The NET effect of novel treatments in lupus nephritis

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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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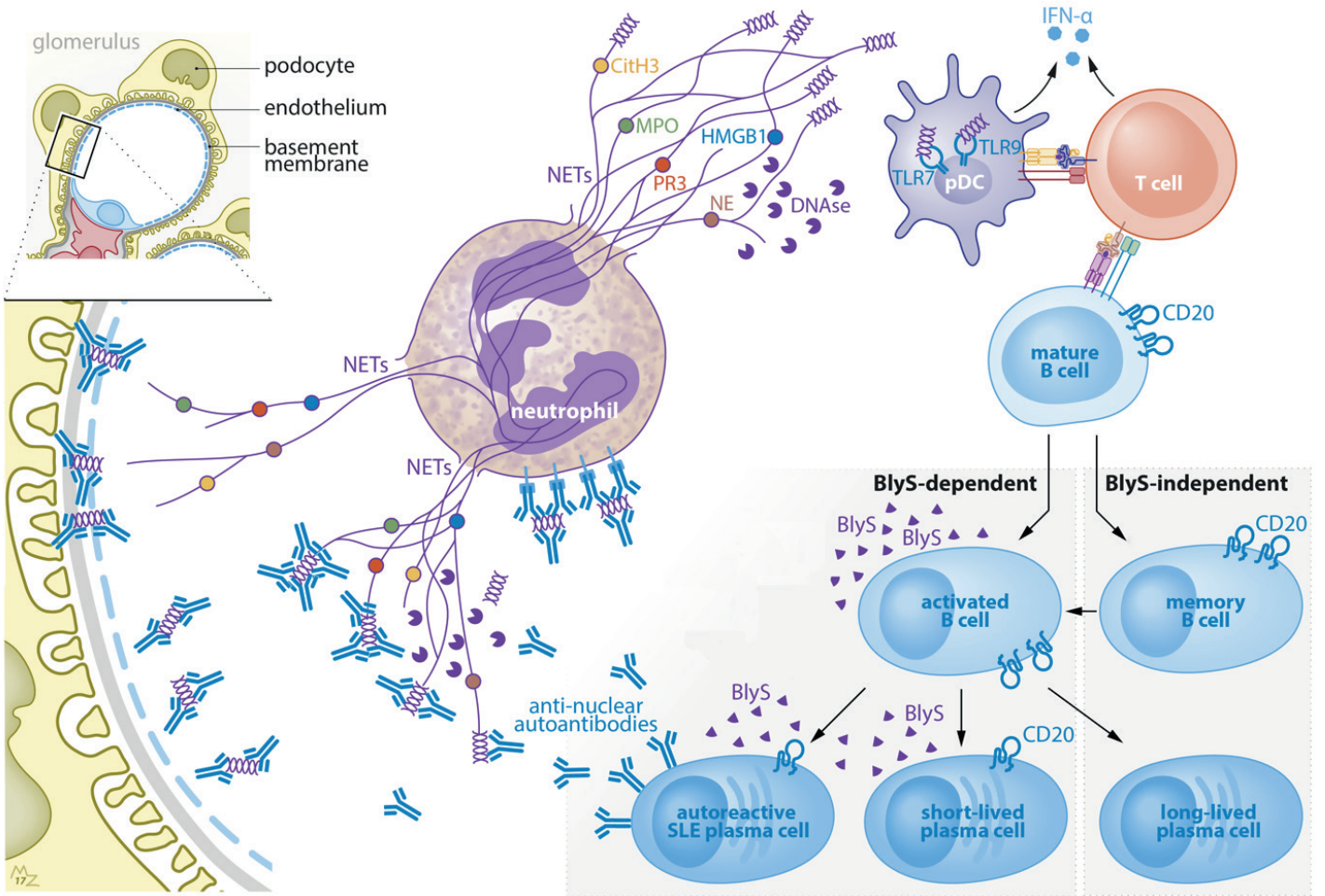
Figure 1. Central role for NETs in SLE.

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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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.

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