CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

ANCA-associated vasculitis

ANCA-associated vasculitides (AAV) are a group of autoimmune diseases characterized by a chronic, and often systemic, inflammation of medium- to small-sized blood vessels [1]. AAV encompasses three clinically defined disorders: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) [1]. In the majority of patients, the disease is hallmarked by the presence of anti-neutrophil cytoplasmic antibodies (ANCA). These autoantibodies are considered to play an important role in the pathogenesis of the diseases [2]. In AAV, ANCA are mainly directed against proteinase-3 (PR3) and myeloperoxidase (MPO) [3].PR3 and MPO are enzymes present in cytoplasmic granules of neutrophils and become accessible for circulating ANCA on the surface of neutrophils after pre-activation (priming) of these cells. Generally, PR3-ANCA are present in the majority of patients with GPA, whereas in MPA patients MPO-ANCA is more prevalent [4]. Patients with PR3-ANCA and MPO-ANCA are also characterized by differences in their clinical presentation. PR3-ANCA is strongly associated with granulomatous inflammation of the upper and lower respiratory tract and a more systemic presentation of the disease frequently involving the kidney whereas patients with MPO-ANCA often present with a renal limited form of vasculitis [5].

Below, I briefly introduce the cellular players that fulfill central roles in the pathogenesis of AAV and discuss how selective targeting of these pathogenic immune cells may hold therapeutic promise for patients with AAV, focusing mainly on GPA patients.

AAV pathogenesis

The etiopathogenesis of AAV is not completely understood. However, multiple cellular players have been proposed to be involved including i) neutrophils, expressing the ANCA target antigens, ii) B cells, being responsible for the production of ANCA, and iii) T cells, mediating the (auto)-inflammatory response in disease development [6].

In AAV pathogenesis, it has been suggested that pro-inflammatory factors e.g. released due to an infection, trigger the disease. In particular, *Staphylococcus aureus* (*S. aureus*) has been shown to be an important risk factor for the occurrence of relapses in AAV and anti-bacterial treatment is beneficial in reducing the relapse rate in these patients [7, 8]. Pro-inflammatory cytokines and chemokines that are released as a result of local or systemic infection cause priming of neutrophils, upregulation of endothelial adhesion molecules, and an expansion of circulating effector T cells. Neutrophil priming results in translocation of the ANCA antigens (i.e. PR3 and MPO) from their lysosomal compartments to the cell surface. Engagement of the ANCA with either PR3 or MPO on the cell surface and interaction of the Fc part of the antibody with Fc receptors activates neutrophils. This causes increased neutrophil adherence to the endothelium and transmigration through the vessel wall. ANCA-mediated neutrophil activation also triggers the production of reactive oxygen species (ROS) and induces neutrophil degranulation of proteolytic enzymes causing vessel wall damage. Meanwhile, the injury to the vessel wall in combination with the pro-inflammatory triggers elicit an adaptive inflammatory immune response recruiting T cells that further contribute to the development of vasculitis. Additional disbalances in the T cell

compartment result in further release of pro-inflammatory cytokines promoting neutrophil priming and persistent activation of T cells that sustain the vascular inflammatory response in AAV.

T cell involvement in AAV

Besides ANCA mediated neutrophil responses, the pro-inflammatory environment that is created will also attract T cells from the adaptive immune system. The involvement of $CD4^+$ T helper (T_H) cells in the pathogenesis of AAV, in particular GPA, is supported by several observations. First, abundant T cell infiltrates can be detected in inflammatory lesions found in AAV and $CD4^+$ T cells are a prominent component of the granuloma frequently observed in GPA [9]. Second, soluble T cell activation markers are elevated in serum and plasma of AAV patients compared to controls and are associated with disease activity [10, 11]. Third, ANCA antigen specific T cells have been detected in the circulation of AAV patients [12, 13]. Fourth, the IgG subclass distribution of ANCA with a predominance of IgG1 and IgG4 subclasses implies isotype switching indicating a T cell dependent immune response [14]. Collectively, these observations make it highly likely that T cell mediated inflammatory responses contribute importantly to AAV pathogenesis.

The $CD4^+$ T cell population can be separated into four distinct subsets based on the surface expression of the phosphatase CD45RO and the lymph node homing chemokine receptor CCR7 [15]. Naïve T cells receiving a relatively weak T cell receptor (TCR) signal and antigen presenting cell-derived co-stimulation will proliferate and differentiate into long lived central memory T (T_{CM}) cells, whereas strong TCR stimulation or prolonged repeated stimulations favors the differentiation into effector memory T (T_{EM}) cells. Naïve T cells and T_{CM} cells express CCR7 and efficiently home to the lymph nodes and exert limited effector functions upon antigen exposure. T_{EM} cells lack the expression of CCR7 but express other chemokine receptors that facilitate migration to non-lymphoid sites of inflammation. These cells are poised for a rapid response to repeated antigen exposure by the production of effector cytokines. Therefore, it is plausible that $CD4^+$ T_{EM} cells may directly contribute to tissue injury and disease progression in GPA [15, 16].

In GPA, a persistent expansion of $CD4^+$ T_{EM} cells has been observed in the peripheral blood of GPA patients in remission, but not in GPA patients with active disease [17]. A follow up study revealed the presence of $CD4^+$ T_{EM} cell in the urine of GPA patients, indicating that $CD4^+$ T_{EM} cells migrate from the circulation to inflammatory lesion during active episodes of the disease [18]. Moreover, the phenotypes of T cells found locally at the inflammatory sites in lung and kidney tissues mainly resemble those of memory T cells [9, 19].

The $CD4^+$ T_{EM} cell population consists of different lineage-committed T_H cell phenotypes that can be distinguished according to surface makers and secreted signature cytokines. These phenotypes include T_H1 cells; characterized by CXCR3 and IFN-γ, T_H2 cells; characterized by CRTh2 and IL-4, T_H17 cells; characterized by CCR6 and IL-17, and, regulatory T (T_{REG}) cells; characterized by CD25 and the transcription factor FoxP3 [20]. In AAV, the T_H cell polarization deviates from the healthy situation depending on disease state (i.e. active disease vs remission) and/or disease category (i.e. localized vs generalized). For instance, a T_H1–type response is predominant in GPA

patients with localized disease, whereas a T_H2-response is associated with more generalized disease [21-24]. Furthermore, increased T_H17-type responses reflected by elevated levels of IL-17 and the presence of auto-antigen specific T_H17 cells are observed in GPA patients [25, 26]. Together, these T_H responses contribute to the pro-inflammatory effector response involved in the pathogenesis of GPA.

In addition, $CD4^+$ T_{EM} cells also display cytotoxic features similar to natural killer (NK) cells [27]. They have been shown to mimic features of NK cells by their surface expression of the natural killer group 2 member D (NKG2D). NKG2D can mediate cytoxic responses and tissue damage through specific interaction with its ligand MICA expressed on target cells [28, 29]. Interestingly, it has been reported that NKGD2 is prefentaily expressed on circulating $CD4^+$ T_{EM} cells and both NKG2D and MICA are expressed in the granulomatous lesion in GPA patients [30]. It is likely that killing mechanisms via NKG2D-MICA interaction contribute to vessel injury and disease progression in AAV-patients.

The observed abnormalities of the expanded $CD4^+$ T_{EM} cell compartment in GPA patients are in part attributed to deregulated expression of cytokines but may also be influenced by aberrant functioning of T_{REG} cells. Under normal physiological conditions T_{REG} cells have the capacity to suppress the activation, proliferation and effector fucntions of $CD4^+$ T_H cells. However, in AAV several studies have reported an impaired functionality of the T_{REG} cells demonstrating that T_{REG} from AAV patients are not able to suppress the proliferation of $CD4^+$ T_{EM} cells [31-33]. Thus, the dysfunctional T_{REG} cells may cause expansion of the $CD4^+$ T_{EM} population and disbalances in effector cells. To date, the underlying mechanisms responsible for the functional impairment of T_{REG} cells in AAV patients remains unclear.

Taken together, the persistent expansion of the $CD4^+$ T_{EM} cells in combination with the lack of inhibitory mechanisms by T_{REG} cells may promote T_{EM} cell effector functions and migration to inflamed tissues in AAV. Therefore, $CD4^+$ T_{EM} cells constitute a potentially interesting cellular target for pharmacological intervention in GPA patients.

B cell involvement in AAV

In AAV pathogenesis B cells are considered crucial because these cells are the precursors for plasma cells that produce the ANCA (like the PR3-ANCA IgG in GPA patients). However, accumulating evidence indicates that beside their antibody-producing role, B cells also exert multiple other functions that influence immune responses. B cells are effective antigen presenting cells (APCs) and can regulate T cell responses by providing co-stimulatory signals and secretion of cytokines [34, 35]. Depending on the cytokines secreted, B cells can be divided into effector cells producing pro-inflammatory cytokines or regulatory B (B_{REG}) cells producing anti-inflammatory cytokines. Effector B cells can stimulate T_H1 and T_H17 cell responses by the secretion of IFN-γ, IL-6, and, TNF-α [36, 37]. These effector cytokines (i.e. TNF-α) are mainly produced by B cells from the memory B cell compartment, including unswitched memory B cells ($CD27^+IgD^+$) and class-switched memory B cells ($CD27^+IgD^-$) [38]. In GPA patients it has been found that both memory B cell phenotypes are decreased in the peripheral blood irrespective of the disease state [39]. Currently the reason for

this decrease remains unclear. However, B cells have been detected in the granulomatous lesions of AAV patients [40]. Therefore, it cannot be excluded that the memory B cells migrate from the circulation to sites of inflammation and exert their effector functions locally similar as the $CD4^+$ T_{EM} cells.

In contrast to their pro-inflammatory effector functions, B cells can also present anti-inflammatory regulatory functions via secretion of IL-10 and TGF-β that inhibit T_H cell responses and modulate the number of T_{REG} cells [41, 42]. Currently, the phenotypic identification of B_{REG} cells remains controversial and relies on the detection of IL-10 production. However, it has been suggested that B_{REG} cells can be identified based on the high expression of surface CD24 in combination with either CD38 or CD27 [43]. Interestingly, studies have shown that alterations in numbers and/or function of $CD24^{hi}CD38^{hi}$ B_{REGS} are associated with progression of several autoimmune diseases and these cells are able to inhibit $CD4^+$ T_H responses [44, 45]. Therefore, similar to $CD4^+$ T_{EM} cells, it could be beneficial to selectively target the pro-inflammatory effector B cells within the memory B cell compartment without impairing the regulatory function of B cells.

Current therapy

The current treatment recommendations in the management of AAV are based on the severity of the disease and the organs involved. High dose glucocorticoids in combination with cyclophosphamide (CYC) is generally used as treatment for induction of remission in AAV patients [46, 47]. Alternatives to CYC such as methotrexate (MTX) or mycophenolate mofetil (MMF) are, compared to CYC, inferior for induction of remission in patients with either non-severe disease or patients that do not tolerate CYC well [48, 49]. More recently, B cell depletion by rituximab (RTX, anti-CD20) treatment has been shown to be equally efficacious as CYC for induction of remission in AAV [50, 51]. Subsequent to the initial therapy aimed to induce remission, patients receive maintenance therapy to prevent disease relapses. The maintenance treatment regimen consists of azathioprine (AZA) or MMF often in combination with low-dose glucocorticoids [52, 53]. MTX is another option for maintenance treatment and has been shown to be similarly effective in sustaining remission compared to AZA but tends to be associated with more severe adverse events in AAV patients [54]. Interestingly, recent data indicate that RTX is superior to AZA in maintaining CYC induced remission [55]. However, optimal dosing regimens, long-term safety and efficacy, as well as cost effectiveness have still to be addressed.

Overall, these current treatment strategies in the management of AAV have changed AAV from fatal diseases to chronic (relapsing) diseases. However, many patients still experience relapses during the course of their disease [56, 57]. PR3-ANCA patients especially are at risk for disease relapse, which in 30-50% of the patients occurs within 5 years after diagnosis [58]. Renewed disease activity exposes patients to more immunosuppressive therapy and accumulating organ damage. Therefore, new treatment options are needed to avoid drug related toxicity and prevent the accumulation of organ damage due to the chronic course of the (frequently) relapsing disease. Preferably, such new treatments should specifically target pathogenic cellular players involved in the pathophysiology of AAV.

Ion channels on Lymphocytes

As described above, neutrophils, T cells and, B cells are closely connected in the pathogenesis of AAV. In particular, the effector T and B cells position themselves as interesting therapeutic targets because of their pro-inflammatory properties. Ion channels comprise a network that perform vital functions in the cellular homeostasis, activation and differentiation of T and B lymphocytes[59]. Of particular interest are potassium (K^+) channels that serve to regulate the membrane potential and calcium signaling in lymphocytes. Human T lymphocytes express two types of K^+ channels, namely the voltage-gate Kv1.3 potassium channel and the calcium-activated KCa3.1 potassium channel. Moreover, the expression of Kv1.3 and KCa3.1 channels on T lymphocytes depends on the state of activation and differentiation of a given T lymphocyte subset [60].

T cells at each differentiation state have either a quiescent or activated state when encountered by an antigen. Patch clamp analysis revealed that quiescent T naïve, T_{CM}, and T_{EM} cells express about 200 – 300 Kv1.3 channels and 5 – 35 KCa3.1 channels per cell (table 1) [61]. The expression-pattern of these channels changes upon T cell activation, leading to altered channel phenotypes in the different T cell subsets. Activated T naïve and T_{CM} cells upregulate KCa3.1 channels to 500 channels per cell, whereas T_{EM} cells increase Kv1.3 expression to 1500 channels per cell with little change in KCa3.1 expression levels [61]. The switch of potassium channel phenotype significantly affects the responsiveness of these cells to Kv1.3 or KCa3.1 blockers. Therefore, T_{EM} cells are highly sensitive to Kv1.3 channels blockers, while T naïve and T_{CM} cells are more sensitive to KCa3.1 channel blockers.

One of the earliest events in T cell activation is the increase in intracellular calcium concentrations [62, 63]. The Kv1.3 channels play a critical role in this process [64]. Antigen presentation to the T cell receptor leads to rapid release of calcium from endoplasmic reticulum (ER) stores. Depletion of the ER Ca^{2+} stores causes Ca^{2+} release-activated calcium (CRAC) channels to open in the membrane ensuring extracellular calcium to enter the cell. The influx of Ca^{2+} raises the intracellular calcium concentration that subsequently culminates in cell activation and proliferation. The large influx of calcium through the CRAC channels induces cell depolarization, which, if left unchecked, induces a reduction in calcium influx. However, the driving force for calcium entry is restored by membrane hyperpolarization induced by the efflux of potassium through the Kv1.3 and KCa3.1 channels. The tight interplay between calcium influx through CRAC channels and potassium efflux through Kv1.3 and KCa3.1 channels underlies the oscillating changes in calcium concentrations necessary for T cell activation.

Similar to T cells, human B cells undergo a comparable differentiation process. Naïve B cells (IgD^+CD27^-) differentiate into unswitched memory B cells (IgD^+CD27^+), and upon repeated stimulation these cells differentiate further into class-switched memory B cells (IgD^-CD27^+) lacking IgD but expressing either IgG, IgA, or IgE on their cell surface. The expression of Kv1.3 and KCa3.1 channels on B cells is identical to the channel expression patterns during differentiation and activation as it does for T cells [65]. Naïve and unswitched memory B cells, like their T cell counterparts (T naïve and T_{CM} cells), up-regulate KCa3.1 channels upon activation,

whereas class-switched memory B cells, like T_{EM} cells, up-regulate Kv1.3 channels upon activation [65]. Interestingly, quiescent class-switched memory B cells express much higher Kv1.3 levels compared to quiescent T_{EM} cells (table 1).

Like T cells, the pharmacological sensitivity of B cells to potassium channel blockers parallels their potassium channel expression pattern. KCa3.1 specific blockers inhibit the proliferation and activation of naïve and unswitched memory B cells, whereas Kv1.3 blockers suppress the proliferation and activation of class-switched memory B cells [65].

Table 1 | Number of Kv1.3 and channels per cell in T and B cell subsets

Lymphocyte	Potassium Channel	Naïve T cells ($CD45RO^-CCR7^+$)		T_{CM} cells ($CD45RO^+CCR7^+$)		T_{EM} cells ($CD45RO^+CCR7^-$)	
		Quiescent	Active	Quiescent	Active	Quiescent	Active
T cell	Kv1.3	300	300	300	300	300	1500
		Naïve B cells (IgD^+CD27^-)		**Unswitched memory B cells (IgD^+CD27^+)**		**Class-switched memory B cells (IgD^-CD27^+)**	
		Quiescent	Active	Quiescent	Active	Quiescent	Active
B cell	Kv1.3	100	90	255	180	2430	3270

T_{EM} cells are main players in mediating the pathophysiological processes of various autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, Type 1 diabetes mellitus, as well as in GPA [61, 66, 67]. As described above, these T_{EM} cells express high numbers of Kv1.3 channels upon activation, which lend themselves to selective targeting by Kv1.3 channel blockers [61, 66]. Targeting these T_{EM} cells without affecting the T naïve and T_{CM} cells represents a promising and more specific way for treating autoimmune diseases avoiding generalized immunosuppression. Furthermore, the Kv1.3 channels expressed as homotetramers, have a functionally restricted tissue distribution for lymphocytes, and therefore represent attractive therapeutic targets in T_{EM} cell mediated autoimmune disorders.

Kv1.3 inhibitors are found in many venoms including that of sea anemones. In 1995, a potent potassium channel blocker was extracted from the Caribbean sea anemone *Stichodactyla helianthus* and termed *Stichodactyla helianthus* K^+ channel toxin (ShK) [68]. Soon after the discovery of the native peptide, the peptide was successfully synthesized, and its three-dimensional structure was determined [69]. Further extensive studies of its structure, selectivity, biological activity in conjunction with the generation of analogs with increased selectivity and stability, resulted in a synthetic form of ShK that blocks the Kv1.3 channels in T_{EM} cells with picomolar affinity [70]. Subsequently, several studies demonstrated that activation of disease-associated (autoreactive) T_{EM} cells can be inhibited by a ShK-mediated Kv1.3 blockade. Selective blockade of the Kv1.3 channels has proven efficacious in preventing and/or treating animal models of delayed type hypersensitivity, type 1 diabetes, rheumatoid arthritis and multiple sclerosis without inducing generalized immunosuppression [66, 71-73].

Aim and outline of this thesis

Advances in the treatment of GPA have led to increased patient survival. However, the prolonged exposure of patients to generalized immunosuppressive therapy carries a heavy burden of adverse events including opportunistic infections and drug related toxic effects. To minimize or circumvent these therapy related adverse effects, tapering or discontinuation of treatment is required. Consequently, GPA patients suffer from frequent disease relapses where each relapse is associated with the risk of cumulative organ damage. This emphasizes the need for improved treatment strategies that are more specific and less toxic for GPA patients. Such improved therapeutic options should preferably be directed to the key cellular players in GPA pathogenesis.

The main aim of this thesis was to investigate the effect of the highly specific Kv1.3 channel inhibitor ShK-186 on the effector functions of $CD4^+$ T_{EM} cells and B cells to provide proof of principle for Kv1.3 blockade as a potential novel treatment strategy for GPA with high specificity towards these pathogenic cellular players. The effector functions of T and B cells were determined in GPA patients and it was investigated whether specific blockade of Kv1.3 channels was effective in reducing the pro-inflammatory functions of these cells. In addition, we characterized the phenotype of circulating $CD4^+$ T_{EM} subsets in GPA patients in relation with the clinical presentation of the disease in these patients. Finally, a potential interplay between regulatory B cells and the expanded T_H17 population in GPA patients was investigated.

In **chapter 2**, we reviewed the literature regarding the role of T cells in systemic autoimmune and chronic inflammation. The current knowledge regarding the behavior of T cells in these two distinct inflammatory conditions was discussed to illustrate the characteristics of T cell features in AAV and atherosclerosis. Particular attention was given to the different T cell phenotypes, the role of effector memory T cell responses and the modulation of the effector T cell responses.

In **chapter 3** we investigated the distribution of differentiated T cell phenotypes based on the co-expression of chemokine receptors. We delineated differences in the distribution of $CD4^+$ T_{EM} phenotypes and analyzed whether these cells associated with the heterogeneous clinical presentation of the disease.

Chapter 4 assessed the effect of the Kv1.3 channel blocker (ShK-186) on the pro-inflammatory properties of $CD4^+$ T_{EM} cells from GPA patients compared to $CD4^+$ T_{EM} cells from healthy individuals *in vitro*.

Besides T cells, Kv1.3 channels are expressed on all B cells among which class-switched memory B cells in particular express high levels of Kv1.3 channels. Therefore, in **chapter 5**, we characterized the distribution of circulating B cell subsets in GPA patients and studied the effect of ShK-186 on B cell cytokine production, proliferation and (PR3-ANCA) IgG production.

Accumulating evidence indicates that B cells modulate T cell responses. In particular, a subset of B cells characterized by high expression of CD24 and CD38, termed regulatory B cells (B_{REG}), has been reported to exert regulatory functions modulating the distribution of $CD4^+$ T_H subsets. In **chapter 6** we hypothesized that numerical alterations in $CD24^{hi}CD38^{hi}$ B_{REG} cells may explain the expansion of the T_H17 population in GPA patients. To test this hypothesis, we assessed the

frequency of circulating $CD24^{hi}CD38^{hi}$ B_{REG} cells and T_H17-cells in GPA patients and investigated the functional impact of these B_{REG} cells on the expanded frequency of the T_H17-cells *in vitro*. Finally, **chapter 7** summarizes and discusses the main findings and future perspectives of the research presented in this thesis in the context of the current literature.

REFERENCES

1. Jennette, J. C. *et al.* 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum.* 65, 1-11 (2013).
2. Jarrot, P. A. & Kaplanski, G. Pathogenesis of ANCA-associated vasculitis: An update. *Autoimmun. Rev.* 15, 704-713 (2016).
3. Kallenberg, C. G., Heeringa, P. & Stegeman, C. A. Mechanisms of Disease: pathogenesis and treatment of ANCA-associated vasculitides. *Nat. Clin. Pract. Rheumatol.* 2, 661-670 (2006).
4. Franssen, C. F. *et al.* Antiproteinase 3- and antimyeloperoxidase-associated vasculitis. *Kidney Int.* 57, 2195-2206 (2000).
5. Hilhorst, M., van Paassen, P., Tervaert, J. W. & Limburg Renal Registry. Proteinase 3-ANCA Vasculitis versus Myeloperoxidase-ANCA Vasculitis. *J. Am. Soc. Nephrol.* 26, 2314-2327 (2015).
6. Chen, M. & Kallenberg, C. G. ANCA-associated vasculitides--advances in pathogenesis and treatment. *Nat. Rev. Rheumatol.* 6, 653-664 (2010).
7. Stegeman, C. A. *et al.* Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis. *Ann. Intern. Med.* 120, 12-17 (1994).
8. Zycinska, K., Wardyn, K. A., Zielonka, T. M., Krupa, R. & Lukas, W. Co-trimoxazole and prevention of relapses of PR3-ANCA positive vasculitis with pulmonary involvement. *Eur. J. Med. Res.* 14 Suppl 4, 265-267 (2009).
9. Weidner, S., Carl, M., Riess, R. & Rupprecht, H. D. Histologic analysis of renal leukocyte infiltration in antineutrophil cytoplasmic antibody-associated vasculitis: importance of monocyte and neutrophil infiltration in tissue damage. *Arthritis Rheum.* 50, 3651-3657 (2004).
10. Stegeman, C. A., Tervaert, J. W., Huitema, M. G. & Kallenberg, C. G. Serum markers of T cell activation in relapses of Wegener's granulomatosis. *Clin. Exp. Immunol.* 91, 415-420 (1993).
11. Sanders, J. S., Huitma, M. G., Kallenberg, C. G. & Stegeman, C. A. Plasma levels of soluble interleukin 2 receptor, soluble CD30, interleukin 10 and B cell activator of the tumour necrosis factor family during follow-up in vasculitis associated with proteinase 3-antineutrophil cytoplasmic antibodies: associations with disease activity and relapse. *Ann. Rheum. Dis.* 65, 1484-1489 (2006).
12. Brouwer, E., Stegeman, C. A., Huitema, M. G., Limburg, P. C. & Kallenberg, C. G. T cell reactivity to proteinase 3 and myeloperoxidase in patients with Wegener's granulomatosis (WG). *Clin. Exp. Immunol.* 98, 448-453 (1994).
13. Seta, N. *et al.* Autoreactive T-cell responses to myeloperoxidase in patients with antineutrophil cytoplasmic antibody-associated vasculitis and in healthy individuals. *Mod. Rheumatol.* 18, 593-600 (2008).
14. Brouwer, E. *et al.* Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders. *Clin. Exp. Immunol.* 83, 379-386 (1991).
15. Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708-712 (1999).
16. Pepper, M. & Jenkins, M. K. Origins of CD4(+) effector and central memory T cells. *Nat. Immunol.* 12, 467-471 (2011).
17. Abdulahad, W. H., van der Geld, Y. M., Stegeman, C. A. & Kallenberg, C. G. Persistent expansion of CD4+ effector memory T cells in Wegener's granulomatosis. *Kidney Int.* 70, 938-947 (2006).
18. Abdulahad, W. H., Kallenberg, C. G., Limburg, P. C. & Stegeman, C. A. Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum.* 60, 2830-2838 (2009).
19. Lamprecht, P. *et al.* CD28 negative T cells are enriched in granulomatous lesions of the respiratory tract in Wegener's granulomatosis. *Thorax* 56, 751-757 (2001).
20. Geginat, J. *et al.* Plasticity of human CD4 T cell subsets. *Front. Immunol.* 5, 630 (2014).
21. Wang, G. *et al.* High plasma levels of the soluble form of CD30 activation molecule reflect disease activity in patients with Wegener's granulomatosis. *Am. J. Med.* 102, 517-523 (1997).
22. Schonermarck, U., Csernok, E., Trabandt, A., Hansen, H. & Gross, W. L. Circulating cytokines and soluble CD23, CD26 and CD30 in ANCA-associated vasculitides. *Clin. Exp. Rheumatol.* 18, 457-463 (2000).
23. Muller, A. *et al.* Localized Wegener's granulomatosis: predominance of CD26 and IFN-gamma expression. *J. Pathol.* 192, 113-120 (2000).
24. Lamprecht, P. *et al.* Differences in CCR5 expression on peripheral blood CD4+CD28- T-cells and in granulomatous lesions between localized and generalized Wegener's granulomatosis. *Clin. Immunol.* 108, 1-7 (2003).
25. Abdulahad, W. H., Stegeman, C. A., Limburg, P. C. & Kallenberg, C. G. Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission. *Arthritis Rheum.* 58, 2196-2205 (2008).
26. Nogueira, E. *et al.* Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis. *Nephrol. Dial. Transplant.* 25, 2209-2217 (2010).
27. Appay, V. The physiological role of cytotoxic CD4(+) T-cells: the holy grail? *Clin. Exp. Immunol.* 138, 10-13 (2004).
28. Bauer, S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285, 727-729 (1999).

29. Allez, M. *et al.* CD4+NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions. *Gastroenterology* 132, 2346-2358 (2007).
30. Capraru, D. *et al.* Expansion of circulating NKG2D+ effector memory T-cells and expression of NKG2D-ligand MIC in granulomaous lesions in Wegener's granulomatosis. *Clin. Immunol.* 127, 144-150 (2008).
31. Abdulahad, W. H. *et al.* Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission. *Arthritis Rheum.* 56, 2080-2091 (2007).
32. Klapa, S. *et al.* Lower numbers of FoxP3 and CCR4 co-expressing cells in an elevated subpopulation of CD4+CD25high regulatory T cells from Wegener's granulomatosis. *Clin. Exp. Rheumatol.* 28, 72-80 (2010).
33. Morgan, M. D. *et al.* Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. *Immunology* 130, 64-73 (2010).
34. Rodriguez-Pinto, D. B cells as antigen presenting cells. *Cell. Immunol.* 238, 67-75 (2005).
35. Lund, F. E. & Randall, T. D. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat. Rev. Immunol.* 10, 236-247 (2010).
36. Menard, L. C. *et al.* B cells amplify IFN-gamma production by T cells via a TNF-alpha-mediated mechanism. *J. Immunol.* 179, 4857-4866 (2007).
37. Barr, T. A., Gray, M. & Gray, D. B cells: programmers of CD4 T cell responses. *Infect. Disord. Drug Targets* 12, 222-231 (2012).
38. van der Geest, K. S. *et al.* Aging-dependent decline of IL-10 producing B cells coincides with production of antinuclear antibodies but not rheumatoid factors. *Exp. Gerontol.* 75, 24-29 (2016).
39. Lepse, N. *et al.* Altered B cell balance, but unaffected B cell capacity to limit monocyte activation in anti-neutrophil cytoplasmic antibody-associated vasculitis in remission. *Rheumatology (Oxford)* (2014).
40. Voswinkel, J. *et al.* B lymphocyte maturation in Wegener's granulomatosis: a comparative analysis of VH genes from endonasal lesions. *Ann. Rheum. Dis.* 65, 859-864 (2006).
41. Mauri, C. & Bosma, A. Immune regulatory function of B cells. *Annu. Rev. Immunol.* 30, 221-241 (2012).
42. Lee, K. M. *et al.* TGF-beta-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. *Eur. J. Immunol.* 44, 1728-1736 (2014).
43. Rosser, E. C. & Mauri, C. Regulatory B cells: origin, phenotype, and function. *Immunity* 42, 607-612 (2015).
44. Blair, P. A. *et al.* CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* 32, 129-140 (2010).
45. Flores-Borja, F. *et al.* CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci. Transl. Med.* 5, 173ra23 (2013).
46. de Groot, K. *et al.* Pulse versus daily oral cyclophosphamide for induction of remission in antineutrophil cytoplasmic antibody-associated vasculitis: a randomized trial. *Ann. Intern. Med.* 150, 670-680 (2009).
47. Harper, L. *et al.* Pulse versus daily oral cyclophosphamide for induction of remission in ANCA-associated vasculitis: long-term follow-up. *Ann. Rheum. Dis.* 71, 955-960 (2012).
48. De Groot, K. *et al.* Randomized trial of cyclophosphamide versus methotrexate for induction of remission in early systemic antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum.* 52, 2461-2469 (2005).
49. Stassen, P. M., Tervaert, J. W. & Stegeman, C. A. Induction of remission in active anti-neutrophil cytoplasmic antibody-associated vasculitis with mycophenolate mofetil in patients who cannot be treated with cyclophosphamide. *Ann. Rheum. Dis.* 66, 798-802 (2007).
50. Stone, J. H. *et al.* Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N. Engl. J. Med.* 363, 221-232 (2010).
51. Jones, R. B. *et al.* Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. *N. Engl. J. Med.* 363, 211-220 (2010).
52. de Joode, A. A., Sanders, J. S., Rutgers, A. & Stegeman, C. A. Maintenance therapy in antineutrophil cytoplasmic antibody-associated vasculitis: who needs what and for how long? *Nephrol. Dial. Transplant.* 30 Suppl 1, i150-8 (2015).
53. Puechal, X. *et al.* Long-Term Outcomes Among Participants in the WEGENT Trial of Remission-Maintenance Therapy for Granulomatosis With Polyangiitis (Wegener's) or Microscopic Polyangiitis. *Arthritis Rheumatol.* 68, 690-701 (2016).
54. Pagnoux, C. *et al.* Azathioprine or methotrexate maintenance for ANCA-associated vasculitis. *N. Engl. J. Med.* 359, 2790-2803 (2008).
55. Guillevin, L. *et al.* Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *N. Engl. J. Med.* 371, 1771-1780 (2014).
56. Flossmann, O. *et al.* Long-term patient survival in ANCA-associated vasculitis. *Ann. Rheum. Dis.* 70, 488-494 (2011).
57. Westman, K., Flossmann, O. & Gregorini, G. The long-term outcomes of systemic vasculitis. *Nephrol. Dial. Transplant.* 30 Suppl 1, i60-6 (2015).
58. Walsh, M. *et al.* Risk factors for relapse of antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum.* 64, 542-548 (2012).
59. Feske, S., Wulff, H. & Skolnik, E. Y. Ion channels in innate and adaptive immunity. *Annu. Rev. Immunol.* 33, 291-353 (2015).
60. Chandy, K. G. *et al.* K+ channels as targets for specific immunomodulation. *Trends Pharmacol. Sci.* 25, 280-289 (2004).

61. Wulff, H. *et al.* The voltage-gated Kv1.3 K(+) channel in effector memory T cells as new target for MS. *J. Clin. Invest.* 111, 1703-1713 (2003).
62. Cahalan, M. D., Wulff, H. & Chandy, K. G. Molecular properties and physiological roles of ion channels in the immune system. *J. Clin. Immunol.* 21, 235-252 (2001).
63. Vig, M. & Kinet, J. P. Calcium signaling in immune cells. *Nat. Immunol.* 10, 21-27 (2009).
64. Cahalan, M. D. & Chandy, K. G. The functional network of ion channels in T lymphocytes. *Immunol. Rev.* 231, 59-87 (2009).
65. Wulff, H., Knaus, H. G., Pennington, M. & Chandy, K. G. K+ channel expression during B cell differentiation: implications for immunomodulation and autoimmunity. *J. Immunol.* 173, 776-786 (2004).
66. Beeton, C. *et al.* Kv1.3 channels are a therapeutic target for T cell-mediated autoimmune diseases. *Proc. Natl. Acad. Sci. U. S. A.* 103, 17414-17419 (2006).
67. Lintermans, L. L., Stegeman, C. A., Heeringa, P. & Abdulahad, W. H. T cells in vascular inflammatory diseases. *Front. Immunol.* 5, 504 (2014).
68. Castaneda, O. *et al.* Characterization of a potassium channel toxin from the Caribbean Sea anemone Stichodactyla helianthus. *Toxicon* 33, 603-613 (1995).
69. Pennington, M. W. *et al.* Chemical synthesis and characterization of ShK toxin: a potent potassium channel inhibitor from a sea anemone. *Int. J. Pept. Protein Res.* 46, 354-358 (1995).
70. Beeton, C., Pennington, M. W. & Norton, R. S. Analogs of the sea anemone potassium channel blocker ShK for the treatment of autoimmune diseases. *Inflamm. Allergy Drug Targets* 10, 313-321 (2011).
71. Beeton, C. *et al.* Selective blocking of voltage-gated K+ channels improves experimental autoimmune encephalomyelitis and inhibits T cell activation. *J. Immunol.* 166, 936-944 (2001).
72. Beeton, C. *et al.* Selective blockade of T lymphocyte K(+) channels ameliorates experimental autoimmune encephalomyelitis, a model for multiple sclerosis. *Proc. Natl. Acad. Sci. U. S. A.* 98, 13942-13947 (2001).
73. Matheu, M. P., Beeton, C., Parker, I., Chandy, K. G. & D Cahalan, M. Imaging effector memory T cells in the ear after induction of adoptive DTH. *J. Vis. Exp.* (18). pii: 907. doi, 10.3791/907 (2008).