B cell phenotype and function in granulomatosis with polyangiitis

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Introduction and aim of the thesis
Vasculitis

Vasculitis is a term used to indicate a spectrum of disorders that present with inflammation of the blood vessel walls. This results in a restricted blood flow owing to damage and changes of the vessel walls and the tissues perfused by these vessels. Vasculitis can present in a single organ, for example the lungs or kidneys, or involve multiple organs. It can be divided in multiple categories, first based on the size of the blood vessels, into large-, medium- and small-vessel vasculitides [1]. The last of these categories contains a group of syndromes characterised by the presence of autoantibodies directed against neutrophil constituents, mainly proteinase 3 (PR3) and myeloperoxidase (MPO). These are termed anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV) [2]. AAV can be subdivided in granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic GPA (EGPA) [1]. The incidence of AAV increases with age, with a peak-age of onset of 64-75 years [3]. AAV has a combined prevalence of 46–184 per million, which has increased over the last 20 years and the gender distribution is fairly equal, with a slight predominance of male patients [4].

Presentation and treatment

AAV patients suffer from nonspecific, systemic inflammation resulting in symptoms that include fever and arthralgia as well as organ specific manifestations. Examples for the latter are pulmonary haemorrhages caused by alveolar capillaritis or glomerulonephritis caused by glomerular capillaritis [5]. Patients with GPA show extravascular necrotising granulomatous inflammation and pauci-immune necrotising crescentic glomerulonephritis. MPA lacks the typical granulomatous inflammation present in GPA and is defined as necrotising vasculitis with few or no immune deposits. EGPA differs clinically from GPA and MPA, and is characterised by asthma, eosinophilia and granulomatosis [6].

Untreated vasculitis has a high mortality rate. However, since the introduction of cyclophosphamide and high dose steroid treatment the prognosis has significantly improved. AAV can now be considered chronic conditions with periodic relapses. However, mortality rates are still substantial, both due to disease manifestations and the toxic effects of extensive immunosuppressive treatment [7]. Currently, B cell depletion treatment with the anti-CD20 monoclonal antibody rituximab has been approved as an alternative induction therapy, as cyclophosphamide is associated with significant toxicity. So far, rituximab treatment does not appear to be inferior to cyclophosphamide in induction of remission of AAV [8, 9].

After the initial therapy to induce remission patients are generally put on a maintenance treatment regime, often azathioprine or mycophenolate mofetil sometimes combined with low-dose steroids. Methotrexate is another option as a remission-maintenance
agent, and this is also used as an alternative to cyclophosphamide in induction therapy for patients with local or early systemic disease, albeit less efficient in prevention of relapse [10]. However, despite maintenance treatment patients still relapse, often during tapering or after discontinuation of medication [7].

**Relapse risk in AAV**

Preventing relapses in AAV is crucial, as each relapse affects the quality of life of the patient and can lead to increased organ damage and even death. Considering the burden of cumulative toxicity associated with the immunosuppressive treatment, it is important to identify which patients are actually at risk for developing a relapse in the near future [11]. Numerous studies have attempted to identify which patients are at risk and which factors should be closely monitored during remission. Patients with GPA have a substantially higher risk for relapse (up to 60% in 5 years) than MPA patients (29%) [12]. PR3-ANCA positivity, lung involvement and chronic nasal carriage of *Staphylococcus aureus* are also indicators for an increased risk for relapse [13, 14]. Conversely, patients with poor renal function and high creatinine values have a decreased risk for exacerbation [11].

One factor that has been thoroughly investigated with regard to monitoring and relapse prediction is the serum ANCA titer, although studies on this topic are not in agreement. While some studies find a strong association between a rise in ANCA titer [15] or persistence of a high ANCA titer [16] and subsequent relapse, others do not [17]. One meta-analysis came to the overall conclusion that monitoring the ANCA titer only has a modest value for predicting relapse in AAV [18]. As such, it is insufficient to exclusively monitor serum ANCA titers in order to guide maintenance treatment regimens.

More recent studies have investigated risk factors related to relapse post-rituximab treatment, mainly aimed at the B cell repopulation. Absence of naive B cell repopulation after six months was associated with an increased risk for relapse [19], and a decreased proportion of CD5+ B cells with a shorter time to relapse [20].

**AAV pathogenesis**

While the aetiology of AAV is currently not fully understood, several pathogenic mechanisms have been described (Figure 1). Observations have linked the onset of AAV to infection, in particular *S. aureus*. A significantly higher percentage of patients with GPA carry this bacterium than healthy individuals [14]. Moreover, antibacterial treatment is beneficial in reducing relapse risk in these patients [21]. Antibodies against other infections agents were also increased in GPA serum, including hepatitis C and *Helicobacter pylori* [22]. One proposed mechanism for this link is molecular mimicry, where autoimmunity is triggered through homology between a human protein and one derived from pathogens [23, 24]. Another link between infection and autoimmunity are the Toll-like receptors (TLRs), a family of receptors capable of recognising microbial
components. Activation through TLRs leads to the production of proinflammatory cytokines and upregulation of costimulatory molecules on antigen presenting cells (APCs). Dysregulation of this system has been related to onset of autoimmunity [25]. In AAV increased expression of TLRs was found on monocytes and natural killer cells, and monocytes from patients with nasal carriage of S. aureus had increased levels of intracellular TLR9 [26]. Expression of TLR2 and TLR4 was detected in glomeruli of AAV patients but not in healthy controls, and expression of TLR4 on the glomeruli was associated with renal injury severity [27].

Release of proinflammatory cytokines such as TNFα leads to priming of neutrophils, as well as upregulation of adhesion molecules on vascular endothelial cells. TLR2 and TLR9 ligands were shown to prime neutrophils to a similar extent as TNFα [28]. Neutrophil
priming results in translocation of PR3 and MPO to the cell surface, increasing their accessibility for the circulating ANCA. The primed neutrophils are recruited to the site of inflammation and bind to ANCA, upon which they become fully activated and adhere to the activated endothelial cells. This leads to production of reactive oxygen species and release of proteolytic enzymes resulting in endothelial damage [29].

The proinflammatory environment that is created will attract mediators from the adaptive immune system. Indeed, in the inflammatory lesions found in AAV, T cells can be detected [30]. Moreover, soluble T cell activation markers are increased in serum or plasma samples from patients compared to controls, and are associated with disease activity [31, 32]. The T-helper (Th) cell polarisation in AAV deviates from the healthy situation. Locally, T cells in granulomatous lesions are predominantly of the Th1 phenotype, and produce interferon (IFN)-γ [33]. Moreover, frequencies of both Th2 and Th17 cells were increased in GPA patients in remission [34].

It has been proposed that Th1, Th2 and perhaps Th17 cells can survive to become lineage-committed effector memory T cells (T_{EM}). The T_{EM} cells lack the lymph node homing receptor CCR7, instead these cells have increased expression of chemokine receptors for migration to inflamed tissues. As such they may be directly recruited to sites of inflammation [35]. In GPA patients in remission a relative increase of CD4+ T_{EM} was observed in the circulation, while during active disease these cells were decreased. Concurrently, CD4+ T_{EM} cell numbers increased in the urine of active vasculitis patients, indicating migration to inflammation sites [36, 37]. T_{EM} cells share certain features with natural killer cells, including surface marker expression and cytotoxic capacity [38]. Specifically targeting the T_{EM} cells could be beneficial in AAV. CD4+ T_{EM} cells are characterised by high surface expression of a specific voltage-gated potassium channel after activation, the Kv1.3 channel [39]. Selective blockade of these channels has proven to be effective in ameliorating autoimmune disease in animal models [40] and could be beneficial in AAV.

**B cells in AAV**

Another cell type crucial in AAV is the B cell, especially considering the likely pathogenic role of the autoantibodies present in these patients. However, while traditionally best known as antibody producing cells, B cells have multiple functions in the immune response (Figure 2). For one, they are effective APCs [41] and studies have shown that antigen presentation by resting B cells can induce T cell tolerance in vivo in animal models [42]. Moreover they are cytokine producing cells, and as such can be divided into effector and regulatory subsets based on which cytokines they produce. Depending on the cytokine milieu present when antigen is first encountered, they produce a polarised array of cytokines that contribute to the effector phase of the immune response. On the other hand, B cells are also capable of producing anti-inflammatory cytokines such as interleukin (IL)10 and transforming growth factor (TGF)-β, and thus contribute to tolerance [43].
Introduction and Aim of the Thesis

In AAV several alterations of the B cell subsets and B cell related markers have been identified compared with healthy controls. GPA patients with active disease have higher levels of B cell activating factor (BAFF) in their serum than controls [52]. BAFF is a critical B cell survival factor and neutralisation of BAFF prevents the persistence of mature B cells [53]. However, excessive production of BAFF in mice rescued self-reactive B cells

Figure 2. The different B cell functions. B cells can, after differentiation into plasma cells, produce a variety of immunoglobulins. In autoimmunity these antibodies will include autoantibodies, for example PR3-ANCA IgG in GPA patients. B cells can present antigens to T cells, they are especially effective at presenting antigens that bind to their surface Ig's which are selectively internalised, processed and presented to T cells [44]. Moreover, B cells produce an array of cytokines. This includes production of the anti-inflammatory cytokines IL10 and TGFβ by regulatory B cells. Regulatory B cells can inhibit differentiation of T helper 1 (Th1) cells and activation of macrophages as well as increase numbers of regulatory T cells (Treg) [45, 46]. Moreover, B cells produce numerous proinflammatory cytokines that affect the different T cell subsets. For example, TNFα can promote Th1 differentiation [47], IFNγ also supports Th1 responses [48], and can promote macrophage activation [49]. B cells can promote Th2 memory responses through production of IL2 [50] and Th17 cell activation through IL6 production [51]. Figure was constructed using Servier Medical Art.

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that would normally be deleted and facilitated migration into otherwise restricted microenvironments, leading to a partial subversion of B cell self tolerance [54]. Multiple studies have identified changes in the B cell distribution in the periphery of AAV patients. These include increased expression of CD38 in patients with active disease [55] and increased proportions of CD25+ and CD86+ B cells in remission patients under glucocorticoid treatment. CD25+ B cells do not secrete immunoglobulins, have increased antigen presenting capacity and have been ascribed with potential regulatory capacities [56]. Peripheral B cells from AAV patients show a decreased frequency of CD27+ memory B cells, and an increased proportion of naive B cells [57]. A number of GPA patients also expressed CTLA-4 (CD152) on the surface of their B cells, while this was not observed in controls. CTLA-4 has an essential inhibitory role in the regulation of the immune response as CTLA-4-deficient mice die at a young age from the consequences of a massive unchecked proliferation of their T cells [58].

Regulatory B cells

The first evidence that B cells could play a regulatory role was obtained from mouse model studies. B cell deficient mice were unable to recover from experimental autoimmune encephalomyelitis (EAE) [59]. Further analysis of the B cells determined that IL10 production by B cells correlated with EAE recovery and that in absence of IL10 the immune response persisted and mice did not recover [60]. Exacerbated arthritis in IL10-depleted mice was accompanied by changes in the T cell compartment, with an increase in Th1 and Th17 cells and a decrease of FoxP3+ regulatory T cells (T_{reg}) [61]. Since then, the expression of the anti-inflammatory cytokine IL10 has defined the regulatory B cell (B_{reg}) subset. IL10 acts through a transmembrane receptor complex composed of IL10R1 and IL10R2, and can regulate the functions of different types of immune cells. It inhibits production of inflammatory mediators and antigen presentation in monocytes and macrophages, and also inhibits the proliferation and the cytokine production of CD4+ T cells [62]. However, IL10 does not solely have a suppressive function, it is also a growth factor that promotes the differentiation of B cells into plasma cells. Inhibition of IL10 signalling could dramatically decrease the frequencies of CD138+CD27^{high} plasmablasts in vitro [63]. This makes the characterisation of the regulatory B cell difficult. Unlike with FoxP3 in T_{reg}, no specific transcription factor has been identified that characterises a distinct B cell subset.

Phenotypical identification of B_{reg} has proven to be a controversial topic and up to date no clear phenotype has been established. As such the production of IL10 remains a key trait for this population. Several proposals have been made for identifying B cell populations that demonstrate an increased production of IL10 after in vitro stimulation. In 2010 two papers were published, each describing a different phenotype for B_{reg} in humans. The first of these showed that B cells with a CD19+CD24^{high}CD38^{high} phenotype produced substantially more IL10 than other B cell populations. B cells with this
phenotype were capable of inhibiting proinflammatory cytokine production by CD4+ T cells [64]. Moreover, CD24^{high}CD38^{high} B cells were able to convert CD4+ T cells into FoxP3+ T_{reg}, a mechanism that was impaired in patients with rheumatoid arthritis [65]. The second paper found that there were low frequencies of IL10 competent cells in the circulation. With additional experiments they identified a so-called B10pro cell that was capable of producing IL10 with sufficient stimulation. Both types of cells were identified to be mainly CD19+CD24^{high}CD27^{+} B cells [66]. While these two phenotypes share the high expression of CD24, they are non-overlapping populations. The CD24^{high}CD38^{high} cells are immature transitional B cells, while the CD24^{high}CD27^{+} cells are categorised in the memory B cell compartment.

**Aims and outline of this thesis.**

One of the main challenges in AAV is the prediction of disease relapse, for which currently no biomarkers are available that can be used in daily clinical practice. When relapses could be predicted, they could potentially be prevented through timely intervention, and non-relapsing patients may be spared intensive immunosuppressive therapy. B cells are critical players in the regulation of immune responses, which is accomplished through a finely regulated balance between effector and regulatory B cell functions and involves multiple B cell populations and antibody-dependent as well as antibody-independent properties. It has become apparent that in AAV alterations in B cell subset distribution and function are present compared with healthy individuals. The aim of this thesis was to determine whether studying the B cell phenotype and function can improve relapse prediction in GPA patients. The distribution of different B cell subsets, production of PR3-ANCA IgG and pro- and anti-inflammatory cytokines were determined in relapsing and non-relapsing GPA patients. Moreover, the potential role for regulatory B cells and production of IL10 in GPA and the production of ANCA was investigated.

While pathogenicity of ANCA is the currently accepted paradigm, there is evidence indicating that not all ANCA are the same and that non-pathogenic ANCA may exist as well. In chapter 2 the evidence for and against pathogenicity of ANCA is reviewed. Moreover, the possibility of specific pathogenic epitopes is highlighted. ANCA are generally measured systemically in serum samples, however, the ANCA titer is not a sufficiently discriminative measure to identify AAV patients about to relapse. The ANCA titer reflects an accumulation of antibodies over time, while in vitro experiments may present a better reflection of the ongoing pathogenic process in the patient. Such an in vitro system is described in chapter 3, where endogenous and exogenous factors are combined to induce production of PR3-ANCA IgG. In order to determine whether this system was more effective at predicting disease relapse in GPA it was subsequently applied in a prospective cohort study, described in chapter 4. Next to the serum ANCA titer and in vitro ANCA production, the B cell subset distribution was investigated,
including two potential phenotypical descriptions of regulatory B cells. Since no clear phenotype for $B_{reg}$ has been established, direct analysis of production of the regulatory cytokine IL10 may be more informative with regard to B cell mediated immune regulation. Moreover, B cells can also produce effector cytokines, and the balance between effector and regulatory cytokines may affect the risk for relapse. Therefore, we investigated whether B cell cytokine production differed in GPA and healthy controls, and could aid in relapse prediction. This is described in a cohort of relapsing patients, non-relapsing patients and healthy controls in chapter 5. IL10 has multiple roles, it can function as a regulatory cytokine and as a plasma cell differentiation factor. As such its effect on the formation of PR3-ANCA IgG is unclear. In general, the cytokine milieu in vitro and in vivo, could affect the formation of PR3-ANCA. To determine this we broadly explored cytokine production in relation to PR3-ANCA production in vitro in chapter 6. This included the direct effect of adding IL10 to cell cultures to determine its effects on ANCA production. Finally, we investigated the effect of the two most used maintenance medications on cytokine production, PR3-ANCA production and plasma cell formation here. The currently used maintenance therapies are not sufficient to keep AAV patients in remission and numerous new treatment strategies are under investigation. In chapter 7 a potential treatment option and its effect on different B cell functions is described. The peptide ShK-186 is capable of blocking specific voltage-gated potassium channels that are key in the activation of effector memory T cells. However, these channels are also highly expressed on B cells, especially switched memory B cells. Therefore, we determined the effect of this peptide on B cell cytokine production, B cell proliferation and (PR3-ANCA) IgG production. Finally, in chapter 8 the work presented in this thesis is summarised and discussed.

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