Effector mechanisms of ANCA-associated glomerulonephritis
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Chapter 8

Summary, discussion and future perspectives
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INTRODUCTION
The ANCA-associated small-vessel vasculitides (ANCA-SVV) Wegener’s granulomatosis, microscopic polyangitis, Churg-Strauss syndrome and renal-limited vasculitis are severe diseases that have a high mortality rate when left untreated. Current treatment protocols are based on non-specific immunosuppression, which is insufficient in preventing relapses and is characterized by severe side-effects. More effective and less toxic therapies are therefore needed. A better understanding of the pathogenic effector mechanisms underlying ANCA-SVV can improve the development of specific therapeutic strategies. The aim of this thesis was therefore to explore further the effector mechanisms involved in ANCA-associated glomerulonephritis, focusing on the discovery of targets for treatment and the testing of experimental therapies. More specifically, we concentrated on the importance of inflammatory stimuli and ANCA IgG characteristics in disease and the therapeutic potential of interfering with leukocyte behaviour.

ANCA-SVV: COMPLEX MULTIFACTORIAL DISEASES
By definition, patients with autoimmune diseases have a loss of immunological tolerance towards an autoantigen, but it is usually less clear why such a break in tolerance occurs. In line with this, it is not known why ANCA autoantibodies develop in ANCA-SVV patients. In recent years, several theories have been proposed to explain the immunogenesis of ANCA-SVV.

The theory of antigen complementarity assumes that antibodies directed against a protein or peptide (antisense) that is complementary to the ANCA antigen (sense) are generated. A subsequent immune response against the antigen-binding part of these antibodies generates idiotypic antibodies that cross-react with the ANCA antigen. The antisense protein or peptide in this theory may be derived from aberrant antisense transcription or from pathogens. A second theory involves molecular mimicry of exogenous proteins with ANCA antigens. This theory assumes that the initial immune response is evoked against pathogen-derived peptides that are highly homologous to peptide sequences within the ANCA-antigens, resulting in a cross-reactive immune response against the ANCA self antigens. Evidence for the occurrence of molecular mimicry in ANCA-SVV is provided by a study showing that circulating autoantibodies against lysosomal associated membrane protein 2 (LAMP-2), a heavily glycosylated type 1 membrane protein involved in cellular adhesion and homeostasis, were highly prevalent in patients with MPO-ANCA- and Pr3-ANCA-positive crescentic glomerulonephritis. These anti-LAMP-2 antibodies were able to activate neutrophils and endothelial cells in vitro, whereas injection of these antibodies into rats induced pauci-immune crescentic glomerulonephritis. The major epitope recognized by anti-LAMP-2 antibodies was highly homologous to FimH-1, a bacterial adhesin of common gram-negative bacteria. When rats were immunized with FimH-1, antibodies directed against FimH-1 were generated that cross-reacted with LAMP-2. Importantly, these rats also developed necrotizing crescentic glomerulonephritis. These data suggest that infections with gram-negative bacteria can induce an autoimmune response to LAMP-2, which in turn causes neutrophil...
activation and vasculitis. A third theory involves the release of so-called neutrophil extracellular traps (NETs), which are chromatin fibers that contain several proteins, including MPO and Pr3. Deposition of NETs was observed in glomerular lesions of ANCA-SVV. The generation of NETs by neutrophils may provide a mechanism by which ANCA autoantigens are continuously exposed to the immune system. None of the three theories has yet been confirmed by other research groups. Mechanisms leading to the break of tolerance, resulting in an autoimmune response, and to induction of disease are still open for research and new theories are highly needed.

Additionally, it is not known why some patients encounter disease relapses, whereas others do not. A better understanding of why relapses occur is relevant for better treatment strategies. Ideally, treatment protocols should be adapted to the individual patient according to the likelihood of getting a relapse and the mechanisms underlying relapse. More research is needed to achieve such individualized therapies, especially aimed at the discovery of biomarkers that can predict relapse. A recent study may have found such a predictor of relapse in the transcriptional signature of T cells. The study defines a subpopulation of patients who were more prone to relapse, based on the gene expression profile of CD8+ T cells. When the predictive value of this transcriptional signature can be confirmed, T cell gene expression analyses may be a step towards therapies based on the likelihood of relapse in an individual.

Despite the fact that we do not know what triggers ANCA development and causes disease relapses, we do know that ANCA-SVV (as are other autoimmune diseases) are complex multifactorial diseases. Disease activity is not only influenced by immunological factors, but also by environmental elements (e.g. infections) and genetic variation (Figure 1). All these factors can contribute to

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**Figure 1** A diagram illustrating that multiple factors – environmental, immunological, and genetic – contribute to the pathogenesis of ANCA-associated small-vessel vasculitides. (color image on page 189)
an individual’s susceptibility for autoimmunity and together they may provide the appropriate conditions for a break in tolerance. The initiation and progression of ANCA-SVV thus involves multiple contributing factors that should be taken into account when studying the immunogenensis and pathogenesis of these diseases. In this chapter, we therefore discuss the results of this thesis in the context of environmental, immunological and genetic factors.

Environmental factors
Several environmental factors can potentially contribute to the development and progression of ANCA-SVV. Exposure to certain drugs or silica has been shown to induce or increase the risk for developing ANCA-associated vasculitis. Evidence exists, though, for the role of infections in the pathogenesis of ANCA-SVV and this will be discussed below. In addition, we will discuss the possible participation of the vascular micro-environment that creates a vascular bed that is susceptible to ANCA-mediated injury.

Infection
The most important reason for assuming that infections contribute to ANCA-associated vasculitis is the fact that neutrophils require a “second hit” to make them susceptible for ANCA-mediated activation. Neutrophils require priming with a pro-inflammatory stimulus, such as TNFα, to increase ANCA antigen availability and to prepare for a full-blown activation. Infections cause production of pro-inflammatory cytokines and other mediators that can exert this priming. At the same time, these pro-inflammatory mediators activate endothelial cells, which increases the interaction of ANCA-bound neutrophils with the endothelium. Because infections can also activate the complement pathway and complement activation plays a pivotal role in ANCA-associated glomerulonephritis, we analyzed in chapter 2 whether the complement factor C5a could act as a pro-inflammatory stimulus that mediates neutrophil priming and endothelial activation. C5a was able to prime human neutrophils for both Pr3-ANCA- and MPO-ANCA-induced respiratory burst in a p38MAPK-dependent manner. In addition, C5a activated glomerular endothelial cells to produce the leukocyte chemoattractants IL-8 and MCP-1. These studies demonstrate that activation of complement and subsequent production of C5a during ANCA-SVV may lead to priming and activation of more neutrophils and endothelial cells and thereby may contribute to vasculitic injury.

Furthermore, bacteria and bacterial endotoxins, such as Mycobacterium tuberculosis and LPS, have been shown to aggravate crescentic glomerulonephritis in animal models of ANCA-associated glomerulonephritis. In chapter 3, we further investigated the underlying mechanism of LPS-mediated aggravation of anti-MPO glomerulonephritis in mice. We found that the presence of TLR4, the receptor for LPS, was required on both bone marrow-derived cells and intrinsic renal cells for maximal neutrophil recruitment and subsequent glomerular injury. This suggests that cells from both compartments become activated upon bacterial infections and contribute to ANCA-associated vasculitis. Whether TLR-4 is involved in human ANCA-associated disease (where LPS may
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not be present) is however not known yet. Interestingly, a recent study described a direct interaction between TLR4 and FcγRs upon stimulation with IgG immune complexes. Activation of neutrophils and macrophages via the IgG immune complex-FcγR interaction was shown to require the presence and integrity of TLR4 in vitro, suggesting that TLR4 may be important for antibody-mediated diseases, even in the absence of TLR4 ligands. As a further step towards understanding human disease, additional studies could investigate the involvement of TLR-4 in animal models that do not include administration of a pro-inflammatory stimulus, e.g. the bone marrow-transplantation mouse model or the rat model of ANCA-SVV. Together, our findings in chapters 2 and 3 add to the evidence for a role of pro-inflammatory stimuli in ANCA-associated vasculitis, through promotion of neutrophil priming and endothelial activation.

Despite the evidence obtained from in vitro and animal experiments, studies on bacterial infections in ANCA-SVV patients have not proven a causative relationship, although a correlation between the presence of S. aureus and the occurrence of relapse has been described for Wegener’s granulomatosis. The fact remains that many patients acquire ANCA-associated vasculitis without having had a preceding infection. Infections are perhaps not required if a combination of other environmental factors and genetic susceptibility provides the appropriate conditions for disease development. This hypothesis is strengthened by the finding that administration of low concentrations of circulating anti-MPO antibodies in rats or mice is only pathogenic in the presence of a pro-inflammatory stimulus, whereas higher concentrations do not require such a trigger. Nevertheless, when an infection is present during active disease, it is likely to substantially contribute to exacerbation of the disease. More studies are required to unambiguously describe the role of infections in ANCA-associated vasculitis and glomerulonephritis.

Vascular micro-environment

An unresolved issue in the pathogenesis of ANCA-SVV relates to the preferential localization of lesions in small- to medium-sized blood vessels. Vasculitic manifestations in ANCA-SVV are largely limited to capillaries, arterioles and venules, whereas larger blood vessels are mostly resistant to ANCA-induced inflammation. In addition, small vessels in certain organs, in particular the kidneys and lungs, are more often affected than those in other organs. Endothelial cells are not only the target for injury, but are also likely to actively participate in the induction and progression of vasculitis. In particular, the coordinated expression of adhesion molecules and the production of cytokines and chemokines by activated endothelial cells are pivotal in driving the inflammatory response. As shown in chapters 2, 3, and 7, glomerular endothelial cells are prominent sources of the chemokines IL-8 and MCP-1 upon stimulation with the pro-inflammatory mediators LPS and C5a. In the context of endothelial heterogeneity, an interesting question would relate to whether endothelial cells derived from other vascular beds would respond to the same extent as glomerular endothelial cells. The endothelial phenotype in a specific vascular bed is dependent on the microenvironment and the cross-talk with neighboring cells, and this may determine its susceptibility for an inflammatory
insult. Therefore, it will be important to investigate whether differences exist between endothelial cells of vessels that are affected in ANCA-SVV and of vessels that are not. One way to achieve this, is to analyze gene and protein expression profiles of affected vascular beds in human tissues and experimental models of ANCA-SVV. This could be achieved by isolating these blood vessels from tissue sections by laser microdissection. Such an analysis of endothelial cell profiles may potentially reveal new targets for treatment.

**Immunological factors**

Besides environmental factors, many immunological factors have been described to be involved in the pathogenesis of ANCA-associated vasculitis. In this section the most prominent factors, ANCA, leukocytes, Fcγ receptors, complement and kinases, are discussed.

**ANCA**

In line with a pathogenic role for ANCA, removal of ANCA from the circulation *via* plasmapheresis has been shown to be beneficial for the treatment of ANCA-SVV patients with alveolar hemorrhage and severe kidney disease. Because plasmapheresis treatment removes not only ANCA IgG molecules but also other plasma components, including circulating cytokines and complement factors, removal of such components may also contribute to the effectiveness of plasmapheresis. Nevertheless, these findings suggest that reducing the pathogenicity of ANCA molecules may be of benefit in the treatment of ANCA-associated glomerulonephritis.

In *chapters 4 and 5* we investigated in more detail some characteristics of ANCA IgG molecules to better understand ANCA pathogenicity and to determine whether we can modulate such characteristics to reduce ANCA pathogenicity. To this end, we modulated the glycosylation status of the ANCA IgG Fc tail in *chapter 4*, because IgG Fc glycosylation is known to be required for activation of FcγRs. Using the bacterial enzyme endoglycosidase S (EndoS) to specifically cleave the Fc glycans from ANCA IgG, we investigated whether IgG Fc deglycosylation could attenuate ANCA-mediated glomerulonephritis. We found that pretreatment of IgG with EndoS reduced ANCA-induced neutrophil activation *in vitro* and prevented induction of anti-MPO IgG/LPS-mediated glomerulonephritis in mice. These results are in line with previous evidence demonstrating an important role of FcγRs in the development of anti-MPO glomerulonephritis (as described below under the subheading Fcγ receptors). It also demonstrates the crucial role that ANCA IgG Fc glycans play in our model. Additionally, systemic treatment of mice with EndoS early after glomerulonephritis induction attenuated the development of disease, thereby indicating that modulation of IgG glycosylation may be a promising strategy to interfere with acute ANCA-mediated inflammatory processes. Future studies should confirm the benefit of treating with EndoS in other models of MPO-ANCA vasculitis, especially in an active model that involves a cellular as well as a humoral anti-MPO immune response (e.g. the MPO-ANCA rat model). Furthermore, the safety and specificity of EndoS needs to be addressed carefully before such a bacterial enzyme can be used in humans.
In the MPO-ANCA mouse model, elicited polyclonal anti-MPO antibodies are pathogenic, but it is not known whether disease induction is dependent on a specific antibody subclass or antigen epitope. To better understand the pathogenic mechanism of ANCA, we investigated in chapter 5 whether a selected combination of anti-MPO monoclonal antibodies (moAbs) can induce crescentic glomerulonephritis in 129S6 mice. We found that a specific combination of three anti-MPO moAbs induced crescentic glomerulonephritis in 129S6 mice when the moAbs were co-administered with LPS. Although there was substantial albuminuria, the number of crescentic glomeruli was low. A higher number of crescents may be achieved by adjusting the anti-MPO moAb dose. This will allow the 129S6 mouse strain to serve as a model to study the pathogenicity of specific ANCA IgG subclasses and antigen recognition patterns via analysis of additional (combinations of) anti-MPO moAbs together with heavy chain switch variants of these moAbs. More information about pathogenic differences between specific ANCA IgG subclasses may for instance be relevant for the potential of EndoS-based therapy, as the interaction of different IgG subclasses with FcγRs may not be equally affected by EndoS treatment.23

In contrast to anti-MPO antibodies, the pathogenicity of anti-Pr3 antibodies is less clear. Several animal models for Pr3-ANCA vasculitis have been developed but they have not convincingly proven that anti-Pr3 antibodies cause vasculitis. A relevant question is whether the pathogenic mechanisms of MPO-ANCA and Pr3-ANCA vasculitis are the same. It is well-known that Pr3- and MPO-ANCA-SVV patients differ to some extent in their clinical presentation and histopathological characteristics of the vasculitic lesions.24 For example, in patients with Pr3-ANCA, extrarenal organ manifestations, respiratory tract granulomas, and disease relapses are much more frequent than in patients with MPO-ANCA. Moreover, passive transfer of anti-Pr3 antibodies in mice does not lead to vasculitic lesions while transfer of anti-MPO antibodies does. These dissimilarities may be caused by differences in the ability of MPO-ANCA and Pr3-ANCA to interact with their target antigens, to activate neutrophils, or to evoke cellular immune responses.24 The discrepancy in pathogenic capacity between anti-Pr3 and anti-MPO antibodies in animal models may also be due to differences in antigen characteristics. In mice and humans, Pr3 is less basic than MPO (isoelectric points of ~7 and >10, respectively).25 This difference could account for differential interactions of the antigen with negatively charged cell structures and could result in differences in pathogenic potential of the antigen-ANCA complex. Human Pr3, on the other hand, is more basic than murine Pr3 (isoelectric points 7.7 and 6.7, respectively), possibly accounting for an increased pathogenicity of anti-Pr3 antibodies in humans. Moreover, murine Pr3 has been shown to have more similarity to human and murine neutrophil elastase than to human Pr3.26 Thus, clinical and experimental findings suggest that MPO-ANCA and Pr3-ANCA do not share a similar pathogenic mechanism. More research is needed, however, to confirm this.

Leukocytes

Because leukocytes are important effector cells in the pathogenesis of ANCA-SVV,14,27 we speculated
that intervention with leukocyte recruitment would provide a potential therapeutic strategy for ANCA-SVV. As chemokines are small cytokines involved in leukocyte recruitment, we analyzed in chapter 6 the spatiotemporal gene expression of chemokines and chemokine receptors in the MPO-ANCA mouse model, to identify potential targets for intervening with leukocyte influx. We found that several chemokines and chemokine receptors were induced or upregulated in anti-MPO antibody-mediated glomerulonephritis. Using laser-dissection microscopy, we localized expression of most chemokines and receptors predominantly to glomeruli as compared to the tubulo-interstitial tissue. The upregulated genes included chemokines and chemokine receptors involved in recruitment of neutrophils and monocytes, suggesting that they may serve to promote recruitment of these specific leukocyte subsets in the development of anti-MPO glomerulonephritis.

Neutrophils are one of the most important types of leukocytes involved in ANCA-SVV. In the absence of neutrophils, glomerulonephritis does not develop in the MPO-ANCA mouse model. In this model, neutrophils are typically recruited and activated in the acute inflammatory phase of glomerulonephritis development. We also found that expression of the chemokine receptor CXCR2, which is known to be predominantly expressed on neutrophils, was induced particularly in the acute phase (chapter 6). In addition, CXCL1 and CXCL2, the main ligands of CXCR2 and homologs of human IL-8, were upregulated during neutrophil recruitment (chapters 3 and 6). These findings suggested that inhibiting either CXCR2 or its ligands would attenuate neutrophil recruitment and subsequent glomerular injury. Indeed, neutralization of the ligands CXCL1 or CXCL2 partly reduced neutrophil recruitment and glomerular injury in vivo (chapter 3). However, when the receptor CXCR2 was inhibited, neutrophil recruitment was increased and glomerular injury was not affected (chapter 6). These contrasting findings are not easily explained, but they may result from differences in experimental setup (i.e. dosing and timing of LPS administration). Another possibility is that CXCL1 and CXCL2 can activate an additional, not yet identified, receptor. These studies suggest that inhibition of a single chemokine-chemokine receptor pair may only work when existence of redundant chemokine-chemokine receptor functions are excluded. More research is needed to clarify the exact role of CXCR2-mediated chemokine signaling in ANCA-associated glomerulonephritis.

Monocytes and macrophages are also likely to play significant roles in the development of ANCA-SVV. ANCA can stimulate monocytes to produce reactive oxygen species and chemokines, and macrophages are present in granulomatous lesions and glomerular crescents. In line with this, we found an increased expression of the monocyte chemoattractants fractalkine and MCP-1 in the crescentic phase of anti-MPO glomerulonephritis (chapter 6). It would be of interest to explore the role of monocytes/macrophages in disease progression. To this end, anti-MPO-mediated glomerulonephritis could be induced in monocyte/macrophage-depleted mice. If monocytes and macrophages are found to contribute substantially to disease in such experimental studies, follow-up experiments could examine whether inhibition of the chemokines MCP-1 and/or fractalkine is able to interfere with recruitment of these cells.
Besides neutrophils and monocytes/macrophages, B and T cells are involved in ANCA-associated glomerulonephritis. B cells can differentiate into plasmacytes to produce ANCA and also function as antigen-presenting cells. Increased activation of B cells was observed in patients with active Wegener’s granulomatosis. Moreover, the efficacy of a B cell-depleting antibody (rituximab) in combination with prednisone has been compared to cyclophosphamide/prednisone as induction therapy for ANCA-associated vasculitis in two randomized controlled trials. The first results from these trials demonstrated that rituximab had comparable efficacy as induction therapy and induced a similar rate of adverse events compared to cyclophosphamide. The efficacy and potential side-effects of rituximab treatment on the long-term are not known and await to be seen. Expansion of activated T effector cells has also been observed in ANCA-SVV patients, possibly caused by dysfunction of regulatory T cells. Activated effector T cells may contribute directly to lesional injury via cytokine production or cytotoxic effects on endothelial cells. A study by Ruth et al demonstrated that anti-MPO-specific T cells in mice indeed migrate to a site of glomerular inflammation induced by co-administration of anti-MPO and anti-glomerular basement membrane (GBM) antibodies and contribute to glomerular injury. However, transfer of MPO-specific T cells from MPO-immunized Mpo-/- mice into immune-deficient Rag2-/- recipients did not induce crescentic glomerulonephritis, suggesting that anti-MPO effector T cells may contribute to anti-MPO antibody-mediated disease but are not pathogenic on their own. The potential contribution of anti-MPO effector T cells requires confirmation by additional studies, particularly in models that do not involve administration of anti-GBM antibodies. Thus, more research is required to establish the therapeutic potential of B and T cell inhibition in ANCA-SVV patients.

**Fcγ receptors**

Fcγ receptors (FcγRs) are effector molecules that are expressed particularly on innate immune cells, including neutrophils, monocytes and macrophages. IgG molecules can bind FcγRs with their Fc tail and activate the receptors. Both activating and inhibiting FcγRs exist, which are in a well-regulated balance under normal circumstances. Under pro-inflammatory conditions, a number of mediators, including C5a, TNFα and LPS, can increase expression and function of activating FcγRs, shifting the balance towards an inflammatory state. In vitro studies in human neutrophils demonstrated that FcγRIIa and FcγRIII are involved in ANCA-mediated neutrophil activation. Furthermore, genetic deletion of activating FcγRs was shown to reduce neutrophil adhesion and glomerulonephritis development in the MPO-ANCA mouse model, demonstrating that FcγRs are important for disease and suggesting that inhibition of FcγRs may be beneficial for the treatment of ANCA-associated vasculitis.

**Complement**

The complement system is a central component of the innate immune system in the defense against bacterial infections. Three pathways exist to activate the complement system, i.e. the classical,
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the lectin and the alternative pathway. A functional alternative pathway is required for induction of glomerulonephritis in the MPO-ANCA mouse model suggesting that activation of complement via the alternative pathway is a pivotal step in ANCA-associated vasculitis.\textsuperscript{10} It has been hypothesized that ANCA-activated neutrophils release components of the alternative pathway, such as properdin and factor B that promote local complement activation (Figure 2 step 1).\textsuperscript{10} Initiation of the alternative pathway relies on the spontaneous conformational alteration of the complement component C3 to generate C3(H₂O), which then binds to properdin and factor B (Figure 2 step 2). Factor B is cleaved to Ba and Bb by the protease factor D, generating the 'initiation C3 convertase' C3(H₂O)Bb. Properdin promotes binding of factor B and stabilizes the multimeric complex. The generated 'initiation C3 convertase' can cleave additional C3 to C3a and C3b. This spontaneous generation of C3 convertase is tightly regulated by complement regulatory factors, restricting activation of the complement cascade under non-inflammatory conditions. Under inflammatory conditions, however, C3b can amplify complement activation by binding to properdin and factor B and generating the 'amplification loop C3 convertase' C3bBb (Figure 2 step 3). Neutrophils contain several complement factors, including factor B and properdin, that are released upon stimulation with pro-inflammatory mediators, such as TNF\textsubscript{α} and C5a.\textsuperscript{49} Possibly, ANCA-mediated activation of neutrophils induces release of properdin and factor B as well, providing all the necessary components for generation

Figure 2 A schematic diagram illustrating the hypothetical mechanism of ANCA-SVV-mediated complement activation via the alternative pathway. ANCA-activated neutrophils release the complement-activating factors properdin (P) and factor B (B). These factors increase complement activation via an amplification loop of the alternative pathway (indicated by 3), resulting in production of chemotactic factors and formation of the membrane attack complex (MAC). See text for details. (color image on page 189)
of the ‘amplification loop C3 convertase’. This increased C3 convertase activity then leads to more complement activation with generation of the chemoattractants C3a and C5a and the formation of the membrane attack complex (MAC). This leads to recruitment and activation of leukocytes as well as endothelial cell injury (Figure 2 step 4). Interestingly, C3b that is complexed to a surface, such as IgG molecules or bacterial glycans, binds properdin more efficiently and is therefore a more potent activator of the alternative pathway than free C3b. In the case of ANCA-SVV, the presence of ANCA IgG molecules and the potential role of bacterial infections may provide the proper conditions for amplification of complement activation. Interestingly, initiation of the alternative pathway by IgG immune complexes requires the presence of IgG Fc glycans \textit{in vitro}, providing a possible link between ANCA IgG glycosylation and activation of the alternative complement pathway.

The finding that activation of the complement pathway is important in ANCA-associated vasculitis suggests that its inhibition can diminish glomerulonephritis development. Indeed, treatment with a monoclonal C5-inhibiting antibody that prevents cleavage of C5 into C5a and C5b markedly reduced glomerulonephritis development in the MPO-ANCA mouse model. Genetic ablation of the C5a receptor (C5aR) also prevented anti-MPO IgG-induced glomerulonephritis in mice.

Whether the complement pathway is also required in human ANCA-associated vasculitis is however not known yet. In \textit{chapter 2} we observed that C5a was able to prime human neutrophils in a p38MAPK-dependent manner and activate human glomerular endothelial cells. In addition, the expression of C5aR was increased in vasculitic lesions of patients with ANCA-associated glomerulonephritis. Our findings advocate the use of C5a or C5aR as potential targets for therapeutic interventions in human ANCA-SVV. Inhibitors of C5 and C5aR are currently being developed by several pharmaceutical companies. Eculizumab, a monoclonal C5-inhibiting antibody has already been shown to be safe and effective for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH). In addition, the experimentally widely used C5aR antagonist and cyclic peptide PMX53 has exhibited safety and tolerability in human clinical trials. Thus, the availability of specific C5 or C5aR inhibitors offers opportunities to test their efficacy for treatment of patients with ANCA-SVV in clinical trials.

\textit{Kinases}

ANCA-induced activation of primed neutrophils \textit{via} MPO/Pr3 binding and FcγRI ligation induces activation of kinase signaling cascades. \textit{In vitro} studies have identified several kinases to be involved in ANCA-induced neutrophil respiratory burst (summarized in Figure 3), including tyrosine kinases (such as src kinases and spleen tyrosine kinase (Syk)), phosphatidylinositol 3 kinase (PI3K), protein kinase B (PKB) / Akt and protein kinase C (PKC). The mitogen activated protein kinases (MAPKs) extracellular signal regulated kinase (Erk) and p38MAPK have been implicated in TNFα-mediated priming for ANCA-induced respiratory burst and diacylglycerol kinase in neutrophil adhesion. The fact that kinases participate in ANCA-induced signaling suggests that inhibition
of a selected kinase in vivo may diminish downstream signaling and thereby diminish neutrophil activation and ANCA disease. This hypothesis can be tested by employing specific kinase inhibitors in MPO-ANCA animal models.

In chapter 7 we tested the potential of inhibition of p38MAPK as a therapy for ANCA-SVV. To this end, we analyzed whether the p38MAPK-specific inhibitor AR-447 reduced the pathogenicity of ANCA in vitro and in vivo. In vitro, AR-447 diminished neutrophil respiratory burst and degranulation induced by patient-derived MPO-ANCA and Pr3-ANCA. In glomerular endothelial cells, AR-447 reduced LPS-induced secretion of the pro-inflammatory cytokine IL-6 and the chemokine IL-8. In the MPO-ANCA mouse model, the formation of glomerular crescents was significantly reduced upon AR-447 treatment, but no effects on urinary abnormalities were observed at that point. Our data showed that inhibition of p38MAPK markedly reduced ANCA-induced neutrophil activation in vitro but only partially reduced pathogenicity in vivo, suggesting that specific inhibition of p38MAPK ameliorates some aspects of the disease, yet does not fully counteract the ANCA-mediated inflammatory response in vivo.

As illustrated in Figure 3, p38MAPK is thought to be involved predominantly in the TNFα-mediated priming phase of neutrophil respiratory burst assays. Inhibition of p38MAPK prevented

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**Figure 3** A schematic representation of TNFα/C5α priming- and ANCA-induced kinase signaling in neutrophils leading to ANCA antigen translocation, respiratory burst and adhesion (see text for details). The numbers near the arrows indicate references. ANCA, antineutrophil cytoplasmic autoantibody; C5aR, C5a receptor; DG kinase, diacylglycerol kinase; Erk, extracellular signal regulated kinase; FcγR, Fcγ receptor; Gi, Gαi protein; MPO, myeloperoxidase; p38MAPK, p38 mitogen activated protein kinase; PI3Kγ, phosphatidylinositol 3-kinase γ; PKB/Akt, protein kinase B/Akt; PKC, protein kinase C; Pr3, proteinase 3; Src kinases, Src family kinases; Syk, spleen tyrosine kinase; TNFα, tumor necrosis factor α; TNFR, TNFα receptor. (color image on page 190)
ANCA antigen translocation to the membrane in response to TNFα and C5a (chapter 2) in vitro. In contrast to the in vitro situation where only one pro-inflammatory stimulus is used for priming, multiple stimuli can contribute to neutrophil priming in vivo. Possibly, some of these pro-inflammatory stimuli induce p38MAPK-independent priming that can circumvent the effect of p38MAPK inhibitors in vivo. Interestingly, a specific inhibitor of the p101/p110γ isoform of PI3K (PI3Kγ) was recently shown to markedly diminish anti-MPO IgG-induced glomerulonephritis in the bone marrow transplantation-based MPO-ANCA mouse model.64 PI3Kγ is activated specifically in response to ANCA Fab binding and Fc ligation. These findings suggest that specific inhibition of kinases involved in ANCA-induced signaling is more effective than inhibition of kinases involved in neutrophil priming.

**Genetic factors**

A number of genetic polymorphisms have been associated with ANCA-associated vasculitis, suggesting that the genetic susceptibility for ANCA-SVV is determined by multiple genes (recently reviewed by Willcocks et al). Polymorphisms associated with ANCA-SVV are usually found in genes encoding for factors involved in the immune response. Several genetic polymorphisms associated with ANCA-SVV are for instance located in genes of the major histocompatibility complex (MHC).65,67 In addition, the PTPN22 gene encoding for protein tyrosine phosphatase non-receptor type 22 (PTPN22) has been implicated in ANCA-SVV. PTPN22 is involved in T cell receptor signaling and possibly also in B cell function.68 Two separate studies demonstrated that the 620W allele of PTPN22 is associated with an increased risk for ANCA-SVV.69,70 Polymorphisms in a second gene encoding for a protein involved in T cell function, i.e. cytotoxic T-lymphocyte antigen 4 (CTLA4), have been associated with ANCA vasculitis.70,71 Other genes that have been associated with ANCA-SVV are genes coding for Fcγ receptors (FCGR2A and FCGR3B),72-74 but more research is needed to firmly establish the contribution of genetic variation in the FCGR locus to ANCA-SVV. Thus, the associations of genetic polymorphisms in several genes with ANCA disease suggest a genetic contribution to the susceptibility for ANCA-SVV.

Also, in MPO-ANCA vasculitis animal models, differences in susceptibility for anti-MPO antibody-induced glomerulonephritis have been observed between strains. WKY rats were shown to be more susceptible for anti-MPO-induced albuminuria and glomerulonephritis compared to Lewis, Wistar Furth and Brown Norway rats.11 Likewise, 129S6 mice were shown to develop more severe glomerulonephritis in response to anti-MPO antibodies when compared to C57BL/6 mice,75 again suggesting that genetic variants may predispose to susceptibility for ANCA-associated glomerulonephritis. Genetic studies using 129S6 and C57BL/6 will reveal more insight into the genes that contribute to ANCA-associated glomerulonephritis and could potentially lead to the discovery of new targets for treatment.
CONCLUDING REMARKS
The effector mechanisms involved in ANCA-associated glomerulonephritis are determined by a combination of environmental, immunological and genetic factors, as discussed in this chapter and summarized in Figure 4. Considering environmental factors, a pro-inflammatory micro-environment contributes to exacerbation of disease through priming of neutrophils and activation of the endothelium. Bacterial infections can potentially create such a pro-inflammatory environment, although a causative role for infections in ANCA-SVV remains to be proven. Several immunological factors, including both cells and stimulatory proteins, are involved in the pathogenesis of ANCA-associated glomerulonephritis and inhibition of these factors may attenuate glomerulonephritis development. In this respect, promising therapeutic strategies for the treatment of ANCA-SVV include inhibition of the C5a/C5aR interaction and disruption of FcγR activation, for instance via enzymatic deglycosylation of ANCA IgG molecules. Clinical studies should be performed to assess the safety and efficacy of such treatments. On the other hand, inflammatory mediators belonging to biologically redundant systems, such as chemokines and mitogen activated protein kinases, may not be as promising as targets for the treatment of ANCA-associated glomerulonephritis. Studies into genetic factors revealed that aberrant regulation of genes, particularly those involved in the immune response may determine susceptibility for development of ANCA-SVV. The mouse strain 129S6 that is highly susceptible for development of glomerulonephritis may provide a model to study, in detail, which genes determine such susceptibility. The 129S6 mouse strain may also provide a good model to test the pathogenicity of different anti-MPO monoclonal antibodies to unravel the importance of ANCA IgG subclasses and epitope specificities for disease.

Environmental factors

Figure 4 A diagram illustrating which environmental, immunological, and genetic factors contribute to the pathogenesis of ANCA-associated small-vessel vasculitides (see text for details). ANCA, antineutrophil cytoplasmic autoantibody; CTLA4, cytotoxic T-lymphocyte antigen 4; FcγR, Fcγ receptor; MHC, major histocompatibility complex; PI3Kγ, phosphatidylinositol 3-kinase γ; PTPN22, protein phosphatase non-receptor type 22. (color image on page 190)


