B cell phenotype and function in granulomatosis with polyangiitis

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chapter 8

Summary, discussion and future perspectives
Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV) are a heterogeneous group of syndromes characterised by small-vessel inflammation and involvement of pathogenic autoantibodies. In patients with granulomatosis with polyangiitis (GPA) the ANCA are mainly directed against proteinase 3 (PR3) and in patients with microscopic polyangiitis (MPA) against myeloperoxidase (MPO) [1]. Both PR3 and MPO are localised in the azurophilic granules of neutrophils and the granules of monocytes [2, 3].

AAV patients generally achieve remission through the use of immunosuppressive treatment. Long-term immunosuppressive treatment is associated with toxicity, and requires that treatment is tapered in patients. AAV patients suffer from frequent disease relapses, often during tapering or after discontinuation of treatment [4]. Each relapse is associated with the risk of cumulative damage [5]. For example, every renal relapse is associated with a decrease in estimated glomerular filtration rate, a measure of kidney function [6]. Therefore, one of the main clinical challenges in AAV is finding accurate methods to predict disease relapse. If biomarkers for relapse are identified this could allow for timely intervention in patients that are about to relapse.

Many types of immune cells are involved in AAV disease pathogenesis, including neutrophils, B cells and T cells. Recently the role of B cells has received more interest. B cells can exert both regulatory and effector functions. Changes in the balance of these functions may influence the risk for relapse in patients. Therefore, in this thesis, we investigated the B cell phenotype and function in GPA patients and related this to relapse risk.

ANCA as a potential biomarker for relapse

Several observations suggest that ANCA play an essential role in the pathophysiology of AAV. As discussed in chapter 2, ANCA are capable of activating neutrophils in vitro [7], resulting in the formation of neutrophil extracellular traps [8] and eventually in endothelial cell injury [9]. Moreover, several animal models have demonstrated the pathogenic role of MPO-ANCA in the development of an MPA phenotype [10, 11]. ANCA are generally measured systemically in patient serum by indirect immunofluorescence and the resulting ANCA titer has often been related to disease activity and the risk for relapse. The overall conclusion has been that monitoring ANCA titers alone is insufficient to guide decisions on treatment [12–14]. In chapter 3 we describe an alternative method to detect the production of PR3-ANCA in GPA patients. A combination of exogenous (CpG-oligodeoxynucleotides) and endogenous (B cell activating factor (BAFF) and interleukin (IL)21) factors could induce production of ANCA in isolated peripheral blood mononuclear cells (PBMCs) from a substantial proportion of GPA patients. These factors synergised in the induction of B cell proliferation and induced formation of plasma cells. Moreover, the use of CpG resulted in upregulation of receptors for BAFF and IL21. Interestingly, in a number of patients PR3-ANCA
production was even detected when PBMC were cultured without stimulation. This was seen in a smaller number of patients, but suggests an increased activation status of autoantibody producing cells or their precursors. The ANCA titer is a reflection of accumulated autoantibodies in serum, while an *in vitro* system may reflect the current capacity for ANCA production better and could be more accurate for relapse prediction. In chapter 4 we investigated both the ANCA titer and *in vitro* ANCA production in relation to relapse in PR3-ANCA positive GPA patients. Eighty-four PR3-ANCA positive GPA patients were prospectively monitored for up to two years and 16 relapses were detected during this period. On group level we did observe an increase in ANCA titer prior to relapse, although this increase was not present in every individual patient. Regarding *in vitro* ANCA production, a number of patients demonstrated a very clear increase before relapse, and this was particularly visible in unstimulated cell culture samples. However, this increase in *in vitro* PR3-ANCA before relapse was not significant on group level. Based on these data, we can conclude that monitoring *in vitro* ANCA production cannot identify all GPA patients that are about to relapse and this method does not appear more accurate for relapse prediction than measuring ANCA titers. Recently it was described that the predictive value of ANCA titers was increased when applied only to AAV patients that presented with renal involvement [15]. Moreover, new-generation methods exist for ANCA measurements in serum that may result in more accurate estimations of ANCA levels [16]. Multiple variants of enzyme-linked immunosorbent assays (ELISA) have become available, for example the anchor ELISA which uses a technique to immobilise PR3 on the ELISA plate surface by employing an anchor molecule as a bridge. This technique demonstrated superior sensitivity for the detection of PR3 antibodies in GPA patients [17]. Another example is the Phadia ImmunoCAP system applied in chapter 4 for cell culture supernatants. In order to fully establish the value of (*in vitro*) ANCA in prediction of relapse in AAV larger prospective studies need to be done in order to detect more relapses. Including more patients will also allow for specific sub analyses based on clinical parameters, and may potentially identify patient groups in which ANCA measurements are more applicable for relapse prediction.

A final point to consider with regard to monitoring ANCA production is that not all ANCA may be equally pathogenic. As discussed in chapter 2, one study found that for MPO-ANCA epitope specificity appeared to define pathogenicity [18]. Low levels of ANCA have been detected in healthy individuals [19], and MPO-ANCA from healthy individuals had epitope specificities that were different from those in patients. Interestingly, ANCA were also detected in patients that were previously thought to be ANCA negative. Further characterisation demonstrated that ANCA in these patients were directed at the same epitope that was associated with active disease in MPA patients [18]. These data do need to be confirmed by further studies and similar analysis of PR3 is warranted.
The level of glycosylation is another mechanism that could affect pathogenicity of antibodies [20]. The IgG subclasses from active GPA patients are hypoglycosylated compared to controls, and a positive correlation has been detected for anti-PR3 specific IgG1 galactosylation and the time to remission [21]. Moreover, the ratio between levels of sialylated anti-PR3 antibodies and levels of total anti-PR3 antibodies was significantly lower in patients with active disease than patients in remission. Desialylation of IgG antibodies in GPA patients resulted in a significantly stronger oxidative burst of neutrophils than nondesialylated IgG antibodies [22]. Collectively, studies defining specific molecular features of the autoantibodies allowing for distinguishing pathogenic ANCA from non-pathogenic ANCA may eventually increase the value of ANCA measurements for relapse prediction.

### B cell phenotype and cytokine production in relapsing GPA patients

B cells are clearly involved in the pathogenesis of AAV, as they are the precursors of the plasma cells that produce ANCA. However, B cells can exert multiple other functions as well, including antigen presentation and production of a variety of pro- and anti-inflammatory cytokines [23, 24]. As such they may be involved in other pathogenic processes as well as in regulation of the immune response. Proinflammatory cytokines including tumour necrosis factor (TNF)α and interferon (IFN)γ can for example promote T helper-1 (Th1) responses [25, 26], while anti-inflammatory cytokines can suppress the immune response. One example of the latter is IL10, known as the signature cytokine for the regulatory B cell (Bregs) population. Bregs have been described to inhibit production of proinflammatory cytokines from monocytes and effector Th cells and support the differentiation of regulatory T cells [27].

Based on its surface markers B cells can be divided into several subsets. While alterations of B cell subsets have been described in multiple autoimmune diseases, it remains unknown whether a specific B cell subset profile is associated with disease relapse. Memory B cells can be identified by their expression of CD27, while naive B cells lack CD27 [28] and transitional B cells have high expression of CD38 [29]. Multiple suggestions have been made to identify Bregs in the circulation based on surface marker expression, including CD24highCD38high [30] and CD24highCD27+ B cells [31]. In AAV patients, a decreased proportion of memory and CD24highCD27+ B cells and an increased percentage of naïve B cells was observed compared to healthy controls [32]. In chapter 4 we confirm these differences in a larger cohort of GPA patients. What we observed further is that patients about to relapse deviate the most from healthy controls, with even lower percentages of memory and CD24highCD27+ and higher percentages of naïve B cells compared to non-relapsing patients. It is unclear whether the decrease in CD24highCD27+ B cells is indicative of decreased B cell regulation or simply a reflection of decreased total CD27+ B cells.
We observed in chapter 5 that GPA patients have increased levels of serum CCL19 compared to healthy controls. CCL19 is a trafficking chemokine that has particularly been associated with memory B cell migration [33]. Increased migration of memory B cells in GPA patients could explain their decreased presence in the periphery and the further decrease in the percentage of memory B cells seen before relapse. To fully prove this, tissue biopsy studies are needed to analyse the presence of different B cell subsets, including memory B cells, in remission patients and compare this to active disease sites, such as the kidneys or lungs. Moreover, we analysed total CD27+ memory B cells in chapter 4, a subset that can be further subdivided in class-switched and non-switched memory B cells. It would be interesting to analyse differences within the memory B cell distribution and determine whether this can improve its value for relapse prediction in GPA.

The balance between pro- and anti-inflammatory cytokine production by B cells could also affect the risk for relapse in patients with GPA. Therefore, the B cell cytokine production profile was assessed in chapter 5 in relapsing patients, non-relapsing patients and healthy controls. Significantly fewer B cells from GPA patients were producing TNFα compared to controls, which was especially visible in patients before relapse. TNFα production correlated positively with other proinflammatory cytokines, IL2 and IFNγ, and the presence of memory B cells in vitro. As such, the reduced presence of memory B cells in the circulation may explain the decreased percentage of TNFα producing B cells. At the moment, data regarding proinflammatory cytokine production by B cells in humans is scarce. It would be interesting to determine whether other autoimmune diseases where memory B cells were found decreased compared to controls, for instance Sjögren’s syndrome [34], display similar differences in effector B cell cytokine production as we found for GPA. Moreover, samples from GPA patients with active disease should be analysed to determine whether circulating memory B cells and TNFα producing B cells decrease further upon relapse.

The association between TNFα production and memory B cells has been described previously. In rheumatoid arthritis (RA) patients TNFα production was threefold greater from memory B cells than from naive B cells [35]. From multiple sclerosis (MS) patients and controls, naive and memory B cells were sorted and stimulated using CD40 engagement and/or B cell receptor (BCR) cross-linking. Proliferation rates upon stimulation were similar in the B cell subsets, but cytokine profiles differed. Memory B cells produced high levels of TNFα upon dual stimulation while naive B cells produced IL10 mainly with CD40 engagement alone [36]. These data suggest the existence of distinct roles for naive and memory B cells in immune regulation.
Regulatory B cells and interleukin-10 production in GPA.

In order to investigate the regulatory capacity of B cells, we also determined IL10 production in chapter 5. No association was observed with this anti-inflammatory cytokine and naive B cells in our analyses, nor did we find differences for IL10 production in B cells from healthy controls, relapsing patients and non-relapsing patients. However, in individual patients prior to relapse IL10 production did decrease significantly, indicating a certain decrease of regulatory capacity may occur prior to relapse. Previous studies have been in disagreement with regard to differences in IL10 production in AAV patients and HC [32, 37, 38]. We can conclude from our results that the potential for IL10 production does not appear to be hampered in GPA patients when B cells are stimulated with the Toll-like receptor (TLR) 9 ligand CpG-ODN. However, it can be argued that CpG stimulation does not sufficiently simulate the in vivo situation.

Other methods used to induce IL10 production in B cells include CD40 signalling, used as a mimic for receiving help from activated T cells in the absence of specific B cell antigens [36]. Addition of CD40L could also further enhance the production of IL10 by TLR stimulated B cells [31]. It is possible that different types of stimulation induce IL10 production through different pathways and in different B cell populations. To determine this, multiple methods of IL10 induction should be directly compared and associated with a wide array of described B cell surface markers. It also appears that a certain degree of plasticity exists with regard to IL10 production in B cells. This is underscored by a mouse model using IL10 reporter mice to track regulatory B cell induction and fate. In this study it was found that regulatory B cells only transiently expressed IL10 prior to terminal differentiation into antibody-secreting plasmablasts and plasma cells [39]. It would be ideal if the B_{reg} could be identified by surface markers, circumventing the need for ex vivo stimulation. However, at the moment the B_{reg} remains an elusive cell. Different surface markers have been associated with the regulatory phenotype. Perhaps the best known phenotypes are the aforementioned CD24^{high}CD38^{high} and CD24^{high}CD27^{+} B cell subsets. An alternatively proposed marker is CD5. In patients with active AAV lower percentages of CD5^{+} B cells were detected, while patients in remission were similar to controls [40]. CD5^{+} B cells correlated positively with the CD24^{high}CD38^{high} subset and were shown to be predictive of relapse after treatment with rituximab [41]. Furthermore, CD5^{+} B cells have been shown to produce more IL10 than CD5^{-} B cells after BCR activation and introduction of CD5 by transfection using vectors encoding the full-length CD5 molecule into a CD5^{-} human B cell line activated synthesis of IL10 mRNA [42]. CD5^{+} B cells also express higher levels of the death inducing ligand FasL than CD5^{-} B cells, and it has been suggested that these B cells may have a specialised regulatory function as so-called killer B cells [43].

In addition, plasmablasts have been ascribed with regulatory function. In an experimental autoimmune encephalomyelitis (EAE) mouse model IL10 producing plasmablasts were
identified, and the EAE phenotype was exacerbated when plasmablasts were lacking. In the same study human plasmablasts, particularly CD27 int plasmablasts derived from naive B cells, were described to preferentially secrete IL10 [44]. A second study found IL10 production enriched in both CD24 high CD27+ B cells and CD27 high CD38 high plasmablasts [45]. Recently the CD24 high CD38 high B cells were characterised in-depth, using multicolour flow cytometry in combination with bioinformatics and functional studies. This analysis provided evidence for the existence of different CD24 high CD38 high transitional B cell subsets with unique phenotypic and regulatory functional profiles suggesting that different regulatory defects in different autoimmune diseases may be due to altered distributions of these subsets. CD27+ transitional B cells could suppress production of proinflammatory cytokines and were demonstrated to produce high levels of IL10. However, production of IL10 was not restricted to any specific B cell subset [46]. Others have similarly suggested that IL10 producing B cells are diffusely scattered throughout the B cell lineage, and are not confined to either the memory (IgD+ CD27+) or the transitional (CD24 high CD38 high) B cell compartment [47]. We also did not find a positive association between production of IL10 by B cells and CD24 high CD38 high, CD24 high CD27+ or CD5+ B cells in chapter 5.

To complicate matters further, IL10 independent B reg mechanisms have been described [48]. Mice with a B cell deficiency had a significant reduction in the number of peripheral regulatory T cells, which was restored by adoptive transfer of B cells. For this result glucocorticoid-induced TNF ligand, but not IL10 expression, by B cells was required [49]. Signalling through the inhibitory receptor programmed death receptor-1 (PD-1) can inhibit population expansion, cytokine production and cytolytic function of T cells [50]. B cells expressing high levels of the ligand PD-L1 can therefore suppress humoral responses and antibody production by attenuating T cell activation [51]. Finally, other cytokines are important in the negative regulation of immunity, for example IL35. Mice with IL35-deficient B cells were unable to recover from EAE. In this model, IL35 was mainly produced by CD138+ plasma cells [52].

In conclusion, regulatory B cells remain difficult to analyse, as different subsets based on differential expression of surface markers have been indicated to produce IL10 and B cells exhibit regulatory functions by multiple mechanisms. It is possible that no specific IL10 producing regulatory B cell subset exists, and IL10 production is induced in different B cells through presence of environmental factors.

**Associations between ANCA and cytokine production**

Production of pro- and anti-inflammatory cytokines could influence the production of autoantibodies in vitro. In chapter 6 we explored cytokine production in cell culture supernatants from GPA patient samples with and without in vitro ANCA production and controls. This demonstrated that in vitro ANCA production correlated with several cytokines known to be involved in plasma cell formation and survival, including IL10.
Moreover, we observed that when in vitro ANCA production increased in individual patients, levels of IL10 in these culture supernatants increased concomitantly. These data suggest that in samples from certain patients a higher level of plasma cells formation and survival occurs, resulting in more PR3-ANCA production. To confirm this, plasma cell formation should be investigated at different time points during the course of an in vitro ANCA culture. Furthermore, it would be interesting to measure circulating plasma cells in GPA patients, based on CD138+ expression, and associate this with ANCA production in vitro. In RA patients the production of anti-citrullinated protein antibodies (ACPA) in vitro was found to be similar when CD20+ cells were depleted, indicating that production ex vivo was dependent on plasmablasts and cells from the circulation [53]. Conversely, when IL10 was added exogenously to cell cultures it could inhibit the production of PR3-ANCA (chapter 6). This inhibitory effect was strongest when IL10 was added directly at the start of cell cultures. As mentioned, aside from its regulatory functions IL10 is known as a plasma cell differentiation and survival factor [54]. Therefore, the effect of IL10 in this setting may be dependent on both timing and the cell composition in vitro. When IL10 is present prior to plasma cell formation it can exert a regulatory function, while IL10 added at a later stage may act as a plasma cell survival factor. IL10 and other cytokines involved in plasma cell formation could be added during different stages of cell cultures to examine their effect on both plasma cell formation and ANCA production.

B cell targeted treatment strategies.

The importance of B cells in autoimmune pathogenesis is underscored by the development of several therapeutic options that target B cells. In Figure 1, several treatment options and their B cell related targets are visualised. The anti-CD20 monoclonal antibody rituximab has been investigated and approved as an alternative induction therapy for AAV. This B cell depleting treatment was not inferior to cyclophosphamide for inducing remission [55, 56]. Moreover, treatment with rituximab has shown promise as maintenance therapy, one study found fewer relapses with rituximab than azathioprine while adverse event rates were similar [57]. However, rituximab treatment has also been associated with increased risk of adverse effects, in particular persistent hypogammaglobulinemia which predisposes patients to recurrent infections [58]. There is also interest in strategies aimed at BAFF and a proliferation inducing ligand (APRIL). BAFF and APRIL are TNF family members, with roles in modulating lymphocyte survival, activation and differentiation [59]. The rationale for such a strategy is that autoreactive B cells appear to have a heightened dependency on BAFF, and excessive BAFF production can rescue autoreactive B cells from peripheral deletion [60]. Moreover, in patients with GPA elevated serum levels of BAFF and APRIL have been detected [61, 62], and in inflamed mucosal biopsies activated B cells were present in
Treatment strategies are aimed at different targets expressed on B cells, or targets with receptors present on B cells. Rituximab targets CD20, present on all stages of B cell development except the early pro-B cells and plasma blasts and cells. Belimumab, tabalumab and atacicept target the TNF cytokine family members B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL). B cells have several receptors for these activating cytokines, BAFF receptor, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). Abatacept inhibits costimulatory signalling. Tocilizumab is a monoclonal antibody aimed at the interleukin (IL)6 receptor present on B cells, inhibiting IL6 binding to IL6R. Finally, ShK-186 targets the Kv1.3 potassium channel, particularly expressed by switched memory B cells.

proximity of the autoantigens, BAFF and APRIL [63]. Treatment with rituximab leads to heightened levels of BAFF [64] and may favour autoreactive B cells. Therefore, neutralisation of BAFF could be particularly beneficial in rituximab treated patients. The anti-BAFF monoclonal antibody belimumab has been approved for treatment of systemic lupus erythematosis (SLE), resulting in improved disease activity scores [65, 66]. In addition to belimumab, other BAFF (and APRIL) neutralising therapeutic agents have been developed, such as tabalumab [67] and atacicept [68]. While targeting BAFF profoundly reduced numbers of naive B cells, it had a markedly smaller effect on memory B cells and long-lived plasma cells [69]. Similarly, memory B cells did not require APRIL for survival or function [70]. This suggests that essential B cell populations involved in autoimmune pathology may be missed with BAFF/APRIL targeted therapy. Abatacept is a fusion protein of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-Ig and the immunoglobulin Fc region, which inhibits costimulatory signalling and consequently inhibits the activation of T cells. In an open-label study in GPA patients, addition of abatacept was well tolerated and associated with high frequency of disease remission and prednisone discontinuation [71]. Treatment with abatacept in RA also demonstrated a clear effect on B cell function, resulting in decreased serum levels.
of Ig and decreasing titers of ACPA and rheumatoid factor (RF). Finally, decreased percentages of post-switch memory B cells were found after abatacept treatment [72]. IL6 receptor inhibition by tocilizumab also affects the B cell compartment. In an open-label study in RA a significantly reduced frequency of memory B cells and serum immunoglobulin levels was detected [73]. Moreover, expression of intracellular TGFβ in CD25\textsuperscript{high} B cells was significantly increased in samples from patients treated with tocilizumab for 3 months, while expression of the activation marker CD69 was reduced [74]. As TGFβ is another immune-regulatory cytokine expressed by B cells, this suggests that tocilizumab may influence the B cell regulatory capacity. In patients with RA and to a lesser extent in patients with giant cell arteritis, tocilizumab has demonstrated benefit [75, 76]. Clinical trials are needed to determine the efficacy of these types of treatment in patients with AAV.

Considering the multiple regulatory mechanisms associated with B cells it remains a question whether indiscriminately targeting B cells, as rituximab does, is the best treatment option. Specifically targeting the effector B cells would leave regulatory mechanisms intact. The voltage-gated potassium channel Kv1.3 is highly present on IgD-CD27+ switched memory B cells, while naive and IgD\textsuperscript{+}CD27\textsuperscript{+} B cells express low levels of this specific channel [77]. Switched memory B cells have been described to be primarily responsible for proinflammatory cytokine production [36], and they can differentiate into (auto)antibody producing plasma cells [78]. The effect of Kv1.3 channel blockade has mainly been described in T cells, as this channel is also highly expressed by effector memory T cells upon activation [79]. Specific Kv1.3 inhibitors have been effective in several animal models of immune-mediated inflammation, including anti-glomerular basement membrane glomerulonephritis [80].

In chapter 7 we examined the effect of Kv1.3 channel blockade on different B cell functions. It was observed that treating PBMCs with a specific Kv1.3 channel blocker resulted in decreased production of both total and PR3-ANCA specific IgG. This reduction was not the result of decreased proliferation of total B cells. Moreover, Kv1.3 channel blockade resulted in a significant reduction of B cell cytokine production. This effect was particularly pronounced on the proinflammatory cytokines TNF\textalpha, IL2 and IFN\gamma, while the effect on IL10 was substantially less prominent. As such the TNF\textalpha/IL10 ratio was significantly decreased with this treatment strategy, indicating that Kv1.3 channel blockade may lead to a relative increase of B cells producing anti-inflammatory cytokines. While the effects we observed were on B cells, they may be the indirect result of decreased T cell activation as PBMC cultures were performed. While the stimulation \textit{in vitro} was specifically aimed at B cells, T cell related effects cannot be excluded as for example T cell help is required in the production of IgG [81]. To fully elucidate the direct effect on B cells experiments with sorted B cells need to be performed. Furthermore it would be interesting to determine the effect of Shk-186 on B cell function \textit{in vivo} by using animal models. Targeting both effector B and T cells makes Kv1.3 channel blockade an intriguing treatment option for AAV, as well as other autoimmune diseases.
Concluding remarks

In this thesis we demonstrate several alterations with regard to B cell function and phenotype in GPA patients compared to healthy controls. Moreover, these differences appear to be most pronounced in patients prior to disease relapse. B cell related markers, particularly the B cell subset distribution, are promising with regard to identification of patients that are at increased risk for disease relapse. Further improvement is needed as we have not identified a biomarker that can be directly applied in daily clinical practice. Future investigations could focus on measuring ANCA with newer methods and attempting to focus specifically on pathogenic ANCA. Finally, investigation of the regulatory function of B cells may be more informative than focusing on phenotypic identification. Especially the regulatory capacity of B cells beyond the production of IL10 requires more attention.

References


11. Little MA, Smyth CL, Yadav R, Ambrose L, Cook HT, Nourshargh S, Pusey CD:


23. Rodríguez-Pinto D: B cells as antigen presenting cells. *Cellular Immunology* 2005, 238:67–75.


relapse risk from B cell phenotype: decreased CD5+ B cells portend a shorter time to relapse after B cell depletion in patients with ANCA-associated vasculitis. Ann Rheum Dis 2015, 74:1784–6


