

New advances in the pathogenesis of ANCA-associated vasculitides

M. Chen^{1,2}, C.G.M. Kallenberg¹

¹Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, University of Groningen, The Netherlands; ²Renal Division, Peking University First Hospital, Beijing 100034, China.

Min Chen, MD

Cees G.M. Kallenberg, MD, PhD

Please address correspondence to:

Cees G.M. Kallenberg,

Department of Rheumatology and Clinical Immunology, AA21,

University Medical Center

Groningen, P.O. Box 30.001,

9700 RB Groningen, The Netherlands.

E-mail: c.g.m.kallenberg@int.umcg.nl

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ABSTRACT

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are a group of autoimmune disorders including Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS) and renal-limited vasculitis (RLV). This paper reviews updated information on the pathogenesis of AAV. Additional clinical evidence for a pathogenic role of ANCA comes from the observation that patients with severe acute renal failure treated with plasma exchange had a lower risk for progression to end-stage renal disease than patients who received intravenous methylprednisolone therapy, both in addition to standard treatment. Recent data also suggest that antibodies to complementary proteinase-3 (cPR3), probably cross-reacting with plasminogen, may induce PR3-ANCA. Furthermore, a new ANCA, directed against human lysosome membrane protein-2 (LAMP-2), concurrent with PR3-ANCA or MPO-ANCA, was described as a sensitive and specific marker for renal AAV. In vitro, ANCA can further activate primed neutrophils to release reactive oxygen species and lytic enzymes, and, in conjunction with neutrophils, damage and lyse endothelial cells. In vivo, transfer of splenocytes from myeloperoxidase-deficient mice immunized with mouse myeloperoxidase into wild-type mice resulted in pauci-immune systemic vasculitis. A similar experiment in PR3-deficient mice did not cause significant vasculitic lesions. In the anti-MPO induced vasculitis mouse model, a critical role of complement activation was suggested. The anti-LAMP-2 antibody can also induce pauci-immune necrotizing crescentic glomerulonephritis in rats. Rats developed both cross-reactive antibodies to LAMP-2 and crescentic glomerulone-

phritis when immunized with FimH, an adhesin from Gram-negative bacteria which has strong homology with human LAMP-2. Together, clinical, in vitro and in vivo data support a pathogenic role for ANCA in AAV, although this role is more evident for myeloperoxidase-ANCA than for PR3-ANCA. The role of anti-LAMP-2 requires further studies.

Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) comprise Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS) and renal-limited vasculitis (RLV). These disorders are characterized by necrotizing small-vessel vasculitis, frequently including the kidneys (1, 2). ANCA are serological hallmarks for the above mentioned small vessel vasculitides. ANCA are predominantly IgG class autoantibodies directed against constituents of granules of neutrophils and lysosomes of monocytes. By indirect immunofluorescence (IIF) on ethanol-fixed neutrophils, two fluorescence patterns of ANCA are distinguished, the cytoplasmic staining pattern (cANCA) and the perinuclear staining pattern (pANCA). Most patients with a cANCA pattern obtained by IIF have ANCA directed against proteinase-3 (PR3), as determined by antigen-specific ELISA. Patients with pANCA mostly have ANCA directed against one of a variety of antigens, but in primary small vessel vasculitis, the target antigen is almost invariably myeloperoxidase (MPO). The combinations of a cANCA pattern by IIF with PR3-ANCA by ELISA and a pANCA pattern by IIF with MPO-ANCA by ELISA are very specific for AAV (3). PR3-ANCA are most frequent in patients with Wegener's granulomatosis and MPO-ANCA in patients with microscopic polyangiitis

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and renal-limited vasculitis (1). However, these associations differ between races as MPO-ANCA predominate, even in patients with WG, in Asian populations and PR3-ANCA in northern Caucasian populations (4-6). Recently, Kain *et al.*: found that individuals with AAV and renal involvement also produce ANCA directed against human lysosomal membrane protein-2 (LAMP-2) – a heavily glycosylated membrane protein (7). Although the pathogenesis of ANCA-associated vasculitides has not yet been fully elucidated, major advances have been made over 20 years since the discovery of ANCA (8, 9). This review will focus on advances in the understanding of the pathogenesis of AAV.

Evidence from clinical studies for a pathogenic role for ANCA

The most direct clinical evidence that ANCA are pathogenic comes from the observation of the development of glomerulonephritis and pulmonary hemorrhage in a neonate shortly after birth from a mother with MPO-ANCA-associated microscopic polyangiitis, apparently caused by transplacental transfer of ANCA IgG (10, 11).

Furthermore, clinical observations on patients with AAV support a pathogenic role for ANCA. Two recent papers on the value of plasma exchange in AAV from the European Vasculitis Study Group described the value of plasma exchange in AAV. Patients with a new diagnosis of ANCA-associated systemic vasculitis and serum creatinine >500 µmol/L were randomly assigned to receive plasma exchange or intravenous methylprednisolone in addition to standard treatment with oral cyclophosphamide and prednisolone. Patients treated with plasma exchange had a lower risk for progression to end-stage renal disease at one year than patients who did not receive plasma exchange (12). A companion paper suggested that even with ominous histologic findings, the chance of renal recovery exceeds the chance of therapy-related death when these patients are treated with plasma exchange as adjunctive therapy (13). The beneficial effect of plasma exchange in AAV supports a pathogenic role for circulating ANCA IgG.

Although debate is still ongoing whether ANCA levels parallel the clinical and histological activity of the disease, many patients show decrease or disappearance of ANCA titers during periods of quiescence (14, 15). A subsequent rise in ANCA titer or reappearance of ANCA has been suggested as being predictive of clinical relapse (16), suggesting that monitoring of changes in ANCA titer might be helpful in predicting relapses (17). However, the consistency between ANCA levels and disease activity remains a controversy (18). A recent multi-centre prospective cohort study with 156 patients with active Wegener's granulomatosis showed that decreases in PR3-ANCA levels are not associated with shorter time to remission, and increases are not associated with relapse. These findings suggest that ANCA levels cannot be used to guide immunosuppressive therapy (19).

As mentioned, Kain *et al.* found that human LAMP-2 is a novel class of autoantigens of ANCA in necrotizing and crescentic glomerulonephritis (NCGN). Specific reactivity of anti-LAMP-2 antibody was detected in about 90% of cases with active phases of NCGN, frequently also in combination with autoantibodies specific for PR3 or MPO. The anti-LAMP-2 antibodies seemed to disappear during quiescent disease (7). Since the observation in 2004 that some patients with PR3-ANCA also have antibodies against a protein coded by the antisense strand of the PR3 cDNA (designated as complementary PR3, cPR3) (20), further progress on the immune response against cPR3 has been made by the Chapel Hill group. Bautz *et al.* investigated potential endogenous targets of anti-cPR3 antibodies. Unexpectedly, plasminogen was identified as a target of anti-cPR3. Of a cohort of patients with PR3-ANCA, nine had documented deep venous thrombotic events, five of whom were positive for antiplasminogen antibodies (21), suggesting that the antibodies might play a role in the occurrence of thrombo-embolic events which are frequently seen in patients with PR3-ANCA (22). Furthermore, about half of the patients with PR3-ANCA had CD4+TH1 memory cells responsive to the cPR3¹³⁸⁻¹⁶⁹-peptide,

and a significant number of patients had T cells simultaneously responsive to this peptide and to heat-inactivated PR3 protein as manifested by proliferation and/or secretion of IFN-γ. There was a significant likelihood that anti-cPR3 antibodies and cPR3-specific T cells coexist in individuals, consistent with an immunological history of encounter with a PR3-complementary protein (23).

Evidence from *in vitro* studies for a pathogenic role for ANCA

ANCA-induced neutrophil activation

In vitro studies suggest that in neutrophils stimulated by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α or IL-18, target antigens of ANCA translocate to the neutrophil surface, thereby allowing binding by circulating autoantibodies. When exposed to ANCA IgG, neutrophils could undergo a respiratory burst and release free oxygen radicals and various proteases, which could play a direct pathogenic role in vasculitic lesions (24, 25). ANCA IgG cause neutrophil activation by ligation of Fc receptors (26, 27) as well as by interaction of their antigen binding sites with ANCA antigens on the cell surface (28). A schematic model is given in Fig. 1.

Interaction between ANCA and their target antigens

The interaction between ANCA and their target antigens has been suggested to play a role in the pathogenesis of AAV. Guilpain *et al.* found that MPO-ANCA positive sera could activate MPO *in vitro* to generate hypochlorous acid. The byproducts of MPO activation exerted a strong cytolytic activity on endothelial cells in culture. Both HOCl production and endothelial lysis were abrogated by N-acetylcysteine (NAC), an antioxidant molecule. The authors suggest that MPO-ANCA could play a pathogenic role *in vivo* by triggering an oxidative burst, leading to severe endothelial damage (29). However, in propylthiouracil-induced AAV, Zhang *et al.* found that MPO-ANCA-positive IgG preparations from most patients could inhibit MPO activity in a dose-dependent manner (30). These results indicate different mechanisms between

drug-induced AAV and primary AAV. Recent findings provide evidence that PR3 might function as endogenous "danger/alarm" signal that communicates the presence of tissue injury to dendritic cells via protease-activated receptor-2 (PAR-2), triggers their maturation and instructs dendritic cells to induce Th1-type cell responses in WG. Furthermore, PR3 has the capacity to bind and activate IL-32, a recently discovered proinflammatory cytokine that has emerged as an important player in innate and adaptive immune response (reviewed by Csernok *et al.* (31)).

Membrane expression of PR3 and MPO

Presence of membrane-bound PR3 (mPR3) is a prerequisite for ANCA-binding and ANCA-mediated vessel damage. Even without priming, PR3 can be detected on the membrane of isolated neutrophils. It has been found by Schreiber *et al.* that mPR3 expression is genetically determined (32). However, increased expression of mPR3 on neutrophils has been observed in several clinical conditions. Neutrophils from patients with PR3-ANCA associated vasculitis and some other chronic inflammatory diseases show higher levels of mPR3 expression than those from healthy controls (33, 34), and the presence of a high proportion of mPR3 expressing neutrophils is associated with more frequent relapse of WG (35). These observations suggest that abnormally expressed mPR3 is involved in the development and severity of WG. It was found that PR3 membrane expression on neutrophils is mediated by CD177, which is coexpressed with PR3 on a subset of neutrophils (36, 37). We recently observed that membrane expression of CD177 on circulating neutrophils is increased in patients with AAV; however, primed neutrophils from CD177-negative individuals also express mPR3 and are susceptible to anti-PR3-mediated oxidative burst, suggesting that recruitment of CD177-independent mPR3 is involved in anti-PR3-induced neutrophil activation (38).

Whether MPO-ANCA-mediated neutrophil activation involves MPO trans-

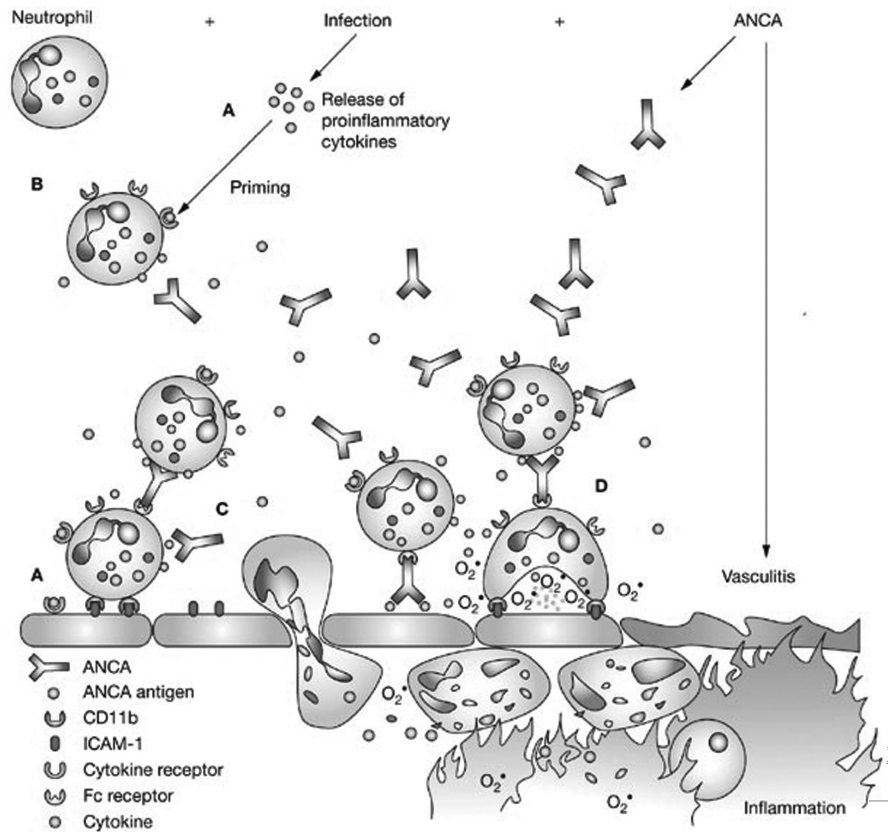


Fig. 1. Schematic representation of the neutrophil responses that are putatively involved in the pathogenesis of ANCA-associated small vessel vasculitis

(A) Proinflammatory cytokines and chemokines (*e.g.* tumor necrosis factor) that are released as a result of local or systemic infection cause upregulation of the expression of endothelial adhesion molecules (*e.g.* selectins, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), and prime neutrophils. (B) Neutrophil priming causes upregulation of the expression of neutrophil adhesion molecules (CD11b) and translocation of the ANCA antigens from their lysosomal compartments to the cell surface. (C) Engagement of dimers of the antigen-binding portion of ANCA with ANCA antigens on the cell surface, and interaction of the Fc part of the antibody with Fc receptors, activates neutrophils and causes increased transmigration and adherence of neutrophils to vessel walls. (D) ANCA-mediated neutrophil activation also triggers production of reactive oxygen radicals and possibly causes neutrophil degranulation. The consequent release of proteolytic enzymes leads to vasculitis. Reproduced with permission from reference (64).

ANCA: antineutrophil cytoplasmic autoantibodies; CD11b: a $\beta 2$ -integrin cell-surface adhesion molecule involved in neutrophil adherence to and migration through vascular endothelial cells; ICAM-1: intercellular adhesion molecule 1; $O_2\cdot$: oxygen radicals.

location to the surface of primed neutrophils has yet to be resolved. Hess *et al.* have shown that resting human neutrophils exposed to supernatants from degranulated autologous neutrophils express MPO, but not PR3, on their cell surface and become responsive to anti-MPO autoantibody (39).

Role of T cells

Most studies on cell-mediated immunity in AAV have focused on T-cell phenotypes in patients with WG since T cells probably participate in granuloma formation in WG. The Th1/Th2 balance

is considered to play a key role in disease development and progression of WG. Analysis of nasal granulomatous lesions from WG revealed a relative increase of cells expressing Th1-associated markers in patients with localized disease, whereas Th2-associated markers dominated in generalized disease, which suggests a shift towards a Th2 response during systemic involvement (40, 41). Recent data have shown that IL-17-producing $CD4^+$ T cells, termed Th17 cells, are probably the main subset implicated in pathogenesis. Abdulahad *et al.* recently found a significant

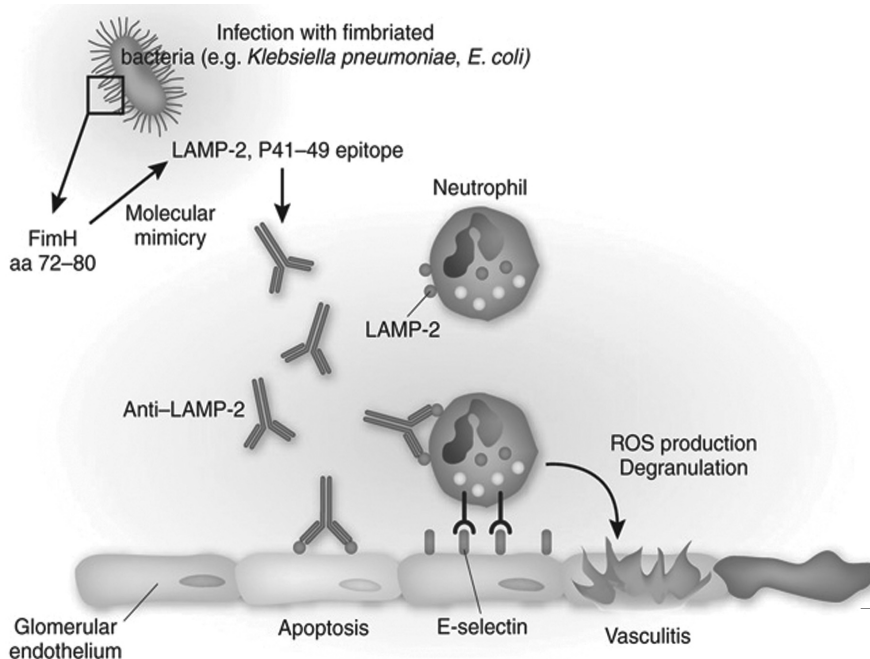


Fig. 2. Human LAMP-2 induces glomerular endothelial injury. Infection with fimbriated bacteria induces generation of antibodies to an epitope shared by the bacterial adhesion FimH and human LAMP-2. Autoantibodies to LAMP-2 bind neutrophils and activate these cells, causing shape changes and degranulation. In addition, autoantibodies to LAMP-2 bind glomerular endothelial cells, resulting in upregulation of E-selectin and eventually causing endothelial cell apoptosis. Ultimately, these processes lead to glomerular capillary injury progressing to focal necrotizing glomerulonephritis. Reproduced with permission from reference (58).

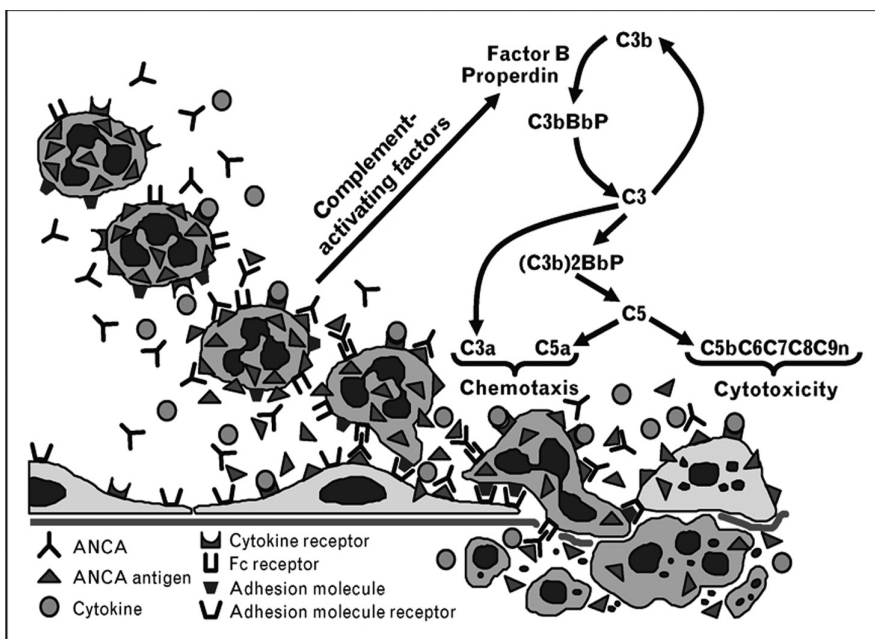


Fig. 3. Diagram depicting hypothetical events in the pathogenesis of ANCA-associated vasculitis that have been observed *in vitro* and are supported by animal models. Beginning in the upper left and moving to the right, cytokines or other priming factors induce neutrophils to express more ANCA antigens at the cell surface where they are available for binding to ANCA, which activate neutrophils by both Fc receptor engagement and direct F(ab)₂ binding to antigen. Neutrophils that have been activated by ANCA interact with endothelial cells via adhesion molecules and release toxic factors that cause apoptosis and necrosis. Neutrophils that have been activated by ANCA also release factors that activate the alternative complement pathway, which generates factors, such as C5a and C3a, which amplify the intensity of ANCA-induced inflammation. Reproduced with permission from reference (59).

increase in the percentages of Th17 cells in *in vitro* stimulated peripheral blood cells from WG patients as compared with healthy controls, and a relative increase in PR3-specific Th17 cells in ANCA-positive WG patients in comparison with ANCA-negative WG patients and controls (42). Other studies suggest that peptidoglycans as well as superantigens from *S. aureus* might have an immunomodulatory effect on dendritic cells by imprinting a strong Th17-polarizing capacity (43, 44). Memory CD4⁺ T cells with the effector cytotoxic phenotype (CD4⁺ TEM) have also been demonstrated to constitute a major effector pathway of tissue injury in patients with WG (45).

Evidence from *in vivo* experimental studies for a pathogenic role for ANCA

Animal models for ANCA-associated vasculitis

The most direct evidence of the pathogenicity of ANCA comes from studies on mice. Xiao *et al.* induced lesions in mice very similar to those in human AAV. Anti-MPO IgG or MPO-reacting splenocytes were obtained from MPO knockout mice immunized with purified mouse MPO, and transferred into recipient mice. In the anti-MPO IgG transfer model, all recipient mice developed pauci-immune focal necrotizing and crescentic glomerulonephritis after 6 days whereas some mice also developed systemic small vessel vasculitis. Mice that received a large dose of anti-MPO splenocytes developed severe necrotizing and crescentic glomerulonephritis and systemic necrotizing vasculitis (46). Depletion of neutrophils in the recipient mice could ameliorate anti-MPO antibody-induced mouse glomerular vasculitic lesions (47), suggesting that neutrophil activation is involved in the pathogenesis of AAV. Moreover, disease severity could be markedly aggravated by the addition of bacterial lipopolysaccharide (LPS) as a proinflammatory stimulus; the disease enhancing effects of LPS could be attenuated, but not fully prevented, by pretreatment of the animals with anti-TNF- α treatment (48). Schreiber *et al.* recently observed that bone

marrow (BM)-derived cells containing MPO are sufficient to cause anti-MPO-induced lesions in the absence of MPO in other cell types, demonstrating that leukocytes are the target of MPO-ANCA (49). Little *et al.* induced focal segmental pauci-immune glomerulonephritis and focal pulmonary capillaritis in rats by immunization with human MPO, which leads to the generation of antibodies against human MPO cross-reacting with rat MPO (50).

The above described animal models all pertain to MPO-ANCA driven disease. Passive transfer of mouse or rat antibodies to PR3 in mice or rats did not result in a relevant animal model of PR3-ANCA-associated disease, *i.e.* Wegener's granulomatosis (51, 52). Immunization of mice deficient for PR3 and neutrophil elastase with murine PR3 led to the generation of antibodies to murine PR3, and subsequent transfer of anti-PR3 positive sera to wild type mice significantly aggravated a local inflammatory response elicited by TNF- α administration in the skin. However, this approach did not induce vasculitis or glomerulonephritis, even not when mice were pretreated with lipopolysaccharide as a proinflammatory stimulus. The discrepancy between the murine model of anti-MPO-IgG-induced necrotizing and crescentic glomerulonephritis and its anti-PR3 equivalent is as yet unexplained, but may indicate functional differences in pathogenic potential between PR3-ANCA and MPO-ANCA.

ANCA-mediated interaction between neutrophils and endothelial cells

In vitro studies have shown that the presence of ANCA could induce interaction between neutrophils and endothelial cells. For example, in the presence of ANCA, incubation with neutrophils resulted in detachment and lysis of endothelial cells (53). In an endothelial-cell-coated flow system, ANCA could convert rolling neutrophils into neutrophils that stably adhered to the endothelial layer (54). ANCA-mediated interaction between neutrophils and endothelial cells has been further investigated in a recent *in vivo* study.

Nolan *et al.* observed, by using intravital microscopy, that in the presence of pro-inflammatory cytokines such as TNF- α , anti-MPO antibodies induced leukocyte adhesion and transmigration across the endothelium. Administration of anti-MPO also led to the recruitment of leukocytes preferentially to the kidney and lung, sites that are often affected in human ANCA-associated vasculitis. Furthermore, their data suggest that Fc γ receptors and β 2 integrins mediate these enhanced leukocyte-endothelial interactions (55). The role of the endothelium in AAV has been well reviewed by Little *et al.* (56).

Anti-LAMP2 autoantibodies

Anti-LAMP2 autoantibodies can activate neutrophils and cause apoptosis of endothelial cells *in vitro*. When injected into rats, the antibodies induced pauci-immune necrotizing crescentic glomerulonephritis. More importantly, the authors showed that a major epitope of human LAMP-2 recognized by the autoantibodies has strong homology with FimH, a bacterial adhesin of common Gram-negative bacteria. When rats were immunized with FimH, most developed both cross-reactive antibodies to LAMP-2 and crescentic glomerulonephritis, which is the renal histological hallmark of AAV (57, 58) (Fig. 2).

ANCA and complement

Recent observations in the anti-MPO induced vasculitis mouse model suggested a critical role of complement activation in ANCA-associated disease. Complement depletion with cobra venom factor completely blocked the development of glomerulonephritis and vasculitis induced by injection of MPO IgG or transfer of anti-MPO splenocytes (59). Subsequently, the role of specific complement activation pathways was studied using mice deficient for the common pathway component C5, the classical and lectin pathway component C4, and the alternative pathway component factor B. These studies revealed that anti-MPO IgG induced NCGN is dependent on an intact alternative pathway. Whereas C4-deficient mice developed NCGN comparable to wild type mice, trans-

genic mice deficient for C5 or factor B were completely protected from disease induction (59). Further support for the role of complement in this model of ANCA disease was reported by Huugen *et al.* (60) who investigated the effects of a C5 inhibiting antibody. Mice received anti-C5 antibody 8hrs before or one day after disease induction with anti-MPO IgG and LPS. None of the mice that received anti-C5 antibody before disease induction developed glomerulonephritis, and anti-C5 antibody administration one day after disease induction also resulted in an 80% reduction in glomerular crescent formation. Schreiber *et al.* investigated the role of C5a in AAV. Supernatants from ANCA-activated neutrophils activated the complement cascade in normal serum resulting in the production of C5a. This conditioned serum primed neutrophils for the ANCA-induced respiratory burst; neutrophil C5a-receptor (C5aR) blockade abrogated this priming. Furthermore, recombinant C5a dosage-dependently primed neutrophils for the ANCA-induced respiratory burst. C5aR-deficient mice could be protected from developing NCGN. This study indicates that C5a and the neutrophil C5aR may compose an amplification loop for ANCA-mediated neutrophil activation (61). Overall, these mouse studies support a crucial role for the alternative pathway of complement activation in AAV.

Two recent studies on renal histopathology also suggested that the complement system, especially the alternative pathway, is involved in renal damage in human AAV (62, 63).

Conclusion

Since ANCA were described in the early 1980s, the association between ANCA and pauci-immune small vessel vasculitides has been well established. There is increasing clinical, *in vitro* and *in vivo* evidence that supports a pathogenic role for ANCA in the pathogenesis of ANCA-associated vasculitis. As our understanding of pathogenic mechanisms becomes clearer, new strategies for more effective and less toxic treatment modalities will hopefully emerge.

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