Targeting autoimmunity in renal diseases: focus on neutrophil extracellular traps and autoreactive B-cells

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1

General introduction and outline

RENAL AUTOIMMUNE DISEASES

Systemic Lupus Erythematosus (SLE) and ANCA-associated vasculitis (AAV) are systemic autoimmune diseases that can affect virtually any organ of the body. Patients can present with a diversity of clinical features ranging from skin and joint involvement to life-threatening renal, hematologic, or central nervous system involvement. These symptoms often occur in combination with constitutional symptoms such as fatigue, fever, myalgia and weight loss. Importantly, both AAV and SLE can lead to glomerulonephritis (GN) which typically manifests as a pauci-immune, crescentic GN in AAV patients, while in SLE patients a proliferative GN with a full-house immunofluorescence pattern is seen. Autoreactive B-cells, autoantibodies and excessive neutrophil extracellular trap (NET) formation play an important role in the pathogenesis of both these renal autoimmune diseases, which were therefore suggested as potential therapeutic targets. Nevertheless, SLE and AAV are two different clinical entities that are distinguished by their clinical phenotypes, histopathology and autoantibody profiles, which will be further discussed in the next paragraphs.

Systemic lupus erythematosus

SLE is a chronic autoimmune disorder of unknown cause that typically occurs in young women of childbearing age[1]. The prevalence of SLE is 1-25 per 100.000 and genetic, infectious, hormonal and environmental factors have been described in its pathogenesis[2]. The disease is characterized by a break of tolerance leading to the development of autoreactive B-cells that produce anti-nuclear autoantibodies (ANAs)[3,4]. About 180 different autoantibodies have been described in SLE patients[4]. Some fluctuated with disease activity (anti-dsDNA, anti-C1q)[5-8], while others were more refractory to immunosuppressive treatments, such as anti-extractable nuclear antigens (ENAs)[9,10], which can be found years before diagnosis. In SLE patients, autoantibodies and their antigens can form immune-complexes (ICx) that deposit in affected tissues leading to complement activation and a systemic inflammatory cascade. A severe manifestation of ICx-mediated inflammation in SLE patients is lupus nephritis (LN), which is histologically represented by a "full-house" immunofluorescence pattern (deposition of IgG, -M, -A and complement proteins) in the glomeruli[11,12]. Additionally, other severe manifestations of SLE include neuropsychiatric, pulmonary and cardiac involvement.

ANCA-associated vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) encompasses granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic GPA, which typically occur in older adults (20 per 1.000.000), more frequently in males than females[13]. The cause of the disease is unknown but infections, genetics, environment

and specific drugs are described as potential triggers of the disease[14]. The disease is characterized by a pauci-immune necrotising vasculitis leading to inflammation and damage in major organs such as the kidneys, lungs, heart, ear-nose-throat (ENT) area and the neurologic system[15]. In GPA and MPA autoantibodies against neutrophil cytoplasmic antigens (ANCAs), including proteinase-3 (PR3) or myeloperoxidase (MPO), develop and are thought to play a pathogenic role in the disease[16].

NEUTROPHIL EXTRACELLULAR TRAPS

Neutrophils are the most abundant (~57%) circulating white blood cells in our body, that act out as first responders of our immune system to fight microbial threats. Upon an infection, neutrophils recruit and activate other immune cells, but also fight pathogens themselves. To exert their primary defense function, neutrophils have the ability to attack pathogens by phagocytosis and by the release of different granules (called degranulation) containing anti-microbial peptides and proteases, such as myeloperoxidase (MPO), neutrophil elastase (NE), LL-37 and matrix metalloproteinases (MMPs)[2]. More recently, it was discovered that neutrophils also have the ability to directly attack and trap pathogens by the release of neutrophil extracellular traps (NETs)[3,4] (Figure 1). Neutrophil extracellular traps (NETs) are immunogenic[17] and toxic[18,19] extracellular DNA structures that serve as an important physiologic defense mechanism but also have been described to be involved in the pathogenesis of both AAV and SLE[17,18,20-28]. AAV patients display both an excessive formation and impaired degradation of NETs[20,23,29]. Secondly, NETs contain the main autoantigens for AAV[30] and NETs directly cause cytotoxicity leading to crescentic lesions in pauci-immune GN in AAV[31]. In SLE patients, NETs are involved in the pathophysiology in multiple ways: NETs encompass different SLE-specific autoantigens and have been shown to induce the formation of SLE-specific autoantibodies. Altogether leading to ICx-formation of autoantibodies with NETs that subsequently will trigger more NET formation and systemic inflammation causing a perpetuating, vicious cycle in SLE patients[17,21,32,33].

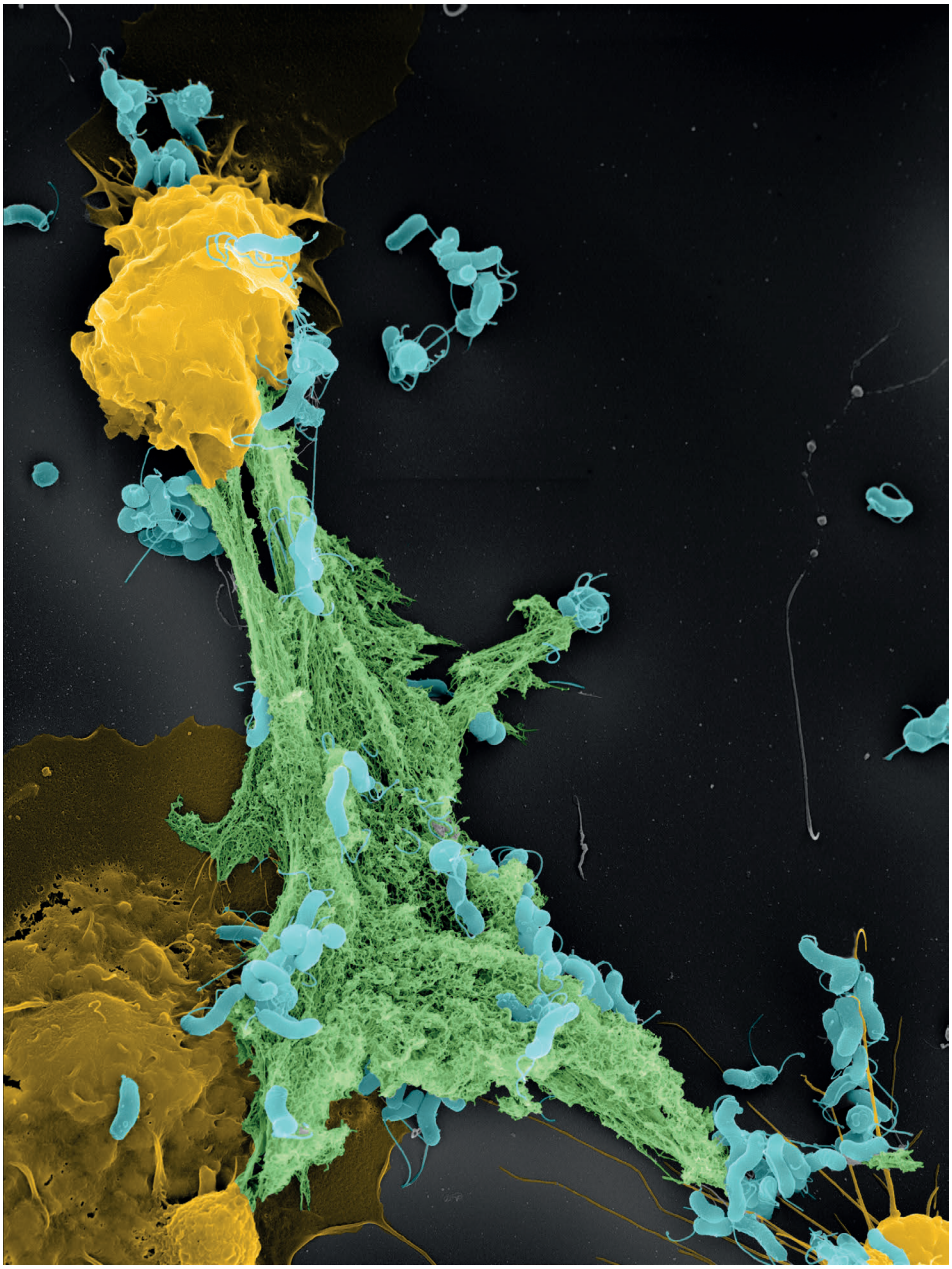

Figure 1. Neutrophil extracellular trap (NET) formation. Scanning electron microscopy of neutrophil (yellow) casting a net (green) entrapping Helicobacter pylori bacteria (blue). Neutrophils were cocultivated with H pylori for 2 h. Image kindly provided by Dr Volker Brinkmann, Max Planck Institute for Infection Biology, Berlin, Germany. Reprinted with permission from Thålin *et al.* Arteriosclerosis, Thrombosis, and Vascular Biology 2019.

AUTOANTIBODIES AND AUTOREACTIVE B-CELLS

B-cells are important contributors to our humoral immunity through the production of antibodies. Antibodies are important for the elimination of pathogens by phagocytic cells and through activation of the complement system. Typically, activation of naive B-cells occurs after antigen encountering usually in combination with T-cell help. Subsequently, these activated B-cells differentiate into plasma cells (PCs) and memory B-cells. The main function of PCs is the production of antibodies. Different classes of antibodies exist whereas IgM is produced early upon an infection, and after class-switching of B-cells IgG is produced, which has a higher affinity and can diffuse into the tissues. On the other hand, the memory B-cells are able to quickly respond to a secondary exposure of the antigen, a mechanism that is broadly used for successful vaccinations.

Upon a break of self-tolerance, autoreactive B-cells will become activated upon encountering self-antigens resulting in the production of IgG autoantibodies (Figure 2). Both in AAV and SLE patients, specific autoantibodies are key hallmarks of the disease and fulfil a pathogenic role. These are respectively PR3- and MPO-ANCAs in AAV and anti-dsDNA, anti-C1q and many others in SLE[4]. A reduction of autoantibodies, or even reversal to negativity, upon immunosuppressive treatment was associated with a beneficial clinical outcome in both AAV[34-37] and SLE[5,38-41] patients. Recently, a specific B-cell subset was identified as an important contributor to the autoimmune responses in SLE patients. This subset was called double negative (DN) B-cells, defined as class switched B-cells lacking expression of CD27 and IgD[42]. These DN B-cells were shown to be the precursors of autoantibody producing plasma cells in SLE patients, and therefore are suggested as important therapeutic targets. Additionally, B-cells and plasma cells also have antibody-independent functions, such as antigen-presentation, T-cell activation and cytokine production, which makes them an even more valuable therapeutic target in autoimmune diseases[43,44]. In conclusion, autoantibodies and autoreactive B-cells are important targets in the treatment of both AAV and SLE patients.

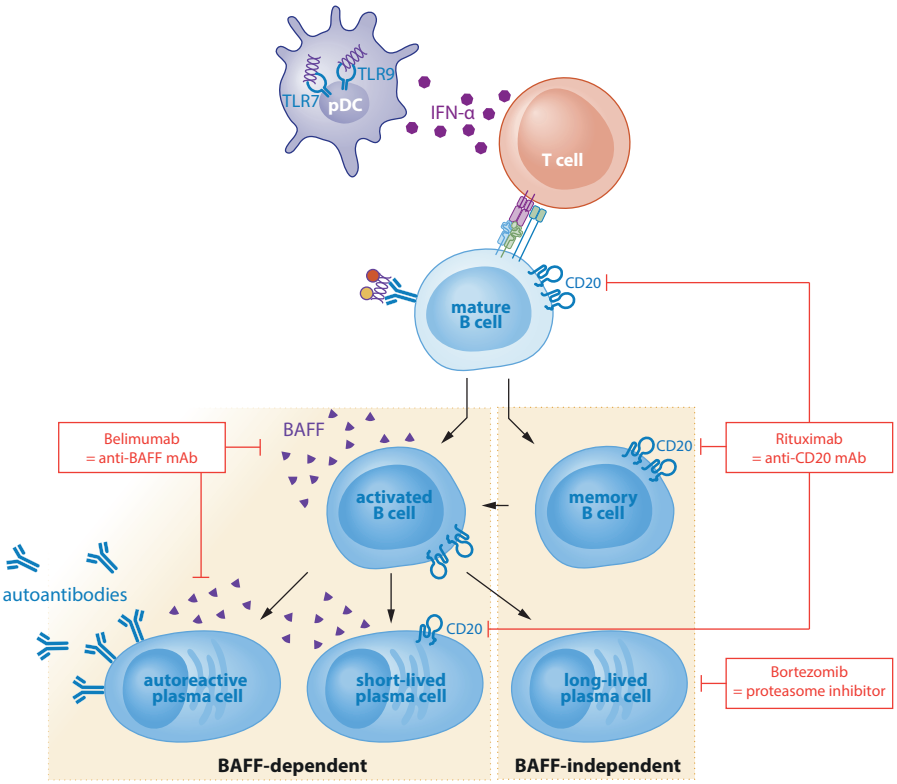

Figure 2. Autoreactive plasma cells produce autoantibodies. After a break of tolerance towards self-antigens and with T-cell help, autoreactive B-cells will become activated and differentiate into autoreactive plasma cells that produce autoantibodies. Different therapeutic approaches that target B-cells in renal autoimmune diseases are shown. BAFF, B-cell activating factor; pDC, plasmacytoid dendritic cell; TLR, toll-like receptor.

TREATMENT OF RENAL AUTOIMMUNE DISEASES

Generally, the treatment goals for patients with SLE[45] or AAV[46] are to achieve long-term survival, improve quality of life, reach long-lasting remission, prevent organ damage and minimize drug-related toxicity. Recommendations for treatment of these patients include (a combination of) immunosuppressive therapies depending on the severity of the disease, the type of organ involvement, response to previous therapies and are preferably tailored to each individual patient.

Treatment of SLE patients

All SLE patients are recommended to be treated with hydroxychloroquine. SLE patients with severe manifestations and major organ involvement are recommended to be treated with intravenous methylprednisolone (usually (500–1000 mg/day for 3 days) in combination with mycophenolate mofetil (MMF) or cyclophosphamide. However, due to its gonadotoxic effects the latter should be used with caution in women and men of fertile age. Both regimens are usually followed by an oral corticosteroid tapering regimen. At the moment, anti-BAFF monoclonal antibody (mAb) belimumab (BLM), is the only officially approved B-cell targeted therapy for SLE patients without renal and/or neurological manifestations, while it has also shown efficacy in lupus nephritis (LN) patients (preliminary results BLISS-LN III). Moreover, other B-cell-targeted therapies are thought to be effective in the treatment of SLE and LN, because different off-label uncontrolled approaches have demonstrated potential clinical benefit, including rituximab[38,47,48], bortezomib[49,50] and the combination of rituximab with belimumab[51], which is further addressed in chapter 5. In addition to immunosuppressive treatments, SLE patients typically receive proton-pump inhibition (if indicated), vitamin D, calcium supplementation and, if indicated, bisphosphonates.

Treatment of AAV patients

Current guidelines recommend to treat AAV patients with severe disease with either cyclophosphamide (CYC) or rituximab (RTX) in combination with corticosteroids as remission-induction therapy[46]. RTX was demonstrated to be as effective as CYC to achieve remission[52,53] while generally being a safer option with less toxicity[54,55]. Notably, combining of RTX+CYC as remission-induction regimen showed also promising results in AAV patients[56-58], but this has not been demonstrated in a randomized controlled trial yet. Remission-induction therapy is typically combined with 1-3 times 500-1000mg methylprednisolone i.v. daily followed by high-dose oral corticosteroids with tapering over 3 months. Additionally, AAV patients typically receive prophylactic treatment with co-trimoxazole 480mg/day, proton-pump inhibition, vitamin D, calcium supplementation and, if indicated, bisphosphonates. Recently, a new approach through C5a receptor

inhibition with Avacopan was effective in replacing high-dose glucocorticoids in treating ANCA-associated vasculitis[59]. Recommendation for maintenance treatment are RTX, azathioprine (AZA), MMF or methotrexate[46]. In the MAINRITSAN-I randomized controlled trial (RCT), RTX was shown to be more effective than AZA to prevent major relapses[60]. Importantly, the choice of RTX maintenance regimen, e.g. fixed 6-monthly, or treatment upon biomarkers, remains a matter of debate. The MAINRITSAN-2 trial demonstrated that the relapse rate was similar for fixed versus individual tailored RTX regimen, with less infusions in the latter[61].

OUTLINE OF THIS THESIS

The aim of this thesis is to gain more insight in the pathogenesis of both AAV and SLE, specifically focussing on the role of NETs, autoantibodies and autoreactive B-cells in the light of B-cell targeted therapies. Additionally, this thesis aimed to understand the humoral autoimmune response and to translate our knowledge to improve the targeting of autoimmunity in AAV and SLE patients. Thereby improving immunomonitoring, individual-tailored therapies and the overall clinical care for these patients.

In **chapter 2** the latest insights on NETs in general and specifically their pathophysiologic role in AAV and SLE are discussed. This chapter provides a translational perspective on the clinical implications of NETs, such as potential therapeutic approaches that target NET formation which are relevant for these renal autoimmune diseases. An important aspect of studies about NET formation is the manner of NET quantification of which a detailed protocol can be found in **chapter 3**. By applying this protocol, NET formation was studied in two large cohorts of AAV and SLE patients demonstrating that in AAV and SLE two distinct forms of NET formation are present, based on findings related to morphology, kinetics, triggers and pathways (**chapter 4**).

Next, different off-label experimental B-cell targeted therapies are currently used to treat SLE patients. In **chapter 5** we investigated the effects of rituximab, rituximab with belimumab, and bortezomib on the humoral autoimmune response in SLE patients by a reversed translational study. This study demonstrated the effects of different B-cell targeted therapies on autoantibodies, NET formation and their clinical consequences.

Nowadays, RTX is the first of choice remission-induction treatment for AAV patients with a new diagnosis or relapse. RTX is an effective regiment but the frequency of relapses is quite high. Therefore, biomarkers are needed that predict relapses and help in clinical decision making. In **chapter 6** the potential of ANCAs and B-cells as biomarkers to predict relapses is evaluated in a large cohort of AAV patients treated with RTX in our center.

Because relapses are frequent after the B-cell depleting agent RTX in AAV patients, minimal residual autoimmunity in the B-cell compartment was investigated by an in-depth flow cytometry study of residing and repopulating B-cells in AAV patients after RTX (**chapter 7**).

Chapter 8 summarises all research discussed in this thesis and further discusses future perspectives on targeting autoimmunity in SLE and AAV.

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