New pathophysiological insights and treatment of ANCA-associated vasculitis

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ANCA-associated-vasculitis (AAV) comprises three different diseases entities: Churg–Strauss syndrome, microscopic polyangiitis, and Wegener's granulomatosis. AAV is an autoimmune disease with complex pathophysiology. Anti-neutrophil cytoplasmic antibodies (ANCAs) with specificity for proteinase-3 (PR3) or myeloperoxidase (MPO) are hallmarks of AAV and have a pivotal role in disease development. In addition to ANCA, the cellular immune system contributes to the pathogenesis of the disease. ANCA-mediated degranulation of neutrophils causes vasculitic damage; T cells drive granuloma formation, promote vasculitic damage by several different pathways, and enhance autoantibody production by B cells. Recently, complementary PR3 and lysosomal membrane protein-2 were suggested as novel autoantigens in AAV. New findings also indicate the importance of complement, danger-associated molecular patterns, and dendritic cells in AAV. This review highlights novel pathophysiological findings in AAV and puts them into context with the current understanding of disease mechanisms. Furthermore, implications for present and new therapeutic strategies are discussed.

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ANCA-associated vasculitis (AAV) is a life-threatening autoimmune disease characterized by necrotizing vasculitis of small- and medium-sized vessels.[1–3] Pauci-immune necrotizing crescentic glomerulonephritis (NCGN) is commonly observed.[3,4] Anti-neutrophil cytoplasmic antibodies (ANCAs) with specificity for either proteinase-3 (PR3) or myeloperoxidase (MPO) are hallmarks of AAV. AAV comprises three disease types: Wegener's granulomatosis (WG), Churg–Strauss syndrome (CSS), and microscopic polyangiitis (MPA). The disease types differ with respect to clinical manifestations and histological findings. Granulomatous inflammation is observed in WG and CSS but not in MPA.[2,5,6] Furthermore, neutrophils are abundantly found in WG-associated inflammation, whereas in CSS eosinophils dominate the inflammatory infiltrate.[2,3] CSS and MPA are mostly associated with ANCAs directed against MPO, whereas WG is more associated with ANCAs with specificity for PR3.[7] The pathophysiology of AAV is complex and the humoral as well as the cellular immune system are involved. ANCAs themselves are thought to be pathogenic. Furthermore, ANCAs promote degranulation of neutrophils and monocytes facilitating endothelial damage.[8] The endothelium is also activated and thereby neutrophil adherence is enhanced.[9–11] The initial damage leads to a cascade of events, resulting in leukocyte tissue infiltration, T-cell-driven granuloma formation, and further damage. This simplified view on disease mechanisms is refined and explained in detail further below. Recent findings on ANCAs, complement, neutrophils, lymphocytes, and dendritic cells (DCs) demand to confine new roles for old players in AAV. Thus, we discuss and illustrate novel insights and the role in the current concept of disease. This review will also highlight and further explain implications for treatment modality.

ANCAs: MODE OF ACTION

The functional characteristics of ANCAs have been studied in different *in vitro* and *in vivo* models providing growing evidence for pathogenicity.[12]

ANCAs bind to neutrophils and endothelial cells having differential but synergistic effects on both cell types. ANCAs bind to membrane-bound PR3/MPO on neutrophils.[12,13] This interaction with ANCAs results in activation and finally in release of cytotoxic superoxide and serine proteases (such

as PR3).[8,14] Membrane-bound MPO/PR3 is expressed constitutively by neutrophils and can be enhanced by pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) ('priming').[13–17] Priming of neutrophils also enhances adhesion to endothelial cells along with a further increase of membrane MPO/PR3 expression.[18,19] Thus, degranulation occurs in close contact with the vascular endothelium, resulting in vasculitic damage (Figure 1). There is ongoing discussion about the role of cytotoxic mediators in endothelial damage. A recent study by Lu *et al.*[20] confirmed former experimental evidence suggesting that serine proteases (like PR3 and elastase) are more important than superoxide radicals in mediating cytotoxic damage.[21] The authors showed *in vitro* that endothelial cell

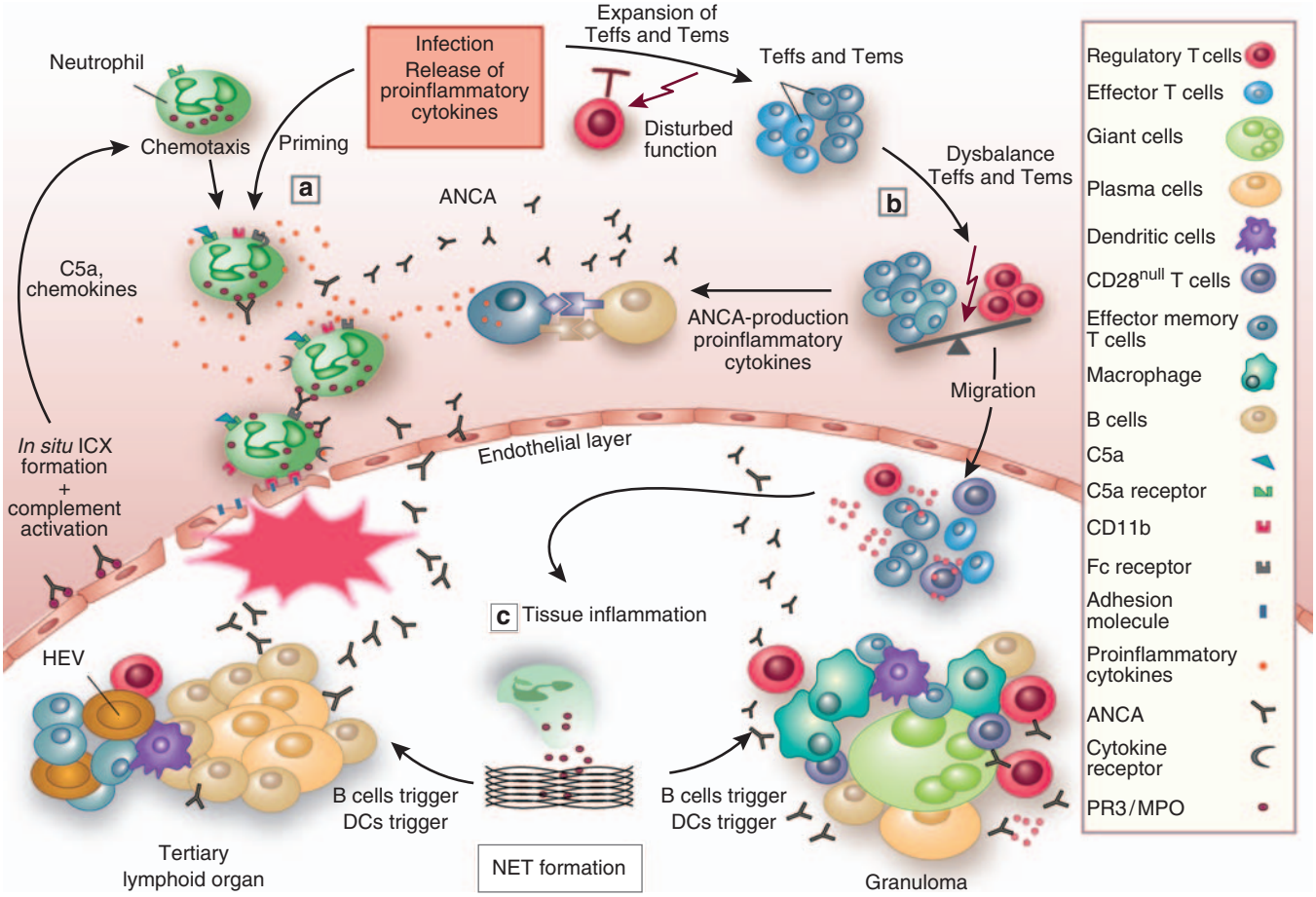


Figure 1 | Two pathways contributing to disease mechanisms in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are depicted. (**a**) The 'classic neutrophil pathway' has been studied and confirmed by several groups. This pathway causes necrotizing vasculitis.[9] (**b**) We propose an additional 'T-cell pathway' that mainly causes granulomatous inflammation and promotes necrotizing vasculitis. Infections are the starting point of both pathways; infections trigger priming of neutrophils (**a**), upregulation of adhesion molecules on endothelial cells, and expansion of circulating effector T cells (**b**). Primed neutrophils show increased surface expression of ANCA antigens and adhesion molecules. ANCA binding activates the neutrophil in the following ways: (1) enhancing vessel wall adherence and transmigration capacity; (2) production and release of oxygen radicals, and (3) degranulation and release of enzymes including myeloperoxidase (MPO) and proteinase-3 (PR3) (**a**). Transient immune complexes are formed locally by binding of ANCA to PR3/MPO sticking to endothelial cells. Subsequently, complement is activated, which further promotes neutrophil degranulation. This all adds to the development of necrotizing vasculitis. Whether this specific cascade is also applicable to disease pathogenesis in ANCA-negative AAV patients remains unclear. The expanded effector memory T cells (Tems) are not sufficiently regulated by regulatory T cells (Tregs, **b**). This leads to dysbalance in the homeostasis of Tregs and Tems, resulting in further release of proinflammatory cytokines promoting neutrophil priming (**a**); moreover, ANCA production is enhanced by further T-cell/B-cell interaction. (**c**) Expanded circulating Tems migrate into target organs such as the lungs or the kidney. Within tissues, Tems drive granuloma formation, which is considered an 'executioner' of tissue destruction. Granulomas are composed of numerous cell types such as T cells, B cells, giant cells, and dendritic cells (DCs). Moreover, ANCA production occurs in granulomas. Possibly, tertiary lymphoid organs (TLOs) are 'local controllers' of tissue inflammation, as induction of Tregs is thought to take place in TLOs. Neutrophil extracellular trap (NET) formation occurs in lesions as a consequence of neutrophil apoptosis and degranulation. DNA and serine proteases are deployed in these NETs. NET-derived products activate DCs and B cells by sensing via Toll-like receptors (TLRs). Interferon (IFN-α) production by DCs might have an impact on local immune regulation; it has been shown to impair Tregs in function.[113] Although major efforts were made to unravel the pathogenesis of AAV, some missing links remain. The origin of ANCA is unexplained so far. If and how genetic background, microbial agents, and/or T-cell dysregulation finally lead to the development of ANCA needs to be investigated further. HEV, high endothelial venules; ICX, immunocomplexes.

injury was not prevented by blocking superoxide release. However, inhibition of serine proteases led to less endothelial cell injury. Therefore, ANCA-induced release of proteases seems to be the most important factor for vasculitic damage. Nevertheless, release of reactive oxygen species enhances the activity of serine proteases by inactivating α_1-anti-trypsin, which is a potent PR3 inhibitor.[22–24] Binding of ANCAs to endothelial cells might occur via PR3/MPO acting as cofactors or via Fc receptors, but the exact mechanism remains controversial.[11,25–27] The interaction of ANCAs with endothelial cells enhances expression of adhesion molecules like E-/P-Selectin and vascular cell adhesion molecule, as shown by several authors.[10,28] Subsequently, neutrophil-endothelial cell adherence is altered as demonstrated in flow models. ANCAs promote firm and sticky attachment of neutrophils to endothelial cells in these models, leading to enhanced transmigration and damage.[18,29–31]

Apart from *in vitro* experiments, ANCA pathogenicity has been investigated in animal models. Although animal models proving MPO-ANCA pathogenicity are well established, similar efforts for PR3-ANCA have not been successful so far. Xiao *et al.*[32] immunized MPO-knockout mice with murine MPO and transferred anti-MPO-IgG to wild-type and immune-deficient ($RAG2^{-/-}$) mice. Wild-type and immune-deficient mice developed NCGN, proving a pathogenic role for MPO-ANCA. The importance of neutrophils and the priming process with proinflammatory agents was confirmed in this model.[33,34] The current rat MPO-vasculitis model shows a varying and inconsistent disease phenotype. Therefore, Little *et al.*[35] recently modified this rat model by using adjuvants to enhance immunization, resulting in experimental vasculitis with robust and reproducible disease expression. Pfister *et al.*[36] could not successfully establish a similar model for PR3-ANCA. Wild-type mice receiving anti-PR3-IgG did not develop glomerulonephritis or human AAV features. Interestingly, in a recent study by Primo *et al.*,[37] anti-PR3-induced immune responses elicited NCGN in mice prone to autoimmunity, demonstrating that PR3 immune responses in general can cause vasculitis and NCGN. However, a certain genetic background predisposing to autoimmunity seems to be indispensable.[37] Hattar *et al.*[38] confirmed the pathogenic potential of anti-PR3 antibodies in an elegant model using isolated rat lungs. Perfusion of these lungs with neutrophils and antibodies against PR3 but not with control IgG resulted in edema formation resembling acute lung injury.

In summary, there is convincing evidence from both *in vitro* and *in vivo* experiments that ANCAs are pathogenic.

ANCAs: EXPANDING RANGE OF SUBTYPES?

In the late 1980s, it was discovered that PR3 was the main antigen for cytoplasmic-ANCA, whereas MPO was shown to be the antigenic target of perinuclear-ANCA in patients with vasculitis.[4,7,39–44] The range of ANCA subtypes expanded and additional autoantigens recognized by ANCAs were found.[45] Recent findings bring up a new hypothesis on the induction

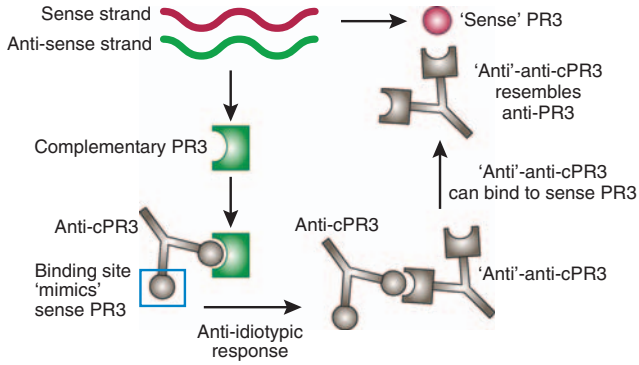


Figure 2 | The principle of the anti-idiotypic response in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). This concept suggests that proteinase-3 (PR3)–ANCA are formed during a secondary immune response to antibodies that have specificity for complementary PR3 (cPR3). The sense strand of the *PR3* gene codes for the corresponding sense protein PR3, whereas the antisense strand of the *PR3* gene codes for the corresponding complementary protein cPR3. According to Pendergraft *et al.*,[46] an immune response against cPR3 is elicited in AAV patients. Subsequently, antibodies with specificity for cPR3 evolve. The antigen-binding region of these antibodies mimics epitopes of the sense protein PR3. An additional immune response against anti-cPR3 antibodies is initiated and defined as 'anti-idiotypic response.' According to this concept, antibodies against anti-cPR3 evolve throughout the disease process. These 'anti'-anti-cPR3 antibodies bind the sense PR3 and resemble PR3-ANCA.

of ANCAs by immune responses against Gram-positive or Gram-negative bacteria.[39,45]

Pendergraft *et al.*[46] investigated the role of complementary peptides in WG. The authors hypothesized that the initial immune response in WG is directed against the complementary protein PR3 (cPR3) and that anti-PR3 antibodies evolve during a secondary anti-idiotypic immune response (Figure 2). According to this hypothesis, antibodies forming the humoral immune response against cPR3 would serve as antigenic target for a secondary immune response ('anti-idiotypic response,' Figure 2).[47] Anti-idiotypic antibodies not only react to anti-cPR3-antibodies but also to the sense protein PR3. Out of 34 WG patients, 7 (20%) had detectable antibodies against cPR3 that indeed formed idiotypic pairs with anti-PR3 antibodies. Injection of cPR3 in mice resulted in anti-cPR3 and anti-PR3 antibodies as predicted. Interestingly, cPR3 mRNA transcripts were only found in leukocytes from WG patients but not from healthy controls or lupus patients. Whether these cPR3 transcripts are of exogenous or endogenous origin remains to be solved. However, pathogens like *Staphylococcus aureus* bear genetic sequences that are complementary to the human *PR3* gene, pointing to an exogenous origin of cPR3 transcripts. Indeed, chronic nasal carriage of *S. aureus* has been demonstrated to increase the risk for disease relapse.[48] Moreover, WG patients treated with cotrimoxazole are less prone to relapse, and in some cases even remission can be induced by applying cotrimoxazole as monotherapy.[49,50] The mechanism proposed by Pendergraft *et al.*[46] defines a pivotal, novel role for a specific ANCA

subtype but needs further confirmation. In addition, the clinical relevance and importance to disease pathogenesis needs to be defined and remains unclear.

Recently, Kain *et al.*[45] reported the discovery of autoantibodies against lysosomal membrane protein-2 (LAMP-2) in patients with AAV-associated NCGN. They provided *in vitro* and *in vivo* evidence for the relevance of these antibodies to disease pathogenesis and linked them with infectious pathogens. Anti-LAMP-2 antibodies were only found in patients with active ANCA-associated NCGN. Interestingly, anti-LAMP-2 antibodies were also detectable in several patients with NCGN lacking PR3-ANCA or MPO-ANCA. Moreover, patients with localized AAV lacking renal involvement were generally negative for anti-LAMP-2 as were disease controls and healthy controls. Furthermore, Wistar Kyoto rats injected with antibodies to LAMP-2 developed crescentic pauci-immune glomerulonephritis. Anti-LAMP-2 crossreacted with FimH, which is part of the fimbriae of Gram-negative pathogens. Accordingly, immunization with FimH led to development of crescentic glomerulonephritis in rats. Altogether, these results suggest that AAV-associated crescentic glomerulonephritis might be triggered by bacterial infection eliciting an immune response to a previously unidentified, novel autoantigen. However, according to our own published observations, disease onset or relapse of AAV is linked to Gram-positive bacteria like *S. aureus* and not to infections with Gram-negative bacteria.[48,51] Thus, the findings by Kain *et al.*[45] need to be confirmed in other patient cohorts.

HNE (human neutrophil elastase) belongs to the chymotrypsin family of serine proteases. ANCAs with specificity to HNE are rarely and infrequently detected in patients with vasculitis.[52] Importantly, HNE-ANCA might be of use for diagnosing cocaine-induced midline destructive disease and/or drug-induced AAV.[53] Dolman *et al.*[54] detected anti-HNE antibodies frequently in patients developing vasculitis during treatment with propylthiouracil. These findings were confirmed by others showing an association of ANCA with the administration of antithyroid drugs.[55]

THE COMPLEMENT SYSTEM AND ITS ROLE IN AAV

Pauci-immunity is a hallmark of ANCA-associated vasculitis, and deposition of immune complexes or complement factors is considered to be absent.[2] However, accumulating evidence suggests that the complement pathway is involved in disease pathogenesis.[56–60]

The complement system has a pivotal role in host defense as well as clearance of immune complexes (Figure 3).

First evidence pointing to the complement system in AAV came from biopsy studies already performed in the late 1970s.[56] In following studies, renal and skin biopsies from patients with AAV were assessed for complement depositions.[58–68] Collectively, more than half of the biopsies from patients with AAV were found to have C3 complement deposition. *In vitro* evidence supports the idea of complement involvement in AAV. Several groups have shown that MPO or oxygen radicals released by degranulation of

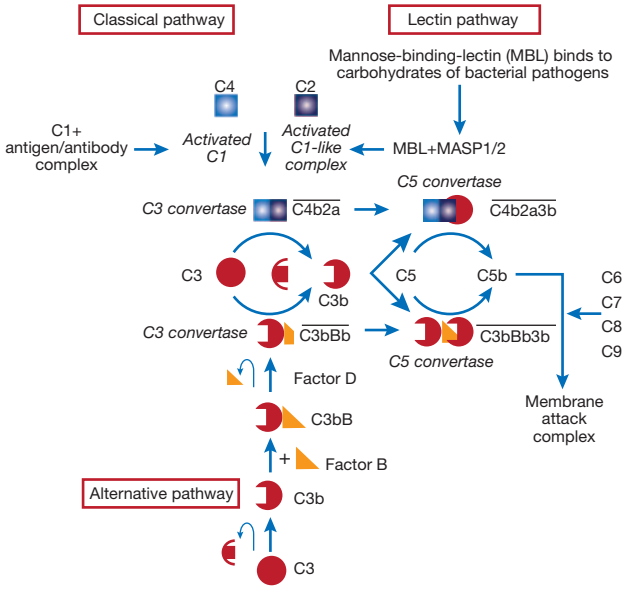


Figure 3 | Overview on the complement system. The complement system has a pivotal role in host defense as well as clearance of immune complexes. Three different activating pathways of the complement cascade have been identified so far. Although the initial activating steps are different, all pathways end up in a common, terminal pathway characterized by cleavage of C5, which finally results in assembly of membrane attack complex (MAC). The classical pathway is initiated by binding of C1q to antigen–antibody complexes, enhancing the formation of a specific C3 convertase (C4b2a). Binding of cleaved C3b to this complex forms C5 convertase. The same convertases are activated by the lectin pathway. In this case, Mannose-binding lectin and ficolins bind to carbohydrates of bacterial pathogens followed by activation of mannose-binding lectin-associated serine proteases (MASPs). These MASPs cleave C4/C2 to the above-mentioned C3 convertase and subsequent C5 convertase activation. The alternative pathway is activated by a different C3 convertase (C3bBb). This C3 convertase is formed by C3 hydrolyzing spontaneously and is stabilized by factors B and D. By further association of C3b to this complex, C5 cleaving convertase (C3bBbC3b) is formed.

neutrophils activate complement factors C3 and C5.[69–71] Moreover, Xiao *et al.*[72] demonstrated that ANCA-induced activation of neutrophils results in complement activation and generation of C3a. Interestingly, complement receptors are also present on neutrophils. Schreiber *et al.*[73] found that C5a is able to prime neutrophils and to enhance ANCA-induced neutrophil activation. Therefore, neutrophils are linked very tightly to complement activation. These *in vitro* data are supplemented by *in vivo* data from animal models. Huugen *et al.*[74] could prevent and/or attenuate MPO-induced NCGN in mice by anti-C5 treatment. Likewise, Xiao *et al.*[72] completely blocked development of MPO-induced NCGN by complement depletion using cobra venom factor. Furthermore, NCGN was absent in factor B knockout mice (an essential factor for alternative pathway) but could be induced in C4 knockout mice (an essential factor for classical/lectin pathway), providing some evidence for pathogenetic involvement of the alternative pathway of complement activation.[72] In conclusion, the complement

system seems to be an important player in AAV. Complement activation and the resulting products might promote inflammation and enhance tissue damage. Although the exact mechanisms are not known yet, the neutrophil–complement axis might be crucial. Neutrophils become activated by complement products and complement is activated by neutrophils. Hence, dysregulation of this axis might lead to sustained, self-perpetuating inflammation and contribute in this way to AAV (Figure 1). Finally, complement activation might also account for increased risk of venous thromboembolism observed in active AAV, as activated complement factors trigger coagulation.[75,76]

T CELLS IN AAV

T cells are usually found within granulomas as well as in other lesions present in AAV.[77–80] In accordance with these findings, elevated levels of markers of T-cell activity such as soluble interleukin-2 (IL-2) receptor, neopterin, and soluble CD30 as measured in the circulation have been shown to be associated with disease activity.[81–83] Furthermore, ANCA IgG subclasses suggest that a T-cell-mediated subclass switch has taken place.[84] Specific T-cell-targeted therapy is occasionally used in refractory cases and has been demonstrated to be beneficial.[85]

In patients with active disease and during remission, T cells are in a persistent state of activation.[81] Furthermore, memory T cells are expanded, whereas naive T cells are decreased.[86,87] Recently, we demonstrated that a specific subset of effector memory T cells (Tems) expressing CD134 and GITR (glucocorticoid-induced TNF-receptor-related protein) is especially expanded in WG patients.[88] $CD134^+$ cells were also found in active lesions, suggesting increased migration to inflamed sites (Figure 1). In line with this, Abdulahad *et al.*[89] reported Tems in the urine, suggesting that Tems migrate from the circulation to inflammatory lesions during active states of the disease. Tems are powerful immune cells that initiate and sustain immune responses. This T-cell population is long lived and responds quickly to adequate triggers. Moreover, granuloma formation is driven by these T cells.[90] Therefore, we believe that Tems have a major pathophysiological role in AAV.

IL-17-producing Th17 effector T cells were shown to be of major importance in autoimmunity.[91] Recently, it was reported that WG patients in remission bear an increased amount of Th17 cells reactive to the autoantigen PR3.[92,93] Moreover, AAV patients harbor an expanded CD45RC T helper cell population that is a source of IL-17.[94] IL-17 facilitates the migration and activation of neutrophils by promoting the secretion of TNF-α and IL-1β.[95] As the influx of neutrophils is a hallmark of AAV, IL-17 might also have a pivotal pathophysiological role in AAV.

Regulatory T cells (Tregs) limit immune responses. In some autoimmune diseases, Treg defects have been described.[96] There are limited data on Tregs in AAV. However, an increase of $FoxP3^+$ Tregs in patients with AAV in remission was described in one study, whereas earlier studies failed to show an increase of circulating $FoxP3^+$ Tregs.[86,97] Subsequently, two studies suggest a functional impairment of Tregs in WG.[98,99] These Tregs fail to inhibit proliferation or cytokine production of effector T cells. Defective Treg function might account for the Tem expansion and the persistent T-cell activation observed in AAV (Figure 1).

TERTIARY LYMPHOID TISSUE IN AAV

At present, it is unknown whether local activation or control of the immune response within the affected tissue itself occurs in AAV. Local control of immune responses is linked to the development of tertiary lymphoid organs (TLOs), also known as lymphoid neogenesis.[100] This has been described in several chronic inflammatory conditions, for example, rejection in the context of organ transplantation and/or inflammation in the context of several autoimmune diseases.[100] TLOs resemble the structure of secondary lymphoid organs and consist of B-cell follicles with a surrounding mantle zone with T cells and DCs. Within these TLOs, T-cell activation by antigen-presenting cell stimulation takes place. It is likely that local, tissue-specific (auto) antigens are presented. Whereas secondary lymphoid organs have organized lymph flow and antigen-presenting cell trafficking, TLOs lack these features, resulting in an unrestricted access of antigens, antigen-presenting cells, and lymphocytes. These conditions might promote persistent and non-physiological T-cell activation in autoimmunity.[100]

What is the evidence for TLOs in AAV? So far, granulomas are regarded as some form of TLOs where immune responses are modified.[78,101] First, Csernok *et al.*[102] revealed that PR3 is abundantly present in granulomas and renders DCs to powerful Th1-cell activators. Indeed, Muller *et al.*[103] found mature DCs in granulomatous lesions of nose biopsies. Moreover, Voswinkel *et al.*[104] demonstrated that affinity maturation of B cells, as is commonly observed in lymphoid tissue, takes place in granulomas. It is suggested that the production of ANCA takes place locally within these granulomas.

Granuloma formation is only rarely found in the kidneys of AAV patients. Some form of lymphoid neogenesis, however, has been observed in renal biopsies of patients with AAV.[105–107] Immature DCs and T cells form aggregates, suggesting a cell–cell interaction. Importantly, these DCs display costimulatory capability by expressing CD80.[106] We hypothesize that in the kidney also, activation of effector T cells and stimulation of the immune response takes place. However, a local induction of Tregs and thus an attenuation of the inflammatory process seems to be another possibility.[106,108] Our own data indicate that $FoxP3^+$ T cells are present in inflammatory lesions of NCGN (B Wilde and JW Cohen Tervaert, unpublished data, 2009). The induction of Tregs is especially confined to places where abundant immature DCs bearing costimulatory properties are present.[109] Recently, Kessenbrock *et al.*[110] added an important piece of the puzzle. They reported formation and renal deposition of neutrophil extracellular traps (NETs) in

AAV. NETs are decondensed chromatin fibers released by neutrophils that contain several cytoplasmic proteins like PR3, MPO, elastase, and LL-37. NET formation is a mechanism of host defense and allows efficient containment as well as killing of microbial invaders.[111] However, NET formation also exerts immune-modulating functions with implications for autoimmunity and AAV. LL-37 deployed in NETs is capable of modifying trapped DNA. The modified DNA then acts as a danger-associated molecular pattern and activates DCs and B cells via Toll-like receptor (TLR)-sensing pathways.[112] In AAV, renal NET deposition triggers IFN-α production of plasmacytoid DCs.[110] IFN-α might sustain inflammation by impairing lesional Tregs in their function.[113] Furthermore, local B-cell maturation and autoantibody production might result from excessive Toll-like receptor triggering.[114] Indeed, Steinmetz *et al.*[107] reported highly complex B-cell follicles suggestive of organized TLOs in renal tissue of AAV patients.

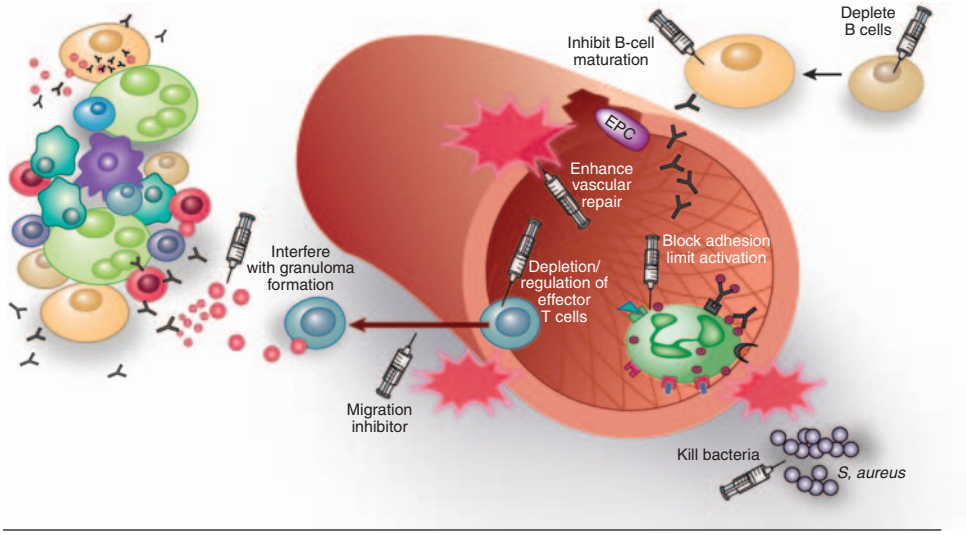
Therefore, both local control of tissue inflammation and activation of immune cells at the site of inflammation are likely to occur in AAV (Figure 1).

DISEASE PATHOGENESIS AND CURRENT THERAPEUTIC CONCEPTS

Summarizing the preceding paragraphs, disease pathogenesis of AAV is complex with a number of overlapping effector limbs. It is clear that ANCAs are of major importance for disease and cause vasculitis, interacting with neutrophils upon specific triggers like infections (Figure 1). At the same time, Tems escaping immune regulation enhance autoantibody production and drive tissue inflammation (Figure 1). Interfering with these pathogenic mechanisms is crucial, as the patients' outcome is fatal if the disease is left untreated.[115] The outcome has improved dramatically since the introduction of cyclophosphamide (CYC) as a therapeutic agent of AAV.[115,116] Most of the drugs used for treatment have a broad spectrum of activity affecting all effector limbs. CYC is one of the most efficient agents available to treat AAV and targets a number of mechanisms described above. It alkylates DNA and thus affects a wide variety of cell types including leukocytes that lack aldehyde dehydrogenase, an essential enzyme breaking down the toxic metabolite of CYC.[117] Thus, B-cell suppression is usually observed and T cells as well as neutrophils are hit by CYC treatment.[118] This results in reduction of pathogenic ANCAs, fewer pathogenic effector memory T cells, and less neutrophils. Azathioprine (AZA), methotrexate (MTX), and mycophenolate mofetile (MMF) interfere with DNA or nucleotide synthesis, affecting dividing cells and thus also pathogenic lymphocytes. Some of the knowledge gained on the pathogenesis of AAV has already been translated into new and specific therapeutic approaches with presumably less side effects. Plasmapheresis and B-cell depletion using rituximab (RTX) are both applied successfully in treating AAV and have the rationale to ameliorate disease by removing autoantibodies or their source.[119–122] Specific T-cell depletion with agents like antithymocyte globulin or Campath-1H (CAMP) has also already been introduced into the clinic but the efficacy is limited so far;[85,123] see Figure 4.

CURRENT THERAPY OF ANCA-ASSOCIATED VASCULITIS: THE EULAR GUIDELINES

Recently, guidelines on treatment made by a working group of the EULAR (European League Against Rheumatism) were published based on data of recent studies.[124] During the initial phase, remission is induced ('induction phase'), whereas thereafter remission is maintained ('maintenance therapy'). The pharmacotherapy of both phases is different. The most effective and best evaluated drug used in the induction phase is CYC. However, it is toxic in a dose-dependent manner and associated with severe side effects affecting long-term morbidity as well as mortality.[125] High cumulative doses above 36 g seem to increase the risk for leukemia and bladder cancer.[125] Thus, adjustment of treatment to disease severity has been suggested (Tables 1 and 2).[124] The disease category reflects the disease extent (Table 1). In case of severe generalized disease, CYC and glucocorticoids (prednisolone (PRED)) in combination are recommended for induction of remission. Oral versus intravenous (i.v.) administration of CYC was studied in the CYCLOPS study by the EUVAS group. Periodical i.v. infusion reduces the cumulative CYC dosage needed and thus toxicity; long-term morbidity and/or mortality because of CYC might be lower.[126] In the randomized controlled trial (RCT) CYCLOPS, a total of 149 WG/MPA patients suffering from new-onset generalized AAV were enrolled: 76 patients were assigned to the i.v. CYC group and 73 were randomized to the oral CYC group. It was seen that 88.1% of the i.v. CYC group and 87.7% of the oral CYC group achieved remission after 9 months. There was no significant difference in median time to remission (3 months each) or improvement of renal function at study end. Although not statistically significant, more relapses occurred in the i.v. CYC group ($n = 13$) than in the oral CYC group ($n = 6$). As expected, the cumulative CYC dosage needed to achieve remission was significantly lower in the i.v. CYC group as opposed to the oral CYC group (8.2 vs 15.9 g, $P < 0.001$). Furthermore, less patients were affected by leucopenia in the i.v. CYC group when compared with the oral CYC group (26 vs 45%, $P = 0.016$). Toxicity as observed during a period of 18 months did not differ. In summary, i.v. CYC seems to be as efficient as oral CYC in inducing remission.[126] The safety profile with possible decreased long-term toxicity because of low CYC doses favors i.v. CYC therapy over oral CYC. The oral CYC regime used in CYCLOPS differed from other studies. In the RAVE and the CYCAZAREM trial, oral CYC was administered for 3 months, and beyond that only until remission was achieved.[121,127] Oral CYC was ceased immediately after having entered remission and then replaced by maintenance therapy. However, in the CYCLOPS trial, oral CYC was not ceased after remission had been achieved but continued for additional 3 months (at a reduced dose of 1.5 mg/kg/day).[126]



Principle	Mechanism	Agent	Evidence	References
Depletion of effector T cells	Antibodies directed against CD25 deplete activated T cells	Basiliximab Daclizumab	Experimental + clinical evidence (RA+Tx) Ongoing RCT in AAV	179, 180 NCT0040248
Regulation of effector T cells	Blockade of CD28/CD80 dependent T cell activation	Abatacept, Belatacept (both CTLA-4 fusion proteins)	Experimental + clinical evidence (RA+Tx) Ongoing trial in AAV	172 NCT00468208
Block adhesion of neutrophils	Blockade of CD11b/ICAM-1 mediated adhesion to endothelium		Experimental evidence	18,178
Limit activation/ recruitment of neutrophils	Inhibition of C5 cleavage. Blockade of C5a receptor on neutrophils	Eculizumab, Pexelizumab (both anti-C5)	Experimental evidence	73, 74
Enhance vascular repair	Promote EPC mobilization and function	EPO Statins	Experimental + clinical evidence	173–176
Inhibition of migration	Blockade of a4-integrins on T cells	Natalizumab	Experimental + clinical evidence in MS	169, 170
Interfere with granuloma formation	Blockade of TNF-α	Infliximab Adalimumab	Experimental + clinical evidence in AAV	90, 134–136
Depletion of B cells	B-cell depletion by antibodies recognizing CD20/CD22	Rituximab, Epratuzumab (both anti-CD20)	Experimental + clinical evidence in AAV	118–122, 166, 177
Inhibition of B-cell maturation	Neutralization of BLys. Blockade of BLys-receptors on B cells	Belimumab (anti-BLys) Atacicept (anti-TACI)	Experimental evidence	118–122, 166, 177
Anti-microbial treatment	Reduction of microbial flora that might trigger disease flares	Cotrimoxazol	Experimental + clinical evidence in AAV	45, 48–51

Figure 4 | Understanding the pathogenesis of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) allows application of targeted therapy. As autoantibodies have a key role in AAV, depletion of B cells or interfering with maturation of these cells might ameliorate disease.[177] Blocking adhesion and activation of neutrophils might also dampen or even prevent vasculitic damage.[178] Vasculitic damage needs to be repaired and endothelial progenitor cells (EPCs) are regarded as an important factor of vascular repair. EPC mobilization and function might be enhanced by additional treatment with erythropoietin (EPO) or statins. T cells drive the disease as well and could be targeted. For this purpose, T-cell activation could be limited by interfering with the costimulatory molecules and it might be beneficial to deplete subsets of effector T cells.[179,180] Next, migration of T cells to tissue sites or T-cell-driven granuloma formation might be inhibited by biologics already available. Finally, treatment with antibiotics might prevent disease flares triggered by bacteria.[48,49,181] BLys, B lymphocyte stimulator; CTLA-4, cytotoxic T-lymphocyte antigen 4; ICAM-1, intercellular adhesion molecule 1; MS, multiple sclerosis; RA, rheumatoid arthritis; RCT, randomized controlled trial; TNF-α, tumor necrosis factor-α. TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor.

This might also account for the large difference in the median cumulative dose between oral and i.v. CYC observed in CYCLOPS. Furthermore, it is not yet clear whether i.v. cycles result in a higher relapse rate. A recent meta-analysis points at an increased risk of relapse in patients treated with i.v. CYC when compared with oral CYC (four studies, relative risk 1.79, confidence interval 1.11–2.87, $P = 0.02$).[128] There was no difference in mortality or remission rates.[128] Nonetheless, a switch to oral CYC should be considered if induction therapy with i.v. CYC fails, and during the last year we observed several i.v. CYC failures (JW Cohen Tervaert, personal observation, 2009).

Removing circulating ANCA might be beneficial considering the pathogenic potential of these autoantibodies.

Table 1 | Disease categories as defined by the European League Against Rheumatism[124]

Disease category	Definition
Localized	Upper and/or lower respiratory tract disease without any other systemic involvement or constitutional symptoms
Early systemic	Any, without organ- or life-threatening disease
Generalized	Organ-threatening disease, if renal involvement: serum creatinine <5.6 mg/dl
Severe	Vital organ failure, if renal involvement: serum creatinine >5.6 mg/dl
Refractory	Disease progression despite therapy with cyclophosphamide and steroids

Table 2 | Recommendations for therapy of AAV by the European League Against Rheumatism[124]

Disease category	Recommended therapy	Grade of recommendation[a]	Level of evidence[b]
Remission induction			
Early systemic/localized disease	Methotrexate+steroids	B	1B
Generalized disease	Cyclophosphamide (i.v. or oral)+steroids	A	$1A^{WG/MPA}$ $1B^{CSS}$
Severe disease with renal failure	Adjunct: plasma exchange	A	1B
Maintenance therapy			
Low-dose steroids +	Azathioprine	A	1B
	Leflunomide	B	1B
	Methotrexate	B	2B

Abbreviations: ANCA-associated vasculitis; CSS, Churg–Strauss syndrome; i.v., intravenous; MPA, microscopic polyangiitis; RCT, randomized controlled trial; WG, Wegener's granulomatosis.
[a]Grade of recommendation: A, based on at least evidence level 1A/B; B, based on at least level 2 evidence or extrapolated recommendations from level 1 evidence.
[b]Levels of evidence: 1A, evidence from meta-analysis of RCT; 1B, from at least one RCT; 2B, from at least one type of quasi-experimental study.

The RCT MEPEX assessed the impact of plasmapheresis on renal/patient survival in a cohort of WG/MPA patients with severe renal vasculitis and acute renal failure.[120] Adjunct plasmapheresis in combination with oral CYC/PRED improved renal recovery when compared with the control arm receiving concomitant courses of i.v. methylprednisolone instead of plasmapheresis. At 3 months, 69% of the AAV patients in the plasmapheresis group were alive and independent of dialysis as opposed to 49% in the control arm. Furthermore, plasmapheresis was associated with a reduction in the risk of progression to end-stage renal disease at 12 months (24%, confidence interval 6.1–41%).There was no difference in patient survival or adverse event rate.[120] A recent meta-analysis by Walters *et al.*[128] confirmed these results in showing that adjunct plasma exchange reduces the risk of requiring dialysis 12 months after induction treatment (Five studies, relative risk 0.47, confidence interval 0.3–0.75, $P=0.002$). Based on these findings, adjunct plasma exchange is advised in patients with severe renal involvement, alveolar hemorrhage, or other life-threatening organ manifestations.[120] A large RCT ('PEXIVAS') is currently underway to assess the impact of plasma exchange on mortality and renal survival in both MPO/PR3 patients with estimated glomerular filtration rate <50 ml/min per 1.73 m^2.[129] If AAV is present without kidney involvement (defined as serum creatinine <120 umol/l), MTX can be used in combination with PRED to spare CYC and to reduce unwanted toxic side effects.[130] In the NORAM study (RCT) enrolling WG/MPA patients, it was demonstrated that MTX was not inferior to CYC/PRED in inducing remission at month 6. However, remission was significantly delayed in patients with extensive disease and pulmonary involvement when treated with MTX. Furthermore, there were more relapses in the MTX limb when compared with the CYC/PRED limb.[130]

INDUCTION THERAPY IN AAV: ALTERNATIVE STRATEGIES

MMF has also been assessed for induction of remission in two open label trials and in a retrospective case review study.[131–133] Complete and partial remission could be achieved in a substantial number of patients by MMF administration. Additional RCTs are needed to answer questions regarding efficacy.[131–133] RTX has recently been studied as an alternative to the standard induction protocol with CYC. Two RCTs provide evidence that RTX/PRED is noninferior to CYC/PRED induction therapy.[119,121] The placebo-controlled RAVE study enrolled 197 patients with MPA/WG and consisted of two trial arms, in which induction therapy with oral CYC/PRED ($n=99$) was compared with RTX/PRED ($n=98$, 4 times 375 mg/m^2).[121] A total of 55% of patients in the CYC arm and 64% in the RTX arm achieved complete remission and were off PRED after 6 months ($P=0.21$). There was no difference in the rate of adverse events or relapses within the first 6 months for patients with new-onset disease, whereas in patients with relapsing disease RTX was significantly better than CYC. A different RCT organized by the EUVAS group ('RITUXVAS') compared the standard CYC induction protocol to RTX in patients with severe generalized WG/MPA.[119] These patients were suffering from more severe disease as indicated by a median glomerular filtration rate of 18 ml/min per 1.73 m^2. Patients were either treated with 6–10 cycles of CYC pulses or with RTX (4 times 375 mg/m^2) in combination with two pulses of

CYC. Maintenance therapy with AZA was only given to patients in the CYC group, and the RTX group did not receive maintenance therapy. A total of 44 patients were enrolled: 33 in the RTX group and 11 in the CYC group. The outcome was comparable in both groups; 76% of patients in the RTX group and 82% of the patients in the CYC arm had entered sustained remission after 12 months ($P = 0.67$). The improvement of renal function during therapy was not different as was the rate of adverse events. These studies provide good evidence for noninferiority of RTX when compared with CYC to induce remission without the burden of long-term toxicity. However, there are not sufficient data on long-term outcome in AAV available yet.

There is a controversy on the use of TNF-α blockade in treatment of AAV. Etanercept was assessed in the WGET trial and serious concerns on safety were raised, as the incidence of malignancies was higher in WG patients treated with etanercept.[134,135] Although this TNF-α blocker has no place in the therapy of AAV, other agents like infliximab or adalimumab are still under evaluation for therapy. The efficacy of adalimumab, which is a humanized anti-TNF-α antibody, was recently studied by Laurino *et al.*[136] in a prospective, uncontrolled phase II trial. A total of 14 patients with new-onset systemic WG/MPA and kidney involvement were enrolled. Adalimumab was administered concomitantly with CYC and PRED, and 79% of all patients entered remission within the first 3 months. Adalimumab administration permitted lower prednisone dosages during the first 3 months when compared with standard induction protocols.[136] Interestingly, CSS patients lacking poor prognosis factors like renal impairment, cardiomyopathy, severe gastrointestinal tract, or central nervous system involvement respond to single treatment with steroids and enter remission as shown by Ribi *et al.*[137] in a prospective, randomized open-label trial. As relapses were common and occurred in 35% of the patients, the efficacy of this approach is clearly limited. Combination with AZA, MMF, or MTX might allow sustained remission if first-line therapy with CYC/PRED is considered to be too toxic.[137,138]

INDUCTION THERAPY: REFRACTORY PATIENTS

There are additional options for induction therapy in patients refractory to standard protocols (Figure 4). Intravenous immunoglobulins were shown in one randomized trial to reduce disease activity of WG/MPA patients.[139] However, the effects were small and short lived, as beyond 3 months there was no additional benefit when compared with placebo infusions. 15-Deoxyspergualin was tested in uncontrolled open-label studies in WG patients with refractory, persistent disease activity.[140,141] In 95%[141] and 70%[140] of these patients, at least partial remission was achieved. Further follow-up data suggest that prolonged treatment with 15-deoxyspergualin is necessary to maintain remission, as relapses were common after withdrawal. A new phase III trial is currently in preparation to further assess the therapeutic value of 15-deoxyspergualin. T-cell-targeted treatment with antithymocyte globulin is also reported to be beneficial in selected patients with severe, refractory WG.[85] Another T-cell-depleting agent, CAMP, has been studied by Walsh *et al.*[123] CAMP is a humanized antibody against CD52 present on lymphocytes and macrophages. This cohort study by Walsh *et al.*[123] on CAMP treatment in AAV patients with refractory or relapsing disease showed high mortality, with 31 deaths out of 71 AAV patients. In all, 60 (85%) patients entered remission but 43 relapsed within a median time of 9.2 months. Adverse events like infections ($n = 28$), thyroid disease ($n = 8$), and malignancies ($n = 3$) were common. Infections or a combination of infection and active disease were the cause of death in 12 cases. Given the mortality and the risk of infection observed in this study, the use of CAMP should be considered carefully and should be restricted to a selected cohort of refractory patients.[123] In selected cases with refractory and/or severe disease, allogeneic hematopoietic stem cell transplantation might allow control of disease activity as recently reported by Bornhauser *et al.*[142] Additionally, two retrospective studies suggest that RTX might be efficient to induce remission in patients with refractory AAV.[143,144] There is one uncontrolled, prospective open-label trial that assessed the value of IFN-α treatment in seven CSS patients refractory to standard therapy.[145] These patients received IFN-α for 6 months along with steroids. Five patients entered complete remission, whereas two were reported to have residual symptoms. No relapses or serious adverse events occurred during 6 months of follow-up. Thus, IFN-α appears to be an option in selected refractory cases; however, because of the small sample size and short follow-up, no certain conclusion on efficacy or safety can be drawn.[145] Furthermore, biologicals like mepolizumab, an anti-IL-5 antibody, and omelizumab, an anti-IgE antibody, might allow induction of remission in refractory CSS. Yet, there are only series of case reports published and evidence is based on only a few patients.[146,147]

MAINTENANCE THERAPY IN AAV

Maintenance therapy should follow induction therapy as relapses frequently occur in AAV patients.[148] Some authors stress that there might be a subset of patients with distinct clinical features not requiring long-term maintenance therapy.[149] This subset of patients is characterized by the presence of MPO-ANCA and vasculitis without involvement of respiratory tract, but specific markers allowing reliable identification are missing so far.[149,150] Thus, at present, we advise to continue maintenance therapy for at least 18–24 months as recommended by the EULAR and the British Society for Rheumatology.[124] Owing to long-term toxicity, CYC should not be used anymore for maintenance of remission, as AZA was proven to be as effective as CYC in preventing relapses of WG/MPA patients.[127] MMF was studied in a number of smaller studies and the efficacy was varying.[131,151,152] A large RCT (IMPROVE) comparing AZA with MMF conducted by the EUVAS study group was recently performed and preliminary results show inferiority of MMF.[153]

MTX might be considered as an alternative to AZA. In a RCT, however, there was a nonsignificant trend to more severe side effects with MTX when compared with AZA.[154] Leflunomide (LEF) was compared with MTX in a controlled trial by Metzler *et al.*[155] This trial had to be terminated early as the rate of major relapses in the MTX limb was too high (46%). However, the relapse rate in the LEF limb (13.1/100 patient-years) was comparable with the one observed for AZA in the CYCAZAREM trial (10.3/100 patient-years), indicating the efficacy of LEF as a therapeutic agent in AAV.[127,155] Importantly, the LEF-associated adverse events are problematic, especially hepatotoxicity with subsequent organ failure.[156] Low-dose PRED should be added to maintenance therapy. The duration is, however, debatable. A meta-analysis found a decreased proportion of relapsing patients in studies with long-term steroid treatment (14%) as opposed to studies with withdrawal of steroids (43%).[157] As relapses are associated with nasal carriage of *S. aureus*, antibiotics might be useful in preventing disease flares.[48] Cotrimoxazole treatment decreases the incidence of relapses in patients with WG and is therefore advised in patients with high relapse rates.[49,51] There are few studies on maintenance treatment in cohorts of CSS patients. Metzler *et al.*[158] conducted a prospective, uncontrolled study to assess the efficacy of IFN-α in maintenance of remission. A total of 13 patients received IFN-α along with steroids. Of these patients, ten relapsed after a median time of 17 months, and adverse events like infectious episodes ($n = 18$) occurred frequently. Therefore, IFN-α cannot be considered as a therapeutic option for maintenance therapy in CSS patients. Mepolizumab, an anti-IL-5 antibody, might arise as an adjunct therapeutic agent to spare steroids in CSS, but evidence is little until now.[159]

The time point at which therapy should be adjusted (for example switching from induction to maintenance treatment) is not well defined. In most studies, induction therapy is switched after 'clinical remission,' defined as the absence of clinical symptoms attributable to active vasculitis.[160,161] Interestingly, we demonstrated that switching to AZA maintenance therapy in patients in clinical remission with a positive PR3-ANCA titer is associated with significantly higher relapse rates when compared with patients being PR3-ANCA negative at the time of switch.[162] Hence, ANCA levels at the time of switch should be studied as a guideline for treatment. Relapses, generally, are treated when clinical symptoms occur. Ideally, biomarkers indicating disease flares before clinical onset should be searched. As a rise in ANCA levels often precedes disease flares,[40,163] we performed more than two decades ago a prospective, randomized, and controlled trial to study a pre-emptive treatment strategy based on 9 months of CYC with a short course of steroids in patients after an ANCA rise.[164] Our study indicated that pre-emptive strategies based on monitoring biomarkers might be used in reducing relapse rates. As our study was done with CYC and as it is considered to be too toxic to be used for this purpose, we do not recommend its use as a pre-emptive therapy.[165] Other therapies such as RTX should be studied for relapse prevention based on ANCA levels.[166] Alternatively, other biomarkers should be considered to predict relapses. Monitoring Treg/Tem ratio might be of interest for this purpose. In the field of transplant immunology, ratios between Tregs and Tems are regarded to be important for establishing and maintaining tolerance.[167] One study assessed the value of these ratios in predicting significant episodes of rejection after a kidney transplant.[168] It was demonstrated that episodes of rejection were preceded by a shift from Tregs to Tems. One could speculate that these ratios are also skewed toward Tems in WG, especially in case of a relapse. However, this has not been investigated in a prospective study at present.

In conclusion, therapy of AAV should be adapted to the phase and severity of disease. For induction therapy of generalized and severe disease, CYC therapy with steroids should be the first choice and plasmapheresis should be considered in cases with renal failure and/or life-threatening disease (Table 2). Alternatively, RTX might be used if CYC is contraindicated. Otherwise, less toxic drugs such as MTX or MMF can be used in systemic disease that is not life threatening. To maintain remission, CYC should be replaced by AZA, MMF, MTX, or LEF.

FUTURE THERAPEUTIC CONCEPTS

Future therapeutic strategies should target specific disease mechanism possibly with reduced toxicity. As depicted above, T cells and T-cell migration are pivotal in AAV pathogenesis. Therefore, blocking T-cell adhesion to endothelial cells and interfering with T-cell trafficking might be beneficial (Figure 4). This approach is currently assessed by using natalizumab in patients suffering from multiple sclerosis.[169] However, the safety profile does not seem to be as good as was hoped, as an increased incidence of multifocal leukoencephalopathy was reported during therapy.[169,170] Another option might be cell-based therapy to modulate T-cell activity (Figure 4). Treg expansion might help to counterbalance persistent T-cell activation in AAV. However, such an approach is very labor intensive and might not be safe, as recent experimental studies revealed that Tregs are able to convert to effector T cells under certain, currently unknown circumstances.[171] In addition, control of T-cell activation might be an attractive therapeutic option. In this regard, blockade of costimulatory pathways such as CD28/CD80 has successfully been used in rheumatoid arthritis and might be a therapeutic opportunity in AAV.[172] Also, 'new players' in the field such as the complement factor C5 and its receptor might be studied as targets for future therapy.[73,74] Repair of vascular damage is an additional critical point that needs to be considered for upcoming therapeutic options. Vascular repair is thought to be mediated by endothelial progenitor cells (EPCs) present in the circulation.[173] AAV is associated with increased cardiovascular morbidity and it has already been demonstrated that AAV patients bear a lower frequency of EPCs than healthy controls.[174–176] Moreover, low amounts of

circulating EPCs seem to increase the probability of disease flares.[175] Thus, promoting EPC mobilization and function might have beneficial impact on disease course (Figure 4). Common drugs like statins and angiotensin receptor blockers enhance EPC mobilization and should be used as an adjunctive therapy in patients with AAV because of the risk of accelerated atherosclerosis.[176] In addition, erythropoietin is able to enhance EPC function and should be studied in AAV.[173] In conclusion, there are several promising candidates that will amend the therapy of AAV in future. This will hopefully lead to improved prognosis and result in less toxic therapeutic regimen.

DISCLOSURE

All the authors declared no competing interests.

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