Chapter 1

General introduction

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**THE GLOMERULAR ENDOTHELIUM**

Blood vessels are involved in a variety of biological processes, including oxygen supply, nutrient transport, waste clearance, blood pressure regulation, inflammation, coagulation, and angiogenesis. To properly exert these functions, endothelial cells of blood vessels play an important role. Endothelial cells are long, flat cells that line the lumens of all blood vessels, forming a highly active and selective barrier for the different blood components. The functions of endothelial cells in a specific vascular bed of an organ are dependent on the function of that organ. Therefore, endothelial cells throughout the body show a large heterogeneity in structure and function.

Endothelial cells of the glomerulus, the blood-filtering unit of the kidney, are for instance particularly involved in permeability. Glomerular capillaries filter blood as they are permeable for water and small molecules (Figure 1A). After reabsorption of essential molecules via the tubular system into the bloodstream, the filtration product will ultimately result in urine. A typical feature of glomerular endothelial cells is that they contain small pores, so-called fenestrae, that are approximately 70 nm in diameter and that enable the diffusion of filtration products out of the bloodstream (Figure 1B). The fenestrated glomerular endothelium, together with the glomerular basement membrane (GBM) and podocyte (glomerular epithelial cell) foot processes, form the glomerular “sieve” that filters the blood.

*Figure 1 Structure of glomerular capillaries.* (A) Unfiltered blood enters the capillaries of the glomerulus via the afferent arteriole. The glomerular capillaries function as a size- and charge-selective barrier that filters water and small solutes from the blood. The filtered blood then leaves the glomerulus via the efferent arteriole. (B) A schematic cross-section of a capillary loop showing that the glomerular filtration barrier consists of fenestrated endothelium, basal lamina (i.e. glomerular basement membrane (GBM)), and podocyte foot processes forming filtration slits. Reproduced with permission from Wolters Kluwer Health from reference 2. (color image on page 178)
ANCA-ASSOCIATED SMALL-VESSSEL VASCULITIS AND GLOMERULONEPHRITIS

Vasculitis is defined as inflammation of blood vessels and is characterized by destruction of the blood vessel wall. Severe damage of the vessel wall causes local vascular leakage and occlusion of the vascular lumen and can lead to organ failure. Vasculitis can occur in blood vessels of any size and type and can either be restricted to a single organ (localized) or affect blood vessels in multiple organs (systemic). Vasculitis in itself is not a disease entity but rather a histopathological criterium that can occur secondary to an existing disease but may also have a primary (unknown) cause. One group of primary vasculitides comprises systemic vasculitides that affect small-to-medium-sized blood vessels and are associated with the presence of anti-neutrophil cytoplasmic autoantibodies (ANCA). Disorders belonging to the group of ANCA-associated small-vessel vasculitides (ANCA-SVV) include Wegener’s granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, and renal-limited vasculitis, which are each characterized by a distinct spectrum of symptoms and histopathological features. ANCA are directed against enzymes stored in the granules of neutrophils and the lysosomes of monocytes. The most common antigenic targets for ANCA are the lysosomal enzymes myeloperoxidase (MPO) and proteinase 3 (Pr3), but other targets have been described as well.

ANCA-SVV are relatively rare diseases (with an annual incidence of ~20 per million in European populations), but these diseases have an extremely high mortality. For instance, the 1-year mortality rate of untreated Wegener’s granulomatosis is 80%, whereas the 5-year mortality rate approaches 100%. Immediate and strong immunosuppressive treatment, generally by a combination of cyclophosphamide and prednisolone, is therefore necessary. The current treatment protocols have substantially reduced 5-year mortality rates to 25% for Wegener’s granulomatosis, 25-55% for microscopic polyangiitis, and 0-32% for Churg-Strauss syndrome. Nevertheless, disease relapses are frequent in ANCA-SVV patients and they therefore require long-term treatment that is often associated with severe side effects.

Although ANCA-SVV can affect multiple organs, the kidneys and the lungs are the primary organs affected. ANCA-SVV manifest themselves in the kidney predominantly as glomerulonephritis with segmental necrotic and fibrotic lesions in conjunction with inflammatory cell infiltrates (Figure 2). The lesions and infiltrates cause disruption of glomerular capillaries which may eventually lead to the formation of crescent-shaped scars of proliferating epithelial cells and macrophages that compress the glomerulus. The glomerular abnormalities are often accompanied by inflammatory infiltrates in the tubulo-interstitial area. Besides histological signs of glomerular damage, ANCA-SVV patients occasionally have urinary abnormalities such as hematuria and mild proteinuria.

Since the discovery of ANCA by Davies et al in 1982, detection of ANCA in patients with vasculitis has become a valuable marker in diagnosis. The original and still widely used method to screen patients for the presence of ANCA is to incubate fixed human neutrophils with patient plasma or serum to evaluate binding of ANCA to the neutrophils using indirect immunofluorescence. When ANCA-containing serum is incubated with ethanol-fixed neutrophils, either a cytoplasmic staining
pattern (termed c-ANCA) or a perinuclear staining pattern (p-ANCA) is observed. Identification of the main target antigens of ANCA revealed that ANCA producing a c-ANCA immunofluorescence pattern recognize Pr3 whereas the vast majority of ANCA producing a p-ANCA staining pattern are directed against MPO.

Despite the fact that ANCA are widely used for diagnostic purposes, it has been uncertain for a long time whether ANCA are pathogenic and cause the underlying vasculitis or whether their presence is a consequence of the severe inflammatory response. A common characteristic of antibody-mediated autoimmune diseases is the deposition of immune products like IgG and
complement factors in affected tissues. In these diseases, initial deposition of autoantibodies at the target site induces an inflammatory reaction that ultimately leads to local tissue injury. In ANCA-SVV, only little immune deposits are found in the vasculitic lesions and ANCA-SVV are therefore considered to be pauci-immune diseases. The absence of immune deposits in the vasculitic lesions led researchers at first to believe that ANCA were not involved in the pathogenesis of ANCA-SVV. Over the last decades, however, several clinical observations and extensive \textit{in vitro} and experimental studies have revealed a pathogenic role for ANCA in ANCA-SVV.\cite{11-13}

\textbf{PATHOGENESIS OF ANCA-ASSOCIATED SMALL-VESSEL VASCULITIS – CLINICAL AND \textit{IN VITRO} OBSERVATIONS}

\textbf{Clinical observations}

The presence of ANCA is strongly correlated with the occurrence of small-vessel vasculitis. Ninety percent of patients with ANCA-SVV have ANCA specific for either MPO or Pr3. MPO-ANCA is most common in microscopic Polyangiitis, Churg-Strauss Syndrome, and renal-limited vasculitis, whereas Pr3-ANCA predominates in patients with Wegener’s granulomatosis. In addition, ANCA titers follow disease activity; titers are low during remission and a rise in titer precedes relapses.\cite{14} Although the second correlation is not strong, these observations suggest a pathogenic role for ANCA. Additional evidence for ANCA pathogenicity comes from case reports in which patients treated with various drugs, including hydralazine and propylthiouracil developed circulating ANCA followed by vasculitic disease, suggesting causation.\cite{15}

The most compelling clinical evidence for ANCA pathogenicity comes from observations in a pregnant woman with a history of microscopic polyangiitis. Because of active disease during pregnancy, MPO-ANCA were transferred across the placenta to the fetus, which caused neonatal pulmonary hemorrhage and renal vasculitis immediately after birth.\cite{16} When the neonate was treated with exchange transfusion and high doses of steroids, the MPO-ANCA level declined below detection level and the symptoms disappeared. However, two cases were recently reported in which transplacental transfer of ANCA during pregnancy did not cause any vasculitic symptoms or other health problems in the neonate.\cite{17, 18} The first case concerned a patient with microscopic polyangiitis having high titers of MPO-ANCA despite being in remission and on maintenance immunosuppressive therapy during the entire pregnancy. The other case concerned a patient with \textit{de novo} development of Wegener’s granulomatosis and high titers of Pr3-ANCA during pregnancy. Several factors could account for the difference in neonatal vasculitis development between these three cases. For instance, the immunosuppressive drugs administered to the mothers may have crossed the placenta and protected the fetuses with different effectiveness. Also, the characteristics of the ANCA IgG, such as subclass and epitope specificities, may have resulted in differences in pathogenic potential of the ANCA IgG between the neonates. In addition, the neonate that developed vasculitic symptoms upon transplacental transfer of ANCA was prematurely delivered
by a cesarian section because of severe preeclampsia of the mother, which may have affected the vulnerability of the neonate for vascular damage. These studies suggest that high ANCA titers alone do not always cause vasculitis and that the pathogenicity of ANCA possibly also depends on other factors, such as ANCA characteristics and inflammatory stimuli.

**In vitro observations**

Our knowledge on the pathogenic mechanisms involved in ANCA-mediated vascular injury is largely obtained from *in vitro* studies. These studies have shown that ANCA bind to and activate neutrophils, which leads to the release of toxic substances that destroy microvascular endothelium.\(^3\) This ANCA-mediated neutrophil activation is completely dependent on cellular antigens as illustrated by the lack of activation of MPO-deficient neutrophils by MPO-ANCA.\(^9\) The pathogenic mechanism of ANCA is considered to involve three players, i.e., an infectious stimulus, neutrophils, and ANCA. The main aspects of the pathogenic mechanisms involved in ANCA-mediated vasculitis are summarized in Figure 3 and will be explained below.

For ANCA to fully activate neutrophils *in vitro*, these cells require priming with a low dose of a pro-inflammatory cytokine such as tumor necrosis factor α (TNFα). TNFα induces translocation of the autoantigens to the neutrophil cell surface, making them accessible for binding to ANCA. In addition, MPO released from activated neutrophils can bind to unstimulated neutrophils, thereby sensitizing these neutrophils for MPO-ANCA-mediated activation.\(^20\) Translocation of MPO and Pr3 to

Figure 3 Proposed scheme for the pathogenic mechanisms involved in ANCA-mediated vasculitis. \(\text{Step 1:}\) Pro-inflammatory cytokines released upon an infection prime neutrophils, leading to increased expression of ANCA antigens (MPO/Pr3), β2-integrins and Fcγ-receptors on the cell membrane. Simultaneously, the cytokines activate endothelial cells to express adhesion molecules (e.g. ICAM-1). \(\text{Step 2:}\) ANCA activate neutrophils by binding with their Fab portion to neutrophil-expressed antigens and with their Fc-tail to neutrophil Fcγ-receptors. This ANCA-mediated cross-linking of neutrophils induces activation of inflammatory signal transduction pathways. \(\text{Step 3:}\) ANCA-bound neutrophils adhere to the activated endothelium via integrin-adhesion molecule interactions. \(\text{Step 4:}\) ANCA-mediated activation of neutrophils causes production of reactive oxygen species and release of lytic enzymes that can directly harm endothelial cells. *(color image on page 179)*
the cell surface upon TNFα priming has been shown to depend on the p38 mitogen activated protein kinase (MAPK) signaling pathway. Furthermore, TNFα priming increases β2-integrin membrane expression, which is required for neutrophil-endothelial interactions. The need for neutrophil priming in vitro suggests a role for some kind of pre-activation trigger in ANCA-SVV patients. Such a trigger could, for instance, be provided by a bacterial infection. This idea is supported by studies in patients with Wegener’s granulomatosis, showing that chronic nasal carriage of *Staphylococcus Aureus* increases the risk of relapse, and that antibacterial (co-trimoxazole) treatment reduces the incidence of relapses in patients in remission. A causative role for infections in ANCA-SVV patients has however not been proven.

The activation of primed neutrophils by ANCA requires both Fab-mediated antigen binding and Fc-mediated Fcγ-receptor (FcγR) ligation. Although binding of ANCA-derived F(ab’)2 fragments to MPO or Pr3 was shown to activate neutrophils in one study, the majority of studies support a crucial involvement of Fcγ-receptors, especially FcγRIIa and FcγRIIIb. FcγRIIa is an Fc-receptor with high affinity for immunoglobulins of the IgG3 subclass. Interestingly, ANCA of the IgG3 subclass are more abundantly present in patients with active disease. ANCA engagement by ANCA leads to activation of several kinases, such as tyrosine kinases, phosphatidylinositol 3 kinase (PI3K), protein kinase B/AKT, and protein kinase C. The signal transduction pathways downstream of ANCA-mediated FcγR ligation are, however, different from conventional FcγR cross-linking, as there is activation of a different subtype of PI3K and no phospholipase D activation. It is not clear how simultaneous binding of the Fab and Fc portions of ANCA occurs in vivo. ANCA can bind either as a monomer to both the antigen and the Fc receptor or as an immune complex to the Fc receptor (i.e. via conventional FcR ligation). Although these mechanisms initiate different signaling pathways, they both result in neutrophil activation. It is conceivable that the two mechanisms occur simultaneously as soon as activation is initiated and MPO and Pr3 are released from already activated neutrophils.

When primed neutrophils become activated by ANCA, they are able to bind to and injure endothelial cells. In vitro flow assays have shown that ANCA directly enhance neutrophil adhesion to and migration through endothelial cell layers that are primed with a low dose of TNFα. The neutrophil-endothelial cell interactions were shown to be β2-integrin dependent. ANCA-activated neutrophils produce reactive oxygen species (ROS) that cause oxidative stress. In addition, they degranulate, releasing enzymes like MPO, Pr3 and elastase, which can directly harm endothelial cells. Pr3 and elastase can induce cell detachment and apoptosis, whereas MPO can be involved in endothelial cell death in at least 3 ways. First, MPO catalyzes the production of hypochlorous acid (HOCl) via oxidation of chloride in the presence of hydrogen peroxide. HOCl is a short-lived but highly reactive compound that can potentially oxidize any oxidizable group in any substrate, including thiol esters and heme groups. Such oxidative modifications may alter protein activity and, as a consequence, may disrupt cellular function and activity. In addition, when anti-MPO antibodies bind to soluble MPO, the ability of MPO to catalyze HOCl production increases even more. Second, since MPO is a highly cationic protein, it can bind to the endothelial cell membrane.
cell-bound MPO can be recognized by MPO-ANCA, and binding may lead to antibody-mediated cellular cytotoxicity. Third, MPO can be internalized upon binding to endothelial cells and has been shown to induce production of ROS in these cells, which may lead to tissue damage. Besides neutrophils and endothelial cells, other cell types play a role in ANCA-SVV. As mentioned above, MPO and Pr3 are also expressed by monocytes, suggesting that monocytes can be activated by ANCA as well. Indeed, ANCA were shown to activate monocytes to produce reactive oxygen species and chemokines (e.g. interleukin (IL)-8) in vitro. In this way, ANCA-activated monocytes contribute directly to the inflammatory process in ANCA-SVV. Since the expression of ANCA antigens decreases during the differentiation of monocytes to macrophages, ANCA does not affect mature macrophages. Mature macrophages and monocytes are, however, present in granulomatous lesions and glomerular crescents in ANCA-SVV. Autoreactive B- and T-cells are probably involved in ANCA-SVV as well, with B-cells producing ANCA and T-cells assisting in ANCA production and ANCA isotype switching.

LESSONS LEARNED FROM ANIMAL MODELS OF ANCA-ASSOCIATED SMALL-VEssel VASCULITIS

The mouse model of MPO-ANCA vasculitis

The first direct evidence for the pathogenicity of MPO-ANCA came from studies in a mouse model developed by Xiao et al in 2002. This model differs from previous models as it is an autologous model that is not hampered by species differences. Xiao et al used purified murine MPO (mMPO) isolated from a murine myeloid cell line to immunize Mpo^−/− C57BL/6 mice. The immunization induced an immune response against mMPO in Mpo^−/− mice, resulting in circulating anti-MPO antibodies. The authors isolated splenocytes from mMPO immunized Mpo^−/− mice, and transferred them to recombinase-activating gene-2-deficient (Rag2^−/−) mice, which lack functioning B- and T-cells. Rag2^−/− mice that received anti-MPO splenocytes developed circulating anti-MPO antibodies within 3 days. Thirteen days after splenocyte transfer, these mice showed severe renal failure, as demonstrated by increased blood urea nitrogen and serum creatinine levels. In addition, all mice developed necrotizing and crescentic glomerulonephritis. Some Rag2^−/− mice that received anti-MPO splenocytes developed circulating anti-MPO antibodies within 3 days. Thirteen days after splenocyte transfer, these mice showed severe renal failure, as demonstrated by increased blood urea nitrogen and serum creatinine levels. In addition, all mice developed necrotizing and crescentic glomerulonephritis. In some Rag2^−/− mice that received splenocytes, vasculitis or granulomatous inflammation in other organs (e.g. spleen and lungs) was observed. Overall, these results suggested that anti-MPO antibodies can cause systemic vasculitis and glomerulonephritis.

To confirm that the anti-MPO antibodies alone were indeed pathogenic, total IgG was isolated from mMPO immunized Mpo^−/− mice, and the purified IgG was passively transferred to both Rag2^−/− and wildtype mice. With this strategy, the contribution of anti-MPO antibodies to the vasculitic lesions could be separated from potential effects of MPO-specific B- and T-cells. Circulating anti-MPO antibodies could still be detected in these mice several days after antibody injection, with titers gradually decreasing over time. Mice were sacrificed after 6 days and, although serum creatinine
levels were not increased, they developed hematuria, proteinuria, and leukocyturia. In addition, all anti-MPO IgG-injected mice displayed a focal glomerulonephritis with capillary necrosis and crescent formation. Only limited amounts of immunoglobulins were detected in glomeruli, which is in agreement with the pauci-immune nature of the glomerular capillary lesions observed in human ANCA-associated glomerulonephritis. In addition to renal abnormalities, vasculitic lesions with striking similarities to lesions in ANCA-SVV patients were occasionally found in lungs and ears. The passive transfer studies showed that anti-MPO IgG can cause vasculitis and glomerulonephritis both in the absence of functional B- and T-cells in Rag2−/− mice and in the presence of an intact immune system in wildtype mice.

The rat model of MPO-ANCA vasculitis
The pathogenicity of anti-MPO antibodies has been confirmed in a rat model of MPO-ANCA vasculitis. In this model, Wistar Kyoto rats were immunized with human MPO, which caused the rats to produce anti-MPO antibodies that recognized both human and rat MPO. As a result of the circulating anti-MPO antibodies, the rats developed a focal necrotizing crescentic glomerulonephritis and pulmonary haemorrhage. Furthermore, glomerular lesions in these rats were pauci-immune in nature and the rats developed hematuria and albuminuria. Importantly, this rat model also confirmed the in vitro finding that MPO-ANCA directly enhance the interactions between primed neutrophils and activated endothelial cells. An intravital microscopy technique was used to study the behaviour of leukocytes in the mesenteric venules. An increased number of adherent leukocytes was found in rats that were immunized with MPO compared to control immunized rats. The increase in number of adherent cells was even higher after topical administration of the chemokine CXCL1. CXCL1 also enhanced the number of cells that transmigrated across the endothelial layer. Besides increased leukocyte-endothelial cell interactions, vascular injury was observed in MPO-immunized rats. The rats had an increased occurrence of haemorrhage in postcapillary venules of the mesenteric vasculature. In addition to active immunization of rats with MPO, IgG purified from MPO-immunized rats was passively transferred to naive rats. Intravital microscopy analysis of the mesenteric venules in these rats revealed that comparable leukocyte responses were induced upon anti-MPO IgGs that were introduced by passive transfer and anti-MPO IgGs that were raised by active immunization. This study demonstrated for the first time that anti-MPO IgG induces increased leukocyte-vessel wall interactions and leukocyte-mediated vascular damage in vivo.

Applications of MPO-ANCA animal models
Although these animal models of MPO-ANCA vasculitis clearly demonstrate the pathogenic potential of MPO-ANCA, it is important to emphasize that these models also have their limitations. Strictly speaking, both models cannot be regarded as auto-immune models, which is mainly due to the fact that the models rely on active immunization strategies for disease induction. As a consequence, high affinity antibodies are induced and the specificity of these antibodies is
most likely not restricted to disease-promoting epitopes. Additionally, the passive transfer model developed by Xiao et al relies on a single injection of anti-MPO IgG. Therefore, the model is useful for studying the acute phase of the disease process but is less suited for studying events in the progression phase of disease development as there is no continuous production of autoantibodies. With respect to this latter issue a modification of the mouse model may have provided a solution to this problem. In this study, immunized Mpo−/− mice were irradiated and received a transplantation with bone marrow (BM) from either MPO-deficient mice or mice that express MPO in their leukocytes. These experiments showed that engraftment of MPO-positive BM cells in mice with circulating anti-MPO antibodies results in crescentic glomerulonephritis in all mice, whereas engraftment of MPO-deficient BM in mice with circulating anti-MPO antibodies does not. These experiments not only demonstrate that MPO-positive BM-derived cells are necessary targets for anti-MPO-mediated glomerulonephritis but it also provides us with a model in which the effects of long-term exposure to anti-MPO responses can be studied, mimicking the human situation more closely.

Despite the limitations, MPO-ANCA vasculitis models have provided us the tools to study effector mechanisms involved in anti-MPO IgG mediated vasculitis, to discover new targets for treatment, and to test experimental therapies (summarized in Table 1).

In particular, the MPO-ANCA mouse model has been used for these purposes. In this model, neutrophils have been identified as the main effector cells, as neutrophil depletion completely prevented vasculitis induction upon transfer of anti-MPO IgG. Additionally, the contribution of pro-inflammatory stimuli to disease development was investigated. Co-administration of lipopolysaccharide (LPS) and anti-MPO IgG severely aggravated glomerulonephritis development, supporting the hypothesis that in a local infectious environment, pro-inflammatory stimuli and MPO-ANCA synergize in causing full-blown vasculitis. Furthermore, the very early effects of anti-MPO antibodies on the interaction of neutrophils with the endothelium have been studied by intravital microscopy analysis of the mouse cremasteric microvasculature. In the presence of a local inflammatory stimulus, anti-MPO IgG reduced neutrophil rolling, while it promoted adhesion and transendothelial migration of neutrophils. However, these effects of anti-MPO IgG were only seen when the cremaster muscle was pretreated with a cytokine (preferably TNFα or CXCL1). Interestingly, increased recruitment of MPO-positive leukocytes was found in renal and pulmonary tissue, suggesting that primed neutrophils and anti-MPO antibodies together can exert systemic effects and affect specific vascular beds. In addition, it was shown that the anti-MPO effects were completely lost when either β2-integrins or Fcγ-receptors were blocked, demonstrating β2-integrin and FcγR involvement in the pathogenesis of anti-MPO mediated SVV in vivo. A similar intravital microscopy study in mice investigated the effects of anti-MPO antibodies on leukocyte-endothelial interactions in a clinically more relevant vascular bed, the glomerulus. This study showed that also in the glomerular capillaries, anti-MPO antibodies induce leukocyte adhesion to endothelial cells under priming conditions (i.e. systemic LPS), in a β2-integrin-dependent manner. Additionally, this study showed that administration of a higher amount of anti-MPO IgG did not require a systemic
Table 1 Summary of findings obtained using MPO-ANCA vasculitis animal models

<table>
<thead>
<tr>
<th>Exploring effector mechanisms</th>
<th>Result</th>
<th>Model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>Neutrophil depletion abrogates crescentic glomerulonephritis</td>
<td>Mouse</td>
<td>56</td>
</tr>
<tr>
<td>Pro-inflammatory stimuli</td>
<td>Lipopolysaccharide aggravates crescentic glomerulonephritis</td>
<td>Mouse</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Pertussis toxin/Mycobacterium tuberculosis aggravates crescentic glomerulonephritis</td>
<td>Rat</td>
<td>60</td>
</tr>
<tr>
<td>Leukocyte-endothelial</td>
<td>Anti-MPO IgG increases leukocyte adhesion and migration in cremasteric venules</td>
<td>Rat</td>
<td>54</td>
</tr>
<tr>
<td>interactions</td>
<td>Anti-MPO IgG increases leukocyte adhesion in glomerular capillaries</td>
<td>Mouse</td>
<td>58</td>
</tr>
<tr>
<td>Genetic susceptibility</td>
<td>Rat strains differ in susceptibility to crescentic glomerulonephritis</td>
<td>Rat</td>
<td>60</td>
</tr>
<tr>
<td>Discovery of targets for</td>
<td>Disruption of alternative complement pathway abrogates crescentic glomerulonephritis</td>
<td>Mouse</td>
<td>62</td>
</tr>
<tr>
<td>treatment</td>
<td>Genetic ablation of C5aR attenuates crescentic glomerulonephritis</td>
<td>Mouse (BM)</td>
<td>63</td>
</tr>
<tr>
<td>PI3Kγ signaling</td>
<td>Genetic ablation of PI3Kγ attenuates crescentic glomerulonephritis</td>
<td>Mouse (BM)</td>
<td>65</td>
</tr>
<tr>
<td>Testing of experimental</td>
<td>Anti-TNFα pretreatment attenuates crescentic glomerulonephritis</td>
<td>Rat</td>
<td>61</td>
</tr>
<tr>
<td>therapies</td>
<td>Anti-CS pretreatment abrogates and treatment attenuates crescentic glomerulonephritis</td>
<td>Mouse</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Treatment with a PI3Kγ inhibitor attenuates crescentic glomerulonephritis</td>
<td>Mouse (BM)</td>
<td>65</td>
</tr>
</tbody>
</table>

BM, bone marrow (refers to the bone marrow transplantation MPO-ANCA mouse model); C5aR, C5a receptor; MPO, myeloperoxidase; PI3Kγ, phosphatidylinositol 3 kinase-γ; TNFα, tumor necrosis factor α.

stimulus to induce glomerular leukocyte adhesion. This higher-concentration-induced leukocyte adhesion was not seen in the cremasteric microvasculature and was dependent on α4-integrins, suggesting that the molecular basis of anti-MPO-induced glomerular leukocyte adhesion can vary according to the amount of circulating anti-MPO IgG and the pre-existing inflammatory state.

A study in the MPO-ANCA rat model confirmed the finding that bacterial products aggravate anti-MPO IgG-mediated glomerulonephritis.60 The same study also showed that different rat strains were not equally susceptible for development of crescentic glomerulonephritis, suggesting that genetic characteristics may contribute to the development of ANCA-associated vasculitis.

The MPO-ANCA models have also been useful for the identification of potential new targets
for treatment and the testing of new experimental therapies. For example, strategies aimed at neutralization of TNFα have been tested in the MPO-ANCA mouse model\(^5\) as well as in the rat model.\(^{61}\) In both models, anti-TNFα treatment attenuated disease development, although this strategy appears to be more effective in rats. Perhaps more promising in this respect is the identification of the unsuspected but crucial role for alternative complement pathway activation in anti-MPO IgG-mediated glomerulonephritis in the mouse.\(^{62}\) In addition, the complement C5a receptor (C5aR) was shown to be involved in the development of anti-MPO IgG-mediated crescentic glomerulonephritis.\(^{63}\) Moreover, administration of a C5-inhibiting antibody markedly attenuated glomerulonephritis development in the model, even when treatment was started after disease induction.\(^{64}\) A recently discovered potential target for treatment is PI3K\(γ\).\(^{65}\) Pharmacological inhibition of PI3K\(γ\) was shown to markedly diminish disease development in the BM transplantation MPO-ANCA mouse model.

**Pr3-ANCA animal models**

In contrast to MPO-ANCA models, the development of Pr3-ANCA vasculitis models has proven difficult. One model that has been described, showed pathogenicity of anti-Pr3 antibodies at local sites of inflammation.\(^{66}\) In this model, anti-Pr3 antibodies were obtained from Pr3/elastase double-knock-out mice upon immunization with murine recombinant Pr3. The anti-Pr3 antibodies were passively transferred to wild-type recipient mice and aggravated subcutaneous panniculitis induced by intradermal injection of TNFα. However, in contrast to anti-MPO antibodies, the presence of circulating anti-Pr3 antibodies did not lead to vasculitic lesions in the lungs or kidneys. By using a double-knock-out, immunological tolerance toward potential epitopes shared by Pr3 and elastase was excluded, whereas in patients, ANCA are very specific for human Pr3 and do not recognize human elastase. Possibly, the use of the double-knock-out resulted in the production of antibodies recognizing a wide spectrum of Pr3 epitopes instead of concentrating on Pr3-specific epitopes that are potentially more relevant for disease. In a different model, immunization of mice and rats with chimeric human/mouse Pr3 induced the production of autoantibodies to mouse Pr3 and rat granulocytes.\(^{67}\) But also in this model, anti-Pr3 antibodies did not cause vasculitic lesions in kidneys or lungs. Conversely, anti-Pr3 antibody-mediated lung injury was observed in a model that is based on perfusion of isolated rat lungs with primed human neutrophils and a monoclonal antibody against human Pr3.\(^{68}\) Perfusion of both neutrophils and anti-Pr3 IgG induced elastase- and oxygen radical-dependent lung edema associated with increased microvascular permeability. Whether this anti-Pr3 IgG-mediated acute lung injury was followed by development of vasculitic lesions could not be studied in such short-time experiments. In an additional model that was recently described, an anti-Pr3 immune response was induced by immunization of autoreactivity-prone non-obese diabetic (NOD) mice with recombinant mouse Pr3.\(^{69}\) Adoptive transfer of splenocytes from these Pr3-immunized mice to NOD-severe combined immunodeficiency (SCID) mice caused development of circulating anti-Pr3 antibodies and crescentic glomerulonephritis in recipient mice. The authors did
not evaluate whether the glomerulonephritis was mediated by the humoral or the cellular (or both) anti-Pr3 response. Together, these experimental findings suggest that under certain conditions anti-Pr3 antibodies can be pathogenic in rodents. However, more research is required in order to draw firm conclusions on the pathogenicity of anti-Pr3 antibodies in ANCA-associated vasculitis.

**AIM AND OUTLINE OF THE THESIS**

In the last decades, several aspects of the pathogenic mechanism of ANCA-associated vasculitis and glomerulonephritis have been elucidated, particularly facilitated by the development of animal models. Although some studies have revealed potential new targets for treatment, patients with ANCA-SVV are still treated with non-selective immunosuppressive agents, which are accompanied by severe side-effects. A better understanding of the pathogenic mechanism underlying ANCA-SVV could potentially lead to the recognition of targets for therapeutic strategies that are more specific and cause less side-effects.

The aim of this thesis was 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. We examined the importance of inflammatory stimuli in ANCA-SVV and considered certain characteristics of ANCA IgG molecules and their roles in disease. In addition, we investigated the therapeutic potential of interfering with leukocyte behaviour in the MPO-ANCA mouse model. For that end, we conducted experiments using human neutrophils and glomerular cells in combination with the adapted mouse model of MPO-ANCA vasculitis, in which co-administration of anti-MPO IgG and LPS causes crescentic glomerulonephritis (Figure 4).

In chapters 2 and 3, we focused on what is considered to be the first step in the pathogenesis of ANCA-mediated vasculitis, i.e. the priming of neutrophils and activation of endothelial cells by an inflammatory stimulus (step 1 in Figure 3). An inflammatory stimulus that is widely used to induce neutrophil priming and endothelial activation *in vitro* is the pro-inflammatory cytokine TNFα. The complement factor and anaphylatoxin C5a is also capable of priming neutrophils *in vitro*. This ability

![Figure 4 Schematic representation of the anti-MPO IgG/LPS-induced glomerulonephritis mouse model.](image)
Chapter 1

of C5a is of particular interest because the complement pathway, and more specifically the receptor for C5a (C5aR), was recently found to be important for the development of glomerulonephritis in MPO-ANCA mouse models. Despite the proof for a pathogenic role of complement in animal models of ANCA-associated vasculitis, evidence for the involvement of complement in human ANCA-associated vasculitis is less clear. In *chapter 2*, we therefore aimed to further explore the contribution of C5a-C5aR interactions to the pathogenesis of ANCA-SVV. We investigated whether C5a-induced priming of human neutrophils is, similar to TNFα-induced priming, dependent on p38MAPK activity and whether C5a is able to activate human glomerular endothelial cells. We also analyzed the expression pattern of C5aR in glomerular lesions of both mice and patients with ANCA-SVV.

An inflammatory stimulus that is used to induce neutrophil priming and endothelial activation *in vivo* is the bacterial endotoxin LPS. Administration of LPS severely aggravates anti-MPO IgG-induced neutrophil activation and crescentic glomerulonephritis in mice. This effect of LPS is relevant, as involvement of bacterial infections in the pathogenesis of human ANCA-SVV has been suggested. It is however not known which cells are primarily responsible for conducting the LPS-induced aggravation of disease in mice. Therefore, we investigated in *chapter 3* whether activation of toll-like receptor 4 (TLR4), the receptor for LPS, on leukocytes or on intrinsic renal cells is required for LPS-mediated aggravation of neutrophil recruitment and glomerular injury.

In *chapters 4 and 5*, some aspects of the pathogenicity of ANCA IgG molecules were addressed (step 2 in Figure 3). As ANCA-SVV are considered to be autoantibody-mediated diseases, inactivation of the autoantibodies seems a promising strategy for treatment. One way to inactivate autoantibodies is to interfere with ligation of the Fc portion of the antibody to Fcγ receptors on leukocytes. The bacterial enzyme Endoglycosidase S (EndoS) deglycosylates the Fc portion of IgG molecules, which reduces the ability of IgG to ligate to Fcγ receptors hampering FcγR-mediated activation of leukocytes and complement. We hypothesized that treatment with EndoS could diminish the pathogenicity of ANCA IgG. To test this hypothesis, we investigated in *chapter 4* whether EndoS treatment inhibits ANCA-mediated neutrophil activation *in vitro* and diminishes glomerulonephritis development in the MPO-ANCA mouse model.

Although ANCA of all IgG subclasses and recognizing different epitope regions are found in patients with ANCA-SVV, it is conceivable that pathogenicity of ANCA is restricted to a specific subset of antibodies. In the mouse model of MPO-ANCA vasculitis, glomerulonephritis is induced by administration of polyclonal anti-MPO IgG to C57BL/6 mice. It is not known whether within this polyclonal anti-MPO preparation certain anti-MPO IgG molecules of a specific subclass or recognizing a specific epitope are more pathogenic than others. Therefore, several anti-MPO monoclonal antibodies of different subclasses and recognizing different epitopes have been generated in a previous study. Administration of these monoclonal antibodies, either alone or in combination, to C57BL/6 mice did not induce crescentic glomerulonephritis. In *chapter 5*, we explored whether a selected combination of monoclonal antibodies directed against MPO induces
crescentic glomerulonephritis in mice from a strain (129S6) that is more susceptible for development of glomerulonephritis.

In chapters 6 and 7, we employed the MPO-ANCA vasculitis mouse model for the discovery of targets for treatment and the evaluation of experimental therapies, focusing on inflammatory mediators and signaling pathways that may be involved in leukocyte recruitment, priming, and activation (steps 1, 2 and 4 in Figure 3). One group of inflammatory mediators that can regulate leukocyte behaviour, particularly recruitment, is the family of chemokines. Chemokines are small chemotactic cytokines that exert their function via chemokine receptors. In chapter 6, we analyzed renal and glomerular gene expression levels of chemokines and chemokine receptors to identify potential targets for intervening with recruitment of leukocytes. The effect of inhibition of one potential target, the chemokine receptor CXCR2, on glomerulonephritis development was also examined.

The inflammatory signaling kinase p38MAPK plays a role in ANCA-mediated neutrophil activation in vitro and is activated in glomerular lesions of ANCA-SVV patients, suggesting that activation of p38MAPK is involved in the pathogenesis of ANCA-SVV. In chapter 7, the potential of p38MAPK inhibition as a therapy for ANCA-SVV was examined. To this end, we analyzed whether inhibition of p38MAPK reduces the pathogenicity of ANCA in vitro in human neutrophils and glomerular cells and in vivo in the MPO-ANCA mouse model.

Finally, the results from chapters 2-7 are summarized and discussed in the context of our current knowledge in chapter 8. Some directions for future research are also given.

REFERENCES


