CHAPTER 7

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Granulomatosis with polyangiitis

Granulomatosis with polyangiitis (GPA) is a rare systemic autoimmune disease characterized by inflammation of the small- and medium-sized blood vessels and is strongly associated with the presence of anti-neutrophil cytoplasmic autoantibodies (ANCA) directed against proteinase 3 (PR3)[1]. The clinical spectrum of GPA is broad comprising localized, early systemic, generalized and severe forms of the disease[2, 3]. In localized GPA, disease manifestations are restricted to the upper and/or lower respiratory tract whereas in the generalized form of the disease the systemic vasculitis affects more organs often including the kidneys. In many cases the disease starts locally and gradually progresses to a more generalized form, whereas only few patients remain in the localized disease stage. Histopathologically, GPA is characterized by necrotizing vasculitis, necrotizing glomerulonephritis and granulomatous inflammation.

To date the etiology of GPA remains unclear but is clearly multifactorial in which environmental factors, genetic predisposition and disturbances in cellular immune responses contribute to disease development and progression[4-7].

Current treatment of GPA consists of two phases of immunosuppressive therapy[8]. The first phase aims at induction of remission and a rapid control of disease activity to prevent irreversible tissue damage. The second phase is directed at preventing disease flares. This treatment strategy exposes patients to long-term broad acting immunosuppressive drugs (e.g. cyclophosphamide and glucocorticoid steroids) which has considerably improved patients' survival but comes at the cost of substantial adverse side effects such as a high rate of infection and drug toxicity[9]. Furthermore, many GPA patients suffer from frequent disease relapses during drug tapering or discontinuation of treatment[10]. Consequently, each relapse is associated with an increased risk of cumulative tissue damage[9] requiring reinitiation of immunosuppressive treatment emphasizing the need for less toxic and more selective treatment approaches in GPA.

To develop a specific and selective therapy that targets effector immune pathways, a better understanding of the immune pathophysiology of GPA is required. Over the past two decades it has become clear that dysregulation in cellular immunity is a critical component of the pathogenesis of GPA. Hence, the role of T and B cells in GPA pathogenesis has received increasing interest. Observational studies on peripheral and lesional T cells in GPA suggest that various T cell subsets may contribute to the pathogenesis of GPA although the underlying effector pathways are only partially understood[7]. Also, more recent studies in GPA patients have demonstrated alterations in peripheral B cell subset distribution which may affect both antibody-independent regulatory and effector B cell functions[11]. Thus, disturbances in both the T and B cell compartment in GPA patients are likely to be involved in GPA pathogenesis.

In this thesis we investigated a potential novel therapeutic strategy in GPA, specifically directed against effector subsets of $CD4^+$ T helper (T_H) cells and B cells while keeping other, beneficial, immune cells unaffected. Since both T_H effector cells and B effector cells are uniquely dependent on Kv1.3 potassium channels for cellular activation, we hypothesized that blocking these channels with the highly potent Kv1.3 channel inhibitor ShK-186 would effectively suppress the pro-inflammatory effector responses of these cells. To further our insights in immune

mechanisms involved in GPA pathogenesis, we also studied the phenotype of circulating $CD4^+$ T_H subsets in GPA patients in more detail and investigated the potential interplay between regulatory B cells and the expanded T_H17 population in GPA patients.

T cell phenotypes in GPA

T cells are critical players in regulating immune responses. Failure of an adequate control of T cell activity may result in unwanted effects such as chronic inflammation and autoimmune disease, both hallmarks of the pathogenesis of GPA. An overview of the T cell mediated immune responses that contribute to chronic vascular inflammation – a manifestation of GPA as well as atherosclerosis – is described in **chapter 2**[7]. In particular, we have discussed the key contribution of distinct $CD4^+$ T_H cell subsets in GPA pathogenesis. The first studies on T_H cells in GPA addressed potential imbalances in the T_H1/T_H2 responses in relation to the stage of the disease since it was hypothesized that a shift in T cell response could be of pathogenic importance in the transformation from localized to generalized GPA. These studies suggested a dominance of T_H1 responses in GPA patients with localized disease whereas in patients with active generalized disease T_H2 responses appeared more prevalent[12-16]. However, later studies indicated that the distinction between localized and generalized GPA based on the type of T_H cell response was less clear than previously thought since IFN-γ producing T_H1 cells could readily be detected in the circulation and lesional airway tissues of GPA patients with active generalized disease as well[17, 18].

In the early 2000's, a distinct novel subset of $CD4^+$ T effector cells was described that was named T_H17 cells due to their characteristic production of pro-inflammatory molecules of the IL-17 family[19]. Subsequent studies revealed a prominent role of T_H17 cells in inflammation and autoimmunity leading to a wealth of studies in various autoimmune diseases including GPA[20]. It has been reported that GPA patients, both during active disease and remission, display an expanded pool of circulating T_H17 cells, elevated levels of serum IL-17 and increased numbers of auto-antigen specific T_H17 cells[21, 22, 23, 24]. The major physiological role of T_H17 cells lies within the defense against bacterial infections, for example *Staphylococcus aureus* (*S. aureus*) infections. Intriguingly, chronic nasal carriage of *S. aureus* has been reported to be a risk factor for disease relapse in GPA patients[25]. Therefore, it has been postulated that chronic carriage of *S. aureus* might drive a T_H17 response in GPA. Moreover, IL-17 facilitates the migration and activation of neutrophils by promoting the secretion of TNF-α and IL-1β[26]. Since the accumulation of neutrophils is a hallmark of the early effector phase of GPA, T_H17 cells might be important drivers of the recruitment and activation of neutrophils during active vasculitis. Together, these data provide important evidence for the involvement of T_H17 cells and its associated cytokine IL-17 in GPA pathogenesis.

T_H effector functions, including those of T_H17 cells, are controlled by regulatory T (T_{REG}) cells. T_{REG} cells are a distinct $CD4^+$ T cell subset characterized by their potent immunosuppressive activity and expression of the transcription factor FoxP3[27]. In addition, both T_H17 and T_{REG} cells are characterized by considerable phenotypic and functional plasticity[28] determined by the cytokine environment. For example, it has been shown that TGF-β alone is able to upregulate

the transcription factor RORγt, which is characteristic for T_H17 cells and necessary for IL-17 production[28]. On the other hand, FoxP3, the signature transcription factor of Tregs, negatively regulates IL-17 production via direct physical interaction with RORγt[29]. Interestingly, the pro-inflammatory cytokine, IL-6 suppresses FoxP3 expression. Consequently, no inhibition of RORγt occurs allowing T cells to differentiate towards T_H17 cells[29, 30]. Therefore, it might be possible that the expanded T_H17 population in GPA is due to a decline in the number of T_{REG} cells or their impaired functionality caused by malfunctioning of FoxP3, both of which have been implicated to contribute to the pathogenesis of GPA[31-33].

The lineage committed effector T_H cells, including T_H1, T_H2, and T_H17 cells can be classified within the antigen-experienced $CD4^+$ effector memory T (T_{EM}) cells. In GPA, studies reported that T cells with a memory phenotype are a major source of IFN-γ and IL-17[18, 34]. Our group previously studied the frequency of $CD4^+$ T_{EM} cells in GPA and found that the circulating $CD4^+$ T_{EM} cells are proportionally increased in GPA patients during remission but are decreased during active disease[35]. Interestingly, the $CD4^+$ T_{EM} cells appear in the urinary sediment of active GPA patients with renal involvement[36]. This observation suggested that $CD4^+$ T_{EM} upon active disease migrate from the circulation to the inflammatory sites in the kidneys. Moreover, T cells with a memory phenotype were also observed in the nasal cavity of GPA patients with localized disease[15]. However, not all $CD4^+$ T_{EM} cells tend to migrate to target tissues. It is possible that the different phenotypes among the circulating $CD4^+$ T_{EM} cells reflect distinct migratory capacities and pathogenic functions in GPA patients related to the various clinical manifestations.

In **chapter 3** we determined the distribution of circulating $CD4^+$ T_{EM} cell subsets in GPA patients based on the co-expression of surface chemokine receptors[37]. Chemokine receptors have been particularly useful for distinguishing $CD4^+$ T_{EM} cell subsets with distinct migratory capacities and effector functions[38]. For instance, CXCR3 is expressed on IFN-γ producing T_H1 cells; CCR6 is expressed on IL-17 producing T_H17 cells; and CRTh2 is expressed on lineage-committed T_H2 cells. Analyzing the expression of these chemokine receptors allowed us to identify distinct $CD4^+$ T_{EM} cell phenotypes ($T_{EM}1$, $T_{EM}2$, $T_{EM}17$, and $T_{EM}17.1$) in the circulation of GPA and healthy individuals. Moreover, T cell phenotype analysis based on chemokine receptor expression allowed us to investigate different $CD4^+$ T_{EM} cell phenotypes without any *in vitro* stimulation or manipulation. This is in contrast to most previous studies in GPA analyzing T cell phenotype distribution of GPA patients using *in vitro* stimulation assays which may have influenced the T cell phenotypes. Therefore, by analyzing the chemokine receptors on the surface of T cells directly after blood withdrawal we expected to obtain a T cell phenotype distribution profile resembling most closely the physiological distribution of circulating T cells in GPA patients. The results presented in **chapter 3** demonstrate a significant increase in the proportion of $T_{EM}17$ cells with a concomitant decrease in the proportion of $T_{EM}1$ cells in the peripheral blood of GPA patients in remission[37]. Furthermore, the increased proportion of $T_{EM}17$ cells was more pronounced in GPA patients with systemic manifestations, whereas GPA patients with local manifestations showed a remarkable increase in $T_{EM}1$ cells. Interestingly, the disturbed balance between $T_{EM}1$ and $T_{EM}17$ cells appeared to be associated with CMV seropositivity.

Human T_H17 cells produce IL-17A and IL-17F, both signature cytokines that define this subset[39]. IL-17A and IL-17F are similar in their biological activity, targeting both immune and non-immune cells, and play an important role in inflammatory responses. IL-17 induces chemokine CXC ligand (CXCL)8 production from epithelial cells, endothelial cells, fibroblast and macrophages leading to the recruitment of neutrophils. In addition, several cell types in chronically inflamed tissues produce chemokine CC ligand (CCL)20 in response to IL-17[40, 41]. CCL20 binds to the chemokine receptor CCR6, which is expressed on T_H17 cells[42]. In GPA patients, *Fagin et al* found increased frequencies of circulating $CCR6^+$ $CD4^+$ T cells and showed that the expression of CCR6 was largely confined to the memory T cell compartment[43]. Furthermore, they found that the $CD4^+CCR6^+$ T cell population contained distinct cytokine-producing cells that mainly produced IL-17. In addition, serum CCL20 has been found up-regulated in GPA patients[24]. Our present findings regarding the increase in the frequency of circulating $T_{EM}17$ cells in GPA patients are in line with previous reports[21-24]. Collectively, these results underscore the critical involvement of T_H17 cells in the pathogenesis of GPA, although it remains unclear which mechanisms initiate the T_H17 response in GPA. As described in **chapter 2**, a possible explanation for the expanded $T_{EM}17$ population might be the presence of chronic nasal carriage of *S. aureus* and the aberrant function of T_{REG} cells in GPA patients[7]. Since T_H17 cells participate in the host defense against fungi and extracellular bacteria such an association might be possible. Indeed, it has been demonstrated that *S. aureus*-specific T_H17 cells produce IL-17, and express RORγt and CCR6[44]. Interestingly, it has also been reported that *in vivo* primed *S. aureus*-specific memory T_H17 cells isolated from healthy donors produce IL-17 and IL-10. The production of IL-10 by T_H17 cells was shown to be regulated by the polarizing cytokines produced by monocytes (i.e. IL-6, IL-23 and IL-1β) exposed to *S. aureus* and appeared to be present only in a narrow time window by strongly activated proliferating T_H17 cells[44]. However, our analysis of $T_{EM}17$ associated chemokine receptors did not show a correlation with *S. aureus* carriage in GPA patients[37]. This lack of association may be due to the fact that the selection of GPA patients in this study was not based on a recurrent activity of *S. aureus*. Therefore, the microenvironment needed to initiate skewing towards $T_{EM}17$ cells was, at the time of sampling, not present.

An additional contribution to T_H17 cell skewing may be provided by T follicular helper (T_{FH}) cells. T_{FH} are characterized by the secretion of IL-21. Initially, it was described that these cells provide help to B cells and stimulate humoral responses. However, in addition to the effects on B cells, IL-21 is now known to promote the generation of T_H17 cells as well[45]. Previously, our group demonstrated increased frequencies of IL-21 producing T cells in GPA patients and found a positive correlation between the frequencies of IL-21 producing T cells and IL-17 producing T cells[46].

The observed expansion of the $T_{EM}17$ population may also be influenced by disturbances in the B cell compartment. In particular regulatory B (B_{REG}) cells have been described to modulate effector T cell responses of T_H1 and T_H17 cells and support the differentiation towards T_{REG} cells[47]. The possible interplay between B_{REG} and the $T_{EM}17$ expansion is described in **chapter 6** and discussed below in more detail.

In addition, it has been reported that a persistent (latent) cytomegalovirus (CMV)-infection is associated with changes in the immune phenotype of lymphocytes. CMV infection primarily results in an altered distribution in the memory T cell compartment[48]. In GPA patients, expansion of $CD4^+CD28^-$ T cells is associated with increased risk of infection and mortality and it has been suggested that this expansion is driven by CMV infection[49]. In line with these observations, we observed that CMV seropositive patients had an altered distribution of the $CD4^+$ T_{EM} cell subsets compared to seronegative patients. Remarkably, the proportion of both $T_{EM}1$ and $T_{EM}17$ cells of CMV seropositive GPA patients equaled the $T_{EM}1$ and $T_{EM}17$ proportions present in CMV seronegative and seropositive healthy controls (**chapter 3**) [37]. In contrast, in CMV seronegative GPA patients the $T_{EM}1$ and $T_{EM}17$ showed a strong disease related phenotype pattern with decreased levels of $T_{EM}1$ cells and increased levels of $T_{EM}17$ cells. Therefore, it seems that persistent (latent) CMV carriage in GPA patients normalizes the distribution of $T_{EM}1$ and $T_{EM}17$ cells towards levels present in healthy controls.

The identification of $CD4^+$ T_{EM} subsets based on chemokine receptor expression revealed an aberrant balance between $T_{EM}1$ and $T_{EM}17$ cells of GPA patients in remission. The disturbed balance was shown to be associated with severity of the disease in terms of organ involvement and tendency to relapse. Interestingly, the imbalance between $T_{EM}1$ and $T_{EM}17$ cells is modulated in CMV seropositive GPA patients in remission. Accordingly, it is of great importance for future studies in GPA to carefully stratify and compare patient groups based on the disease activity, disease category (i.e. localized, early systemic, generalized or severe), duration of remission and persistent presence of (latent) infections such as CMV, as these factors may influence the T_H cell repertoire. Eventually, longitudinal studies in properly stratified patients groups that monitor changes in the $CD4^+$ T_H cell profile could be informative to obtain a better understanding of the T_H1, T_H17, and T_{REG} balances when GPA disease progresses.

Selective targeting of effector memory T cells in GPA by a Kv1.3 blocker

Increased $CD4^+$ T_{EM} cells in the urine of GPA patients with active renal involvement and the aberrant distribution of $CD4^+$ T_{EM} cell subsets in the circulation indicates their contribution to disease pathogenesis and their involvement in tissue damage. Therefore, selective targeting of these $CD4^+$ T_{EM} cells may have great added value in the treatment of GPA. Interestingly the activation of the $CD4^+$ T_{EM} cells is uniquely dependent on the voltage-gate Kv1.3 potassium channels[50]. In **chapter 4** we investigated whether inhibiting the activation of $CD4^+$ T_{EM} cells via specific blockade of the Kv1.3 channel using ShK-186 protects against pro-inflammatory effector functions of $CD4^+$ T_{EM} cells in GPA patients.

The voltage-gated Kv1.3 potassium channels are the most prevalent potassium channels expressed by human T lymphocytes[50]. The Kv1.3 channels are important for the biological activity of T_{EM} cells, which are considered major mediators in autoimmune diseases[51, 52]. The engagement of the T cell receptor (TCR) by antigen presenting cells (APC) results in an influx of calcium into the cytoplasm, initially from the endoplasmic reticulum (ER) and subsequently from the extracellular space via the Ca^{2+} release–activated Ca^{2+} channels[50]. Opening of the Kv1.3 channel

in the T cell membrane and the resulting efflux of potassium promotes the entry of calcium to sustain the intracellular calcium levels at concentrations necessary for T cell activation[50]. Resting T cells express low levels of Kv1.3 channels. However, upon activation T_{EM} cells uniquely up-regulate Kv1.3 channel expression whereas the expression level of Kv1.3 on activated naïve T cells and central memory T cells (T_{CM}) equals that of their resting state[51].

Previously, it has been shown that in autoimmune diseases T_{EM} cells expressing high levels of surface Kv1.3 channels are present at sites of inflammation, such as in the synovial fluid of rheumatoid arthritis (RA) patients, and in the circulation as demonstrated in patients with multiple sclerosis (MS) and type 1 diabetes (T1D)[51, 52]. In addition, specific Kv1.3 channel inhibitors have been found to be effective in ameliorating inflammation in numerous animal models of autoimmune diseases including chronic relapse-remitting experimental autoimmune encephalomyelitis (CR-EAE), adoptive EAE, pristane-induced arthritis (PIA), delayed type hypersensitivity (DTH), and anti-glomerular basement membrane (anti-GBM) glomerulonephritis[52-56]. This motivated several research groups to focus on the development of specific and potent Kv1.3 channel inhibitors for the treatment of inflammation and autoimmune diseases. In particular, the identification of the Kv1.3 channel blocker extracted from the Caribbean Sea anemone *Stichodactyla helianthus* toxin termed ShK[57] has led to extensive studies regarding its therapeutic applicability. One striking aspect of ShK is its extreme potency to block voltage-gated potassium channels. In fact, it is the most potent Kv1.3 blocker described thus far[58], but it also potently blocks other channel isotypes expressed in various tissues like the Kv1.1 (cardiac tissue) and Kv1.6 (brain tissue) channels[59-61], raising concerns about potential cardiac and neuronal toxic side effects. However, development of chemically synthesized analogs of ShK has improved the selectivity and stability of the peptide tremendously. The main lead peptide ShK-186 has a > 100-fold increased selectivity for Kv1.3 over Kv1.1 and >1000-fold over Kv1.6[60]. The relative safety of ShK-186 may also be due to the structural composition of Kv1.3 channels on T cells. The Kv1.3 channels expressed on T cells are present as homotetramers whereas Kv1.3 channels expressed on other cell types are present as heteromultimers in which Kv1.3 forms heteromeric channels with other Kv1 subfamilies such as the Kv1.1, Kv1.2, Kv1.4 and Kv1.6 channels as it does for instance in neurons[62, 63]. Furthermore, toxicity and safety studies of ShK-186 showed it to have an excellent safety prolife in animal models[52, 53, 64]. In additon, ShK-186 was reported to exhibit no perceptible *in vitro* toxicity, was negative in the ames test, and had no effect on cardiac parameters[53]. Furthermore, repeated subcutaneous administration of ShK-186 in rats did not cause clinical toxicity as indicated by normal blood cell counts and serum chemistry parameters, and it did not cause any histopathological changes in various tissues examined (brain, heart, lung, kidney) [52, 53]. Moreover, *in vivo* studies have demonstrated that the efficacy of ShK-186 can be achieved without causing general immunosuppression[64]. In rats, administration of ShK-186 did not compromise the protective immune response to acute viral (Influenza) or bacterial (*Chlamydia*) infections at pharmacological doses that did ameliorate autoimmune disease manifestations[64].

In **chapter 4** we demonstrated that the increased pro-inflammatory cytokine production in $CD4^+$ T_H cells from GPA patients was effectively suppressed by ShK-186 *in vitro*. These data are

in line with previous *in vitro* studies showing that ShK-186 preferentially inhibited IL-2, IFN-γ, and TNF-α production by T_{EM} cells derived from the synovial fluid from RA patients, blood derived myelin antigen–specific T cells from MS patients or islet antigen–specific T cells from type 1 diabetes mellitus patients[52]. Additionally, *ex vivo* stimulation of whole blood from cynomolgus monkeys treated with ShK-186 resulted in reduced expression of IL-2, IFN-γ and IL-17[65]. Next, *Chi et al* showed similar *ex vivo* results using human whole blood treated with ShK-186[60]. Remarkably, they observed that ShK-186 was most effective in suppressing the production of IL-2 followed by IFN-γ and IL-17 but was less effective in suppressing IL-4. We observed a similar pattern after *ex vivo* stimulation of whole blood of GPA patients and healthy controls (**chapter 4**). Interestingly, it has been shown that TCR induced Ca^{2+} signaling is lower in T_H2 cells compared to T_H1, T_H17 and, naïve T cells[66, 67]. This suggests that Kv1.3 mediated T cell activation is differentially regulated between T cell subsets which might explain why ShK-186 mediated Kv1.3 channel blockade has a more pronounced effect on the production of the pro-inflammatory cytokines IFN-γ and IL-17 compared to IL-4. However, it has also been reported that ShK-186 significantly reduced IL-4 and IL-5 production by allergen-stimulated peripheral T cells from subjects with asthma, suggesting selectivity towards T_H2 cells in asthma patients[68]. In addition, recent data suggest that antigen-specific T cell are programmed towards Kv1.3 dependency which is enforced by chronic antigen stimulation[69]. Thus, irrespective of the T cell phenotype, the susceptibility to Kv1.3 blockade is a property of repeatedly stimulated antigen-specific T cells. Therefore, T cells derived from autoimmune disease patients chronically exposed to the auto-antigens contain a pool of T_{EM} cells amenable to Kv1.3 inhibition. Future studies in GPA should assess whether PR3 specific autoreactive T cells are more susceptible for Kv1.3 channel blockade compared to conventional $CD4^+$ T_{EM} cells.

Modulation of B cell effector function in GPA by Kv1.3 blocker

Similar to the T cell lineage, Kv1.3 channels may serve as therapeutic targets for modulation of B cell function in autoimmune disorders. Four human B cell subsets can be distinguished based on the expression of IgD and CD27[70]. Mature naive B cells express IgD but not CD27. Acquisition of CD27 following somatic hypermutations results in a $CD27^+IgD^+$ memory B cells subsets[71]. Next, during Ig class switching replacement of the surface IgD with other Ig isotypes yields $CD27^+IgD^-$ class switched memory B cells. These class switched memory B cells are the precursors of the (auto)antibody producing plasma cells and contribute importantly to antibody independent B cell effector functions via secretion of pro-inflammatory cytokines[72]. Hence, class switched memory B cells have been shown to contribute to the aberrant immune response via (auto)antibody-dependent and (auto)antibody-independent functions in MS, T1D, and RA[73, 74, 75]. Interestingly, Kv1.3 channels are highly expressed on the class switched memory B cells while naive and switched memory B cells express low levels of Kv1.3 channels[76].

In **chapter 5** we investigated the effect of Kv1.3 channel blockade using ShK-186 on B cell functions including proliferation, cytokine and (auto)antibody production. We observed that PBMCs from GPA patients treated with ShK-186 have a decreased production of both total and

PR3-ANCA specific IgGs. The reduction in IgG and ANCA was not due to decreased proliferation of total B cells. Furthermore, Kv1.3 channel blockade resulted in a significant decrease in cytokine production of B cells. More specifically, a pronounced effect was observed on the production of the pro-inflammatory cytokines TNF-α, IFN-γ and IL-2, whereas production of the anti-inflammatory cytokine IL-10 was less affected. Consequently, the TNF-α/IL-10 ratio was significantly decreased in the presence of ShK-186 indicating that Kv1.3 blockade skews the B cell response towards a more pronounced regulatory phenotype. However, since these effects were observed on B cells during *in vitro* stimulation of total PBMCs, we cannot exclude an indirect effect of T cells, especially because T cell help is required for the production of IgG. To unravel a direct effect of ShK-186 on B cells, experiments with sorted B cell subsets should be performed.

Effector and regulatory B cells

B cells are central to the pathogenesis of GPA as they are the precursors of the ANCA producing plasma cells. Besides ANCA production, B cells exhibit multiple other functions including antigen presentation and the production of various pro- and anti-inflammatory cytokines[77, 78]. Thus, B cells are involved in the pathogenic effector processes in GPA but may also participate in the regulation of (auto)immune responses. Indeed, cytokine production by B cells influences the T_H responses and it has been reported that pro-inflammatory cytokines (e.g. IL-4 and IFN-γ) regulate T_H1 and T_H2 responses while the anti-inflammatory cytokines (e.g. IL-10) can suppress immune responses and skew T cell towards a regulatory phenotype[47, 79-81]. As such, the so-called regulatory B (B_{REG}) cells have been described to inhibit the production of pro-inflammatory cytokines from monocytes and effector T_H cells and to support the function of regulatory T cells[82, 83]. Therefore, considering the effector and regulatory function of B cells, the question remains whether complete depletion of B cells following rituximab (RTX) treatment is the best treatment option. It might be desirable to specifically target the effector B cells without impairing the regulatory B cell compartment.

Although B and T cells have their unique effector function in physiological immune responses, these cells do not act separately. To mount an optimal immune response, a close interplay between B and T cells, either via direct cell contact or cytokine secretion, is necessary. In this context, B cells with immune regulatory properties, termed regulatory B (B_{REG}) cells, have gained increasing interest in recent years. In particular, B_{REG} cells that can be phenotypically identified as IL-10 producing $CD24^{hi}CD38^{hi}$ B cells were demonstrated to exert immune-regulating properties[82]. The mechanism of B_{REG}-mediate suppression occurs primarily via the production of IL-10. As such, IL-10 producing B_{REG} cells have been shown to inhibit the activation of T_H1 responses, inhibit the differentiation into T_H17 cells, and to convert $CD4^+$ T cells into regulatory T cells[83, 84]. In addition, besides the anti-inflammatory mediator IL-10, engagement of costimulatory molecules like CD80 and CD86 on B_{REG} cells also enhances the inhibition of T_H1 responses[83]. Furthermore, an aberrant distribution and function of $CD24^{hi}CD38^{hi}$ B_{REG} cells is associated with progression of several autoimmune disease including SLE and RA[83, 84]. These studies have demonstrated that these impaired B_{REG} cells from SLE and RA patients also failed to suppress T_H1 and T_H17 responses

and to convert $CD4^+$ T cells into T_{REG} cells. Therefore, B_{REG} cells may be important in dampening the inflammatory responses in autoimmune diseases.

In AAV it has been reported that the frequency of circulating B_{REG} cell is significantly decreased, although their function in terms of IL-10 production and suppression of immune cell activity is not compromised[85]. In **chapter 6** we investigate whether the decreased proportion of B_{REG} cells may explain the enhanced T_H17 cell response in GPA patients. We showed a significant inverse correlation between $T_{EM}17$ cells and B_{REG} in GPA patients, which suggests that a decrease B_{REG} cell populations allows the T_H17 cells to expand. Moreover, in co-culture experiments we demonstrated a significant increase in the frequency of $IL\text{-}17^+$ producing T_H cells in B_{REG} depleted cultures compared to the undepleted B_{REG} cultures. This observation is in line with other studies that indicate similar interactions between B_{REGs} cells and T_H cells[83, 84]. These studies reported that B_{REG} cells were able to decrease cytokine production of T_H cells and suppress their differentiation. Furthermore, a possible link between B_{REG} and T_H17 cells may be derived from data of rituximab treated patients. It has been demonstrated that RTX reduces the T_H17 response in RA patients, but does not influence T_H1 cells, T_{REG} cells, and TNF-α responses[86]. In addition, the inhibition of the T_H17 response by RTX was lost in the absence of B cells, supporting the contention that B cells directly affect T_H17 responses. When B cells reconstitute following B cell depletion by RTX, $CD24^{hi}CD38^{hi}$ B_{REG} form the initial (immature) B cell subset to repopulate the B cell compartment and may even become the dominant circulating B cell subset[87, 88]. Thus, enrichment of the $CD24^{hi}CD38^{hi}$ B_{REG} subset upon B cell reconstitution after RTX treatment may affect T_H cell distribution with a major effect on T_H17 cells[86]. However, the way in which B_{REG} cells inhibit T_H17 responses in inflammatory conditions remains to be elucidated. Yet, the suppressive functions of B_{REG} cells are most likely mediated via the engagement of a combination of several molecules including CD40 TLR and BCR signaling as well as CD80, CD86, and the production of immune regulatory cytokine such as IL-10, TGF-β and IL-35[89].

Of note, the $CD24^{hi}CD38^{hi}$ B_{REG} cells can be identified within the naïve or transitional B cell compartment[70, 83]. These B cells lack the expression of CD27. Interestingly, it has been demonstrated that the $CD27^-$ B cells have lower numbers of Kv1.3 channels compared to the class switched memory B cells and therefore are likely to be less sensitive to Kv1.3 channels blockade[76]. Future studies are required to elucidate whether indeed B_{REG} cells escape the effects of ShK-186 due to lower numbers of Kv1.3 channels expressed and continue to exert their suppression function.

B and T cell targeted therapies in GPA: Implications and future perspectives

The importance of targeting the effector $CD4^+$ T_{EM} cells and effector memory B cells is emphasized by the fact that current treatments are directed against either the full spectrum of the immune system by using broad acting immune suppressive drugs, or T / B cell directed therapies that block the activation and/or migration of all T / B cell populations. In GPA, several T cell directed therapies are currently under investigation including drugs that either deplete T cells, block the pro-inflammatory effect of TNF-α, or inhibit co-stimulatory signals needed for

T cell activation. For example, alemtuzumab is a humanized anti-CD52 monoclonal antibody (CAMPATH-1H) capable of selectively depleting peripheral circulating T lymphocytes, monocytes and macrophages. Alemtuzumab has been studied in a small uncontrolled trial of AAV patients in whom all immunosuppressant drugs had been discontinued (except for low dose (<10mg/day) prednisolone) [90]. The majority of patients achieved clinical remission, but a significant proportion of these patients relapsed at a median time interval of 9 months after completion of the therapy. Also, adverse events such as severe infection and malignancies were reported in the alemtuzumab treated group[90].

Another approach to influence T cells responses in GPA is via interference with co-stimulatory molecules. The costimulatory blocker abatacept is a fusion protein of the protein cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and the immunoglobulin Fc region. Abatacept inhibits costimulatory signaling and consequently inhibits the activation of T cells. In a small, open-label prospective trial in GPA patients addition of abatacept to standard maintenance treatment with glucocorticoids was well tolerated and associated with a high frequency of disease remission[91]. An additional clinical trial with abatacept for the treatment of relapsing and non-severe GPA patients is currently running (ClinicalTrials.gov, NCT02108860).

Besides targeting the T cells, trials have been conducted against individual cytokines that are considered to play a major role in the pathogenesis of AAV. For instance, the efficacy of anti-TNF-α treatment using etanercept (ETA) or infliximab (IFX) has been investigated in AAV[92, 93]. In the Wegener's Granulomatosis Etanercept Trail (WGET), ETA was evaluated as adjuvant treatment to standard therapy. This trial showed that ETA was not effective for the maintenance of remission in patients with GPA[92]. In addition, there was a high rate of treatment-related complications in the group that had received ETA including a higher incidence of solid malignancies[92, 94]. Although AAV patients treated with IFX have been reported to enter clinical remission, an additional clinical benefit of IFX over standard therapy was not reached[93]. As such the addition of IFX to standard therapy did not influence remission rates, adverse events, or relapse rates. Furthermore, a comparison between IFX with RTX for remission induction in patients with severe refractory GPA favors the use of RTX[95]. These data together with the results of the WGET study results have led to the abandonment of both ETA and IFX for the treatment of AAV.

Treatment strategies aimed to affect B cells in autoimmune diseases comprise anti-BAFF with or without simultaneous blockade of a proliferation-inducing ligand (APRIL) (i.e. belimumab, tabalumab and atacicept) but, so far, have failed to show clinical efficacy in trials in SLE, MS, and RA patients[96-100]. Recently, a trial in AAV patients with anti-BAFF therapy has been completed but results have not been reported yet (ClinicalTrials.gov, NCT01663623). Although these agents are directed against mature B cells and short-lived plasma cells, the memory B cells are not affected[101]. Especially class switched memory B cells appear to be resistant to BAFF depletion[102].

Anti-CD20 B cell depleting studies using rituximab show beneficial outcomes for patients with GPA and RA[103-105]. However, disease flares have been observed after reconstitution of B cells, accompanied by an increase in ANCA levels[106]. Also, a concern of long-term RXT treatment is the increased risk of adverse effects. In particular persistent hypogammaglobulinemia and low

$CD4^+$ cell counts have been linked to recurrent infections in RTX-treated patients[107]. Overall, the therapeutic agents currently under investigation, some more promising than others, do not adequately target either the effector $CD4^+$ T_{EM} or the effector class switched memory B cells.

In contrast to the therapeutic strategies described above, ShK-186 is unique in blocking a subset of the chronically activated T cell population, the $CD4^+$ T_{EM} cells, and effector memory B cells, the class switched memory B cells, both involved in the pathogenesis of GPA. Moreover, the use of ShK-186 in *in vitro* and *in vivo* models showed that other T and B cell populations (i.e. naïve T cells, T_{CM}, naïve B cells, and unswitched memory B cells) remain largely unaffected[52, 76]. Treatment with ShK-186 inhibits the production of pro-inflammatory cytokines and therefore may have a more potent effect than therapies directed against a single cytokine. It may also constitute a much more specific therapy by targeting the most potent pathogenic T and B cell subsets in GPA (**chapter 4 and 5**). Currently, ShK-186 has been given the FDA approved name dalazatide and has been studied in a phase 1a clinical trial in healthy volunteers (ClinicalTrials.gov, NCT02446340) and in a phase 1b clinical trial with psoriasis patients (ClinicalTrials.gov, NCT02435342). Both trials were designed to evaluate the safety, tolerability, and pharmacodynamics of dalazatide. These first clinical trials with dalazatide indicate that the drug is safe and well tolerated. In the phase 1b trial, psoriasis patients treated twice weekly with dalazatide showed improved skin lesions associated with reduced plasma levels of multiple inflammatory markers and a reduced expression of T cell activation markers on the peripheral blood memory T cells of these patients[108]. This observation highlights that twice weekly administration of ShK-186 is sufficient to achieve blood levels required for suppressing $CD4^+$ T_{EM} cells. Moreover, the study showed that the therapeutic potential of dalazatide, which is an unconjugated small molecular mass peptide, is viable. This avoids additional engineering costs to enhance the pharmacokinetic and pharmacodynamic properties keeping the overall expenses low in comparison to other currently developed biologicals. In addition, based on the durable pharmacological responses and therapeutic effects of ShK-186, treatment could be paused in the event of an acute infection which would be an added benefit compared to current treatments in GPA (i.e. cyclophosphamide, high dose corticosteroids and rituximab) of which the effects take several months to subside. To date, dalazatide awaits further phase 2 trials for several systemic autoimmune diseases.

Although the list of treatment possibilities for GPA and autoimmune diseases in general offers interesting alternatives for these patients, the disease-causing cells such as the auto reactive T cells and especially the auto reactive B / plasma cells responsible for ANCA production remain largely unaffected. Interestingly, intriguing developments are taking place at present in the field of immune-oncology with regard to the use of cell-based immunotherapies. Studies show that the anti-tumor function of T cells can be genetically enhanced via the addition of chimeric antigen receptors (CARs). CARs are immunoreceptors consisting of the antigen-recognition domain of an antibody linked to the cytoplasmic T-cell signaling domain and co-stimulatory domains. The CAR-T cells are designed to recognize extracellular tumor-associated antigens and eliminate the tumor cells. Currently, the most notable example of CAR-T cell therapy are the CAR-T cells that target CD19. These CAR-T cells have shown remarkable antitumor activity in

patients with refractory B-cell malignancies such as acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL) [109, 110]. Theoretically, a similar strategy could also be applied to target and eliminate autoreactive B cells. In a first proof of principle study, *Ellebrecht et al.* reengineered CAR T cells into chimeric autoantigen receptor (CAAR) T cells designed to specifically eliminate desmoglein 3 (Dsg3)-specific autoreactive B cells responsible for the blistering autoimmune disease pemphigus vulgaris *in vivo*[111]. Using a murine model, the authors demonstrated that Dsg3 CAAR-T cells exhibited specific cytotoxicity against B cells bearing anti-Dsg3 B cell receptors *in vitro* and specifically eliminated Dsg3-specific B cells *in vivo*. Since for GPA the auto-antigens (i.e. PR3 or MPO) are known it is intriguing to consider such a CAAR T cell approach to eliminate PR3 or MPO autoreactive B cells in AAV as well.

Overall, future therapeutic strategies in AAV, whether they make use of small peptides, biologicals or live T cell-based therapies, should target either specific cellular players or other disease specific mechanisms aimed to improve specificity and reduce treatment related toxicity.

Concluding remarks

The data presented in this thesis contribute to our knowledge on the T and B cell effector functions in GPA. We demonstrate an aberrant balance between the T_H1 and T_H17 cells within the $CD4^+$ T_{EM} cell compartment in GPA patients compared to healthy controls. The disturbed balance of T_H1 and T_H17 cells appears to be associated with severity of the disease. In addition, we showed that a diminished B_{REG} cell number may contribute to the increased T_H17 response in GPA patients.

Considering the high expression of Kv1.3 channels on the surface of $CD4^+$ T_{EM} cells and switched memory B cells combined with their involvement in the pathogenesis of GPA, specific Kv1.3 blockade could be an attractive therapeutic option. Here, we showed that the Kv1.3 blocker ShK-186 (dalazatide) modulates pro-inflammatory effector functions of blood derived $CD4^+$ T cells and B cells from GPA patients *in vitro*. For $CD4^+$ T cells, ShK-186 predominantly inhibited cytokine production of $CD4^+$ T_{EM} cells, whereas for B cells we showed that ShK-186 skews these cells towards a more pronounced regulatory B cell response. However, given the limited data available to date, future studies should investigate whether and how ShK-186 treatment affects the function of regulatory T and B cells directly. Also, the long-term immunomodulatory effects of ShK-186 should be studied since long-term ShK-186 treatment could polarize effector T and B towards an anti-inflammatory regulatory T and B cell profile. In this context, *in vivo* studies using the experimental autoimmune vasculitis rat model for ANCA-associated vasculitis might be helpful to study long-term immunomodulatory effects of ShK-186[112]. Such studies may provide valuable information on whether an enhanced regulatory immune profile is safe.

In conclusion, the results presented in this thesis support the contention that selective Kv1.3 channel blockade using ShK-186 may be an attractive therapeutic option for T and B effector subset-selective immunomodulation in GPA. Further *in vitro* and *in vivo* studies are necessary however, to firmly establish that Kv1.3 blockade is an effective and safe treatment strategy for GPA.

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