, significantly restored NET formation in cultures with SLE patient serum (mean ± SEM fold induction 8.77 ± 1.99; $P < 0.001$ versus healthy controls), but not in cultures with IgG derived from AAV patient serum (mean ± SEM fold induction 2.73 ± 1.03; $P = 0.1$ versus healthy controls) (Figure 2D).

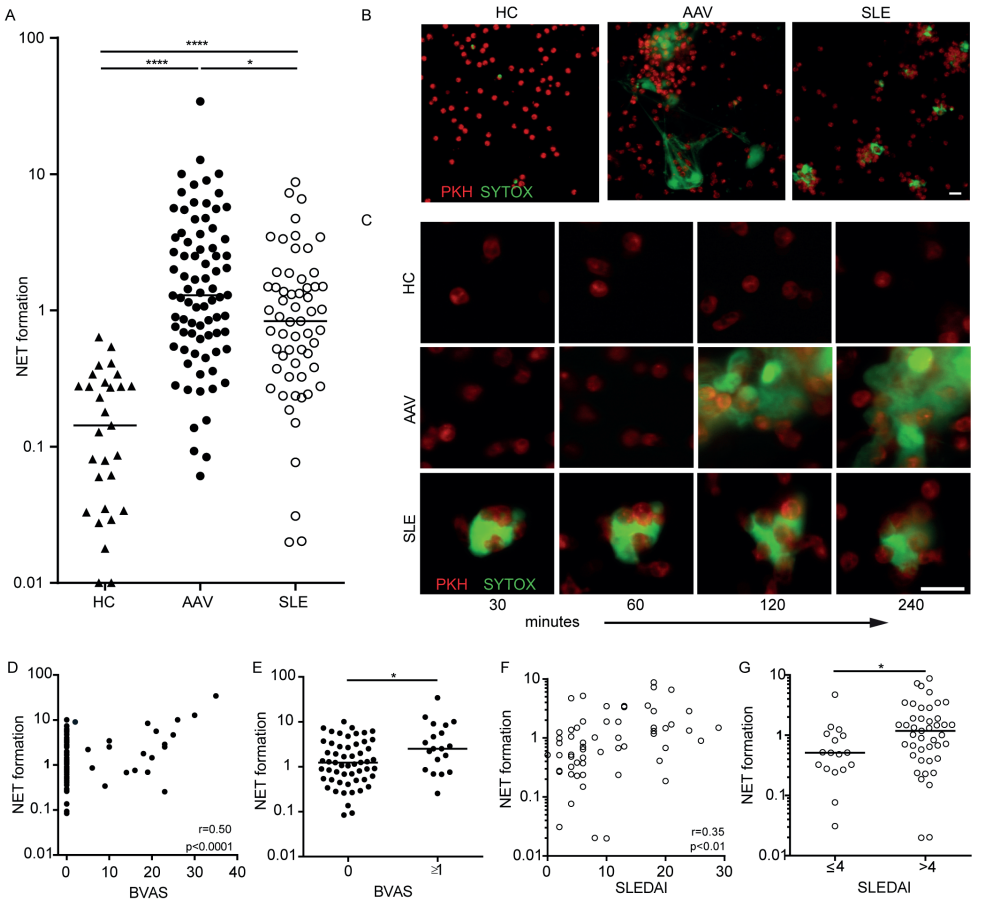

Figure 1. Lytic neutrophil extracellular trap (NET) formation in patients with antineutrophil cytoplasmic antibody–associated vasculitis (AAV) compared to non-lytic NET formation in patients with systemic lupus erythematosus (SLE). **(A)**, Ex vivo NET formation was measured in the serum of 80 patients with AAV, 59 patients with SLE, and 29 healthy controls (HCs), using a highly sensitive NET quantification assay (see ref. 26). Symbols represent the NET area per imaged neutrophil in each sample; horizontal lines show the median. **(B)**, Representative confocal microscopy images show the results of the NET quantification assay in PKH-labeled and Sytox green–stained neutrophils. Original magnification × 10; bar = 20 μm. **(C)**, Live-cell imaging shows representative examples of AAV- and SLE-induced NET formation over time (for full movies, see Supplementary Movies 1, 2, and 3 [http://onlinelibrary.wiley.com/doi/10.1002/art.41047/abstract]). Original magnification × 20; bar = 20 μm. **(D)** and **(E)**, In patients with AAV, the correlation of NET formation with disease activity as measured by the Birmingham Vasculitis Activity Score (BVAS) was assessed **(D)**, and NET formation was compared between AAV patients with a BVAS of 0 and those with a BVAS of >0 **(E)**. **(F)** and **(G)**, In patients with SLE, the correlation of NET formation with disease activity as measured by the SLE Disease Activity Index (SLEDAI) was assessed **(F)**, and NET formation was compared between SLE patients with a SLEDAI score of <4 and those with a SLEDAI score of ≥4 **(G)**. Symbols represent individual samples; horizontal lines show the median. Statistical correlations were assessed using Pearson's r test. * = $P < 0.05$; **** = $P < 0.0001$ by Mann-Whitney U test.

To further study the signaling cascade of ICx-induced NET formation, the spleen tyrosine kinase (Syk) inhibitor R406, an inhibitor of IgG-mediated activation of FcγR signaling (28), was employed. After inhibition of Syk, only NET formation induced by SLE patient serum was diminished (mean ± SEM ratio 0.53 ± 0.17; P = 0.04), whereas NET formation induced by AAV patient serum was unaffected by the Syk inhibitor (mean ± SEM ratio 1.30 ± 0.18; P = 0.09) (Figure 2E). Taken together, these data demonstrate that SLE ICx induce excessive NET formation through, at least partially, FcγR signaling, whereas AAV-induced NET formation occurs independent of IgG ANCAs.

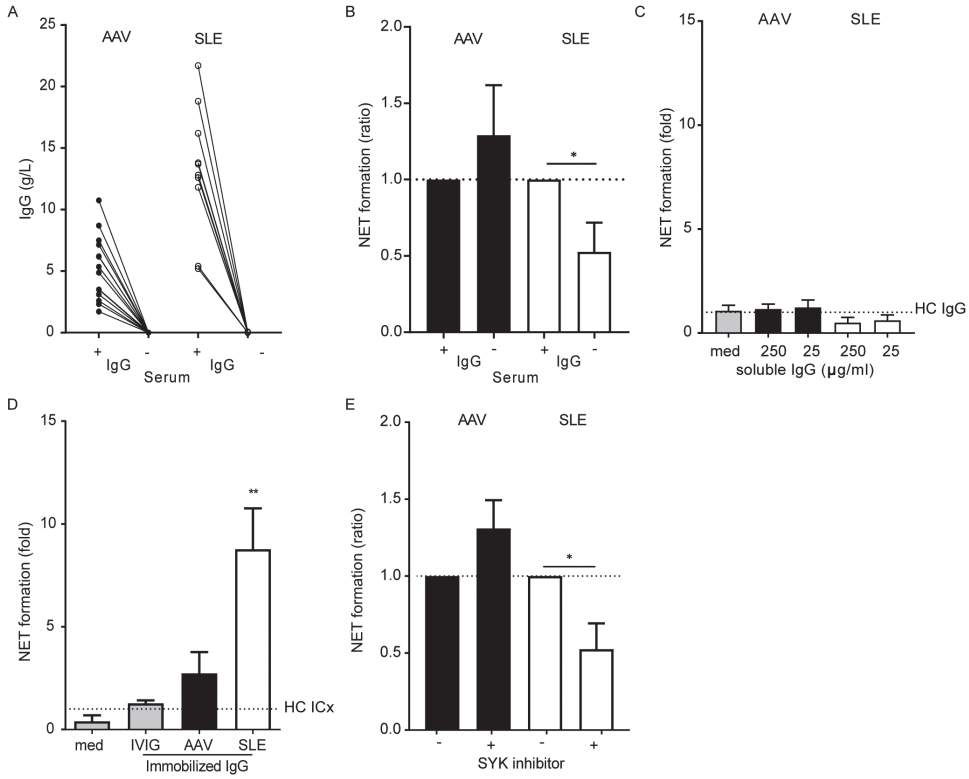

Figure 2. NET formation is triggered by SLE immune complexes (ICx) in an Fcγ receptor (FcγR)–dependent manner, but not by antineutrophil cytoplasmic antibody (ANCA IgG). **(A)**, IgG levels were compared before and after depletion of IgG from the serum of patients with ANCA-positive AAV (n = 14) and patients with antinuclear antibody–positive SLE (n = 10). IgG was depleted by protein G–agarose beads. **(B)**, Ex vivo NET formation in IgG-depleted patient serum was measured. Results are the mean ± SEM ratio compared to NET formation in the whole serum (broken horizontal line). **(C)**, Ex vivo NET formation in the presence of isolated soluble IgG from patient serum was measured. Results are the mean ± SEM fold increase relative to NET formation in the presence of soluble IgG from HC serum (n = 6) (broken horizontal line). **(D)**, Ex vivo NET formation in the presence of immobilized IgG from patient serum or intravenous immunoglobulin (IVIG)

was measured. Results are the mean ± SEM fold increase compared to NET formation in the presence of immobilized IgG from HC serum (n = 6) (broken horizontal line). **(E)**, Ex vivo NET formation was measured after FcγR signaling blockade by the Syk inhibitor R406. Results are the mean ± SEM ratio compared to untreated neutrophils stimulated with the same serum sample (broken horizontal line); representative data from 3 experiments are shown. * = $P < 0.05$; ** = $P < 0.01$ by Mann-Whitney U test. med = medium (see Figure 1 for other definitions).

Enrichment of citrullinated histone 3 (CitH3) on AAV-induced NETs and exclusive presence of HMGB-1 on SLE-induced NETs

Because the morphologic features, kinetics, and triggers of NET formation were different between AAV and SLE, we next compared the composition of NETs, by investigating the presence of NET-associated proteins. AAV-induced NETs were significantly enriched for CitH3 (29) ($P < 0.0001$) (Figures 3A and F). In contrast, SLE-induced NETs were significantly enriched for neutrophil elastase ($P < 0.0001$) (Figures 3B and F) and exclusively contained HMGB-1 ($P < 0.0001$) (Figures 3C and F). On unstimulated neutrophils, CitH3, neutrophil elastase, and HMGB-1 were absent (Figures 3A–C), whereas MPO and PR3 were present (Figures 3D and E).

MPO was found both on AAV-induced NETs and on SLE-induced NETs, whereas PR3 was localized on the cell membrane of SLE NET-ting neutrophils (Figures 3D, E, and G). Taken together, these data demonstrate that NET-related proteins are different on AAV-induced NETs compared to SLE-induced NETs.

Binding of immunoglobulins to SLE-induced NETs, implicating NETs as a substrate for ICx

Because many of the above-mentioned NET-related proteins are potential sources of autoantigens for disease-relevant autoantibodies, we next investigated whether NETs are substrates for ICx. We therefore investigated the presence of AAV and SLE autoantibodies on AAV-and SLE-induced NETs (Figures 4A and B). On AAV-induced NETs, colocalization of IgG was a mean ± SEM 2.4 ± 0.63%, whereas colocalization of IgM was 9.9 ± 2.5% and IgA was 0 ± 0% (Figures 4A and C). In comparison, on SLE-induced NETs, the colocalization of IgG (mean ± SEM 33.3 ± 3.6%; $P < 0.0001$), IgM (26 ± 3.3%; $P < 0.0001$), and IgA (6.7 ± 1.4%; $P < 0.0001$) was significantly higher (Figures 4B and C). These data provide evidence of the binding of SLE-related autoantibodies to NETs, suggesting that SLE-induced NETs are capable of formation of ICx. In contrast, in AAV-induced NETs, there was a paucity of immunoglobulin binding to NETs.

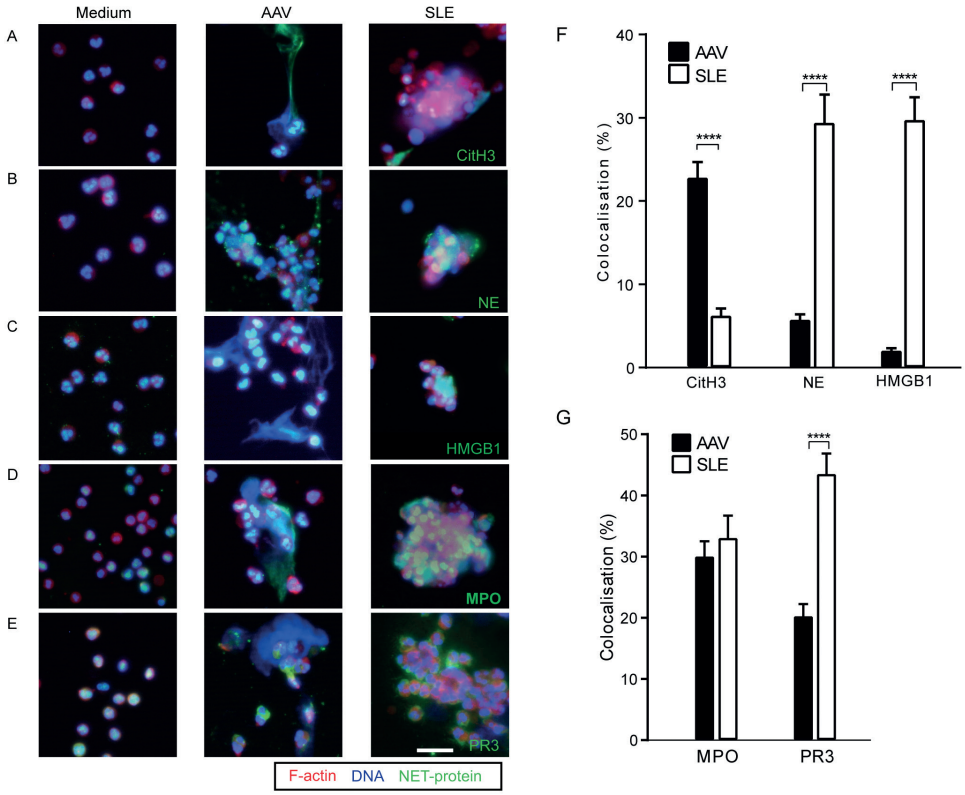

Figure 3. Citrullinated histone 3 (CitH3) is enriched on AAV-induced NETs, whereas high mobility group box chromosomal protein 1 (HMGB-1) is exclusively present on SLE-induced NETs. **(A–E)**, Immunofluorescence staining was used to assess NET-related proteins on AAV-induced and SLE-induced NETs. Representative overlay images show the presence of CitH3 **(A**, neutrophil elastase (NE) **(B)**, HMGB-1 **(C)**, myeloperoxidase (MPO) **(D)**, and proteinase 3 (PR3) **(E)** on unstimulated neutrophils (Medium) compared to AAV-induced and SLE-induced NETs. Cells were stained for F-actin (phalloidin red), DNA (Hoechst blue), and different NET-related proteins (green). Original magnification × 20; bar = 20 μm. **(F)** and **(G)**, The percentage colocalization of CitH3, neutrophil elastase, and HMGB-1 **(F)** and MPO and PR3 **(G)** was determined on DNA from AAV- induced and SLE-induced NETs. Results are the mean ± SEM. **** = $P < 0.0001$ by Mann-Whitney U test. See Figure 1 for other definitions.

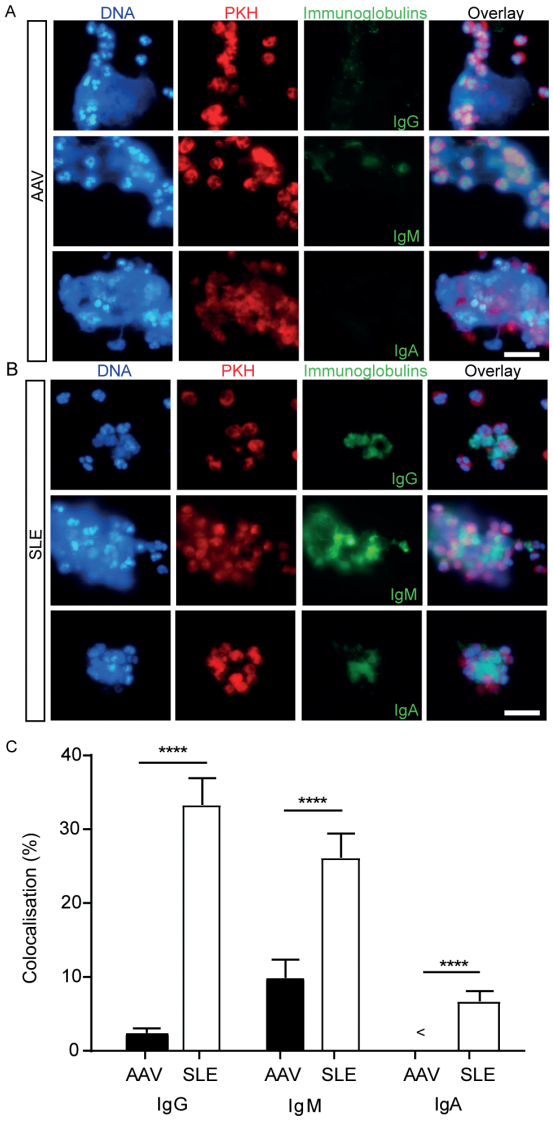

Figure 4. Immunoglobulins bind to SLE-induced NETs, implicating NETs as a substrate for immune complexes. The potential binding of serum autoantibodies from patients with AAV (n = 4) and patients with SLE (n = 3) to AAV- and SLE-induced NETs was studied by immunofluorescence staining of IgG, IgM, or IgA. **(A)** and **(B)**, Representative images show IgG, IgM, and IgA autoantibody binding on AAV-induced NETs**(A)** and SLE-induced NETs **(B)**. Original magnification × 20; bars = 20 μm. **(C)**, The percentage colocalization of IgG, IgM, and IgA on AAV-induced and SLE-induced NETs was determined. Results are the mean ± SEM percentage of total DNA area. **** = $P < 0.0001$ by Mann-Whitney U test. < = not detectable (see Figure 1 for other definitions).

Enrichment of oxidized mitochondrial DNA (mtDNA) in SLE-induced NETs

Oxidized mtDNA has previously been shown to enhance IFN responses in SLE through the cyclic GMP-AMP synthase/stimulator of IFN genes (cGAS/STING) pathway, supporting a critical role of mtDNA in the pathogenesis of autoimmunity in SLE (10). We therefore assessed the presence of mtDNA in AAV-and SLE-induced NETs by quantifying the colocalization of TOMM20 (a marker of the mitochondrial outer membrane protein) and MitoSOX Red (a red fluorescent indicator of specific binding to mitochondria after oxidation) as markers of (oxidized) mtDNA (Figure 5A). TOMM20 colocalization was significantly lower on AAV-induced NETs (mean $\pm$ SEM 1.7 $\pm$ 1.5%) compared to SLE-induced NETs (7.0 $\pm$ 1.3%; $P < 0.001$) (Figure 5B). Similarly, MitoSOX colocalization was significantly lower on AAV-induced NETs compared to SLE-induced NETs (mean $\pm$ SEM 0.8 $\pm$ 0.2% versus 4.2 $\pm$ 0.8%; $P < 0.001$) (Figure 5C). Thus, TOMM20 and MitoSOX were both significantly enriched on SLE-induced NETs but were virtually absent on AAV-induced NETs, thereby confirming that SLE-induced NETs are enriched for oxidized mtDNA.

Differing molecular pathways of NET formation in AAV and SLE

To get further insight into the mechanisms of AAV and SLE-induced NET formation, different canonical pathways of NET formation were studied. AAV-induced NET formation was significantly decreased by chloramidine, an inhibitor of peptidyl arginine deaminase (PAD) enzymes (mean $\pm$ SEM ratio 0.63 $\pm$ 0.09; $P = 0.03$), whereas SLE-induced NET formation remained unaffected by this PAD inhibitor (mean $\pm$ SEM ratio 1.4 $\pm$ 0.39; $P = 0.58$) (Figure 6A). As a consequence of PAD inhibition, the presence of CitH3 became undetectable on both AAV- and SLE-induced NETs (Figure 6B).

AAV-induced NET formation was also significantly decreased after inhibition of NADPH oxidase by diphenyleneiodonium (DPI) (mean $\pm$ SEM ratio 0.29 $\pm$ 0.08; $P = 0.002$), whereas SLE-induced NET formation remained unaffected by this DPI inhibitor (mean $\pm$ SEM ratio 0.86 $\pm$ 0.19; $P = 0.5$) (Figure 6C). Therefore, taken together, these findings indicate that excessive NET formation in AAV, but not in SLE, is dependent on PAD enzymes and NADPH oxidase.

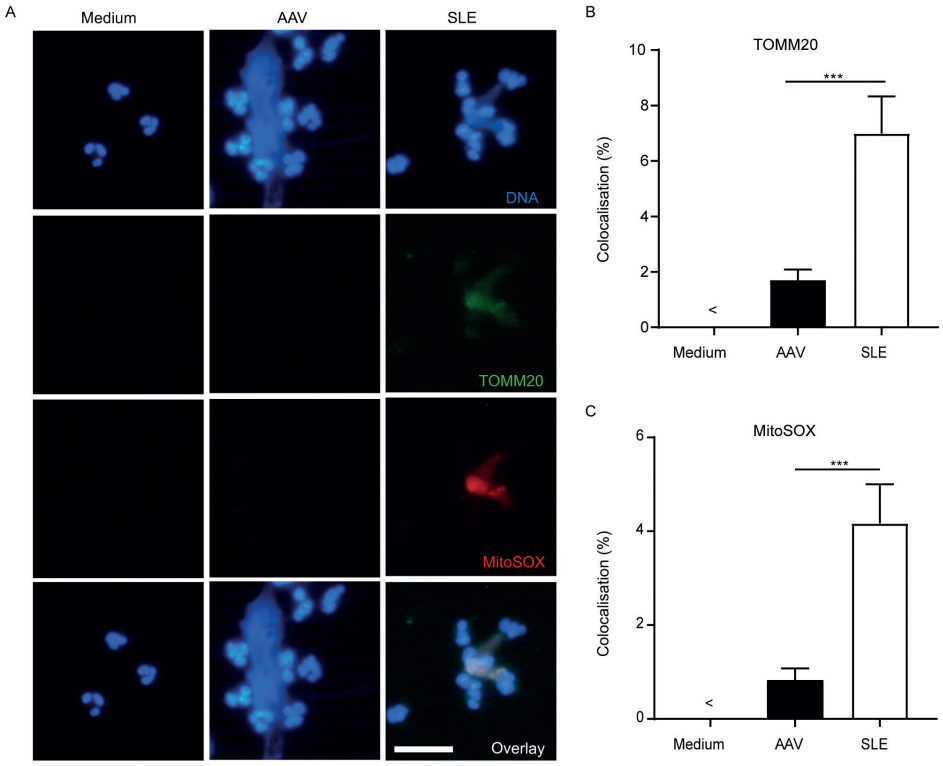

Figure 5. Oxidized mitochondrial DNA is enriched in SLE-induced NETs compared to AAV-induced NETs. **(A)**, Immunofluorescence microscopy analysis of unstimulated neutrophils (Medium), AAV-induced NETs, and SLE-induced NETs was carried out with MitoSOX Red labeling of the neutrophils after staining for TOMM20 (green) and DNA (Hoechst blue). Representative images are shown. Original magnification × 20; bar = 20 μm. **(B)** and **(C)**, The percentage colocalization of TOMM20 **(B)** and MitoSOX Red **(C)** was determined on NETs. Results are the mean ± SEM percentage of total DNA area per image. *** = $P < 0.001$ by Mann-Whitney U test. < = not detectable (see Figure 1 for other definitions).

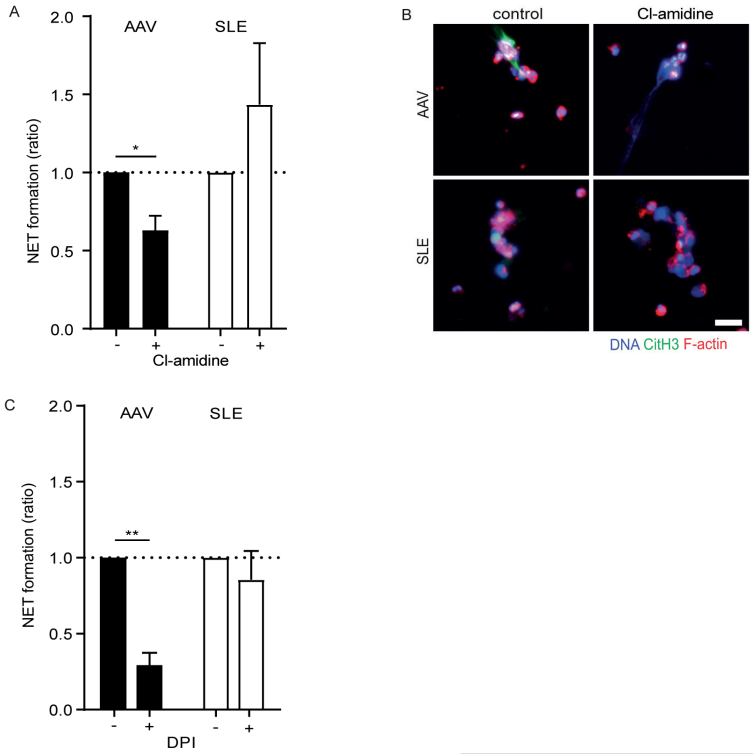

Figure 6. The regulators of NET formation differ between AAV and SLE. **(A)**, AAV-induced and SLE-induced NET formation was quantified after peptidyl arginine deaminase (PAD) inhibition with 200 μM chloramidine (Cl-amidine), a pan-PAD inhibitor. Results are the mean ± SEM ratio of NET formation before and after PAD inhibition in serum from patients with AAV (n = 6) and patients with SLE (n = 8) as compared to its paired control serum sample (broken horizontal line). **(B)**, Representative immunofluorescence images show PKH-labeled neutrophils (red), DNA (Hoechst blue), and citrullinated histone 3 (CitH3) colocalization (green) on AAV- and SLE-induced NETs with and without PAD inhibition by Cl-amidine. Original magnification × 20; bar = 20 μm. **(C)**, AAV-induced and SLE-induced NET formation was quantified after NADPH oxidase inhibition with 1 μM diphenyleneiodonium (DPI). Results are the mean ± SEM ratio of NET formation before and after NAPDH oxidase inhibition in serum from patients with AAV (n = 10) and patients with SLE (n = 9) as compared to its paired control serum sample (broken horizontal line). * = $P < 0.05$; ** = $P < 0.01$ by Wilcoxon's matched pairs test. See Figure 1 for other definitions.

Discussion

In separate studies, NETs have been reported to play a role in the pathophysiology of both AAV and SLE, although each is a clinically distinct systemic autoimmune disease [3,4]. The present study is the first to directly compare the characteristics of ex vivo NET formation induced by serum from patients with AAV to that induced by serum from patients with SLE. We were able to confirm that both patients with AAV and patients with SLE displayed excessive NET formation, and that this was correlated with the extent of disease activity. Most importantly, we demonstrated that AAV and SLE induce 2 distinct forms of NET formation, based on morphology, kinetics, triggers, and pathways. In AAV, an IgG- independent lytic expulsion of NETs containing citrullinated histones, and involving PAD enzymes and NADPH oxidase, was observed. In contrast, in SLE, a non-lytic, ICx-induced NET formation was observed, with concomitant clustering of neutrophils independent of PAD enzymes and NADPH oxidase. In addition, SLE-induced NETs had immunogenic properties, including enrichment for HMGB-1 and oxidized mtDNA, and the ability to form ICx, all of which were not observed in AAV-induced NETs. Taken together, our findings show that the differences in ex vivo NET formation between AAV and SLE, reflecting lytic versus non-lytic NET formation, respectively, and the distinct characteristics of NETs in each disease are indicative of the versatile role of NETs in the pathophysiology of AAV and SLE.

Mainly 2 processes of NET formation have recently been described, lytic and non-lytic NET formation, which is also referred to as “suicidal” and “vital” NET formation, respectively, and primarily depends on the type of stimulus used [6,30]. Typically, lytic NET formation depends on NADPH oxidase, takes a few hours, and involves recruitment of pore-forming units to disrupt the plasma membrane [31]. It can be induced by phorbol 12-myristate 13-acetate (PMA) (a robust chemical compound) [32], specific microorganisms [33], and crystals [34]. In contrast, non-lytic NET formation is typically induced within minutes, and can be triggered by lipopolysaccharide (LPS) [33], specific bacterial products [35], Toll-like receptor 4 (TLR-4)–activated platelets [33], or complement proteins together with TLR-2 ligands [36] and SLE-specific ICx. Non-lytic NET formation can be independent of NADPH oxidase [30,33,35,37], but some studies have shown that NADPH oxidase can be involved [10,36]. Importantly, the plasma membrane is not disrupted and NETs are released via vesicular transport while remaining viable, and typically contain oxidized mtDNA [10,35,36].

These pivotal features of lytic and non-lytic NET formation corresponded to the properties of NET formation respectively observed on AAV- and SLE-induced NETs [6]. AAV-induced NET formation is accompanied by neutrophil lysis after more than 1 hour, and depends on NADPH oxidase, indicative of a lytic form of NET formation [30]. Even though we demonstrated that AAV-induced NET formation was independent of IgG ANCAs, the exact factors in AAV serum that

trigger NET formation remain unknown. In a previous study, our group showed that neither IgA depletion nor C5 blockade affected AAV-induced NET formation, and there was no correlation with the serum levels of tumor necrosis factor (TNF), interleukin-8, or C-reactive protein [20]. Interestingly, the necroptosis pathway was recently demonstrated to be involved in ANCA-mediated NET formation [13]. Necroptosis can be induced by various triggers, including TNF, LPS, TLRs 1/2, 3, 4, and 7/8, and different chemical, toxic, and viral factors [38]. In addition, Heeringa et al suggested that other complement factors, such as C3a, could contribute to NET formation in AAV [39], which points toward the notion that there may be multifactorial triggers for lytic NET formation in the serum of AAV patients.

In comparison, SLE-induced NETs display features of non-lytic NET formation, referring to the rapid extrusion of NETs enriched with oxidized mtDNA within minutes, independent of NADPH oxidase. Of note, the involvement of NADPH oxidase in non-lytic NET formation is still under debate [10,36]. A cumulating number of studies have suggested that the trigger to induce NET formation in SLE deserves important considerations. Highly purified ICx generated from isolated anti-RNP autoantibodies mixed with its substrate, snRNP (small nuclear RNP), can induce NETs in a manner that is dependent on NADPH oxidase [10]. The characteristics of these "pure" RNP ICx, which are distinct from the heterogenous autoantibodies and ICx derived from SLE patient serum investigated in the present study, likely explain the discrepancy with regard to whether or not NADPH oxidase is involved. Indeed, our finding of NADPH oxidase–independent formation of NETs in SLE was recently confirmed by others in studies that utilized a novel method for discriminating between NADPH oxidase– dependent and –independent NET formation [37].

Importantly, the viability of the neutrophils stimulated with SLE patient serum in our study could be confirmed by our observations of their persistent motility, leading to neutrophil clustering, and the lack of observable plasma membrane lysis [35,40]. Taken together, the results of the present study directly comparing NET formation between AAV and SLE provide further evidence that AAV- and SLE-induced NETs have important and distinct immunologic characteristics.

Previous studies have shown that certain features of NETs play a pathogenic role in AAV and SLE. Studies in murine models revealed that NET-derived histones can cause crescentic GN. Crescentic GN is typically seen in AAV [3,19], emphasizing the relevance of the currently reported observation that citrullinated histones are predominantly found on AAV-induced NETs, implicating their contribution to the renal pathophysiology of AAV [13,41]. Another relevant feature was the detection of HMGB-1 on SLE-induced NETs. HMGB-1 facilitates the endosomal uptake of self-DNA via the receptor for advanced glycation end products/TLR-9 pathway [42], which is an important premise for the formation of anti-DNA autoantibodies, as demonstrated in nonautoimmune mice, in which HMGB-1–nucleosome complexes induced anti– double-

stranded DNA responses in a TLR-2–dependent manner [43]. Therefore, the presence of HMGB-1 on SLE-induced NETs indicated the ability of SLE-specific NET–derived extracellular DNA to be taken up, intracellularly processed, and presented as a relevant autoantigen in SLE [11]. Moreover, previous studies have demonstrated that NET-bound SLE autoantibodies and C1q resulted in activation of the classical and lectin complement pathways [12], while AAV-induced NETs activated the alternative pathway of the complement system [13,41].

Furthermore, SLE-induced NETs were also enriched for oxidized mtDNA, which augment their potential to induce a pro-inflammatory type I IFN response via the cGAS/STING pathway [10]). Importantly, SLE NETs are enriched for mtDNA, but do not solely consist of mtDNA. This was supported by the finding that "NET-ting" neutrophils containing LL37–DNA complexes, implying that they were chromosomal NETs, could activate autoreactive B cells in lupus [17]. SLE-induced NETs were demonstrated to contribute to ICx formation and, as such, can be implicated in the renal pathology of SLE. Indeed, the typical histopathologic finding in lupus nephritis is that of a "full house" GN detected by immunofluorescence, indicating that deposition of ICx and complement activation have occurred.

The direct comparison of ex vivo NET formation between AAV and SLE in the present study reveals several interesting observations. First, the clustering of neutrophils observed in SLE-induced NET formation is not new to the field. The SLE serum–induced clustering of neutrophils was demonstrated as far back as 1990, and this was found to be dependent on C1q-containing ICx and correlated with SLE disease activity, which preceded the discovery of NETs in 2004 [44,45]. Interestingly, we demonstrated that in both AAV patients and SLE patients, excessive NET formation was associated with active disease. However, in AAV and SLE patients during times of disease remission, the levels of NET formation were heterogeneous. It is possible that excessive NET formation in these patients who were without active disease could be an indication of the potential for a future disease relapse.

Second, citrullination appeared to be not essential for NET formation in both patients with AAV and patients with SLE, which is consistent with previous observations that confirmed the independence of PAD4, i.e., citrullination, for the formation of NETs in humans [46,47]. These observations are in contrast to those in studies that demonstrated the involvement of PAD enzymes in murine lupus [48–50]. It is noteworthy that in those studies, NET formation was induced with calcium ionophores [48], PMA [49,50], or imiquimod [48], and NET quantification was based on CitH3 positivity. Importantly, PMA-induced NET formation is truly independent of PAD enzymes, while calcium ionophore–induced NET formation is completely dependent on PAD enzymes [47]. Furthermore, it needs to be taken into account that PAD inhibition will prevent histone citrullination of NETs, resulting in CitH3-negative NETs [46,47]. Conversely, in a murine

model of lupus in MRL.Faslpr mice, lupus and proliferative nephritis were not affected by PAD4 inhibition [51].

Third, we confirmed that CitH3, generally reported as a key marker of NETs [14,29], is a highly specific NET marker, since it was absent on healthy and necrotic neutrophils (data not shown). However, CitH3 was not a sensitive marker, as illustrated by the high load of NET-derived DNA that did not colocalize with CitH3. Similarly, the sensitivity of MPO, but not its specificity, as a NET marker was demonstrated in our study. Taken together, these data indicate that careful considerations should be made when identifying neutrophil-derived extracellular DNA as NETs, which cannot be easily disentangled from necrosis [52].

Moreover, we noted the paucity of immunoglobulin binding on AAV-induced NETs, while several AAV- and SLE-relevant autoantigens, as detected by immunohistochemistry using monoclonal antibodies, were present on NETs (Figure 3). These data do not prove that the presence of these autoantigens on NETs is actually relevant, because it is well-known that in vivo autoantibodies detected in AAV and SLE patients are heterogenous and polyclonal. Indeed, previous studies have investigated anti-NET antibodies in AAV, although it remains uncertain as to whether these anti-NET antibodies were the same as ANCAs [3,7]. Of note, it is important to realize that these studies reported on antibodies reacting with PMA-induced NETs, which might be unrelated to either in vivo or ex vivo NET formation in humans. Previously, it was demonstrated that neutrophil-derived MPO and NETs were prominently deposited in the glomerular, periglomerular, and tubulointerstitial regions of kidney biopsy tissue from patients with MPO-specific AAV [14]. On the other hand, it is known that ANCA-associated GN is typically a crescentic, pauci-immune GN, implying that there are no deposits of autoantibodies or complement in the kidney. Thus, despite the abundant presence of MPO, anti-MPO ANCAs were not abundantly detected, which does support our findings. Alternatively, we speculate that the paucity of immunoglobulins on AAV-induced NETs could be related to the serum concentration of ANCAs as compared to the concentrations of the monoclonal antibodies used for immunohistochemistry. Unfortunately, we were unable to investigate whether epitope variation could also explain this observation.

There are some limitations to our study that need to be acknowledged. First, we used a relatively novel method to quantify NET formation, which has an intrinsically high interassay variability due to the use of freshly isolated neutrophils from healthy subjects each time (20), but simultaneously has several advantages compared to currently reported assays (20,26,52). To minimize variability in the present study, AAV and SLE sera were tested simultaneously within the same experiments.

Another potential limitation of this assay is the use of neutrophils from healthy donors instead of neutrophils from AAV or SLE patients. However, we have demonstrated previously that

spontaneous NET release by AAV-derived neutrophils was not significantly different from spontaneous NET release by healthy neutrophils. More importantly, AAV-induced NET formation by neutrophils derived from healthy donors was also not significantly different from NET formation by neutrophils derived from AAV patients [20]. Similar results were obtained for neutrophils derived from SLE patients (data not shown).

Second, regarding the results of our immunohistochemical studies of NET-associated proteins, we were unable to dissect whether the colocalization of NET-associated proteins either originated from the serum or were derived from the neutrophils.

In conclusion, this study provides compelling support for the observation that NET formation in AAV and SLE are disease-specific processes, with each encompassing their own unique properties and implications for the distinct pathophysiology of AAV and SLE. These excessively formed NETs in AAV and SLE have distinct features and depend on intrinsically distinct processes, thereby implicating a unique role for NETs in each separate autoimmune disease. Thus, the recognition of the diversity of NET formation in systemic autoimmune diseases has important implications when evaluating NET as a potential therapeutic target.

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References

1. Jennette JC, Wilkman AS, Falk RJ. Anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and vasculitis. Am J Pathol 1989;135:921–30.
2. D'Cruz DP, Khamashta MA, Hughes GR. Systemic lupus erythematosus. Lancet 2007;369:587–96.
3. Kessenbrock K, Krumbholz M, Schönermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 2009;15:623–5.
4. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med 2011;3:73ra20.
5. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med 2011;3:73ra19.
6. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. Nat Med 2017;23:279–87.
7. Nakazawa D, Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. J Am Soc Nephrol 2014;25:990–7.
8. Hakkim A, Fürnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A 2010;107:9813–8.
9. Söderberg D, Kurz T, Motamedi A, Hellmark T, Eriksson P, Segelmark M. Increased levels of neutrophil extracellular trap remnants in the circulation of patients with small vessel vasculitis, but an inverse correlation to anti-neutrophil cytoplasmic antibodies during remission. Rheumatology (Oxford) 2015;54:2085–94.
10. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. Nat Med 2016;22:146–53.
11. Sangaletti S, Tripodo C, Chiodoni C, Guarnotta C, Cappetti B, Casalini P, et al. Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. Blood 2012;120:3007–18.
12. Leffler J, Martin M, Gullstrand B, Tydén H, Lood C, Truedsson L, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol 2012;188:3522–31.
13. Schreiber A, Rousselle A, Becker JU, von Mässenhausen A, Linkermann A, Kettritz R. Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis. Proc Natl Acad Sci U S A 2017;114:E9618–25.

14. O'Sullivan KM, Lo CY, Summers SA, Elgass KD, McMillan PJ, Longano A, et al. Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. Kidney Int 2015;88:1030–46.
15. Yaniv G, Twig G, Shor DB, Furer A, Sherer Y, Mozes O, et al. A volcanic explosion of autoantibodies in systemic lupus erythematosus: a diversity of 180 different antibodies found in SLE patients. Autoimmun Rev 2015;14:75–9.
16. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog 2009;5:e1000639.
17. Gestermann N, Di Domizio J, Lande R, Demaria O, Frasca L, Feldmeyer L, et al. Netting neutrophils activate autoreactive B cells in lupus. J Immunol 2018;200:3364–71.
18. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, et al. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. PLoS One 2012;7:e32366.
19. Kumar SV, Kulkarni OP, Mulay SR, Darisipudi MN, Romoli S, Thomasova D, et al. Neutrophil extracellular trap-related extracellular histones cause vascular necrosis in severe GN. J Am Soc Nephrol 2015;26:2399–413.
20. Kraaij T, Kamerling SW, van Dam LS, Bakker JA, Bajema IM, Page T, et al. Excessive neutrophil extracellular trap formation in ANCA-associated vasculitis is independent of ANCA. Kidney Int 2018;94:139–49.
21. Yoshida M, Sasaki M, Sugisaki K, Yamaguchi Y, Yamada M. Neutrophil extracellular trap components in fibrinoid necrosis of the kidney with myeloperoxidase-ANCA-associated vasculitis. Clin Kidney J 2013;6:308–12.
22. Kraaij T, Kamerling SW, de Rooij EN, van Daele PL, Bredewold OW, Bakker JA, et al. The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus. J Autoimmun 2018;91:45–54.
23. Van der Linden M, van den Hoogen LL, Westerlaken GH, Fritsch-Stork RD, van Roon JA, Radstake TR, et al. Neutrophil extracellular trap release is associated with antinuclear antibodies in systemic lupus erythematosus and anti-phospholipid syndrome. Rheumatology (Oxford) 2018. E-pub ahead of print.
24. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, et al. Nomenclature of systemic vasculitides: proposal of an international consensus conference. Arthritis Rheum 1994;37:187–92.
25. Hochberg MC, for the Diagnostic and Therapeutic Criteria Committee of the American College of Rheumatology. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus [letter]. Arthritis Rheum 1997;40:1725.
26. Kraaij T, Tengström FC, Kamerling SW, Pusey CD, Scherer HU, Toes RE, et al. A novel method for high-throughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes. Autoimmun Rev 2016;15:577–84.

27. Arends EJ, van Dam LS, Kraaij T, Kamerling SW, Rabelink TJ, van Kooten C, et al. A high-throughput assay to assess and quantify neutrophil extracellular trap formation. J Vis Exp 2019;e59150.
28. Braselmann S, Taylor V, Zhao H, Wang S, Sylvain C, Baluom M, et al. R406, an orally available spleen tyrosine kinase inhibitor blocks Fc receptor signaling and reduces immune complex-mediated inflammation. J Pharmacol Exp Ther 2006;319:998–1008.
29. Wang Y, Li M, Stadler S, Correll S, Li P, Wang D, et al. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol 2009;184:205–13.
30. Yipp BG, Kubes P. NETosis: how vital is it? Blood 2013;122:2784– 94.
31. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 2007;176:231–41.
32. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303:1532–5.
33. Pieterse E, Rother N, Yanginlar C, Hilbrands LB, van der Vlag J. Neutrophils discriminate between lipopolysaccharides of different bacterial sources and selectively release neutrophil extracellular traps. Front Immunol 2016;7:484.
34. Desai J, Kumar SV, Mulay SR, Konrad L, Romoli S, Schauer C, et al. PMA and crystal-induced neutrophil extracellular trap formation involves RIPK1-RIPK3-MLKL signaling. Eur J Immunol 2016;46:223–9.
35. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol 2010;185:7413–25.
36. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ 2009;16:1438–44.
37. Pieterse E, Rother N, Yanginlar C, Gerretsen J, Boeltz S, Munoz LE, et al. Cleaved N-terminal histone tails distinguish between NADPH oxidase (NOX)-dependent and NOX-independent pathways of neutrophil extracellular trap formation. Ann Rheum Dis 2018;77:1790–8.
38. Vanden Berghe T, Hassannia B, Vandenabeele P. An outline of necrosome triggers. Cell Mol Life Sci 2016;73:2137–52.
39. Heeringa P, Rutgers A, Kallenberg CG. The net effect of ANCA on neutrophil extracellular trap formation. Kidney Int 2018;94:14–6.
40. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med 2012;18:1386–93.
41. Wang H, Wang C, Zhao MH, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. Clin Exp Immunol 2015;181:518–27.
41. Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, et al. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nat Immunol 2007;8:487–96.

43. Urbonaviciute V, Fürnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, et al. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. J Exp Med 2008;205:3007–18.
44. Sturfelt G, Jonsson H, Hellmer G, Sjöholm AG. Clustering of neutrophil leucocytes in serum: possible role of C1q-containing immune complexes. Clin Exp Immunol 1993;93:237–41.
45. Jonsson H, Sturfelt G. A novel assay for neutrophil clustering activity of human sera: relation to disease activity and neutropenia in systemic lupus erythematosus. Ann Rheum Dis 1990;49:46–50.
46. Konig MF, Andrade F. A critical reappraisal of neutrophil extracellular traps and NETosis mimics based on differential requirements for protein citrullination. Front Immunol 2016;7:461.
47. Kenny EF, Herzig A, Krüger R, Muth A, Mondal S, Thompson PR, et al. Diverse stimuli engage different neutrophil extracellular trap pathways. eLife 2017;6:e24437.
48. Liu Y, Lightfoot YL, Seto N, Carmona-Rivera C, Moore E, Goel R, et al. Peptidylarginine deiminases 2 and 4 modulate innate and adaptive immune responses in TLR-7-dependent lupus. JCI Insight 2018;3:124729.
49. Knight JS, Subramanian V, O'Dell AA, Yalavarthi S, Zhao W, Smith CK, et al. Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice. Ann Rheum Dis 2015;74:2199–206.
50. Knight JS, Zhao W, Luo W, Subramanian V, O'Dell AA, Yalavarthi S, et al. Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. J Clin Invest 2013;123:2981–93.
51. Gordon RA, Herter JM, Rosetti F, Campbell AM, Nishi H, Kashgarian M, et al. Lupus and proliferative nephritis are PAD4 independent in murine models. JCI Insight 2017;2:92926.
52. Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, et al. NETosis markers: quest for specific, objective, and quantitative markers. Clinica Chim Acta 2016;459:89–93.

Supplementary material

Supplemental Patients & Methods

Supplemental information on study population

14 AAV serum samples were selected for the experiments in Figure 2, which had an average NET formation of 3.2 ± 2.0 (SD). The serum level of anti-PR3 ANCA was 30.8 ± 28.0 IU/ml and anti-MPO ANCA was 32.8 ± 10.0 IU/ml in these AAV patients. 3 out of 14 patients had active disease (BVAS > 0) with BVAS scores ranging from 19 to 25, the other patients did not have active disease. We did not detect a difference between the active patients versus inactive patients for these set of experiments. 10 SLE serum samples were selected, which had an average NET formation of 2.4 ± 1.4. Serum level of anti-dsDNA was 57,4 ± 55 IU/ml and 9 out of 10 patients had active disease (SLEDAI >4), with SLEDAI scores ranging from 6 to 24.

Supplemental methods on the quantification of ex vivo NET formation

12 z-stacked images of 25 predefined high power fields (HPFs) at a 20x magnification were automatically captured. The HPFs were evenly spread throughout the well by a standardized 5x5 zig-zag pattern with 400 μm (length) and 500 μm (width) spaced between each high HPF. Hereby, a total of 11.1% of the well is imaged. The microscope was programmed to automatically focus on PKH26 membrane staining. For each image, PKH26 (Cy3) and Sytox green (Alexa488) was visualized. The same exposure time was applied to all images in the same experiment. Acquired images were semi-automatically analyzed by ImageJ image analysis software (NIH, Bethesda, USA) by determining the threshold area of PKH and extracellular DNA.

IgG depletion

IgG was depleted from sera of ANCA-positive AAV (n=14), ANA-positive SLE patients (n=10) and from healthy donors (n=7) using protein G agarose beads (Sino Biological, Beijing, China). During each experiment, a sham depletion and isolation was performed with TRIS buffer to control for contaminating factors. Briefly, the beads were washed three times with binding buffer (50 mM TRIS, 100 mM NaCl, pH 8.0) and 500 μl of serum was mixed overnight with 500 μl of the protein G agarose beads at 4°C. Samples were centrifuged to separate the upper fraction (containing the IgG-depleted serum) from the agarose beads. The total protein in the IgG-depleted serum was measured with a Pierce assay (Thermo Scientific, Waltham, MA, USA), total IgG was measured with an IgG ELISA (Bethyl Laboratories, Montgomery, Texas, USA).

IgG separation

Protein G agarose beads containing IgG were loaded on a 1 ml micro column and washed three times with binding buffer. The IgG was then eluted from the beads with 0.5 ml acidic elution buffer (0.1 M Glycine, pH 2–3). IgG fractions were collected into tubes with neutralizing

buffer (1 M Tris-HCl, pH 8–9) and the pH of the eluted fractions was immediately adjusted to a physiological pH of 7.5. Fractions containing IgG were pooled, dialyzed against PBS overnight at 4°C and concentrated with a centrifugal filter unit with a 30-kDa cut-off (Merck Millipore, Darmstadt, Germany). Total IgG was measured with an IgG ELISA (Bethyl Laboratories).

Live-cell imaging

PKH-labelled (2 μM, Sigma-Aldrich, Saint-Louis, USA) neutrophils were plated in a 96-well culture plate (Falcon, Tewksbury, MA, USA), 42.000 per well. Neutrophils were stimulated with medium (negative control), 10% serum of patients or healthy controls together with 1 μM Sytox green. NET formation was visualized by confocal laser scanning microscopy (CLSM) by recording a picture every 7-11 minutes with 20x magnification in a standardized 3x3 zigzag stitched pattern. For each image, PKH26 (Cy3) and Sytox green (Alexa488) was visualized. The same exposure time was applied to all images in the same experiment. All images were converted to a movie with ImageJ.

Immunofluorescence staining of NETs

Fixed neutrophils were blocked with 1% BSA and 20% normal goat serum (NGS) in PBS with 5 mM EDTA to inhibit nuclease activity. Slides were stained with 5 μg/ml polyclonal rabbit anti-human citrullinated histone-3 (Abcam, Cambridge, UK), 5 μg/ml polyclonal rabbit neutrophil elastase (NE) (Abcam, Cambridge, UK), 5 μg/ml polyclonal rabbit anti-human high mobility group box protein 1 (HMGB1) (Abcam, Cambridge, UK), 2 μg/ml TOMM20 (Sigma-Aldrich, Darmstadt, Germany), 10 μg/ml monoclonal mouse IgG1-anti-human MPO (Hycult, Plymouth Meeting, USA) 10 μg/ml mouse IgG1-anti-human PR3 (Abcam, Cambridge, UK) or corresponding polyclonal rabbit IgG isotype control or monoclonal mouse IgG1 isotype control (Dako, Santa Clara, USA) in PBS and 1% NGS with 5 mM EDTA and incubated for two hours. Then, neutrophils were washed and incubated for 1 hour with 1:500 secondary Alexa-488 labelled goat anti-rabbit IgG or goat anti-mouse IgG1 antibodies (Thermo Fisher, Waltham, USA) and 1:100 phalloidin (Sigma-Aldrich, Saint-Louis, USA), The secondary antibodies were pre-incubated for 30 minutes with 5% normal human serum (NHS) to bind anti-human immunoglobulins. Hereafter neutrophils were washed and stained with 1 μg/ml Hoechst 33258 (Thermo Fisher, Waltham, USA). Presence of immunoglobulins was detected directly without blocking with 5 μg/ml of cross-absorbed secondary goat-anti-human -IgG or -IgM (Alexa-488) (Thermofisher, Waltman, USA) or preadsorbed secondary goat-anti-human-IgA (Dylight 488) (Abcam, Cambridge, UK), medium and normal healthy serum was used as control condition to check for aspecific binding, all were negative. 10 images per well were acquired with the Leica DMI6000 inverted microscope using a 20x magnification.

NET inhibition experiments

Spleen tyrosine kinase (SYK) was inhibited by R406 (Rigel Pharmaceuticals Inc., San Francisco, USA). The concentration of R406 was adopted according to previous reports [26], but also titrated on the capacity to inhibit IL-8 production of neutrophils stimulated with immobilized immune complexes (IVIG). Neutrophils were incubated with 1 μM R406 at 37°C for 30 minutes and then ANCA-positive AAV serum (n=6) and ANA-positive SLE serum (n=8) was added to the plate. Because R406 was diluted in DMSO, there was a control condition in which DMSO was pre-incubated with cells. Cl-amidine (EMD Millipore, Billerica, USA), a pan-PAD inhibitor, was used to inhibit PAD4 in neutrophils. The concentration of Cl-amidine was adopted according to previous reports[27], and titrated on the capacity to inhibit NET formation in neutrophils stimulated with calcium ionophore (10 μM A23187, Sigma Aldrich, Saint-Louis, USA). Healthy neutrophils were pre-incubated with 200μM Cl-amidine for 15 minutes to inhibit PAD enzymes and then AAV (n=10) and SLE (n=9) sera were used to induce NET formation. Diphenyleneiodonium (DPI) (Sigma-Aldrich, Saint-Louis, USA) was used to inhibit ROS production through NADPH oxidase. The concentration of DPI was titrated on the capacity to inhibit NET formation in neutrophils stimulated with PMA (Sigma-Aldrich, Saint-Louis, USA). Healthy neutrophils were pre-incubated with 1 μM DPI and then AAV (n=7) and SLE (n=11) sera were used to induce NET formation. For all inhibition experiments, NET formation was quantified as described before and expressed as ratio of inhibited versus control per patient serum sample.

Table S1. Patient characteristics.

Patient Characteristics	AAV (n=80)	SLE (n=59)	HCs (n=29)
Male (%)	70%	16%	35%
Age (years)	63 ± 13	39 ± 14	34 ± 8
Disease involvement			
Renal involvement (%)	72	79	-
ENT-involvement (%)	63	-	-
Lung involvement (%)	43	24	-
Joint involvement (%)	49	48	
Disease duration (years)	9 ± 8	10 ± 8	-
BVAS/SLEDAI*	5 ± 10	*10 ± 8	-
DEI	6.7 ± 3.1	-	-
eGFR	47 ± 20.3	73 ± 20	-
CRP	20 ± 34	7 ± 8	-
BSE	43.3 ± 43.3	43 ± 29	-
MPO positive patients (%)	29	-	-
MPO titer (IU/ml)	21,9 ± 23,7	-	-
PR3 positive patients (%)	71%	-	-
PR3 titer (IU/ml)	38,5 ± 49,5	-	-
Anti-dsDNA positive patients	-	83%	-
Anti-dsDNA ab titer (IU/ml)	-	87 ± 117	-
Complement consumption (%)	-	70	
C3 serum level (g/L)	-	0.86 ± 0.33	
Immunosuppressive medication			-
Prednison (%)	62	74	-
Mean dosage	10.8 ± 18	17 ± 17	-
Azathioprine (%)	34	10	-
Cellcept (%)	4	51	
HCQ (%)	0	75	-

Values are given as percentage or mean ± SD

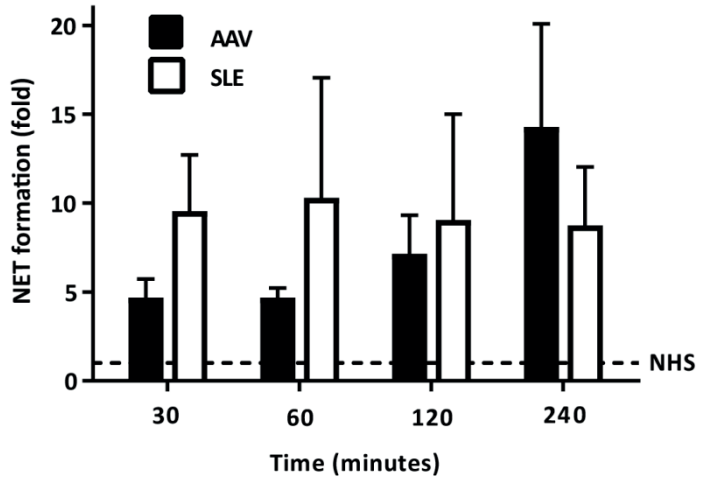

Figure S1. Kinetics of AAV- and SLE-induced NET formation. Quantification of NET formation for AAV (n=3) and SLE (n=3) after 30, 60, 120 and 240 minutes of stimulation. Data are expressed as mean ± SEM fold induction as compared to normal human serum (dotted line) within the same experiment.

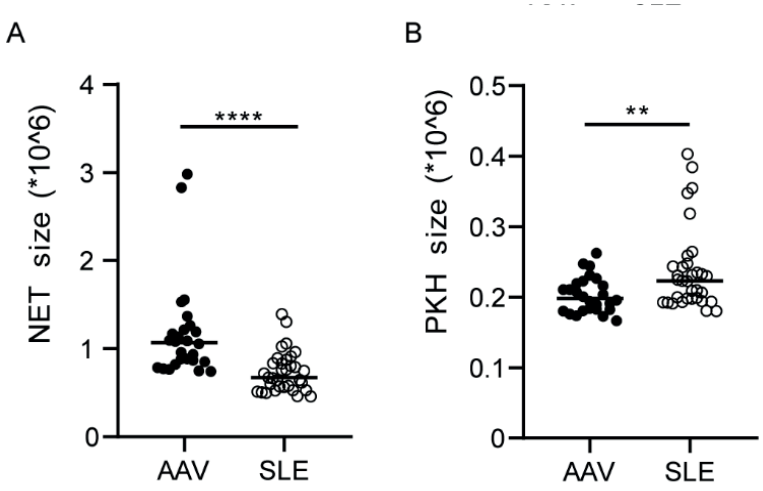

Figure S2. Quantification of relevant morphologic appearances of AAV- and SLE-induced NET formation. PKH-labelled neutrophils were stimulated with AAV sera (n=26, black dots) and SLE sera (n=30, open dots) to induce NET formation, as stained by SYTOXgreen and detailed analysis of images was performed to quantify **(A)** the average size of individual NETs and **(B)** the average size of neutrophil areas. Median of the AAV and SLE cohorts are depicted by the horizontal black line. For statistical analysis between two groups the Mann-Whitney U test was used. **$p < 0.01$, ****$p < 0.0001$. AAV – ANCA-associated vasculitis, NET – neutrophil extracellular trap, SLE – systemic lupus erythematosus.

Chapter 4

Excessive neutrophil extracellular trap formation in ANCA-associated vasculitis is independent of ANCA

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Abstract

Neutrophil extracellular traps (NETs) are auto-antigenic strands of extracellular DNA covered with myeloperoxidase (MPO) and proteinase3 (PR3) that can be a source for the formation of anti-neutrophil cytoplasmic autoantibodies (ANCAs). The presence of NETs was recently demonstrated in renal tissue of patients with ANCA-associated vasculitis (AAV). NET formation was enhanced in AAV, suggesting that MPO-ANCA could trigger NET formation, supporting a vicious circle placing NETs in the center of AAV pathogenesis. Here we investigated NET formation in 99 patients with AAV by a novel highly sensitive and automated assay. There was a significant excess of ex vivo NET formation in both MPO-ANCA– and PR3-ANCA– positive patients with AAV compared to healthy individuals. Excessive NET formation did not correlate with serum ANCA levels. Likewise, immunoglobulin G depletion had no effect on excessive NET formation in patients with AAV, indicating an ANCA-independent process. Next, we explored the relation of excessive NET formation to clinical disease in ten patients with AAV and showed that excessive NET formation was predominantly found during active disease, more so than during remission. Excessive NET formation was found in patients with AAV hospitalized for disease relapse but not during severe infection. Thus, excessive NET formation in AAV is independent of ANCA, and an excess of ex vivo NET formation was related to active clinical disease in patients with AAV and a marker of autoimmunity rather than infection.

Introduction

Activated neutrophils have been shown to release neutrophil extracellular traps (NETs), a novel antimicrobial mechanism coexisting with the classical and well-known enzyme degranulation and reactive oxygen species (ROS) production. NETs are net-like structures extruded by neutrophils and consist of decondensed DNA with nuclear and granular proteins, such as histones, proteinase 3 (PR3), and myeloperoxidase (MPO)1. They can be released upon stimulation with pathogens [1–4], immune complexes [5,6], and chemical compounds and are able to trap and kill pathogens [1,7,8].

Although NETs clearly act as an important first-line immune defense mechanism, they express potential autoantigens and have therefore been implicated in the pathogenesis of ANCA-associated vasculitis (AAV). Anti-neutrophil cytoplasmic antibodies (ANCAs) against MPO and PR3 have been shown to induce NET release [8,9], and NETs are present in kidney biopsies of AAV patients [8,10]. Furthermore, in MPO-ANCA–positive AAV a reduced degradation of NETs has been observed, suggesting that ANCAs arise from a possible break of tolerance against NET-derived antigens such as MPO and PR3 [9]. The latter was further corroborated by showing that myeloid dendritic cells (mDCs) loaded with extracellular DNA derived from NETotic neutrophils led to MPO-ANCA and PR3-ANCA production with concomitant vasculitis-like renal lesions in lpr/lpr mice [11]. In addition, the pathogenicity of NETs was demonstrated by NET-mediated damage to the vascular endothelium and its surrounding tissues [12–14]. Lastly, levimasole has recently been shown to induce NETs resulting in drug-induced systemic vasculitis strongly resembling AAV [15]. Taken together, these studies provide evidence for a central role for NETs in the pathophysiology of AAV.

Supported by the accumulating evidence that NETs are important to the initiation and perpetuation of vasculitis, the present study applied a newly developed, highly sensitive, and automated assay [16] to quantify ex vivo NET formation in PR3- and MPO-ANCA–positive AAV patients to study the interaction between ANCAs, NET formation, and clinical disease in AAV.

Materials and methods

Study population

Samples were obtained from AAV patients who were monitored at the outpatient clinic for Lupus, Vasculitis, and Complement-mediated Systemic Diseases at the Leiden University Medical Center (LUMC). Freshly frozen serum samples were collected from 63 PR3-ANCA–positive and 36 MPO-ANCA–positive AAV patients. From an additional 14 AAV patients, multiple sequential

samples were available. In 10 patients, samples were obtained during active disease as well as during remission. Clinical data were extracted from patients' electronic records, and disease status of remission, flare, or active disease was based on the treating physician's judgement. Fifteen samples from 7 patients were obtained at the time of hospitalization. As a control group, serum samples were obtained from 18 healthy subjects who consented to participate in the healthy donor biobank of the LUMC. Fifty-six percent of healthy donors were female, with a mean age SD of 39 ± 12 years. Both biobanking studies were approved by the local ethical committee at the LUMC.

Preparations of neutrophils

Twenty milliliters of whole blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes (BD; Franklin Lakes, NJ) from healthy donors. Neutrophils were isolated by density gradient centrifugation with Ficoll-amidotrizoate (LUMC, Leiden, the Netherlands) followed by erythrocyte lysis at 4°C. Cells were counted using trypan blue, labeled with PKH26 (2 mM; Sigma-Aldrich, St. Louis, MO), and 37.500 neutrophils per well were seeded into a 96-well culture plate (Falcon, Tewksbury, MA) in phenol red-free RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 2% heat-inactivated fetal calf serum. Neutrophils were stimulated during 3.75 hours with 1 of the following stimuli: medium (negative control), 10% serum, 10% IgG-depleted serum, 10% IgA- and IgG-depleted serum, 250 or 25 μg/ml healthy and patient IgG, phorbol 12-myristate 13-acetate (Sigma-Aldrich), or calcium ionophore A23187 (Sigma-Aldrich). Dose-effect studies were performed for serum (20%-10%-5%), healthy and patient IgG (750-250-83-25 μg/ml), calcium ionophore (1000-500-250-125-62.5 mM), and phorbol 12-myristate 13-acetate (100-50-25-12.5-6.25-3.125-1.6 mM) in relation to the effect on NET formation. Hereafter, 1 mM impermeable DNA dye Sytox green (Thermo Fisher, Waltham, MA) was added for 15 minutes, after which neutrophils were fixed with 4% paraformaldehyde (Added Pharma, Oss, the Netherlands).

Neutrophil priming and inhibition experiments

For priming experiments, 5 ng/ml TNF-α (Biolegend, San Diego, CA), 1 mM diphenyleneiodonium (Sigma-Aldrich), 200 mM Cl-amidine (EMD Millipore, Billerica, MA), or 10 mM C5aR antagonist (EMD Millipore) was added to neutrophils. Next, cells were seeded in a 96-well plate and incubated for 15 or 30 minutes at 37°C. After priming, cells were stimulated during 3.75 hours with any given stimulus. For inhibition of C5, healthy and patient serum was incubated with 10 μg/ml or 100 μg/ml eculizumab (Alexion Pharmaceuticals, New Haven, CT) for 30 minutes at room temperature on a rotation device and subsequently added to healthy neutrophils as described above. Neutralization of serum C5 by eculizumab was confirmed with the C5b-9 Wieslab kit (Euro-diagnostics, Malmö, Sweden).

NET visualization and quantification

Immediately following fixation, NETs were visualized by confocal laser scanning microscopy using the automated BD Pathway 855 (BD Biosciences, San Jose, CA), as described previously16 or the Image Xpress Micro Confocal (Molecular Devices, Sunnyvale, CA). Briefly, 12 z-stacked images of 25 predefined high-power fields (HPFs) at 20 magnification were automatically captured. The HPFs were evenly spread throughout the well by a standardized 5 × 5 zigzag pattern with 400 mm (length) and 500 mm (width) spaced between each HPF. Thereby, a total of 11.1% of the well was imaged. The microscope was programmed to automatically focus on PKH26 membrane staining. For each image, PKH26 (Cy3) and Sytox green (Alexa 488) were visualized. The same exposure time was applied to all images within 1 experiment. Acquired images were automatically analyzed by ImageJ image analysis software (NIH, Bethesda, MD) by determining the area of PKH and the area of extracellular DNA, using a pixel threshold to exclude potential intracellular staining. Extracellular DNA of NETs was quantified as the cumulative area of positive Sytox green. To correct for the number of neutrophils, the mean area of positive PKH26 staining was quantified. Thus, the ratio of both areas was calculated, representing the NET area corrected for the amount of imaged neutrophils. A higher ratio indicates a larger NET area present. For comparison of values between different experiment, a fold increase compared with 3 normal human serum controls taken in each experiment is calculated. The intra-assay variability is 26% (based on 30 serum samples), and the inter-assay variability is 39% (based on 2 identical experiments). To circumvent this variation, when making direct comparisons (for instance when using inhibitors or studying IgG depletion), experiments were always performed within one plate to reliably assess the effects on NET formation. Of note, only serum was used in our studies because sodium citrate, EDTA, and heparin interfered with the NET quantification assay (Supplementary Figure S1D).

Fluorescence immunocytochemistry of NETs

PKH-labeled neutrophils were seeded onto chambered coverslips (ibiTreat; Ibidi, Martinsried, Germany), and NETs were induced according to the protocol mentioned above. Fixed neutrophils were blocked with 1% BSA and 20% normal goat serum in phosphate-buffered saline with 5 mM EDTA to inhibit nuclease activity and stained with 1 μg/ml polyclonal rabbit anti-human citrullinated histon3 (Abcam, Cambridge, UK), 5 μg/ml polyclonal rabbit neutrophil elastase (Abcam) or a polyclonal rabbit IgG isotype control (Dako, Santa Clara, CA) in phosphate-buffered saline and 1% normal goat serum with 5 mM EDTA and incubated for 2 hours. Then neutrophils were washed and incubated with a 1 per 500 secondary goat anti-rabbit Alexa 488 antibody (Thermo Fisher). The secondary antibodies were pre-incubated for 30 minutes with 5% normal human serum to bind anti-human Igs. After 60 minutes of incubation with the secondary antibodies and 1 per 100 phalloidin (Sigma-Aldrich), neutrophils were washed and stained with 1 ug/ml Hoechst 33258 (Thermo Fisher). Images were acquired with the Leica DMI6000 inverted microscope using a ×20 magnification.

IgG and IgA depletion from serum

IgG was depleted from sera using protein G agarose beads (Sino Biological, Beijing, China). During each experiment, a sham depletion and isolation was performed with tris(hydroxymethyl) aminomethane buffer to control for (bacterial) contaminating factors. Briefly, beads were washed 3 times with binding buffer (50 mM tris[hydroxymethyl] aminomethane, 100 mM NaCl, pH 8.0) and 500 ml of serum was mixed overnight with 500 ml of the protein G agarose beads at 4°C. Then, the mix was centrifuged to separate the upper fraction, containing the IgG-depleted serum, from the agarose beads. Total protein in the IgG-depleted serum was measured with a Pierce assay (Thermo Scientific), and IgG was measured with an IgG ELISA (Bethyl Laboratories, Montgomery, TX). Selected IgG-depleted sera were subsequently depleted for IgA using an IgA affinity column (sepharose beads conjugated with mAb HisA43 anti-human IgA) and mixed overnight at 4°C. Next, the mix was centrifuged and the upper fraction, containing IgG- and IgA-depleted serum, was collected. IgG and IgA concentration was measured with ELISA (LUMC, the Netherlands).

IgG separation

Protein G agarose beads containing IgG were loaded on a 1-ml micro-column and washed 3 times with binding buffer. Next, IgG was eluted from the beads with 0.5 ml acidic elution buffer (0.1 M Glycin, pH 2–3). IgG fractions were collected in tubes with neutralizing buffer (1 M tris[hydroxymethyl] aminomethane–HCl, pH 8–9) to immediately adjust eluted fractions to physiological pH of 7.5. Fractions containing IgG were pooled, dialyzed against phosphate-buffered saline overnight at 4°C, and concentrated with a centrifugal filter unit with a 30 kDa cut-off (Merck Millipore, Darmstadt, Germany). Lastly, total IgG concentration and specific anti-PR3 IgG and anti-MPO IgG was measured by fluorescence ELISA using a Phadia250 system (Thermo Scientific).

Flow cytometry

Isolated healthy neutrophils were primed with 5 ng/ml TNF-α in RPMI medium supplemented with 2% heat-inactivated fetal calf serum for 15, 30, or 60 minutes. Next, cells were washed and incubated at 4°C with the primary antibodies: 10 μg/ml mouse monoclonal anti-human MPO antibody (Hycult Biotech, Plymouth Meeting, PA), 10 μg/ml mouse monoclonal anti-human PR3 antibody (Abcam), or 10 μg/ml isotype control, a mouse anti-human IgG1 antibody (Agilent Technologies, Santa Clara, CA). After 30 minutes, cells were washed and incubated for 30 minutes at 4°C with 5 ug/ml secondary antibody goat anti-mouse Ig F(ab)2 antibody with PE label (Agilent Technologies). After washing, surface expression of PR3 and MPO was measured on the BD LSR 2 flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Ashland, OR).

Statistical analysis

Data are expressed as mean NET area per imaged neutrophil, as mean fold induction SEM or median interquartile range, calculated relative to the mean normal human serum value within the same experiment. Statistical difference between 2 groups was determined with a Student's t-test and paired t-test, if applicable. Correlations were assessed using Pearson and Spearman correlation coefficients. All statistical analyses were performed with GraphPad software (La Jolla, CA).

Results

Ex vivo visualization of NET formation in AAV

Upon stimulation with serum from AAV patients, the formation of NETs was visualized by the expression of NET-specific citrullinated histon3 (citH3) and neutrophil elastase (NE) on Hoechst-positive extracellular DNA from healthy neutrophils (Figure 1a). Additionally, inhibition of reduced NAD phosphate oxidase with diphenyleneiodonium (DPI) and peptidyl arginine deiminase (PAD) with Cl-amidine confirmed that AAV-induced extracellular DNA were part of NETs: co-incubation with DPI demonstrated a significant reduction of AAV-associated NET induction (mean reduction SEM: 61% ± 10%, $P < 0.01$; Figure 1b) as well as co-incubation with Cl-amidine, which led to a significant reduction of AAV-derived NET formation (mean reduction T SEM: 40% T ± 11%, $P < 0.05$; Figure 1c).

Excessive ex vivo NET formation in AAV does not correlate with serum ANCA levels

Ex vivo NET formation was determined in a large cohort of AAV patients positive for anti-PR3 or anti-MPO. To measure NET formation by serum from AAV patients, we used a novel, highly sensitive, and automated method [16]. Experimental studies supporting the assay's approach to use healthy neutrophils as the basis to quantify excessive NET formation are summarized in Supplementary Figure S1A–C. As such, Figure 2a shows excessive NET formation in PR3-ANCA– positive patients (median fold induction ± interquartile range: 13.5 ± 52) as well as MPO-ANCA–positive patients (38.8 ± 160.1) compared with healthy controls. The difference between MPO-ANCA– and PR3-positive ANCA patients was statistically significant ($P < 0.01$). Unexpectedly, neither PR3-ANCA titers ($R = –0.17$, $P = 0.19$; Figure 2b) nor MPO-ANCA titers ($R = 0.22$, $P = 0.21$; Figure 2c) correlated with the amount of NET formation. NET formation was not found to correlate with serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, or IL-8 (data not shown) or classical markers of inflammation (i.e., C-reactive protein and erythrocyte sedimentation rate; Supplementary Figure S2).

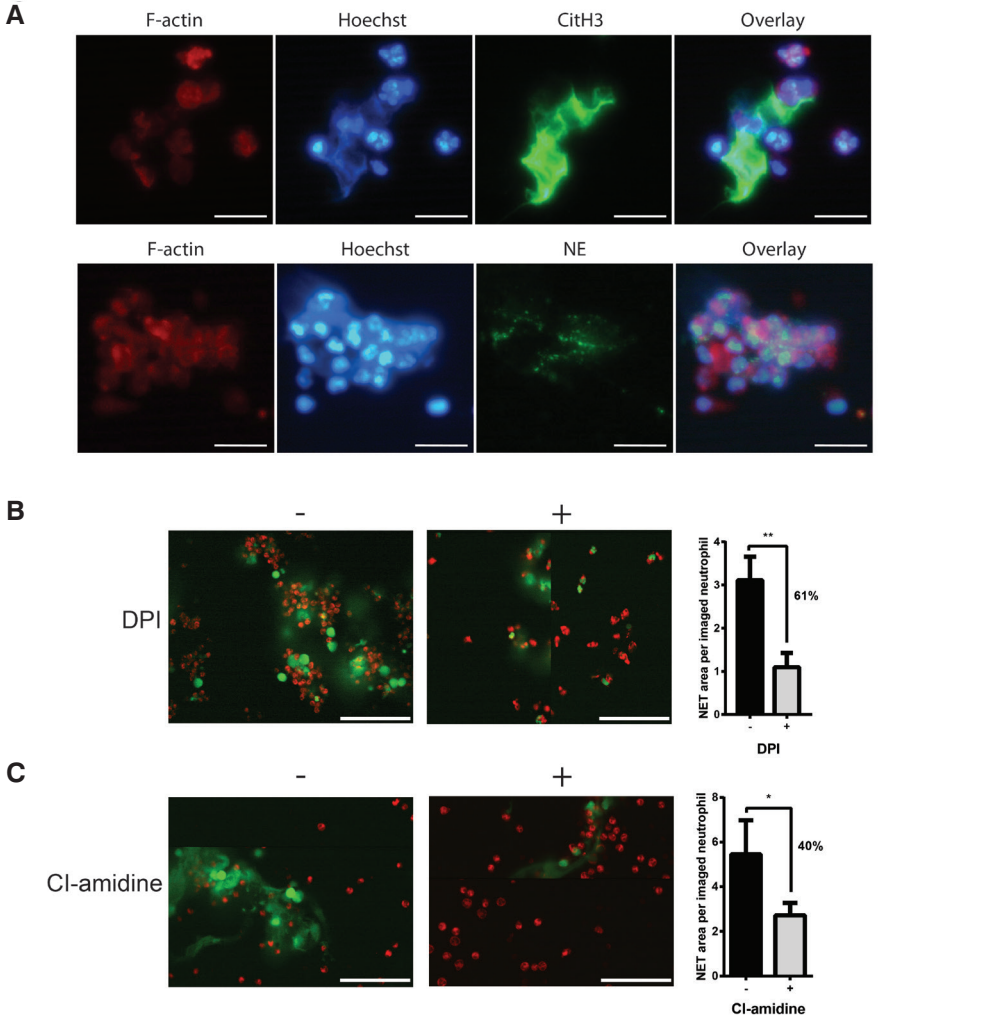


Figure 1. Neutrophil extracellular trap (NET) formation in antineutrophil cytoplasmic autoantibody-associated vasculitis (AAV) patients. **(A)** Representative images of NETs, induced with 10% serum from AAV patients, stained with NET-specific antigens citrullinated histon3 (citH3; top panel) or neutrophil elastase (NE; lower panel). DNA was stained with Hoechst (blue), and F-actin (red) was used as a cell marker. Bar 30 mm. **(B)** Representative images of AAV-induced NETs with and without pre-incubation of neutrophils with 1 mM diphenyleneiodonium (DPI). Neutrophils were labeled with PKH26 (red), and extracellular DNA (green) was stained with Sytox green. Quantitative results are based on 9 AAV patient samples. Bar 125 mm. **(C)** Representative images of AAV-induced NETs with and without pre-incubation of neutrophils with 200 mM Cl-amidine. Neutrophils were labeled with PKH26 (red), and extracellular DNA (green) was stained with Sytox green. Quantitative results are based on 7 AAV patient samples. Bar 125 mm. *$P < 0.05$; **$P < 0.01$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

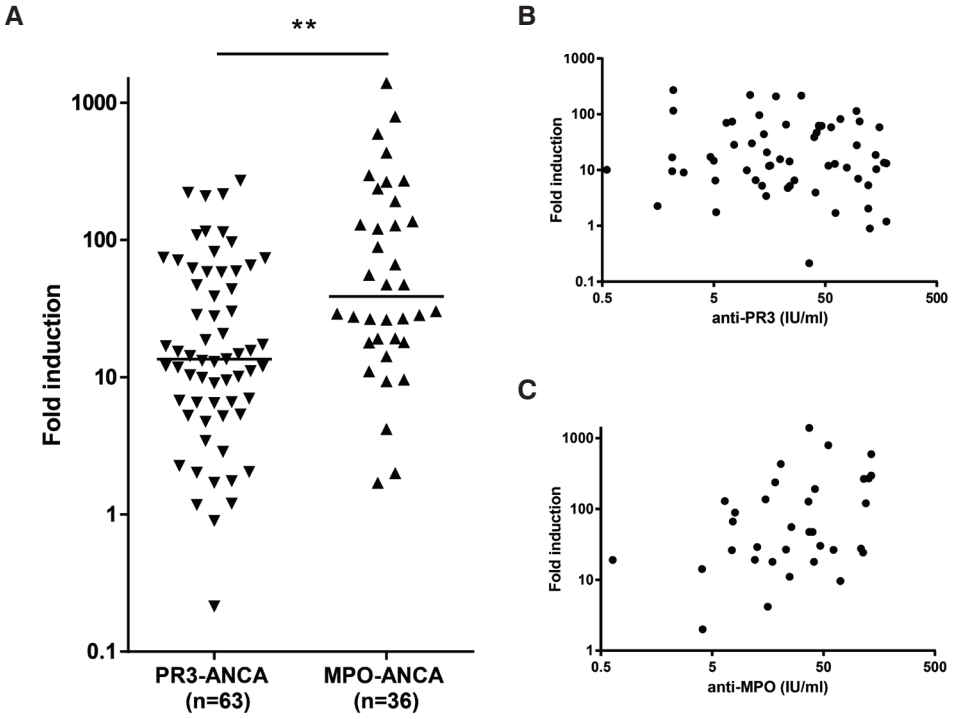


Figure 2. Excessive neutrophil extracellular trap (NET) formation in antineutrophil cytoplasmic autoantibody (ANCA)–associated vasculitis does not correlate with ANCA titers. **(A)** Quantification of ex vivo NET induction in 63 proteinase3 (PR3)-ANCA– and 36 myeloperoxidase (MPO)-ANCA–positive ANCA-associated vasculitis patients. Results are shown as median fold induction relative to healthy controls. **(B)** No correlation was observed between NET formation and PR3-ANCA IgG. **(C)** No correlation was observed between NET formation and MPO-ANCA IgG.

ANCA IgG is not responsible for excessive NET formation in AAV

To investigate whether ANCA IgG was responsible for the excessive NET formation by AAV serum, we next depleted IgG, and later also IgA, from serum of 10 PR3-ANCA-positive patients, 5 MPO-ANCA–positive patients, and 3 healthy controls. Figure 3a summarizes the serum characteristics demonstrating IgG depletion, including depletion of ANCA IgG. Also, after elution of IgG from the depleting beads, IgG including PR3-ANCA and MPO-ANCA were retrieved. Figure 3b illustrates for 1 representative patient that despite IgG depletion, excessive NET formation remained present and IgG alone was not able to induce NET formation. Quantifying all patients together, for PR3-ANCA–positive patients we observed no significant difference in NET formation after IgG depletion (mean ± SEM fold induction: 8.1 ± 1) compared with whole serum (9.3 ± 2.4; $P = 0.60$). For MPO-ANCA–positive patients we also observed no significant difference in NET formation after IgG depletion (18.3 ± 5.4) compared with whole serum (25.9 ± 6; $P = 0.06$). Moreover, isolated IgG from PR3-ANCA–positive patients was unable to induce NET formation (fold induction ± SEM: 1.5 ± 0.4 for 250 μg/ml and 1.3 ± 0.6 for 25 μg/ml), and neither was isolated IgG from MPO-ANCA–positive patients (1.4 ± 0.7 for 250 μg/ml and 1.1 ± 0.3 for 25 μg/ml) (Figure 3c and d).

To exclude that IgA-derived ANCA was responsible for the observed NET formation in IgG-depleted sera, IgA was subsequently depleted. IgA depletion was confirmed with enzyme-linked immunosorbent assay (ELISA). No significant difference was found after both IgA and IgG depletion (mean fold induction SEM: 17 5.5) compared with corresponding serum samples (20.2 ± 10.1; $P = 0.74$; Figure 3e and f).

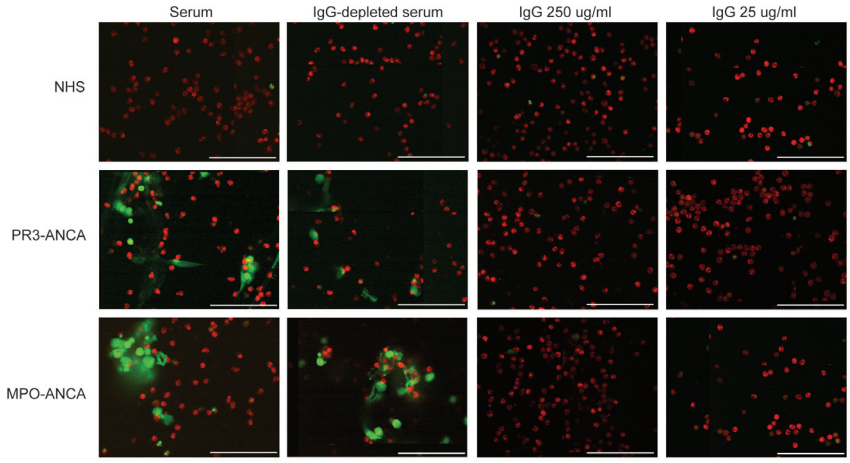
Next, we investigated whether upregulation of PR3 and MPO expression on neutrophils could influence NET formation by ANCA IgG. To do so, neutrophils were primed with TNF-α, after which PR3 and MPO upregulation was confirmed by flow cytometry (Figure 4a). Incubation of unprimed and TNF-α-primed neutrophils with isolated IgG from AAV patients did not show a significant difference: using 25 ug/ml IgG, the mean fold NET induction ± SEM in unprimed cells was 1.5 ± 0.5 versus 1.5 ± 0.3 ($P = 0.69$) in primed cells. Also, when increasing IgG concentration to 250 ug/ml, the mean fold NET induction SEM in primed cells (2.4 ± 0.4) was not significantly different from that in unprimed cells (2.8 ± 1.2, $P = 0.67$). As a positive control, NET formation was observed with whole serum from AAV patients before and after IgG depletion in unprimed as well as primed cells (Figure 4b).

A

	Serum	IgG-depleted serum	IgG
IgG (g/l)	8.2 ± 1	<0.008[a]	2 ± 0.3
IgA (g/l)	6.3 ± 2.2	1.9 ± 0.7	<0.05[a]
PR3-ANCA IgG (IU/ml)	33 ± 8.5	<0.2[a]	58.8 ± 25.4
MPO-ANCA IgG (IU/ml)	40 ± 19.6	<0.2[a]	81.1 ± 26

Data are presented as mean ± SEM; [a]Below the detection limit of ELISA

B



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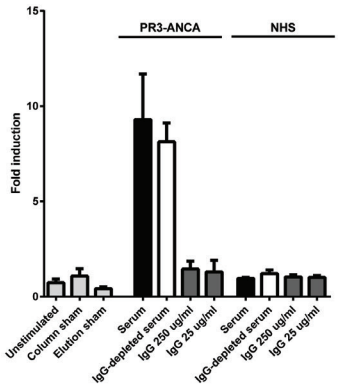

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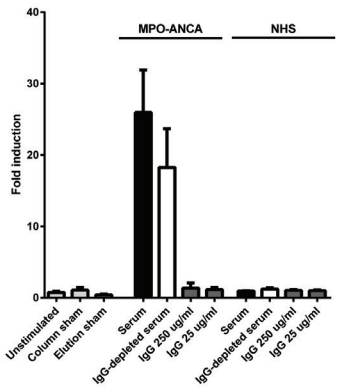

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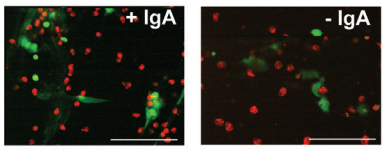

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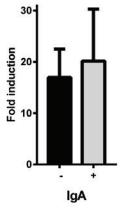

Figure 3. Excessive neutrophil extracellular trap (NET) formation is independent of myeloperoxidase (MPO)-antineutrophil cytoplasmic autoantibody (ANCA) and proteinase3 (PR3)-ANCA IgG. **(A)** Summary of serum characteristics used to investigate NET induction in ANCA-associated vasculitis (AAV). Mean SEM levels of total IgG, total IgA, PR3-ANCA IgG, and MPO-ANCA IgG are depicted from whole serum (n = 15), serum after IgG depletion (n = 5), and isolated IgG (n = 5). **(B)** Representative images of NET formation with whole serum, IgG-depleted serum, and isolated IgG of 1 AAV patient and a healthy control. Neutrophils were labeled with PKH26 (red), and extracellular DNA (green) was stained with Sytox green. **(C,D)** Quantification of NET formation is shown for PR3-ANCA (n = 10) and MPO-ANCA–positive patients (n = 5). An unstimulated control, column sham (representing tris[hydroxymethyl] aminomethane [TRIS] buffer that was incubated overnight with protein G agarose beads), and elution sham (representing elution of TRIS buffer from the beads) were taken along as controls. **(E)** Representative images of NET formation by serum before and after both IgG and IgA depletion. **(F)** Quantification of NET formation shown for serum with corresponding IgG- and IgA-depleted serum (n = 5). Bar = 125 mm. NHS, normal human serum. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

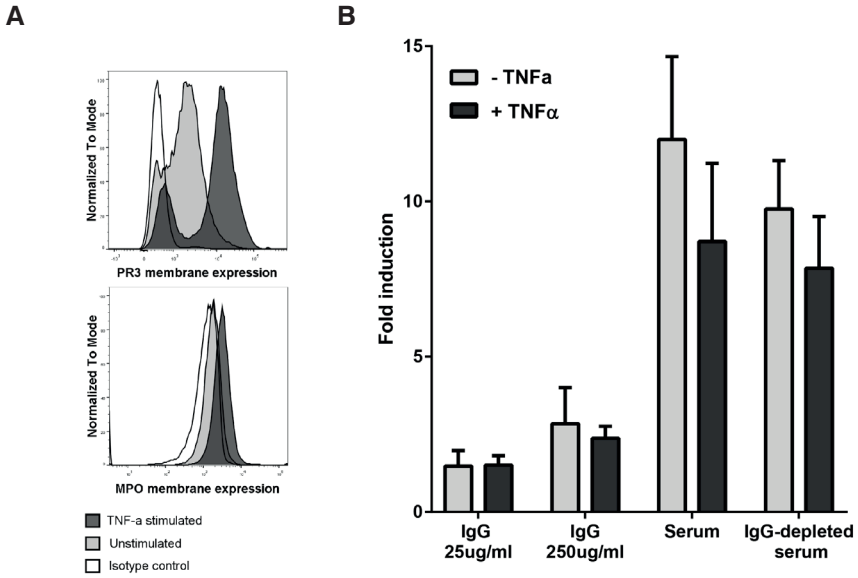

Figure 4. Ex vivo neutrophil extracellular trap (NET) formation by tumor necrosis factor (TNF)-α–primed neutrophils is independent of myeloperoxidase (MPO)-antineutrophil cytoplasmic autoantibody (ANCA) and proteinase3 (PR3)-ANCA IgG. **(A)** Expression of MPO and PR3 on healthy neutrophils after TNF-α priming (15 minutes with 5 ng/ml). Light gray histograms represent MFI of unprimed neutrophils; dark gray histograms represent TNF-a–primed neutrophils; white histogram represent the isotype control. **(B)** Quantification of NET formation shown for unprimed (light gray bars) and TNF-α–primed neutrophils (black bars) upon incubation with isolated IgG 25 ug/ml, isolated IgG 250 ug/ml, whole serum, and IgG-depleted serum (bars represent mean SEM of 8 ANCA-associated vasculitis patients). MFI, mean fluorescence intensity.

Activated complement pathways are not involved in NET formation in AAV

Because neutrophils of AAV patients are activated through C5a receptor triggering,17,18 we investigated whether complement activation had a role in excessive NET formation in AAV. Therefore, we pre-incubated healthy neutrophils with a C5aR antagonist, after which NET formation was induced with serum from ANCA-positive AAV patients. Figure 5a shows that excessive NET formation was independent of C5aR inhibition (mean ± SEM of 11 ± 3.8 without and 12.1 ± 3.6 with C5aR inhibition). Next, we pre-incubated serum from ANCA-positive AAV patients with eculizumab, a C5 inhibitor, after which NETs were induced in healthy neutrophils. Neutralization of C5 by 100 ug/ml eculizumab was functionally confirmed (Supplementary Figure S3); however, the abrogation of activated complement with eculizumab did not result in a significant change in NET formation (mean ± SEM of 11 ± 3.8 without and 13.5 ± 4.5 with 10 μg/ml eculizumab and 13.4 ± 4 with 100 μg/ml eculizumab) (Figure 5b). From these data it can be concluded that activated complement pathways were not involved in NET formatio in AAV.

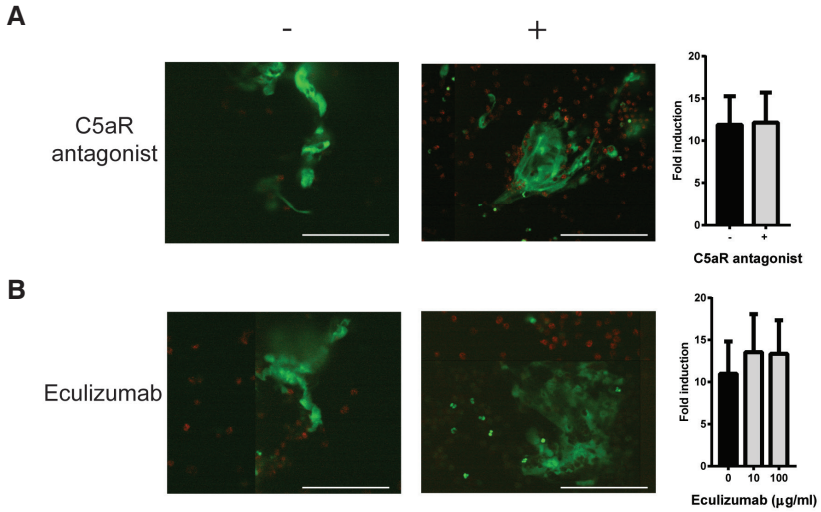

Figure 5. Excessive neutrophil extracellular trap (NET) formation in antineutrophil cytoplasmic autoantibody–associated vasculitis (AAV) is independent of complement activation. **(A)** Representative images of AAV-induced NET formation with and without pre-incubation of healthy neutrophils with 10 mM C5aR antagonist. Neutrophils were labeled with PKH26 (red), and extracellular DNA (green) was stained with Sytox green. Quantification of NET release is shown for 7 AAV samples. **(B)** Representative images of NET induction by AAV serum incubated with or without 10 and 100 μg/ml of the C5 inhibitor eculizumab. Neutrophils were labeled with PKH26 (red), and extracellular DNA (green) was stained with Sytox green. Bars represent mean ± SEM of 5 AAV patients. Bar = 125 mm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

NET formation in relation to clinical disease in AAV

Because NET formation appeared to be independent of the presence of circulating ANCA, a well-established biomarker in AAV, we explored whether excessive NET formation related to clinical disease in AAV. We found excessive NET formation in paired samples from 10 AAV patients during active disease (mean fold induction SEM was 9.8 ± 2.5), which was significantly less when disease was in remission (2.2 ± 0.3, $P < 0.01$), suggesting excessive NET formation associated with clinical disease activity (Figure 6a). Moreover, at time of hospitalization of patients with an established AAV diagnosis, we observed a significant excess in NET formation (mean fold induction SEM of 10 ± 3.2) in patients who were admitted with relapsing AAV disease compared with patients who were admitted with a severe infection (2.9 ± 0.5; $P < 0.05$) (Figure 6b). Details of the clinical characteristics and infectious events are summarized in Supplementary Table S1. Taken together, we observed that excessive NET formation was associated with clinical disease activity in AAV patients and possibly distinguished autoimmunity from infection.

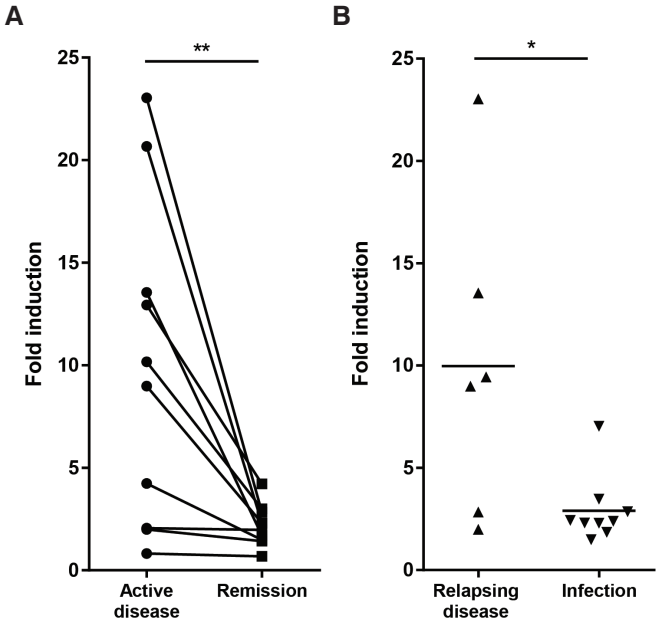

Figure 6. Excessive neutrophil extracellular trap (NET) formation is related to clinical disease activity and could distinguish autoimmunity from infection**. (A)** Paired NET induction in 10 antineutrophil cytoplasmic autoantibody–associated vasculitis (AAV) patients at time of active disease and disease remission. **(B)** NET induction at time of hospitalization of patients with an established AAV diagnosis (n = 15). Excessive NET induction in patients admitted for relapsing AAV disease as compared with patients admitted for infection. *$P < 0.05$, **$P < 0.01$.

Discussion

This study demonstrated that excessive NET formation in a large cohort of MPO-ANCA– and PR3-ANCA–positive AAV patients was independent of the presence of PR3- and MPO-ANCA. Additionally, we observed that excessive NET formation was predominantly seen in AAV patients during active disease rather than in remission or during infection. Thus, the present study conceptualized measuring excessive NET formation in AAV patients, which could be instrumental to increase our understanding of NETs in relation to clinical disease.

Although several studies have previously described NET formation in MPO-ANCA–positive patients [8,9], the present study is the first to describe excessive NET formation in a large number of PR3-ANCA–positive patients. In contrast to the pioneering studies on NETs in AAV, we demonstrate by using a nonbiased automated quantification method of NETs that ANCAs do not contribute to the induction of NETs in AAV. Thus far, it was a matter of debate whether ANCAs mediated NET formation in AAV because Söderberg et al. [19] demonstrated that circulating NET remnants were higher in AAV patients with an inverse correlation to serum ANCA levels in AAV patients in remission and a negative correlation in active MPO-ANCA–positive patients. Additionally, ANCA-negative patients showed higher levels of circulating NET remnants compared with ANCA-positive patients, supporting the notion that mechanisms other than ANCA might be responsible for excessive NET formation in AAV. Until now, methods for NET quantification (e.g., using fluorescence microscopy [8] or ELISA measurements of NET components [8,19–21]) incorporated some limitations, such as the inability to dissect in vivo NET formation from NET degradation and the inability to correct for extracellular DNA derived from other death pathways (such as necrosis), and depended upon the relatively low-frequency expression of specific markers such as MPO or citH3. The method used in this study overcame these issues by using automated laser scanning microscopy, enabling us to detect and quantify large series of imaged neutrophils, optimizing the reliability and reproducibility of the amount of NET released by neutrophils [16]. A potential limitation of our assay was the use of healthy neutrophils instead of (autologous) neutrophils from AAV patients. Besides the obvious challenge to perform a NET quantification with freshly obtained AAV patients, we addressed this issue of intrinsic differences between healthy neutrophils compared with AAV neutrophils in 2 ways. First, we observed that spontaneous NET release in AAV-derived neutrophils was not significantly different from healthy neutrophils (Supplementary Figure S1A). Second, with respect to quantifying ex vivo NET formation, we observed no significant differences in the excessive NET formation induced by serum from AAV patients (Supplementary Figure S1B). These studies supported the approach used for the reported NET assay to use healthy neutrophils as the basis to quantify serum factors that induce excessive NET formation. Thus, our study provided unique, novel insights in the dynamics of excessive NET formation in AAV, notably that it was

independent of ANCAs. Because our study could exclude ANCA-IgA or complement activation as a potential factor to induce NET formation in AAV, future research will be directed at identifying the exact molecular triggers and pathways underpinning this process. Because we observed higher NET formation during active disease than during remission, it is tempting to speculate that excessive NET formation is induced by a combination of pro-inflammatory cytokines and/or damage-associated molecular patterns. In recent years, additional molecules have been described to be enhanced in AAV, including calprotectin [22], GM-CSF, and others [23]. For future research, it will be critical to investigate these factors, either alone or in combination, in relation to their effect on NET formation.

ANCAs play an important role in the pathogenesis of ANCA-associated vasculitis [24]. In vitro, ANCAs can activate neutrophils via Fcγ receptors and binding of ANCA to the ANCA target antigens, leading to the release of reactive oxygen species (ROS) and granule enzymes [25], mediating damage to the endothelium and inducing more neutrophil activation via complement activation. In addition, AAV patients have higher availability of ANCA target antigens on the resting neutrophil membrane [26,27]. Also, neutrophil adhesion molecules are upregulated on ANCA-stimulated neutrophils and migration, and translocation of neutrophils is enhanced [28]. In vivo, anti-MPO splenocytes cause pauciimmune crescentic glomerulonephritis in mice [29]. From a clinical perspective, support for pathogenicity of ANCAs has come from the positive predictive value of ANCA serology [30], its association with disease activity [31], and the beneficial effects of B-cell–targeted therapies and plasma exchange [32–34]. Although we show that ANCA has only a minor role in NET formation in AAV, our current data do not discredit the vast literature on ANCA pathogenicity.

There is an unmet need for biomarkers for disease monitoring in AAV. ANCAs are present in 90% [35} of patients with active disease and are commonly used as a biomarker for diagnosis but not for disease activity. Research on the value of ANCA titers to predict relapse is controversial and evolving [31,36]. In a cohort of 201 patients, [37] an ANCA rise was significantly associated with a relapse in patients with renal involvement, but less so in patients without renal involvement. Therefore, several studies have attempted to investigate whether NETs can be used as a biomarker in AAV. Nakazawa et al. [9] showed a correlation between Cit H3-positive NETs and Birmingham Vasculitis Activity Score (BVAS) in MPO-ANCA–positive patients only. Wang et al. [20] found no association between ELISA-measured cell-free DNA or MPO-DNA complexes and disease activity in AAV patients, likely because the quantification measured the net result of NET formation and NET degradation in vivo. The present study explored a novel and more sensitive assay for NET formation, a potential autoantigen for the production of ANCA, in relation to clinical disease in AAV. Based on our study, it remains speculative whether quantifying excessive NET formation can truly serve as a biomarker in AAV because our study was limited by retrospective

design lacking contemporary BVAS, and patient numbers with longitudinal sampling were small. However, despite these important limitations, this study presents for the first time intriguing associations between excessive NET formation and disease activity, as well as the ability to distinguish between disease relapse and severe infection. Both illustrate the need to better establish the potential of measuring excessive NET formation for diagnostic as well as clinical purposes in larger, prospective studies.

Lastly, the serendipitous finding that excessive NET formation was significantly higher in MPO-ANCA– compared with PR3-ANCA–positive patients is of interest. Intriguingly, we know from genome-wide associated studies that there is a distinct genetic association between GPA and MPA [38]. Also, a neutrophil gene signature has been shown in AAV that strongly correlated with disease activity [39]. This gene signature overlapped the low-density granulocyte signature described previously in systemic lupus erythematosus [40,41], where low-density granulocytes have been shown to release NETs spontaneously. It is tempting to speculate that because the clinical manifestations of GPA and MPA can vary widely [35] and are strongly associated with the presence of PR3-ANCA and MPO-ANCAs, respectively, the role of NET formation in GPA and MPA is indeed different.

In conclusion, this study demonstrated that excessive NET formation observed in AAV is for the most part independent of ANCA and seems to be related to disease activity. Our preliminary data emphasize the need to further investigate whether quantifying excessive NET formation can indeed guide clinical decision-making in AAV patients.

Acknowledgements

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References

1. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303:1532–1535.
2. Pilsczek FH, Salina D, Poon KKH, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol. 2010;185:7413–7425.
3. Urban CF, Ermert D, Schmid M, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog. 2009;5:e1000639.
4. McCormick A, Heesemann L, Wagener J, et al. NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. Microbes Infect. 2010;12:928–936.
5. Behnen M, Leschczyk C, Möller S, et al. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgRIIIB and Mac-1. J Immunol. 2014;193:1954–1965.
6. Chen K, Nishi H, Travers R, et al. Endocytosis of soluble immune complexes leads to their clearance by FcgammaRIIIB but induces neutrophil extracellular traps via FcgammaRIIA in vivo. Blood. 2012;120:4421–4431.
7. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med. 2013;5:178ra40.
8. Kessenbrock K, Krumbholz M, Schönermarck U, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009;15:623–625.
9. Nakazawa D, Shida H, Tomaru U, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. J Am Soc Nephrol. 2014:1–8.
10. O'Sullivan KM, Lo CY, Summers SA, et al. Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. Kidney Int. 2015;88:1030–1046.
11. Sangaletti S, Tripodo C, Chiodoni C, et al. Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. Blood. 2012;120:3007–3018.
12. Saffarzadeh M, Juenemann C, Queisser MA, et al. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: A predominant role of histones. PLoS One. 2012;7.
13. Villanueva E, Yalavarthi S, Berthier CC, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol. 2011;187:538–552.
14. Nakazawa D, Kumar SV, Marschner J, et al. Histones and neutrophil extracellular traps enhance tubular necrosis and remote organ injury in ischemic AKI. J Am Soc Nephrol. 2017;28:1753–1768.
15. Lood C, Hughes GC. Neutrophil extracellular traps as a potential source of autoantigen in cocaine-associated autoimmunity. Rheumatology (Oxford). 2016;56:638–643.

16. Kraaij T, Tengström FC, Kamerling SWA, et al. A novel method for highthroughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes. Autoimmun Rev. 2016;15:577–584.
17. Xing GQ, Chen M, Liu G, et al. Complement activation is involved in renal damage in human antineutrophil cytoplasmic autoantibody associated pauci-immune vasculitis. J Clin Immunol. 2009;29:282–291.
18. Wang H, Wang C, Zhao MH, et al. Neutrophil extracellular traps can activate alternative complement pathways. Clin Exp Immunol. 2015;181:518–527.
19. Söderberg D, Kurz T, Motamedi A, et al. Increased levels of neutrophil extracellular trap remnants in the circulation of patients with small vessel vasculitis, but an inverse correlation to anti-neutrophil cytoplasmic antibodies during remission. Rheumatology (Oxford).2015;54:2085–2094.
20. Wang H, Sha LL, Ma TT, et al. Circulating level of neutrophil extracellular traps is not a useful biomarker for assessing disease activity in antineutrophil cytoplasmic antibody-associated vasculitis. PLoS One. 2016;11:1–10.
21. Surmiak M, Hubalewska-Mazgaj M, Wawrzycka-Adamczyk K, et al. Neutrophil-related and serum biomarkers in granulomatosis with polyangiitis support extracellular traps mechanism of the disease. Clin Exp Rheumatol. 2016;34:S98–S104.
22. Ehrchen JM, Sunderkötter C, Foell D, et al. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol. 2009;86:557–566.
23. Monach PA, Warner RL, Tomasson G, et al. Serum proteins reflecting inflammation, injury and repair as biomarkers of disease activity in ANCA-associated vasculitis. Ann Rheum Dis. 2013;72:1342–1
24. Jennette JC, Falk RJ. Pathogenesis of antineutrophil cytoplasmic autoantibody-mediated disease. Nat Rev Rheumatol. 2014;10:463–473.
25. Falk RJ, Terrell RS, Charles L, et al. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. Proc Natl Acad Sci U S A. 1990;87:4115–4119.
26. Schreiber A, Luft FC, Kettritz R. Membrane proteinase 3 expression and ANCA-induced neutrophil activation. Kidney Int. 2004;65:2172–2183.
27. van Rossum AP, Limburg PC, Kallenberg CGM. Membrane proteinase 3 expression on resting neutrophils as a pathogenic factor in PR3-ANCAassociated vasculitis. Clin Exp Rheumatol. 2003;21:S64–S68.
28. Radford DJ, Savage COS, Nash GB. Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion. Arthritis Rheum. 2000;43:1337–1345.
29. Xiao H, Heeringa P, Hu P, et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J Clin Invest. 2002;110:955–963.

30. Christiaan Hagen E, Daha MR, Hermans J, et al. Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis for the EC/BCR Project for ANCA Assay Standardization. Kidney Int. 1998;53:743–753.
31. Tomasson G, Grayson PC, Mahr AD, et al. Value of ANCA measurements during remission to predict a relapse of ANCA-associated vasculitis-a meta-analysis. Rheumatology (Oxford). 2012;51:100–109.
32. Jayne DRW, Gaskin G, Rasmussen N, et al. Randomized trial of plasma exchange or high-dosage methylprednisolone as adjunctive therapy for severe renal vasculitis. J Am Soc Nephrol. 2007;18: 2180–2188.
33. Stone JH, Merkel PA, Spiera R, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. N Engl J Med. 2010;363:221–232.
34. Guillevin L, Pagnoux C, Karras A, et al. Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. N Engl J Med. 2014;371: 1771–1780.
35. Kallenberg CGM. Key advances in the clinical approach to ANCA-associated vasculitis. Nat Rev Rheumatol. 2014;10:484–493.
36. Yates M, Watts RA, Bajema IM, et al. EULAR/ERA-EDTA recommendations for the management of ANCA-associated vasculitis. Ann Rheum Dis. 2016;75:1583–1594.
37. Kemna MJ, Damoiseaux J, Austen J, et al. ANCA as a predictor of relapse: useful in patients with renal involvement but not in patients with nonrenal disease. J Am Soc Nephrol. 2015;26:537–542.
38. Lyons PA, Rayner TF, Trivedi S, et al. Genetically distinct subsets within ANCA-associated vasculitis. N Engl J Med. 2012;367:214–223.
39. Grayson PC, Carmona-Rivera C, Xu L, et al. Neutrophil-related gene expression and low-density granulocytes associated with disease activity and response to treatment in antineutrophil cytoplasmic antibody-associated vasculitis. Arthritis Rheumatol. 2015;67:1922–1932.
40. Carmona-Rivera C, Kaplan MJ. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. Semin Immunopathol. 2013;35:455–463.
41. Garcia-Romo GS, Caielli S, Vega B, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med. 2011;3:73ra20.

Supplementary material

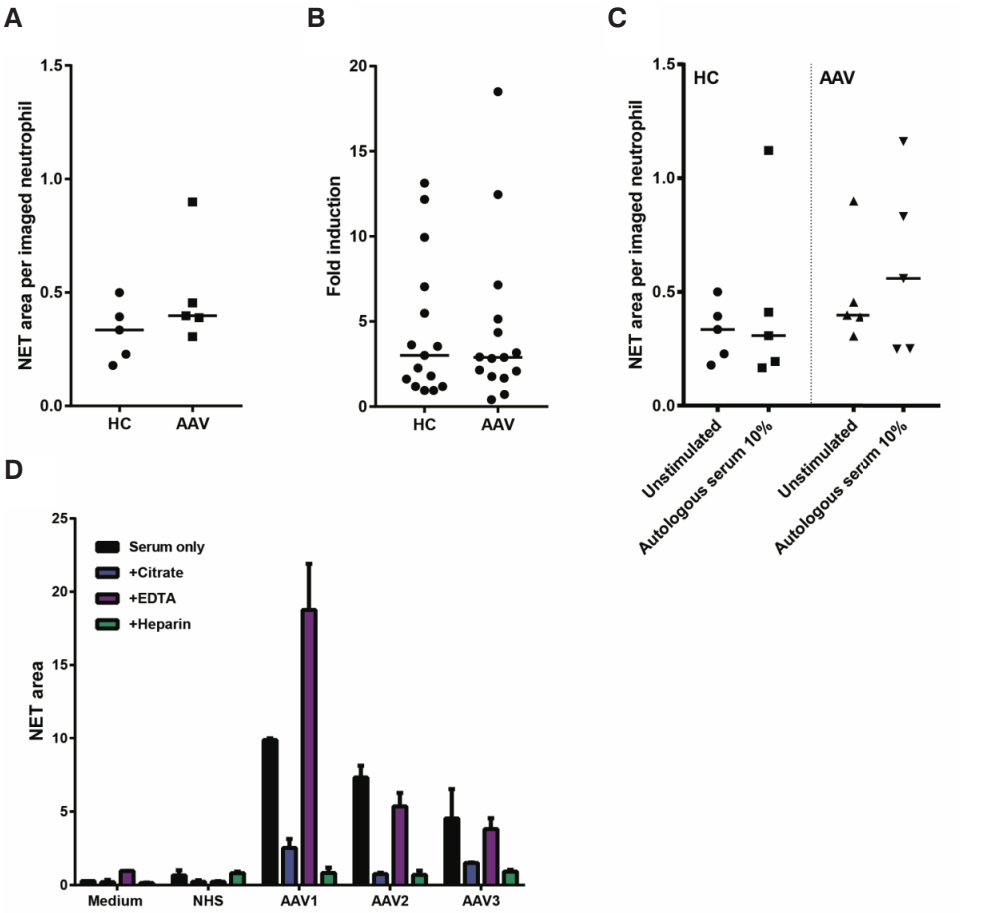


Figure S1. Overview of a highly sensitive automated assay for quantification of neutrophil extracellular traps. Several experiments were performed quantifying excessive NET formation using healthy versus AAV-derived neutrophils. **(A)** Spontaneous NET formation after 4 hours in healthy (n ¼ 5) and AAV-derived neutrophils (n ¼ 5). **(B)** Quantifying excessive NET formation induced by 3 NET-inducing AAV sera co-incubated with 5 different sources of healthy neutrophils (HC) and 5 different AAV-derived neutrophils (n ¼ 5). In order to compare NET formation between the 15 experiments, excessive NET formation was calculated as "fold increase" from spontaneous NET formation in the corresponding neutrophil donor. **(C)** Autologous serum experiments in which neutrophils derived from 5 healthy donors and 5 AAV patients were left unstimulated or stimulated with autologous serum. These experiments demonstrated an increased variability in NET formation by AAV-derived neutrophils compared with healthy neutrophils. **(D)** Neutrophils were stimulated with anticoagulants (17 USP/ml heparin, 0.105 M sodium citrate, 0.7 mg/ml EDTA) and NET-inducing AAV serum to investigate the effect of anticoagulants on the NET-inducing capacity of these samples, revealing an inhibitory effect of sodium citrate and heparin. HC, healthy control; NHS, normal human serum.

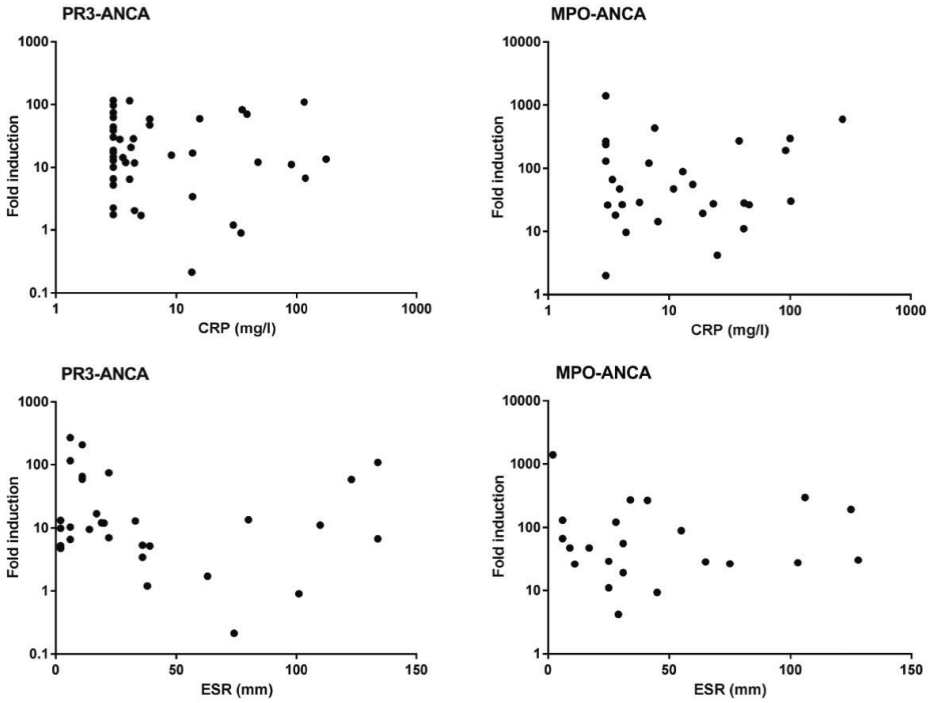


Figure S2. Excessive NET formation in AAV does not correlate with the inflammatory mediators CRP and ESR. No correlation was observed between CRP and NET induction as well as BSE and NET induction in both PR3-ANCA samples (n = 43 and n = 30 pairs, respectively) and MPO-ANCA samples (n = 29 and n = 22 pairs, respectively). CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

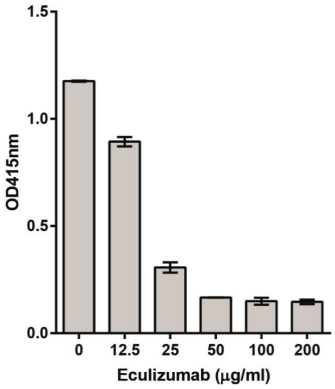


Figure S3. Eculizumab neutralizes C5 in a concentration of 100 mg/ml inhibiting functional complement activation. Representative result of the Wieslab assay determining the formation of C5b-9 via the classical complement pathway in serum of a normal human serum control. The experiment was performed at least 3 times.

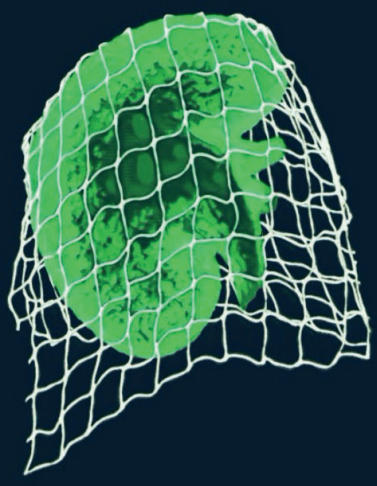
Table S1a. Concurrent clinical data of AAV patients for whom excessive NET formation was investigated longitudinally (as depicted in Figure 6A).

Table S1b. Overview of infections in 9 patients hospitalized with infectious disease from whom excessive NET formation was investigated as depicted in Figure 6B of the manuscript. We obtained 9 samples from hospitalized patients. Of note, several hospitalized patients were diagnosed with more than 1 infectious micro-organism.

Urinary tract infections	
Candida Albicans	2
Enterococcus Fecalis	1
Culture negative	1
Respiratory tract infection	
Stapyloccocus Aureus	1
Rhinovirus	1
Influenza A	1
Culture negative	2
Gastro-enteritis	
Culture negative	1

PART 2

New therapeutic options for SLE



Chapter 5

Belimumab after rituximab as maintenance therapy in lupus nephritis

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Rheumatology (Oxford). 2014 Nov;53(11):2122-4.

Letter to the editor

Sir, belimumab has been approved for autoantibody positive SLE with persistent disease activity despite standard treatment [1]. With respect to LN, European League Against Rheumatism (EULAR) guidelines state that the position of belimumab needs further definition [2]. Previously another biologic, rituximab, failed to be superior over placebo when added to MMF and corticosteroids in LN [3]. However, retrospective cohort data suggest a role for rituximab in refractory LN [4]. Here we report our experience with rituximab followed by belimumab as maintenance treatment in two refractory LN patients.

Patient 1, a 32-year-old female, was diagnosed with SLE 3 years earlier based on a butterfly exanthema, discoid lupus, photosensibility, lymphadenopathy, GN and positive ANA (3+) and anti-dsDNA (3+). A kidney biopsy revealed diffuse and global proliferative GN class IV-S(A). Since her diagnosis she had received two induction regimens (MMF and Eurolupus CYC), despite which her disease flared. The latter manifested with discoid lupus, arthralgias, diffuse alopecia and persisting GN with urinary red blood cell (RBC) casts and proteinuria (SLEDAI score 22). She was re-treated with MMF induction and corticosteroids during which progressive proteinuria (8 g/day) developed (Fig. 1). Concomitant angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) induced symptomatic hypotension. At this time, pulse steroids and rituximab followed by MMF and prednisolone maintenance was initiated. This led to a partial response with a reduction of proteinuria to 3.5 g/day. Disease amelioration was hampered because MMF caused intractable nausea and weight loss that challenged her treatment adherence. Seven months after rituximab she was started on belimumab. The initiation coincided with the time B cells started to fully repopulate. In the following months her skin lesions, alopecia and arthralgia resolved. Moreover, she independently tapered her prednisolone to zero because of tremors that interfered with her daily work requiring highly developed fine motor skills. Currently, after 18 months, she remains in remission on belimumab monotherapy with proteinuria of 0.9 g/day, a reduction in ANA (1+) and anti-dsDNA (1+), C3 normalization (from a nadir of 0.7 to 1.0; normal range >0.9 g/l) and increasing C4 levels (from a nadir of 103 to 194; normal range >95 mg/l) and a reduced number of circulating B cells (SLEDAI score 6).

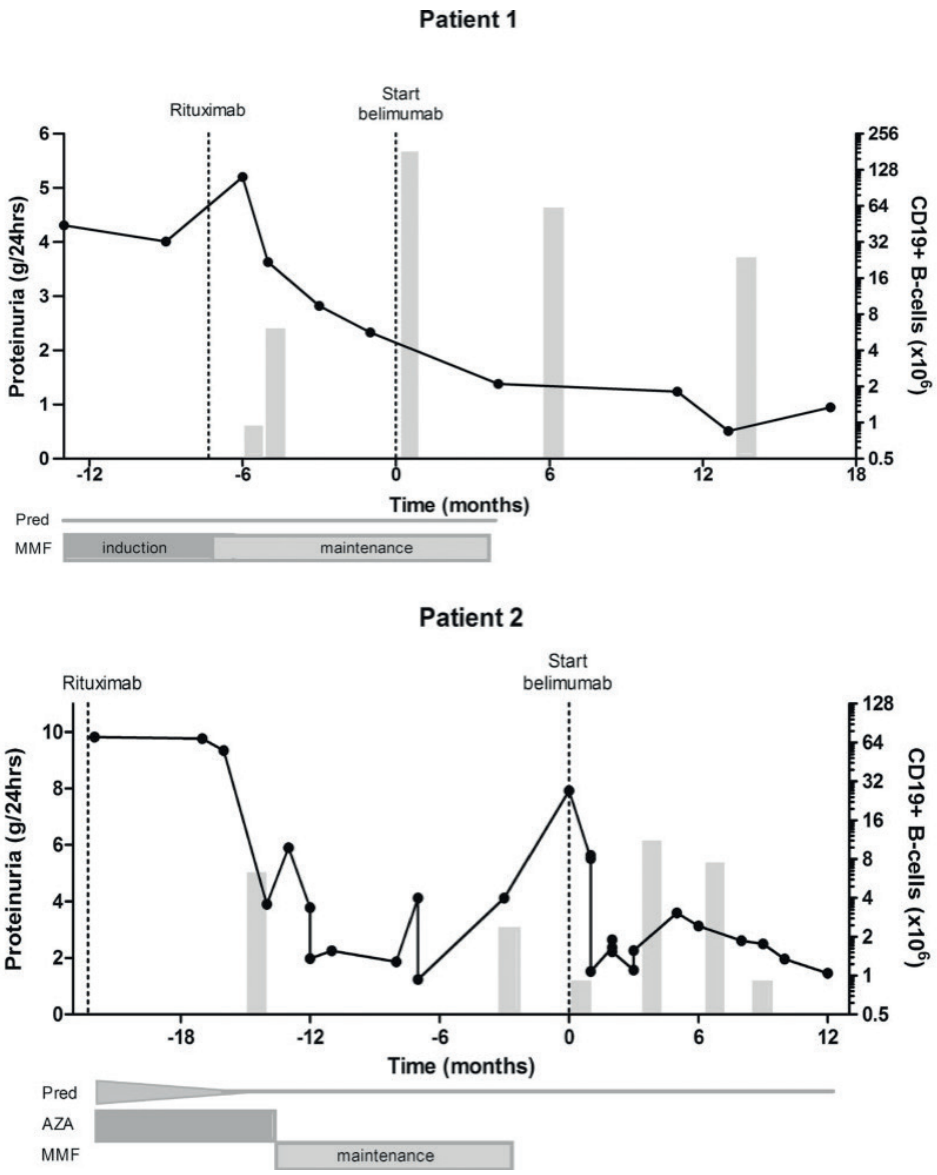

Figure 1. Overview of the two reported patients with respect to proteinuria and CD19+ B cells. The vertical dotted lines represent the start of two courses of 1 g rituximab and the start of belimumab treatment (three 2-weekly infusions followed by monthly infusions of 10 mg/kg). Time scale is related to the start of belimumab treatment, i.e. T = 0 months, so the time interval between rituximab and belimumab initiation as well as the time to disease amelioration is illustrated. The vertical grey bars represent the absolute number of circulating CD19+ B cells (right y-axis) after rituximab treatment (normal range for CD19+ B cells: 60-1000 x 10^6 cells/l).

The horizontal bars below the graph represent the (tapering of) concomitant immunosuppressive treatments through time. MMF dosages were titrated by measuring the area under the curve of serum levels, aimed at 60-90 mg/h/l during induction treatment and 45-60 mg/h/l during maintenance. Prednisone dosage was tapered from 1 mg/kg/day during induction (triangle) to 7.5 mg/day during maintenance treatment (line). Pred: prednisolone (triangles illustrate a tapering schedule).

Patient 2, a 42-year-old male, was diagnosed with SLE 7 years earlier, presenting with nephrotic syndrome, urinary RBC casts, class IV-G(A) LN, positive autoantibodies (ENA 2+), complement usage, alopecia, anorexia and auditory hallucinations with psychosis, including cerebral white matter lesions confirming neuropsychiatric involvement. He was referred to our hospital 2 years ago with a therapy-refractory nephrotic syndrome (proteinuria 9.8 g/ day, creatinine 150 mmol/l) while taking a combination of ACE inhibitors and ARBs. He was already treated with two previous induction treatments (CYC and MMF), each followed by MMF maintenance with concomitant prednisolone and HCQ. Another kidney biopsy confirmed the LN class IV-G(A) diagnosis with full-house immunofluorescence and additional focal global sclerosis and arteriosclerosis. The patient was treated with pulse steroids and rituximab followed by MMF and prednisolone, leading to partial remission with a nadir of his proteinuria at 1.3 g/day (Fig. 1). Unfortunately, due to MMF-related gastrointestinal side effects he was unable to adhere to his anti-proteinuric and immunosuppressive therapy. A relapse occurred with nephrotic proteinuria (7.9 g/day), erythrocyturia and complement usage (SLEDAI score 10) while circulating CD19+ B cells started to repopulate 20 months after rituximab initiation. To improve therapy adherence, MMF was tapered and belimumab was added to prednisolone. Thereafter proteinuria improved (1.5 g/day), ENA became negative, C3 normalized (from a nadir of 0.6 to 0.9 g/l), C4 increased (from a nadir of 210 to 386 mg/l) and circulating B cells remained reduced. At the 12-month follow-up he had low disease activity (SLEDAI score 4) while continuing belimumab and tapering prednisolone to zero.

These cases illustrate the potential added value of belimumab after rituximab treatment in active LN. It should be noted that both patients continue to have low disease activity while on belimumab monotherapy. To our knowledge, this is the first report of LN patients treated with two consecutive B cell targeted treatments. In both patients, belimumab halted the full repopulation of circulating B cells after rituximab. From a pathophysiological point of view, it is well appreciated that autoantibody-positive disease is found specifically in LN and that B cell hyperactivity is a landmark in SLE [5]. It is tempting to speculate that the clinical improvement of these patients is due to a synergic effect of rituximab and belimumab.

Previously, two randomized trials (BLISS-52 AND BLISS-76) showed beneficial effects of belimumab in reducing concomitant immunosuppression in autoantibody-positive SLE without

major organ involvement and without previous rituximab treatment. Recently one patient with active LN was reported to have a beneficial response to belimumab, albeit in conjunction with pulse steroids [6]. Currently we await the results of a randomized trial assessing belimumab's efficacy in LN (NCT01639339). This report describes belimumab as rescue treatment in refractory LN due to commonly seen gastrointestinal intolerance to MMF [7]. The present report encourages further research into the clinical yield of combining B cell targeted treatment in the difficult population of SLE patients with major organ involvement or refractory disease.

References

1. Hahn BH. Belimumab for systemic lupus erythematosus. N Engl J Med 2013;368:1528-35.
2. Bertsias GK, Tektonidou M, Amoura Z et al. Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. Ann Rheum Dis 2012;71:1771-82.
3. Rovin BH, Furie R, Latinis K et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum 2012;64: 1215-26.
4. Diaz-Lagares C, Croca S, Sangle S et al. Efficacy of rituximab in 164 patients with biopsy-proven lupus nephritis: pooled data from European cohorts. Autoimmun Rev 2012;11:357-64.
5. Dorner T, Jacobi AM, Lee J, Lipsky PE. Abnormalities of B cell subsets in patients with systemic lupus erythematosus. J Immunol Methods 2011;363:187-97.
6. of a mycophenolate mofetil-refractory proliferative lupus nephritis with belimumab in a 19-year-old woman. Lupus 2013;22:1523-5.
7. Bunnapradist S, Lentine KL, Burroughs TE et al. Mycophenolate mofetil dose reductions and discontinuations after gastrointestinal complications are associated with renal transplant graft failure. Transplantation 2006;82: 102-7.

Chapter 6

The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus

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Abstract

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. 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. 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. 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.

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.

Materials and methods

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).

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.

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°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).

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.

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.

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.

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.

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).

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).

Results

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.

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 [$21–302\times10^6$] at baseline to a nadir of 2.43×10^6 cells/liter [$1.17–8.0\times10^6$] ($P<0.0001$) equal to 98% decrease from baseline. At 24 weeks, the B-cells had repopulated to a level of 11×10^6 cells/liter [$0.5–64.7\times10^6$] ($P=0.0002$) (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 [$0–0.15\times10^6$] ($P=0.0002$) representing 100% decrease. Circulating plasma cells were depleted from 2.13×10^6 cells/liter [$0.07–10.5\times10^6$] at baseline to a nadir of 0.11×10^6 cells/liter [$0–11\times10^6$] ($P=0.104$) 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 [$0.31–49.5\times10^6$] equal to 76% decrease from baseline. Circulating plasma cells repopulated to 1.26×10^6 cells/liter [$0–18.3\times10^6$] equal to 72% decrease from baseline. Transitional B-cells did not repopulate and remained depleted at a level of 0×10^6 cells/liter [$0–6.22\times10^6$] 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.

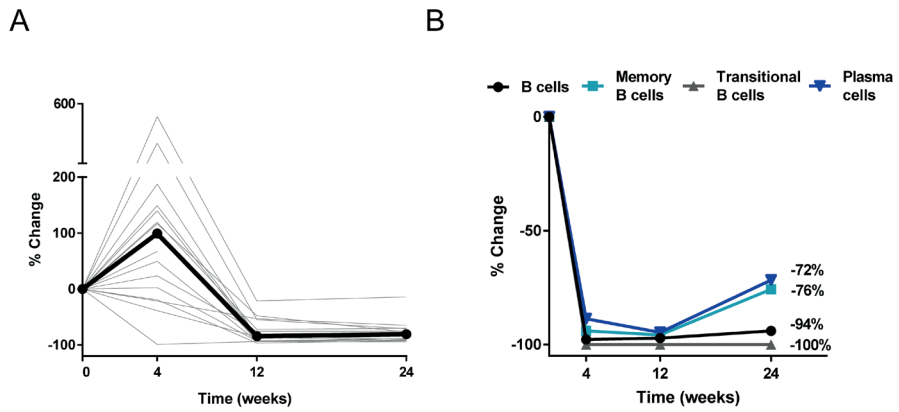

Figure 1. RTX+BLM specifically reduces humoral autoimmune phenomena in refractory SLE patients. **(A)** The percentage change of free serum BlyS in individual patients throughout the 24-week study. The thick black line represents the median and thin grey lines represent individual data. **(B)** The median 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.

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.

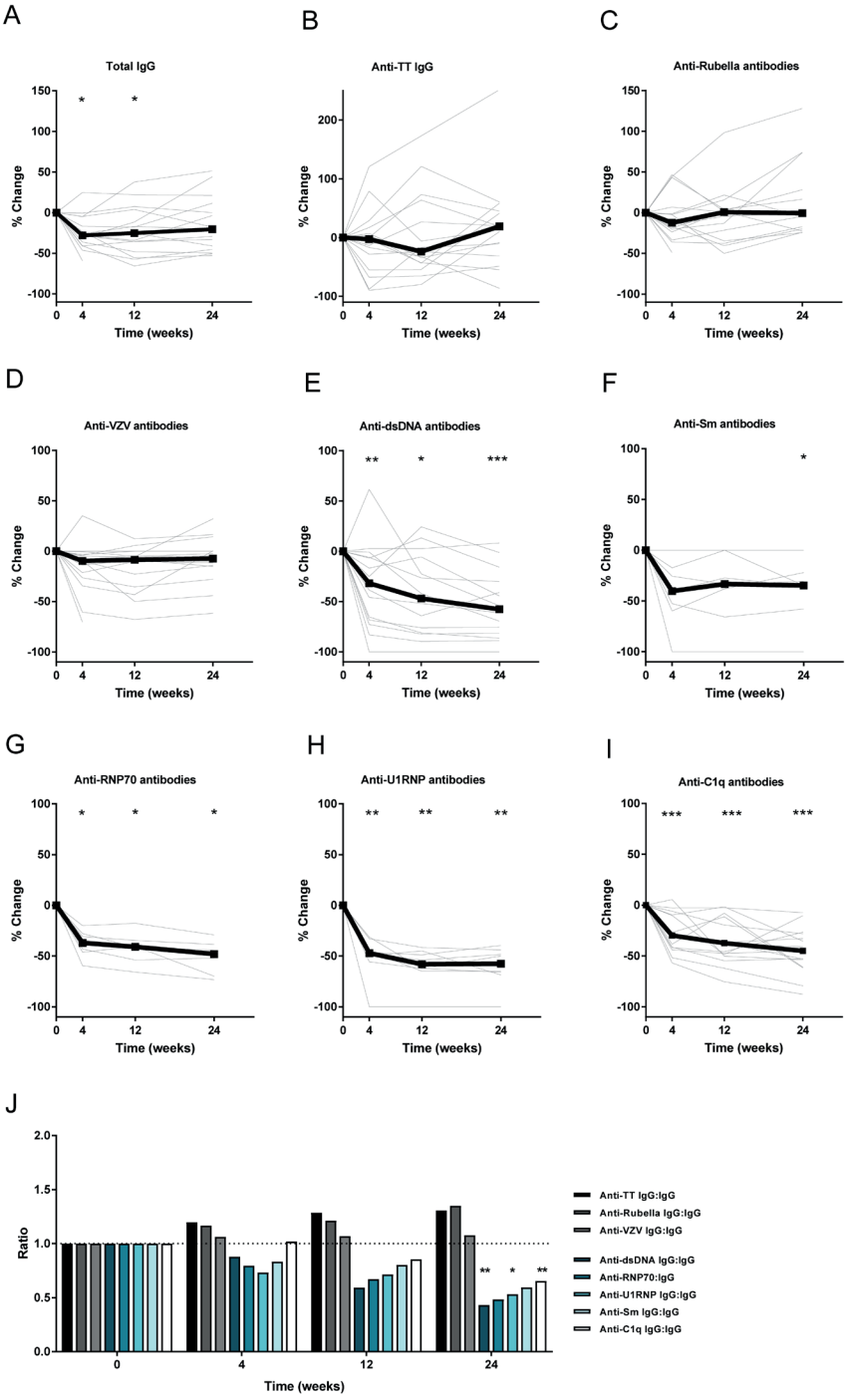
A
Total IgG
% Change
Time (weeks)
B
Anti-TT IgG
% Change
Time (weeks)
C
Anti-Rubella antibodies
% Change
Time (weeks)
D
Anti-VZV antibodies
% Change
Time (weeks)
E
Anti-dsDNA antibodies
% Change
Time (weeks)
F
Anti-Sm antibodies
% Change
Time (weeks)
G
Anti-RNP70 antibodies
% Change
Time (weeks)
H
Anti-U1RNP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

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.

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.

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

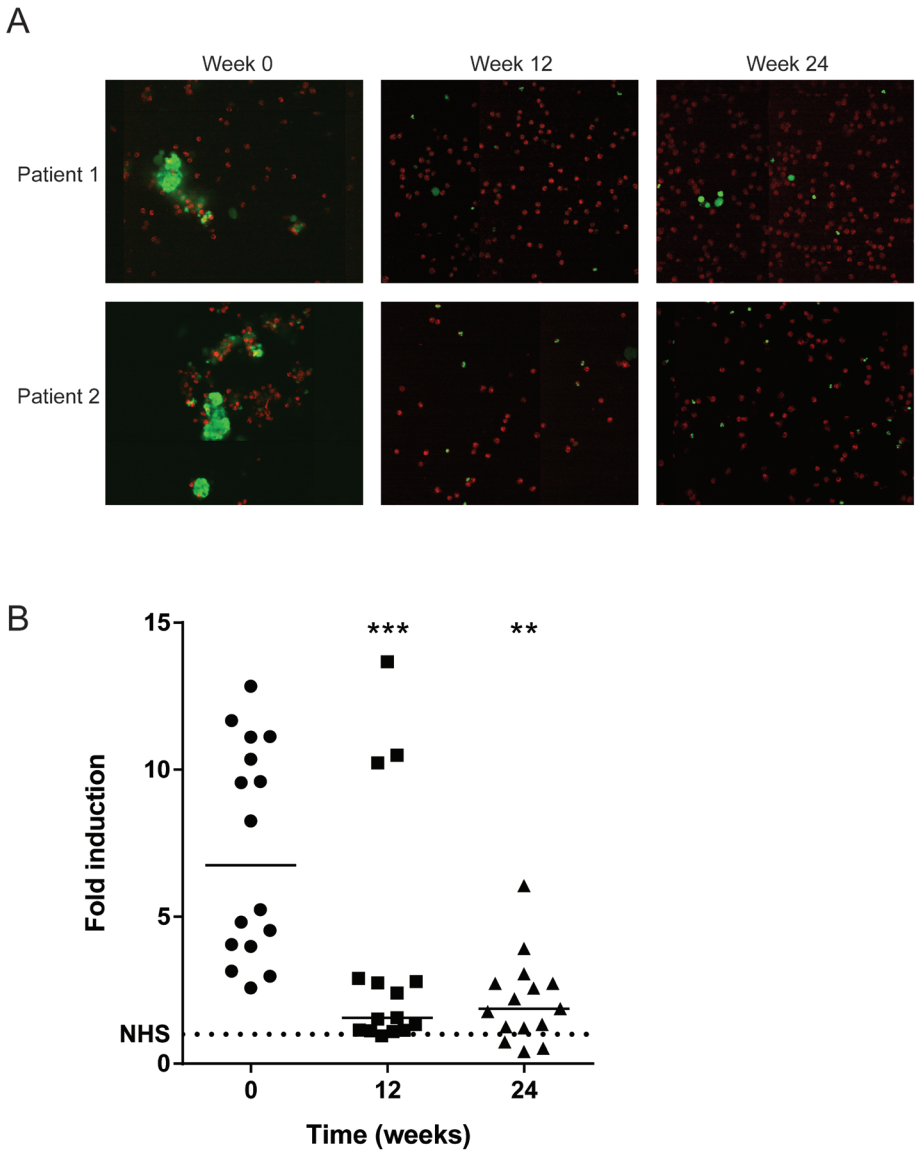

Figure 3. RTX+BLM treatment significantly reduces excessive *ex vivo* NET formation. **(A)** Representative images of ex vivo NET formation at week 0, week 12 and week 24 in 2 patients. Extracellular DNA is stained with SYTOX (green) and cells are labelled with the membrane stain PKH26 (red). Bars = 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. $^{**}P<0.01$, $^{***}P<0.001$.

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).

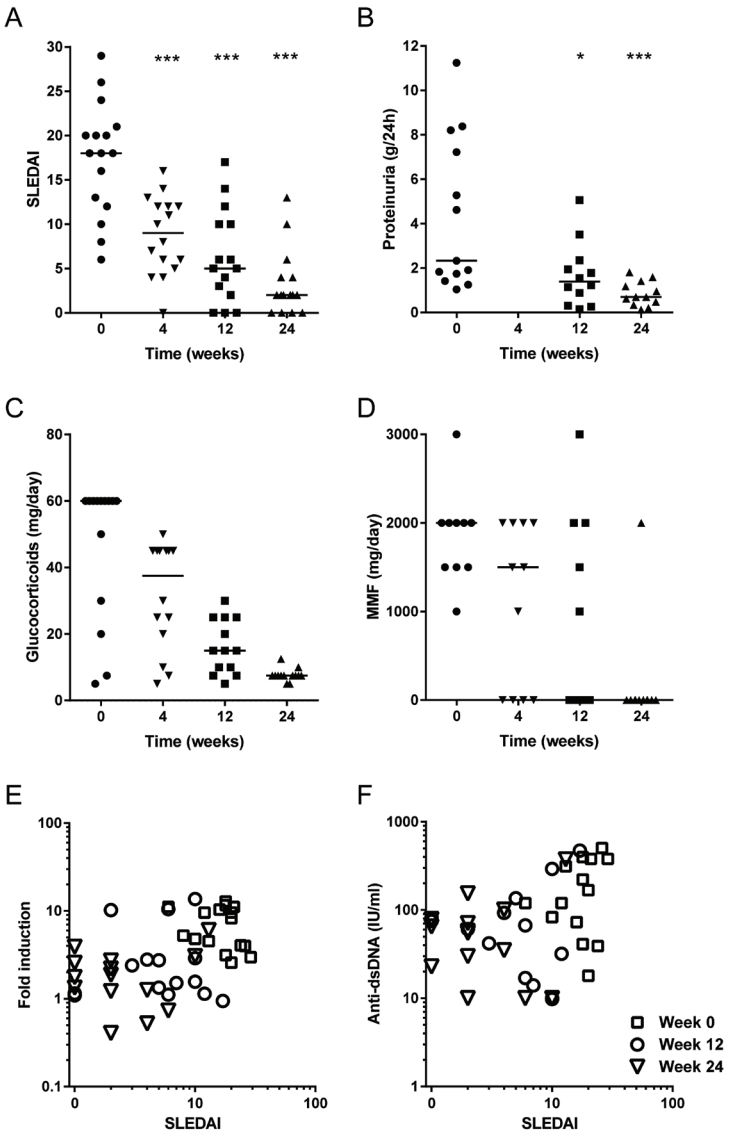

Figure 4. Beneficial clinical responses in refractory patients treated with RTX+BLM. **(A)** SLE Disease Activity Score (SLEDAI) at weeks 0, 4, 12, and 24. **(B)** Proteinuria (g/day) at weeks 0, 12, and 24 in patients with active lupus nephritis at baseline (n=13). **(C)** Successful tapering of glucocorticoids 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. *$P<0.05$, ***$P<0.001$.

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 (hospitalization) , 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.

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.

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.

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.

Supplementary material

Supplementary Table S1. Biological markers of systemic lupus erythematosus (SLE) disease activity throughout the SynBioSe study.

	Week 0 (n=16)		**Week 4 (n=16)**		**Week 12 (n=15)**		**Week 24 (n=15)**		**P –value***
ESR	47	(6-129)	42	(2-104)	25	(6-120)	19	(2-77)	**0.015**
Leucocytes	4.9	(2.4-8.1)	6.8	(1.9-13)	5.8	(1.6-13)	5.7	(2.4-15.2)	0.188
IgG (g/l)	11.5	(4.8-23.6)	8.9	(3.1-14.1)	8.11	(2.6-14.4)	8.9	(3.4-16.4)	0.121
Anti-tetanus toxoid IgG (AE/ml)	13.4	(1-77.8)	9.4	(0.1-75.6)	12.6	(0.2-44.4)	10.2	(1.1-93.4)	0.890
Anti-rubella IgG (mIU/ml)	60.7	(6.9-340.4)	63.9	(5.5-276.7)	59	(6-343.5)	66	(5.8-338.3)	0.639
Anti-VZV IgG (mIU/ml)	3128	(301-4000)	2850	(89.5-4000)	2716	(251-4000)	2837	(443-4000)	0.110
ANA positivity (%)†	100		100		100		100		
Anti-dsDNA positivity (%)†	75		50		40		33		
Anti-dsDNA titer (IU/ml)	144	(18-505)	77.5	(10-501)	64	(10-471)	57	(10-374)	**0.000**
Anti-RNP70 positivity (%)†	38		38		40		40		
Anti-RNP70 titer (IU/ml)	146	(26-2375)	85	(18-1712)	81	(17-1394)	71	(16-1322)	**0.031**
Anti-U1RNP positivity (%)†	56		44		40		40		
Anti-U1RNP titer (IU/ml)	196	(10-3679)	198	(11-2465)	128	(6-2148)	127	(8-2060)	**0.016**
Anti-Sm positivity (%)†	44		38		40		40		
Anti-Sm titer (IU/ml)	39	(6-3419)	19	(1-1376)	23	(1-2120	16	(1-2662)	**0.031**
Anti-C1q positivity (%)	88		75		73		53		
Anti-C1q titer (U/ml)	58	(10-135)	45	(7-90)	42	(7-69)	24	(9-72)	**0.000**
Free Blys (ng/ml)	1.08	(0.14-3.22)	1.17	(0.02-4.81)	0.2	(0.03-0.37)	0.15	(0.05-0.4)	**0.000**
Complement activation (%)‡	100		88		60		53		
C3 (g/l)§	0.6	(0.3-0.8)	0.7	(0.4-1)	0.8	(0.4-1.2)	0.8	(0.5-1.3)	**0.002**
C4 (mg/l)¥	54	(21-80)	69	(36-167)	89	(29-283)	110	(39-292)	**0.039**

CP (%activation)#	31	(5-71)	56	(17-88)	70	(9-101)	82	(22-114)	**0.001**
CD19+ B cells (x10^6/l)	100	(21-302)	2.4	(1.2-8)	2.4	(0.52-31)	10.6	(0.53-64.7)	**0.000**
Memory B cells (x10^6/l)	17.5	(6.2-142)	1.34	(0.15-4.40)	1.44	(0.19-9.47)	4.46	(0.31-49.5)	**0.035**
Transitional B cells (x10^6/l)	0.93	(0.08-3.43)	0	(0-0.15)	0	(0-0.71)	0	(0-6.22)	0.068
Plasma cells (x10^6/l)	2.13	(0.07-10.5)	0.24	(0-3.44)	0.11	(0-11)	1.26	(0-18.3)	0.268

Numbers depicted are median (range) unless specified otherwise.

**P*-values represent non-parametric testing comparing baseline values (week 0) versus values at week 24, i.e. time of primary endpoint. †Positivity is defined as semiquantative score of positive or strongly positive on immunofluorescence. ‡Complement activation is defined as decreased levels of C3, C4 or decreased classical pathway (CP) activity. §C3 values in patients with a decreased concentration at baseline (n=14). Normal C3: 0.9-2.0 g/l. ¥C4 values in patients with a decreased concentration at baseline (n=8). Normal C4: 95-415 mg/l. #Classical pathway (CP) activation in patients with a decreased CP activation at baseline (n=14). Normal CP: >74 %activation. ESR, erythrocyte sedimentation rate; VZV, varicella zoster virus.

Chapter 7

Measuring plasma C4D to monitor immune complexes in lupus nephritis

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Abstract

Because currently available assays that measure circulating immune complexes (ICx) are suboptimal, a novel assay was recently developed measuring C4d, a stable product of activation of the classical complement pathway. The present study aimed to establish the value of measuring plasma C4d levels in a longitudinal cohort of patients with severe refractory SLE who were treated with a combination therapy of rituximab with belimumab (RTX+BLM). Fifteen patients with SLE who were treated with RTX+BLM in a phase 2A, open label study were included to sequentially measure plasma C4d levels and correlated to well-established markers of ICx-formation, that is, autoantibodies against double-stranded (ds) DNA, autoantibodies against C1q and proteinuria. The performance of plasma C4d measurements, C4 measurements and the ratio of C4d over C4 (C4d:C4) was evaluated. After establishing that on RTX+BLM treatment kinetics of C4d levels was distinct from traditional C3 and C4 levels, we found strong correlation of C4d:C4 with anti-dsDNA ($R=0.76$, $p<0.001$) and anti-C1q ($R=0.65$, $p<0.001$) autoantibody levels, which outperformed both stand-alone C4 and C4d levels. Additionally, changes in C4d:C4 over time correlated strongly with changes in proteinuria ($R=0.59$, $p<0.001$) as well as anti-dsDNA ($R=0.46$, $p=0.003$) and anti-C1q ($R=0.47$, $p=0.002$). In patients with severe SLE, plasma C4d levels in relation to C4 levels is useful for longitudinal monitoring after RTX+BLM treatment to reflect amelioration of classical complement activation by ICx as well as proteinuria.

Introduction

Kidney involvement in SLE is typically defined by immunoglobulin deposition at the glomerular basement membrane (GBM) leading to membranoproliferative glomerulonephritis with the typical deposition of various complement components together with immunoglobulins referred to as 'full-house immunofluorescence' [1]. The deposition of immunoglobulins in the kidney are caused by DNA or nucleosome containing immune complexes that can bind to the GBM and cause inflammation and damage through local as well as systemic complement activation [2] and as such complement activation products (such as C3d and C5b-9) can be detected in the urine of patients with SLE nephritis [3]. The major cause of complement activation in SLE is thought to be the formation of immune complexes that activate complement via the classical pathway.

In clinical practice, immune complex (ICx)-mediated inflammation in patients with SLE is demonstrated by the consumption of complement components. Complement consumption is generally quantified with circulating C3 or C4 levels or functionally assessed by measuring complement classical pathway activity (CH50). Complement consumption has been found in approximately 75% of patients with SLE with focal proliferative glomerulonephritis and in 90% of patients with diffuse proliferative glomerulonephritis [4]. However, a number of other factors not related to immune complexes or SLE disease activity per se may influence the degree of reduction of serum levels of complement components, including complement activation of the lectin or alternative pathway [5], the rate of complement production versus catabolism and the presence of autoantibodies directed against complement proteins, such as C1q. Therefore, several assays that (in-)directly measure circulating ICx have been developed with the intent to aid clinicians in their routine evaluations of ICx-mediated inflammation in patients with SLE. Since the currently available assays have thus far been insufficient to reliably and reproducibly detect immune complexes [6], a novel assay was recently developed measuring C4d, a stable product of activation of the classical complement pathway [7]. We previously showed that plasma C4d levels is a functional readout for ICx-mediated classical pathway complement activation and associated with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), particularly nephritis and could forecast impending renal flares in patients with previous renal involvement [8].

In the present study, we aimed to further establish the value of measuring plasma C4d levels in longitudinal cohort of patients with severe, refractory SLE who were treated with a combination therapy of rituximab (RTX) with belimumab (BLM) [9]. The cohort was selected because based on previous studies, the most severe patients with SLE were anticipated to have high C4d levels and a novel, synergetic B-cell targeted treatment was postulated to reduce, and even

eradicate, ICx-forming autoantibodies. Therefore, optimizing the clinical conditions to investigate sequentially measured plasma C4d levels in patients with SLE.

Methods

Study participants

Patients with severe, refractory SLE who participated in a phase 2A, single-arm, proof-of-concept study investigating combination therapy of rituximab with belimumab (SynBioSe study, registered at ClinicalTrials.gov NCT02284984) were selected for this study.9 Patients were treated with 1000 mg rituximab at weeks 0 and 2 and with monthly 10 mg/kg belimumab from week 4 and onwards. Only patients who completed 24 weeks of follow-up with serial serum sampling were selected. Details of the study participants, treatment and clinical efficacy were published elsewhere [9]. All patients provided written informed consent.

ELISA detecting soluble C4d

Maxisorp 96-well plates (Nunc) were coated with rabbit anti-human C4d neoepitopes-specific antibody.7 After blocking with washing buffer (50 mM Tris–HCl, 0.15 M NaCl, 0.1% Tween, pH 7.5) supplemented with 3% fish gelatin (Norland Products), patient plasma as well as pooled plasma from healthy volunteers (lacking C4d) supplemented with Escherichia coli expressed C4d standard in serial dilutions, were diluted to 4% in phosphate-buffered saline+0.02% Tween-20+0.02 M Na_2EDTA and added to the plate for 1 hour incubation at 37°C. Detection was achieved using mouse anti-human C4d antibody (Quidel, #253) followed by horseradish peroxidase-conjugated goat antimouse secondary antibody (Dako, #P0447). Plates were developed using OPD (Kem-En-Tec) as substrate and absorbance was measured at 490 nm using Cary50 microplate reader (Varian).

Reference values

In order to assess complement normalization, clinical routine reference values were applied for C3 (normal >0.9 g/L) and C4 (normal >95 mg/L). For C4d, reference values were based on previous published C4d levels and were categorized according to receiver operating characteristic curve analyses in a longitudinal cohort for lupus nephritis (normal <1.1 mg/L).

Statistical analyses

For patients' characteristics before and after treatment, descriptive analysis was used and median values reported on the basis of the small cohort size. Non-parametric, two-tailed, paired samples Wilcoxon tests were used to compare treatment effects. To investigate the assay's performance, non-parametric Spearman's correlation coefficients were analyzed.

Results

Table 1 describes the patients' disease characteristics illustrating severe and refractory SLE and establishing that RTX+BLM indeed resulted in significant reductions in SLE relevant autoantibody levels. Anti-double stranded (ds) DNA levels declined from a median of 144 IU/mL (range 18–505) at baseline to 56.5 (1–374) at week 24 ($p < 0.001$) and anti-C1q levels declined from 75.1 U/mL (25.1–135) to 27.6 (9.8–71.3) ($p < 0.001$).

Table 1. Patient characteristics.

	N=15 patients
Age (years)	31 [19-51]
Sex (F/M)	13 / 2
Disease duration (years)	9 [2-24]
No. of relapses	4 [1-6]
Before treatment	
SLEDAI	18 [6-29]
Urine PCR (mg/mmol)	107 [11-852]
C4 (mg/L)	79 [21-260]
C3 (g/L)	0.6 [0.3-1.3]
C4d (mg/L)	2.05 [0.98-6.40]
Anti-dsDNA (IU/ml)	144 [18-505]
Anti-C1q (U/ml)	75.1 [25.1-135]
After treatment (24 weeks)	
SLEDAI	2 [0-13]*
Urine PCR (mg/mmol)	46 [9-134]*
C4 (mg/L)	151 [39-339]*
C3 (g/L)	1.0 [0.5-1.4]*
C4d (mg/L)	1.25 [0.17-3.62]*
Anti-dsDNA (IU/ml)	56.5 [1-374] *
Anti-C1q (U/ml)	27.6 [9.8-71.3]*

Depicted are median [range]; *statistically significant ($p < 0.05$) difference from baseline

With respect to immune complex formation, the solid-phase C1q binding test was positive in 5 of 15 patients. As a surrogate, serum levels of C3 and C4 were reduced at baseline and improved significantly on treatment from, respectively, median 0.6 g/L (0.3–1.3) to 1.0 g/L (0.5–1.4) ($p < 0.001$) and 80 mg/L (21–260) to 151 (39–339) ($p = 0.008$). Plasma C4d levels were high at

baseline with a median of 2.05 mg/L (0.98–6.40), in comparison to previously published C4d levels in patients with SLE [8] and decreased significantly on treatment to 1.25 mg/L (0.17–3.62) ($p = 0.01$) (table 1). We observed that at baseline, C4d levels were abnormal in 14 out of 15 patients (93%) which was significantly more than C4 (abnormal in nine patients, equal to 60%) and comparable to C3 (abnormal in 13 patients, equal to 87%) (table 2). There was no significant difference for normalization within 24 weeks of C4d (occurred in six patients (43%)), of C4 (in six patients (67%)) and of C3 (in six patients (46%)). Of interest, out of six patients whose C4d normalized, four patients had normalized C3 levels. Vice versa, out of six patients whose C3 normalized only half had normalized C4d levels suggestive of continued classical complement activation despite normalization of C3 levels.

Table 2. Number (%) of patients with complement activation before and normalization after RTX+BLM

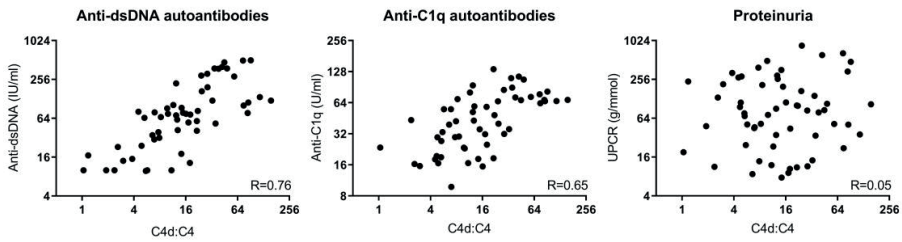
Complement activation	**C4d**	**C4**	**C3**	**Total**
C4d	14	9 (64%)	13 (93%)	14/15 (93%)
C4	9 (100%)	9	8 (89%)	9/15 (60%)
C3	13 (100%)	9 (69%)	13	13/15 (87%)
Complement normalization	**C4d**	**C4**	**C3**	**Total**
C4d	6	6 (100%)	4 (67%)	6/14 (43%)
C4	3 (50%)	6	4 (67%)	6/9 (67%)
C3	3 (50%)	6 (100%)	6	6/13 (46%)

We next correlated C4d levels to C3 and C4 levels to establish that C4d levels reflected distinct aspects of complement activation. Indeed, we found no correlation between levels of C4d with either C4 levels ($R = 0.25$, $p = 0.38$) nor C3 levels ($R = -0.12$, $p = 0.66$). Also, reduction of C4d levels did not correlate to increases in C4 ($R = 0.02$, $p = 0.90$) or C3 ($R = -0.20$, $p = 0.20$).

Since levels of total C4 showed individual variation, we calculated the ratio of C4d activation product and total C4 (C4d:C4). The performance of these complement markers as a reflection of ICx-mediated inflammation was determined by comparison with other well-established markers of ICx-formation, that is, antibodies against dsDNA (anti-dsDNA) and C1q (anti-C1q) and proteinuria. We observed (figure 1A) the strongest correlations for C4d:C4 with anti-dsDNA ($R = 0.76$, $p < 0.001$) and anti-C1q ($R = 0.65$, $p < 0.001$) autoantibodies which outperformed both C4 (respectively, $R = -0.43$, $p = 0.001$ and $R = -0.62$, $p < 0.001$) and C4d (respectively, $R = 0.67$, $p < 0.001$ and $R = 0.35$, $p = 0.01$). Of note, none of the complement markers correlated with proteinuria. Additionally, we investigated whether changes in complement markers accompanied changes in markers of ICx-formation (figure 1B). Again, we found that

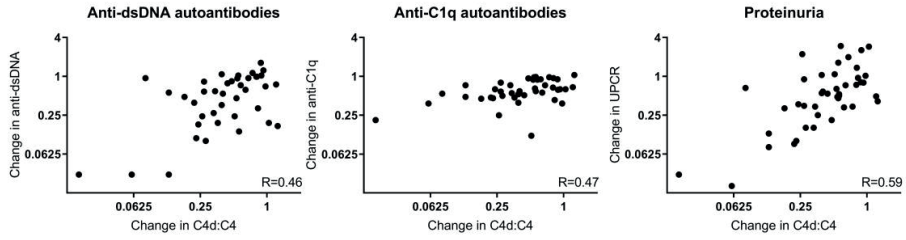
changes in C4d:C4 over time had the strongest correlation with changes in anti-dsDNA (R = 0.46, p = 0.003), anti-C1q (R = 0.47, p = 0.002) and proteinuria (R = 0.59, p < 0.001). Likewise, changes in C4d:C4 outperformed the stand-alone measurements of C4 and C4d.

A



	C4		C4d		C4d:C4	
	R	P	R	P	R	P
Anti-dsDNA	-0.43	0.001	0.67	**<0.001**	0.76	**<0.001**
Anti-C1q	-0.62	**<0.001**	0.35	0.01	0.65	**<0.001**
Proteinuria (UPCR)	*0.17*	*0.19*	*0.19*	*0.15*	*0.05*	*0.73*

B



Change in	C4		C4d		C4d:C4	
	R	P	R	P	R	P
Anti-dsDNA	*-0.21*	*0.18*	*0.22*	*0.17*	0.46	0.003
Anti-C1q	*-0.08*	*0.61*	**0.49**	**<0.001**	0.47	0.002
Proteinuria (UPCR)	**-0.53**	**<0.001**	0.30	0.048	**0.59**	**<0.001**

Figure 1. Significant association of plasma C4d levels with traditional, surrogate markers for immune complex formation. **(A)**. Levels of C4d and C4 were measured simultaneously from which the C4d:C4 ratio was derived and correlated to circulating levels of autoantibodies directed against double stranded (ds) DNA and C1q and proteinuria (measured by the urine protein:creatinine ratio (UPCR)). The correlation plots of the ratio of C4d:C4 illustrate strong correlation with levels of anti-dsDNA and anti-C1q autoantibodies (R=Spearman's correlation coefficient). The table reports the correlation coefficients separately for C4d, C4 and C4d:C4 where strong correlations (R≥0.5) are depicted in dark grey filled boxes; moderate correlation (0.3<R<0.5) are depicted in light grey filled boxes; no or weak correlations (R≤0.3) are depicted in non-filled boxes. P values were considered significant when below 0.05. **(B)**. Change in levels of C4d, C4 and

C4d:C4 in comparison to baseline levels were calculated. The relative changes of C4d, C4 and C4d:C4 were correlated to the relative changes in circulating levels of autoantibodies directed against dsDNA, C1q and proteinuria. Depicted graphs illustrate the correlation of changes in C4d:C4 with changes in anti-dsDNA ($R=0.46$; $p=0.003$) and anti-C1q ($R=0.47$; $p=0.002$) autoantibody levels and, most relevantly, changes in proteinuria ($R=0.59$; $p<0.001$). In the table, correlation coefficients are reported separately for changes in C4d, C4 and C4d:C4.

Discussion

The present study demonstrated that in patients with severe SLE measuring plasma C4d levels, and quantifying C4d:C4, was well correlated with current widely employed, surrogate measurements for ICx-formation (ie, anti-dsDNA, anti-C1q levels and proteinuria). In a cohort of patients with SLE with severe lupus nephritis, changes in C4d:C4 was strongly correlated with the renal outcome criterium of proteinuria and outperformed stand-alone C4d and C4 measurements. Also, on treatment, in some cases, C4d levels normalized, while C3 levels were still abnormal. Taken together, this study suggests that C4d quantification could be of added value to conventional complement measurements and warrants further validation for its use in the evaluation of immune complex-mediated diseases, including the wide spectrum of patients with mild to severe SLE, cryoglobulinemic glomerulonephritis or antibody-mediated humoral rejection.

Thus far, assays for measuring circulating immune complexes are insufficient because of the multiplicity of different techniques available for detecting immune complexes, differences in results when using different techniques, lack of reproducibility between the same techniques performed in different laboratories, and lack of studies demonstrating clear clinical predictive value of immune complex measurements [6]. As such, the lack of detectable circulating immune complexes does not exclude the presence of immune complex-mediated diseases, including involvement of complement activation [10]. Therefore, there is a clinical need for the development of more specific biomarkers of classical pathway complement activation [3, 11]. The assay for quantifying plasma C4d levels employs neoantigen-specific antibodies to nascent cleavage epitope found only in C4d but not its parent molecules such as C4 and C4b [7]. Of note, patients with a genetic variation at the C4 null alleles can impact the rate of production and catabolism of C4 and C4d. To correct this phenomenon, the ratio of C4d:C4 can be calculated or serial measurements within the same patient can be performed, as exemplified in this study.

Previously, in patients with SLE, the deposition of C4d on reticulocytes and platelets was shown to associate with SLEDAI [12] and was suggested as useful biomarker for monitoring disease activity [13]. A prospective pilot study showed that levels of C4d on circulating cells correlated

with C4d staining in lupus nephritis biopsies [14]. An assay panel combining anti-dsDNA, ANA, anti-mutated citrullinated vimentin, erythrocyte C4d and B-cell C4d was shown to be sensitive and specific for the diagnosis of SLE [15]. The present study is the first to add to this body of evidence that C4d levels in relation to C4 levels is a useful marker for longitudinal monitoring in the setting of treatment reflecting amelioration of classical complement activation by immune complexes. Future studies will need to elucidate how clinical decision-making can benefit from routine C4d:C4 measurements.

References

1. Rijnink EC, Teng YKO, Wilhelmus S, et al. Clinical and histopathologic characteristics associated with renal outcomes in lupus nephritis. Clin J Am Soc Nephrol 2017;12:734–43.
2. van der Vlag J, Berden JHM. Lupus nephritis: role of antinucleosome autoantibodies. Semin Nephrol 2011;31:376–89.
3. Manzi S, Rairie JE, Carpenter AB, et al. Sensitivity and specificity of plasma and urine complement split products as indicators of lupus disease activity. Arthritis Rheum 1996;39:1178–88.
4. Valentijn RM, van Overhagen H, Hazevoet HM, et al. The value of complement and immune complex determinations in monitoring disease activity in patients with systemic lupus erythematosus. Arthritis Rheum 1985;28:904–13.
5. Chen M, Daha MR, Kallenberg CGM. The complement system in systemic autoimmune disease. J Autoimmun 2010;34:J276–J286.
6. Wener MH. Tests for circulating immune complexes. Methods Mol Biol 2014;1134:47–57.
7. Blom AM, Österborg A, Mollnes TE, et al. Antibodies reactive to cleaved sites in complement proteins enable highly specific measurement of soluble markers of complement activation. Mol Immunol 2015;66:164–70.
8. Martin M, Smoląg KI, Björk A, et al. Plasma C4d as marker for lupus nephritis in systemic lupus erythematosus. Arthritis Res Ther 2017;19.
9. Kraaij T, Kamerling SWA, de Rooij ENM, et al. The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus. J Autoimmun 2018;91:45–54.
10. Use and abuse of laboratory tests in clinical immunology: critical considerations of eight widely used diagnostic procedures. Report of a joint IUIS/WHO meeting on assessment of tests used in clinical immunology. Clin Immunol Immunopathol 1982;24:122–38.
11. Buyon JP, Tamerius J, Belmont HM, et al. Assessment of disease activity and impending flare in patients with systemic lupus erythematosus. Comparison of the use of complement split products and conventional measurements of complement. Arthritis Rheum 1992;35:1028–37.
12. Liu C-C, Manzi S, Kao AH, et al. Reticulocytes bearing C4d as biomarkers of disease activity for systemic lupus erythematosus. Arthritis Rheum 2005;52:3087–99.
13. Kao AH, Navratil JS, Ruffing MJ, et al. Erythrocyte C3d and C4d for monitoring disease activity in systemic lupus erythematosus. Arthritis Rheum 2010;62:837–44.
14. Batal I, Liang K, Bastacky S, et al. Prospective assessment of C4d deposits on circulating cells and renal tissues in lupus nephritis: a pilot study. Lupus 2012;21:13–26
15. Kalunian KC, Chatham WW, Massarotti EM, et al. Measurement of cell-bound complement activation products enhances diagnostic performance in systemic lupus erythematosus. Arthritis Rheum 2012;64:4040–7.

Chapter 8

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

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Abstract

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). 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). 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 repeated minor flare. 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. 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 benefits in a therapy-refractory SLE population prompt further studies on RTX+BLM.

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 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 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, 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 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. higher BAFF levels available) can underpin resistance to anti-CD20 therapy. The importance of BAFF levels in anti-CD20 therapy is further illustrated in a study using an invitro 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) 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 previously12. 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. A flow diagram of the patients is included in Figure 1.

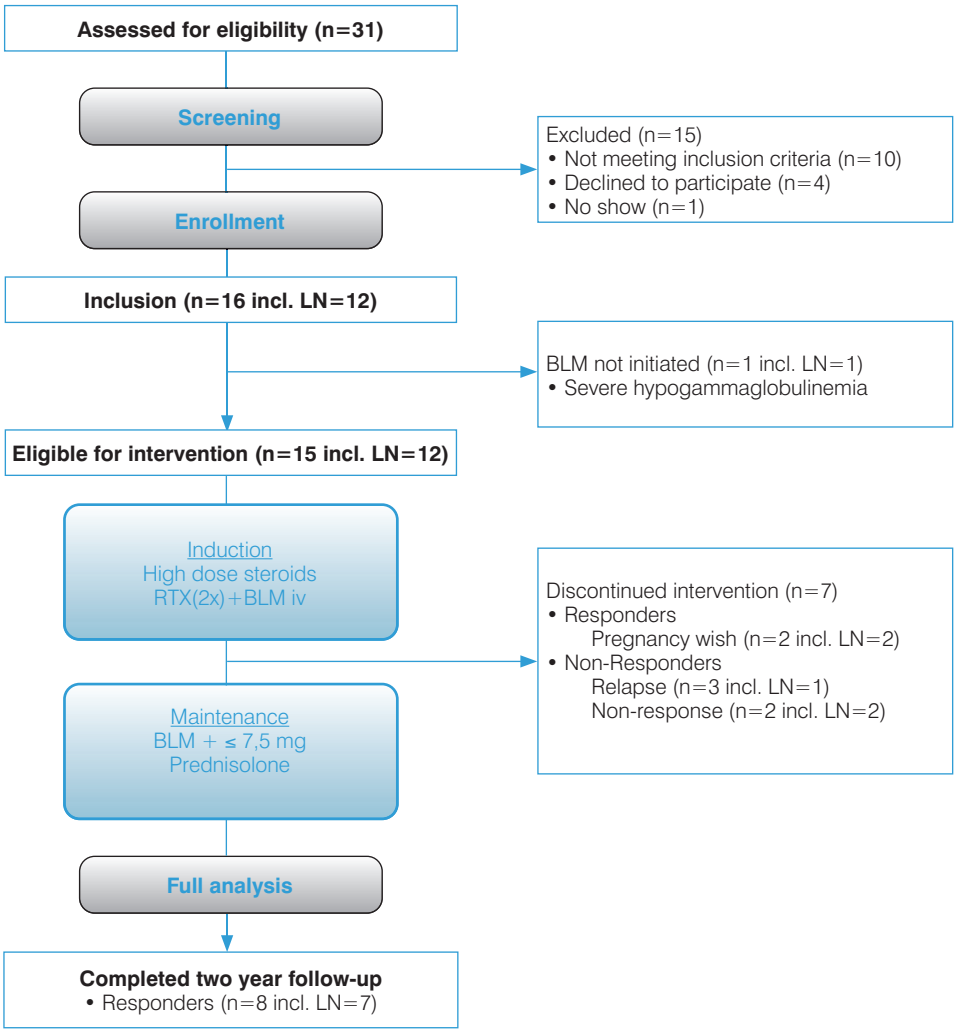

Figure 1. Flow diagram of patients

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 2). Clinical response is illustrated in Figure 2A 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 2B). In

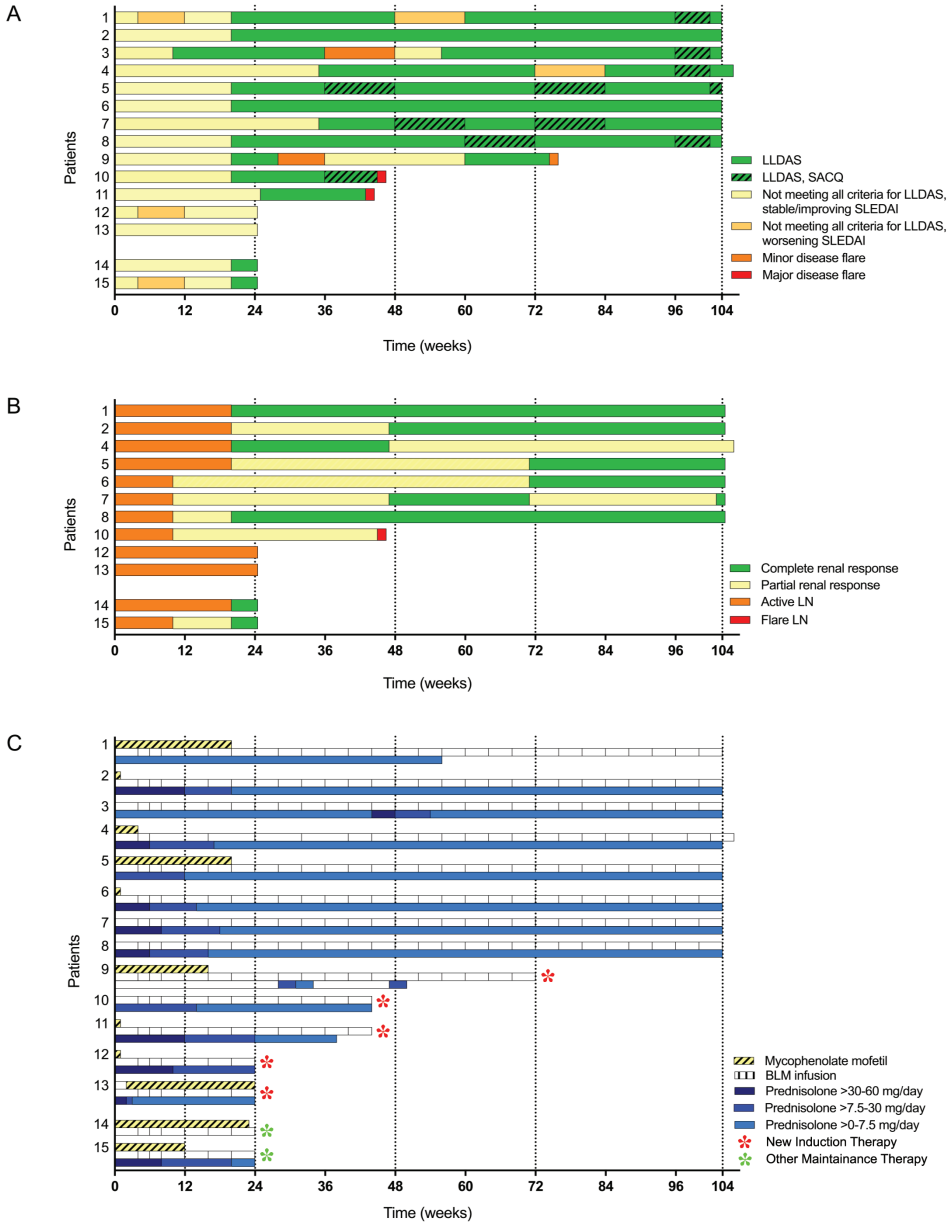

Figure 2. Overview of the clinical responses, renal responses and concomitant immunosuppression upon RTX+BLM treatment. **(A)** Achievement of lupus low disease activity state (LLDAS) over time. **(B)** Achievement of a renal response in patients included with active lupus nephritis (n=12). Complete 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.

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 2C).

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 0.5 grams/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 0.7 grams/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.

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 Origen	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	7	(88)	3	(60)
Transverse myelitis	0	(0)	1	(20)
Persistent disease activity despite treatment	1	(13)	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[a] (g/24h), median (range)	4.6	(1.3-11.2)	1.9	(1.0-8.4)
Treatment at disease flare				
Glucocorticoids[b], 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[c] (AU/ml), median (range)	268	(50-827)	479	(33-1123)
Complement consumption[d] (%)	100		100	

C3[e] (g/l), median (range)	0.6	(0.3-0.8)	0.6	(0.5-1.3)
C4[f] (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]Proteinuria did not differ significantly between both groups, P-value 0,67. [b]Patients were treated with the glucocorticoid equivalent prednisolone. [c]Normal anti-dsDNA IgG $<$10 IU/ml. [d]Complement consumption is defined as decreased CP (classical pathway) activation, decreased C3 or decreased C4. [e]Normal C3: 0.9-2 g/l. [f]Normal C4: 95-415 mg/l.

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 3A,B) 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% ($0.66*10^6$cells/L [$<10^4$;$18.3*10^6$]) and in lesser extent repopulation of switched memory B-cells up to a median decrease of 71% ($2.03*10^6$cells/L [$<10^4$;$41.9*10^6$]) compared to baseline values. Only from 48 weeks onwards, the resurge of immature B-cells occurred with return of transitional B-cells (+52%,$0.78*10^6$cells/L [0.19;$2.23*10^6$]) and non-switched memory B-cells (-19%,$1.53*10^6$cells/L [1.02;$3.19*10^6$]) at week 104. Interestingly, continuous BLM treatment prevented complete repopulation of naive B-cells (-81%,$6.07*10^6$cells/L [0.70;$25.8*10^6$]) as well as double negative (DN) B-cells (-82%,$2.48*10^6$cells/L [0.46;$4.25*10^6$]) at 104 weeks (Figure 3C,D).

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.

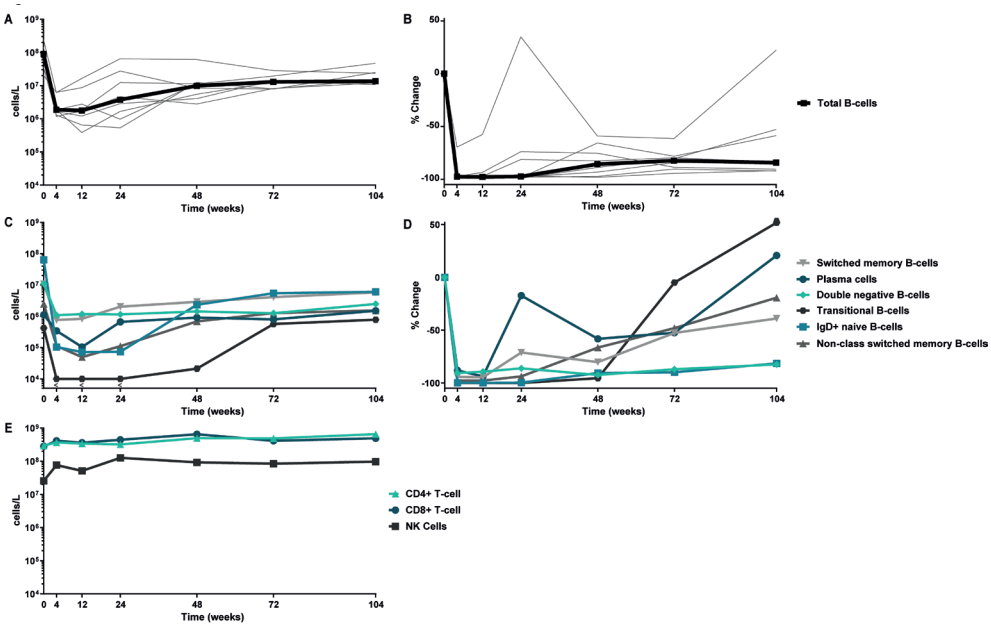

Figure 3. Longitudinal kinetics of circulating immune cells over 2 years of follow-up after RTX+BLM treatment (n=8 responders). **(A,B)** RTX+BLM prevents the complete repopulation of circulating B-cells. Depicted are individual values of all responders with the median in bold representing change of $CD19^+$B-cells in (A) absolute numbers and (B) the percentage of change from baseline. **(C,D)** Repopulation of B-cell subsets upon RTX+BLM. Depicted are the median change from baseline in (C) absolute numbers and (D) the percentage of change of the following B-cell subsets: plasmablasts (CD3-D38brightCD27brightCD19+), 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 ($CD3^-CD19^+$ $CD38^{bright}CD24^{bright}$). **(E)** 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 in absolute numbers.

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 2C). As a consequence, the observed low levels of T- and NK-cells at baseline significantly increased over time. Circulating $CD4^+$T-cells increased 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 [12,1;$418*10^6$] 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 3E).

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 11.3 g/L [5;23.6]) initially decreased at 12 weeks (7.8 g/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 4A). 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 4B+C) while anti-varicella zoster virus IgG (anti-VZV IgG) showed a significant decrease (Figure 4D) 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 4E). 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 4F-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 4J 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

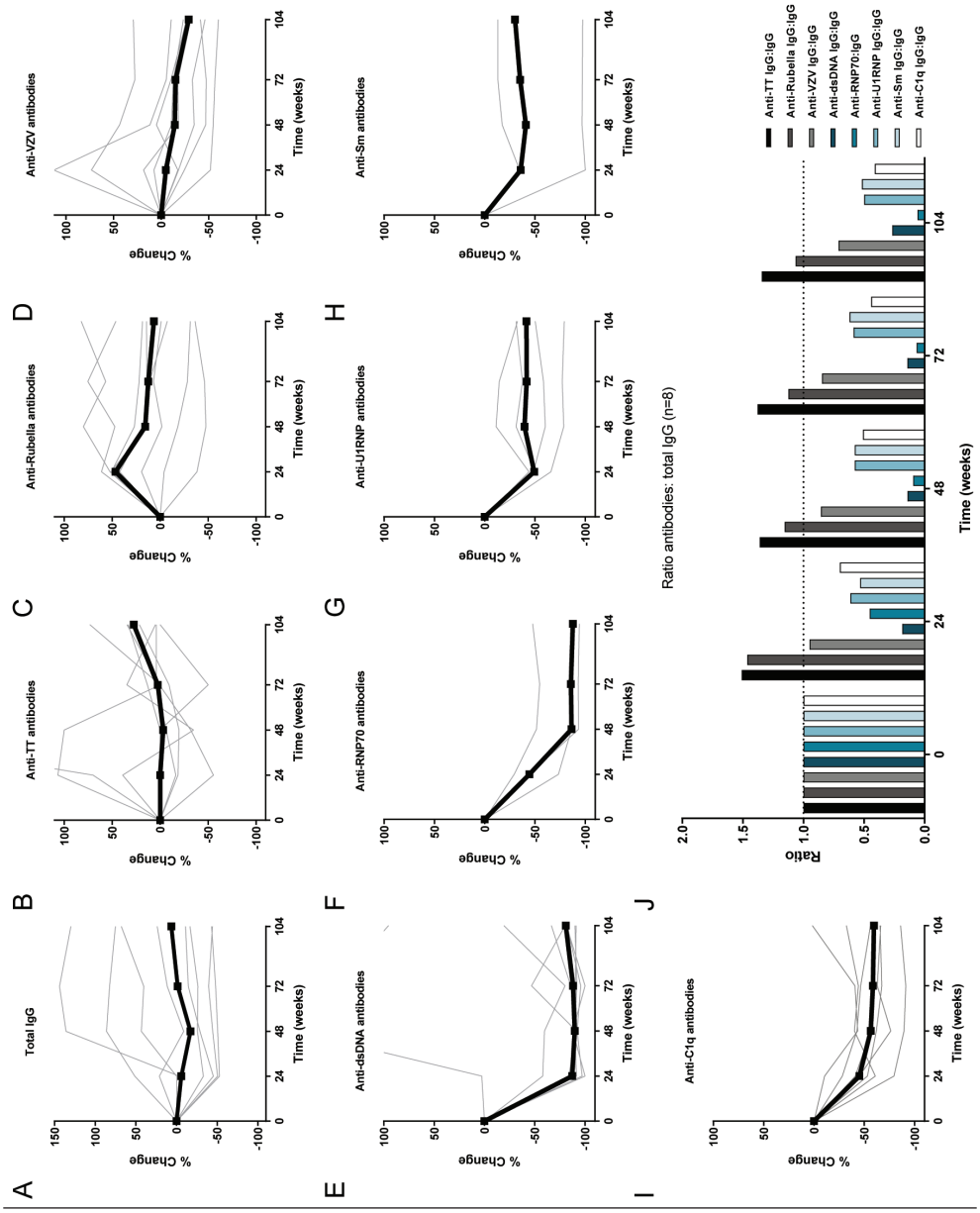

Figure 4. RTX+BLM resulted in prolonged, specific reduction of autoantibody levels over 2 years follow-up (n=8 responders). **(A-D)** Percentage change of physiological antibody levels are depicted, i.e. total IgG, anti-tetanus toxoid, anti-rubella and anti-varicella zoster antibodies **. (E-G)** Percentage 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.

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 0.97 ng/ml [0.48-1.4] vs 0.44 ng/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 (0.15 ng/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 [16-18]. We found that only transitional B cells and plasma cells repopulated to baseline levels and that naïve B cell repopulation present at 48 weeks remained suppressed for the duration of the follow-up period. Even more interesting are the double negative B cells that showed a less profound depletion but did not repopulate throughout 104 weeks. Additionally, we 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 (10 mg/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 ≤0.7 g/24hours, this in comparison to ≤0.5 g/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 0.5 g/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.

References

1. Lech M, Anders HJ. The pathogenesis of lupus nephritis. *Journal of the American Society of Nephrology : JASN* 2013;24(9):1357-66. doi: 10.1681/ASN.2013010026 [published Online First: 2013/08/10]
2. Almaani S, Meara A, Rovin BH. Update on Lupus Nephritis. *Clinical journal of the American Society of Nephrology : CJASN* 2017;12(5):825-35. doi: 10.2215/CJN.05780616 [published Online First: 2016/11/09]
3. Parikh SV, Rovin BH. Current and Emerging Therapies for Lupus Nephritis. *Journal of the American Society of Nephrology : JASN* 2016;27(10):2929-39. doi: 10.1681/ASN.2016040415 [published Online First: 2016/06/11]
4. Hanly JG, O'Keeffe AG, Su L, et al. The frequency and outcome of lupus nephritis: results from an international inception cohort study. *Rheumatology (Oxford)* 2016;55(2):252-62. doi: 10.1093/rheumatology/kev311 [published Online First: 2015/09/06]
5. Merrill JT, Neuwelt CM, Wallace DJ, et al. 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 and rheumatism* 2010;62(1):222-33. doi: 10.1002/art.27233
6. Rovin BH, Furie R, Latinis K, et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. *Arthritis and rheumatism* 2012;64(4):1215-26. doi: 10.1002/art.34359
7. Ehrenstein MR, Wing C. The BAFFling effects of rituximab in lupus: danger ahead? *Nat Rev Rheumatol* 2016;12(6):367-72. doi: 10.1038/nrrheum.2016.18 [published Online First: 2016/02/19]
8. Cambridge G, Stohl W, Leandro MJ, et al. Circulating levels of B lymphocyte stimulator in patients with rheumatoid arthritis following rituximab treatment: relationships with B cell depletion, circulating antibodies, and clinical relapse. *Arthritis and rheumatism* 2006;54(3):723-32. doi: 10.1002/art.21650
9. Gong Q, Ou Q, Ye S, et al. Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. *J Immunol* 2005;174(2):817-26. doi: 10.4049/jimmunol.174.2.817 [published Online First: 2005/01/07]
10. Saito Y, Miyagawa Y, Onda K, et al. B-cell-activating factor inhibits CD20-mediated and B-cell receptor-mediated apoptosis in human B cells. *Immunology* 2008;125(4):570-90. doi: 10.1111/j.1365-2567.2008.02872.x [published Online First: 2008/06/11]
11. Lin W, Seshasayee D, Lee WP, et al. Dual B cell immunotherapy is superior to individual anti-CD20 depletion or BAFF blockade in murine models of spontaneous or accelerated lupus. *Arthritis Rheumatol* 2015;67(1):215-24. doi: 10.1002/art.38907 [published Online First: 2014/10/11]
12. Kraaij T, Kamerling SWA, de Rooij ENM, et al. The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus. *Journal of autoimmunity* 2018 doi: 10.1016/j.jaut.2018.03.003

13. Group AT. Treatment of lupus nephritis with abatacept: the Abatacept and Cyclophosphamide Combination Efficacy and Safety Study. *Arthritis Rheumatol* 2014;66(11):3096-104. doi: 10.1002/art.38790 [published Online First: 2014/11/19]
14. Furie R, Dooley MA, Wofsy D, et al. OP0253 A phase iii randomised, double-blind, placebo-controlled study to evaluate the efficacy and safety of abatacept or placebo on standard of care in patients with active class iii or iv lupus nephritis. *Annals of the rheumatic diseases* 2018;77(Suppl 2):176-77. doi: 10.1136/annrheumdis-2018-eular.3451
15. Appel GB, Contreras G, Dooley MA, et al. Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. *Journal of the American Society of Nephrology : JASN* 2009;20(5):1103-12. doi: 10.1681/ASN.2008101028 [published Online First: 2009/04/17]
16. Tsang ASMW, Bultink IE, Heslinga M, et al. Both prolonged remission and Lupus Low Disease Activity State are associated with reduced damage accrual in systemic lupus erythematosus. *Rheumatology (Oxford)* 2017;56(1):121-28. doi: 10.1093/rheumatology/kew377 [published Online First: 2016/11/03]
17. Golder V, Kandane-Rathnayake R, Hoi AY, et al. Association of the lupus low disease activity state (LLDAS) with health-related quality of life in a multinational prospective study. *Arthritis research & therapy* 2017;19(1):62. doi: 10.1186/s13075-017-1256-6 [published Online First: 2017/03/23]
18. Morand EF, Trasieva T, Berglind A, et al. Lupus Low Disease Activity State (LLDAS) attainment discriminates responders in a systemic lupus erythematosus trial: post-hoc analysis of the Phase IIb MUSE trial of anifrolumab. *Annals of the rheumatic diseases* 2018;77(5):706-13. doi: 10.1136/annrheumdis-2017-212504 [published Online First: 2018/02/09]
19. Gonzalez-Echavarri C, Ugarte A, Ruiz-Irastorza G. Rituximab-refractory lupus nephritis successfully treated with belimumab. *Clinical and experimental rheumatology* 2016;34(2):355-6. [published Online First: 2016/02/18]
20. Kraaij T, Huizinga TW, Rabelink TJ, et al. Belimumab after rituximab as maintenance therapy in lupus nephritis. *Rheumatology (Oxford)* 2014;53(11):2122-4. doi: 10.1093/rheumatology/keu369 [published Online First: 2014/09/11]
21. Simonetta F, Allali D, Roux-Lombard P, et al. Successful treatment of refractory lupus nephritis by the sequential use of rituximab and belimumab. *Joint Bone Spine* 2017;84(2):235-36. doi: 10.1016/j.jbspin.2016.01.008 [published Online First: 2016/05/31]
22. Psarelis S, Nikiphorou E, Boumpas DT. Successful use of sequential B-cell depletion therapy in lupus. *Lupus* 2018;27(2):345-46. doi: 10.1177/0961203317717084 [published Online First: 2017/06/24]
23. Gualtierotti R, Borghi MO, Gerosa M, et al. Successful sequential therapy with rituximab and belimumab in patients with active systemic lupus erythematosus: a case series. *Clinical and experimental rheumatology* 2018;36(4):643-47. [published Online First: 2018/03/14]
24. Ramskold D, Parodis I, Lakshmikanth T, et al. B cell alterations during BAFF inhibition with belimumab in SLE. *EBioMedicine* 2019;40:517-27. doi: 10.1016/j.ebiom.2018.12.035 [published Online First: 2018/12/30]

25. Scholz JL, Crowley JE, Tomayko MM, et al. BLyS inhibition eliminates primary B cells but leaves natural and acquired humoral immunity intact. *Proc Natl Acad Sci U S A* 2008;105(40):15517-22. doi: 10.1073/pnas.0807841105 [published Online First: 2008/10/04]
26. Woodland RT, Schmidt MR, Thompson CB. BLyS and B cell homeostasis. *Semin Immunol* 2006;18(5):318-26. doi: 10.1016/j.smim.2006.06.001 [published Online First: 2006/08/26]
27. Jacobi AM, Huang W, Wang T, et al. Effect of long-term belimumab treatment on B cells in systemic lupus erythematosus: extension of a phase II, double-blind, placebo-controlled, dose-ranging study. *Arthritis and rheumatism* 2010;62(1):201-10. doi: 10.1002/art.27189
28. Stohl W, Hiepe F, Latinis KM, et al. Belimumab reduces autoantibodies, normalizes low complement levels, and reduces select B cell populations in patients with systemic lupus erythematosus. *Arthritis and rheumatism* 2012;64(7):2328-37. doi: 10.1002/art.34400 [published Online First: 2012/01/26]
29. Tipton CM, Fucile CF, Darce J, et al. Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus. *Nat Immunol* 2015;16(7):755-65. doi: 10.1038/ni.3175
30. Jenks SA, Cashman KS, Zumaquero E, et al. Distinct Effector B Cells Induced by Unregulated Toll-like Receptor 7 Contribute to Pathogenic Responses in Systemic Lupus Erythematosus. *Immunity* 2018;49(4):725-39 e6. doi: 10.1016/j.immuni.2018.08.015 [published Online First: 2018/10/14]
31. Lazarus MN, Turner-Stokes T, Chavele KM, et al. B-cell numbers and phenotype at clinical relapse following rituximab therapy differ in SLE patients according to anti-dsDNA antibody levels. *Rheumatology (Oxford)* 2012;51(7):1208-15. doi: 10.1093/rheumatology/ker526
32. Dias SS, Rodriguez-Garcia V, Nguyen H, et al. Longer duration of B cell depletion is associated with better outcome. *Rheumatology (Oxford)* 2015;54(10):1876-81. doi: 10.1093/rheumatology/kev036 [published Online First: 2015/06/06]
33. ital EM, Dass S, Buch MH, et al. B cell biomarkers of rituximab responses in systemic lupus erythematosus. *Arthritis and rheumatism* 2011;63(10):3038-47. doi: 10.1002/art.30466
34. Anolik JH, Campbell D, Felgar RE, et al. The relationship of FcgammaRIIIa genotype to degree of B cell depletion by rituximab in the treatment of systemic lupus erythematosus. *Arthritis and rheumatism* 2003;48(2):455-9. doi: 10.1002/art.10764 [published Online First: 2003/02/07]
35. Ng KP, Leandro MJ, Edwards JC, et al. Repeated B cell depletion in treatment of refractory systemic lupus erythematosus. *Annals of the rheumatic diseases* 2006;65(7):942-5. doi: 10.1136/ard.2005.044487 [published Online First: 2005/11/05]
36. Kraaij T, Arends EJ, Dam Lv, et al. OP0042 LONG-TERM EFFECTS OF SYNERGETIC B CELL IMMUNOMODULATION WITH RITUXIMAB AND BELIMUMAB COMBINATION TREATMENT IN SEVERE, REFRACTORY SLE: TWO YEAR RESULTS. *Annals of the rheumatic diseases* 2019;78(Suppl 2):91-92. doi: 10.1136/annrheumdis-2019-eular.5664

Supplementary material

Supplemental file S1

Methods

Clinical parameters

Patients were followed for 2 years during which SLEDAI-SELENA and LLDAS was assessed every 3 months and renal response every 6 months. LLDAS was defined according to the following definition[37]: 1) SLEDAI≤4, with no activity in major organ systems; 2) no new clinical features of lupus disease activity compared with previous assessment(serological activity was allowed in patients during LLDAS, referred to with the term 'serologically active clinically quiescent'(SACQ)); 3) physician global assessment ≤1; 4) prednisolone dose ≤7.5mg per day; and 5) well-tolerated treatment with immunosuppressive drugs and/or biological agents. In patients with LN, renal responses were defined as follows: a complete renal response was achieved when proteinuria decreased to ≤0.7g/24h and normal serum albumin, stable kidney function(<25% decline in serum creatinine compared to baseline) and a normal urinary sediment were achieved. Partial renal response was achieved when proteinuria: >0.7–2.9g/24h with a decrease in proteinuria of ≥50% from baseline, serum albumin >30g/L and a stable kidney function as measured by serum creatinine. Urine sediment did not necessarily have to be normalized for achieving a partial renal response. All other patients were considered to be renal non-responders. A minor flare was defined as a situation in which 1 or more of the following criteria were met: 1) increase in SLEDAI with ≥3 points;2) new or worse SLE-related activity, i.e.: skin manifestations(discoid LE, photosensitivity, lupus profundus, cutaneous vasculitis, bullous lupus), nasopharyngeal ulcers, pleuritis, pericarditis, arthritis and/or lupus fever. A major flare was defined as a situation in which 1 or more of the following criteria are met: 1) increase in SLEDAI with ≥ 12 points; new or worse SLE-related activity of major organs, i.e.: CNS-SLE(includes NPSLE), vasculitis, nephritis, myositis, thrombocytopenia $<60.10^9$/L, hemolytic anemia <4.4mmol/L(=7.0g/dL). According to the predefined study protocol, patients who experienced a major flare dropped out of the study. Due to the need for new induction therapy these patients were no longer followed according to our study protocol.

High sensitivity flow cytometry

High sensitivity flow cytometry(HS-FACS) was performed every 3 months on isolated peripheral blood mononuclear cells(PBMCs) from fresh blood collected in EDTA-coated tubes using density gradient centrifugation with Ficoll-amidotrizoate(LUMC). On average 1.8 million[0.4;6.7] cells were stained with the following antibodies divided over 6 different panels for FACS staining: CD3(SK7), CD8(SK1), CD11B(ICRF44), HLA-DR(G46-6), all from BD Biosciences, and CD4(SK3), CD14(M†P9), 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 detected using a LSRII flow cytometer(Becton Dickinson, San Jose, CA, USA) and analyzed using Flowjo software(Ashland, Oregon, USA). A minimum of 20 events was necessary to be counted as a population. B-cells were defined

as $CD3^-CD19^+$cells, wherein non-switched memory B-cells were $CD27^+IgD^+$cells, switched memory B-cells were $CD27^+IgD^-$, naive cells were $CD27^-IgD^+$cells, double negative(DN) B-cells were $CD27^-IgD^-$cells, transitional B-cells were $CD24^{bright}CD38^{bright}$cells, and circulating plasmablasts were $CD38^{bright}CD27^{bright}$cells. Absolute cell numbers were calculated using the absolute lymphocyte counts.

Autoantibody measurements

Anti-dsDNA autoantibodies were semi-quantitatively measured using the Crithidia luciliae indirect fluorescent test(CLIFT). For quantitative measurements of anti-dsDNA, an in-house ELISA was used. Briefly, 96-well plate microtiter ELISA plates(Nunc Maxisorb, Thermo Fisher, Waltham, USA) were coated with 2,5 μg/ml ultrapure calf thymus DNA solution(Thermofisher, Waltham, USA) diluted in reacti-bindTM DNA coating solution(Thermofisher, Waltham, USA) After coating, plates were washed three times with PBS/0.05% Tween 20 and blocked with 100 μl/well PBS/Casein 2%(Sigma, Saint-Louis, USA) 1 hour at 37 C. Wells were washed three times and sera of SLE patients or healthy controls were diluted in PBS/0.05% Tween 20/Casein 2%(Tween 20, Sigma, Saint-Louis, USA) for 1 hour at 37 C. Autoantibodies in sera were detected with 1:12000 diluted polyclonal rabbit-ήhuman-IgG/HRP secondary antibody(Dako, Jena, Germany, stock: 1.3 g/L). 100 μl/well 3,3',5,5'-Tetramethylbenzidine(TMB)(Sigma, Saint-Louis, USA) was used as a substrate for the revelation of HRP for approximately 20 – 30 minutes in the absence of light. The optical density(OD) was measured at 450 nm wavelength using an automated microplate reader spectrophotometer(Bio-Rad, Hercules, USA). The color reaction was stopped with 100 μl/well sulfuric acid, H2SO4, TMB-stop solution(Merck, Darmstadt, Germany). A serum of SLE patient with a high titer was chosen as a positive standard for the ELISA and was given an Arbitrary unit/ml(AU/ml). Anti-U1RNP, anti-RNP70 and anti-Sm autoantibody serum levels were measured using the Phadia 250(Thermo Fisher, Waltham, MA, USA). 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.

Statistical analysis

Data are reported as median[range] unless otherwise specified. Differences between responders and non-responders were compared using the Mann Whitney test. Predictors of response, i.e. autoantibody and complement consumption and changes in B-cell subsets, were determined by comparing the percentage change between groups using the Mann Whitney test. Differences between week 0 and end of study were compared using paired t-tests. All analyses were performed with GraphPad, version 7.02(La Jolla, CA, USA).

Supplemental file S2 Detailed overview of adverse events

Treatment-emergent adverse events	# events (%)	
All adverse events	86	
Severe adverse events (hospitalization)	5	(5.8)
Major infection	4	(4.7)
Gastro-intestinal infection	4	
Culture negative	4	
Cholelithiasis	1	(1.2)
Minor infection	29	(33.7)
Upper respiratory tract	15	(17.4)
Rhinovirus	4	
Metapneumovirus	1	
Respiratory syncytial virus	1	
Culture negative	6	
No available culture	3	
Lower respiratory tract	4	(4.7)
Haemophilus influenzae	1	
Culture negative	2	
No available culture	1	
Urinary tract	4	(4.7)
Escherichia coli	1	
Culture negative	3	
Urogenital infection	2	(2.3)
Neisseria gonorrhoeae	1	
Chlamydia trachomatis	1	
Sinusitis	1	(1.2)
Rhinovirus	1	
Influenza	1	(1.2)
Herpes simplex	1	(1.2)
Skin	1	(1.2)
HACA formation	4	(4.7)
Symptomatic	1	(1.2)
Asymptomatic	3	(3.5)
Hypogammaglobulinemia (<4.0 g/l)	2	(2.3)
Infusion-related reaction	2	(2.3)
Myalgia	7	(8.1)
Diarrhoea	8	(9.3)

Headache	3	(3.5)
Pyrexia	3	(3.5)
Nausea	2	(2.3)
Mood disorder	2	(2.3)
Fatigue	2	(2.3)
Other	17	(19.8)
Not-lupus related skin lesions	4	
Flank pain	2	
Iron deficiency anemia	1	
Onycholysism	1	
Hyperkalemia	1	
Dry eyes	1	
Dysuria	1	
Bacteriuria	1	
Tendinitis	1	
Palpitations	1	
Gastroesophageal reflux disease	1	
Dizziness	1	
Onychomycosis	1	

Supplemental file S3. Biological markers of systemic lupus erythematosus (SLE) disease activity in responders

	Week 0		Week 12		Week 24		Week 36	
	Responders (n=8)		Responders (n=8)		Responders (n=8)		Responders (n=8)	
ESR (mm)	45	(14-109)	27	(6-65)	17	(2-77)	25	(2-56)
Leucocytes (*10^9 cells/liter)	5.1	(3.6-7.2)	7.4	(3.6-13)	6	(4.1-15.2)	7.6	(4.8-10.4)
Thrombocytes (*10^9 cells/liter)	281	(168-345)	282	(234-417)	279	(234-408)	276	(243-332)
IgG (g/l)	11.5	(5-23.6)	7.8	(2.6-14.4)	9.7	(3.4-16.4)	11.5	(5.1-16.3)
IgA (g/l)	3	(1.2-4.5)	2.5	(1.1-3.5)	2.8	(1.1-3.6)	2.8	(1.1-3.7)
IgM (g/l)	0.7	(0.3-1.1)	0.5	(0.3-0.7)	0.4	(0.3-0.7)	0.4	(0.3-0.6)
Anti-tetanus toxoid IgG (IU/ml)	0.40	(0.02-1.84)	N/A		0.36	(0.02-2.24)	N/A	
Anti-rubella IgG (IU/ml)	61	(13.1-262.9)	51.2	(13.4-172)	59.7	(16.8-198.5)	N/A	
Anti-VZV IgG (mIU/ml)	3435	(441-4000)	2725	(251-4000)	2853	(443-4000)	N/A	
ANA positivity (%)†	100		100		100		87.5	
Anti-dsDNA positivity (%)†	87.5		25		13		25	
Anti-dsDNA titer (AE/ml)	268	(50-1430)	N/A		30	(0-105)	N/A	
Anti-RNP70 positivity (%)†	37.5		37.5		37.5		37.5	
Anti-RNP70 titer (IU/ml)	161	(130-2375)	107	(55-1394)	92	(43-1322)	N/A	
Anti-U1RNP positivity (%)†	50		50		50		50	
Anti-U1RNP titer (IU/ml)	288	(16-3679)	125	(6-2148)	132	(8-2060)	N/A	
Anti-Sm positivity (%)†	37.5		37.5		37.5		25	
Anti-Sm titer (IU/ml)	93	(23-176)	56	(0-128)	15	(0-113)	N/A	
Anti-C1q positivity (%)	87.5		62.5		62.5		N/A	
Anti-C1q titer (U/ml)	82	(25-115)	43	(16-69)	32	(10-59)	N/A	
Complement activation (%)‡	100		62.5		50		50	
C3 (g/l)§	0.6	(0.5-0.8)	0.9	(0.4-1.2)	1	(0.5-1.3)	0.9	(0.5-1.4)
C4 (mg/l)¥	55	(35-80)	94	(76-283)	128	(73-292)	150	(28-279)
CP (%activation)#	21	(12-70)	70	(44-101)	83	(32-91)	83	(6-111)

Week 48		Week 60		Week 72		Week 84		Week 96		Week 104	
Responders (n=8)		Responders (n=8)		Responders (n=8)		Responders (n=8)		Responders (n=8)		Responders (n=8)	
22	(2-36)	16	(2-36)	17	(2-36)	17	(2-36)	18	(2-34)	17	(2-45)
7.4	(4.4-13.2)	5.3	(3-9.1)	6.1	(3.6-11.7)	5.3	(2.8-12.7)	5.7	(4.1-11.6)	5.7	(4.2-8.4)
273	(221-308)	268	(198-285)	285	(208-315)	286	(212-308)	276	(212-341)	299	(202-401)
10.3	(4.4-19.2)	10.7	(4.6-17.9)	11.1	(5.3-18.8)	10.4	(5.6-19.5)	10.3	(6.4-20.3)	11.3	(5.5-22.5)
2.9	(0.9-3.7)	2.9	(0.9-3.3)	2.8	(0.8-3.6)	2.8	(0.8-3.4)	3	(0.6-3.4)	3	(0.7-3.8)
0.3	(0.2-0.6)	0.3	(0.3-0.4)	0.3	(0.2-0.6)	0.3	(0.2-0.7)	0.4	(0.2-0.7)	0.4	(0.2-0.7)
0.46	(0.02-1.48)	N/A		0.43	(0.01-2.17)	N/A		N/A		0.46	(0.02-2.24)
70.6	(16.2-262.8)	N/A		73.4	(17.2-249.7)	N/A		N/A		74	(19.2-2.24)
2433	(472-3826)			2356	(468-4000)			N/A		2436	(404-3625)
100		100		100		100		100		100	
12.5		12.5		25		12.5		12.5		12,5	
29	(6.8-195)	N/A		25	(0-195.7)	N/A		N/A		51.8	(22.7-132.3)
37.5		37.5		37.5		37.5		37.5		37.5	
63	(22-160)	N/A		59	(23-167)	N/A		N/A		68	(20-139)
50		50		50		50		50		50	
97	(13-3264)	N/A		102	(12-3146)	N/A		N/A		115	(11-2480)
25		25		25		25		25		25	
19	(3-104)	N/A		20		N/A		N/A		20	(2.4-123)
50		N/A		50		N/A		N/A		50	
20	(7-47)	N/A		21	(10-44)	N/A		N/A		36	(10-38)
62.5		62.5		50		37.5		75		50	
1	(0.8-1.3)	0.9	(0.7-1.4)	0.9	(0.7-1.4)	0.9	(0.8-1.3)	0.8	(0.7-1.3)	1	(0.8-1.3)
155	(100-285)	155	(67-286)	128	(94-279)	146	(101-232)	137	(85-225)	147	(74-279)
73	(20-107)	70	(49-103)	75	(53-116)	83	(63-103)	72	(13-94)	76	(51-96)

†Positivity is defined as semi-quantitative score of positive or strongly positive on immunofluorescence, titers are shown of patients with positive IF at week 0.

‡Complement consumption is defined as decreased levels of C3, C4 or decreased classical pathway (CP) activity.

§C3 values in patients with a decreased concentration at baseline. Normal C3: 0.9-2.0 g/l.

¥C4 values in patients with a decreased concentration at baseline. Normal C4: 95-415 mg/l.

#Classical pathway (CP) activation in patients with a decreased CP activation at baseline (9 responders; 5 non-responders). Normal CP: >74 %activation.

ESR, erythrocyte sedimentation rate; VZV, varicella zoster virus.

Supplemental file S4. Biological markers of systemic lupus erythematosus (SLE) disease activity in non-responders

	Week 0		Week 12		Week 24	
	Non responders (n=5)		Non responders (n=5)		Non responders (n=5)	
ESR (mm)	79	(39-129)	22	(6-120)	33	(6-70)
Leucocytes (*10^9 cells/liter)	3.1	(2.4-8.1)	5.7	(1.6-5.8)	3.75	(2.4-5.8)
Thrombocytes (*10^9 cells/liter)	281	(115-327)	309	(124-413)	257	(146-373)
IgG (g/l)	12.9	(4.9-16.6)	9.6	(4.3-13.8)	7.6	(6.5-13.2)
IgA (g/l)	2.9	(1.6-6.3)	2.7	(1.3-6.1)	2.1	(1.7-5.3)
IgM (g/l)	0.8	(0.4-1.1)	0.5	(0.2-0.8)	0.4	(0.2-0.7)
Anti-tetanus toxoid IgG (IU/ml)	0.22	(0.05-1.13)	N/A		0.19	(0.10-1.19)
Anti-rubella IgG (IU/ml)	93	(6.9-340.4)	88.7	(6-344)	92.6	(5.8-338.3)
Anti-VZV IgG (mIU/ml)	2859	(1382-3925)	2716	(446-3525)	2790	(530-3473)
ANA positivity (%)†	100		100		100	
Anti-dsDNA positivity (%)†	80		60		60	
Anti-dsDNA titer (AE/ml)	478	(33-1122)	N/A		92.1	(19.3-549.2)
Anti-RNP70 positivity (%)†	40		40		40	
Anti-RNP70 titer (IU/ml)	745	(102-1388)	437	(47-827)	234	(49-418)
Anti-U1RNP positivity (%)†	40		40		40	
Anti-U1RNP titer (IU/ml)	1401	(365-2437)	734	(128-1340)	446	(127-764)
Anti-Sm positivity (%)†	60		60		60	
Anti-Sm titer (IU/ml)	39	(6-3419)	26	(6-2120)	27	(6-2662)
Anti-C1q positivity (%)	100		80		40	
Anti-C1q titer (U/ml)	68	(40-135)	43	(18-68)	19	(17-72)
Complement activation (%)‡	100		60		60	
C3 (g/l)§	0.6	(0.5-0.8)	0.7	(0.5-1)	0.6	(0.5-1.1)
C4 (mg/l)¥	41	(21-68)	43	(29-108)	41	(39-117)
CP (%activation)#	33	(5-74)	56	(9-97)	44	(22-114)

Week 36		Week 48	Week 60	Week 72
Non responders (n=3)		Non responders (n=1)	Non responders (n=1)	Non responders (n=1)
28	(25-29)	11	11	11
6.2	(2.6-6.3)	2.4	2.5	2.5
276	(121-292)	139	140	109
8.6	(7-11)	11.9	12.3	10
1.9	(1.3-2.2)	2.5	2.4	
0.6	(0.2-0.7)	0.5	0.4	0.5
N/A		0.19	N/A	0.17
N/A		77.2	N/A	75.2
N/A		2713		2597
100		100	100	100
67		100	100	100
N/A		288	N/A	306.8
20		0	0	0
N/A				
33		0	0	0
N/A				
33		0	0	0
N/A				
N/A		0		0
N/A				
67		100	100	100
0.8	(0.7-0.8)	0.7	0.6	0.7
85	(61-108)	75	40	50
91	(54-100)	26	30	37

†Positivity is defined as semi-quantitative score of positive or strongly positive on immunofluorescence, titers are shown of patients with positive IF at week 0.

‡Complement consumption is defined as decreased levels of C3, C4 or decreased classical pathway (CP) activity.

§C3 values in patients with a decreased concentration at baseline. Normal C3: 0.9-2.0 g/l.

¥C4 values in patients with a decreased concentration at baseline. Normal C4: 95-415 mg/l.

#Classical pathway (CP) activation in patients with a decreased CP activation at baseline (9 responders; 5 non-responders). Normal CP: >74 %activation.

ESR, erythrocyte sedimentation rate; VZV, varicella zoster virus.

Chapter 9

TAC-TIC use of tacrolimus-based regimens in lupus nephritis

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Abstract

Current guidelines do not mention tacrolimus (TAC) as a treatment option and no consensus has been reported on the role of TAC in lupus nephritis (LN). The present study aimed to guide clinical judgement on the use of TAC in patients with LN. A meta-analysis was performed for clinical studies investigating TAC regimens in LN on the basis of treatment target (induction or maintenance), concomitant immunosuppression and quality of the data. 23 clinical studies performed in patients with LN were identified: 6 case series, 9 cohort studies, 2 case-control studies and 6 randomized controlled trials (RCTs). Of the 6 RCTs, 5 RCTs investigated TAC regimens as induction treatment and 1 RCT as maintenance treatment. Five RCTs investigated TAC in combination with steroids and 2 TAC with mycophenolate plus steroids. All RCTs were performed in patients of Asian ethnicity. In a meta-analysis, TAC regimens achieved a significantly higher total response (relative risk (RR) 1.23, 95% CI 1.12 to 1.34, $p<0.05$) and significantly higher complete response (RR 1.48, 95% CI 1.23 to 1.77, $p<0.05$). The positive outcome was predominantly defined by the largest RCT investigating TAC with mycophenolate plus steroids. Regarding safety, the occurrence of leucopoenia was significantly lower, while the occurrence of increased creatine was higher. Clinical studies on TAC regimens for LN are limited to patients of Asian ethnicity and hampered by significant heterogeneity. The positive results on clinical efficacy of TAC as induction treatment in LN cannot be extrapolated beyond Asian patients with LN. Therefore, further confirmation in multiethnic, randomized trials is mandatory. Until then, TAC can be considered in selected patients with LN.

Introduction

Lupus nephritis (LN) occurs in up to 60% [1] of all patients with systemic lupus erythematosus (SLE) and is associated with increased mortality rates [2]. Current guidelines on the treatment for LN recommend corticosteroids in combination with cyclophosphamide or mofetil mycophenolate (MMF) as induction treatment and azathioprine or MMF as maintenance treatment [3, 4]. Nevertheless, there is a persistent need for new therapeutic options since the cumulative renal flare rate is 50% within 10 years upon the first-choice conventional treatments [5]. For these refractory patients, guidelines are less specific in their recommendations: Rituximab is most often recommended to be considered despite the negative results in randomized trials [6, 7]. Interestingly, no consensus was reached on the role of calcineurin inhibitors (CNIs) [3, 4] despite two recently published, large randomized controlled trials (RCTs) showing a positive signal on the efficacy of a tacrolimus (TAC)-based treatment in LN [8, 9]. Moreover, an attractive aspect of TAC is that it also can be given during pregnancy [10, 11], which is a frequent dilemma in young women with SLE. Also, TAC is a readily available agent and commonly used in kidney transplantation. Taken together, systematically analyzing the potential role of TAC as treatment for LN is necessary.

TAC is a macrolide CNI frequently used in solid organ transplantation to prevent rejection [12]. Calcineurin inhibition by TAC prevents dephosphorylation of the nuclear factor of activated T cells and thereby reduces activity of genes coding interleukin 2 and related cytokines [13], leading to inhibition of T cell activation. Besides its immunosuppressive effect TAC, as well as its calcineurin-inhibiting predecessor ciclosporine, are both known for their antiproteinuric effects in treating a variety of renal pathologies [14]. In an SLE mouse model [15], treatment with TAC in animals with spontaneous LN shows inhibition of the progression of glomerular hypercellularity, crescent formation, proteinuria development and suppression of serum anti-dsDNA antibody elevation. Thus, from an immunological point of view, TAC might have potential as treatment for LN. The present study aimed to guide clinical judgement on the use of TAC in patients with LN. Therefore, we systematically reviewed all the published clinical studies that investigated a TAC regimen in LN and performed a meta-analysis on the efficacy of TAC regimens and assessed available safety parameters.

Methods

Pubmed, Embase, Web of Science and Cochrane databases were searched for all human studies on treatment of LN with TAC. The following search terms were used: (((‘Tacrolimus’[Mesh] OR ‘tacrolimus’[tw] OR tacrolimus*[tw] OR ‘Prograf’[tw] OR ‘Prograft’[tw] OR ‘FR-900506’[tw]

OR 'FR 900506'[tw] OR 'FR900506'[tw] OR 'FK-506'[tw] OR 'FK 506'[tw] OR 'FK506'[tw] OR 'WM0H WNM'[all fields]) AND ('Nephritis'[Mesh] OR 'nephritis'[tw] OR nephrit*[tw] OR 'Glomerulonephritis'[tw] OR 'Anti-Glomerular Basement Membrane Disease'[tw] OR 'Glomerulosclerosis'[tw] OR 'Balkan Nephropathy'[tw] OR 'Pyelonephritis'[tw] OR 'Pyelitis'[tw] OR 'Pyelocystitis'[tw]) AND ('Lupus Erythematosus, Systemic'[Mesh] OR 'Systemic Lupus Erythematosus'[tw] OR 'SLE'[tw] OR 'lupus'[tw])) OR (('Tacrolimus'[Mesh] OR 'tacrolimus'[tw] OR tacrolimus*[tw] OR 'Prograf'[tw] OR 'Prograft'[tw] OR 'FR-900506'[tw] OR 'FR 900506'[tw] OR 'FR900506'[tw] OR 'FK-506'[tw] OR 'FK 506'[tw] OR 'FK506'[tw] OR 'WM0H WNM'[all fields]) AND ('Lupus Nephritis'[Mesh] OR 'Lupus Nephritis'[tw] OR 'Lupus Glomerulonephritis'[tw]))) AND ('Clinical Trial'[publication type] OR random*[tw] OR 'trial'[tw] OR 'RCT'[tw] OR placebo*[tw] OR 'double blind'[tw]). According to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria,16 titles and abstracts of search results were evaluated for suitability based on the following criteria: (1) published as a clinical trial in human subjects; (2) included patients had an established diagnosis of SLE in accordance with the American College of Rheumatology revised criteria; (3) the presence of LN and persistent clinical findings such as elevated serum creatine, proteinuria >0.5 g or active urine sediment; (4) for controlled studies: well defined renal complete, partial and non-response criteria. The studies were judged and selected independently by two investigators (TK and YKOT). Consensus was achieved on studies that were selected by only one of two investigators.

All studies were labelled according to their design, that is: 'case series' when 10 or less patients were reported, 'uncontrolled cohort' when more than 10 patients were studied, 'case-control study' (CCS) or 'randomized controlled trial' (RCT). Study characteristics were summarised by descriptive statistics and ordered on the basis of type and goal of TAC treatment leading to four categories: (1) studies applying a TAC regimen as induction treatment for new LN or flare of LN; (2) studies applying a TAC regimen as maintenance treatment for patients with LN who had received any given induction treatment; (3) studies applying a TAC regimen applied as induction treatment and followed by (lower dosages of) TAC as maintenance treatment; (4) studies switching conventional treatment to a TAC regimen during the maintenance phase. The quality of randomized controlled trials was assessed with the Delphi list [17].

From all controlled studies relevant variables were extracted, that is, baseline characteristics, trial design characteristics, TAC regimen characteristics, renal responses, dropouts and adverse events. With respect to renal response criteria, the definitions for complete, partial and no response were adapted from the individual studies.

Statistical analysis

Descriptive statistics were used to summarize baseline, trial and TAC regimen characteristics. Data from five RCTs were used in a meta-analysis, to compare renal response and adverse events between TAC-based regimens and control therapy. The meta-analysis was performed with Stata, V.10 (Statacorp, Texas, USA). The relative risk (RR) and 95% CI for each outcome was calculated for each study using the Mantel-Haenszel fixed effects model. Heterogeneity was determined by the χ^2 and I^2 tests. An outcome of $p<0.05$ was considered a significant difference.

Results

Summary of the literature search

Our search strategy resulted in 239 articles of which 23 relevant clinical studies were selected based upon the predefined quality criteria (figure 1). As depicted in table 1, we found that the majority of clinical studies consisted of uncontrolled case series (26%) and uncontrolled cohort studies (39%). Controlled studies encompassed 2 (9%) CCS and 6 (26%) RCTs. From all selected studies, 87% were exclusively performed in Asian LN populations, leaving 3 (13%) uncontrolled studies in non-Asian patients. The most frequently studied TAC regimen combined TAC with steroids (65%), also termed 'duo therapy'. Six (26%) studies combined TAC with steroids plus MMF, also termed 'triple therapy'. A majority of 13 (57%) studies investigated TAC as induction treatment, 5 (22%) as maintenance treatment, 3 (13%) used TAC as induction and subsequent maintenance treatment and 2 (9%) studies investigated a switch of conventional maintenance to a TAC maintenance regimen.

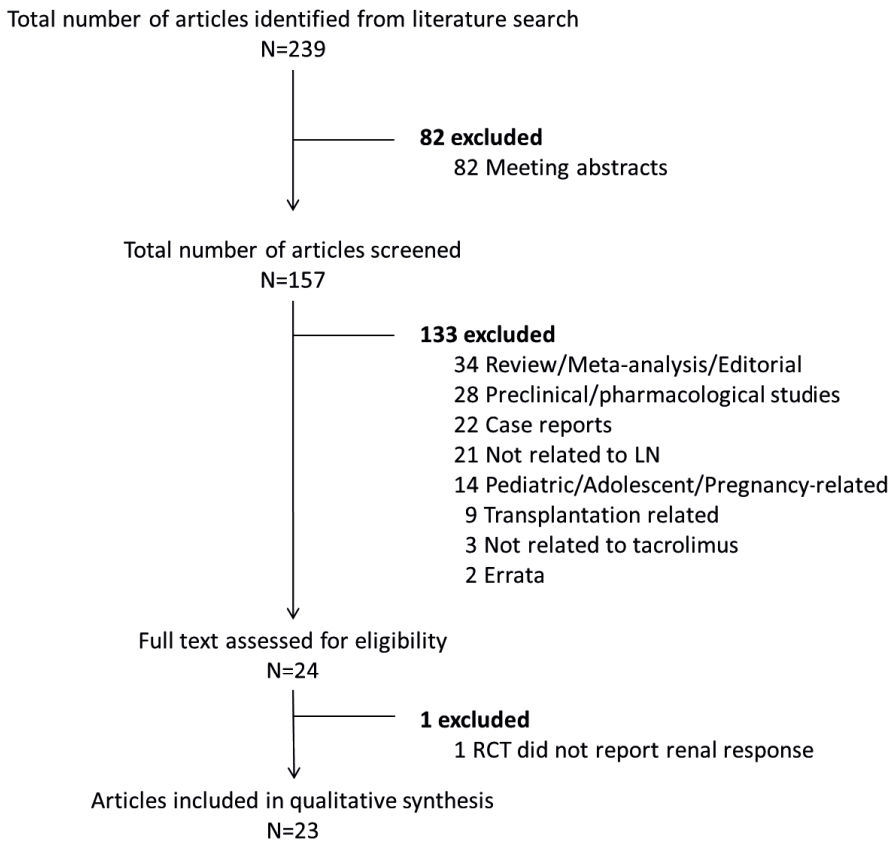

Figure 1. Flow chart of the literature search. LN, lupus nephritis; RCT, randomized controlled trial.

Table 1. Summary of study characteristics.

Study characteristics (N=23)	**(%)**
Design	
Case series (N ≤ 10)	6 (26)
Uncontrolled cohort (N > 10)	9 (39)
Case-control study (CCS)	2 (9)
Randomized controlled trial (RCT)	6 (26)
Subjects	
Asian alone	20 (87)
Non-Asian	3 (13)
Regimen	
Tacrolimus + steroids	15 (65)
Tacrolimus + steroids + Mycophenolate	6 (26)
Tacrolimus + steroids + Mizoribine	2 (9)
Tacrolimus used as	
Induction therapy	13 (57)
Maintenance therapy	5 (22)
Induction & maintenance therapy	3 (13)
Therapy switch*	2 (9)

*Study was designed to switch patients from conventional treatment to a tacrolimus-based regimen.

Table 2 summarizes the controlled studies grouped by treatment goal and on the basis of their treatment regimen. This overview illustrates the heterogeneity of the published studies.

To better understand the studied TAC regimens in the controlled studies, the quality score (only applicable in RCT), study designs and TAC dosing were summarized in table 3. Overall, the quality of studies was poor to average (median score 4, range 3–5) as measured by the standardized Delphi scoring for RCTs. Importantly, one shared characteristic was that all studies investigating induction treatment with TAC regimens defined their renal response end point at 6 months. The definition of renal response, however, was different for each study (see online supplementary table S2). With respect to dosing, we could not find any coherence between any of the studies nor within studies investigating duo therapy or triple therapy TAC regimens. Seven (88%) studies measured TAC trough levels to guide their dosing, however target trough levels varied per study (table 3).

Table 2. Number of studies stratified by treatment intention.

	Tacrolimus + steroids	Tacrolimus + steroids + Mycophenolate	Tacrolimus + steroids + Mizoribine
Induction therapy	7 *3 RCTs**	4 *2 RCTs**	2
Maintenance therapy	4 *1 RCT**	2	0
Induction & maintenance therapy	2 *2 CCS†*	0	0
Therapy switch*	2	0	0

* RCT = randomized controlled trial; † CCS = case-control study

Table 3. Overview of the studies fulfilling the predefined selection criteria for analysis of tacrolimus-based regimens in lupus nephritis patients.

	Type of study*	Quality score (0-9)†	No. of patients	Time to endpoint	Treatment regimen‡
Induction with duo-therapy					
Chen et al. 2011 (18)	RCT	5	81	6 months	TAC: blood concentration of 5-10 ng/ml Pred: initial dose 1 mg/kg/d (max. 60mg/d) tapered until 10 mg/d
Li et al. 2012 (19)	RCT	4	60	6 months	TAC: blood concentration of 6-8 ng/ml Pred: initial dose 1 mg/kg/d (max. 60mg/d), tapered until 10 mg/d
Mok et al. 2014 (8)	RCT	4	150	6 months	TAC: 0,1 mg/kg/d reduced to 0,06 mg/kg/d at 3 months if clinical response is satisfactory Pred: initial dose 0,6 mg/kg/d for 6 weeks, tapered until <10 mg/d

Induction with triple therapy					
Bao et al. 2008 (20)	RCT	5	40	6 months	TAC: blood concentration of 5-7 ng/ml MMF: 1,0 g/d, AUC 20-45mg.h/l Intravenous methylprednison: 0,5 g/d for 3 days Pred: pred 0,6-0,8 mg/kg/d for 4 weeks, tapered until maintenance dose 10mg/d
Liu et al. 2015 (9)	RCT	5	362	6 months	TAC: adjusted according to blood concentration measured throughout study MMF: according to AUC measured throughout study Pred: similar between treatment groups, gradually tapered
Maintenance with duo-therapy					
Chen et al. 2012 (21)	RCT	4	70	6 months	TAC: blood concentrations of 4-6 ng/ml Pred: 10 mg/d
Induction and maintenance with duo-therapy					
Yap et al. 2012 (22)	CCS	NA	16	24 months	TAC: blood concentration of 6-8 ng/ml in the first 6 months; 5-5,9 ng/ml in the next 6 months; 3,0-4,9 in the last year Pred: 0,8 mg/kg/d (max. 50 mg/d), tapered to 7,5 mg/d until end of study (in patients <50 kg reduced to 5 mg/d)
Wang et al. 2012 (23)	CCS	NA	40	12 months	TAC: blood concentration of 6-8 ng/ml during induction, 4-6 ng/ml during maintenance Pred: 0,8 mg/kg/d (max. 50mg/d), tapered until 10-15 mg/d during maintenance

* RCT = randomized controlled trial; †Quality assessed with the Delphi score; NA = not applicable; CCS = case-control study ‡TAC = tacrolimus; MMF = mycophenolate mofetil; pred = prednisone

Patient characteristics

Overall, 693 patients were included in the meta-analysis on renal response and most frequently reported adverse events. Patient characteristics were summarized in table 4.

In short, 90% of the subjects were female. The mean age was 32 years and 100% were of Asian ethnicity. Histopathologically, 84% had an LN class III/IV±V and 16% LN class V.

Of note, for non-Asian subjects we did not find any controlled trials. Two case series [24, 25] and one uncontrolled cohort study26 with a total of 32 patients using TAC regimens have been published and none met the selection criteria.

Table 4. Baseline characteristics of lupus nephritis patients from the selected RCTs that are used in the meta-analysis for renal response and adverse events.

	All	Induction therapy	
		Duo therapy	Triple therapy
N*	693	291	402
Age*	32	33	32
Female (%)	90	89	90
Disease duration (years)*	1,6	3,2	0,5
Asian ethnicity (%)	100	100	100
LN class (%)			
I/II			
III/IV ± V	84	85	83
V	16	15	17

*Data are expressed as the mean; TAC = tacrolimus; LN = lupus nephritis

Meta-analysis of renal responses upon induction treatment with TAC-based regimens

The results of the meta-analysis are shown in figure 2. Five RCTs investigated TAC regimens in the induction treatment phase and were used for data extraction. Again, all studies reported renal response rate as a primary end point at 6 months. Three RCTs used intravenous cyclophosphamide in the control arm, [9, 18, 20] one study mycophenolate [8] and one study [19] contained two control arms using either mycophenolate or cyclophosphamide. TAC-based induction treatment led to a significantly higher total renal response (RR 1.23, 95% CI 1.12 to 1.34, $p < 0.05$) with significantly higher complete renal response (RR 1.48, 95% CI 1.23 to 1.77, $p < 0.05$) and equivalent partial renal response (RR 0.98, 95% CI 0.79 to 1.21, p = not significant (NS)). The RR for total, complete and partial response was also assessed for studies using duo therapy and triple therapy separately. In RCTs using duo therapy, TAC-based induction treatment led to equivalent total renal response (RR 1.06, 95% CI 0.94 to 1.19, p = NS) with equivalent complete renal responders (RR 1.15, 95% CI 0.92 to 1.44, p = NS) as well as partial responders (RR 0.91, 95% CI 0.62 to 1.34, p = NS). For RCTs using triple therapy, TAC-based induction treatment led to a significantly higher total renal response (RR 1.37, 95% CI 1.21 to 1.56, $p < 0.05$), with more complete responders (RR 1.94, 95% CI 1.45 to 2.61, $p < 0.05$), and equivalent partial responders (RR 1.01, 95% CI 0.78 to 1.31, p = NS).

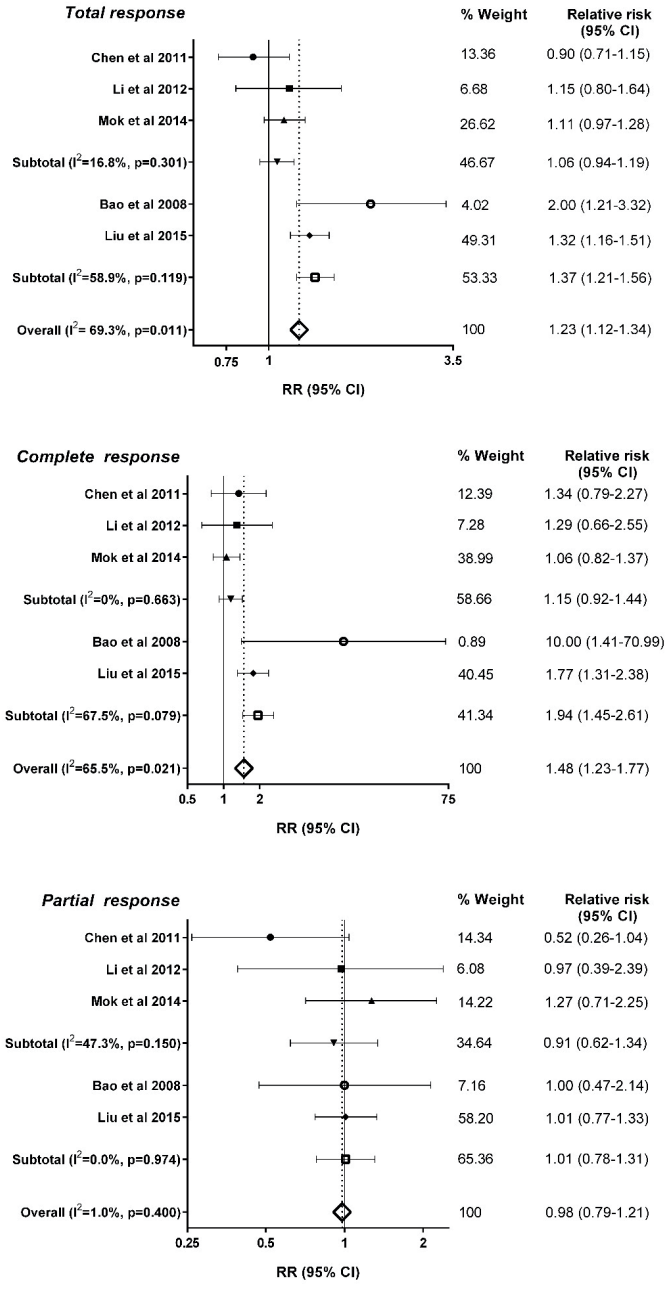

Figure 2. Forest plots of the relative risks (RRs) and 95% CIs of the total (complete plus partial), complete and partial renal response rates in the selected randomized controlled trials (RCTs) upon induction tacrolimus-based treatment versus conventional treatment. A fixed-effects meta-analysis was performed. The meta-

analysis was performed for studies using duo therapy (adapted from Mok et al [8], Chen et al [18], Li et al [19]) and for studies using triple therapy (adapted from Liu et al [9], Bao et al [20]) separately as well. The vertical solid line represents an RR of 1 and the dotted line illustrates the overall RR. The p value of the test for heterogeneity is shown for subtotal and overall analyses.

Renal responses upon maintenance treatment with TAC and steroids

Only one study [21] met our quality criteria to evaluate the effect of maintenance treatment with a TAC regimen. This study reported an equivalent response of 100% vs 95% to TAC versus control treatment after 6 months: 56% achieved a complete remission (19 out of 34) and 44% achieved a partial remission (15 out of 34). No flares were observed during this period. In the control group, where patients received azathioprine, 64% achieved complete remission (23 out of 36) and 31% a partial remission (11 out of 36). Two flares were observed in the control arm.

Meta-analysis of adverse events upon induction treatment with TAC and steroids

From the five RCTs investigating TAC regimens in the induction phase, the most frequently reported adverse events were included for meta-analysis (figure 3). Leukopenia was significantly less reported in the TAC-based treatment group (RR 0.21, 95% CI 0.08 to 0.54, $p<0.05$). A rise of serum creatine was higher in the TAC-based treatment group (RR 6.29, 95% CI 1.79 to 22.09, $p<0.05$). Infectious complications were comparable between the TAC-based treatment group and control group (RR 0.91, 95% CI 0.69 to 1.19, p=NS).

Although severe infections (RR 0.90, 95% CI 0.48 to 1.69, p=NS) and hyperglycemia (RR 1.40, 95% CI 0.78 to 2.52, p=NS) were more often reported in the TAC-based treatment group, these results did not reach statistical significance. Relative risks for the most reported adverse events were also compared between duo therapy and triple therapy separately. Overall, results between studies using duo therapy or triple therapy did not differ. Importantly, the TAC-based treatment in the RCTs using duo therapy showed a lower, non-significant rate for severe infection (RR 0.42, 95% CI 0.17 to 1.03, p=NS), whereas a trend to a higher rate of severe infections was seen with triple therapy (RR 2.83, 95% CI 0.92 to 8.72, p=NS).

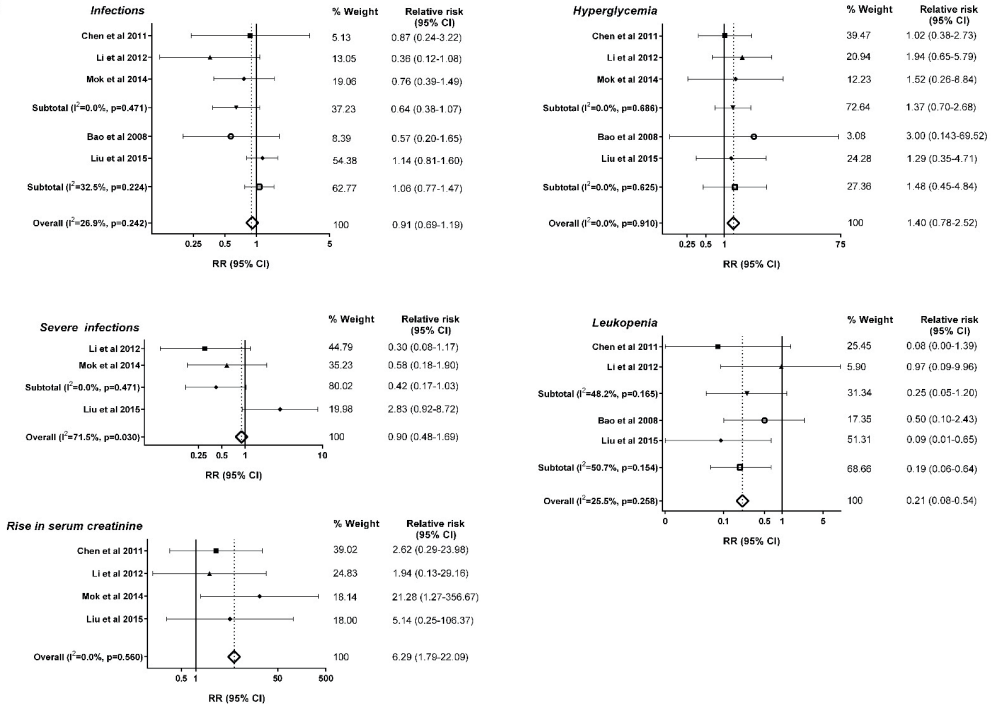

Figure 3. Forest plots of the relative risks (RRs) and 95% CIs for the five most commonly reported adverse events in the selected randomized controlled trials (RCTs) on tacrolimus-based treatment versus conventional treatment. Overall infections, severe infections, hyperglycemia, leucopoenia and rise in serum creatine were used in a meta-analysis, using a fixed-effects model. For infections, hyperglycemia and leucopoenia, a meta-analysis was performed for studies using duo therapy (adapted from Mok et al [8], Chen et al [18], Li et al [19]) and for studies using triple therapy (adapted from Liu et al [9], Bao et al [20]) separately as well. The vertical solid line represents an RR of 1 and the dotted line illustrates the overall RR. The p value of the test for heterogeneity is shown for subtotal and overall analyses.

Discussion

The present study was performed to better guide clinical judgement on the use of TAC in patients with LN. Selecting only the highest quality studies for meta-analyzing the clinical efficacy of TAC-based regimen, we demonstrated that the currently available studies are predominantly non-randomized, uncontrolled studies. Our systematic meta-analysis of randomized trials comparing TAC-based regimens with conventional treatment demonstrated superior efficacy in Asian patients with LN, mainly determined by studies evaluating triple therapy [9, 20]. Safety

profiles of TAC-based regimens were comparable to conventional treatment. These results cannot be extrapolated to the general LN population. Therefore, taken all together, current evidence supports the use of TAC-based regimens in a selected group of patients with LN of Asian ethnicity with a preference for using triple therapy (TAC, MMF and steroids) as induction treatment. The latter said, long-term safety of TAC-based regimens is not established.

The goal of this study was to translate published study results on TAC in LN to current clinical practice. Based on our study and previous meta-analyses [27, 28] there is level 1A evidence [29] to support the clinical efficacy of TAC in the subgroup of Asian patients with LN. However, our study illustrated that a 'grade A' recommendation for TAC is hampered by the heterogeneity of TAC-based regimens studied in this subgroup of patients with LN. In this view it is important to note that the positive result of our meta-analysis was predominantly determined by the study of Liu et al [9] that investigated a TAC-based regimen using 'triple' therapy combining steroids, mycophenolate and TAC. Altogether, it is self-evident that a randomized, multiethnic study is mandatory to further expand our knowledge and evidence of TAC treatment in LN.

To further guide clinicians in the use of TAC, it is reasonable to extrapolate the level 1A evidence (see online supplementary table S1) described above to the subgroup of refractory patients with LN. Generally, refractory LN is defined as a failure on two conventional treatments (being either mycophenolate or cyclophosphamide) [3, 4, 30]. Several treatment suggestions are made in LN treatment guidelines for refractory LN such as rituximab, CNIs, intravenous immunoglobulins, plasmapheresis and tumor necrosis factor (TNF) blockade [31]. Thus, with respect to TAC, a grade B positive recommendation can be formulated. Our data at the least suggest that the use of TAC is not inferior to conventional treatment. Moreover, we and others [27, 28, 32] showed that the safety profile of TAC is very good in LN. Therefore, we would recommend TAC to be considered as a treatment option in patients with refractory LN.

TAC is a safe drug during pregnancy and its continuation is commonly recommended in the setting of pregnant patients who have received solid organ transplantation [10, 33–35]. From this perspective, the level 1A evidence on the efficacy of TAC in Asian patients with LN should also be considered for extrapolation to this special subgroup of patients with LN. Although TAC is non-teratogenic, there is an increased risk of gestational diabetes and hypertension [36]. Currently, there are no controlled studies available investigating TAC for LN in pregnant patients. In a case series on nine patients with LN [33], TAC was successfully used to maintain remission in three patients and to treat a lupus flare in six patients. All pregnancies resulted in live births with birth weights according to gestational age and no congenital abnormalities. At present, azathioprine is considered the first choice of treatment in pregnant patients with LN [37]. However, in those

patients with LN who are azathioprine-resistant or azathioprine-intolerant, TAC can be considered as a treatment option.

CNIs were studied in LN before. Early exploratory studies on the efficacy of ciclosporine in LN resulted in comparable efficacy to conventional treatments, at the cost of unacceptably higher adverse events rates [38]. However, a small RCT in 40 patients (Cyclofa-Lune trial) [39] demonstrated that after approximately 8 years of follow-up, ciclosporine was non-inferior to high-dose cyclophosphamide as induction treatment for proliferative LN. A second RCT [40] in class V membranous LN, showed faster remission with ciclosporine compared with cyclophosphamide with comparable remission rates. Long-term follow-up of 5 years showed increased relapse rates in the ciclosporine treated arm. Only one study that investigated ciclosporine as maintenance therapy observed equal efficacy to azathioprine in preventing disease flares [41]. On a histopathological level, ciclosporine was unable to reduce chronic activity in lupus kidney biopsies, supporting the hypothesis that the antiproteinuric effects of ciclosporine were predominantly attributable to hemodynamic rather than immunological changes [42]. We know from the vast literature on transplantation that ciclosporine and TAC are different with respect to immunological efficacy as well as safety profile. Ciclosporine binds cyclophilin while TAC binds FK506, resulting in different immunosuppressive effects [43]. Furthermore, both ciclosporine and TAC have small therapeutic widths, causing small variations in dosing to potentially imply large differences in efficacy and toxicity. Therefore, irrespective of the available data on ciclosporine, further investigations into the efficacy of TAC on clinical as well as histopathological end points are clearly warranted.

There are important limitations to consider in the present meta-analysis. First, the quality of the controlled studies was low as defined by the Delphi score, mainly because of the incomplete blinding procedures in all studies. Second, as mentioned before, from five RCTs the largest RCT performed by Liu et al9 determined 49% of the overall total response. Third, TAC regimens were heterogeneous across all studies: target trough levels varied or were not used and also concomitant steroid dosing differed (see online supplementary table S2). This notion hampers a general recommendation on the optimal dosing of TAC. Fourth, no long-term results could be investigated in this meta-analysis. Only one study [8] reported long-term results (i.e., 5 years of follow-up) after induction treatment with TAC and prednisone during 6 months followed by azathioprine and prednisone as maintenance treatment. Of note, a higher rate of renal relapses was observed in the TAC-based treatment arm, which did not reach statistical significance ($p=0.13$). Lastly, it needs to be emphasized that all included studies were performed in Asian patients. The importance of ethnicity has been demonstrated by the ethnicity-based subgroup analysis of the Aspreva Lupus Management Study (ALMS) trial [44]. Superiority of mycophenolate over cyclophosphamide was predominantly determined by its efficacy in African-American and

Hispanic patients. In addition, genome-wide association studies revealed different genetic susceptibility loci for SLE between ethnicity groups [45]. Also, the CYP3A5 polymorphism determines the metabolism of TAC, and a lower bioavailability of TAC in African-American kidney transplant recipients [46, 47] is well described. Altogether emphasizing that the extrapolation of these data to other ethnic groups is not self-evident. Of note, we found only three non-controlled case series treating non-Asian subjects with TAC-based regimens [24–26]. Despite these limitations, this comprehensive analysis of all published studies illustrated that TAC-based therapy in selected patients with LN can be efficacious without major safety concerns. Therefore, these data emphasize the importance to further investigate the efficacy of TAC for patients with active LN.

Indeed, the international Lupus Trial Nephritis Network has recently initiated the design of a trial with a TAC-based regimen. In this respect a few considerations could be deducted from our current study. Based on the efficacy results in our meta-analysis, it would be plausible to investigate triple therapy randomizing a multiethnic patient population with LN. Regarding safety of such an RCT, a possible higher risk for severe infections in the triple therapy arm needs to be monitored closely. The general dosing in a TAC regimen is roughly estimated at 3–4 mg twice daily during the induction phase, based on the summary of studies. Monitoring of trough levels is not mandatory although it can help to exclude low exposition in patients. Most importantly, the definition of the primary renal end point needs much attention: due to the hemodynamic effects of TAC on reducing proteinuria, as discussed above, the classic LN renal end point which is mainly based on proteinuria improvement is intrinsically biased. Briefly, patients with LN with TAC have a quick reduction of proteinuria within the first weeks of treatment (most probably due to hemodynamic effects) and therefore empirically a higher chance of achieving a partial or even complete response. Also, in the current meta-analysis, we could not exclude whether a hemodynamic effect is (partly) responsible for the positive effects of TAC regimens. While a hemodynamic effect is undoubtedly present, it seems unlikely that this effect could fully explain the beneficial effect of TAC-based therapies. Thus, it seems wise to consider a less biased renal end point such as a repeat renal biopsy. Although timing of this renal biopsy needs careful consideration, it would confirm treatment efficacy in a more objective manner plus help to identify whether, if any, there is a risk for CNI toxicity on the renal tissue level. Of note, it is important to take into account that all included studies in this study used proteinuria as an important remission criterion (see online supplementary table S2). Taking these considerations into account, a multicenter, international RCT defining the role of TAC in LN treatment should be feasible and eagerly embraced by the scientific community.

In conclusion, we recommend the use of a TAC-based regimen in the selected group of Asian patients with LN. In addition, we recommend considering a TAC-based regimen in the subgroups of patients with refractory LN and (pre)pregnant patients with LN. Although long-term efficacy and safety results are lacking, it seems reasonable to conclude that when patients with LN are to be treated with TAC, this strategy seems not to be inferior to conventional treatment and has a good safety profile. In the future, the place of TAC in the therapeutic armamentarium for LN can only be established when a multicenter, international RCT is performed as now proposed by the international Lupus Nephritis Trial Network.

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References

1. Seligman VA, Lum RF, Olson JL, et al. Demographic differences in the development of lupus nephritis: A retrospective analysis. Am J Med 2002;112:726–9.
2. Bernatsky S, Boivin JF, Joseph L, et al. Mortality in systemic lupus erythematosus. Arthritis Rheum 2006;54:2550–7.
3. Hahn BH, McMahon MA, Wilkinson A, et al., American College of Rheumatology. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. Arthritis Care Res (Hoboken) 2012;64:797–808.
4. Bertsias GK, Tektonidou M, Amoura Z, et al. Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. Ann Rheum Dis 2012;71:1771–82.
5. Tamirou F, D'Cruz D, Sangle S, et al. Long-term follow-up of the MAINTAIN Nephritis Trial, comparing azathioprine and mycophenolate mofetil as maintenance therapy of lupus nephritis. Ann Rheum Dis 2016;75:526–31.
6. Rovin BH, Furie R, Latinis K, et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum 2012;64:1215–26.
7. Merrill JT, Neuwelt CM, Wallace DJ, et al. 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 2010;62:222–33.
8. Mok CC, Ying KY, Yim CW, et al. Tacrolimus versus mycophenolate mofetil for induction therapy of lupus nephritis: a randomised controlled trial and long-term follow-up. Ann Rheum Dis 2016;75:30–6.
9. Liu Z, Zhang H, Liu Z, et al. Multitarget Therapy for Induction Treatment of Lupus Nephritis. Ann Intern Med 2015;162:18–26.
10. EBPG Expert Group on Renal Transplantation. European best practice guidelines for renal transplantation. Section IV: Long-term management of the transplant recipient. IV.10. Pregnancy in renal transplant recipients. Nephrol Dial Transplant 2002;17(Suppl 4):50–5.
11. Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group. KDIGO clinical practice guideline for the care of kidney transplant recipients. Am J Transplant 2009;9(Suppl 3): S1–155.
12. Mendez R. FK 506 and mycophenolate mofetil in renal transplant recipients: six-month results of a multicenter, randomized dose ranging trial. FK 506 MMF Dose-Ranging Kidney Transplant Study Group. Transplant Proc 1998;30:1287–9.
13. Scott LJ, McKeage K, Keam SJ, et al. Tacrolimus: a further update of its use in the management of organ transplantation. Drugs 2003;63:1247–97.

14. Naesens M, Kuypers DRJ, Sarwal M. Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol 2009;4:481–508.
15. Entani C, Izumino K, Iida H, et al. Effect of a novel immunosuppressant, FK506, on spontaneous lupus nephritis in MRL/MpJ-lpr/lpr mice. Nephron 1993;64:471–5.
16. Moher D, Liberati A, Tetzlaff J, et al. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. Ann Intern Med 2014;151:264–9.
17. Verhagen AP, De Vet HCW, De Bie RA, et al. The Delphi list: a criteria list for quality assessment of randomized clinical trials for conducting systematic reviews developed by Delphi consensus. J Clin Epidemiol 1998;51:1235–41.
18. Chen W, Tang X, Liu Q, et al. Short-term outcomes of induction therapy with tacrolimus versus cyclophosphamide for active lupus nephritis: A multicenter randomized clinical trial. Am J Kidney Dis 2011;57:235–44.
19. Li X, Ren H, Zhang Q, et al. Mycophenolate mofetil or tacrolimus compared with intravenous cyclophosphamide in the induction treatment for active lupus nephritis. Nephrol Dial Transplant 2012;27:1467–72.
20. Bao H, Liu ZH, Xie HL, et al. Successful treatment of class V+IV lupus nephritis with multitarget therapy. J Am Soc Nephrol 2008;19:2001–10.
21. Chen W, Liu Q, Chen W, et al. Outcomes of maintenance therapy with tacrolimus versus azathioprine for active lupus nephritis: a multicenter randomized clinical trial. Lupus 2012;21:944–52.
22. Yap DY, Yu X, Chen XM, et al. Pilot 24 month study to compare mycophenolate mofetil and tacrolimus in the treatment of membranous lupus nephritis with nephrotic syndrome. Nephrology (Carlton) 2012;17:352–7.
23. Wang S, Li X, Qu L, et al. Tacrolimus versus cyclophosphamide as treatment for diffuse proliferative or membranous lupus nephritis: a non-randomized prospective cohort study. Lupus 2012;21:1025–35.
24. Lanata CM, Mahmood T, Fine DM, et al. Combination therapy of mycophenolate mofetil and tacrolimus in lupus nephritis. Lupus 2010;19:935–40.
25. Gordon S, Denunzio T, Uy A. Success using tacrolimus in patients with proliferative and membranous lupus nephritis and refractory proteinuria. Hawaii J Med Public Health 2013;72:18–23. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3764582&tool=pmcentrez&rendertype=abstract
26. Cortés-Hernández J, Torres-Salido MT, Medrano AS, et al. Long-term outcomes—Mycophenolate mofetil treatment for lupus nephritis with addition of tacrolimus for resistant cases. Nephrol Dial Transplant 2010;25:3939–48.
27. Lee YH, Song GG. Relative efficacy and safety of tacrolimus, mycophenolate mofetil, and cyclophosphamide as induction therapy for lupus nephritis: a Bayesian network meta-analysis of randomized controlled trials. Lupus 2015;24:1520–8.

28. Hannah J, Casian A, D'Cruz D. Tacrolimus use in lupus nephritis: a systematic review and meta-analysis. Autoimmun Rev 2016;15:93–101.
29. Shekelle P, Woolf S, Eccles M, et al. Clinical guidelines: developing guidelines. BMJ 1999;318:593–6. http://www.ncbi.nlm.nih.gov/pmc/ articles/PMC1115034/pdf/593.pdf
30. van Tellingen A, Voskuyl AE, Vervloet MG, et al., Dutch Working Party on Systemic Lupus Erythematosus. Dutch guidelines for diagnosis and therapy of proliferative lupus nephritis. Neth J Med 2012;70:199–206.
31. Tunnicliffe DJ, Singh-Grewal D, Kim S, et al. Diagnosis, monitoring and treatment of systemic lupus erythematosus: a systematic review of clinical practice guidelines. Arthritis Care Res (Hoboken) 2015;67:1440–52.
32. Singh JA, Hossain A, Kotb A, et al. Risk of serious infections with immunosuppressive drugs and glucocorticoids for lupus nephritis : a systematic review and network meta-analysis. BMC Med 2016;14:137.
33. Webster P, Wardle A, Bramham K, et al. Tacrolimus is an effective treatment for lupus nephritis in pregnancy. Lupus 2014;23: 1192–6.
34. Christopher V, Al-Chalabi T, Richardson PD, et al. Pregnancy outcome after liver transplantation: a single-center experience of 71 pregnancies in 45 recipients. Liver Transplant 2006;12:1138–43.
35. Kainz A, Harabacz I, Cowlrick IS, et al. Review of the course and outcome of 100 pregnancies in 84 women treated with tacrolimus. Transplantation 2000;70:1718–21.
36. Bramham K, Soh MC, Nelson-Piercy C. Pregnancy and renal outcomes in lupus nephritis: an update and guide to management. Lupus 2012;21:1271–83.
37. Grootscholten C, Ligtenberg G, Hagen EC, et al. Azathioprine/ methylprednisolone versus cyclophosphamide in proliferative lupus nephritis. A randomized controlled trial. Kidney Int 2006;70: 732–42.
38. Isenberg DA, Snaith ML, Morrow WJ, et al. Cyclosporin A for the treatment of systemic lupus erythematosus. Int J Immunopharmacol 1981;3:163–9.
39. Zavada J, Pesickova S, Rysava R, et al. Cyclosporine A or intravenous cyclophosphamide for lupus nephritis: the Cyclofa-Lune study. Lupus 2010;19:1281–9.
40. Austin HA, Illei GG, Braun MJ, et al. Randomized, controlled trial of prednisone, cyclophosphamide, and cyclosporine in lupus membranous nephropathy. J Am Soc Nephrol 2009;20:901–11.
41. Moroni G, Doria A, Mosca M, et al. A randomized pilot trial comparing cyclosporine and azathioprine for maintenance therapy in diffuse lupus nephritis over four years. Clin J Am Soc Nephrol 2006;1:925–32.
42. Radhakrishnan J, Kunis CL, D'Agati V, et al. Cyclosporine treatment of lupus membranous nephropathy. Clin Nephrol 1994;42:147–54. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7994932
43. Almawi WY, Melemedjian OK. Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A. Nephrol Dial Transplant 2000;15:1916–8.

44. Appel GB, Contreras G, Dooley MA, et al. Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. J Am Soc Nephrol 2009;20:1103–12.
45. Sánchez E, Comeau ME, Freedman BI, et al. Identification of novel genetic susceptibility loci in African American lupus patients in a candidate gene association study. Arthritis Rheum 2011;63:3493–501.
46. Dirks NL, Huth B, Yates CR, et al. Pharmacokinetics of immunosuppressants: a perspective on ethnic differences. Int J Clin Pharmacol Ther 2004;42:701–18.
47. Provenzani A, Santeusanio A, Mathis E, et al. Pharmacogenetic considerations for optimizing tacrolimus dosing in liver and kidney transplant patients. World J Gastroenterol 2013;19:9156–73.

Supplementary material

Supplemental Table 1. Classification scheme of the strength of evidence.

Classification schemes	
Category of evidence:	
Ia	Evidence for meta-analysis of randomised controlled trials
Ib	Evidence from at least one randomised controlled trial
IIa	Evidence from at least one controlled study without randomisation
IIb	Evidence from at least one other type of quasi-experimental study
III	Evidence from non-experimental descriptive studies, such as comparative studies, correlation studies, and case-control studies
IV	Evidence from expert committee reports or opinions or clinical experience of respected authorities, or both
Strength of recommendation:	
A	Directly based on category I evidence
B	Directly based on category II evidence or extrapolated recommendation from category I evidence
C	Directly based on category III evidence or extrapolated recommendation from category I or II evidence
D	Directly based on category IV evidence or extrapolated recommendation from category I, II or III evidence

(Adopted from: Shekelle P, Woolf S, Eccles M, Grimshaw J. Clinical guidelines: developing guidelines. BMJ 999;318:593–596.)

Supplemental Table 2. Overview of available RCTs on the effect of TAC-based regimens in lupus nephritis.

Reference	No. of patients	Type of study*	Duration	Study protocol †	Remission criteria ‡	Outcome (CR/PR/NR)
Chen et al 2011[18]	81	RCT	6 months	TAC-based regimen (n=42): Pred: initial dose 1 mg/kg/d tapered until 10 mg/d TAC: initally 0.05 mg/kg/d (through 5-10 ng/ml) Control arm IVCYC (n=39): Pred: initial dose 1 mg/kg/d tapered until 10 mg/d IVCYC: 0.75 g/m2 for the 1st month, then adjusted to 0.5-1 g/m2	CR: proteinuria <0.3 g/24h, normal urinary sediment, serum albumin ≥3.5 g/dl, stable kidney function (normal range or not >15% more than baseline PR: proteinuria 0.3-2.9 g/24h and a decrease of at least 50% of baseline level, serum albumin ≥3 g/dl, stable kidney function NR: proteinuria >3 g/24h or 0.3-2.9 g/24h, serum albumin <3 g/dl, increase in serum creatinine >30% of baseline	TAC-based regimen: 52/22/26 Control arm IVCYC: 38/43/19
Li et al 2012[19]	60	RCT	6 months	TAC-based regimen (n=20): Pred: initial dose 1 mg/kg/d tapered until 10 mg/d TAC: 0.08-0.1 mg/kg/d (through 6-8 ng/ml) Control arm MMF (n=20): Pred: initial dose 1 mg/kg/d tapered until 10 mg/d MMF: 1.5-2 g/d Control arm IVCYC (n=20): Pred: initial dose 1 mg/kg/d tapered until 10 mg/d IVCYC: 0.5-0.75 g/m^2 monthly	CR: proteinuria <0.3 g/24h with normal urine sediment, serum albumin >3.5g/dl and stabilization (±15%) or improvement of serum creatinine PR: proteinuria 0.3-2.9 g/24h, having decreased by at least 50% from baseline values, with a serum albumin of at least 3 g/dl and relative stabilization (±30%) in serum creatinine	TAC-based regimen: 45/30/25 Control arm MMF: 30/30/40 Control arm IVCYC: 45/30/25

Supplemental Table 2.Continued

Mok et al 2014[8]	150	RCT	6 months	TAC-based regimen (n=74): Pred: initial dose 1 mg/kg/d tapered until 10 mg/d TAC: initally 0.1 mg/kg/d to 0.06 mg/kg/d if clinical response was satisfactory at month 3 Control arm MMF (n=76): Pred: initial dose 1 mg/kg/d tapered until 10 mg/d MMF: 2-3 g/d	CR: proteinuria <1 g/24h or uP/Cr <1 with stabilization (within 25%) or improvement in serum creatinine, resolution of urinary sediment abnormalities, persistent improvement in C3 and anti-dsDNA levels PR: reduction of proteinuria; if nephrotic at baseline, a 50% decrease but <3 g/24h or uP/Cr <3; if non-nephrotic, a decrease to ≤50% of pre-treatment value but >1 g/24h (or uP/Cr >1), with stabilization (within 25%) or improvement in serum creatinine, improvement of urinary sediment abnormalities (>50% reduction in haematuria and urine RBC (<10/HPF) NR: deterioration of serum creatinine (>25%), an increase in proteinuria, or a reduction in proteinuria but not to the extent of CR of PR	TAC-based regimen: 62/27/11 Control arm MMF: 59/21/20
Bao et al 2008[20]	40	RCT	6 months	TAC-based regimen (n=20): Methylprednisone 0.5 g/d for 3d + pred taper until 10 mg/d TAC: 4 mg/d BID (through 5-7 ng/ml) MMF: 1.5-2 g/d (through AUC 20-45 mg*h/l) Control arm IVCYC (n=20): Methylprednisone 0.5 g/d for 3d + pred taper IVCYC: 0.75 g/m^2 for the 1st month, then adjusted to 0.5-1.0 g/m^2 monthly	CR: proteinuria 0.4g/24h, normal urinary sediment, serum albumin ≥3.5 g/dl, normal serum creatinine or no more than 15% above baseline PR: normal or at least a 50% improvement in proteinuria and haematuria, serum albumin ≥3 g/dl, normal serum creatinine or no more than 15% above baseline	TAC-based regimen: 50/40/10 Control arm IVCYC: 5/40/55

Liu et al 2015[9]	362	RCT	6 months	TAC-based regimen (n=181): Methylprednisone 0.5 g/d for 3d + pred taper until 10 mg/d TAC: 4 mg/d MMF: 1g/d Control arm IVCYC (n=181): Methylprednisone 0.5 g/d for 3d + pred taper until 10 mg/d IVCYC: 0.75 g/m2 for the 1st month, then adjusted to 0.5-1 g/m2	CR: proteinuria <0.4 g/24h, normal urinary sediment, serum albumine ≥3.5 g/dl, normal serum creatinine PR: proteinuria <3.5 g/24h and ≥50% reduction, serum albumin ≥3 g/dl, normal or ≤25% increase in serum creatinine from baseline	TAC-based regimen: 46/38/17 Control arm IVCYC: 26/37/37

* RCT = randomized controlled trial; † TAC = tacrolimus; IVCYC = intravenous cyclophosphamide; MMF = mycophenolate mofetil; pred = prednisone; ‡ CR = complete response; PR = partial response; NR = no response

Chapter 10

Summary, general discussion and future perspectives

Summary

The first part of this thesis investigated different aspects of neutrophil extracellular traps (NETs) in autoimmune disease. The quantification of NETs was studied and the method developed by us, was used to study the characteristics of NETs in systemic lupus erythematosus (SLE) and ANCA-associated vasculitis (AAV). We further used it to investigate whether NET formation can function as a biomarker in SLE and AAV. In the second part of this thesis, new treatments for patients with severe, refractory SLE were studied. We put forward a new therapeutic strategy combining rituximab (RTX) and belimumab (BLM) and we studied humoral immune responses after treatment with RTX+BLM.

In **chapter 2** we described a method to quantify NETs which has the potential to monitor autoantigen load in the setting of autoimmune diseases where NETs play a role in its pathophysiology. This method was set up to measure 'low level NET release', as was observed by stimulation of neutrophils with immune complexes. The use of confocal microscopy with multiple z-stacks, makes it a sensitive method, in particular in contrast to methods that have been developed using PMA-induced NET release [1].

In **chapter 3 and 4** we provided a context of how NETs can be quantified in SLE and AAV. We demonstrated that not all NETs are created equally and translation of NET formation to a digital quantification creates a narrow view. Indeed, we showed that important qualitative differences can underpin the formation of NETs, which are not captured by simply quantifying the amount of NETs via methods based on plasma or supernatant NET-related protein measurements with enzyme-linked immunosorbent assay (ELISA). We attempted to correlate ex vivo NET formation with clinical measures and showed a moderate correlation with the Birmingham Vasculitis Activity Score (BVAS) as well as significantly higher NET formation in active AAV patients (BVAS≥1) compared to patients in remission (BVAS=0). Further, we showed higher ex vivo NET formation in AAV patients with active disease compared to AAV patients with an underlying infection supporting that excessive NET formation is an autoimmune phenomenon. Also, we demonstrated that the observed excessive NET formation is independent of ANCAs (IgG and IgA isotype), complement component 5 (C5) and C5a receptor activation.

In the next part of this thesis, we focus on new treatments in lupus nephritis (LN). In **chapter 5**, we present two patients with refractory LN that were treated with BLM after RTX. In both patients, this led to beneficial clinical and immunological effects, putting forward the combination RTX+BLM as an interesting therapeutic option in SLE, which is further explored in the Synbiose study (Synergetic B cell immunomodulation in SLE).

Chapter 6 and 8 describe the results of the Sybiose study, a phase 2 proof-of-concept study that included 15 patients with severe, refractory SLE treated with RTX+BLM. We showed that RTX+BLM has the ability to reduce autoantibodies, thereby indirectly reducing excessive NET formation in SLE, presumably due to the targeting of autoreactive B cells. Further, we observed a clinical response in our patients while tapering immunosuppressive medication. After the primary endpoint at week 24, 7 patients discontinued; 2 due to a pregnancy wish and 5 were non-responders of whom 2 experienced a disease relapse. We showed a further decrease in autoantibodies while complete B cell depletion was not achieved and early repopulation of B cells was dominated by memory B cells and plasma cells. Eight patients that completed 104 weeks of follow up, showed lasting lupus low disease activity state and LN patients all showed a renal response.

Besides NET formation, we studied plasma C4d as a functional measure of circulating immune complexes in Synbiose patients in **chapter 7**. Plasma C4d, representing activation of the classical complement pathway, and especially the ratio C4d over total C4, correlated well with traditional markers for immune complex formation in LN; anti-dsDNA and anti-C1q autoantibodies, as well as with change in proteinuria. This study suggests that C4d measurement could be of value in immune complex-mediated diseases.

Tacrolimus (TAC) has been investigated as induction treatment for LN in RCTs, all performed in Asian patients, as duo therapy with steroids or as triple therapy with mycophenolate. Our meta-analysis in **chapter 9** shows superior efficacy of the TAC-based regimens compared to conventional treatment, mainly determined by studies evaluating triple therapy. We recommend the use of TAC in Asian LN patients and use of TAC can be considered in refractory LN patients and pregnant LN patients.

General Discussion and future perspectives

We have known for a long time that there is a role for neutrophils in the pathogenesis of SLE. In 1949, the clumping of leukocytes was detected in bone marrow preparations upon stimulation with SLE plasma [2]. Further, a granulopoiesis signature in SLE was described by Bennett et al. [3], related to presence of highly granular cells, at different stages of maturation. Also, high numbers of low density granulocytes (LDG) were detected in SLE, correlating with disease activity [4]. Then in 2004, NETs came along. NET release upon stimulation with LPS and cytokines as a defense mechanism was described for the first time by the group of Zychlinsky [5], after which many articles were published on the topic. Scientists soon realized the dangers of NETosis as well. A pathological role for NETs has been described in sepsis, thrombosis, atherosclerosis, cancer and autoimmune diseases.

In SLE, there is a disbalance of NETs caused on the one hand by excessive NET release as shown in this thesis and by others, while on the other hand, clearance of NETs is defective [6]. We and others have shown [7–9] that immune complexes induce NET release in SLE. By measuring ex vivo NET release in response to patient serum, NET-inducing circulating particles are measured, i.e. immune complexes containing nuclear antigens. In the Synbiose cohort, we were able to prospectively measure ex vivo NET formation and we found diminished excessive NET formation after dual B cell therapy, better corresponding with disease activity than anti-dsDNA antibodies. This result indicates that RTX+BLM indirectly affects NET release by decreasing autoreactive B cells and autoantibodies, leading to lower amounts of circulating immune complexes. This novel concept, put forward in this thesis, is illustrated in Figure 1. Important studies are currently performed to further investigate the potential of RTX+BLM in autoimmune diseases, in particular in severe SLE.

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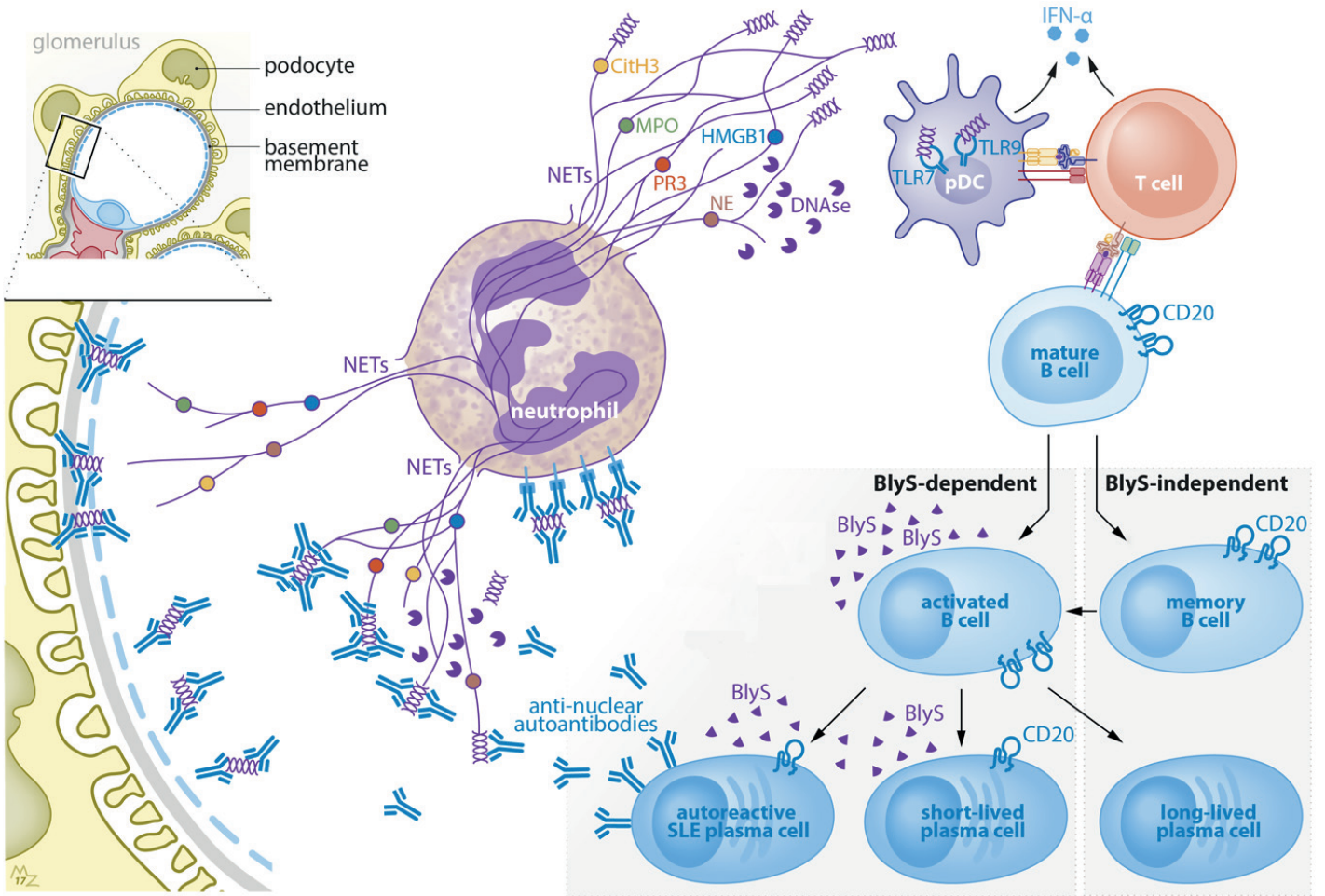
Figure 1. Central role for NETs in SLE.

NETs are released by neutrophils when stimulated with immune complexes. NETs by itself cause damage to endothelium and cells, and are a source of nuclear antigens, leading to immune complex formation and immune complex deposition in the glomerulus, illustrated on the left. Further, NETs activate pDCs via TLR7 receptor, thereby initiating the production of large amounts of IFN alpha. This process leads to the presentation of self-nuclear antigen to T cells and in the end the production of autoantibodies, directed against nuclear components, by autoreactive B cells. NETs; neutrophil extracellular traps, TLR7; toll like receptor 7, pDCs; plasmacytoid dendritic cells, CitH3; citrullinated histon3, MPO; myeloperoxidase, NE; neutrophil elastase, PR3; proteinase3, HMGB1; high mobility group box.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memory B cell
CD20
anti-nuclear autoantibodies
BlyS
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CD20
autoreactive SLE plasma cell
short-lived plasma cell
long-lived plasma cell

Quantification of neutrophil extracellular traps

Generally, NET quantification methods are based on enzyme-linked immunosorbent assay (ELISA), flow cytometry and fluorescence microscopy. With our method, we quantify NETs 'ex vivo' upon stimulation of healthy neutrophils with patient sera, thus reflecting circulating NET-inducing factors. Another functional approach is the measurement of NET degradation by patient sera [6], further discussed below. Assessment of NET degradation could also reflect circulating factors, e.g. 'anti-NET antibodies', that protect NETs from being degraded [10]. Our method was initially set up with the confocal microscope BD Pathway (BD Biosciences, CA, USA). Recently, our protocol was updated due to availability of a new confocal microscope (Image Xpress, Molecular Devices, CA, USA) [11]. Recently, other imaging-based methods have introduced automatic NET quantification by real time imaging. Gupta et al. [12] used a real-time technique, thereby including all neutrophils and not only the NETting neutrophils imaged at time of analysis in 'fixed time' assays. Importantly, this technique makes it possible to study kinetics of NET release, also described by Van der Linden et al. [13]. The latter study further shows the difference between fixed time NET quantification and real time quantification. Both studies show the applicability of this technique for investigating kinetics of NET release.

Methods based on ELISA are relatively fast and straightforward, however, we learned from our own experience as well as from others [14–17], that the presence of NET-associated proteins varies dependent on the used stimulus and in chapter 3 we further showed different morphology and different timing of NET expulsion between AAV-induced and SLE-induced NET formation. These results are important to consider when interpreting methods based on measuring circulating NET-related proteins.

Methods based on flow cytometry techniques are less often implemented for NET quantification. Gavillet et al. developed a flow cytometry-based method with citrullinated histon3 (CitH3) and MPO staining and used it to quantify circulating NETs in whole blood samples from septic patients, showing more circulating NETs in septic patients compared to healthy controls [18]. Sytox green has been used as well in a flow cytometry-based method, developed with PMA-induced NET release but not yet used in patient studies [19]. These protocols are relatively simple and objective as well.

In summary, it appears that different methods have a different applicability because they are largely based on different aspects of NET formation. Our method reflects the amount of NET-inducing circulating factors e.g. autoantigen load in SLE patient serum. Thus, in this case, NET formation is a functional measure for circulating immune complexes. In contrast, measurement of circulating factors such as DNA-MPO complex might reflect circulating NETs in (patient) serum. We now know that presence of NET-associated proteins on NETs vary widely, which is

an essential factor to consider in NET-related research. Real-time imaging is useful for studying kinetics of NET formation. Since our assay reflects autoantigen load in patients, which makes it a functional assay, we used it to study ex vivo NET release as a biomarker to monitor immune complexes and possibly disease activity in autoimmune disease.

Neutrophil extracellular traps as a biomarker

In AAV, four studies [20–23] investigating NET release as a biomarker were performed showing divergent results, presumably due to the use of different methods and clinical outcomes. Circulating mitochondrial DNA [23] and CitH3 positive NETs [20] correlate with Birmingham Vasculitis Activity Score (BVAS), a score for assessing disease activity in AAV, but circulating NET remnants [21], cell-free DNA, MPO-DNA and CitH3 complex did not correlate with BVAS [22]. A distinction between active AAV and remission, however, could be made with measurement of NET remnants [21]. In chapter 4, we demonstrated that the observed excessive NET release is independent of ANCAs (IgG and IgA isotype), complement component 5 (C5) and C5a receptor activation. Recently, it was shown that high levels of serum myeloperoxidase (MPO) are present in patients with active AAV and inhibition of MPO with the drug AZM198 (a 2-thioxanthine-based MPO inhibitor) decreased ex vivo NET release upon stimulation with PR3-ANCA [24]. Whole kidney, glomerular and extra-leucocytic MPO was associated with more crescents in renal biopsy and with clinical disease activity. In vivo, in a nephrotoxic nephritis model, AZM198-treated mice showed reduced glomerular inflammation, less proteinuria and lower creatinine. Thus, (extracellular) MPO might be involved in triggering excessive NET release in AAV and its potency to function as a biomarker in AAV should be studied further.

In SLE, multiple studies investigating NET release as a biomarker were performed as well. NET degradation correlates well with presence of renal disease [6], SLEDAI and low levels of complement proteins C3 and C4 [25]. Reduced NET degradation could be an effect of the presence of DNase inhibitors [6] or the presence of factors that protect NETs from degradation, such as anti-NET antibodies and C1q [25], suggesting that NET degradation could reflect autoantigen load. Further, cell-free DNA measurements were associated with active renal disease, with increased 24-hour proteinuria and with a lower albumin/creatinine ratio [26]. In the study of Cheng et al. [27], serum human neutrophil peptide 1-3 (HNP1-3), which are antimicrobial proteins found in e.g. granules of neutrophils and also present in immune complexes of SLE patients [28], was higher in LN patients (n=40) compared to SLE patients without renal disease (n=40) and controls (healthy controls and IgA nephropathy (IgAN) and minimal change disease (MCD) patients). The group with the highest HNP1-3 levels had higher proteinuria and overall, the HNP levels had a moderate correlation with urinary protein excretion and with the activity index of the renal biopsy (2003 International Society of Nephrology (ISN)/ Renal Pathology Society (RPS) classification [29]), but in this study there was no correlation with SLEDAI or with

autoantibodies. Another study found NETting neutrophils in renal biopsies were associated with a higher activity index [30], further indicating the pathological role of NETs. This is also illustrated by the finding of NETs in lupus skin [31,32]. In a recently published study by our group, van Dam et al. [33] compared humoral immune responses in three cohorts of patients with severe, refractory SLE; patients treated with RTX, proteasome inhibitor bortezomib (BTZ) and our cohort of patients treated with RTX+BLM. After treatment with RTX (n=16), median overall reduction of ex vivo NET formation was 42% compared to baseline, for RTX+BLM (n=15) treatment, this was a reduction of 75%. BTZ treatment did not influence NET release. Interestingly, the largest decrease in autoantibody levels was seen after RTX+BLM therapy as well compared to the other groups.

Overall, based on this thesis and described literature, it seems that amount of NET release in SLE is associated with severity of clinical disease (e.g. correlation with SLEDAI and presence of LN) and autoantibodies. Interestingly, an association with the activity index of LN patients was found as well. Unfortunately, in the studies described in this thesis, we were only able to use a small number of patient samples. A large number of samples from a well-defined patient cohort are necessary to establish whether NET formation can indeed function as a biomarker in SLE and AAV.

New therapeutic options in lupus nephritis

Current guidelines for LN

Currently, for lupus nephritis, mycophenolate mofetil (MMF) and cyclophosphamide (CYC) are treatment of choice for the induction phase. For maintenance, MMF and azathioprine (AZA) are current treatment options [34]. Also, a role for calcineurin inhibitors (CNIs) has been described in the current guidelines, as it might be considered as a second line treatment.

Calcineurin inhibitors

The results of tacrolimus (TAC) in multitarget therapy with MMF and steroids compared to AZA in the maintenance phase of LN treatment were published and showed the relapse rate in the multitarget group was comparable to the relapse rate in the AZA groups during 1.5 years of follow-up [35]. Recently, the results of an international phase 2 study (AURA-LV) were published comparing the new CNI Voclosporin (VCS) in 2 doses combined with standard of care (SOC), MMF and steroids, to placebo in combination with SOC for induction therapy in LN patients [36]. After 24 and 48 weeks, complete renal remission (CR) was significantly higher in patients receiving low dose VCS compared to placebo. Of note, in all study groups, oral steroids were used in low doses and were rapidly tapered. VCS is structurally very similar to cyclosporine but

due to a modification the binding to calcineurin is different which leads to a 4-fold increased potency. Also, the metabolism is different leading to lower metabolite exposure and therefore drug level monitoring is not required. The results of the phase 3 study (AURORA-1) using VCS with standard of care compared to placebo with SOC, were very recently announced. The study demonstrates superiority of VCS over placebo, i.e. renal response at 52 weeks and secondary endpoints were all achieved, making VCS a new option in the therapeutic armamentarium for LN.

Anti-CD20 monoclonal antibodies

Many potential biological targets have been and are being studied in SLE, of which targeting B cells was studied first. RTX is a chimeric type I anti-CD20 monoclonal IgG1 antibody that targets the pan B cell marker CD20 and is the first biological used in SLE. RTX currently is described only as a treatment option in organ-threatening, refractory SLE [34,37].

A drawback of the use of RTX is the development of human anti-chimeric antibodies (HACAs), which occurs in up to 30% of SLE patients [38–40]. Although their clinical relevance is not fully understood, Bayer et al. [41] described a higher rate of serum sickness in SLE patients compared to patients with hematological malignancies receiving RTX. We also observed development of HACAs and serum sickness in the Synbiose study. Currently, this side effect has not been described with use of humanized anti-CD20 antibodies.

Currently, new anti-CD20 monoclonal antibodies are explored in treatment for SLE. Ocrelizumab is a humanized anti-CD20 IgG and its efficacy in combination with SOC was compared to placebo in an RCT with LN patients [42] and ocrelizumab-treated groups showed higher renal response rates, although not statistically significant. Importantly, this study (BELONG) was terminated early due to serious infections in the ocrelizumab-treated patients and currently no further RCTs are performed with ocrelizumab in SLE. Another humanized anti-CD20 monoclonal antibody is Obinutuzumab (Obi). In vitro, this type II antibody showed more B cell toxicity compared to RTX [43]. The most important characteristic of type I compared to type II antibodies is their ability to compartmentalize CD20 in 'lipid rafts', of the plasma membrane. It is thought that the lipid distribution at the cell membrane is important for anti-CD20 antibody efficacy [44]. It potentially leads to complement-dependent cellular cytotoxicity (CDC), which is seen to a lesser extent by type II antibodies. Binding of type II antibodies seems to induce more direct cell death. Other cytotoxic mechanisms of anti-CD20 antibodies are Fc gamma receptor (FcyR)-mediated depletion through antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis. Recently, the results of the NOBILITY study were presented [45], a study comparing SOC+OBI with SOC in LN. Overall renal response at week 52 was significantly higher in the Obi group and no important safety issues were reported. Interestingly, in this study the percentage of B cell depleted patients was compared between the NOBILITY and LUNAR study,

showing more profound B cell depletion in the NOBILITY study. In conclusion, Obi might be an attractive option for LN treatment.

Anti-BAFF monoclonal antibodies

The BLISS studies were performed in non-renal SLE patients, described in the introduction of this thesis, and we are now awaiting the publication of the BLISS-LN study (NCT01639339). 448 LN patients were enrolled in this phase 3 study and received SOC with Belimumab (BLM) or placebo for 104 weeks. The sponsor of this study recently announced that the primary endpoint, a renal response at week 104, was achieved: 43% versus 32% patients treated with BLM and placebo, respectively, achieved a renal response over 104 weeks follow-up [46]. Further, after 104 weeks, BLM-treated patients more often achieved a complete renal response and time to death or renal-related event was better in the BLM arm. Thus, BLM as add-on therapy in LN is clinically effective and will have a place in the future of LN therapy.

BLM is not the only studied anti-BAFF antibody. Atacicept, a recombinant fusion protein, targets both survival factors BAFF and APRIL. APRIL binds TACI and BCMA while BAFF binds strongly to BAFFR and TACI and less so to BCMA [47,48]. Atacicept was studied in LN but this study was terminated after 6 patients were included, of whom 4 received atacicept, due to occurrence of severe infections (pneumonia) in 2 atacicept treated patients, simultaneous with a severe fall in serum IgG (<3 g/l). Use of MMF and steroid treatment and the nephrotic range proteinuria presumably affected the rate of hypogammaglobinemia as well [49]. A phase 2 RCT investigating 2 doses of atacicept with SOC versus placebo with SOC in patients with mild-moderate SLE, LN patients were excluded, showed a significant difference in SLE Responder Index 4 (SRI-4) at week 24 between the low dose atacicept and placebo group, though the primary endpoint was not reached (SRI-4 in both atacicept groups) [50]. However, in the high disease activity (HDA) subgroup, based on high SLEDAI scores, and in the serologically active subgroup, significantly higher SRI-4 response rates with both doses were found. Blisibimod is a BAFF inhibitor composed of a tetrameric BAFF binding domain fused to a human IgG1 Fc region which selectively inhibits soluble BAFF as well as membrane bound BAFF. The phase 3 study evaluated the efficacy and safety in SLE patients with high disease activity but it did not meet its endpoint, though blisibimod was associated with steroid reduction, decreased proteinuria and biomarker responses [51]. Currently, no clinical studies are being performed with either atacicept or blisibimod. The place of these drugs is currently unclear in the future of SLE treatment.

In conclusion, many new treatment options including biologicals targeting molecules involved in LN pathophysiology have been studied lately. Most recently, studies with Voclosporin, Obinutuzumab and Belimumab in LN showed positive results and will therefore impact the future treatment strategies of LN.

Another important step in LN treatment is that steroids are increasingly avoided [52] or lower doses of steroids are being used [36,53]. High-dose steroids are associated with adverse events and long-term damage and the use of less steroids would therefore be a great step. An RCT evaluating this viable treatment option is necessary to further investigate avoiding steroids in LN patients.

Dual B cell targeted therapy

Human data on dual B cell therapy

Several case reports were published on combined RTX and BLM treatment in LN [54–57], including the case report in chapter 5, all showing beneficial clinical effects of dual B cell therapy in refractory LN patients. The Synbiose study, described in this thesis, did not raise safety issues and showed beneficial immunological and clinical effects. The CALIBRATE study [58] which is a phase 2 randomized study that compared CYC+RTX and methylprednisolone with or without BLM in 43 patients with LN, also did not raise any safety concerns. Indeed, CALIBRATE's primary endpoint, was occurrence of serious infectious adverse events at 48 weeks and was not different between treatment groups. A non-significant trend was seen with respect to renal response in 52% and 41% of patients in the BLM and the placebo group, respectively, was observed after 48 weeks. These two studies were the first evaluating dual B cell therapy in patients with severe SLE. Most importantly, both studies showed that the combination therapy is generally well-tolerated. These studies are the first important stepping stones towards larger studies studying clinical efficacy of RTX+BLM for SLE in a randomized setting.

Currently, multiple studies investigating dual B cell therapy in SLE are conducted. First, BLISS-BELIEVE, which is a phase 3, multicenter, randomized controlled trial that involves 3 treatment arms; BLM+placebo, BLM+RTX and BLM+SOC [59]. Patients with an SLEDAI of ≥6 will be included and the goal is to include at least 200 patients. After 52 weeks, disease control will be assessed, based on SLEDAI and concomitant immunosuppressive therapy. Second, Synbiose-2 (NCT03747159) is a follow-up study of the Synbiose study described in this thesis. In this randomized, open label study, patients with severe SLE will start weekly subcutaneous BLM before treatment with RTX, hypothesizing that tissue residing B cells will migrate to the circulation and will be targeted by subsequent RTX treatment as well. The control arm of the study will receive SOC. The primary outcome is the reduction of pathological autoantibodies after 28 weeks. Clinical response will be assessed as well together with further experimental secondary endpoints focused on humoral immune responses. Third, BEAT Lupus is a randomized phase 2 study that will include up to 56 SLE patients and evaluate the effect of RTX+BLM on anti-dsDNA

antibodies after 52 weeks [60]. After treatment with RTX, patients will be randomized to receive BLM or placebo.

These studies are crucial for us to first of all learn more about efficacy of dual B cell treatment in all SLE patients. Larger and more divergent patient groups will be studied mainly in the BLISS-BELIEVE study and BEAT Lupus as well. Further, it is important to learn more about humoral immune responses upon this treatment, to get a better understanding of SLE pathophysiology and to detect possible new biomarkers. Synbiose-2 is designed to evaluate this and, in this study, the humoral immune responses can be compared to patients receiving SOC. These studies will further determine the applicability of dual B cell therapy in SLE.

Dual B cell therapy is investigated in other autoimmune diseases as well, since a rise in BAFF after RTX treatment is also observed in other autoimmune diseases such as AAV [61] and Sjogren's syndrome [62]. In Sjogren's syndrome (SS), a case report was published supporting the beneficial effect of RTX+BLM [63] in a patient with severe SS and currently an RCT (NCT02631538) is performed investigating the clinical efficacy of dual therapy compared to placebo and RTX and BLM monotherapy. Elevated BAFF levels have also been implicated in the pathogenesis of immune thrombocytopenia [65], membranous nephropathy [66] and systemic sclerosis [67]. Studies evaluating safety and efficacy of RTX+BLM are conducted in these patient groups as well (NCT03154385, NCT03949855 and NCT03844061, respectively).

The BREVAS study included 105 AAV patients that received induction therapy with RTX or CYC with steroids and were then randomized to receive azathioprine, steroids and BLM or placebo [64]. The primary endpoint included time to a protocol-specified event; a BVAS ≥6, presence of ≥1 major BVAS item and treatment failure. BLM maintenance treatment did not reduce the risk of relapse. Overall, the number of relapses was low (in 11 versus 10 patients receiving placebo and BLM, respectively), but no relapses were seen in patients that had received RTX for induction and BLM during maintenance in comparison to 3 relapses in patients that had received RTX and placebo afterwards. Obviously, these numbers are very low and a new RCT should be carried out to specifically study the clinical efficacy of RTX+BLM in AAV. Currently, the COMBIVAS study (NCT03967925) is recruiting GPA patients to compare RTX+BLM and RTX+placebo. This phase 2 study will evaluate the effect of dual therapy on biological endpoints such as autoantibodies.

Dual B cell therapy and B cells

In the Synbiose study, we showed repopulation of mainly switched memory B cells and plasma cells after 24 and 104 weeks and transitional B cells as well after 104 weeks while naïve B cells and CD27-IgD-, or double negative (DN) B cells, remained suppressed. Importantly, overall, there was a persistent reduction of CD19+ B cells (a median of 85% reduction compared to

start of study). This early rise in memory B cells after BLM therapy was shown by others as well; the BLISS study showed an early rise of memory B cells after BLM treatment [68] as well as previous BLM studies in SLE [69,70]. In other patients groups this effect was seen as well; such as in the previously mentioned BREVAS study [64], in kidney transplantation recipients treated with BLM [71], in rheumatoid arthritis [72], in SS [73], in myasthenia gravis [74] and in primary membranous nephropathy [75]. This observation led to the hypothesis that belimumab induces the migration of CD20+ B cells into the circulation and thus, by treating with RTX after BLM, more efficient B cell depletion could be established possibly also leading to more efficient depletion of autoreactive B cells. This approach is implemented in Synbiose-2 (NCT03747159), BLISS-BELIEVE [59] and in the RCT investigating RTX+BLM in SS (NCT02631538).

Another interesting finding is the long-term suppression of double negative B cells since they were previously shown to be a major source of autoantibody secreting cells (ASCs) [76] and perhaps could be an interesting biomarker in SLE [77,78]. In the study by Wang et al. [79], B cells were studied in more than 200 SLE patients. They found high amounts of $CD11c^{high}$ B cells that expressed low CD27 and IgD. These cells were sorted and cultured, as well as memory (CD27+) and naïve (CD27-CD11c-) B cells, and high levels of plasma cells derived from $CD11c^{high}$ B cells were found after 7 and 11 days that produced significantly more autoantibodies than plasma cells derived from naïve or memory B cells. Further, $CD11c^{high}$ B cells correlated with SLEDAI and presence of LN. In conclusion, these cells are presumably important in the development of autoreactive B cells and it would be interesting to further study these cells in response to dual B cell therapy, which we were unfortunately unable to do in our study.

In conclusion, times are changing for LN treatment. The development of biologicals is mainly responsible for the changing landscape of LN treatment. Dual B cell targeted therapy with RTX+BLM, presented in this thesis, shows promising effects on humoral immune responses by targeting autoreactive plasma cells and diminishing excessive NET formation. Meanwhile the treatment seems safe, well-tolerated and leads to beneficial clinical effects in patients with severe, refractory SLE. Therefore, the studies in this thesis have led to further studies investigating and developing RTX+BLM treatment in autoimmune diseases. These studies will further identify the effects on humoral immune responses and ultimately will need to prove the clinical efficacy of RTX+BLM.

References

1. J.H.O. Hoffmann, K. Schaekel, M.R. Gaiser, A.H. Enk, E.N. Hadaschik, Interindividual variation of NETosis in healthy donors: Introduction and application of a refined method for extracellular trap quantification, Exp. Dermatol. 25 (2016) 895–900. doi:10.1111/exd.13125.
2. J.R. Haserick, D.W. Bortz, A NEW DIAGNOSTIC TEST FOR ACUTE DISSEMINATED LUPUS ERYTHEMATOSUS*, n.d.
3. L. Bennett, a K. Palucka, E. Arce, V. Cantrell, J. Borvak, J. Banchereau, V. Pascual, Interferon and granulopoiesis signatures in systemic lupus erythematosus blood., J. Exp. Med. 197 (2003) 711–723. doi:10.1084/jem.20021553.
4. E. Hacbarth, A. Kajdacsy-Balla, Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever, Arthritis Rheum. 29 (1986) 1334–1342. doi:10.1002/art.1780291105.
5. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria., Science (80-.). 303 (2004) 1532–1535. doi:10.1126/science.1092385.
6. 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.
7. 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.
8. C. Lood, L.P. Blanco, M.M. Purmalek, C. Carmona-Rivera, S.S. De Ravin, C.K. Smith, H.L. Malech, J.A. Ledbetter, K.B. Elkon, M.J. Kaplan, Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease, Nat. Med. (2016) 1–11. doi:10.1038/nm.4027.
9. 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.
10. J. Leffler, B. Gullstrand, A. Jönsen, J.Å. Nilsson, M. Martin, A.M. Blom, A.A. Bengtsson, Degradation of neutrophil extracellular traps co-varies with disease activity in patients with systemic lupus erythematosus, Arthritis Res. Ther. (2013). doi:10.1186/ar4264.
11. E.J. Arends, L.S. Van Dam, T. Kraaij, S.W.A. Kamerling, T.J. Rabelink, C. Van Kooten, Y.K.O. Teng, A high-throughput assay to assess and quantify neutrophil extracellular trap formation, J. Vis. Exp. 2019 (2019). doi:10.3791/59150.

12. S. Gupta, D.W. Chan, K.J. Zaal, M.J. Kaplan, A High-Throughput Real-Time Imaging Technique To Quantify NETosis and Distinguish Mechanisms of Cell Death in Human Neutrophils, J. Immunol. 200 (2018) 869–879. doi:10.4049/jimmunol.1700905.
13. M. van der Linden, G.H.A. Westerlaken, M. van der Vlist, J. van Montfrans, L. Meyaard, Differential Signalling and Kinetics of Neutrophil Extracellular Trap Release Revealed by Quantitative Live Imaging, Sci. Rep. 7 (2017) 6529. doi:10.1038/s41598-017-06901-w.
14. M.F. Konig, F. Andrade, A critical reappraisal of neutrophil extracellular traps and NETosis mimics based on differential requirements for protein citrullination, Front. Immunol. 7 (2016). doi:10.3389/fimmu.2016.00461.
15. E.F. Kenny, A. Herzig, R. Krüger, A. Muth, S. Mondal, P.R. Thompson, V. Brinkmann, H. von Bernuth, A. Zychlinsky, Diverse stimuli engage different neutrophil extracellular trap pathways, Elife. 6 (2017). doi:10.7554/eLife.24437.
16. C.L. Holmes, D. Shim, J. Kernien, C.J. Johnson, J.E. Nett, M.A. Shelef, Insight into Neutrophil Extracellular Traps through Systematic Evaluation of Citrullination and Peptidylarginine Deiminases, J. Immunol. Res. 2019 (2019). doi:10.1155/2019/2160192.
17. C.M. de Bont, W.J.H. Koopman, W.C. Boelens, G.J.M. Pruijn, Stimulus-dependent chromatin dynamics, citrullination, calcium signalling and ROS production during NET formation, Biochim. Biophys. Acta - Mol. Cell Res. 1865 (2018) 1621–1629. doi:10.1016/j.bbamcr.2018.08.014.
18. M. Gavillet, K. Martinod, R. Renella, C. Harris, N.I. Shapiro, D.D. Wagner, D.A. Williams, Flow cytometric assay for direct quantification of neutrophil extracellular traps in blood samples, Am. J. Hematol. 90 (2015) 1155–1158. doi:10.1002/ajh.24185.
19. S. Masuda, S. Shimizu, J. Matsuo, Y. Nishibata, Y. Kusunoki, F. Hattanda, H. Shida, D. Nakazawa, U. Tomaru, T. Atsumi, A. Ishizu, Measurement of NET formation in vitro and in vivo by flow cytometry, Cytom. Part A. 91 (2017) 822–829. doi:10.1002/cyto.a.23169.
20. D. Nakazawa, H. Shida, U. Tomaru, M. Yoshida, S. Nishio, T. Atsumi, A. Ishizu, Enhanced Formation and Disordered Regulation of NETs in Myeloperoxidase-ANCA-Associated Microscopic Polyangiitis., J. Am. Soc. Nephrol. (2014) 1–8. doi:10.1681/ASN.2013060606.
21. D. Söderberg, T. Kurz, A. Motamedi, T. Hellmark, P. Eriksson, M. Segelmark, Increased levels of neutrophil extracellular trap remnants in the circulation of patients with small vessel vasculitis, but an inverse correlation to anti-neutrophil cytoplasmic antibodies during remission, Rheumatology (Oxford). 54 (2015) 2085–2094. doi:10.1093/rheumatology/kev217.
22. H. Wang, L.L. Sha, T.T. Ma, L.X. Zhang, M. Chen, M.H. Zhao, Circulating level of neutrophil extracellular traps is not a useful biomarker for assessing disease activity in antineutrophil cytoplasmic antibody-associated vasculitis, PLoS One. 11 (2016) 1–10. doi:10.1371/journal.pone.0148197.
23. M.P. Surmiak, M. Hubalewska-Mazgaj, K. Wawrzycka-Adamczyk, W. Szczeklik, J. Musiał, M. Sanak, Circulating mitochondrial DNA in serum of patients with granulomatosis with polyangiitis, Clin. Exp. Immunol. 181 (2015) 150–155. doi:10.1111/cei.12628.

24. M. Antonelou, E. Michaëlsson, R.D.R. Evans, C.J. Wang, S.R. Henderson, L.S.K. Walker, R.J. Unwin, A.D. Salama, Therapeutic myeloperoxidase inhibition attenuates neutrophil activation, anca-mediated endothelial damage, and crescentic GN, J. Am. Soc. Nephrol. 31 (2020) 350–364. doi:10.1681/ASN.2019060618.
25. 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.
26. S. Zhang, X. Lu, X. Shu, X. Tian, H. Yang, W. Yang, Y. Zhang, G. Wang, Elevated Plasma cfDNA May be Associated with Active Lupus Nephritis and Partially Attributed to Abnormal Regulation of Neutrophil Extracellular Traps (NETs) in Patients with Systemic Lupus Erythematosus, Intern. Med. (2014). doi:10.2169/internalmedicine.53.2570.
27. F.J. Cheng, X.J. Zhou, Y.F. Zhao, M.H. Zhao, H. Zhang, Human neutrophil peptide 1-3, a component of the neutrophil extracellular trap, as a potential biomarker of lupus nephritis, Int. J. Rheum. Dis. 18 (2015) 533–540. doi:10.1111/1756-185X.12433.
28. 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). doi:10.1126/scitranslmed.3001180.
29. J.J. Weening, V.D. D'Agati, M.M. Schwartz, S. V. Seshan, C.E. Alpers, G.B. Appel, J.E. Balow, J.A. Bruijn, T. Cook, F. Ferrario, A.B. Fogo, E.M. Ginzler, L. Hebert, G. Hill, P. Hill, J.C. Jennette, N.C. Kong, P. Lesavre, M. Lockshin, L.M. Looi, H. Makino, L.A. Moura, M. Nagata, The Classification of Glomerulonephritis in Systemic Lupus Erythematosus Revisited, J. Am. Soc. Nephrol. 15 (2004) 241–250. doi:10.1097/01.ASN.0000108969.21691.5D.
30. 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.
31. R. Safi, J. Al-Hage, O. Abbas, A.G. Kibbi, D. Nassar, Investigating the presence of neutrophil extracellular traps in cutaneous lesions of different subtypes of lupus erythematosus, Exp. Dermatol. 28 (2019) 1348–1352. doi:10.1111/exd.14040.
32. F. E., C. A., M. A., K. K., A. S., A. I., A. A., G. H., B. G.K., V. P., R. K., B. D.T., E. Frangou, A. Chrysanthopoulou, A. Mitsios, K. Kambas, S. Arelaki, I. Angelidou, A. Arampatzioglou, H. Gakiopoulou, G.K. Bertsias, P. Verginis, K. Ritis, D.T. Boumpas, REDD1/autophagy pathway promotes thromboinflammation and fibrosis in human systemic lupus erythematosus (SLE) through NETs decorated with tissue factor (TF) and interleukin-17A (IL-17A), Ann. Rheum. Dis. 78 (2019) 238–248. doi:10.1136/annrheumdis-2018-213181 LK - http://WT3CF4ET2L.search.

serialssolutions.com?sid=EMBASE&issn=14682060&id=doi:10.1136%2Fannrheumdis-2018-213181&atitle=REDD1%2Fautophagy+pathway+promotes+thromboinflammation+and+fibrosis+in+human+systemic+lupus+erythematosus+%28SLE%29+through+NETs+decorated+with+tissue+factor+%28TF%29+and+interleukin-17A+%28IL-17A%29&stitle=Ann.+Rheum.+Dis.&title=Annals+of+the+Rheumatic+Diseases&volume=78&issue=2&spage=238&epage=248&aulast=Frangou&aufirst=Eleni&auinit=E.&auf.

33. L.S. van Dam, Z. Osmani, S.W.A. Kamerling, T. Kraaij, J.A. Bakker, H.U. Scherer, T.J. Rabelink, R.E. Voll, T. Alexander, D.A. Isenberg, C. van Kooten, Y.K.O. Teng, A reverse translational study on the effect of rituximab, rituximab plus belimumab, or bortezomib on the humoral autoimmune response in SLE, Rheumatology. (2020). doi:10.1093/rheumatology/kez623.
34. A. Fanouriakis, M. Kostopoulou, A. Alunno, M. Aringer, I. Bajema, J.N. Boletis, R. Cervera, A. Doria, C. Gordon, M. Govoni, F. Houssiau, D. Jayne, M. Kouloumas, A. Kuhn, J.L. Larsen, K. Lerstrøm, G. Moroni, M. Mosca, M. Schneider, J.S. Smolen, E. Svenungsson, V. Tesar, A. Tincani, A. Troldborg, R. Van Vollenhoven, J. Wenzel, G. Bertsias, D.T. Boumpas, 2019 Update of the EULAR recommendations for the management of systemic lupus erythematosus, Ann. Rheum. Dis. 78 (2019) 736–745. doi:10.1136/annrheumdis-2019-215089.
35. H. Zhang, Z. Liu, M. Zhou, Z. Liu, J. Chen, C. Xing, H. Lin, Z. Ni, P. Fu, F. Liu, N. Chen, Y. He, J. Liu, C. Zeng, Z. Liu, Multitarget therapy for maintenance treatment of lupus nephritis, J. Am. Soc. Nephrol. 28 (2017) 3671–3678. doi:10.1681/ASN.2017030263.
36. B.H. Rovin, N. Solomons, W.F. Pendergraft, M.A. Dooley, J. Tumlin, J. Romero-Diaz, L. Lysenko, S. V. Navarra, R.B. Huizinga, I. Adzerikho, E. Mikhailova, N. Mitkovskaya, S. Pimanov, N. Soroka, B.I. Bogov, B. Deliyska, V. Ikonomov, E. Tilkiyan, R. Almeida, F. Jimenez, F. Teran, I. Tchokhonelidze, N. Tsiskarishvili, M. Herrera Mendez, N.N. Chavez Perez, A.R. Loaeza, S.R. Gutierrez Urena, J. Romero Diaz, R. Araiza Casillas, M. Madero Rovalo, S. Niemczyk, A. Sokalski, A. Wiecek, M. Klinger, O. V. Bugrova, T.M. Chernykh, T.R. Kameneva, L. V. Lysenko, T.A. Raskina, O. V. ReshEtko, N.N. Vezikova, T. V. Kropotina, A.N. Maksudova, V. Marasaev, V.A. Dobronravov, I. Gordeev, A.M. EssAian, A. Frolov, R. Jelacic, D. Jovanovic, B. Mitic, G. Pekovic, M. Radovic, G. Radunovic, P. Carreira, F. Diaz Gonzalez, X. Fulladosa, E. Ucar, S. De Silva, C. Herath, A. Hewageegana, A.L.M. Nazar, A.W.M. Wazil, I. Dudar, O. Godlevska, S. Korneyeva, V.I. Vasylets, N. Sydor, M. Kolesnyk, S. V. Parikh, N. Olsen, E.M. Ginzler, J.A. Tumlin, A. Saxena, R. Saxena, R.A. Lafayette, W.F. Pendergraft, A.S. Podoll, A.A. Arrey-Mensah, M. Bubb, J. Grossman, A.I. Oporta, A. Nami, M.M. Rahman, S.A. Haq, T.M.D. Chan, M.M.Y. Temy, H.M.P. Gomez, J. Bermas, B.H. Reyes, L.T. Hao, L.C. Roberto, E. Amante, A.E. Lanzon, J.Y. Choe, T.Y. Kang, Y.S. Kim, S.G. Lee, J.S. Lee, J.C.C. Jun, A. Vasudevan, S.F. Luo, T.T. Cheng, B. Satirapoj, K. Noppakun, A randomized, controlled double-blind study comparing the efficacy and safety of dose-ranging voclosporin with placebo in achieving remission in patients with active lupus nephritis, Kidney Int. 95 (2019) 219–231. doi:10.1016/j.kint.2018.08.025.

37. F. Catapano, A.N. Chaudhry, R.B. Jones, K.G.C. Smith, D.W. Jayne, Long-term efficacy and safety of rituximab in refractory and relapsing systemic lupus erythematosus, Nephrol. Dial. Transplant. 25 (2010) 3586–3592. doi:10.1093/ndt/gfq256.
38. K.G.C. Smith, R.B. Jones, S.M. Burns, D.R.W. Jayne, Long-term comparison of rituximab treatment for refractory systemic lupus erythematosus and vasculitis: Remission, relapse, and re-treatment, Arthritis Rheum. 54 (2006) 2970–2982. doi:10.1002/art.22046.
39. D. Albert, J. Dunham, S. Khan, J. Stansberry, S. Kolasinski, D. Tsai, S. Pullman-Mooar, F. Barnack, C. Striebich, R.J. Looney, E.T. Luning Prak, R. Kimberly, Y. Zhang, R. Eisenberg, Variability in the biological response to anti-CD20 B cell depletion in systemic lupus erythaematosus, Ann. Rheum. Dis. 67 (2008) 1724–1731. doi:10.1136/ard.2007.083162.
40. R.J. Looney, J.H. Anolik, D. Campbell, R.E. Felgar, F. Young, L.J. Arend, J.A. Sloand, J. Rosenblatt, I. Sanz, B cell depletion as a novel treatment for systemic lupus erythematosus: A phase I/II dose-escalation trial of rituximab, Arthritis Rheum. 50 (2004) 2580–2589. doi:10.1002/art.20430.
41. G. Bayer, M.S. Agier, B. Lioger, M. Lepelley, M. Zenut, M.C. Lanoue, F. Maillot, A.P. Jonville-Bera, Rituximab-induced serum sickness is more frequent in autoimmune diseases as compared to hematological malignancies: A French nationwide study, Eur. J. Intern. Med. 67 (2019) 59–64. doi:10.1016/j.ejim.2019.06.009.
42. E.F. Mysler, A.J. Spindler, R. Guzman, M. Bijl, D. Jayne, R.A. Furie, F.A. Houssiau, J. Drappa, D. Close, R. Maciuca, K. Rao, S. Shahdad, P. Brunetta, Efficacy and Safety of Ocrelizumab in Active Proliferative Lupus Nephritis: Results From a Randomized, Double-Blind, Phase III Study, Arthritis Rheum. 65 (2013) 2368–2379. doi:10.1002/art.38037.
43. V. Reddy, C. Klein, D.A. Isenberg, M.J. Glennie, G. Cambridge, M.S. Cragg, M.J. Leandro, Obinutuzumab induces superior B-cell cytotoxicity to rituximab in rheumatoid arthritis and systemic lupus erythematosus patient samples., Rheumatology (Oxford). 56 (2017) 1227–1237. doi:10.1093/rheumatology/kex067.
44. M.J.E. Marshall, R.J. Stopforth, M.S. Cragg, Therapeutic antibodies: What have we learnt from targeting CD20 and where are we going?, Front. Immunol. 8 (2017) 1245. doi:10.3389/fimmu.2017.01245.
45. R. Furie, G. Aroca, A. Alvarez, H. Fragoso-Loyo, E.Z. Santillan, B. Rovin, T. Schindler, I. Hassan, M. Cascino, J. Garg, A. Malvar, A Phase II Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Efficacy and Safety of Obinutuzumab or Placebo in Combination with Mycophenolate Mofetil in Patients with Active Class III or IV Lupus Nephritis [abstract], Arthritis Rheumatol. 71 (2019). https://acrabstracts.org/abstract/a-phase-ii-randomized-double-blind-placebo-controlled-study-to-evaluate-the-efficacy-and-safety-of-obinutuzumab-or-placebo-in-combination-with-mycophenolate-mofetil-in-patients-with-active-class-iii/ (accessed January 26, 2020).
46. GSK announces positive headline results in phase 3 study of Benlysta in patients with lupus nephritis | GSK, (n.d.). https://www.gsk.com/en-gb/media/press-releases/gsk-announces-positive-headline-results-in-phase-3-study-of-benlysta-in-patients-with-lupus-nephritis/ (accessed April 2, 2020).

47. W. Hoffman, F.G. Lakkis, G. Chalasani, B cells, antibodies, and more, Clin. J. Am. Soc. Nephrol. (2016). doi:10.2215/CJN.09430915.
48. W. Stohl, D.M. Hilbert, The discovery and development of belimumab: The anti-BLyS-lupus connection, Nat. Biotechnol. 30 (2012) 69–77. doi:10.1038/nbt.2076.
49. E.M. Ginzler, S. Wax, A. Rajeswaran, S. Copt, J. Hillson, E. Ramos, N.G. Singer, Atacicept in combination with MMF and corticosteroids in lupus nephritis: results of a prematurely terminated trial, Arthritis Res. Ther. 14 (2012) R33. doi:10.1186/ar3738.
50. J.T. Merrill, D.J. Wallace, S. Wax, A. Kao, P.A. Fraser, P. Chang, D. Isenberg, Efficacy and Safety of Atacicept in Patients With Systemic Lupus Erythematosus, Arthritis Rheumatol. 70 (2018) 266–276. doi:10.1002/art.40360.
51. J.T. Merrill, W.R. Shanahan, M. Scheinberg, K.C. Kalunian, D. Wofsy, R.S. Martin, Phase III trial results with blisibimod, a selective inhibitor of B-cell activating factor, in subjects with systemic lupus erythematosus (SLE): Results from a randomised, double-blind, placebo-controlled trial, Ann. Rheum. Dis. 77 (2018) 883–889. doi:10.1136/annrheumdis-2018-213032.
52. M.B. Condon, D. Ashby, R.J. Pepper, H.T. Cook, J.B. Levy, M. Griffith, T.D. Cairns, L. Lightstone, Prospective observational single-centre cohort study to evaluate the effectiveness of treating lupus nephritis with rituximab and mycophenolate mofetil but no oral steroids, Ann. Rheum. Dis. (2013). doi:10.1136/annrheumdis-2012-202844.
53. S. Oon, M. Huq, T. Godfrey, M. Nikpour, Systematic review, and meta-analysis of steroid-sparing effect, of biologic agents in randomized, placebo-controlled phase 3 trials for systemic lupus erythematosus, Semin. Arthritis Rheum. 48 (2018) 221–239. doi:10.1016/j.semarthrit.2018.01.001.
54. 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).
55. 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.
56. 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.
57. R. Gualtierotti, M.O. Borghi, M. Gerosa, T. Schioppo, P. Larghi, J. Geginat, P.L. Meroni, Successful sequential therapy with rituximab and belimumab in patients with active systemic lupus erythematosus: a case series, Clin. Exp. Rheumatol. 36 (2018) 643–647.
58. M. Dall'Era', C. Aranow, M. Byron, L. Ding, D. Smilek, B. Diamond, D. Wofsy, Phase 2 Trial of Induction Therapy with Anti-CD20 (Rituximab) Followed By Maintenance Therapy with Anti-BAFF (Belimumab) in Patients with Active Lupus Nephritis [abstract]., Arthritis Rheumatol. 70 (2018) https://acrabstracts.org/abstract/phase-2-trial-of.

59. Y.K. Onno Teng, I.N. Bruce, B. Diamond, R.A. Furie, R.F. Van Vollenhoven, D. Gordon, J. Groark, R.B. Henderson, M. Oldham, P.P. Tak, Phase III, multicentre, randomised, double-blind, placebo-controlled, 104-week study of subcutaneous belimumab administered in combination with rituximab in adults with systemic lupus erythematosus (SLE): BLISS-BELIEVE study protocol, BMJ Open. 9 (2019) e025687. doi:10.1136/bmjopen-2018-025687.
60. A. Jones, P. Muller, C.J. Dore, F. Ikeji, E. Caverly, K. Chowdhury, D.A. Isenberg, C. Gordon, M.R. Ehrenstein, Belimumab after B cell depletion therapy in patients with systemic lupus erythematosus (BEAT Lupus) protocol: A prospective multicentre, double-blind, randomised, placebo-controlled, 52-week phase II clinical trial, BMJ Open. 9 (2019). doi:10.1136/bmjopen-2019-032569.
61. H.T. Maecker, J.P. McCoy, R. Nussenblatt, Standardizing immunophenotyping for the Human Immunology Project, Nat. Rev. Immunol. (2012). doi:10.1038/nri3158.
62. R.P.E. Pollard, W.H. Abdulahad, A. Vissink, N. Hamza, J.G.M. Burgerhof, J.M. Meijer, A. Visser, M.G. Huitema, F.K.L. Spijkervet, C.G.M. Kallenberg, H. Bootsma, F.G.M. Kroese, Serum levels of BAFF, but not APRIL, are increased after rituximab treatment in patients with primary Sjögren's syndrome: data from a placebo-controlled clinical trial, Ann. Rheum. Dis. 72 (2013) 146–148. doi:10.1136/annrheumdis-2012-202071.
63. 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].
64. D. Jayne, D. Blockmans, R. Luqmani, S. Moiseev, B. Ji, Y. Green, L. Hall, D. Roth, R.B. Henderson, P.A. Merkel, J.A. Lozano, H. Becker, A.C. Quiroz, S. Carette, S. Carrillo-Vazquez, M.C. Cid, D. D'Cruz, A. Deodhar, O. Flossman, G. Garibotto, L. Gesualdo, S. Hall, T. Hauser, B. Hellmich, D. Kidder, M. Kimmel, M. Little, M. Majdan, K. Maksimowicz-McKinnon, G. Marder, G. Matsievskaia, A.S. Meneses, E. Molloy, R. Mueller, C. Neuwelt, J.R. Hernandez, U. Specks, V. Tesar, M. Walsh, Efficacy and Safety of Belimumab and Azathioprine for Maintenance of Remission in Antineutrophil Cytoplasmic Antibody–Associated Vasculitis: A Randomized Controlled Study, Arthritis Rheumatol. 71 (2019) 952–963. doi:10.1002/art.40802.
65. X.J. Zhu, Y. Shi, J. Peng, C.S. Guo, N.N. Shan, P. Qin, X. Bin Ji, M. Hou, The effects of BAFF and BAFF-R-Fc fusion protein in immune thrombocytopenia, Blood. 114 (2009) 5362–5367. doi:10.1182/blood-2009-05-217513.
66. S.S. Han, S.H. Yang, H.A. Jo, Y.J. Oh, M. Park, J.Y. Kim, H. Lee, J.P. Lee, S.H. Lee, K.W. Joo, C.S. Lim, Y.S. Kim, D.K. Kim, BAFF and APRIL expression as an autoimmune signature of membranous nephropathy, Oncotarget. 9 (2018) 3292–3302. doi:10.18632/oncotarget.23232.
67. T. Matsushita, M. Hasegawa, K. Yanaba, M. Kodera, K. Takehara, S. Sato, Elevated serum BAFF levels in patients with systemic sclerosis: Enhanced BAFF signaling in systemic sclerosis B lymphocytes, Arthritis Rheum. 54 (2006) 192–201. doi:10.1002/art.21526.

68. W. Stohl, F. Hiepe, K.M. Latinis, M. Thomas, M.A. Scheinberg, A. Clarke, C. Aranow, F.R. Wellborne, C. Abud-Mendoza, D.R. Hough, L. Pineda, T.S. Migone, Z.J. Zhong, W.W. Freimuth, W.W. Chatham, Belimumab reduces autoantibodies, normalizes low complement levels, and reduces select B cell populations in patients with systemic lupus erythematosus, Arthritis Rheum. 64 (2012) 2328–2337. doi:10.1002/art.34400.

69. 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.

70. A.M. Jacobi, W. Huang, T. Wang, W. Freimuth, I. Sanz, R. Furie, M. Mackay, C. Aranow, B. Diamond, A. Davidson, Effect of long-term belimumab treatment on B cells in systemic lupus erythematosus: Extension of a phase II, double-blind, placebo-controlled, dose-ranging study, Arthritis Rheum. 62 (2010) 201–210. doi:10.1002/art.27189.

71. G.D. Banham, S.M. Flint, N. Torpey, P.A. Lyons, D.N. Shanahan, A. Gibson, C.J.E. Watson, A.-M. O'Sullivan, J.A. Chadwick, K.E. Foster, R.B. Jones, L.R. Devey, A. Richards, L.-P. Erwig, C.O. Savage, K.G.C. Smith, R.B. Henderson, M.R. Clatworthy, Belimumab in kidney transplantation: an experimental medicine, randomised, placebo-controlled phase 2 trial., Lancet (London, England). 391 (2018) 2619–2630. doi:10.1016/S0140-6736(18)30984-X.

72. W. Stohl, J.T. Merrill, J.D. McKay, J.R. Lisse, Z.J. Zhong, W.W. Freimuth, M.C. Genovese, Efficacy and safety of belimumab in patients with rheumatoid arthritis: A phase II, randomized, double-blind, placebo-controlled, dose-ranging study, J. Rheumatol. 40 (2013) 579–589. doi:10.3899/jrheum.120886.

73. R. Seror, G. Nocturne, T. Lazure, H. Hendel-Chavez, F. Desmoulins, R. Belkhir, P. Ravaud, M. Benbijja, V. Poirier-Colame, Y. Taoufik, X. Mariette, Low numbers of blood and salivary natural killer cells are associated with a better response to belimumab in primary Sjögren's syndrome: Results of the BELISS study, Arthritis Res. Ther. 17 (2015). doi:10.1186/s13075-015-0750-y.

74. K. Hewett, D.B. Sanders, R.A. Grove, C.L. Broderick, T.J. Rudo, A. Bassiri, M. Zvartau-Hind, V. Bril, Randomized study of adjunctive belimumab in participants with generalized myasthenia gravis, Neurology. 90 (2018) E1425–E1434. doi:10.1212/WNL.0000000000005323.

75. C. Barrett, L.C. Willcocks, R.B. Jones, R.M. Tarzi, R.B. Henderson, G. Cai, S.I. Gisbert, A.S. Belson, C.O. Savage, Effect of belimumab on proteinuria and anti-phospholipase A2 receptor autoantibody in primary membranous nephropathy, Nephrol. Dial. Transplant. (2019). doi:10.1093/ndt/gfz086.

76. C. Tipton, C. Fucile, J. Darce, A. Chida, … T.I.-N., undefined 2015, Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus, Nature.Com. (n.d.).

77. C. Wei, J. Anolik, A. Cappione, B. Zheng, A. Pugh-Bernard, J. Brooks, E.-H. Lee, E.C.B. Milner, I. Sanz, A New Population of Cells Lacking Expression of CD27 Represents a Notable Component of

the B Cell Memory Compartment in Systemic Lupus Erythematosus, J. Immunol. 178 (2007) 6624–6633. doi:10.4049/jimmunol.178.10.6624.

78. C.M. Tipton, J.R. Hom, C.F. Fucile, A.F. Rosenberg, I. Sanz, Understanding B-cell activation and autoantibody repertoire selection in systemic lupus erythematosus: A B-cell immunomics approach, Immunol. Rev. 284 (2018) 120–131. doi:10.1111/imr.12660.

79. S. Wang, J. Wang, V. Kumar, J.L. Karnell, B. Naiman, P.S. Gross, S. Rahman, K. Zerrouki, R. Hanna, C. Morehouse, N. Holoweckyj, H. Liu, K. Casey, M. Smith, M. Parker, N. White, J. Riggs, B. Ward, G. Bhat, B. Rajan, R. Grady, C. Groves, Z. Manna, R. Goldbach-Mansky, S. Hasni, R. Siegel, M. Sanjuan, K. Streicher, M.P. Cancro, R. Kolbeck, R. Ettinger, IL-21 drives expansion and plasma cell differentiation of autoreactive CD11chiT-bet+ B cells in SLE, Nat. Commun. 9 (2018). doi:10.1038/s41467-018-03750-7.

Chapter 11

Samenvatting
List of publications
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Samenvatting

Systemische lupus erythematosus (SLE) is een auto-immuunziekte waarbij er autoantilichamen worden gevormd tegen nucleaire componenten zoals DNA, wat leidt tot inflammatie van verschillende organen zoals de huid, nieren, longen, hart, gewrichten en de hersenen. SLE komt vooral voor bij jonge vrouwen tussen 20-40 jaar. De geschatte incidentie in Noord-Amerika is 23.2 per 100.000 persoon-jaren. Lupus nefritis (LN) wordt bij 29-82% van de SLE patiënten gezien en is nog altijd lastig te behandelen. Een jaar na LN behandeling is de complete renale respons 10 tot 40% en 10% van de LN patiënten krijgt eindstadium nierfalen. Volgens de huidige richtlijn bestaat inductiebehandeling uit corticosteroïden met mycofenolaat mofetil (MMF) of cyclofosfamide en onderhoudsbehandeling uit azathioprine of MMF. Nieuwe behandelingen voor LN zijn echter noodzakelijk gezien 50% van LN patiënten binnen 10 jaar na de eerste inductiebehandeling een LN flare doormaakt. Momenteel worden er voor deze refractaire patiënten geen duidelijke opties genoemd in de huidige richtlijnen. Het is van essentieel belang om allereerst meer kennis over pathofysiologische mechanismen die een rol spelen in SLE op te doen om nieuwe behandelopties te kunnen ontwikkelen.

Centraal in de pathofysiologie van SLE staat het concept van autovaccinatie met nucleaire antigenen zoals DNA waardoor autoantilichamen worden gevormd (bijvoorbeeld anti-dsDNA) en vervolgens immuuncomplexen die kunnen neerslaan in de nieren en daarmee glomerulonefritis veroorzaken. Extracellulair DNA is een zeer potente stimulus voor het immuunsysteem. Er zijn verschillende defecten beschreven die leiden tot verhoogde hoeveelheden extracellulair DNA in SLE zoals C1q deficiëntie (C1q bindt apoptotische cellen wat leidt tot het opruimen hiervan), verminderde fagocytose door macrofagen en defecten in de vroege fase van apoptose, maar ook versnelde celdood speelt een rol.

Een nieuwe vorm van celdood werd beschreven in 2004 door Zychlinsky et al., genaamd NETosis, waarbij neutrofielen doodgaan na het uitscheiden van “neutrophil extracellular traps” (NETs). Het uitscheiden van NETs is een nieuw verdedigingsmechanisme van neutrofielen. NETs zijn strengen van extracellulair DNA met antimicrobiële eiwitten waarmee pathogenen worden gevangen (als in een net) en geëlimineerd. Echter, NETs spelen ook een rol in de pathofysiologie van auto-immuunziekten. In vitro onderzoek heeft aangetoond dat het opruimen van NETs verminderd is bij SLE patiënten en verder dat neutrofielen van SLE patiënten meer NETs uitscheiden. Ook kunnen NETs leiden tot interferon alfa productie via de stimulatie van plasmacytoïde dendritische cellen. Daarnaast kunnen NETs het complementsysteem activeren. Concluderend lijken NETs ook te kunnen fungeren als bron van extracellulaire nucleaire antigenen en zo bij te dragen aan het verlies van tolerantie van die autoantigenen en de vorming van autoantilichamen.

Ook in de pathofysiologie van antineutrofiele cytoplasmatische antistoffen (ANCA) vasculitis spelen NETs een rol. Zo is in een in vivo studie aangetoond dat injectie van dendritische cellen 'geladen' met NETs in muizen leidt tot de productie van ANCAs en ook werden NETs aangetoond in nierbiopten van ANCA-vasculitispatiënten.

Er zijn reeds klinische studies uitgevoerd gericht op beïnvloeden van productie van pathologische autoantilichamen in SLE, zoals met het anti-CD20 monoklonale antilichaam rituxumab (RTX). Twee grote klinische studies, een met non-renale SLE en een met LN patiënten, lieten beide geen klinische effectiviteit zien t.o.v. placebo. Dit zou onder andere te maken kunnen hebben met productie van B-cel activerende factor (BAFF of Blys) door B-cel depletie na RTX behandeling. BAFF is een cytokine dat belangrijk is voor B-cel overleving. Belimumab (BLM) is een anti-BAFF monoklonaal antilichaam dat is goedgekeurd als add-on behandeling bij serologisch actieve SLE. Daarnaast lijkt de combinatiebehandeling van RTX en BLM in meerdere muismodellen een betere klinische respons te geven.

In het eerste deel van dit proefschrift worden verschillende aspecten van NETs in auto-immuunziekten beschreven. Met een nieuwe methode, in het LUMC ontwikkeld, worden karakteristieken van NETs in SLE en ANCA-vasculitis (AAV) beschreven. Verder wordt onderzocht of NETs als biomarker in SLE en AAV kunnen fungeren. In het tweede deel van het proefschrift worden nieuwe behandelingen voor patiënten met ernstige, refractaire SLE onderzocht. In dit deel wordt de nieuwe behandelstrategie beschreven waarbij RTX en BLM worden gecombineerd voor behandeling van patiënten met ernstige refractaire SLE, waarmee potentieel de productie van pathologische antilichamen kan worden beïnvloed. Met name de effecten op de humorale immuunrespons worden weergegeven.

In **hoofdstuk 2** wordt de nieuwe NET kwantificatiemethode gepresenteerd. De hoeveelheid NETs die middels geautomatiseerde microscopie wordt gemeten, weerspiegelt de hoeveelheid NET-inducerende factoren in de gebruikte stimulus, zoals SLE serum. Deze methode werd initieel opgezet om de 'low level NET release' te meten die werd gezien door stimulatie van neutrofielen met humane immuuncomplexen. Door gebruik te maken van geautomatiseerde confocaal microscopie met meerdere z-stacks, is het een sensitieve en objectieve methode. Deze nieuwe methode zou bijvoorbeeld kunnen dienen in het kader van autoantigeen monitoring in auto-immuunziekten waarin NETs een rol spelen.
In **hoofdstuk 3 en 4** maken we gebruik van de nieuwe methode voor NET kwantificatie om NET uitscheiding te onderzoeken in SLE en AAV. Hier wordt laten zien dat niet alle NETs er hetzelfde uit zien; SLE- en AAV-geïnduceerde NETs bevatten verschillende eiwitten en hebben verschillende morfologische kenmerken. Ook is de timing van NET uitscheiding is verschillend en er zijn mechanistische verschillen. Kortom, er zijn belangrijke kwalitatieve verschillen tussen SLE-

en AAV-geïnduceerde NETs, die niet gedetecteerd kunnen worden door de hoeveelheid NETs te kwantificeren middels NET-gerelateerde eiwit metingen met enzyme-linked immunosorbent assay (ELISA) in neutrofiel supernatant of plasma. Deze studies impliceren dat NETs in beide auto-immuunziekten een andere rol spelen en geven een beter begrip van de pathofysiologische rol van NETs. De hoeveelheid NET uitscheiding wordt tevens gecorreleerd aan ziekteactiviteit scores. Er wordt een gematigde correlatie gezien van de hoeveelheid AAV-geïnduceerde NETs met de Birmingham Vasculitis Activity Score (BVAS) en is er significant meer NET uitscheiding in patiënten met actieve ziekte vergeleken met patiënten die in remissie zijn. Verder is het opvallend dat NET uitscheiding in AAV onafhankelijk gebeurt van ANCAs (IgG en IgA isotype), complement component 5 (C5) en C5a receptor activatie.

Het volgende onderdeel gaat over nieuwe behandelingen voor LN. In **hoofdstuk 5** laten we data zien van twee patiënten met refractaire LN die werden behandeld met BLM na RTX. Bij beide patiënten leidt de behandeling tot gunstige klinische en immunologische effecten, wat deze combinatietherapie een interessante behandeling maakt voor SLE. De combinatiebehandeling wordt verder onderzocht in de Synbiose (Synergetic B cell immunomodulation in SLE) studie.

In **hoofdstuk 6 en 8** worden de resultaten laten zien van de Synbiose studie, een fase 2 "proof-of-concept" studie waarin 15 patiënten met ernstige refractaire SLE worden behandeld met RTX en BLM. De behandeling leidt tot reductie van autoantilichamen, waardoor indirect NET uitscheiding verminderd wordt, vermoedelijk via de reductie van autoreactieve B-cellen. Ook wordt een klinisch effect gezien en kan verdere immunosuppressiva worden afgebouwd. Het primaire eindpunt is gedefinieerd na 24 weken, waarna 7 patiënten stoppen; 2 vanwege een zwangerschapswens en 5 patiënten laten geen respons zien waarvan bij 2 patiënten sprake is van een flare. Acht patiënten voltooien de follow-up van 104 weken in totaal; bij alle 8 patiënten wordt aanhoudende LLDAS (lupus low disease activity state) gezien en de LN patiënten hebben een langdurige renale respons.

Naast de uitscheiding van NETs, hebben we in **hoofdstuk 7** gekeken naar complement component C4d als maat voor circulerende immuuncomplexen in de Synbiose patiënten. C4d is een stabiel product dat wordt gevormd bij complementactivatie via de klassieke route. We laten zien dat C4d en in het bijzonder de ratio C4d:C4 goed correleert met de traditionele markers van aanwezig van immuuncomplexen zoals anti-dsDNA, anti-C1q antistoffen en verandering in proteïnurie. Concluderend zou C4d waardevol kunnen zijn in de monitoring van immuuncomplex-gemedieerde ziekten.

Tacrolimus (TAC) is reeds onderzocht in RCTs als potentiële inductietherapie in LN. Deze studies zijn met name uitgevoerd bij Aziatische SLE patiënten, als duo therapie met corticosteroïden of

als triple therapie met MMF. In **hoofdstuk 9** hebben we de verschillende regimes onderzocht en een meta-analyse uitgevoerd die superieure effectiviteit van TAC-gebaseerde regimes laat zien vergeleken met conventionele behandeling. Dit effect wordt vooral bepaald door de studies die triple therapie toepassen. Op basis van deze meta-analyse kan TAC worden gebruikt bij Aziatische LN patiënten en kan TAC overwogen worden bij refractaire LN patiënten of LN patiënten die zwanger zijn of een zwangerschapswens hebben.

Al met al zijn de tijden aan het veranderen voor de behandeling van LN. Dit komt met name door de ontwikkeling van biologicals. De duale B-cel therapie met RTX en BLM die in dit proefschrift wordt gepresenteerd, laat veelbelovende effecten zien op de humorale immuunrespons via reductie van autoreactieve B-cellen en NET uitscheiding. Tegelijkertijd lijkt de behandeling veilig en wordt deze over het algemeen goed verdragen door patiënten en leidt het tot significante klinische effecten bij patiënten met ernstige, refractaire SLE. De studies beschreven in dit proefschrift hebben dan ook geleid tot verdere ontwikkeling van klinische studies waarin deze combinatietherapie verder onderzocht zal worden in verschillende auto-immuunziekten. Deze studies zullen verdere immunologische effecten moeten identificeren en uiteindelijk zullen grotere klinische studies de klinische effectiviteit van RTX+BLM moeten aantonen.

List of publications

T. Kraaij, E.J. Arends, L.S. van Dam, S.W.A. Kamerling, P.L.A. van Daele, O.W. Bredewold, A. Ray, J.A. Bakker, H.U. Scherer, T.J.W. Huizinga, T.J. Rabelink, C. van Kooten, Y.K.O. Teng, Long-term effects of combined B-cell immunomodulation with rituximab and belimumab in severe, refractory systemic lupus erythematosus: 2-year results, Nephrol. Dial. Transplant. (2020). doi:10.1093/ndt/gfaa117.

L.S. van Dam, Z. Osmani, S.W.A. Kamerling, **T. Kraaij**, J.A. Bakker, H.U. Scherer, T.J. Rabelink, R.E. Voll, T. Alexander, D.A. Isenberg, C. van Kooten, Y.K.O. Teng, A reverse translational study on the effect of rituximab, rituximab plus belimumab, or bortezomib on the humoral autoimmune response in SLE, Rheumatology. (2020). doi:10.1093/rheumatology/kez623.

E.J. Arends, L.S. Van Dam, **T. Kraaij**, S.W.A. Kamerling, T.J. Rabelink, C. Van Kooten, Y.K.O. Teng, A high-throughput assay to assess and quantify neutrophil extracellular trap formation, J. Vis. Exp. 2019 (2019). doi:10.3791/59150.

L.S. van Dam, **T. Kraaij**, S.W.A. Kamerling, J.A. Bakker, U.H. Scherer, T.J. Rabelink, C. van Kooten, Y.K.K.O. Teng, Intrinsically Distinct Role of Neutrophil Extracellular Trap Formation in Antineutrophil Cytoplasmic Antibody–Associated Vasculitis Compared to Systemic Lupus Erythematosus, Arthritis Rheumatol. 71 (2019) 2047–2058. doi:10.1002/art.41047.

T. Kraaij, S.C. Nilsson, C. Van Kooten, M. Okrój, A.M. Blom, Y.O. Teng, Measuring plasma C4D to monitor immune complexes in lupus nephritis, Lupus Sci. Med. 6 (2019). doi:10.1136/lupus-2019-000326.

T. Kraaij, S.W.A. Kamerling, L.S. van Dam, J.A. Bakker, I.M. Bajema, T. Page, F. Brunini, C.D. Pusey, R.E.M. Toes, H.U. Scherer, T.J. Rabelink, C. van Kooten, Y.K.O. Teng, Excessive neutrophil extracellular trap formation in ANCA-associated vasculitis is independent of ANCA, Kidney Int. 94 (2018). doi:10.1016/j.kint.2018.01.013.

T. Kraaij, S.W.A. Kamerling, E.N.M. de Rooij, P.L.A. van Daele, O.W. Bredewold, J.A. Bakker, I.M. Bajema, H.U. Scherer, R.E.M. Toes, T.J.W. Huizinga, T.J. Rabelink, C. van Kooten, Y.K.O. Teng, The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus, J. Autoimmun. (2018). doi:10.1016/j.jaut.2018.03.003.

E.C. Rijnink, Y.K.O. Teng, **T. Kraaij**, O.M. Dekkers, J.A. Bruijn, I.M. Bajema, Validation of the Systemic Lupus International Collaborating Clinics classification criteria in a cohort of patients with full house glomerular deposits, Kidney Int. 93 (2018). doi:10.1016/j.kint.2017.07.017.

E.C. Rijnink, Y.K.O. Teng**, T. Kraaij**, R. Wolterbeek, J.A. Bruijn, I.M. Bajema, Idiopathic non-lupus full-house nephropathy is associated with poor renal outcome, Nephrol. Dial. Transplant. 32 (2017). doi:10.1093/ndt/gfx020.

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. 15 (2016). doi:10.1016/j.autrev.2016.02.018.

T. Kraaij, O.W. Bredewold, S. Trompet, T.W.J. Huizinga, T.J. Rabelink, A.J.M. de Craen, Y.K.O. Teng, TAC-TIC use of tacrolimus-based regimens in lupus nephritis, Lupus Sci. Med. 3 (2016) e000169. doi:10.1136/lupus-2016-000169.

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.

Curriculum vitae

Tineke Kraaij was born on the 14th of June 1988, 5 minutes after her beloved twin sister Marjan Kraaij, in Drachten, Friesland. She started medicine at the Rijksuniversiteit Groningen in 2007. During her studies she was involved in the transplantation summer school for many years, a time she has fond memories of. This gave her the opportunity to do a scientific internship in the lab of Prof. Benito Yard in Mannheim, Germany, co-supervised by Prof. Willem van Son, where she worked on a project involving ischemic reperfusion injury. After this period she started her clinical rotations in the University Medical Center Groningen (UMCG) and later in the Medical Center Leeuwarden. Then she decided to spend some time in Nanatha, Mozambique, her favorite country, where she learned about global health. Late 2013 she came back to the UMCG as an intern in the nephrology department where she further discovered her love for nephrology and immunology. She obtained her medical degree in 2013. She left Groningen and the UMCG with bittersweet feelings, to move to Amsterdam and work as a PhD candidate in the nephrology department in the Leiden University Medical Center (LUMC), where Dr. Onno Teng and Prof. Cees van Kooten gave her the opportunity to combine clinical research with lab work. Her activities within the Platform AIOS nephrology (PLAN) and Leiden Oxford Transplantation Summer school (LOTS) made her time as a PhD candidate extra special. In 2017, she was accepted for internal medicine residency in the LUMC (supervisor: Prof. Hans de Fijter) which she started in May 2018 in the Haaglanden Medical Center (supervisors: Dr. Aart Bootsma, Dr. Marielle de Vreede, Dr. Yvo Sijpkens). In the future she hopes to become a successful nephrologist and continue to work in the field of nephrology-immunology.

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