General Introduction and Aims of this Thesis
Anti-Neutrophil Cytoplasmic Autoantibody-associated Vasculitides

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is a rare, but severe autoimmune disease that affects 10-20 individuals per million annually\(^1\). AAV usually affects elderly individuals with a peak-age of disease onset between 64-75 years\(^1\). The disease can be fatal if left untreated. AAV is a form of small vessel vasculitis that is characterized by inflammation of small- to medium-sized blood vessels\(^2\) and the presence of circulating ANCA. Necrotizing inflammation, thickening, and scarring of the blood vessel walls that disturbs normal blood flow are characteristics of vascular inflammation in AAV. Obstruction of blood vessels will result in diminished oxygen exchange and can eventually lead to tissue necrosis and severe organ failure. AAV can manifest itself in all small- and medium-sized blood vessels in the body, but has a predilection for the upper airways, kidneys, lungs, and skin and often affects multiple organs or tissues simultaneously.

AAV can be divided, based on clinical and pathological symptoms, into granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic GPA\(^2\). GPA patients show, often in addition to necrotizing vasculitis and/or glomerulonephritis, extravascular necrotizing granulomatous inflammation, whereas MPA patients lack (extravascular) granulomatous inflammation. In addition, while frequent in GPA, destructive upper airway involvement in MPA is absent. ANCA in AAV are directed against proteinase (PR) 3 or myeloperoxidase (MPO), which are enzymes found in granules of neutrophils and monocytes\(^3\). Although anti-PR3- and anti-MPO-ANCA can be found across the spectrum of AAV, ANCA of GPA patients are mainly directed against PR3\(^4\), whereas MPA patients more often present with ANCA directed against MPO\(^5\).

AAV Pathogenesis

The etiology of AAV is unknown but is considered to be multifaceted involving genetic predisposition, environmental exposure (e.g. infections, toxic exposures) and acquired alterations in the immune system. Two genome-wide association studies in AAV patients found single nucleotide polymorphisms in the genes coding for the major histocompatibility complex (MHC) II related to the disease. These associations segregated along the ANCA specificity, with human leucocyte antigen (HLA) DP associated with PR3-ANCA positivity and HLA-DQ with MPO-ANCA positivity\(^6,7\). This indicates that antigen (Ag) presentation by specific major histocompatibility complex (MHC)-II molecules might predispose individuals to develop specific ANCA and AAV. Other single nucleotide polymorphisms associated with AAV susceptibility include mutations in the PRTN3 and SERPINA1 genes\(^8\). PRTN3 encodes the ANCA-Ag PR3, and SERPINA1 encodes a null allele of the enzymatic inhibitor α1-antitrypsin\(^9\), allowing increased PR3 levels to persist in AAV patients.
The current line of thought is that *Staphylococcus aureus* infection or exposure is an important driver for onset and relapse of AAV. The majority (± 60%) of GPA patients are nasal carriers of *S. aureus*. This nasal presence of *S. aureus* correlates with higher relapse rates\(^9,10\) that can be prevented by oral antibiotic treatment\(^11\). To date, the exact role of *S. aureus* in the disease pathogenesis is unknown but it has been proposed that *S. aureus* infections may induce tolerance breakdown to self-Ags in susceptible individuals and contribute to triggering of disease relapses. With respect to tolerance breakdown to self-Ags by *S. aureus*, at least two mechanisms have been put forward. First, it has been reported that *S. aureus* contains proteins highly homologous to a complementary (c) form of human PR3\(^12\). cPR3 is the protein translated from the antisense DNA strand of the PR3 gene. Immunization of mice with human cPR3 or a synthetic homolog resulted in the production of antibodies (Abs) directed against cPR3 and, via idiotypic-anti-idiotypic responses, ANCA directed against PR3, indicating that these homologous proteins can contribute to breakdown of tolerance\(^12\). However, the relevance of this finding remains controversial since in the original study only seven out of 34 PR3-ANCA positive patients presented with cPR3 Abs\(^12\), and increased reactivity against cPR3 could not be confirmed in an independent cohort of AAV patients\(^13\). Second, other *S. aureus* derived proteins, e.g. extracellular adherence protein (Eap) and staphylococcal peroxidase inhibitor (SPIN), could also indirectly contribute to autoimmune disease onset\(^14\). Eap and SPIN are recently identified enzyme inhibitors produced by *S. aureus* that target and form complexes with PR3 and MPO, respectively\(^15,16\). Importantly, natural (low-affinity) Abs directed against PR3/MPO (natural ANCA) have been demonstrated in sera of healthy controls (HCS)\(^17,18\), indicative of the presence of natural ANCA-producing B cells. Hypothetically, these Eap:PR3 or SPIN:MPO complexes may be recognized by those natural ANCA-producing B cells and become internalized. Since B cells are professional Ag-presenting cells, processing of these complexes followed by presentation of bacterial peptides by MHC-II molecules may activate EAP- or SPIN-specific T helper (Th) cells. In turn, these EAP- or SPIN-specific Th cells provide help to the natural ANCA-producing B cell, allowing isotype switching and affinity maturation, leading to the production of high affinity pathogenic IgG ANCA and thus initiation of autoimmune disease\(^19\). In summary, several mechanisms have been proposed by which *S. aureus* may cause a breakdown of tolerance in AAV patients but direct evidence pinpointing *S. aureus* as the instigator of autoimmunity in AAV is lacking.

*S. aureus* also seems to play a role in triggering relapses in AAV patients. Using similar mechanisms as described for disease onset, Eap:PR3 or SPIN:MPO complexes could also play a role in inducing disease flares via reactivation of PR3- or MPO-specific B cells. In addition, *S. aureus*-derived constituents such as CpG motifs and superAgs may also reactivate the autoinflammatory response in AAV patients\(^20,21\). This notion is supported by studies showing that peripheral blood mononuclear cells (PBMCs) derived from GPA
patients stimulated with CpG and IL-2\textsuperscript{22} or IL-21 and B cell activating factor (BAFF) trigger \textit{in vitro} PR3-ANCA production. In addition, superAgs derived from \textit{S. aureus} may activate autoreactive immune cells by forcing cell contact between T- and B cells or T cells and Ag-presenting cells\textsuperscript{24}. Thus, \textit{S. aureus}-derived constituents may contribute to tolerance breakdown and triggering inflammatory responses in AAV patients in various ways.

The pathogenesis of AAV involves a complex interplay between several immune cells. As described before, it is believed that the AAV pathogenesis is induced by (super) Ags of \textit{S. aureus} at the respiratory epithelium. Ag-presenting cells present (\textit{S. aureus}-derived) Ags to naïve T cells and produce interleukin (IL) 23, resulting in naïve T cell activation and generation of effector Th17 cells. These Th17 cells secrete their signature cytokine IL-17 that activates innate immune cells such as macrophages to produce the pro-inflammatory cytokines IL-1β and tumor necrosis factor (TNF) α. IL-1β and TNFα prime neutrophils to express adhesion molecules and enzymes such as PR3 and MPO on their surface\textsuperscript{3}. PR3 and MPO are also released upon neutrophil priming and are sampled by dendritic cells and presented to Th cells. Th cells play an important role in AAV, as demonstrated by the dominant ANCA isotypes (i.e. IgG1 and IgG4)\textsuperscript{25} that are only formed after isotype switching of the autoreactive B cell interacting with a Th cell. Next to these effector T cells, regulatory T cells (Tregs) are considered to fundamentally contribute to the AAV pathogenesis. These cells are crucial for sustaining tolerance but have been found to be defective in function in AAV\textsuperscript{26}. Moreover, perturbations in the distribution of circulating Th cell subsets in GPA patients have been reported\textsuperscript{25,27,28}. For example, an increase in a subset of memory CD4\textsuperscript{+} T cells, termed effector memory T (T\textsubscript{EM}) cells, has been detected in peripheral blood of GPA patients in remission compared to HCs\textsuperscript{29}. Interestingly, these circulating CD4\textsuperscript{+} T\textsubscript{EM} cells decrease during active disease which could be consistent with their migration towards inflamed tissues (e.g. the kidneys) where these cells may contribute to tissue damage\textsuperscript{30}. Moreover, aberrant Th cell responses have been reported in GPA in terms of skewing towards a Th17 cell phenotype as demonstrated by an increased circulating T\textsubscript{EM}17 cell frequency\textsuperscript{31} and serum IL-17A levels\textsuperscript{32}, and an expanded circulating population of PR3-specific Th17 cells\textsuperscript{20,32}. Collectively, these observations indicate that effector Th cells, mainly T\textsubscript{EM}17 cells, play an important role in the AAV disease pathogenesis.

Th cells also have an important function in the activation of B cells. In the AAV pathogenesis, help of these Th cells is needed for B cell activation and differentiation, ultimately leading to the formation of ANCA-producing plasma cells. The interaction between Th- and B cells takes place in germinal centers of secondary lymphoid organs. The T-B cell interplay triggers B cells to undergo isotype switching and somatic hypermutation to increase their Ab affinity\textsuperscript{33}. Ag-specific plasmablasts (i.e. the precursors of plasma cells)/plasma cells typically migrate to the bone marrow after finishing the germinal center reaction and are rarely found in the circulation\textsuperscript{34}. However, recent
studies suggest that PR3-specific plasmablasts can be detected in the circulation of GPA patients. Plasmablasts and plasma cells need several growth factors to survive and provide long-term humoral memory. One of these growth factors is B cell activating factor (BAFF) which is found in increased abundance in serum of GPA patients. This has led to the hypothesis that increased B cell survival in the periphery of AAV patients enhances ANCA production initiating the effector phase of AAV that ultimately causes tissue injury. In the effector phase of the disease, release of pro-inflammatory cytokines, due to for example infection with \textit{S. aureus}, primes neutrophils to express PR3 and MPO on their cell surface and induces expression of adhesion molecules on both neutrophils and endothelial cells. These adhesion molecules allow neutrophils to bind to endothelial cells of small blood vessels. Next, ANCA bind to PR3 or MPO on the surface of the adherent neutrophils inducing full-blown neutrophil activation leading to the release of their granular contents and production of reactive oxygen species causing endothelial cell injury. Furthermore, upon neutrophil degranulation, the ANCA-Ags PR3 and MPO are deposited in the blood vessel wall which may further activate the Ag-specific cellular immune response and ultimately result in the formation of granulomas. More recently, ANCA have been found to induce the formation of neutrophil extracellular traps (NETs). NET formation is a form of regulated cell death termed NETosis in which neutrophils discharge nuclear chromatin decorated with granule proteins forming an extracellular web to trap and kill bacteria. Since NETs are covered with various potentially injurious and immune stimulating proteins, including the ANCA Ags PR3 and MPO, it has been proposed that ANCA-induced NET formation contributes to the vessel wall damage and promotes the autoimmune response in AAV.

**B cells in AAV**

The most convincing evidence that B cells are crucially involved in the pathogenesis of AAV comes from studies involving B cell depletion as a treatment for AAV. In clinical trials, B cell depletion in peripheral blood using rituximab was shown to be an effective therapy for remission induction and maintenance in AAV patients. Rituximab is a mouse-human chimeric monoclonal Ab that targets CD20 on B cells, thereby effectively depleting all B cells, except CD20- plasma cells. Depletion of circulating B cells in AAV patients by rituximab results in a reduction of total IgG and also ANCA levels. This indicates that rituximab indirectly reduces (auto)Ab levels by depleting other B cells or directly via depletion of ANCA-producing plasma cell precursors. However, B cells possess multiple other functions that can potentially contribute to AAV pathogenesis, such as Ag presentation and cytokine production. In the context of AAV, little is known about the involvement of those Ab-independent properties of B cells in disease pathogenesis. Therefore, it is of interest to further investigate B cell distribution and
function in AAV. Analyses of the circulating B cell repertoire of GPA patients revealed several alterations in B cell subset distribution when compared to HCs. One of these alterations is that GPA patients show decreased circulating regulatory B cell (Breg) frequencies. Like Tregs, these cells play a role in maintaining immune tolerance. Importantly, their exact role and function in GPA pathogenesis is not yet elucidated. Other alterations in the circulating B cell compartment of GPA patients that have been reported include higher naïve B cell and lower memory B cell frequencies compared to HCs. Additionally, circulating B cells of active GPA patients are in a heightened activated state compared to circulating B cells of GPA patients in remission and HCs.

It is currently unknown why these B cells of GPA patients are more activated. In other autoimmune diseases (i.e. primary Sjögren’s syndrome (SS) and rheumatoid arthritis (RA)) important differences in the B cell receptor (BCR) signaling pathway have been demonstrated to be the basis of increased B cell activation. In both SS and RA patients, protein levels of Bruton’s tyrosine kinase (BTK) in B cells are increased and related to increased B cell activation in these patients. BTK is critically involved in the BCR signaling pathway. The BCR signaling pathway is initiated upon Ag binding and cross-linking of two BCRs triggering an intracellular signaling cascade that starts with the activation of the Src tyrosine kinase Lyn (Figure 1). Lyn phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of Igα and Igβ BCR co-receptors (also known as CD79). Spleen tyrosine kinase (SYK) becomes activated upon binding to these phosphorylated ITAMs, and this protein continues to phosphorylate B cell linker protein (BLNK) and BTK. The direct downstream target of BTK is phosphatidylinositol-specific phospholipase C (PLC) γ2. PLCγ2 phosphorylation occurs when both BTK and PLCγ2 bind to phosphorylated tyrosines on BLNK. Eventually, PLCγ2 induces the mitogen-activated protein kinase (MAPK) pathway, resulting in the activation of the extracellular signal-regulated kinase (ERK). However, PLCγ2 also induces transformation of phosphatidylinositol-bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG), resulting in increased cytoplasmic calcium levels. These increased calcium levels allow DAG to ultimately activate S6 kinase, which induces nuclear factor κ-light chain enhancer of activated B cells (NF-κB) activation. NF-κB is a transcription factor that regulates DNA transcription resulting in cytokine production and mediating cell survival. NF-κB activation also induces B cell survival, differentiation, cytokine production and Ab secretion (Figure 1). Since B cells of AAV patients persist in an activated state and play an important role in the disease pathogenesis, protein levels or phosphorylation of molecules in the BCR signaling pathway are likely to be increased. Therefore, characterization of the BCR signaling cascade in AAV may contribute to our understanding of B cell activation in AAV pathogenesis and could lead to novel and more specific treatment targets.
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Figure 1. BCR signaling cascade. Simplified overview of the BCR signaling cascade after the BCR becomes cross-linked by Ag. Lyn phosphorylates ITAMs of the BCR as well as Igα and Igβ (CD79). Phosphorylated CD79 mediates activation of SYK, which continues to phosphorylate CD19, BTK, and BLNK. Next, PLCγ is phosphorylated that activates the MAPK pathway, involving ERK and increasing intracellular calcium levels. PLCγ also induces PIP2 conversion into IP3 and DAG, which activate the AKT pathway in which S6 is involved. Eventually, both pathways result in NF-κB activation that ultimately results in Ab production, B cell survival, differentiation, and cytokine production.

Therapies Used in AAV

Since the introduction of cyclophosphamide and glucocorticoids, the prognosis for patients with AAV has significantly improved53. More recently, rituximab was introduced to treat AAV patients and found to be as efficacious as cyclophosphamide in inducing remission54,55. After induction therapy with rituximab or cyclophosphamide, patients switch to maintenance therapy consisting of either azathioprine (AZA), methotrexate, or mycophenolate mofetil (MMF) combined with corticosteroids, which are tapered over time56. Additionally, a randomized controlled trial compared the effectiveness of rituximab and AZA maintenance therapy after induction of remission with cyclophosphamide, demonstrating a lower relapse rate in patients treated with rituximab54. A second rituximab maintenance study demonstrated that the intensity of the B cell depleting therapy can be guided by monitoring circulating peripheral blood B cells and the level of ANCA57.

Current induction and subsequent maintenance therapies successfully induce remission in most patients and reduce subsequent relapse rate. However, AAV patients are still at risk for cumulative tissue damage resulting from disease relapses and toxic side effects of immunosuppressive treatment58. Patients who receive MMF as maintenance
therapy have a higher relapse risk compared to AZA-treated patients. Both treatments are non-specific and target proliferation of leucocytes (AZA) and lymphocytes (MMF). This discrepancy between the relapse rates in AAV patients treated with these immunosuppressive drugs and the effects of these therapies on immune cell functioning remains poorly understood. Therefore, there is a need to develop more specific treatments to limit side effects. As described before, B cell depletion using rituximab might be more effective for remission maintenance. However, depleting all circulating B cells (including Bregs) seems an over-rigorous method and long-term effects of this therapy are to date unclear. Thus, there is still a need to further understand the pathogenic mechanisms involved in AAV in order to develop more specific and effective treatment.

**Predicting Disease Relapses**

To date, treatment strategies for AAV are not completely effective in preventing disease relapses. During the course of their disease, ±60% of GPA patients experience disease relapses compared to around 25% of MPA patients. Each episode of active disease in AAV patients causes, in addition to renewed treatment toxicity, additional tissue injury and can eventually result in severe organ failure. Therefore, prediction of disease relapses is pivotal for patient care, so that relapses can be treated at an early stage or even prevented. To predict ensuing AAV disease relapses, a (bio)marker, i.e. a measurable and validated indicator correlated to an upcoming disease relapse, is needed. These markers can include a single marker, a set of (bio)markers or a method to assess the patients’ immune status. Previously, multiple studies aimed at identifying biomarkers that are related to future disease relapses in AAV. Markers to predict disease relapses proposed so far include pulmonary involvement during active disease, PR3-ANCA positivity and chronic nasal carriage of *S. aureus*. However, none of these markers proved to be accurate indicators for disease relapses. Other suggested biomarkers for AAV are the serum ANCA titer and changes herein during follow-up. Although in some studies correlations between future relapses and persistence or a rise in ANCA titers have been reported, other studies could not confirm such associations. Indeed, a meta-analysis by Tomasson *et al.* showed that rises in serum ANCA titer over time only weakly predicted disease relapses and were ineffective in predicting disease relapses for all AAV patients. ANCA titers are determined by immunofluorescent techniques, and recently novel and more quantitative methods for measuring PR3-ANCA levels have been introduced (e.g. Phadia ImmunoCAP analyzer). Recently, variation in *in vitro* PR3-ANCA production was assessed as a potentially sensitive approach for predicting disease relapses in GPA patients. In this cell culture assay, PBMCs are cultured in the presence of CpG, BAFF and IL-21 and PR3-ANCA levels are measured in supernatants.
using the Phadia analyzer. Prospective monitoring of \textit{in vitro} PR3-ANCA levels turned out to be very variable between patients and did not aid in predicting future disease relapses better than ANCA titers\textsuperscript{21}. Thus, there is an urgent need for further prospective studies to discover markers or develop methods to more accurately identify those patients at risk for relapse.

**Aims of this Thesis**

The research presented in this thesis focuses on the role of B cells in the disease pathogenesis of AAV (Figure 2), with a major focus on GPA. B cells play a crucial role in the GPA pathogenesis as demonstrated by the efficacy of B cell depletion therapy. However, the exact involvement of regulatory and effector B cell functions in the disease pathogenesis is not yet fully understood. To further expand our understanding of B cell activation, function and response to treatment in AAV, studies were performed on B cells from GPA patients to map the BCR signaling pathway and assess the effects of BCR activation and commonly used immunosuppressive drugs on B cell function. In addition, the B cell phenotype distribution and its relation to disease relapses in AAV was explored.

Besides the possible pathogenic role that B and T cells can exert in AAV, these cells can also play an important role in the suppression of inflammation. It has been proposed that the suppressive function of Tregs and Bregs could be decreased in AAV, thereby allowing continuation of autoimmune inflammation. Therefore, our aim in **Chapter 2** was to review the literature on Tregs and Bregs focusing on the role both regulatory immune cell subsets could play in the AAV disease pathogenesis. We propose that an imbalance between regulatory and effector functions underlies the pathogenic process in AAV, and that this imbalance is responsible for decreased suppression by Bregs and Tregs.

The exact involvement of Bregs and potential aberrancies in Breg-mediated suppression in the disease pathogenesis of AAV is not yet elucidated. A decreased Breg frequency has been demonstrated in the circulation of AAV patients, but no clear functional deviance of Bregs has been established in AAV. Since Th17 cells play an important role in the AAV pathogenesis, we hypothesized in **Chapter 3** that Bregs of GPA patients are less effective at suppression of the Th17 response. To address this hypothesis, we first assessed the frequency of CD24\textsuperscript{hi}CD38\textsuperscript{hi} Bregs and Th17 cells in the circulation of GPA patients and subsequently studied the functional effect of Bregs on Th17 cell expansion \textit{in vitro} (Figure 2).
B cells are considered crucial players in the AAV pathogenesis but their exact role is not completely understood. In this thesis, several aspects concerning the contribution of B cells to the AAV pathogenesis were investigated including functional implications (e.g. effector and regulatory functions) in disease development (Chapters 2, 3 and 4), as treatment target (Chapters 4 and 5) and as a dynamic circulating immune cell population related to relapses (Chapter 6). See the text for a more detailed explanation.

Although B cells seem to persist in an activated state in GPA, little is known about the status of the BCR signaling pathway (Figure 1). In other autoimmune diseases, it has been demonstrated that BCR sensitivity and signaling is increased and correlates with autoAb levels. Possibly, deviations in this pathway could contribute to GPA pathogenesis as well (Figure 2). Therefore, in Chapter 4, we studied the BCR signaling pathway in B cells from GPA patients focusing on the levels and phosphorylation status of BTK, a tyrosine kinase essential in downstream BCR signaling (Figure 1). Protein levels and phosphorylation status of BTK, as well as various other signaling molecules up- and downstream of BTK, were determined in B cells from active and remission GPA patients and HCs. In addition, we also investigated the effects of inhibition of
BTK phosphorylation on in vitro B cell function to assess whether BTK blockade could constitute a novel treatment option in GPA.

Currently, several immunosuppressive drugs are used to treat GPA patients. AZA and MMF are two drugs frequently used to maintain remission in AAV patients. Interestingly, MMF-treated patients have been reported to relapse more frequently than AZA-treated patients which may be due to differential effects of these drugs on immune cell function.

In Chapter 5, we hypothesized that MMF, in contrast to AZA, inhibits the regulatory function of B cells, and that this might be the underlying mechanism for increased relapse rates in MMF-treated GPA patients. To this end, ex vivo and in vitro effects of AZA and MMF on B cell phenotype and function were studied.

After remission induction in GPA, immunosuppressive drugs are tapered. During tapering or ceasing immunosuppressive therapies, the majority (±60%) of GPA patients experience a disease relapse. Relapses significantly increase morbidity and contribute to mortality in AAV. So far, some markers predicting future disease relapses have been identified, however, none proved to be sufficiently reliable to identify patients at risk for future disease relapse. Since B cells are important in the AAV pathogenesis, both as Ab- and cytokine producing cells, we hypothesized that plasmablasts, i.e. the precursors of (ANCA-producing) plasma cells, are related to future disease relapses. The research described in Chapter 6 assessed frequencies of several B cell subsets, including plasmablasts, and differences herein as possible (bio)marker for future disease relapses (Figure 2).

Finally, in Chapter 7, we summarize and discuss the results presented in this thesis. We propose how these findings contribute to the understanding of the GPA pathogenesis and could aid future research on novel treatments and predictors of disease relapses.