The NET effect of novel treatments in lupus nephritis

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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.

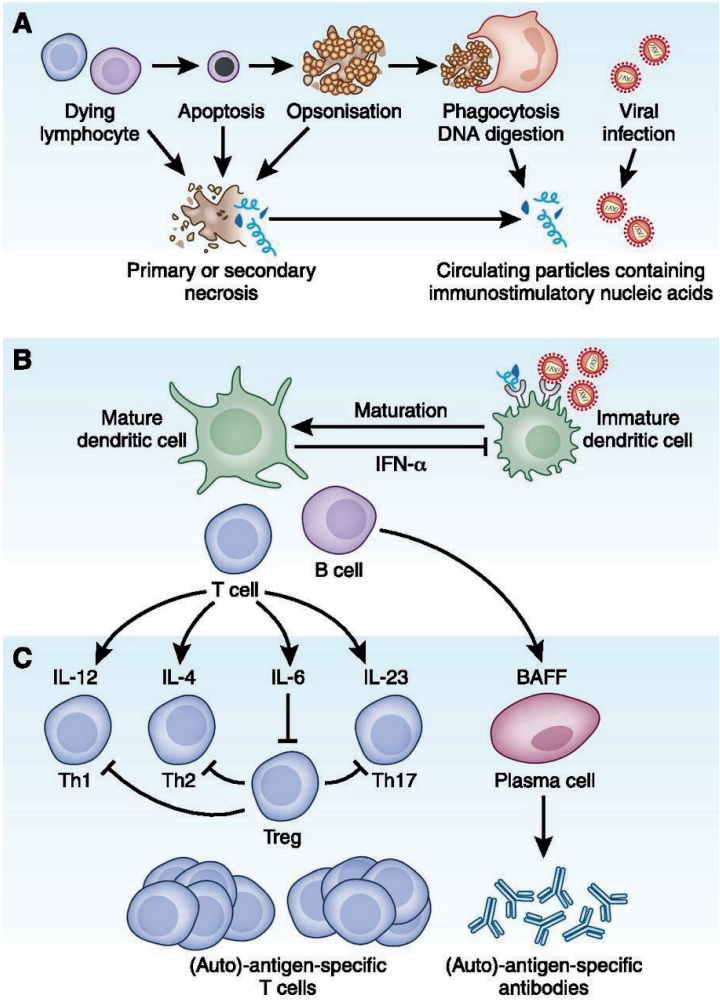

Figure 1. Autovaccination with autoantigen.
In **(A)**, cell death by apoptosis and primary or secondary necrosis leads to circulating particles containing immunostimulatory nucleic acids. In **(B)**, dendritic cells take up this autoantigenic material in a similar way as viral particles are taken up 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.

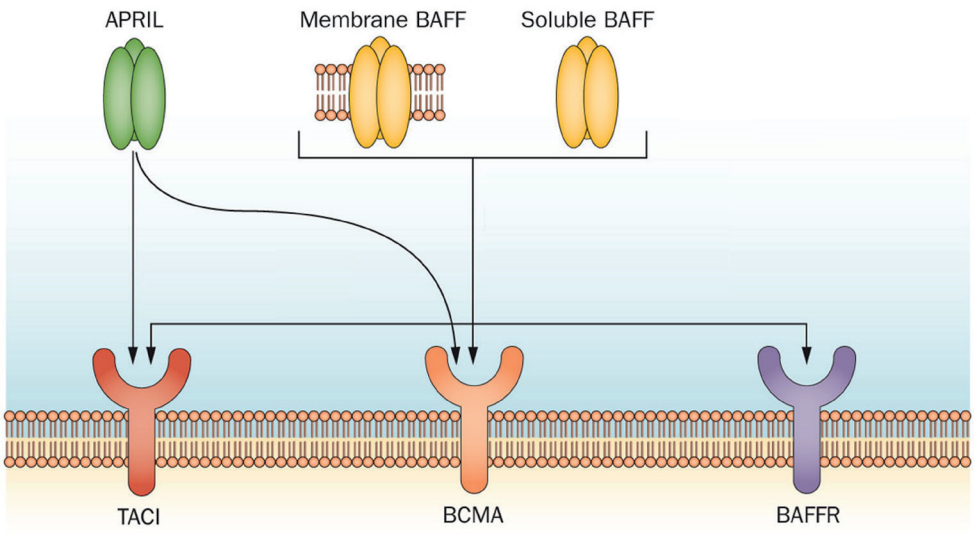

Figure 2. BAFF axis.
Figure adapted from Stohl et al. [95] with permission.

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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.

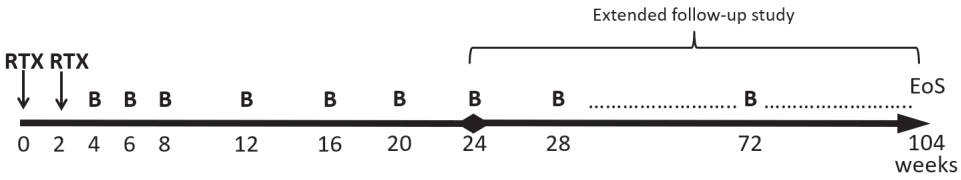

Figure 3. Flowchart Synbiose study.
RTX; Rituximab, B; Belimumab, EoS; end of study.

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