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## Review

# Cellular immune regulation in the pathogenesis of ANCA-associated vasculitides



Anouk von Borstel <sup>a,\*</sup>, Jan Stephan Sanders <sup>a</sup>, Abraham Rutgers <sup>b</sup>, Coen A. Stegeman <sup>a</sup>, Peter Heeringa <sup>c</sup>, Wajel H. Abdulahad <sup>b,c</sup>

<sup>a</sup> Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, Groningen 9713 GZ, The Netherlands

<sup>b</sup> Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, Groningen 9713 GZ, The Netherlands

<sup>c</sup> Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, Groningen 9713 GZ, The Netherlands

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## ABSTRACT

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) are systemic autoimmune diseases characterized by necrotizing inflammation of small- to medium-sized blood vessels, affecting primarily the lungs and kidneys. Both animal and human studies show that the balance between inflammatory- and regulatory T- and B cells determines the AAV disease pathogenesis. Recent evidence shows malfunctioning of the regulatory lymphocyte compartment in AAV. In this review we summarize the immune regulatory properties of both T- and B cells in patients with AAV and discuss how aberrations herein might contribute to the disease pathogenesis.

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## 1. Introduction

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) are characterized by systemic inflammation of small- to medium-sized blood vessels [1]. A hallmark of AAV is the presence of ANCA [2], predominantly directed against the enzymes proteinase 3

(PR3) [3] and myeloperoxidase (MPO) [4]. Both enzymes are stored in both primary granules of neutrophils and lysosomes of monocytes and are released into the extracellular environment upon cell activation [5]. Based on clinical and histopathological features, AAV is divided into three disease categories: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic GPA (EGPA) [6]. GPA is characterized by pauci-immune necrotizing granulomatous inflammation, particularly in the upper airways and the kidneys [7,8]. In MPA necrotizing vasculitis is also common, however, without granulomatous inflammation [8]. ANCA are most commonly directed against PR3 [9] and MPO [10] in GPA and MPA respectively. In contrast to GPA

\* Corresponding author at: Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen, IPC EA11, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.

E-mail address: [a.vonborstel@umcg.nl](mailto:a.vonborstel@umcg.nl) (A. von Borstel).

and MPA, EGPA is, in addition to small vessel vasculitis, typically characterized by eosinophilia and asthma and is an infrequent AAV disease type [6]. Because EGPA is histopathologically and clinically different from GPA and MPA, this disease subtype will not be further discussed in this review.

Although the pathogenesis of AAV has not been fully elucidated, the pathogenic potential of ANCA in the early effector phase of the disease is fairly well established (reviewed in [11]). The generally accepted mechanism includes exposure of neutrophils to pro-inflammatory cytokines (e.g. interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ ) causing translocation of the ANCA-antigens (Ags) to the cell surface. Following binding of ANCA, neutrophils are fully activated, resulting in the production of reactive oxygen species and release of their granular contents, which include the ANCA-Ags PR3 and MPO. More recently, an important role for alternative complement pathway activation in the disease induction has been established and C5a/C5a receptor interactions have been demonstrated to be important primers of neutrophils for activation by ANCA (reviewed in [12]).

AAV are autoimmune diseases implicating a breach of tolerance to self, but why autoimmunity to ANCA-Ags develops and why the immune system fails to suppress autoreactive lymphocytes in these patients is unknown. In homeostasis, our immune system is well balanced and regulatory mechanisms exist to dampen effector immune responses to limit tissue damage and maintain tolerance. Autoreactivity usually does not lead to autoimmune disease since regulatory cells efficiently suppress the immune response against autoAgs. Failure of immune suppression due to defects of regulatory immune cells might thus cause a break in self-tolerance, leading to autoimmune disease. Consequently, resolution of chronic inflammation often requires immunosuppressive medication in order to regain control over the autoreactive response [13]. Two immune regulatory cell types essential for peripheral tolerance are regulatory T cells (Tregs) and regulatory B cells (Bregs). Tregs were first discovered by Sakaguchi and coworkers, who showed that this T cell subset was critical in preventing autoimmunity [14]. More recently, immunosuppressive capacity has been demonstrated in the B cell lineage as well and the existence of so-called Bregs has been proposed [15].

In recent years, evidence has emerged that the delicate balance between immune effector responses and immune regulation in AAV is disturbed and malfunctioning of immune regulatory cells in AAV patients has been described. In this review, we will discuss the current knowledge on the role of Tregs and Bregs in the AAV pathogenesis and how aberrations in their immune regulatory properties might contribute to disease progression.

## 2. T cell involvement in AAV

AAV is generally considered an autoantibody (autoAb)-mediated disease due to the central role of ANCA in the effector phase of the disease. However, this does not exclude an important pathogenic role for T cell responses in AAV as well. The onset of AAV is postulated to arise from repeated exposure to an (super) Ag, resulting in persistent T cell activation. Malfunctioning of the Treg compartment results in failure to suppress the activation of autoAg-specific T cells [16]. Indeed, activated T cells can be readily detected in inflammatory lesions in the affected organs of AAV patients, particularly in the granulomas, which are a characteristic histopathological feature of GPA. Moreover, in AAV patients alterations in circulating T cell subsets and increased levels of soluble factors indicative of T cell activation in the serum have been described [17–19]. For example, increased levels of soluble T cell activation markers such as sCD25 and sCD30 can be detected in the plasma of GPA patients when compared to healthy controls (HCs) [17]. Also, increased numbers of persistently activated T cells (i.e. T cells with high HLA-DR expression) are present in AAV patients and are positively associated with disease severity [18,19]. This strongly suggests that the T cell compartment in AAV is in a persistently activated state.

In GPA, the contribution of CD4<sup>+</sup> T helper (Th) cells to the immune pathology might depend on whether the disease is localized or generalized. Initially, research in GPA patients focused on the disturbed balance between Th1 and Th2 cells. Studies revealed that CD4<sup>+</sup> T cells of GPA patients with active disease displayed a profound Th1 cytokine profile, characterized by increased interferon (IFN)  $\gamma$  production but normal IL-4 production [20,21]. Subsequently it was found that Th1-associated markers in the circulation and in nasal granulomatous lesions dominate in patients with localized disease, while Th2-associated markers are more prominent in patients with generalized disease [22,23]. More recently, it was demonstrated that in GPA patients Ag-specific Th17 cells are expanded, irrespective of disease activity and maintenance therapy [24]. In active GPA patients elevated serum IL-17 levels have been found [25], which remained elevated when patients entered remission [26]. Moreover, *in vitro* stimulation of PBMCs from GPA patients with PR3 resulted in increased IL-17 and ROR $\gamma$ T (i.e. transcription factor (TF) of Th17 cells) expression [25].

In summary, in AAV persistent T cell activation, especially of Th17 cells, is well established and likely contributes to the autoimmune pathogenesis of AAV. However, the mechanisms that initiate and maintain T cell activation in AAV remain unclear. Interestingly, emerging evidence indicates that in AAV Tregs do not function properly, suggesting that disturbed immune regulation is an important factor in persistent T cell activation in AAV. Following a brief introduction on Tregs and their mechanisms of action, the existing evidence for Treg malfunctioning in AAV will be discussed.

### 2.1. Regulatory T cells: Phenotype and mechanism of action

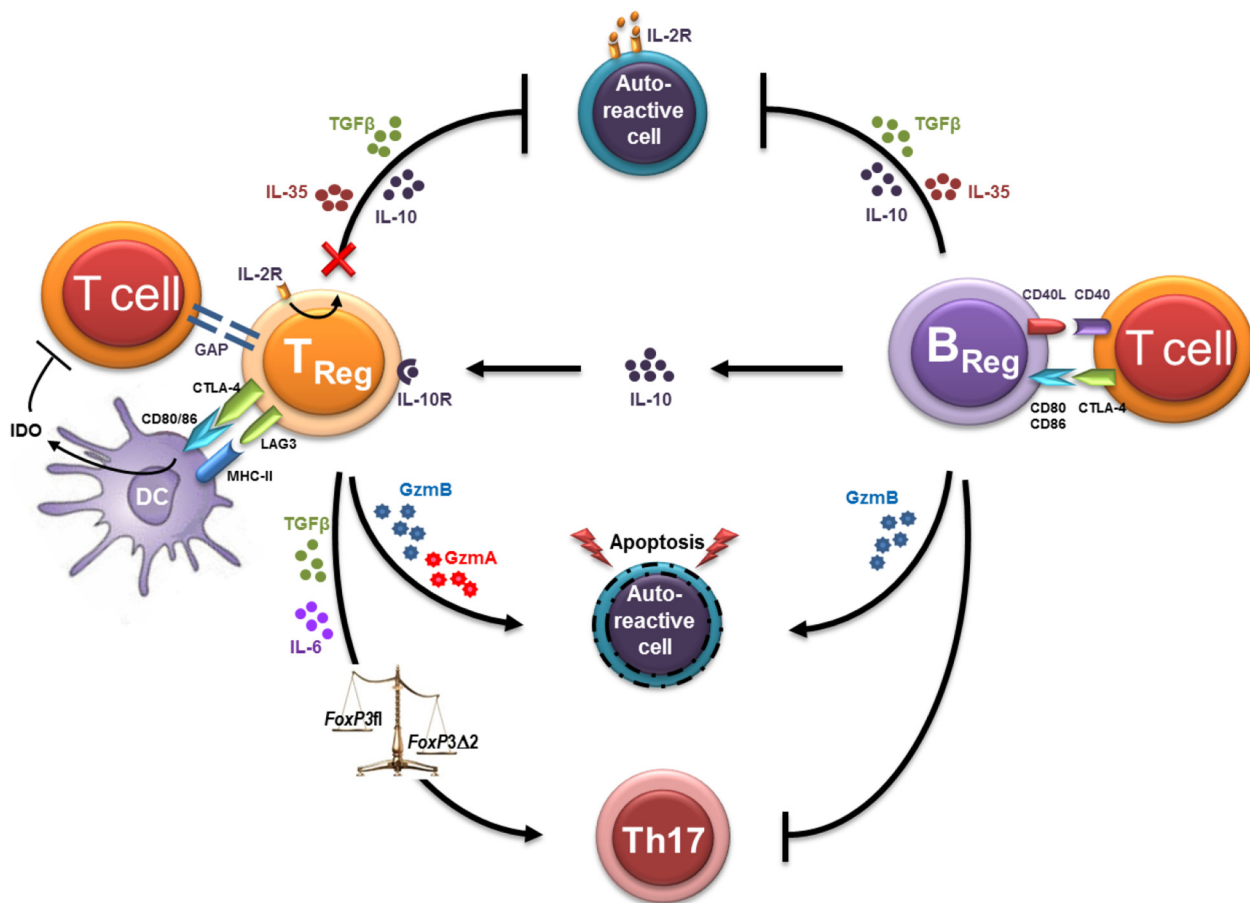
The immunosuppressive ability of T cells was first discovered >40 years ago [27,28]. However not until 1995, a subset of circulating CD4<sup>+</sup> Th cells characterized by the expression of the IL-2 receptor  $\alpha$ -chain (i.e. CD25) was identified and shown to be critical for the prevention of autoimmunity [14]. Depletion of CD4<sup>+</sup> CD25<sup>+</sup> T cells in wild type (WT) mice and transfer of the remaining T cells into syngeneic athymic nude mice induced autoimmunity and multi-organ injury [14]. Interestingly, transfer of the CD4<sup>+</sup> CD25<sup>+</sup> T cell subset into the same mice prevented the development of autoimmunity. Later, similar CD4<sup>+</sup> suppressor T cells or Tregs were also found in humans. In co-cultures of CD4<sup>+</sup> CD25<sup>hi</sup> T cells with CD4<sup>+</sup> CD25<sup>-</sup> responder T cells, it was shown that the CD4<sup>+</sup> CD25<sup>hi</sup> T cell subset was potent in directly suppressing proliferation of and cytokine production by CD4<sup>+</sup> CD25<sup>-</sup> responder T cells [29]. These results provided the first evidence for the existence of human Tregs and identified a phenotype whereby these cells can be detected.

Subsequent research identified a key marker expressed in Tregs, which distinguishes them from other CD4<sup>+</sup> T cells: the TF FoxP3. FoxP3 encodes the scurfin protein and was first detected in the scurfy mouse strain. These mice have X-linked recessive mutations in the FoxP3 gene, leading to mortality of hemizygous male mice at the age of 16–25 days. These hemizygous male mice show excess lymphoproliferation combined with infiltration of CD4<sup>+</sup> T cells in multiple organs that overproduce cytokines [30]. A mutated form of FoxP3 was later also described in patients with X-linked syndrome (i.e. IPEX), a disease characterized by immunodysregulation, polyendocrinopathy, and enteropathy [31–33]. Additional research in both scurfy mice and IPEX patients showed that CD4<sup>+</sup> CD25<sup>+</sup> Tregs were absent. Interestingly, retroviral transduction of FoxP3 into naïve CD25<sup>-</sup> CD4<sup>+</sup> T cells induced T cells that phenotypically and functionally resembled potent Tregs [34]. These results indicate that FoxP3 is necessary for both Treg development and function. According to the expression level of FoxP3 in naïve and memory CD4<sup>+</sup> Th cells, different Treg subsets have been described by Miyara and coworkers [35]. In total, three subpopulations of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in the peripheral blood of humans were identified: CD45RA<sup>+</sup> FoxP3<sup>lo</sup> resting Tregs (rTregs), CD45RA<sup>-</sup> FoxP3<sup>hi</sup> activated Tregs (aTregs) and IFN $\gamma$ - and IL-2-secreting Tregs that are

CD45RA<sup>-</sup>FoxP3<sup>lo</sup>CD25<sup>+</sup>. The first two Treg subpopulations (i.e. rTregs and aTregs) had potent suppressive function *in vitro*, whereas cytokine-secreting CD45RA<sup>-</sup>FoxP3<sup>lo</sup>CD25<sup>+</sup> Tregs did not suppress effector T cells [35]. This indicates that expression of FoxP3 alone is insufficient to identify truly suppressive Tregs. Accordingly, different isoforms of FoxP3 have been investigated in human Tregs [36]. The first isoform represents the full-length isoform, and the second represents an isoform lacking the exon 2. It has been shown that different FoxP3-isoforms impact Treg function and lineage commitments. More specifically, the full-length FoxP3 (FoxP3fl), but not the isoforms lacking exon 2 (FoxP3Δ2), interact with the Th17 TF RORγt and inhibit the expression of genes that define Th17 cells [37–39]. Interestingly, a reciprocal relationship in the development of Tregs and Th17 cells has been described [40–42]. In line with this, it has been demonstrated that, under neutral conditions *in vitro*, TGFβ can shift the balance towards functional FoxP3<sup>+</sup> Tregs, whereas in the context of an inflammatory cytokine milieu (i.e. IL-1β, IL-2, IL-15) functional Tregs convert into IL-17-producing, non-functional Tregs. Thus, assessment of FoxP3-isoforms in combination with the expression of CD25 and CD45RA are essential to delineate the truly suppressive Tregs.

The exact mechanisms by which Tregs regulate immune responses are not fully understood. It is assumed that these cells use different mechanisms to exert their function, namely secretion of anti-inflammatory cytokines, cytotoxicity, metabolic disruption and modulation

of dendritic cell (DC) maturation and/or function (reviewed in [43]) (Fig. 1). Tregs are potent producers of anti-inflammatory cytokines such as IL-10, IL-35 and TGFβ, all of which can suppress responses of activated immune cells (reviewed in [43]). Cytotoxicity is a mechanism in which Tregs release enzymes that induce apoptosis of effector cells (e.g. granzyme A or B). Tregs are equipped with a third mechanism, namely metabolic disruption. An example of metabolic disruption by Tregs is IL-2 deprivation that induces cell death in effector cells. Another example is that Tregs can inhibit effector cell function via transfer of soluble mediators (e.g. cyclic AMP) via gap junctions into target cells (e.g. effector T cells and DCs) [43]. In target cells, cAMP induces inducible cAMP early repressor (ICER), which is thought to inhibit cell proliferation and IL-2 production [44]. Lastly, Tregs can modulate DC maturation and function via physical interaction through inhibitory surface molecules such as lymphocyte activation gene (LAG) 3 and cytotoxic T lymphocyte-associated Ag (CTLA)-4. When DCs sense these molecules, production of indoleamine 2,3-dioxygenase (IDO) is initiated, which in turn suppresses other immune cells [43]. The mechanism by which Tregs initiate suppression is dependent on the Ag dose. A high Ag dose elicits potent inhibition via induction of FAS-mediated apoptosis or T cell receptor (TCR) ligation, which both result in T cell anergy, whereas a low Ag dose only induces secretion of IL-10 or TGF-β [45]. Tregs can prevent autoimmunity by inhibiting the activation of T effector cells via both inhibitory co-stimulation and secretion of anti-inflammatory



**Fig. 1.** Overview of immune suppressive mechanisms used by Tregs and Bregs. Bregs and Tregs inhibit autoreactive cells and Th17 cells via production of anti-inflammatory cytokines (e.g. IL-10, IL-35, TGFβ) and induce apoptosis of autoreactive cells via production of granzymes. Bregs also exert suppressive function via direct cell contact mediated via CD40-CD40L and CD80/86-CTLA-4 interaction. Tregs can suppress autoreactive cells via CTLA4-CD80/86-mediated induction of IDO, which is an immune suppressive molecule produced by DCs. In addition, Tregs can indirectly inhibit activated autoreactive cells via IL-2 deprivation or via transfer of soluble mediators (e.g. cyclic AMP) through gap junctions. The suppressive capacity of Treg cells can be enhanced by IL-10 secreted by Bregs. In AAV, aberrancies in both Tregs and Bregs contribute to the disease pathogenesis. The suppressive function of Tregs in AAV seems to be diminished. Due to decreased (functional) IL-2R expression, less IL-2 can be bound mediating decreased suppressive cytokine production (i.e. IL-10, TGFβ and IL-35) that inhibit autoreactive cells. Moreover, the relative ratio of the full length FoxP3 isoform (FoxP3fl) over the FoxP3 isoforms lacking the exon 2 (FoxP3Δ2) may impact Treg function and lineage commitments. Lower ratio of FoxP3fl/FoxP3Δ2 isoforms will favor conversion of Tregs into IL-17-secreting cells, which may contribute to the AAV disease pathogenesis. Furthermore, a lower Breg frequency was found in AAV, which seems so far the most important reason for decreased suppressive function by Bregs.

cytokines (reviewed in [46]). In conclusion, Tregs employ multiple mechanisms to exert their immune suppressive effect and play a pivotal role in the suppression of autoimmune responses.

## 2.2. Regulatory T cells in AAV

In AAV defective immune suppression of Tregs may contribute to persistent immune cell activation and development of chronic autoimmune inflammation. To date, most research on immune regulation in AAV has focused on the aberrant function and/or altered frequency of Tregs. Although most studies agree on the fact that Treg-mediated immune suppression is impaired in AAV, some controversy exists on whether this defect is caused by numerical or functional changes or both. These aspects will be discussed in more detail in the next paragraphs. Key publications on Tregs in AAV are summarized in Table 1.

### 2.2.1. Numerical and functional alterations of Tregs in AAV

There is some discrepancy in literature regarding Treg proportions in AAV: our group previously assessed the frequency of Tregs in peripheral blood of GPA patients in remission and observed that the frequency of CD25<sup>hi</sup>FoxP3<sup>+</sup> Tregs was increased in AAV patients compared to HCs [47], as did others [19,48]. In contrast, others have reported a decreased CD4<sup>+</sup>CD25<sup>hi</sup> Treg frequency in GPA patients [26,49–51]. In one study, the decrease in the CD4<sup>+</sup>CD25<sup>hi</sup> Treg frequency was associated with the conventional immunosuppressive therapy the patients received (i.e. CYC + pred or MMF/MTX/AZA + pred) [49]. Interestingly, in the same study it was observed that the circulating Treg frequency in patients receiving B cell depletion therapy (i.e. Rituximab (RTX)) was similar to HCs, whereas it was significantly increased compared to conventionally treated patients [49]. However, in this study, FoxP3 expression within the CD4<sup>+</sup>CD25<sup>hi</sup> T cell compartment was not assessed, which is important since it identifies activated Tregs as demonstrated by Miyara et al. [35]. Another study found that the percentage of Tregs significantly expanded during extended remission ( $\geq 1$  year), independently of immunosuppressive treatment. The increase in Treg frequency was accompanied by an increased Th2 frequency. The authors speculated that decreased Treg numbers in active AAV allow expansion of Th17 cells during remission, whereas immunosuppressive medication inhibits Th17 cells and allows Treg expansion resulting in a shift in Th

cell balance [26]. Combined, these results indicate that immunosuppressive medication restores the Treg frequency of AAV patients in remission.

Although there is no consensus concerning Treg frequency in AAV, all studies conducted so far, but one [52], report a reduced suppressive function of Tregs in the majority of AAV patients [47,48,50,51]. Previously, our group showed that despite an increased frequency of FoxP3<sup>+</sup> Tregs in GPA patients, their suppressive function was diminished as demonstrated by increased proliferation of responder T cells in co-cultures with Tregs from AAV patients [33], which was subsequently confirmed by others [48,50,51].

Together, these results indicate that the discrepancy in the literature with respect to Treg frequencies in AAV patients may, at least in part, be explained by differences in markers or gating strategies used to identify Tregs between the studies. Nevertheless, most studies agree on the fact that Treg function is diminished in AAV.

### 2.2.2. Possible underlying causes for Treg malfunctioning in AAV

Although most studies demonstrate diminished suppressive function of Tregs in AAV patients, the exact mechanisms underlying impaired Treg function are unknown. The first mechanism that might induce decreased suppression by Tregs is the apparent plasticity of these cells in a pro-inflammatory microenvironment. Evidence exists of Tregs converting into Th1 cells in diabetes patients [53,54]. Two studies involving type 1 diabetes patients showed that Th1-like Tregs expressed Tbet and CXCR3 besides FoxP3 and produced IFN $\gamma$ , indicating that these Tregs lost their suppressive function [53,54]. Interestingly, in 2008 it was demonstrated that CD25<sup>hi</sup>FoxP3<sup>+</sup> T cells could convert into IL-17-producing T cells when stimulated with allogeneic Ag presenting cells (APCs) [40]. This observation was later also confirmed by others [42,55]. Subsequent studies in rheumatoid arthritis (RA) [56] and cancer [57] demonstrated that Th17-like Tregs expressing FoxP3 were specifically more abundant at inflammatory sites. Also in AAV there seems to be increased skewing of Tregs towards IL-17-producing cells at sites of inflammation [58], which was hypothesized to be due to increased IL-6 and TGF $\beta$  production in the inflammatory microenvironment [59]. These findings suggest that the impaired suppressive capacity of Tregs could be due to extensive exposure to TGF $\beta$  and IL-6 at inflammatory sites. Such a mechanism may be operative in AAV as well, given the reported increase in Th17 levels and diminished Treg function in AAV patients [25]. IL-6 seems to be of great importance as it was recently demonstrated that Tregs lose their suppressive function, accompanied by decreased Helios expression, when exposed to IL-6 [60]. Helios is a TF expressed in Tregs that can be induced *in vitro* by TGF $\beta$  stimulation, and has previously shown to support suppressive function of Tregs. Interestingly, in RA patients treatment with Tocilizumab, a monoclonal antibody (Ab) blocking the IL-6-receptor, was associated with an increased frequency of circulating Helios<sup>+</sup>FoxP3<sup>+</sup>CD4<sup>+</sup> T cells compared to HCs or RA patients that did not receive Tocilizumab [60]. Moreover, forced Helios expression in murine Tregs enhanced their suppressive function in conjunction with increased expression of Treg markers (i.e. CD103, GITR, GARP, FR4 and IL-10) [60]. Collectively, the observations described above suggest that the plasticity of Tregs may contribute to an amplification loop in autoimmune diseases such as AAV. In AAV a pro-inflammatory environment with high levels of IL-6 converts Tregs into IL-17-producing T cells, lose their suppressive function and promote ongoing effector cell activation. Moreover, since these non-suppressive Th17-like Tregs do express FoxP3, it again emphasizes the need for additional markers to identify genuine suppressive Tregs.

As a second mechanism, Wilde and colleagues proposed that perhaps the diminished suppressive capacity of Tregs in AAV is related to reduced responsiveness to IL-2. They demonstrated significantly decreased expression of the  $\beta$ -chain of the IL-2 receptor (IL-2R; CD122) on activated Th cells and Tregs in AAV patients compared to HCs [61]. IL-2 is a cytokine that all T cells need for their functioning under both homeostatic and inflammatory conditions (reviewed in [62]). If Tregs

**Table 1**  
Key publications on Tregs in AAV.

| Authors, journal & year                    | Main conclusions   | Reference |
|--|--|-----------|
| Marinaki et al. Clin. Exp. Immunol. (2005) | - Persistent state of T cell activation, accompanied by increased T cell proliferation<br>- Decreased IL-10 production of PBMCs after <i>in vitro</i> stimulation    | [18]      |
| Abdulhad et al. Arthr. Rheum. (2007)       | - GPA patients have an expanded frequency of CD4 <sup>+</sup> CD25 <sup>hi</sup> Tregs<br>- CD4 <sup>+</sup> CD25 <sup>hi</sup> Tregs in GPA are functionally defect | [47]      |
| Klapa et al. Clin. Exp. Rheumatol. (2010)  | Decreased frequency FoxP3 <sup>+</sup> Tregs in GPA.   | [50]      |
| Morgan et al. Immunology (2010)            | Relative deficiency (1) and functional impairment (2) of FoxP3 <sup>+</sup> Tregs in GPA.  | [51]      |
| Free et al. Arthr. Rheum. (2013)           | CD4 <sup>+</sup> CD25 <sup>hi</sup> CD127 <sup>lo</sup> FoxP3 <sup>+</sup> Tregs with the FoxP3 $\Delta 2$ isoform have diminished suppressive function.             | [48]      |
| Wilde et al. Dis. Markers (2014)           | CD122 expression is decreased on CD4 <sup>+</sup> CD25 <sup>+</sup> FoxP3 <sup>+</sup> Tregs in AAV, leading to decreased suppressive function.                      | [61]      |
| Zhao et al. Rheumatology (2014)            | B cell depletion normalizes balance in the T cell compartment.   | [49]      |

are less responsive to IL-2, Tregs possibly cannot exert their suppressive function.

Aberrant expression in FoxP3-isoforms could be a third explanation for Treg malfunctioning in AAV. As mentioned before, the FoxP3 $\beta$ , but not FoxP3 $\Delta$ 2, interacts with ROR $\gamma$ t and inhibits the expression of genes that define Th17 cells. Thus, FoxP3 $\Delta$ 2 may result in a dominant expression of ROR $\gamma$ t in Tregs, ensuing production of IL-17 and skewing of Tregs towards pathogenic Th17 cells. Interestingly, the FoxP3 $\Delta$ 2 isoform in Th cells was increased in both active and inactive AAV patients compared to HCs. In addition, the frequency Tregs with FoxP3 $\beta$  was decreased in the same patients in comparison to HCs. A positive correlation was found between decreased suppressive capacity of Tregs of AAV patients and the frequency of exon 2-deficient Tregs [48]. Taken together, these results indicate that expression of the splice variant of FoxP3 may be responsible for decreased suppressive function.

A fourth explanation focuses on epigenetic aberrancies in Tregs. The CpG motifs in the FoxP3 promoter, also called Treg-specific demethylated region (TSDR), are usually demethylated which allows transcription of the FoxP3 gene [63]. In resting conventional T cells the same motifs are only weakly demethylated. Acetylated histones were more strongly associated with the FoxP3 promoter in Tregs compared to conventional T cells [63,64], which might makes the FoxP3 promoter more accessible to RNA polymerase. This would result in transcription of FoxP3 specifically in Tregs and thereby activating their suppressive function. Strikingly, a study confirmed the existence of an inactive Treg population that lost both FoxP3 expression and their suppressive function, which was associated with a demethylated TSDR indicating commitment to the Treg lineage. Intriguingly, FoxP3 expression, and therewith suppressive function, could be reinforced via TCR stimulation in these inactive Tregs [65]. Further studies are necessary to confirm whether histones are less acetylated in AAV, causing increased methylation of the FoxP3 promoter and resulting in more inactive Tregs.

In summary, several mechanisms have been proposed to mediate decreased suppressive function of Tregs including IL-6 mediated Treg-Th17 conversion, reduced IL-2 responsiveness, increased expression of FoxP3 splice variants and the FoxP3 promoter methylation status. All of these may be involved in the reported reduced suppressive capacity of Tregs in AAV as well. However, the exact mechanism of their impairment in AAV remains to be determined.

### 3. B cell involvement in AAV

B cells play an important role in the pathogenesis of AAV as they are the precursors of plasma cells that produce ANCA. However, B cells exert multiple other functions including Ag presentation and production of a variety of pro- and anti-inflammatory cytokines. These properties of B cells suggest that these cells may contribute to the pathogenic and immune regulatory processes in AAV in an Ab-independent manner as well [66,67].

Evidence for an Ab-independent pathogenic role of B cells in AAV was first described in 1999. In this study it was shown that the frequency of activated B cells, identified as B cells with high expression levels of CD38, was significantly increased during active disease compared to patients in remission and HCs [68]. Interestingly, no correlation between activated B cells and ANCA levels was found, whereas the proportion of activated B cells did significantly correlate with disease activity [68].

Additional evidence for an Ab-independent role of B cells in AAV comes from two major clinical trials that demonstrated that treatment with the B cell-depleting Ab RTX is as efficacious in inducing disease remission in AAV as standard immunosuppressive therapy [66,67]. RTX is a chimeric monoclonal anti-CD20 Ab that depletes B- but not plasma cells. Upon RTX treatment, ANCA in the circulation were detectable in patients in remission and only decreased after six months after start of RTX treatment [66,67], indicating that clinical improvement in RTX-treated patients can precede the reduction in autoAb titers. Moreover,

relapses did occur in RTX-treated AAV patients, but only after B cell repopulation [67]. This indicates that besides eliminating the precursors of Ab-generating cells, the therapeutic effects of RTX also involve modulation of Ab-independent properties of B cells.

The Ab-independent functions of B cells and their potential role in autoimmune disease pathogenesis have gained considerable interest in recent years. In particular, the fact that B cells can produce pro- and anti-inflammatory cytokines that can shape both T cell and innate immune responses and act as drivers or regulators of (auto)immune responses respectively, is increasingly recognized [69]. Moreover, in mice and humans specific B cell subsets termed Bregs, have been identified that display immune regulatory properties. Bregs are defined by their capacity to suppress pathological immunity primarily via provision of IL-10 and in mouse models of various autoimmune disorders IL-10-producing B cells have been shown to suppress disease development [15,70]. This has led to the concept that in autoimmune disorders such as AAV, the balance between B effector cells and Bregs may be disturbed, driving the pathological autoimmune response.

#### 3.1. Regulatory B cells: Phenotype and mechanism of action

The theory that B cells are able to suppress immune responses was first postulated by both Katz and Neta in 1974 [71,72]. They showed that adoptive transfer of B cell-depleted splenocytes were unable to suppress delayed type hypersensitivity in guinea pigs, which suggested that B cells may suppress the activity of T cells in delayed skin hypersensitivity. However, the phenotype and the mechanism of action of these suppressor B cells remained uncharacterized. In 2002, studies in animal models of colitis and experimental autoimmune encephalitis (EAE) showed that a subset of B cells could suppress inflammation and that IL-10 is a hallmark of these suppressive B cells [15,73]. In 2008, Yanaba and coworkers reintroduced the paradigm of suppressor B cells in mice by identifying a subset of peripheral B cells expressing surface CD5 and a high level of CD1d, which were termed Bregs [74]. Bregs were also identified in humans and characterized by the production of IL-10 upon *in vitro* stimulation [75,76]. Since *in vitro* identification of human Bregs via IL-10 is labor intensive (i.e. PBMC isolation and cell culture is needed), there was a clear need for immunophenotypic surface markers that classify Bregs. The search for a specific Breg immunophenotype has so far led to multiple studies that claimed to have identified (different) Breg-specific markers [75–78].

Firstly, Bregs have been identified in the immature or transitional B cell subset [75]. These Bregs were characterized by high expression of both CD24 and CD38. Flores-Borja et al. showed that this CD24<sup>hi</sup>CD38<sup>hi</sup> Breg subset successfully inhibited the differentiation of naïve T cells into both Th1 and Th17 cells. Production of IL-10 was mainly found in the CD24<sup>hi</sup>CD38<sup>hi</sup> Breg compartment, and these Bregs were potent suppressors of multiple activated cell types and played an important role in activating Tregs [79]. Additional surface markers have been reported to be expressed by immature Bregs to identify truly suppressive Bregs that include CD1d [78] and CD5 [75]. However, the exact phenotype by which suppressor CD24<sup>hi</sup>CD38<sup>hi</sup> Bregs can be recognized is not yet elucidated.

In addition to the immature/transitional Bregs, a second Breg subset was identified within the CD27<sup>+</sup> memory B cell compartment [76]. These memory CD24<sup>hi</sup>CD27<sup>+</sup> Bregs were also enriched for IL-10 production and suppressed cytokine production in CD4<sup>+</sup> T cells *in vitro* [76]. It seems that the immunosuppressive property of Bregs is mainly mediated via IL-10 secretion (Fig. 1).

In addition to IL-10, studies have shown that activated Bregs can express other immune regulatory cytokines including IL-35 and TGF $\beta$ . Studies in EAE mice showed that B cells lacking IL-35 expression mediated exacerbated EAE and these mice lost the ability to recover from the disease [80]. Another study demonstrated that adoptive transfer of IL-35-producing Bregs inhibited experimental uveitis [81]. It has also been shown that B cells down-regulate pathogenic autoimmune responses via expression of TGF $\beta$  [82]. Treatment of prediabetic non-

obese diabetic mice with activated TGF $\beta$ -secreting Bregs inhibited spontaneous T cell autoreactivity to  $\beta$  cell autoAgs, enhanced mononuclear cell apoptosis in the peripheral lymphoid tissue, and temporarily impaired the function of APCs. TGF $\beta$ -secreting Bregs have also been identified in humans, where this subset was characterized by high expression of CD25, a signature marker of Tregs. TGF $\beta$  produced by these Bregs promoted FoxP3 and CTLA-4 expression in T cells [83].

All the aforementioned findings demonstrate that Bregs mediate their suppressive effect through the expression of immune regulatory cytokines. In addition to expression of immunomodulatory cytokines, a new cytotoxic marker in Bregs was identified: granzyme B (GzmB) production [77]. GzmB production by these Bregs seems to be an additional mechanism of Breg-mediated suppression to exert their cytotoxic activity.

Besides the secretion of cytokines and cytotoxic proteins, Bregs need direct cell-cell interaction to suppress other immune cells. It was shown that direct cell-cell contact of Bregs with T cells, mediated via CD40-CD40L interaction, was a crucial pathway for IL-10 induction in B cells and also induced CTLA-4 and FoxP3 expression in T cells [15]. Interestingly, IL-10 production by Bregs also impacts the suppressive function by Tregs. It has been shown that Tregs lacking the IL-10 receptor do not suppress activated Th17 cells [84], indicating that IL-10 signaling in Tregs is crucial for maintaining certain immunosuppressive functions of this subset. This could be an additional mechanism of Bregs by which they impact the suppressive function of Tregs.

Altogether, Bregs play an important role in suppressing immune responses and multiple mechanisms of suppression have been demonstrated. Besides IL-10, Bregs can exert their suppressive function via TGF $\beta$ - and IL-35 secretion and the expression of cytotoxic proteins. Multiple studies clearly show that Bregs are needed for both the suppression of activated immune cells and induction of differentiation of naïve cells into regulatory cells. However, more research is needed to clarify the exact underlying mechanism for each Breg subset.

### 3.2. Regulatory B cells in AAV

Studies involving Bregs in autoimmune diseases have focused predominantly on abnormalities in their numbers (based on their surface markers) and/or function (based on *in vitro* IL-10 expression and suppression of immune cells in co-culture). In this section we will review studies on Bregs in AAV. Key publications on B cell suppression in AAV are summarized in Table 2.

Using the phenotypical markers CD24 and CD38 to distinguish circulating Bregs, contradictory results were found in patients with AAV. The study by Todd and coworkers demonstrated a significant reduction in the frequency of circulating immature CD24<sup>hi</sup>CD38<sup>hi</sup> Bregs in both MPO- and PR3-AAV patients with quiescent disease, whereas during active disease only in PR3-AAV patients a decreased Breg frequency was found [85]. Another study by Aybar and coworkers showed that the

frequency of circulating CD24<sup>hi</sup>CD38<sup>hi</sup> Bregs, additionally defined by CD5, was decreased during active AAV and normalized during disease remission [86]. In accordance we found a decreased frequency of circulating CD24<sup>hi</sup>CD38<sup>hi</sup> Bregs in GPA patients with active disease, whereas no difference was observed in quiescent patients in comparison to HCs [87]. In addition we analyzed the frequency of memory CD24<sup>hi</sup>CD27<sup>+</sup> Bregs and we found a significant decrease in the frequency of these memory Bregs in both active and quiescent AAV patients compared to HCs [87]. Overall, all studies indicate a reduction in circulating Breg frequency during active AAV, which highlights their role in disease progression.

To further evaluate the relationship between Bregs and states of remission and relapse in AAV, O'Dell Bunch and colleagues examined circulating Bregs (identified by CD5 expression) in AAV patients that received RTX and consequently underwent B cell depletion [88]. They found that these Bregs were decreased during active disease, and normalized during remission. A significant shorter time to relapse (i.e. 17 months) was observed in patients with low CD5<sup>+</sup> B cell repopulation (i.e. <30%) after treatment with RTX combined with a low dose (i.e. <1 g/day) mycophenolate mofetil (MMF). In contrast, in patients with >30% CD5<sup>+</sup> B cell repopulation an increased time to relapse (i.e. 31 months) was found [88,89]. Moreover, an inverse correlation was found between the percentage of CD5<sup>+</sup> B cells and disease activity in RTX-treated patients only. These results indicate that Bregs are protective in AAV patients. Contrastingly, in the study by Unizony and coworkers no relation was found between the percentage CD5<sup>+</sup> B cells and relapse rate [90]. Further studies are needed to clarify whether Bregs identified by CD5 expression are protective for disease relapses in AAV and how the frequency of these cells is influenced by treatment.

As IL-10 is considered the functional marker of Bregs, studies have analyzed the frequency of IL-10-producing Bregs upon *in vitro* stimulation. In AAV, multiple groups have studied the frequency of IL-10-producing Bregs after *in vitro* stimulation. One study showed that the IL-10<sup>+</sup> B cell frequency was decreased in active and remission AAV patients compared to HCs [91]. Interestingly, others only found a decreased IL-10<sup>+</sup> B cell frequency in active patients and a normal frequency in remission patients [86]. Contrastingly, we and others found no difference in the IL-10<sup>+</sup> B cell frequency in AAV patients compared to HCs [85,87]. Together these results show that there is no consensus yet about the IL-10<sup>+</sup> B cell frequency in AAV and more research should focus on these cells in AAV.

As mentioned before, Bregs can also exert their suppressive function by producing other immunomodulatory cytokines, such as IL-35 and TGF $\beta$ , and the cytotoxic protein GzmB. Not much knowledge is available about the expression of IL-35 and TGF $\beta$  in Bregs from AAV patients. In a preliminary study, a decrease in GzmB production was found in B cells derived from quiescent AAV patients after *in vitro* stimulation compared to HCs [92], suggesting that cytotoxic Breg function is decreased in AAV patients.

**Table 2**  
Key publications on Bregs in AAV.

| Authors, journal & year                     | Patients       | Breg subset studied  | Main conclusion  | Reference |
|---|----------------|--|--|-----------|
| O'Dell Bunch et al. Ann. Rheum. Dis. (2013) | PR3- & MPO-AAV | CD5 <sup>+</sup> B cells   | A decreased CD5 <sup>+</sup> B cell frequency after B cell depletion shortens the time to relapse in AAV.  | [88]      |
| Wilde et al. Ann. Rheum. Dis. (2013)        | PR3- & MPO-AAV | IL-10 <sup>+</sup> B cells   | IL-10 producing B cells are diminished in AAV.   | [91]      |
| Lepse et al. Rheumatology (2014)            | PR3- & MPO-AAV | CD24 <sup>hi</sup> CD38 <sup>hi</sup> & CD24 <sup>hi</sup> CD27 <sup>+</sup> Bregs | In AAV, the B cell balance is altered, but B cell function is not impaired reflected as the inhibition of TNF $\alpha$ production from monocytes.            | [87]      |
| Todd et al. Rheumatology (2014)             | GPA & MPA      | CD24 <sup>hi</sup> CD38 <sup>hi</sup> Bregs  | Regulatory B cells are numerically but not functionally deficient in AAV.  | [85]      |
| Aybar et al. Clin. Exp. Immunol. (2015)     | PR3- & MPO-AAV | CD5 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> Bregs                       | Reduced CD5 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> and IL-10 <sup>+</sup> regulatory B cells in active AAV permit increased circulating autoAbs. | [86]      |
| Unizony et al. Arthr. Rheum. (2015)         | AAV            | CD5 <sup>+</sup> B cells   | The percentage CD5 <sup>+</sup> B cells correlates inversely with disease activity after RTX therapy.  | [90]      |

In addition to quantifying circulating Bregs and determining their cytokine pattern, researchers have also assessed their suppressive capacity *in vitro*. Interestingly, no deficiency in Breg suppressive function was found so far in AAV. One study co-cultured CD24<sup>hi</sup>CD38<sup>hi</sup> Bregs and CD4<sup>+</sup> T cells and found significant suppression when cultured in a 1:1 ratio. The CD24<sup>hi</sup>CD38<sup>hi</sup> Bregs from AAV patients prevented both TNF $\alpha$ - and IFN $\gamma$  production by T cells to the same extent as the same Bregs from HCs did [85]. We previously co-cultured total CD19<sup>+</sup> B cells with monocytes (1:1 ratio) and activated the B cells with CpG and CD40L and monocytes with LPS. We showed that the inhibition of monocyte TNF $\alpha$  production by AAV Bregs was not different from HCs Bregs since the same frequencies of TNF $\alpha$  were found [87]. Both studies indicate that Bregs from AAV patients are not inferior in suppressing pro-inflammatory cytokine production in both T cells and monocytes.

Overall, the available data described here indicate that an imbalance between regulatory and effector functions of B cells is an important aspect contributing to disease pathogenesis in AAV (Fig. 1). Decreased Breg numbers were most prominent in patients with active disease, which may contribute to decreased immune suppression in AAV patients. However, to date there is no evidence for impaired Breg functioning in AAV.

#### 4. Concluding remarks

In AAV patients elimination or adequate suppression of autoreactive T- and B cells fails. This allows initiation and propagation of the immune response against self-proteins leading to the development of autoreactive effector T cells and pathogenic autoAb production. To date, multiple studies in AAV focusing on the immune regulatory cell types essential for peripheral tolerance (i.e. Tregs and Bregs) have demonstrated numerical and functional aberrations in these immune regulatory subsets, although discrepancies between studies exist. For Tregs, data on numerical changes in AAV patients are inconsistent but in most studies the suppressive function of Tregs was found to be diminished. For Bregs, decreased numbers of this subset in AAV patients have been described but so far no deficiencies in suppressive function have been reported. Collectively, these data indicate that a dysbalance between effector and regulatory cells in both the T- and B cell compartment is an important aspect of AAV pathogenesis suggesting that (therapeutic) ways of restoring this balance could be of benefit for AAV patients.

However, many questions still remain. First, it is currently unknown whether the observed aberrations in immune regulatory subsets are a cause or consequence of the disease and relatively little attention has been paid to the (long-term) effects of induction and maintenance therapy on these subsets. Second, further clarification of the phenotypes of both Bregs and Tregs is needed. For Bregs, multiple markers are currently used to define these cells and this has led to the description of multiple Breg subsets. Although a more discriminative phenotype would enable more accurate elucidation of the role of Bregs in AAV, it is also likely that immunosuppression in the B cell compartment is not a trait of a separate Breg lineage with a specific phenotype. As proposed by Mauri and colleagues, immunosuppression by B cells could also rather be the outcome of the dynamic balance between multiple B cell subsets and other cells of the immune system suggesting that Bregs emerge as a consequence of the inflammatory response when immunosuppression is most needed [93]. Regarding Tregs, the challenge will be to find markers that specifically delineate true suppressive Tregs. Third, little is known about the underlying mechanisms that mediate decreased suppressive function of Tregs in AAV. In AAV patients there are indications that the proportion of Tregs expressing a FoxP3-isoform that inhibits their suppressive ability is increased. It may be worthwhile to perform more detailed studies on the expression of genes potentially contributing to diminished suppressive function of Tregs by for example (single cell) RNA sequencing. Such studies might reveal differentially regulated genes in Tregs of AAV patients. In

conjunction with functional analyses of these genes, such studies may pinpoint important mechanisms contributing to the functional impairment of Tregs in AAV and potentially identify targets amenable for therapeutic intervention.

#### Disclosure

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