Neutrophil-endothelial interaction in ANCA-associated vasculitis

Nan Hu
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Neutrophil-endothelial interaction in ANCA-associated vasculitis

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Niels van der Geest

致我的父母，孙迪和橙橙
To my parents, Sun Di and Yaochen
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<tr>
<td>ANCA</td>
<td>Anti-neutrophil cytoplasmic autoantibody</td>
</tr>
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<td>AAV</td>
<td>ANCA-associated systemic vasculitides</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid</td>
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl)benzenesulfonyl fluoride</td>
</tr>
<tr>
<td>AECA</td>
<td>Antiendothelial cell autoantibody</td>
</tr>
<tr>
<td>ANGPT</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>Ars</td>
<td>Adenosine receptors</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>BM-PMN</td>
<td>Bone marrow neutrophils</td>
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<tr>
<td>BPI</td>
<td>Bactericidal/permeability-increasing protein</td>
</tr>
<tr>
<td>BVAS</td>
<td>Birmingham vasculitis activity score</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
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<tr>
<td>CEC</td>
<td>Circulating endothelial cell</td>
</tr>
<tr>
<td>CG</td>
<td>Cathepsin G</td>
</tr>
<tr>
<td>CiGenC</td>
<td>Conditionally immortalized human glomerular endothelial cell</td>
</tr>
<tr>
<td>CSS</td>
<td>Churg Strauss syndrome</td>
</tr>
<tr>
<td>CYC</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>DHR 123</td>
<td>Dihydrorhodamine 123</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>EGM2-MV</td>
<td>Endothelial growth medium 2-microvascular</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENT</td>
<td>Ear, nose and throat</td>
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<tr>
<td>ESL-1</td>
<td>E-selectin ligand-1</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucylphenylalanine</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GEnC</td>
<td>Glomerular endothelial cell</td>
</tr>
<tr>
<td>GP</td>
<td>Granule protein</td>
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<tr>
<td>GPA</td>
<td>Granulomatosis with polyangiitis</td>
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<td>GPI</td>
<td>Glycosyl phosphatidylinositol</td>
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<tr>
<td>HAGG</td>
<td>Heat-aggregated goat immunoglobulin</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<td>HC</td>
<td>Healthy controls</td>
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<tr>
<td>hEGF</td>
<td>Human epidermal growth factor</td>
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<tr>
<td>hFGF</td>
<td>Human fibroblast growth factor</td>
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<tr>
<td>HLE</td>
<td>Human leukocyte elastase</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical cord endothelial cell</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAM-A</td>
<td>Junctional adhesion molecule-A</td>
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<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genome</td>
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<tr>
<td>LAD</td>
<td>Leukocyte adhesion deficiency</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
</tr>
<tr>
<td>MB</td>
<td>Myeloblast</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<tr>
<td>MMF</td>
<td>Mycophenolate mophetil</td>
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<td>MPA</td>
<td>Microscopic polyangiitis</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td>mPR3</td>
<td>Membrane-bound PR3</td>
</tr>
<tr>
<td>MY</td>
<td>Metamyelocyte</td>
</tr>
<tr>
<td>NETs</td>
<td>Neutrophil extracellular traps</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>PLSCR1</td>
<td>Phospholipid scramblase 1</td>
</tr>
<tr>
<td>PM</td>
<td>Promyelocyte</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>PR3</td>
<td>Proteinase 3</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>R 123</td>
<td>Rhodamine 123 (R 123)</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-------------</td>
<td>---------------------------------------------------</td>
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<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans-endothelial electrical resistance</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl-benzidin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>u-PAR</td>
<td>Urokinase-type plasminogen activator receptor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WG</td>
<td>Wegener’s granulomatosis</td>
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Introduction to the thesis
ANCA-associated systemic vasculitides
Antineutrophil cytoplasmic autoantibodies (ANCA) associated vasculitides comprise three disease phenotypes: granulomatosis with polyangiitis (GPA), formerly Wegener’s Granulomatosis, microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS). ANCA-associated systemic vasculitis (AAV) is a relatively rare disease, with an annual incidence of approximately 20/million in Europe. There seems to be an upward trend of incidence during the last decades, which, however, is perhaps due to an increase of disease awareness and improved diagnosis amongst physicians. A central feature of AAV is fibrinoid necrosis affecting small- to medium-sized vessels with little or no deposition of immunoglobulins or complement, in a so-called pauci-immune pattern. By electron microscopy, subendothelial edema, microthrombosis and degranulation of infiltrated neutrophils are observed. Vascular lesions may be limited to a single organ, but commonly affect multiple organ systems. When lungs or kidneys are involved, often presenting with aggressive renal failure or pulmonary haemorrhage, the disease becomes life-threatening. AAV has a universally poor prognosis with mortality approaching 100% within 5 years when left untreated. The current standard treatment of AAV is based on cyclophosphamide and glucocorticoids, which is effective in 70–90% of patients and transforms AAV from a rapidly fatal disease to a state of chronic relapsing disease. Also due to the broad and severe side effects of these regimens, AAV is still in need of improvements in therapeutic strategies.

Pathogenesis of AAV
The pathogenesis of AAV has not been fully understood. A widely accepted paradigm holds ANCA, primed neutrophils and activated vascular endothelium as key players.
During infection, proinflammatory cytokines up-regulate expression of adhesion molecules on endothelial cells (ECs), transforming ECs into a pro-adhesive status. Meanwhile, upon stimulation of neutrophils by these cytokines, proteinase 3 (PR3) or myeloperoxidase (MPO), both of which are stored in neutrophil granules, are translocated to the cell surface as part of priming of neutrophils. Adhesion molecules on neutrophils are also upregulated in this process. When ANCA are present, they bind to autoantigens on primed neutrophils, converting slow rolling into firm adhesion of neutrophils and leading to neutrophil degranulation and respiratory burst. Released proteolytic enzymes and reactive oxygen species (ROS) may cause necrosis, apoptosis and detachment of adjacent endothelial cells.
Hence, ANCA-mediated activation of adherent neutrophils may directly attack the vessel wall.¹⁰
In addition, granuloma formation, particularly occurring in PR3-ANCA AAV, suggests involvement of cellular immunity. Increased numbers of effector memory T cells have been observed in peripheral blood in AAV patients during remission and have been detected in urine during active disease, suggesting a pathogenic role in AAV.¹¹,¹² Furthermore, it has been shown in a mouse model of MPO-ANCA associated vasculitis that complement activation via the alternative pathway is also required for AAV development.¹³

**Interaction of neutrophils and endothelial cells in AAV**

It is intriguing that ANCA-mediated vascular lesions preferentially occur in the microvasculature, the loci where neutrophil trafficking takes place. This would suggest that close interaction of neutrophils and endothelial cells is important for disease development. Indeed, it has been shown that infiltrated neutrophils during acute vasculitis are at or within glomerular capillary loops with rather poor penetration into the interstitial tissue or peri-tubular region where their chemoattractants are detectable, suggesting that neutrophils are retained within the microvascular compartment by certain mechanisms.¹⁴ To gain insight into the interaction between neutrophils and endothelial cells, mouse cremasteric microvasculature was studied via intravital microscopy. In the presence of local inflammatory stimuli, anti-MPO antibodies were found to reduce neutrophil rolling while increasing firm adhesion of leukocytes.¹⁵ Also, ANCA-mediated neutrophil activation requires an adhesive state of neutrophils, suggesting that ANCA not only induce close interaction of neutrophils and endothelial cells, but also that ANCA-associated events are dependent on this close contact.¹⁶

**Aim and outline of the thesis**

In this thesis, the mechanisms underlying retaining of neutrophils within the vascular wall and the ensuing vascular damage were investigated. Secondly, up-regulated expression of ANCA antigens, such as membrane-bound protease 3 (mPR3), is an important event for ANCA-induced neutrophil activation. The mechanisms of PR3 expression on the neutrophil membrane were, therefore, also studied and presented in this thesis.¹⁷

In Chapter 2, an overview is given on the mechanisms regulating neutrophil-endothelial interaction during inflammation and specifically in AAV. Current knowledge on the effector mechanisms taking place in this process, which induce
endothelial damage in AAV, is summarized. As mentioned before, persistent inflammation within the vessel wall suggests perturbed neutrophil trafficking of activated neutrophils through the endothelium. CXCR1 and CXCR2, being major chemokine receptors on neutrophils, are largely responsible for neutrophil recruitment, and are, therefore, studied in Chapter 3. In this chapter, we tested the hypothesis that down-regulated expression of CXCR1/2 retains neutrophils within the vessel wall and, consequently, leads to persistence of neutrophils in the microvasculature. Anti-endothelial cell autoantibodies (AECA) have been described in AAV as one of the effector mechanisms causing vascular damage. The presence of AECA in patients with AAV has been reported by several groups with conflicting data on prevalence ranging from 8% to 100%. Types of substrate cells used for AECA testing partially explain this variation. In Chapter 4, we investigated AECA prevalence in AAV using a human glomerular endothelial cell line in comparison with primary human umbilical vein endothelial cells, which have frequently been used for AECA detection.

Membrane expression of the ANCA-antigens, such as PR3, allows ANCA binding and is a crucial step of ANCA-mediated neutrophil activation. Indeed, up-regulation of PR3 on the neutrophil membrane has been shown during neutrophil adhesion. Since the PR3 molecule does not contain a transmembrane domain in its sequence, the mechanisms of membrane expression of PR3 and the proposed signal transduction, therefore, become interesting and are reviewed in Chapter 5. CD177 glycoprotein has been demonstrated as a receptor of PR3 on the neutrophil membrane. The expression profile of CD177 and its role in PR3-ANCA-mediated neutrophil activation is studied in Chapter 6. We found an enlarged neutrophil subset with CD177 expression in patients with AAV. The molecular function of CD177 is largely unknown, so the functional differences between CD177\(^+\) and CD177\(^-\) neutrophils cannot be easily deduced. Therefore, in Chapter 7, we performed a gene microarray-based study to investigate differences between CD177\(^+\) and CD177\(^-\) neutrophils, which may help to clarify the pathophysiologic significance of the enlarged CD177\(^+\)/mPR3\(^{\text{high}}\) neutrophil subset in order to better understand the role of neutrophils in the pathogenesis of AAV.
References


Dysregulated neutrophil-endothelial interaction in antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides
implications for pathogenesis and disease intervention

Nan Hu
Johanna Westra
Cees GM Kallenberg

*Autoimmun Rev, in press*
Abstract
The interplay between neutrophils and endothelial cells allows ANCA to become pathogenic and results in uncontrolled inflammation in the vessel wall. This review presents an overall view on neutrophil-endothelial interaction during inflammation with a focus on ANCA-associated vasculitis, and summarizes the effector mechanisms which cause vascular damage in AAV. In addition, potential elements for disease intervention based on this process are discussed.
Introduction
The anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) are classified into granulomatosis with polyangiitis (GPA, renamed from Wegener’s granulomatosis [WG]), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS). These potentially life-threatening diseases are characterized by necrotizing damage to small- and medium-sized vessels in various organs including lungs and kidneys. Pathogenesis of AAV has not been fully understood, and a widely accepted paradigm considers ANCA, neutrophils and vascular endothelium as key players. During infection, proinflammatory cytokines up-regulate expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1), on endothelial cells (ECs), transforming ECs into a pro-adhesive state. Besides, these proinflammatory cytokines prime neutrophils resulting in translocation of ANCA antigens, proteinase 3 (PR3) or myeloperoxidase (MPO), to the cell surface, and up-regulated expression of β2-integrins. ANCA cross-linking PR3/MPO and the Fc-receptor FcyRIIa leads to neutrophil degranulation and respiratory burst. Released proteolytic enzymes and reactive oxygen species (ROS) may cause apoptosis, necrosis and detachment of ECs. Thus, activation of adherent neutrophils directly causes damage to the vessel wall. Structural or functional features of endothelial cells in small vessels might underlay the distinct pattern of lesion distribution in AAV. Capillaries and venules are the loci where leukocyte trafficking takes place during inflammation, and the endothelium of these vessels is particularly responsive to proinflammatory signals. No direct evidence has shown that ANCA from AAV patients bind to and activate their own neutrophils in the circulation. Even if true, ROS or proteolytic enzymes released into the circulation by activated neutrophils would be rapidly diluted by blood flow or blocked by circulating inhibitors. Thus, these non-specific reagents only attack the vessel wall when they get in close contact with vascular ECs. Therefore, the synapse-like interaction between neutrophils and the endothelial surface is the initial and primary event in necrotizing vasculitis. In this review, the pathogenesis of AAV will be reviewed, with focus on neutrophil-endothelial interaction.

1. Neutrophil recruitment during inflammation
Rolling
Neutrophil emigration into sites of inflammation involves multiple steps and a complex sequence of molecular and cellular responses (Figure 1). The initial
attachment of neutrophils to endothelial cells, called rolling, is determined by ECs, which respond to stimuli such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or IL-17. These stimuli are generated during infection or inflammation and result in up-regulation of P-selectin and E-selectin on the luminal surface of ECs. Ligands of these selectins on neutrophils, L-selectin and P-selectin ligand-1 (PSGL-1), are constitutively expressed on the tips of neutrophil microvilli. E-selectin also binds to E-selectin ligand-1 (ESL-1) and CD44, which are expressed on microvilli and the cell body of neutrophils, respectively. Bsg/CD147 has recently been reported as another counterpart of E-selectin on neutrophils, which mediates neutrophil recruitment and contributes to the tissue damage caused during renal ischemia/reperfusion. Binding of PSGL-1 to P- and E-selectin is the initial interaction of neutrophils and activated ECs, while E-selectin interaction with ESL-1 mediates slowing of rolling, and E-selectin binding to CD44 induces redistribution of L-selectin and PSGL-1 to form clusters leading to further reduction of the rolling speed.

**Firm adhesion**
Selectin-mediated neutrophil-EC interaction only lasts seconds and is reversible. This step is followed by firm adhesion mediated by the β₂-integrins, LFA-1 (α₅β₃) and Mac-1 (α₅β₃), on neutrophils and their ligands, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, present on the EC surface. The role of β₂-integrins has been demonstrated in adhesion molecule knock-out mice and in patients with leukocyte adhesion deficiency (LAD), who are deficient in CD18 expression, by their inability to establish firm adhesion and emigration of neutrophils. β₂-integrins do not interact with their ligands until the former are activated. Various agonists have been found inducing Mac-1 activation on neutrophils, such as platelet-activating factor (PAF), IL-8, fMLP, TNF-α, or bacterial products, like LPS. Meanwhile, an extended, but not a high affinity, form of LFA-1 is induced during neutrophil rolling on E- or P-selectin, which only assists neutrophil rolling, not firm adhesion, on ECs. Full activation of LFA-1 is dependent on activation of chemokine receptors on neutrophils, which sense their ligands secreted by ECs or present on the EC surface as solid form via glycosaminoglycans or the Duffy antigen-receptor. Thus, LFA-1, together with Mac-1, mediates arrest of rolling and firm adhesion of neutrophils to ECs.
Two routes have been described for neutrophils migrating through the endothelium: the transcellular migration, whereby neutrophils penetrate the body of ECs; and the paracellular migration used by the majority of neutrophils, whereby they squeeze between two adjacent ECs. Regardless of the route taken by neutrophils during emigration, LFA-1 and Mac-1 and their ligands, ICAM-1 and ICAM-2, are generally key players involved in guidance of neutrophils towards

**Figure 1. Neutrophil recruitment during inflammation**

Proinflammatory cytokines, such as TNF-α or IL-1β, activate ECs, resulting in upregulation of P- and E-selectin on ECs. PSGL-1, ESL-1 and CD44 are constitutively expressed on the neutrophil surface and mediate rolling of neutrophils along activated ECs. Chemokine receptors, like CXCR2, on neutrophils are activated during slow rolling by their ligands secreted by ECs or inflammatory cells or present on the EC surface in solid form, leading to upregulation of β2-integrins. Rolling is subsequently converted to firm adhesion, which is mainly mediated by β2-integrins (LFA-1 and Mac-1) and their ligands (ICAM-1 and ICAM-2) on ECs. VCAM-1 expression is also upregulated on activated ECs and probably binds to β1-integrins and mediates firm adhesion as well. Most neutrophils use the paracellular pathway to migrate through endothelium. Mac-1 (aMβ2 integrin) binds to ICAM-1 and ICAM-2 and guides neutrophils towards the EC-EC junction. Subsequently, hemophilic binding, between neutrophil- and EC-JAM-A and between neutrophil- and EC-PECAM-1, forms apart the EC-EC junction. Heterophilic binding between neutrophil-Cd77 and EC-PECAM-1 may also be involved in neutrophil transendothelial migration. Thus, neutrophils make their way through endothelium and migrate towards a chemoattractant gradient sensed by their chemokine receptors.
signals through ICAM-1 leading to formation of membrane structures called apical cups with concentrated expression of ICAM-1 and VCAM-1, suggesting that β₁-integrins, which are the binding partners for VCAM-1, may also be involved in neutrophil migration.²⁰⁻²⁴ As mentioned before, Mac-1 and LFA-1 share the same ligands, ICAM-1 and ICAM-2, however, a recent study showed that LFA-1 is probably more essential for firm adhesion than Mac-1 and that Mac-1 has a distinct function mediating intravascular crawling of neutrophils along ECs towards the sites of paracellular migration.²⁸,²⁵

Mechanisms regulating paracellular migration of neutrophils continuously relate to ICAM-1, ICAM-2, junctional adhesion molecule-A (JAM-A) and platelet endothelial cell adhesion molecule-1 (PECAM-1) on ECs. Experiments with blocking or depletion of ICAM-1or ICAM-2 revealed that they are both involved in guiding neutrophils to enter the EC-junctions.²⁶ JAM-A is associated with further penetration of neutrophils, since, in JAM-A⁻/⁻ mice, neutrophils were observed to accumulate deeper down between ECs of cremaster muscles after treatment with IL-1β than in ICAM-2⁻/⁻ mice.²⁷ Finally, PECAM-1 performs the last step of neutrophil transendothelial migration, which is demonstrated by the observation that neutrophils trapped between ECs and basement membrane in PECAM-1⁻/⁻ mice.²⁷ Besides, recent studies reported that CD177 (NB1-glycoprotein, discussed in detail later) is a heterotypic binding partner for PECAM-1 on neutrophils.²⁸,²⁹ The functional relevance of CD177-PECAM-1 interaction needs further investigation.

Molecular mechanisms of transcellular migration are less clear than those of the paracellular route. Mac-1 might promote migration through the paracellular route, since the proportion of neutrophils taking the transcellular route increases significantly in the absence of Mac-1.³⁰ CD99 may play a similar role in transcellular migration as PECAM-1 does in the paracellular route.³¹ The aforementioned steps in neutrophil recruitment are common pictures seen in various in vitro or in vivo experimental settings, however, particular processes may differ according to the involved tissue or microenvironment due to the diversity of ECs.³² The exact mechanisms of neutrophil trafficking, for instance, in inflamed glomeruli, have not been delineated.

2. Vascular damage in AAV
The characteristic glomerular lesion in AAV is focal and segmental necrosis and thrombosis formation in glomeruli, which may progress to crescentic glomerulonephritis. Immunohistochemistry studies of kidney samples in AAV showed little or no glomerular staining for immunoglobulins or complement, a
so-called pauci-immune staining pattern. More recent research, however, demonstrated that the alternative complement pathway is actually crucial to the pathogenesis in animal models of MPO-ANCA disease, and that a few immune complexes are detectable after careful examination. By electron microscopy, subendothelial edema, microthrombosis and degranulation of neutrophils are observed.

Different vascular lesions are generally resolved via a common pathway resulting in fibrosis; therefore, many studies have focused on the early lesions that may better represent the pathogenesis of endothelial damage in AAV. In renal biopsies, electron microscopy has shown that the earliest change occurring in the vascular endothelium is swelling and loss of cytoplasmic organelles. Necrosis and detachment of glomerular endothelial cells (GECs) are present. At a cellular level, neutrophils, eosinophils, monocytes, macrophages, giant cells and lymphocytes are seen in various degrees and in different stages of disease development. However, the early lesions are mainly accompanied by neutrophil influx and accumulation in the glomeruli.

Circulating endothelial cells (CECs) will become detectable in the peripheral blood once they have detached from the endothelium after damage. Therefore, methodologies counting CECs in blood have been adopted to quantify vascular damage in many vascular diseases and found to be reliable markers correlating well with clinical evolutions. In an early study by Woywodt et al., it has been shown that the CEC count is significantly higher in patients with AAV compared to healthy controls and that it correlates positively with disease activity. The authors later on demonstrated that AAV patients with limited disease (restricted to the upper-airway) with granulomatous inflammation had only slightly elevated CEC counts that were comparable with those seen in remission. As disease controls, they showed that patients with infection did not have elevated CEC numbers. These results not only prove that the vascular damage is the central feature of AAV, but also suggest that the mechanisms that lead to detachment of ECs from vascular beds are of significant relevance for disease pathogenesis. Besides, levels of soluble adhesion molecules, reflecting the degree of damage or activation of ECs, have been measured in patients with AAV, and soluble VCAM-1 showed increased levels in the circulation of patients with any phenotype of AAV.

3. Dysregulated neutrophil-endothelial interactions in AAV

By careful examination of renal biopsies from AAV patients, Cockwell et al. have located infiltrated neutrophils during acute vasculitis at or within the glomerular capillary loops with rather poor penetration into the interstitial tissue or the
proximal tubular epithelial region where IL-8 expression is detectable, suggesting that neutrophils are retained within the microvascular compartment by certain mechanisms.\(^{43}\) A similar pattern of neutrophil distribution with enrichment in the intravascular compartment has also been observed in renal biopsies of patients with severe ANCA-associated glomerulonephritis in an earlier study.\(^{44}\) Neutrophil influx into glomeruli during early vascular lesions has been observed in animal models of MPO-ANCA associated vasculitis.\(^{45}\) To gain insight into the very early events of neutrophil-EC interaction, mouse cremasteric microvasculature was monitored via intravital microscopy.\(^{46}\) In the presence of local inflammatory stimuli, including TNF-\(\alpha\), IL-\(\beta\) and keratinocyte-derived chemokine (KC, functional homologue of human IL-8), anti-MPO antibodies reduced neutrophil rolling while they increased firm adhesion and transendothelial migration of leukocytes. Blocking of \(\beta_2\)-integrins or Fc\(\gamma\) receptors (Fc\(\gamma\)R) reversed these effects.

4. Affected adhesion molecules and related modulators
Since neutrophil trafficking through the endothelium is a synergic process determined by cell adhesion molecules (CAMs) and their counterparts present on either neutrophils or ECs, dysregulation in neutrophil-endothelial interaction is likely reflecting modified expression or activity of these CAMs. From the side of ECs, E-selectin and P-selectin are normally absent in glomeruli, which has been proposed as a protective mechanism against glomerulonephritis.\(^{47}\) In cultured glomeruli, E-selectin, however, can be induced by stimulation with IL-\(\beta\), TNF-\(\alpha\) or interferon-\(\gamma\).\(^{48}\) P-selectin is stored in the Weibel-Palade bodies in not only ECs but also platelets, which hampers to assess to what extent P-selectin, which has been detected in the glomeruli during glomerulonephritis, is involved in this process.\(^{49}\) Besides, P-selectin mRNA has been detected in interstitial endothelium instead of glomerular endothelium during inflammation.\(^{50}\) In renal biopsies from patients with ANCA-associated glomerulonephritis, the expression of ICAM-1 and VCAM-1, both of which are essential for neutrophil firm adhesion and early steps during emigration, has been demonstrated to be increased, although this expression was not restricted to glomeruli or glomerular lesions.\(^{51}\) In vitro stimulation of mouse glomerular ECs with anti-MPO IgG induced upregulation of ICAM-1, VCAM-1 and E-selectin at the mRNA level with only ICAM-1 showing sustained increased expression at the protein level.\(^{52}\) Whether JAM-A, PECAM-1 or CD99, involved in transendothelial migration of neutrophils, also play a pathogenic role in AAV has not been investigated so far.
β₂-integrins are the major adhesion molecules expressed by neutrophils, responsible for neutrophil firm adhesion and partially for transendothelial migration. TNF-α-activated neutrophils are prone to adhere to an EC monolayer and to induce EC detachment, while blocking CD18 may reduce adhesion of neutrophils to ECs.⁵³ Effects of TNF-α on neutrophil adhesion can be further enhanced when ANCA are present and can be reversed by blocking of CD18 or the FcγIIa receptor, as shown in a flow-based system by Radford et al.⁵⁴ Later on, these researchers observed that ANCA exposure of TNF-α-primed neutrophils also promoted neutrophil migration through EC monolayers.⁵⁵ The molecular mechanisms involved were further clarified by a recent study that showed that ANCA-mediated neutrophil adhesion was dependent on further activation of β₂-integrins accompanied by activation of diacylglycerol kinase which might be a target for therapeutic intervention.⁵⁶ Alpha-4-integrins are not readily detected on the neutrophil membrane, but become detectable after stimulation with C5a, N-formyl-methionyl-leucylphenylalanine (fMLP) or leukotriene B₄ (LTB₄) requiring pre-treatment with cytochalasins.⁵⁷ Requirement of cytochalasins suggests that cellular events involving cytoskeleton rearrangement are important for making α₄-integrins visible, such as fast internalization. Mechanisms of α₄-integrin-dependent neutrophil-endothelial interaction are unknown. A recent study investigated neutrophil recruitment by renal intravital microscopy and observed that low-dose anti-MPO and LPS treatment induced β₂-integrin-dependent neutrophil adhesion, whereas high-dose anti-MPO alone led to rapid upregulation of α₄-integrin and α₄-integrin-dependent neutrophil adhesion.⁵⁸ These results suggest that ANCA might induce glomerular neutrophil adhesion via multiple pathways.⁵¹,⁵²

CD177 has recently been reported as the receptor of membrane-bound PR₃, an autoantigen of ANCA, on the neutrophil membrane. Increased proportions of CD177-expressing neutrophils were observed in patients with AAV and SLE compared to healthy controls.⁵⁹,⁶⁰ Functions of this molecule are largely unknown, although a recent study showed that CD177 could be a heterogeneous binding partner for PECAM-1, suggesting a role in neutrophil migration.²⁸,²⁹ However, our unpublished data showed that CD17⁷⁺ and CD17⁷⁻ neutrophil subsets do not differ in neutrophil migration towards IL-8 in a transwell-based system, and the proportions of CD17⁷⁺ neutrophils in synovial fluid from patients with rheumatoid arthritis were shown to be identical to those in peripheral blood from the same individuals, indicating minor effects of CD177 on neutrophil migration. The role of chemokines and their receptors in the development of AAV has not been clearly delineated. IL-8, the most potent neutrophil chemoattractant, has
been found elevated in the circulation of AAV patients in remission and increased further during active disease. The cellular sources of IL-8 could be active ECs, as well as neutrophils stimulated with ANCA. However, ligation of circulating neutrophils with IL-8 may frustrate neutrophils within the intravascular compartment. It has been shown that intravascular administration of IL-8 or manipulation of liver and intestinal gene expression in transgenic mice resulting in elevated serum levels of IL-8, leads to neutrophil sequestration in the microvasculature and inhibition of neutrophil migration towards extravascular tissues enriched with IL-8 or other chemoattractants. In our ongoing study, patients with AAV indeed showed decreased CXCR1 and CXCR2 expression on circulating neutrophils, and levels of these IL-8 receptors negatively correlated with serum levels of IL-8. Functional blockade of CXCR1 and CXCR2 significantly increased neutrophil adhesion and inhibited migration through glomerular EC monolayers.

5. Effectors of vascular damage

ANCA and neutrophil activation

Primed neutrophils activated by ANCA undergo the respiratory burst and degranulation, with release of ROS and proteases, such as PR3 and elastase, which may cause necrosis and apoptosis of ECs. However, a protective mechanism in which adenosine inhibits superoxide production by ANCA-stimulated neutrophils has been proposed recently, arguing the pathogenic role of ANCA-mediated ROS production in vivo. During cellular distress, such as hypoxia or inflammation, ATP and ADP are released into the extracellular space from either resident tissue cells or neutrophils transmigrating through the endothelium. CD39 and CD73, which are phosphohydrolytic enzymes present in most cell types, process these nucleotides into AMP and adenosine subsequently. Adenosine receptors (ARs), including A₁AR, A₂AAR, A₂BAR and A₃AR, are G-protein coupled receptors. Activation of A₂AAR on neutrophils may attenuate neutrophil adhesion and inhibit ROS production, indicating its anti-inflammatory effect. It has been shown, in a recent study, that neutrophils isolated from AAV patients can be inhibited for ROS production by co-culture with human umbilical cord endothelial cells (HUVECs) to the same level as those from healthy donors. This effect is dependent on the activity of adenosine. Investigating possible deficiencies in this protective mechanism in locally activated ECs as well as the expression profile of A₂AAR on neutrophils from AAV patients, may further clarify the role of ROS production in vascular injury.
Serine proteases released during ANCA-mediated neutrophil activation may also be responsible for EC damage, probably depending on their enzymatic activity, since administration of diisopropylfluorophosphate, a serine protease inhibitor, in a coculture system, could abolish EC injury. Elastase and PR3 have been shown to be potent inducers of EC apoptosis. Released PR3 from activated neutrophils can be taken up by adjacent ECs, and, subsequently, induce their apoptosis. How PR3 initiates this programmed cell death is not clearly understood. Besides, indirect effects of PR3 on EC injury are also extensive. In vitro incubation of HUVECs with PR3 induced release of monocyte chemoattractant protein-1 (MCP-1), significantly increased ICAM-1 and slightly increased VCAM-1 expression on the EC surface. Functional studies showed that, after 24-hour incubation with PR3, EC monolayers showed remarkable increase in neutrophil adhesion, which could be blocked by anti-ICAM-1 or anti-CD18 antibodies.

**Neutrophil extracellular traps**

Neutrophil extracellular traps (NETs) are structures formed by chromatin fibers that are released by activated neutrophils. ANCA-activated neutrophils produce NETs in vitro and NETs can be found in the renal biopsies from AAV patients. It has recently been found that NETs cause damage to activated ECs in culture. Deficiency in NETs degradation, due to decreased DNase-1 levels in plasma, leads to increased risk of nephritis in a subset of patients with systemic lupus erythematosis, suggesting a proinflammatory role of NETs. When zooming in on the structure of NETs, MPO and PR3 are present on NETs, suggesting an alternative mechanism for membrane display of ANCA-antigens. It may also explain the observation that membrane-bound PR3 expression is significantly upregulated during neutrophil adhesion to ECs and is even further increased when ANCA are present, whereas, expression of CD177, as a PR3 receptor, is not increased in this situation. A recent study showed that CXCR2 activation not only promotes neutrophil adhesion but also induces NETs formation, establishing a link between neutrophil adhesion, activation and vascular damage. It is tempting to speculate that neutrophils stimulated by IL-8 firmly adhere to locally activated ECs and are activated by ANCA, resulting, amongst others, in production of NETs and concentration of PR3 and MPO on their surface. The released ROS and proteolytic enzymes are concentrated adjacent to ECs and lead to uncontrollable inflammation in the vessel wall (Figure 2).

**Anti-endothelial cell autoantibodies**
Antiendothelial cell autoantibodies (AECA) in AAV have been detected in several studies using Western Blotting on endothelial lysates or cell-based ELISA. However, the prevalence of AECA in AAV varies very much among reported tests, probably due to different techniques used or various origins of the EC substrate used, which still waits for standardization. AECA have been suggested to be pathogenic in AAV and to be associated with

Figure 2. Neutrophil-endothelial interaction in AAV
A. ICAM-1 is constitutively expressed on ECs, as are the E-selectin ligands, CD177 and CXCR2 on resting neutrophils. ANCA might recognize ANCA-antigens planted or produced on the membrane of ECs. B. After neutrophil priming by circulating proinflammatory cytokines, ANCA-antigens, such as PR3, and β2-integrins are up-regulated on circulating neutrophils. Ligation of CXCR2 with IL-8 in the circulation induces CXCR2 internalization. Cross-linking of PR3 and FyRIIa by PR3-ANCA may transduce signals through β2-integrins and further activate β2-integrins. ROS production from activated neutrophils is inhibited by adenosine. ANCA may induce α4-integrin expression as well. C. Meanwhile, proinflammatory cytokines activate ECs and induce upregulated expression of P- and E-selectin, ICAM-1 and VCAM-1 on the EC surface. E-selectin, possibly also P-selectin, and their ligands on neutrophils mediate neutrophil rolling along the EC surface. D. Beta2-integrins and ICAM-1, together with α4/β1-integrins and VCAM-1, mediate neutrophil firm adhesion and guide neutrophils to the site of transendothelial migration. Neutrophils are defective in sensing extravascular IL-8, due to decreased CXCR1/2 expression, and are frustrated in the vascular compartment. ANCA activate neutrophils during this process. Released ROS and proteolytic enzymes concentrated by NETs cause neutrophil necrosis and apoptosis, respectively. In addition, released PR3 stimulates ECs to produce MCP-1 and ICAM-1, further promoting leukocyte recruitment.
propylthiouracil-induced AAV.\textsuperscript{81} Investigations on the pathophysiological effects of AECA in AAV has largely been hampered by the fact that specific antigens of AECA have not been defined yet, although it has been shown that AECA might induce IL-1β production from ECs and cause EC apoptosis.\textsuperscript{88,89} It has also been shown that AECA stabilize neutrophil adhesion on ECs.\textsuperscript{90} Thus, AECA could play a role additive to ANCA and augment vascular damage, but this needs further investigation.

6. Modulation of neutrophil-endothelial interactions in vascular diseases

Intervention in neutrophil-endothelial interaction by modulating adhesion molecules, including selectins, integrins and chemokine receptors or their ligands, may protect ECs from ANCA-induced damage and present novel therapeutic strategies.

Chemical compounds, such as fucoidan and glycomimetics, baring similar structures as selectin ligands, have been shown to block selectin binding in vitro and demonstrated to be useful tools for inhibition of leukocyte migration in a variety of pulmonary diseases and injury models.\textsuperscript{91-93} Anti-CD18 therapies might be more promising in the treatment of AAV, because of the crucial role of β2-integrins in neutrophil adhesion, which is highlighted in the pathogenesis of AAV. Tools for specific blocking of β2-integrins are not yet available. Leumedins are a class of pharmaceuticals with low molecular weight, which have been shown to inhibit CD18-dependent neutrophil adhesion.\textsuperscript{94} The underlying mechanism, however, is not clear. NPC15669 is one of the well-characterized leumedins, which is effective in blocking pulmonary leukocyte recruitment during bacterial pneumonia, probably due to its ability to inhibit the leukocyte response to C5a and fMLP.\textsuperscript{95,96} Alpha-4 integrins are newly defined pathogenic factors in AAV, although a recombinant monoclonal antibody against α4-integrins, natalizumab, has already been used in clinical trials for the treatment of multiple sclerosis and Crohn’s disease.\textsuperscript{97,98} Administration of natalizumab in animal models of MPO-ANCA associated vasculitis may further clarify the role of α4-integrins in the pathogenesis of AAV and potentially lead to a novel therapy.

Repertaxin, an allosteric inhibitor of CXCR1 and CXCR2, has proved to be effective in inhibiting neutrophil infiltration and neutrophil-mediated tissue damage in many inflammatory diseases.\textsuperscript{99,100} However, persistent inflammation within the vessel wall in AAV suggests frustration of activated neutrophils within the intravascular compartment. Blocking IL-8 receptors, that means inhibition of neutrophil emigration, may worsen disease outcome. Indeed, in an animal model
of MPO-ANCA vasculitis, treatment with anti-CXCR1 or anti-CXCR2 antibodies did not improve hematuria, proteinuria or crescent formation, but induced further accumulation of neutrophils in glomeruli. These results confirm the relationship of dysregulation of chemokine receptors and neutrophil frustration in the microvasculature and suggest that blocking of circulating chemoattractants, other than chemokine receptors, may be effective in AAV therapy.

In summary, accumulating evidence points to neutrophil-endothelial interaction as a spatiotemporally crucial step for ANCA-induced EC damage in AAV. It is a complex process and involves many adhesion molecules, chemoattractants and their ligands, and immune modulators, which, on the other hand, offers plenty of opportunities for disease intervention.

References

NEUTROPHIL-ENDOTHELIAL INTERACTION

Decreased CXCR₁ and CXCR₂ expression on neutrophils in ANCA-associated vasculitides potentially increases neutrophil adhesion and impairs migration

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Abstract

Objectives: In anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV), persistent inflammation within the vessel wall suggests perturbed neutrophil trafficking leading to accumulation of activated neutrophils in the microvascular compartment. CXCR1 and CXCR2, being major chemokine receptors on neutrophils, are largely responsible for neutrophil recruitment. We speculate that down-regulated expression of CXCR1/2 retains neutrophils within the vessel wall and, consequently, leads to vessel damage.

Methods: Membrane expression of CXCR1/2 on neutrophils was assessed by flow cytometry. Serum levels of IL-8, TNF-α, angiopoietin 1 and angiopoietin 2 from quiescent and active AAV patients and healthy controls (HC) were quantified by ELISA. Adhesion and transendothelial migration of isolated neutrophils was analyzed using adhesion assays and Transwell systems, respectively.

Results: Expression of CXCR1 and CXCR2 on neutrophils was significantly decreased in AAV patients compared to HC. Levels of IL-8, which dose-dependently down-regulated CXCR1 and CXCR2 expression on neutrophils in vitro, were significantly increased in the serum of patients with active AAV and correlated negatively with CXCR1/CXCR2 expression on neutrophils, even in quiescent patients. Blocking CXCR1 and CXCR2 with repertaxin increased neutrophil adhesion and inhibited migration through a glomerular endothelial cell layer.

Conclusion: Expression of CXCR1 and CXCR2 is decreased in AAV, potentially induced by circulating IL-8. Down-regulation of these chemokine receptors could increase neutrophil adhesion and impair its migration through the glomerular endothelium, resulting in neutrophil accumulation and, consequently, persistent inflammation within the vessel wall.
Introduction
AAV comprises granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and Churg Strauss syndrome (CSS), which share a spectrum of clinical manifestations reflecting necrotizing damage to small- and medium-sized vessels. A role for neutrophils as effector cells in AAV is supported by a large body of evidence from in vitro and in vivo studies. After being primed by proinflammatory cytokines such as TNF-α, neutrophils can be activated by ANCA and release oxygen radicals and proteolytic enzymes, which have been shown to lyse endothelial cells in in vitro co-cultures. In vivo, neutrophil accumulation in glomeruli has been observed in the early phase of crescentic glomerulonephritis and neutrophil depletion could completely prevent disease development in experimental models.

Migration of neutrophils is largely regulated by the concentration gradient of CXC-chemokines which contain a glutamic acid-leucine-arginine (ELR) motif and are the most powerful chemoattractants for neutrophils. Interleukin 8 (IL-8) is the most potent member of the CXC family with high affinity for both of its receptors, CXCR1 and CXCR2, which are co-expressed on the membrane of neutrophils. Thus, binding of IL-8 to CXCR1/2 is a major element in neutrophil recruitment.

Neutrophils function in immune surveillance. Their activation, in terms of degranulation and oxidative burst, arms neutrophils with microbicidal activity, which normally appears only after they have migrated from the circulation and reach inflamed tissues. However, in the pathology of AAV, necrotizing damage within the vessel wall suggests that ANCA-mediated neutrophil activation is already triggered during neutrophil recruitment. This may be caused by impaired neutrophil trafficking retaining activated neutrophils within the microvascular compartment.

Although consensus is still lacking whether ANCA bind to neutrophils in suspension, it has been well demonstrated that membrane bound-PR3 on neutrophils is significantly upregulated during neutrophil adhesion, and full neutrophil activation not only requires ANCA-antigen cross-linking but also adherence of neutrophils. Here, we hypothesize that IL-8-CXCR1/CXCR2-mediated neutrophil recruitment is hampered in AAV, which leads to firm adhesion of neutrophils to the endothelium without further transmigration. Retained neutrophils within the microvascular compartment may be activated by ANCA, which will result in persistent inflammation of the vessel wall. To test this hypothesis, we investigated expression profiles of CXCR1 and CXCR2 on
neutrophils in patients with AAV, related expression to levels of chemoattractants, and analyzed effects on transmigration.

Materials and Methods
Patients and healthy controls
For measurement of CXCR1 and CXCR2 expression, 37 patients with quiescent AAV and 5 AAV patients with active disease were recruited from our out-patient clinic. Thirty healthy donors recruited from laboratory personnel were included as a normal control population. Demographic characteristics of patients and controls are summarized in Table 1.

A diagnosis of granulomatosis with polyangiitis (GPA), Churg Strauss syndrome (CSS) or microscopic polyangiitis (MPA) was based on the Chapel Hill definitions. ANCA specificity for PR3 or MPO was determined by capture ELISA. Patients in remission were receiving none or minimal treatment. Treatment of active patients is listed in Table 1. Disease severity was quantified using the Birmingham Vasculitis Activity Score (BVAS).

Sera from AAV patients and healthy volunteers were collected for cytokine profiling. For patients in remission, samples were drawn on the same day for CXCR1/2 measurement and cytokine testing. For patients who were in remission but had suffered from active disease before (n=18), serum samples from active disease were retrieved from a stored serum bank. All sera were stored at -80°C before testing.

All subjects gave their informed consent and the study was approved by the hospital medical ethical committee.

Antibodies used for flow cytometry
Peridin chlorophyll protein (PerCP)/Cy5.5-conjugated anti-CD14 (HCD14; Biolegend, San Diego, CA) was used to discriminate neutrophils from monocytes in whole blood staining. Phycoerythrin (PE)-conjugated anti-CD181 (CXCR1, 5A12; BD Bioscience, Alphen aan de Rijn, The Netherlands), allophycocyanin (APC)-conjugated anti-CD182 (CXCR2, 6C6; BD bioscience, Alphen aan de Rijn, The Netherlands) and fluorescein isothiocyanate (FITC)-conjugated anti-CD177 (NB1, MEM166; abCAM, Cambridge, UK) were used to detect membrane expression of CXCR1, CXCR2 and CD177, respectively. Irrelevant antibodies of the same isotype were used as negative controls.

Cell culture
Human conditionally immortalized glomerular endothelial cells (CiGENC)\textsuperscript{13} were cultured in endothelial growth medium 2-microvascular (EGM2-MV; Cambrex-Lonza, Breda, The Netherlands) containing 5\% fetal calf serum and growth factors, without vascular endothelial growth factor (VEGF), as supplement. CiGENC up to passage 40 were propagated at 33°C to keep cells in a proliferative

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* HC= healthy control; AAV= ANCA-associated vasculitides; GPA= granulomatosis with polyangiitis; MPA= microscopic polyangiitis; NCGN= necrotizing crescentic glomerulonephritis; PR3= proteinase 3; MPO= myeloperoxidase; ENT= ear, nose and throat; BVAS= Birmingham Vasculitis Activity Score; MMF= mycophenolate mofetil; CYC= cyclophosphamide. \textsuperscript{†}, Active patients included in CXCR1 and CXCR2 measurement by flow cytometry. \textsuperscript{‡}, Active patients included in cytokine profiling by ELISA.
state. Experiments were carried out on cells that had grown into a confluent monolayer and had been incubated for 5 days at 37°C in order to acquire a non-proliferative/quiescent phenotype.

**Neutrophil isolation and stimulation**

Neutrophils were isolated from peripheral blood according to routine procedures as described previously.\(^4\) Briefly, heparinized venous blood was centrifuged on Lymphoprep (Axis-Shield, Oslo, Norway). Contaminating erythrocytes were lysed with ice-cold ammonium chloride buffer. Afterwards, cells were washed with cold Hanks’ balanced salt solution (HBSS) without Ca\(^{2+}\)/Mg\(^{2+}\) (HBSS\(^{-/-}\)) and resuspended in HBSS with Ca\(^{2+}\)/Mg\(^{2+}\) (HBSS\(^{+/+}\); Gibco/Life Technologies, Breda, The Netherlands) to obtain \(10^7\) cells/ml.

Where indicated, cells were incubated with serial doses of interleukin-8 (IL-8; R&D Systems, Minneapolis, MN USA), angiopoietin-1 (ANGPT-1; R&D Systems, Minneapolis, MN USA), angiopoietin-2 (ANGPT-2; R&D Systems, Minneapolis, MN USA), recombinant human tumor necrosis factor-α (rhTNF-α; R&D Systems, Minneapolis, MN USA) or repertaxin (Sigma-Aldrich, USA) at 37°C for 30 min. Non-stimulated cells were incubated with control medium under the same condition.

**Membrane staining of neutrophils for flow cytometry**

Membrane expression of CXCR1, CXCR2 and CD\(_{177}\) on neutrophils was assessed by flow cytometry. According to the manufacturer’s instructions, 100 µl of whole blood from patients and controls or \(10^6\) of purified neutrophils after in vitro stimulation were incubated with antibodies at room temperature, in the dark, for 15 min, followed by fixation and lysing of erythrocytes with 20× volume of FACS lysis solution (BD Bioscience, San Jose, CA). Fluorescence intensity was measured by FACS Calibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and calibrated using CellQuest\textsuperscript{TM} software (Becton Dickinson). Results were analyzed using Win-List software package (Verity Software House, Topsham, ME). Mean fluorescence intensity (MFI) of \(5.10^4\) counted cells was measured in each sample. Neutrophils were gated as forward scatter\(^{\text{high}}\) (FSC\(^{\text{high}}\))-side scatter\(^{\text{high}}\) (SSC\(^{\text{high}}\))-CD\(_{14}\)\(^{\text{low}}\) cells. Expression levels were presented as MFI corrected for nonspecific binding of isotype control antibodies on neutrophils from the same donor.

**MPO activity assay**
Numbers of adherent or migrated neutrophils in adhesion or migration assay were quantified by myeloperoxidase (MPO) activity assay. Briefly, cells were lysed with 0.5% Triton-X-100 at 4°C for 20 min and acidified by adding 1M citrate buffer (pH=4.2, 50 µl/1 ml of cell lysate) just prior to the reaction. Aliquots (75 µl) from each sample were transferred into wells of a flat-bottomed 96-well plate. 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) at a concentration of 0.05% in 100 mM citrate with 0.03% H₂O₂ was loaded in an equal volume as substrate for MPO. After the reaction, OD₄₀₅ was measured using an ELISA plate reader. Numbers of neutrophils in each well was determined as based on standards generated from known concentrations of neutrophils from the same isolation.

Neutrophil adhesion
Neutrophil adhesion to endothelial monolayers was quantified by adhesion assay as described previously with modifications. CiGENCs were seeded in 24-well plates and allowed to proliferate to confluency. Cell monolayers were carefully washed with HBSS⁺/⁻ and stimulated with 10 ng/ml TNF-α at 37°C for 4 hrs. Cells were then washed three times before 0.5 × 10⁶ neutrophils were loaded. Where indicated, neutrophils were pretreated with serial doses of IL-8, TNF-α or repertaxin which is a noncompetitive allosteric inhibitor of CXCR1 and CXCR2. After incubation of endothelial cells with neutrophils at 37°C for 30 min, loosely adherent or non-adherent neutrophils were washed off with HBSS⁺/⁻ and the endothelial monolayers plus adherent neutrophils were lysed in 0.5% Triton-X-100. All experiments were run in duplicate. The numbers of neutrophils were quantified, as described above, by MPO activity assay.

Neutrophil migration
Transwell inserts (Croning, NY, USA), with 3.0 µm pores in 12-well plates, were precoated with CiGENCs. Confluence of the endothelial monolayers was determined by continued monitoring trans-endothelial electrical resistance (TEER) which plateaued at 20-25 Ω on the 5th day after seeding. Histological staining of the membrane showed that the formation of monolayers on inserts required 6 × 10⁴ cells and 5 days of culture. Therefore, all the Transwell inserts were coated with CiGENCs following this standard procedure. Isolated neutrophils from healthy donors were preincubated with repertaxin, IL-8 or TNF-α, and 1.10⁶ cells were loaded in the upper chamber. 10ng/ml of IL-8, which showed optimal efficiency as chemoattactant in titration in preliminary experiments (data not shown), was placed in the lower chamber. Wells without
IL-8 in the lower chamber or neutrophils without any treatment in the upper chamber were included as controls. All experiments were run in duplicates. After coincubation of neutrophils with the inserts at 37°C for 2 hrs, the plates with inserts within the wells were centrifuged for 5 min at 50g and 4°C to dislodge adherent neutrophils on the lower surface of the inserts. The cells in the lower chamber were collected and quantified by MPO activity assay.

**Serum levels of IL-8, TNF-α, angiopoietin-1 (ANGPT-1) and angiopoietin-2 (ANGPT-2)**

Sera collected from AAV patients in remission (n=37), patients with active disease (n=18) and healthy blood donors (n=30) were used for measurement of IL-8, TNF-α, ANGPT-1 and ANGPT-2. IL-8 and TNF-α levels were measured by ELISA (R&D Systems, Minneapolis, MN) as described previously. After sample incubation and binding of the biotinylated detecting antibodies, color reaction was performed with streptavidin-poly-HRP (Sanquin, Amsterdam, the Netherlands) and tetramethyl-benzidin (TMB, Roth, Karlsruhe, Germany). Levels of angiopoietin-1 and angiopoietin-2 in sera were measured using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Normal levels of these cytokines in serum were defined as the mean±2SD of healthy controls.

**Statistical analysis**

Results of CXCR1 and CXCR2 expression are presented as medians. Serum levels of IL-8, TNF-α, ANGPT-1 and ANGPT-2 and membrane expression of adhesion molecules are presented as means. Data were analyzed by Mann-Whitney test and Spearman’s rank correlation test using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA). P values less than 0.05 were considered significant.

**Results**

**Expression of CXCR1 and CXCR2 on the neutrophil membrane is decreased in AAV**

We first evaluated expression levels of CXCR1 and CXCR2 on neutrophils in AAV patients (remission, n=37; active, n=5) in comparison with healthy blood donors (n=30). Both receptors were highly expressed on the membrane of neutrophils. Neutrophils from patients in remission (median 1153, range 640–1986) showed lower, although not statistically significant, expression of CXCR1 than those from HC (1163, 790–1790, p=0.3), while neutrophil CXCR1 levels were significantly decreased in patients with active disease (533, 416–1071) compared to either HC
(p=0.003) or patients in remission (p=0.0023) (Figure 1A). CXCR2 expression was significantly down-regulated in AAV patients in remission (453, 201–817) compared to HC (589, 290–941, p=0.0004) and further decreased in active patients (323, 242–564) as compared to HC (p=0.0086) (Figure 1B). A significant correlation was observed between the expression of CXCR1 and CXCR2 on neutrophils of AAV patients, regardless of disease activity (Figure 1C, spearman r=0.87, p<0.0001).

Figure 1. Down-regulated expression of CXCR1 and CXCR2 on neutrophils in AAV.

Levels of CXCR1 (A) and CXCR2 (B) expression on the membrane of neutrophils were measured by flowcytometry and compared between AAV patients in remission (n=37), AAV patients with active disease (n=5) and healthy controls (HC, n=30). Expression levels are presented as MFI corrected for nonspecific binding of isotype control antibodies on neutrophils. Horizontal lines represent medians. * , p<0.05; **, p<0.01 ***, p<0.001. Correlation between CXCR1 and CXCR2 expression (C) on neutrophils was analyzed in patients in remission and with active disease (n=42, Spearman r=0.87, p<0.0001). Dotted line represents the best-fit correlation between CXCR1 and CXCR2 expression.
IL-8 and TNF-α down-regulate CXCR1/CXCR2 from the neutrophil membrane

IL-8 and TNF-α are proinflammatory cytokines reported to be elevated in the serum of AAV patients. Serum levels of ANGPT-2 have also recently been reported to be increased in AAV and to correlate with disease activity. IL-8 is also produced by endothelial cells stimulated with ANGPT-1. Therefore, we tested the influences of these cytokines on CXCR1 and CXCR2 expression. As a result, both IL-8 and TNF-α down-regulated CXCR1 and CXCR2 expression on isolated neutrophils in a dose-dependent manner. IL-8, at a concentration of 5ng/ml, reduced CXCR2 levels to 61% compared to control incubation with normal medium, while IL-8 down-regulated membrane expression of CXCR1 to 71% at 20ng/ml (Figure 2A). TNF-α significantly reduced CXCR1/CXCR2 expression already at a dose of 1ng/ml (Figure 2B). When tested in parallel,
Levels of IL-8 (A), ANGPT-1 (B) and ANGPT-2 (C) in sera of AAV patients in remission \( (n=37) \) and active disease \( (n=18) \) were measured by ELISA and compared to HC \( (n=30) \). Results are presented as the concentration of each cytokine in serum. Bars denote medians, *, \( p<0.05 \); ***, \( p<0.001 \).

CD\textsubscript{177} (NB\textsubscript{1}) expression was slightly increased by IL-8 and significantly up-regulated by TNF-\( \alpha \), as reported in earlier studies.\textsuperscript{14-24} ANGPT-1 and ANGPT-2 did not affect CXCR\textsubscript{1}/CXCR\textsubscript{2} or CD\textsubscript{177} expression on isolated neutrophils (Figure 2C and 2D).

**Serum levels of IL-8, TNF-\( \alpha \), ANGPT-1 and ANGPT-2 in AAV**

Levels of the cytokines mentioned above, which may have direct or indirect effects on CXCR\textsubscript{1}/CXCR\textsubscript{2} expression, were evaluated in our patient cohort in comparison with HC.

Defining the normal range as mean\( \pm \)2SD of the IL-8 concentration in 30 healthy controls, 5 out of 37 (14\%) patients in remission and 10 out of 18 (56\%) active patients showed increased IL-8 levels. Levels of IL-8 were not significantly different between patients with quiescent AAV (median 2.81 pg/ml, range 0.0~19.64 pg/ml) and healthy controls (2.30 pg/ml, 0~15.43 pg/ml). However, a negative correlation was present between CXCR\textsubscript{1}/CXCR\textsubscript{2} expression on neutrophils and levels of IL-8 in patients in remission (Figure 4). A negative correlation was not observed between IL-8 and CXCR\textsubscript{1}/2 in active AAV, probably due to the small sample size. Nevertheless, in concert with further decreased
Figure 4. CXCR1/CXCR2 expression correlates with IL-8 levels in quiescent AAV.
Expression of CXCR1, CXCR2 and levels of IL-8 were measured in parallel in patients in remission (n=37). Membrane expression of CXCR1 and CXCR2 is presented as MFI corrected for nonspecific binding of isotype control antibodies on neutrophils. IL-8 levels are presented as concentration in serum. A. Correlation between CXCR1 expression on neutrophils and serum levels of IL-8 (Spearman r=-0.34, p=0.04). B. Correlation between CXCR2 expression on neutrophils and serum levels of IL-8 (Spearman r=-0.41, p=0.01). Dotted lines represent the best-fit correlation between expression of CXCR1/2 and IL-8 levels.

CXCR1/2 expression, IL-8 levels increased remarkably during active disease (8.67 pg/ml, 3.96~51.55 pg/ml), and were significantly higher than levels in HC and patients in remission (Figure 3A). These data suggest that IL-8 in the circulation, also during remission, may be responsible for decreased expression of CXCR1/CXCR2 on the membrane of neutrophils in AAV.
As for levels of ANGPT-1, no significant difference was observed between HC and patients in remission. The median level of ANGPT-1 in active patients was lower than in HC (Figure 3B). Levels of ANGPT-2 were not increased in patients in remission nor in patients with active disease (Figure 3C). TNF-α was not detectable in sera of either HC or patient groups.

Neutrophil recruitment after CXCR1/CXCR2 blockade
To further clarify possible consequences of CXCR1 and CXCR2 down-regulation on neutrophils, we investigated neutrophil adhesion and transmigration through a glomerular endothelial monolayer. Neutrophils were pretreated with repertaxin, mimicking decreased efficiency of IL-8 receptors, or stimulated with IL-8/TNF-α, both of which lead to decline of CXCR1 and CXCR2 expression.
Glomerular endothelial cell layers were stimulated with 10 ng/ml of TNF-α for 4 hours prior to the adhesion or migration assay to mimic activated glomerular endothelial cells as occurs in AAV. Preincubation with repertaxin inhibited neutrophil migration in a dose-dependent way. 100nM of repertaxin reduced migration to 76%, significantly lower than control incubated neutrophils. IL-8 preincubation reduced the number of migrated neutrophils to 39%. Although
incubation with TNF-α resulted in a significantly reduced expression of CXCR1/CXCR2 on the membrane of neutrophils, migration was not significantly influenced, and numbers of migrated neutrophils were comparable with control incubation (Figure 5B). Neutrophils preincubated with 2 ng/ml TNF-α showed enhanced adhesion to glomerular endothelium compared to control incubation. IL-8 (up to 20 ng/ml), however, did not show a remarkable effect on neutrophil adhesion. Blocking

Figure 5. CXCR1/CXCR2 blockade enhances neutrophil adhesion and inhibits neutrophil transmigration through an endothelial cell monolayer.
Isolated neutrophils were pre-incubated with serial doses of repertaxin, IL-8 (10 ng/ml) or TNFα (2 ng/ml) before being loaded onto a glomerular endothelial monolayer or a transwell insert. After co-incubation of pretreated neutrophils with glomerular endothelial cells for 30 min for the adhesion assay (A, n=5) or with transwell inserts coated with endothelial cells for 2 hrs (B, n=6), the number of adherent or migrated neutrophils were quantified by MPO activity assay. Results are presented as the percentages of adherent or migrated neutrophils after stimulation compared to control incubation. *, p<0.05

CXCR1 and CXCR2 with repertaxin also increased the number of adherent neutrophils dose-dependently, from 135% with 10 nM of repertaxin up till more than 300% at 0.5 μM as compared to cells without stimulation (Figure 5A).

Discussion
This is the first study, to our knowledge, reporting decreased expression of CXCR1 and CXCR2 on neutrophils in AAV, potentially induced by IL-8. Functional deficiency of CXCR1/CXCR2, induced by specific inhibition of these receptors with repertaxin, increased neutrophil adhesion and impaired migration through a glomerular endothelial monolayer. Down-regulation of CXCR1 and CXCR2 may retain activated neutrophils within the vessel wall, allowing interaction with circulating ANCA, so leading to vascular damage in situ. These findings highlight a chemokine-receptor-based mechanism of inflammation confined within the vessel wall and would further explain the role of neutrophils in the pathogenesis of AAV.
CXCR1 and CXCR2 are not stably expressed on the neutrophil membrane. During chemotaxis, ligand-mediated internalization and recycling of these chemokine receptors is proposed as a self-limiting mechanism allowing neutrophils, by desensitization to their ligands, to retain in situ, once they reach their targets.\(^{25}\) However, this physiological process is probably abused in AAV, and down-regulated CXCR1/CXCR2 expression may desensitize circulating neutrophils to chemotactic signals before they migrate into the tissue resulting in accumulation of neutrophils within the vessel wall.

Our in vitro experiments confirmed this hypothesis by showing significantly increased adhesion and decreased migration of neutrophils through glomerular endothelium after CXCR1 and CXCR2 blockade. This is in line with our previous observation that, in a model of MPO-ANCA-associated vasculitis, repertaxin treated mice were not protected from haematuria, albuminuria or glomerular crescent formation. Instead, we observed a larger number of neutrophils accumulating in glomeruli in comparison to untreated mice.\(^{26}\) Besides, CXCR1/CXCR2-knockout mice showed deficiency in neutrophil migration and bacterial clearance in a urinary tract infection model, and decline in CXCR1/CXCR2 expression may worsen outcome of infections due to impaired neutrophil recruitment.\(^{27-31}\)

Notably, down-regulation of CXCR1 and CXCR2 from the neutrophil surface may not be specific for AAV. As IL-8 is a potential inducer of decreased CXCR1/2 expression, increased levels of circulating IL-8 in other clinical conditions may also result in a similar decline of CXCR1/2 expression. In addition, our results may reflect a state of neutrophil activation mediated by IL-8 or other proinflammatory cytokines, such as TNFα, as has been described in AAV by Muller Kobold et al. previously.\(^{32}\) In the presence of ANCA, however, pre-activated neutrophils lacking CXCR1/2 expression can be confined in the microvasculature and fully activated by ANCA.

The role of CXCR1 and CXCR2 in neutrophil adhesion has not been clearly delineated. Blocking of IL-8-receptors showed an inhibitory effect on IL-8-induced neutrophil adhesion in an early study.\(^{33}\) The underlying mechanisms are not fully elucidated so far. A recent study conducted by Cohen-Hillel et al. showed that migratory desensitization triggers over-activation of focal adhesion kinase (FAK), which leads to increased neutrophil adhesion.\(^{34}\) The latter mechanism is likely operative in AAV, where neutrophils are migratory desensitized under continuous exposure to low-dose IL-8.\(^{20}\) Besides, in our experiments, neutrophils pretreated with repertaxin did show significantly increased adhesion on an activated CiGENC monolayer. As endothelial cells might
also be actively involved in neutrophil-endothelial interaction during inflammation, this adhesive effect may also depend on endothelial phenotypes and microenvironment, explaining why Coelho et al. observed short-term suppression of neutrophil adhesion to synovial endothelium after repertaxin treatment in a mouse model of arthritis.\textsuperscript{35} Glomerular endothelial cells (ECs) have unique features and differ from synovial ECs in many ways, such as expression profile of adhesion molecules.\textsuperscript{36} Besides, neutrophil recruitment in an in vitro model of rheumatoid arthritis only occurs in co-cultures of ECs with synovial fibroblasts but not with control tissues,\textsuperscript{37} suggesting that immune regulators in the microenvironment also play an important role in this process.

Since CXCR1 and CXCR2 are internalized upon ligation and IL-8 has been shown to be significantly elevated in AAV patients,\textsuperscript{20} we speculate that IL-8 is one of the factors inducing decreased expression of these receptors. Indeed, we found that CXCR1 and CXCR2 expression was remarkably lower in patients with active AAV than in HC. Decreased expression was accompanied by significantly elevated serum levels of IL-8 in active AAV. Moreover, a negative correlation was observed between CXCR1/CXCR2 expression and serum IL-8 levels in patients with quiescent disease. As an indirect evidence of this relationship, a negative effect of serum IL-8 on neutrophil recruitment has been demonstrated in earlier studies, which was supposed to be caused by down-regulation of IL-8 receptors.\textsuperscript{38,39} Cockwell et al. proposed a similar scenario in AAV by showing that ANCA-activated neutrophils produce IL-8 and that neutrophils accumulate locally in the glomerular capillary loops but poorly penetrate into IL-8 enriched tissues as observed in renal biopsies of AAV patients.\textsuperscript{40} From the data of the present study, a relationship is suggested between circulating IL-8 levels, decrease in CXCR1/CXCR2 expression and neutrophil accumulation in the microvasculature in AAV.

Circulating IL-8 can be produced by ANCA-activated neutrophils and endothelial cells stimulated by IL-1, TNF-α or PR3-ANCA.\textsuperscript{40-42} Although circulating IL-8 levels were not significantly elevated in patients during remission, it should be noted that a low-level of immune activation is present during quiescent disease as well, including minor increase in IL-8 levels.\textsuperscript{43} CXCR1 and CXCR2 expression can also be regulated through enzymatic cleavage via metalloproteinases,\textsuperscript{44} by other immunomodulators such as TNF-α which have been detected at inflammatory sites in AAV and prime neutrophils for ANCA-induced activation.\textsuperscript{20,45-47} Technical limitations to measure TNFα in serum could have hampered to reveal the relationship between TNFα levels and CXCR1/2 expression. As shown in the current study, TNF-α, up till a concentration of 20
ng/ml, was more efficient than IL-8 in down-regulating CXCR1/CXCR2 in vitro. Therefore, although TNF-α could not be detected in sera, there is still the possibility that increased levels of TNF-α, especially during active disease, lead to a decrease in CXCR1 and CXCR2 expression.

It needs to be mentioned that the number of patients with active AAV included in the current study was relatively small, so not allowing firm conclusions as to the correlation between neutrophil CXCR1 and CXCR2 expression and disease activity. Such a correlation is, however, suggested by the further decrease of CXCR1 and CXCR2 expression that we observed during active disease. Furthermore, immunohistochemical studies are needed to show lack of CXCR1 and CXCR2 expression on neutrophils recruited to the vascular wall in AAV. Such data could further strengthen the role of CXCR1 and CXCR2 down-regulation in AAV.

In conclusion, expression of CXCR1 and CXCR2 on neutrophils is down-regulated in AAV patients, potentially induced by circulating IL-8. Decreased expression of these chemokine receptors may enhance neutrophil adhesion and impede neutrophil migration through activated glomerular endothelium, which may account for neutrophil accumulation and persistent ANCA-induced inflammation in the vessel wall.

References


Autoantibodies against glomerular endothelial cells in ANCA-associated systemic vasculitis

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Coen A Stegeman
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Abstract
Objectives: The prevalence of anti-endothelial cell autoantibodies (AECA) in patients with ANCA-associated systemic vasculitis (AAV) has been reported by several groups with conflicting results ranging from 8% to 100%. Types of substrate cells used for AECA testing partially explain this variation. Endothelial cells from kidney origin have been reported to be predominant in AECA binding. Therefore, we investigated AECA prevalence using a human glomerular endothelial cell (GENC) line compared to primary human umbilical vein endothelial cells (HUVEC) which have frequently been used for AECA detection.

Methods: Sera from 43 AAV patients (29 WG, 14 MPA) with active disease were assessed for AECA positivity using cell-based ELISA. Forty serum samples from healthy controls were tested in parallel. To evaluate endothelial activation levels, soluble intercellular cell adhesion molecule 1 (sICAM-1) and vascular cell adhesion molecule 1 (sVCAM-1) were measured with capture ELISA.

Results: AECA were detected in 4 of 29 WG patients (14%) but none of 14 MPA patients were positive for AECA using GENC as a substrate, while AECA were positive in 10% of WG patients and 14% of MPA patients on HUVEC. No significant differences were found between AAV patients and controls in AECA test. Serum levels of sVCAM-1 and sICAM-1 in AAV patients were significantly higher than in controls. However, there were no differences between AECA positive and negative patients for both of the activation markers.

Conclusion: AECA, directed against glomerular endothelial cells, have a low prevalence in AAV patients with active disease and are not correlated with endothelial activation.
Introduction
ANCA-associated systemic vasculitides (AAV) are a group of vascular disorders consisting of Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) which share many clinical manifestations, including pulmonary vasculitis, pauci-immune focal necrotizing and/or crescentic glomerulonephritis, and presence of anti-neutrophil cytoplasmic autoantibodies (ANCAs) in the circulation.\textsuperscript{1,2} ANCA are important diagnostic markers for AAV, and the pathogenic role of ANCA has been demonstrated by in vitro and in vivo studies.\textsuperscript{3-5} However, the involvement of blood vessel inflammation in these diseases also evoked interest into the role of anti-endothelial autoantibodies (AECA) in the pathophysiologic process. The prevalence of AECA in AAV has been reported by several groups with conflicting data ranging from 8\% to 100\% of AAV patients.\textsuperscript{6-11} In a study of Holmen et al., increased binding of AECA to endothelial cells isolated from nose, kidney and lungs, which are the most frequently involved organs in AAV, was demonstrated.\textsuperscript{9} These results suggest that AECA in AAV patients are organ-specific and could imply that, as substrates, endothelial cells from relevant organs should be used in AECA detection. Therefore we investigated the prevalence of AECA in ANCA-associated vasculitis using a human glomerular endothelial cell (GEnC) line in comparison with primary human umbilical vein endothelial cells (HUVEC) which are frequently used in AECA detection. Further, since AECA may induce endothelial activation,\textsuperscript{12,13} serum levels of adhesion molecules, as markers of endothelial activation, were also analyzed in this study.

Methods
Sera of patients and healthy controls
A diagnosis of WG or MPA was established when the patients fulfilled the Chapel Hill definitions for WG or MPA.\textsuperscript{14} Serum samples were collected at the time of diagnosis, before immunosuppressive therapy started, and stored at -80°C until AECA testing. Sera of 43 patients (29 WG/14 MPA) with active disease diagnosed between 2005 and 2007 were selected randomly and included in this study. All the 43 serum samples were tested for AECA and endothelial activation markers. The sera of 40 healthy volunteers, age and sex matched, were tested as controls. More information of patients and controls is given in Table 1.
Table 1. Characteristics of patients and controls

<table>
<thead>
<tr>
<th></th>
<th>AAV patients</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WG</td>
<td>MPA</td>
</tr>
<tr>
<td><strong>Total number, n</strong></td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td><strong>Gender, male (%)</strong></td>
<td>17 (59)</td>
<td>10 (71)</td>
</tr>
<tr>
<td><strong>Age, median (range)</strong></td>
<td>62 (34-84)</td>
<td>70 (40-84)</td>
</tr>
<tr>
<td><strong>ANCA-specificity, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR3</td>
<td>24 (83)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>MPO</td>
<td>2 (7)</td>
<td>12 (86)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (7)</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td><strong>BVAS at diagnosis, median (range)</strong></td>
<td>22 (7-33)</td>
<td>18 (9-27)</td>
</tr>
<tr>
<td><strong>Organ involvement at diagnosis, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENT</td>
<td>29 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>16 (55)</td>
<td>8 (57)</td>
</tr>
<tr>
<td>Kidney</td>
<td>19 (66)</td>
<td>12 (86)</td>
</tr>
<tr>
<td>Skin</td>
<td>6 (21)</td>
<td>4 (29)</td>
</tr>
<tr>
<td>Nervous system</td>
<td>11 (38)</td>
<td>6 (43)</td>
</tr>
</tbody>
</table>


Cell culture
CiGenC The conditionally immortalized human glomerular endothelial cell (CiGenC) line (provided by the Academic Renal Unit, University of Bristol, UK) was used for AECA detection. Tumor antigen transfection and characteristics of the cell line have been described.\textsuperscript{5} CiGenC were seeded on 96-well micro titer plates (Costar, Corning Inc., Badhoevedorp, The Netherlands) and cultured in endothelial growth medium 2–microvascular (EGM2-MV, Cambrex, Wokingham, UK), containing growth factors and supplements provided by the supplier, including human fibroblast growth factor (hFGF), human recombinant long-insulin-like growth factor-1 (R3-IGF), human epidermal growth factor (hEGF), gentamicin and amphotericin (GA-1000), hydrocortisone, heparin, ascorbic acid and 5% fetal bovine serum (FBS), at 33°C. At the permissive temperature, the cells were allowed to proliferate for about 48 hours, until confluence. Afterwards, the plates were transferred to 37°C where the transgene was inactivated, rendering cells non-proliferative and quiescent. At the non-permissive temperature cells were cultured for another 5 days before experiments. To investigate the influence of endothelial cell stimulation on AECA binding, some of the GEnC were
incubated with 10 ng/ml tumor necrosis factor alpha (TNF-α; R&D systems, Abingdon, UK) for 4 hours prior to AECA detection.

HUVEC The human umbilical vein endothelial cells (HUVEC), isolated as described, were also used as substrate for AECA detection for comparison. Second passage HUVEC cells were cultured in gelatin pre-coated 96-well micro titer plates with RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) containing 10% FCS at 37°C. Cells were cultured for about 2 days until confluence.

AECA detection

AECA in serum samples were measured using a cell-based enzyme-linked immunosorbent assay (ELISA) with unfixed CiGEnC and HUVEC as described previously. Briefly, endothelial cell coated plates were washed with Hanks balanced salt solution (HBSS; GIBCO, Life Technologies, Breda, The Netherlands). Non-specific binding was inhibited by incubating cells with blocking buffer (HBSS/0.5% BSA) for 60 min at 37°C. After additional washing, cells were exposed to the samples 1:20 diluted in blocking buffer. After incubation at room temperature for 1 hr, cells were washed again and incubated with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma, Zwijndrecht, The Netherlands) for 1 hr at room temperature followed by 3 washes. The substrate p-nitrophenylphosphate disodium (Sigma, Zwijndrecht, The Netherlands) was added to obtain proper color reaction. After 20 min, optical density (OD) was scanned at 405 nm. In each experiment, CD31 was measured as a positive control. Samples were run in duplicate and the absolute OD values were obtained by subtracting the mean OD readings of blank wells. IgG class of AECA were considered positive when levels exceeded the mean±1SD value of samples from healthy controls (n=40).

Evaluation of endothelial activation

As endothelial activation markers, serum levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) (R&D Systems, Abingdon, UK) and soluble intercellular adhesion molecule-1 (sICAM-1) (R&D Systems, Abingdon, UK) were measured by capture ELISA according to the manufacture’s instructions. The results were expressed as the concentration calculated from a standard curve.

Statistical analysis

Values of AECA titers (OD) and concentrations of sVCAM-1 and sICAM-1 were expressed as medians and analyzed for statistical differences by the two-tailed Mann-Whitney U test. Probability values of <0.05 were considered significant.
Table 2. Prevalence of AECA in AAV detected on GEnC and HUVEC

<table>
<thead>
<tr>
<th></th>
<th>HC (n=40)</th>
<th>All patients (n=43)</th>
<th>WG (n=29)</th>
<th>MPA (n=14)</th>
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<tbody>
<tr>
<td>AECA-prevalence n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CiGEnC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean+1SD †</td>
<td>3 (8)</td>
<td>4 (9)</td>
<td>4 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Mean+2SD †</td>
<td>2 (5)</td>
<td>1 (2)</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td>HUVEC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean+1SD †</td>
<td>8 (20)</td>
<td>5 (12)</td>
<td>3 (10)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Mean+2SD †</td>
<td>1 (3)</td>
<td>1 (2)</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td>sICAM</td>
<td>median (range)</td>
<td>111 (8-252)</td>
<td>214 (72-438)</td>
<td>214 (72-401)</td>
</tr>
<tr>
<td>sVCAM</td>
<td>median (range)</td>
<td>296 (102-494)</td>
<td>753 (413-2138)</td>
<td>630 (413-1322)</td>
</tr>
</tbody>
</table>

†: Values over Mean+1 standard deviation (SD) of 40 healthy controls’ results were considered positive,
‡: Values over Mean+2 SD of 40 healthy controls’ results were considered positive. sICAM: concentrations of soluble intercellular adhesion molecule in sera, sVCAM: concentrations of soluble vascular cell adhesion molecule in sera.

Results

AECA were detected in 4 of the 29 WG patients (14%) but not in the MPA patients when GEnC were used as substrate, while 10% of sera from WG patients and 14% from MPA patients tested positive using HUVEC-based ELISA (summarized in Table 2). Pre-stimulation of the endothelial cells with TNF-α did not show obvious influence on AECA binding (data not shown). Although a few patients were positive for AECA defined as exceeding mean+1SD value of samples from healthy controls, there was no significant difference between AAV patients and controls in AECA titer when GEnC were used as substrate and levels in patients were even lower than controls with HUVEC as substrate (Figure 1).

Serum levels of sVCAM-1 and sICAM-1 were measured to evaluate in vivo endothelial activation. AAV patients with active disease had increased serum levels of sVCAM-1 and sICAM-1 when compared with healthy controls. AECA positive patients did not show higher levels of endothelial activation markers than AECA negative patients (Figure 2). No correlation could be found between levels of sVCAM-1/sICAM-1 and AECA titers.
Figure 1. AECA titers (OD) in ASV patients (n=43) and healthy controls (n=40) were detected on GEnC and HUVEC. Results are expressed as absolute OD values, by subtracting readings of blank wells. Lines denote the medians which were analyzed by two-tailed Mann-Whitney U test for statistical differences.*: p<0.05, ns: not significant.

![AECA titers graph](image)

Figure 2. Elevated levels of endothelial activation markers in ASV patients compared to healthy controls. Serum levels of sICAM-1 (A) and sVCAM-1 (B) were compared between controls (n=40) and ASV patients (n=43), or between AECA negative (n=38) and AECA positive (n=4) patients. Results were expressed as the concentration (ng/ml) calculated from a standard curve. Lines denote the medians which were analyzed by two-tailed Mann-Whitney U test for statistical differences. ***: p<0.0001, ns: not significant.

![sICAM-1 and sVCAM-1 levels graph](image)

**Discussion**

AECA have been studied for more than three decades since the first detection in human serum. Diversity of techniques were used for testing AECA, such as immunofluorescence, radioimmunoassay, fluorescence-activated cell sorting, and immunoblotting. The cell-based ELISA, which was used in our study, is a reference approach for AECA detection and has been used in most AECA screening studies recently. However, even using the same method, different groups reported conflicting AECA prevalence in AAV patients, which varied from 8% to 100%. This difference could be partially explained by the use of fixation of cells. In some early studies, cultured HUVEC were fixed before being incubated with serum samples, to avoid non-specific immunoglobulin binding and loss of
cells. Fixation, on the other hand, makes cells permeable and, at least in part, AECA positivity could be due to binding to intracellular compounds. Another reason for the variation could be the difference in endothelial cells used as substrate in the AECA test. AAV is a group of diseases characterized by necrotizing damage of small-sized vessels.\textsuperscript{1,2} Holmen et al. showed in their study that AECA binding was, to some extent, organ specific, by isolating endothelial cells from kidney, nose or lung in order to use these cells as a substrate for AECA detection. AECA bound differentially to these substrates suggesting a differential pathologic role of AECA in WG.\textsuperscript{9,23} Therefore, in comparison with HUVEC, which is considered macrovascular EC, renal microvascular EC (such as GEnC) could be a better candidate for AECA detection in AAV. However, our data did not show a high prevalence of AECA which was reported to be 71% in Holmen’s study using human kidney endothelial cells.\textsuperscript{9} To our knowledge, no specific antigens have been demonstrated for AECA, and AECA recognize a wide rage of autoantigens such as constitutively expressed or cytokine-induced cryptic antigens, adhesion molecules and extracellular matrix components.\textsuperscript{24} In Holmen’s study, the endothelial cells that they used were isolated from whole kidney, instead of glomeruli, which may display a broader spectrum of antigens for AECA binding resulting in a higher percentage of positive results. Moreover, they used flow cytometry, other than cell-based ELISA, to measure AECA which includes different ways for cell preparation and different methods to set the cut-off value for a positive result, making the results of both methods incomparable.

The pathogenic role of AECA in AAV is controversial. It has been suggested that, in patients with WG or MPA, AECA cause EC damage through antibody-dependent cell-mediated cytotoxicity (ADCC). In the same way, ANCA could play a central role in case they recognize planted or in situ expressed proteinase 3 (PR3) or myeloperoxidase (MPO) on the EC surface, resulting in ADCC of endothelial cells.\textsuperscript{30,25} Expression of PR3 on EC has been shown by Mayet et al., but their results have not been confirmed by other groups.\textsuperscript{36} Ballieux et al. even denied a prominent role of ANCA mediated ADCC of EC in WG pathogenesis by showing that cultured HUVEC, pre-incubated with PR3 or MPO, followed by ANCA or rabbit anti-PR3 or anti-MPO, were not susceptible to ADCC.\textsuperscript{27} Triggering of EC activation was otherwise considered as a pathogenic effect of AECA in AAV. Some in vitro studies showed that AECA induce production of IL-1\textbeta, IL-6, IL-8 and monocyte chemotactic factor-1, and activated EC increase leukocyte adhesion by up-regulating the expression of adhesion molecules.\textsuperscript{12,13} Our data showed increased levels of sVCAM-1 and sICAM-1 in the circulation of AAV patients with active disease, however, levels of these adhesion molecules did not correlate with
AECA titers and there were no significant differences between AECA positive and negative patients with regard to endothelial activation markers. These results suggest that either AECA detected with the current methods are not pathogenic, or the presence of AECA is not the only factor influencing endothelial activation. In conclusion, AECA against glomerular endothelial cells have a low prevalence in AAV patients with active disease and are not correlated with endothelial activation. It could be helpful to use more than one type of substrate cells for AECA testing in order to increase their detection rate. However, elucidating the antigens of AECA in AAV is a prerequisite for further assessing their diagnostic and pathogenetic role.

References
Membrane-bound Proteinase 3 and its receptors
relevance for the pathogenesis of Wegener’s Granulomatosis

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Abstract

Wegener's Granulomatosis (WG) is a life-threatening autoimmune disease. A pathogenic role for anti-neutrophil cytoplasmic autoantibodies (ANCAs) by inducing necrotizing damage to the vessel wall has been strongly suggested by in vitro and in vivo experimental data. Proteinase 3 (PR3), a serine protease mainly stored in the azurophilic granules of neutrophils, has been identified as a major ANCA-antigen in WG. Elevated expression levels of membrane-bound PR3 (mPR3) has been observed in WG and some other chronic inflammatory diseases, suggesting a pathogenic role of mPR3 by allowing interaction with PR3-ANCA. Recent studies revealed CD177 as a receptor for mPR3 on the neutrophil membrane. However, we recently showed that CD177 negative neutrophils also express mPR3 and are susceptible to PR3-ANCA induced neutrophil activation. Therefore, it is of interest to further investigate the functional consequences of binding of mPR3 to CD177, to explore other binding partners for mPR3 on the neutrophil membrane, and to study the relevance of colocalization of these molecules for disease pathogenesis. This review gives updated information on the mechanism of mPR3 expression and the relevance of colocalization of mPR3 with other molecules on the neutrophil membrane for the pathophysiological events occurring in WG.
Introduction
Proteinase 3 (PR3) is a neutrophil-derived serine protease, homologous to leukocyte elastase (HLE), cathepsin G (CG) and inactive azurocidin. PR3 is mainly stored in azurophilic (primary) granules, and, to a lesser extent, in specific (secondary) granules and secretory vesicles, and differentially expressed on the plasma membrane of neutrophils.¹² These serine proteases share similar structural elements and biological functions, but PR3 is unique in many aspects. PR3, together with myeloperoxidase (MPO), are major autoantigens of anti-neutrophil cytoplasmic autoantibodies (ANCAs), and ANCA directed to PR3 (PR3-ANCA) have been detected in more than 70% of patients with Wegener’s Granulomatosis (WG).³
WG is a life-threatening disease characterized by granuloma formation in the upper/lower airways, glomerulonephritis and necrotizing small-vessel vasculitis.⁴⁵ Although an animal-model for PR3-ANCA associated vasculitis is not available, the pathogenic role of PR3-ANCA has been well established in vitro. The central mechanism of vessel-damage starts with ANCA-binding to their antigens expressed on the surface of cytokine-primed neutrophils resulting in neutrophil activation, in terms of neutrophil degranulation and oxidative burst. Released proteolytic enzymes and reactive oxygen species further cause necrotizing damage to the vessel wall. Therefore, presence of membrane-bound PR3 (mPR3) is a prerequisite for ANCA-binding and ANCA-mediated vessel damage.³⁵ Deficiency of the PiZ-allele of α-antitrypsin, which is the endogenous inhibitor of PR3, is associated with WG, stressing the pathogenic role of PR3 in the development of WG.⁶ This article reviews current knowledge on the mechanism of PR3 membrane-binding and the relevance of colocalization of PR3 with other molecules on the neutrophil membrane for the pathophysiological events occurring in WG.

PR3 expression in neutrophils
Even though large amounts of PR3 are stored in the granules and vesicles of neutrophils, low expression of PR3 can be detected as well on the membrane of isolated neutrophils. Priming of neutrophils with low-dose of TNF-α, which brings neutrophils to a preactivated state, may translocate PR3 to the plasma membrane and raise the expression level up to two- to three- folds of that on resting neutrophils.⁷ IL-8, TGF-β and GM-CSF have also been reported to upregulate mPR3 expression on neutrophils.⁸⁹ Interestingly, mPR3 is not uniformly expressed on the whole population of neutrophils from most individuals. Primed neutrophils can be divided into two
subsets according to the amount of mPR3 expressed on their membrane. One subset shows a rather low level of mPR3 expression and the other subset expresses a substantial amount of mPR3, indicating a bimodal-pattern of membrane staining for PR3 by flowcytometry.¹ This phenomenon is seldom seen for membrane proteins of neutrophils, and has not been found for other family members of neutrophil serine proteases. The percentage of mPR3high expressing neutrophils, namely the size of the mPR3high subset, ranges from 0% to 100% of the total number of neutrophils in a healthy population and remains strikingly constant within one individual over time.¹ The influence of genetic factors has been verified by Schreiber et al., by showing a strong correlation of the percentages of mPR3high neutrophils between monozygotic twins but not in dizygotic twins.¹⁰ 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,¹¹¹² and a high proportion of mPR3 expressing neutrophils is associated with more frequent relapse of WG.¹³ These observations suggest that abnormally expressed mPR3 is involved in the development and severity of WG.

To explain the differential expression of mPR3, Gencik et al. identified 10 polymorphisms in the promoter region of the PR3 gene, but only one of them is possibly associated with a high percentage of mPR3high neutrophils.¹⁴ However, Abdgawad et al. were not able to confirm this result in their cohort.¹⁵ Later, Vietinghoff et al. found that HLA antigen matched siblings showed comparable percentages of mPR3high neutrophils, which in fact was similar to the correlation between monozygotic twins, suggesting that the HLA region is responsible for the genetic influence on the percentage of mPR3 presenting neutrophils.¹⁶ In normal conditions, transcription of proteins stored in azurophilic granules is in silence once a granulocyte matures and is transferred into the circulation.¹⁷ However, Yang et al. found that the gene encoding PR3 is reactivated in circulating neutrophils and monocytes from patients with ANCA-associated vasculitis.¹⁸ Therefore, it seems that abnormal PR3 expression in WG is being regulated from various stages of neutrophil maturation and PR3 production. Taken together, the mechanism underlying the regulation of mPR3 expression still needs further elucidation.

**Interaction of mPR3 with other molecules on the neutrophil membrane**
The membrane-binding mechanism of PR3 is unknown. In early studies, Witko-Sarsat et al. demonstrated that mPR3 binding did not occur in a charge-
dependent manner, by showing that mPR3 could not be eluted off the membrane by drastic pH changes,\(^2\) while Goldmann et al. showed that purified PR3 interacted with lipid bilayers by hydrophobic insertion.\(^9\) Along with observations on the membrane-binding mechanism of HLE and CG, which are homologues to PR3, David et al. recognized CD1\(_b/CD18\) (Mac-1, \(\beta_2\)-integrin) as a binding-partner of mPR3.\(^20\) Later on, they found that Fc\(\gamma\)RIIIB also colocalizes with PR3 on the neutrophil membrane.\(^21\) However, both CD1\(_b/CD18\) and Fc\(\gamma\)RIIIB are universally expressed on neutrophils; therefore, differential expression of mPR3 could not be fully explained until Vietinghoff et al. revealed that a substantial amount of PR3 was exclusively expressed on CD177 (NB1) expressing neutrophils.\(^22\)

CD177 is the coding gene for the NB1 glycoprotein. Characteristics of CD177 and NB1gp have in depth been reviewed by Stroncek et al.,\(^23\) and it is of interest that CD177 also shows differential expression on the neutrophil surface. The percentage of CD177 expressing neutrophils, similar to mPR3, ranges from 0% to 100% in a healthy population.\(^23\) Vietinghoff et al. showed that CD177 is the receptor of mPR3 and mediates PR3 expression on the neutrophil membrane.\(^22\) Our group confirmed this finding by showing an elevated percentage of CD177 expressing neutrophils in patients with ANCA-associated vasculitis and SLE, which may account for the increased expression of mPR3 in these clinical conditions.\(^24\) The mechanism involved in the physical binding between mPR3 and CD177 is largely unknown. Active PR3, but not proPR3 can bind to the surface of CD177-transfected HEK293 cells, suggesting that N-terminal processing is important for binding of PR3 to CD177.\(^25\) Meanwhile, Korkmaz et al. predicted that the unique hydrophobic cluster of PR3, as compared to HLE and CG, probably mediates PR3-CD177 interaction.\(^26\)

It should be mentioned that mPR3-expression has been observed on also the mPR3low subset in previous studies.\(^5\) Data from our group also showed that neutrophils from CD177 negative individuals express low levels of PR3, but not CD177, on their membrane after priming with TNF-\(\alpha\), and these primed neutrophils were also susceptible for PR3-ANCA induced neutrophil activation.\(^24\) These results indicate that CD177 is not an exclusive binding partner of mPR3, and other binding site(s) are present as well on neutrophils and mediate a low amount of mPR3 expression, such as CD1\(_b/CD18\) and Fc\(\gamma\)RIIIB mentioned before. In addition, HLE and CG have recently been shown to bind to chondroitin sulfate- and heparin sulfate-containing proteoglycans, which provide low affinity but high capacity binding sites for cationic proteins on the neutrophil membrane. PR3 competes with HLE and CG for these binding sites.\(^27\) Moreover, Hajjar et al.
Figure 1. Relevance of mPr3-coexpressing molecules in the pathophysiology of PR3-ANCA-mediated neutrophil activation. (A) TNF-α primes neutrophils and translocates PR3 to the cell membrane. PR3-CD177 binding might activate β2-integrins and promote neutrophil firm adhesion. (B) PR3-ANCA cross-link mPr3 and FcγRIIa, which further induces the oxidative burst of neutrophils. Released proteolytic enzymes and reactive oxygen species cause vessel damage. (C) FcγRIIIb engagement activates β2-integrin, and the latter binds to ICAM-1 expressed on endothelial cells and, on the other hand, mediates PR3-ANCA induced neutrophil activation. EC: endothelial cell; PMN: polymorphonuclear neutrophil.

presumed that PR3 is a peripheral membrane protein that directly interacts with the neutrophil membrane through its hydrophobic region, based on studies on a membrane model using molecular dynamics simulation.28 Researchers from the same group also demonstrated that PR3 is externalized during neutrophil apoptosis independent of degranulation. This process is mediated by phospholipid scramblase 1 (PLSCR1), a protein related to the bidirectional movement of plasma-membrane phospholipids.29 On the whole, CD177, as a receptor of mPr3, accounts for substantial membrane-expression of PR3, while the expression of smaller amounts of mPr3 is probably mediated by other mechanism(s) mentioned before. These latter mechanisms allow the subset of mPr3low neutrophils to be involved in the pathophysiology of PR3-ANCA associated vasculitis as well.
Relevance of mPR3-coexpressing molecules in the pathophysiology of WG

Binding partners of mPR3 might have a role in the process of PR3-ANCA induced vessel damage. The signal transduction pathways mediating PR3-ANCA induced neutrophil activation have been reported, and it is well accepted that crosslinking of PR3-ANCA/MPO-ANCA with FcyRIIa on primed neutrophils leads to the oxidative burst (Figure 1B). However, neutrophils, generally, are not activated by ANCA in the circulation, but TNF-α induced neutrophil adhesion to the endothelium has been shown to be a requirement for inducing the neutrophil oxidative burst. Reumaux et al. found that PR3- or MPO-ANCA induced neutrophil activation is strongly impaired when neutrophils are not allowed to adhere by persistent stirring or by blocking CD18. On the other hand, FcyRIIIb is a glycosyl phosphatidylinositol (GPI)-linked protein which is not capable of mediating transmembrane signals, and signal transduction here probably occurs by molecules colocalizing with FcyRIIIb, such as β2-integrins. It has been shown that engagement of FcyRIIIb by immune complexes in the circulation may activate β2-integrins and lead to a proadhesive phenotype likely to promote systemic vascular damage (Figure 1C).

In contrast to FcyRIIIb and β2-integrins, little is known about the function of CD177. CD177 is also a GPI-anchored protein lacking the capability of mediating signal transduction. We have shown that CD177-deficient neutrophils also could be activated by PR3-ANCA in vitro, indicating that CD177 is not necessary for this signaling. Urokinase-type plasminogen activator receptor (uPAR) is a homologue of CD177, and the uPA-uPAR system has a role in angiogenesis and tumor cell migration in cancer. The function of uPAR in cell-cell interaction has been well established. A series of studies have identified a specific binding site for uPAR in the CD11b/CD18 complex, and the binding of uPA to uPAR, similar to mPR3-CD177 interaction, may lead to conformational changes in β2-integrins (Figure 1A). Moreover, in a mouse model of Pseudomonas aeruginosa infection, it was observed that recruitment of neutrophils to the lung occurred rapidly in uPAR+/+ mice, and was drastically reduced in uPAR-/- mice, suggesting a role of uPAR in neutrophil transendothelial migration which is probably dependent on β2-integrins. In addition, Sachs and colleagues recently showed that CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31) which is predominantly expressed on the membrane of endothelial cells. These data support the hypothesis that mPR3 binds to CD177 on the neutrophil membrane upon priming and, consequently, activates β2-integrins co-expressed in the same complex, and that the complex further accelerates neutrophil firm adhesion and transmigration.
Conclusion

The percentage of mPR3 expressing neutrophils is genetically determined. The presence of an elevated proportion of neutrophils expressing a substantial amount of mPR3 in patients with ANCA-associated vasculitis could also be related to genetic factors. With respect to the mechanisms underlying membrane expression of PR3, a complex of molecules that colocalize with PR3 on the neutrophil membrane appears to be involved. This complex probably functions in neutrophil recruitment bringing these effector cells close to the endothelium and further mediate PR3-ANCA induced neutrophil activation and vessel damage. Either blocking the membrane presentation of PR3 or neutralizing the functions of its binding partners might generate novel therapeutic strategies for ANCA-associated vasculitis.

References


Co-expression of CD177 and membrane Proteinase 3 on neutrophils in ANCA-associated vasculitis

anti-PR3 mediated neutrophil activation is independent of CD177 expression

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Abstract
Objectives. Wegener's Granulomatosis (WG) is strongly associated with anti-neutrophil cytoplasmic autoantibodies (ANCAs) directed against Proteinase 3 (PR3). Recent studies have shown that membrane-bound PR3 (mPR3) is differentially expressed and colocalizes with CD177/NB1 on circulating neutrophils. We undertook this study to assess differential expression of CD177 on neutrophils from ANCA-associated systemic vasculitides (AAV) patients in comparison to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients and healthy individuals, and to investigate whether colocalization of mPR3 and CD177 affects anti-PR3-mediated neutrophil activation.
Methods. CD177 and mPR3 expression on isolated neutrophils from patients with AAV (n=53), SLE (n=30), RA (n=26), and healthy controls (n=31), was analyzed by flow cytometry. Neutrophil activation mediated by anti-PR3 antibodies was assessed by measuring the oxidative burst using dihydrorhodamine assay.
Results. Percentages of CD177-expressing neutrophils were significantly higher in patients with AAV and SLE than in healthy controls. In three healthy donors, CD177 expression was not detected and neutrophils remained negative after priming while mPR3 expression was induced. Neutrophils from CD177 negative donors or CD177 negative neutrophils sorted from donors with bimodal expression were susceptible to anti-PR3-mediated oxidative burst. Variation in the extent of anti-PR3 mediated neutrophil activation among different donors appeared independent of the percentage of CD177-expressing neutrophils.
Conclusion. Membrane expression of CD177 on circulating neutrophils is increased in AAV and SLE patients but not in RA patients. 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.
Introduction

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated systemic vasculitis (AAV) comprises Wegener's Granulomatosis (WG), microscopic polyangiitis (MPA) and Churg Strauss syndrome (CSS), which share a spectrum of clinical manifestations reflecting necrotizing damage of small- and medium-sized vessels. The pathogenic role of ANCA in AAV is supported by a large body of in vitro and in vivo evidence, and the presence of ANCA in circulation is an important serologic marker for the diagnosis and follow-up of AAV. Proteinase 3 (PR3) and myeloperoxidase (MPO), both of which are mainly stored in primary granules of neutrophils, have been identified as ANCA antigens. Although either specificity can occur with any AAV phenotype, PR3-ANCA are most frequently detected in sera of WG patients.

In resting neutrophils, PR3 is contained intracellularly in azurophilic granules. However, in many individuals a membrane bound form of PR3 (mPR3) can also be detected in a subset of neutrophils making it accessible for ANCA binding. In the general population, the percentage of mPR3 expressing neutrophils ranges from 0 to 100%. Within a given individual, the percentage of mPR3\textsuperscript{high} neutrophils is constant in time and is not affected by neutrophil activation, disease activity or therapy, suggesting involvement of genetic factors in the regulation of mPR3 expression.

The mechanism of membrane PR3 expression is still under investigation. Colocalization of mPR3 with other membrane proteins, such as β2-integrin and FcyRIIIb, has been reported previously. In recent studies, CD177 (NB1) has been proposed as the mPR3 receptor on the neutrophil surface. CD177 is a neutrophil specific, GPI-anchored glycoprotein, compartmentalized in secondary (specific) granules. Concurrent with mPR3, CD177 also shows differential expression on the neutrophil surface, with percentages of CD177\textsuperscript{+} neutrophils ranging from 0 to 100%. It has also been observed that mPR3 colocalizes with CD177 on the neutrophil membrane and the subpopulation of neutrophils expressing CD177 is identical to that expressing mPR3. Interestingly, the intracellular content of PR3 is similar in all neutrophil subsets regardless of mPR3 expression status, while CD177 molecules are only present in lysates of neutrophils expressing CD177 on their membrane. Collectively, these observations suggest that CD177 may be a determinant of membrane expression of PR3 and, as such, influences the potential of neutrophils to be activated by PR3-ANCA. Indeed, increased percentages of mPR3 expressing neutrophils have been observed in AAV patients, and a high percentage of mPR3-expressing cells is a risk factor for relapse in WG. However, expression levels of CD177 have not
been consistently studied in patient populations, and its influence on ANCA-induced neutrophil activation has not been described. Therefore, we assessed the differential expression of CD177 and mPR3 on neutrophils from AAV patients in comparison with disease and healthy controls, and investigated the role of CD177 expression in neutrophil activation by PR3-ANCA.

**Patients and Methods**

**Study population**

For the assessment of neutrophil CD177 expression, consecutive patients with ANCA-associated systemic vasculitis (n=53), rheumatoid arthritis (n=26) and systemic lupus erythematosus (n=30) recruited from our out-patient clinic, as well as healthy controls (n=31) recruited from laboratory personnel, were included. Individuals with pregnancy, G-CSF treatment or bacterial infection were excluded.

ANCA-associated systemic vasculitis (AAV): A diagnosis of Wegener’s granulomatosis (WG), Churg Strauss syndrome (CSS) or microscopic polyangiitis (MPA) was based on the Chapel Hill definitions.\(^\text{20}\) ANCA specificity for PR3 or MPO was determined by capture ELISA. ANCA titers were determined by indirect immunofluorescence (IIF) assay on ethanol-fixed neutrophils.\(^\text{21,22}\)

Rheumatoid arthritis (RA): A diagnosis of RA was established on the American College of Rheumatology (ACR) criteria for definite RA.\(^\text{23}\)

Systemic lupus erythematosus (SLE): A diagnosis of SLE was based on the revised ACR-criteria for this disease.\(^\text{24}\)

Demographic characteristics of patients and controls are given in Table 1.

All patients and controls gave informed consent and the study was approved by the Medical Ethical Committee of the hospital.

**Antibodies**

PR3 was detected using three murine monoclonal antibodies; PR3G-3,\(^\text{25}\) 4A3 (Wieslab, Lund, Sweden), and CLB12.8 (CLB, Amsterdam, The Netherlands). MEMi66 (BD Pharmingen, San Diego, CA) was used for CD177 detection. Irrelevant isotype IgGl (MCG1; IQProducts, Groningen, The Netherlands) and IgG2a (MCG2a; IQProducts, Groningen, The Netherlands) antibodies were used as control. FITC- and PE-conjugated goat anti-mouse secondary antibodies (Southern Biotechnology Associates Inc., Birmingham, USA) were used for FACS analysis. Human IgG fractions were isolated from PR3-ANCA-positive sera of WG patients, anti-glomerular basement membrane (GBM)-positive sera of patients
with Goodpasture syndrome, and sera of age-matched healthy donors using a protein G column (MabTrap G II; Pharmacia Biotech, Uppsala, Sweden) following manufacturer’s instructions.

Table 1. Characteristics of patients and healthy controls included in the analysis of CD177 expression

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HC: Healthy Control; PR3-ANCA: PR3-ANCA-associated vasculitis; MPO-ANCA: MPO-ANCA associated vasculitis; SLE: Systemic Lupus Erythematosus; RA: Rheumatoid Arthritis.

Isolation and priming of neutrophils

Neutrophils were isolated from peripheral blood and primed following routine procedures published previously. Briefly, neutrophils were isolated from heparinized venous blood by centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Contaminating erythrocytes were lysed with ice-cold ammonium chloride buffer. Cells were washed and resuspended in cold Hanks’ balanced salt solution (HBSS) without Ca²⁺/Mg²⁺ (HBSS⁻/⁻; Gibco/Life Technologies, Breda, The Netherlands). Next, cells were resuspended in RPMI 1640 (Cambrex BioScience, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS; BioWhittaker Europe, Verviers, Belgium) and 50 µg/ml of gentamicin (Gibco, Paisley, UK), to obtain 10⁶ cells/ml.

Where indicated, cells were primed with 2 ng/ml recombinant human tumor necrosis factor-α (rhTNF-α; Boehringer Mannheim, Germany) at 37°C for 15 min, and non-primed cells were incubated with control medium under the same condition.

Measurement of membrane-bound PR3 and CD177 expression

Membrane expression of PR3 and CD177 was measured by flowcytometry. The complete procedure was performed on ice to avoid cell activation. After priming, each sample, containing 1x10⁶ isolated neutrophils, was washed with ice-cold HBSS⁻/⁻ / 1% FCS and centrifuged at 1,200xg, 4°C for 3 min. Cell pellets were
incubated with 0.5 mg/ml heat-aggregated goat immunoglobulin (HAGG; Sigma, Zwijndrecht, The Netherlands) for 15 min to block Fc receptors on the cell surface. Next, cells were loaded with saturating dose of monoclonal antibodies against human PR3 (PR3G-3) or CD177 (MEM166). An irrelevant isotype IgG1 mouse monoclonal antibody (MCG1) was used as control. Where indicated, 4A3 and CLB12.8 were also used for PR3 detection. After 30 min, unbound antibodies were washed off and cells were resuspended in PE-conjugated secondary antibody diluted with 0.5 mg/ml HAGG, and incubated in the dark for another 30 min. Next, cells were washed and resuspended in washing buffer. Fluorescence intensity was measured immediately by FACS Calibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and calibrated using CellQuestTM software (Becton Dickinson). The results were analyzed using WinList software package (Verity Software House, Topsham, ME), and figures were edited with FlowJo™ analysis software (Treestar, Ashland, OR). Expression levels are presented as mean fluorescence intensity of 10,000 counted cells corrected for nonspecific binding of isotype control antibodies. Bimodal expression was defined as the presence of 10–90% mPR3\textsuperscript{high}/CD177\textsuperscript{+} cells, respectively, while percentages out of this range were defined as monomodal. Definitions for monomodal-mPR3\textsuperscript{high} and monomodal-mPR3\textsuperscript{low} were based on data from a previous study.\textsuperscript{99}

**Detection of intracellular level of PR3 and CD177**
Isolated neutrophils were suspended in HBSS\textsuperscript{−/-} to obtain a concentration of 1×10\textsuperscript{6} cells/ml. The cell suspensions were incubated with 10 μg/ml cytochalasin B at 37°C for 5 min, and primed with 2 ng/ml rhTNF-α at 37°C for 15 min. Next, all neutrophils were fixed and permeabilized with Fix&Perm kit (Caltag Laboratories, Burlingame, CA) following the manufacturer’s instructions. Next, intracellular levels of PR3 and CD177 were detected by incubation with anti-PR3 (PR3G-3) or anti-CD177 antibody (MEM166) at saturating concentrations. MCG1 was used as negative control. After 30 min incubation at room temperature, unbound antibodies were washed off and cells were incubated with PE-conjugated secondary antibody diluted with 0.5 mg/ml HAGG in the dark for another 30 min. After a next washing step, cells were resuspended in washing buffer. Fluorescence intensity was measured immediately by FACS Calibur and calibrated with CellQuestTM software. Levels of PR3 and CD177 expression were given as MFI and corrected for non-specific binding as detected from isotype control antibody incubation.
Dihydrorhodamine 123 oxidation assay
Intracellular hydrogen peroxide produced during neutrophil activation was measured by the dihydrorhodamine 123 (DHR 123) oxidation assay as described previously. Isolated neutrophils from healthy donors were suspended in HBSS with Ca\(^{2+}\)/Mg\(^{2+}\) to a concentration of 2.5x10^6 cells/ml and incubated with cytochalasin B (5 µg/ml, Serva Electrophoresis, Heidelberg, Germany) at 37°C, to enhance oxygen radical production. Next, cells were loaded with 1 µg/ml DHR 123 (Molecular Probes Europe) and kept at 37°C for 15 min. Sodium azide (NaN_3, 2 mM) was added in order to prevent intracellular breakdown of H_2O_2 by catalase. Part of the DHR 123 loaded cells were incubated for 15 min in the presence of a priming concentration of rhTNF-α (2 ng/ml). Next, neutrophils were stimulated for 1 hour with anti-PR3 antibody (PR3G-3, 5 µg/ml) or an irrelevant mouse IgG1 monoclonal antibody (MCG1) at the same concentration. Where indicated, neutrophils were also stimulated with 200 µg/ml of PR3-ANCA\(^+\) or irrelevant human IgG fractions (anti-GBM or normal IgG). Stimulation with 0.2 µM phorbol myristate acetate (PMA; Sigma, Zwijndrecht, The Netherlands) for 30 min was performed as positive control. Reaction was stopped with ice-cold HBSS/-. Finally, cells were resuspended in washing buffer and fluorescence intensity, resulting from the intracellular oxidation of DHR 123 into the fluorescent rhodamine 123 (R 123), was measured using a FACS Calibur and calibrated using CellQuestTM software.

Statistical analysis
Results were expressed as median values. Statistical analysis was performed using Mann-Whitney U test, Kruskal-Wallis test and Spearman nonparametric correlation with GraphPad Prism, version 4.03 (GraphPad Software, San Diego, CA). Probability values of <0.05 were considered significant.

Results
Expression of CD177 correlates with mPR3 expression on primed neutrophils
Combining data from healthy controls and patient groups (n=140), 120 donors were identified that displayed a bimodal expression of mPR3. In agreement with other studies, CD177 and mPR3 expression on primed neutrophils showed a strong correlation (data not shown). However, neutrophil priming was an essential procedure for assessing the correlation between mPR3 and CD177 expression, as priming with TNF-α is necessary for standardized assessment of mPR3 expression as shown before. In 19 of the 31 healthy donors (61%), isolated
neutrophils showed monomodal-negative expression of mPR3 without priming, while, after incubation with TNF-α for 15 min, 12 of these donors turned into bimodal patterns with a low- and a high-expression subset (Figure 1A). Percentages of mPR3^high neutrophils ranged from 38 to 85%, and remained constant in repeated tests within a given individual (data not shown). The amount of mPR3 on the neutrophil surface, given as mean fluorescence intensity (MFI), was also remarkably upregulated by TNF-α stimulation (Figure 1B). Effect of neutrophil priming on mPR3 recruitment was comparable between patient groups (Table 2 and Figure 1B). Therefore, priming with TNF-α revealed the potential of neutrophils to increase expression of mPR3. In contrast to mPR3 expression, percentages of CD177^+ neutrophils were not modified by neutrophil priming (Table 2). The amount of membrane expressed CD177 was only slightly upregulated after priming, with a mean increase of MFI of 21±19% of the expression level on non-primed neutrophils (Figure 1B).
Table 2. Bimodal expression of mPR3 and CD177 induced by TNF-α priming

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<th>HC</th>
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HC: Healthy Control; PR3-ANCA: PR3-ANCA-associated vasculitis; MPO-ANCA: MPO-ANCA-associated vasculitis; SLE: Systemic Lupus Erythematosus; RA: Rheumatoid Arthritis.

**mPR3 expression on the neutrophil surface can be independent of CD177 expression**

In 3 healthy donors, CD177 expression was not detected on the membrane of isolated neutrophils either before or after priming. However, after incubation with TNF-α, neutrophils from these CD177 negative donors expressed mPR3.

![Figure 2. TNF-α-induced mPR3 expression is detected on CD177 negative neutrophils.](image)

Membrane expression of mPR3 and CD177 on neutrophils was measured by flowcytometry in parallel. (A) CD177 and mPR3 expression on non-primed and primed neutrophils from a CD177 negative donor. Figure shows a representative of duplicated experiments with 3 CD177’ donors. (B) mPR3 on neutrophils from CD177’ donors was detected with 4A3, PR3G-3 and CLB12.8 anti-PR3 antibodies, and PR3-ANCA’ IgG fractions, in comparison with CD177 expression detected with MEM166 (anti-CD177). Results show mean±SD of mPR3 and CD177 expression level (MFI) of 3 CD177’ donors after correcting for nonspecific binding. (C) Histograms showing intracellular content of CD177 (grey-colored) and PR3 (solid line) in CD177 negative and positive donors measured by flowcytometry. An isotype control IgG, (dashed line) and anti-MPO antibody (dotted line) were used as negative and positive control, respectively. The figure shows a representative example of 3 independent experiments with different donors.
which was detected using three different monoclonal antibodies (Figure 2A and 2B). Absence of CD177 expression was confirmed by intracellular FACS staining. In contrast, PR3 and MPO were uniformly detected in these neutrophils (Figure 2C). These results demonstrate that mPR3 on the neutrophil surface can be independent of CD177 expression.

**Percentages of mPR3\textsuperscript{high} and CD177 expressing neutrophils in inflammatory diseases**

Percentages of CD177\textsuperscript{+} and mPR3\textsuperscript{high} neutrophils were compared among AAV (n=53), SLE (n=30) and RA (n=26) patients and healthy donors (n=31). In this analysis, donors with monomodal expression were also included. Median percentages of mPR3\textsuperscript{high} neutrophils were significantly higher in AAV (67\%, range 16–100\%) and SLE (80\%, range 25–100\%) but not in RA (61\%, range 0–100\%) patients compared with healthy controls (51\%, range 0–100\%) (Figure 3A). Furthermore, percentages of CD177 expressing neutrophils were also significantly

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**Figure 3. Percentages of CD177 expressing neutrophils are increased in ASV and SLE patients.**

Membrane expression of CD177 and mPR3 were measured on isolated neutrophils by flowcytometry. Results are presented as percentages of CD177\textsuperscript{+}/mPR3\textsuperscript{high} neutrophils in the total neutrophil population. Percentages of mPR3\textsuperscript{high} (A) and CD177\textsuperscript{+} (B) neutrophils were compared among healthy controls (n=31), and patients with ASV (n=53), SLE (n=30) and RA (n=26). (C) Percentages of CD177\textsuperscript{+} neutrophils were compared between MPO-ANCA\textsuperscript{+} (n=22) and PR3-ANCA\textsuperscript{+} (n=32) ASV patients, and between WG (n=33) and MPA (n=19) ASV phenotypes. (D) Comparison of CD177\textsuperscript{+} neutrophils between PR3-ANCA positive patients with or without treatment with cyclophosphamide (CYC) or prednisolone and patients with or without relapsing disease. ns: not significant, *: p<0.05, **: p<0.01, ***: p<0.001. Horizontal line denotes the median.
higher in AAV (71%, range 18–97%) and SLE (82%, range 33–94%) patients than in healthy controls (62%, range 0–87%) and RA patients (61%, range 0–93%) (Figure 3B). Within the AAV patient group, the expression profile was also analyzed according to ANCA specificity and AAV phenotypes. There were no statistical differences in the percentages of CD177+ neutrophils between patients with MPO-ANCA (77%, range 25–94%) and PR3-ANCA (67%, range 18–97%) associated vasculitis, or between patients with WG (67%, range 18–97%) and MPA (80%, range 25–94%) (Figure 3C).

Immunosuppressive treatment or prednisolone administration did not increase the proportion of CD177+ neutrophils in patients with SLE and MPO-ANCA associated vasculitis. However, in patients with PR3-ANCA associated vasculitis, patients treated with cyclophosphamide or prednisolone had higher percentages of CD177 expressing neutrophils than patients without these treatments (Figure 3D). We did not find any correlation between the percentages of CD177 expressing neutrophils and disease activity or disease duration in PR3-ANCA associated vasculitis (data not shown). However, neutrophils from patients who suffered relapsing disease showed significantly higher percentages of CD177+ neutrophils than those patients without relapses (Figure 3D). Concurrently, 4 out of 6 patients treated with cyclophosphamide and 10 out of 12 patients treated with prednisolone had relapsing disease.

**Neutrophil activation by PR3-ANCA**

Neutrophil activation induced by a mouse anti-human PR3 monoclonal antibody (PR3G-3) or by PR3-ANCA+ IgG fractions was evaluated by measuring the oxidative burst using the dihydrorhodamine assay. In agreement with previous studies, oxidative burst was detected only when neutrophils were primed with TNF-α (data not shown). 26,27 Neutrophils from CD177 negative donors (n=3) could be activated by anti-PR3 antibody and showed comparable levels of neutrophil activation as neutrophils from donors with moderate expression of CD177 (40–70% positive; n=3) and high expression of CD177 (>70% positive; n=3) (Figure 4A). Moreover, no correlation was observed between the levels of neutrophil oxidative burst induced by anti-PR3 antibody and percentages of CD177+ neutrophils (data not shown). Next, CD177 positive and negative neutrophils from donors with bimodal expression of CD177 were sorted by flow cytometry. The purity of each sorted sample was more than 95% (Figure 4B). These subsets were used for induction of the oxidative burst by anti-PR3 antibody. The results were comparable for the CD177 negative and positive subpopulations (Figure 4C).
Figure 4. Anti-PR3-antibody-mediated neutrophil activation is independent of CD177 expression. Oxidative burst was assessed by DHR 123 oxidation assay and activation level is presented as MFI of Rhodamine 123. (A) Levels of neutrophil activation were compared among individuals with CD177 negative neutrophils (0% CD177+, n=3), neutrophils with moderate expression of CD177 (40–70% CD177+, n=3) and high expression of CD177 (>70% CD177+, n=3). (B) CD177 negative and positive neutrophils from the same donor were sorted by flow cytometry, purity of each sorted sample was more than 95%. Figure shows a representative example of 3 independent experiments. (C) Levels of neutrophil activation induced by anti-PR3 antibody were compared between CD177− and CD177+ neutrophils from 3 individual donors.

Discussion
In this study, we describe the profile of CD177 expression in AAV and the contribution of CD177 to PR3-ANCA mediated neutrophil activation. In agreement with results reported by others, mPR3 and CD177 expression were highly correlated on primed neutrophils from donors with a bimodal expression pattern of both molecules.14,15 Percentages of mPR3high/CD177+ neutrophils were increased in AAV and SLE patients compared to healthy individuals. However, colocalization of mPR3 and CD177 was not crucial for PR3-ANCA mediated neutrophil activation which is considered a central event in disease pathogenesis leading to vessel wall damage.28 After priming, mPR3 was expressed on CD177 negative neutrophils, suggesting additional mechanisms of membrane presentation of PR3 independent of CD177. Anti-PR3 antibody mediated
CD177 expression in AAV

neutrophil activation, as analyzed by DHR 123 oxidation, could also be induced in CD177 negative neutrophils, and, among healthy individuals, there was no correlation between the level of neutrophil activation and the percentage of CD177 expressing neutrophils. Although the pathogenesis of AAV is still unclear, it has been shown that ANCA are pathogenic in animal models and that the antibodies in vitro activate cytokine-primed neutrophils leading to oxidative burst and neutrophil degranulation.[^3][^4] Membrane-bound PR3, which is accessible for ANCA binding, shows heterogeneous expression on the neutrophil surface.[^9] The percentage of mPR3[^3][^4] neutrophils is increased in AAV patients and is a risk factor for relapse of WG.[^14][^18][^29] Recent studies have suggested that CD177 acts as a receptor of mPR3 by showing that mPR3 expression is highly correlated with CD177 expression and mPR3 colocalizes with CD177 on the neutrophil membrane.[^14][^15] It is tempting to speculate that increased proportions of mPR3 expressing neutrophils in AAV patients reflect upregulated expression of CD177. Furthermore, it is relevant in view of the pathogenesis of AAV whether CD177[^3][^4] neutrophils act differently from CD177[^14][^15] mPR3[^3][^4] neutrophils when encountering PR3-ANCA, for instance, in PR3-ANCA mediated neutrophil activation.

It has been reported that the percentage of CD177[^3][^4] neutrophils is constant within a given individual.[^30] However, enlarged CD177[^3][^4] subsets have been observed in some clinical conditions with altered neutrophil production. The percentage of CD177[^3][^4] cells increased to 90% in healthy individuals receiving G-CSF treatment, which can mobilize myeloid progenitor cells into the peripheral blood and is used for stem cell collection.[^31][^32] Women in early- and late-stage pregnancy have approximately 10% more CD177 expressing neutrophils and increased neutrophil counts than healthy female blood donors.[^33][^34] To this point, the current study is the first to report that percentages of CD177 expressing neutrophils are also increased in AAV and SLE patients compared to healthy individuals, which could account for the elevated expression of mPR3 in both patient groups.

It has been shown that the percentage of mPR3-expressing neutrophils cannot be modified by disease activity or treatment.[^14][^15] In agreement, we did not find any effect of treatment on the proportion of CD177[^3][^4] neutrophils in patients with SLE and MPO-ANCA associated vasculitis. However, in patients with PR3-ANCA associated vasculitis, patients treated with cyclophosphamide (CYC) or prednisolone showed significantly higher percentages of CD177 expressing neutrophils than patients without these treatments (Figure 3D). In our disease control groups, treatment with prednisolone did not increase the percentage of CD177[^3][^4] neutrophils in patients with SLE, and neither CYC nor prednisolone
induced upregulation of CD177 in patients with MPO-ANCA+ vasculitis, suggesting that increased CD177 expression is not a consequence of treatment, but probably associated with other factors. In patients with PR3-ANCA+ vasculitis, we did not find any correlation between the percentages of CD177+ neutrophils and disease activity or disease duration. However, we found that patients who had suffered from relapsing disease showed significantly higher percentages of CD177+ neutrophils than patients without relapses. Concurrently, four out of six patients treated with CYC and 10 out of 12 patients treated with prednisolone had relapsing disease, probably explaining differences in CD177 expression between patients on CYC or prednisolone and those without these treatments. Therefore, treatment with immunosuppressives and corticosteroids has, probably, in itself no effect on CD177/mPR3 expression. Moreover, the current study confirmed our previous observation that a high proportion of mPR3 (and CD177) expressing neutrophils is associated with relapse in WG.\textsuperscript{18}

Whereas increased mPR3 expression on neutrophils in AAV has been linked to PR3-ANCA induced neutrophil activation in vitro\textsuperscript{29} and associated with relapsing disease in patients with AAV,\textsuperscript{18} the pathogenetic significance of increased expression of mPR3 and CD177 in SLE is not clear. As ANCA in SLE are, in most of the cases, not directed to PR3,\textsuperscript{35-37} a role for mPR3 expression on neutrophil activation by ANCA can not be substantiated. Otherwise, neutrophils do play a role in the pathogenesis of SLE, by interacting with immune complexes or by inducing or exacerbating autoimmune responses when these cells accumulate in an apoptotic state.\textsuperscript{38} Indeed, accelerated apoptosis of neutrophils and decreased uptake of apoptotic neutrophils has been observed in SLE.\textsuperscript{39,40} Whether the increased expression of PR3 on the membrane of primed neutrophils in SLE is related to an increased level of activation or even to a pre-apoptotic state remains speculative.

CD177 is a heterophilic binding partner of CD31 which is expressed on endothelial cells suggesting a role of CD177 in neutrophil migration.\textsuperscript{41} Apart from this, the biological function of CD177 is largely unknown. Furthermore, CD177 negative individuals, about 3% of Caucasians, are healthy, although detailed studies on neutrophil function in these individuals are lacking. In contrast, PR3 executes multiple biological functions such as regulation of granulopoiesis, microbicidal activity, degradation of extracellular matrix, and modulation of inflammatory factors.\textsuperscript{32-43} It has been shown, in vivo, that PR3 is essential for immune complex-mediated neutrophil activation by cleaving anti-inflammatory progranulin into its inactive form, and both PR3 and elastase deficiency diminish neutrophil infiltration into the inflammatory site.\textsuperscript{44} Apparently, enzymatic activity of PR3 is
required in most of its important actions, and it is probably protected from endogenous inhibitors by anchoring at the neutrophil membrane instead of being secreted in a soluble form.\textsuperscript{45,46} Therefore, one may expect that mPR3 expression also occurs in healthy persons with CD177 deficiency. In our study, PR3 was detected by 3 different MoAbs on the surface of primed neutrophils from CD177 negative donors. Membrane-bound PR3 expression on mPR3$^{\text{low}}$ neutrophils has also been observed in other studies,\textsuperscript{9,19,47} and we have shown, previously, that the extent of TNF-\(\alpha\)-induced mPR3 upregulation on mPR3$^{\text{low}}$ neutrophils is comparable with that of neutrophil elastase.\textsuperscript{19} These results suggest that CD177 is not an exclusive receptor of mPR3 and the binding site(s) for CD177-independent mPR3 expression are still open for discussion. It has been shown that mPR3 colocalizes with activated \(\beta_2\)-integrin and Fc\(\gamma\)R in the neutrophil membrane.\textsuperscript{12,13,48} PR3 can insert into the membrane lipid bilayer via the hydrophobic region.\textsuperscript{49} Elastase, a proteinase homologous to PR3, binds to chondroitin sulphate (CS)- and heparin sulphate (HS)- containing proteoglycans in the neutrophil membrane, and PR3 is competitive for these binding sites.\textsuperscript{50} In a recent study, Witko-Sarsat et al. assumed mPR3 to be a peripheral membrane protein and predicted an interfacial binding site in the hydrophobic region, through molecular dynamics simulation on a membrane model.\textsuperscript{46} All these data suggest that mPR3 binds to the neutrophil membrane via its cationic nature and functionally colocalizes with some receptor molecules, such as integrins, Fc\(\gamma\)R and CD177, for signal transduction.

Although mPR3 is differentially expressed on neutrophils, the oxidative burst mediated by anti-PR3 antibody has been uniformly induced in neutrophils from donors with bimodal mPR3 expression.\textsuperscript{26} In the present study, we showed that the level of neutrophil activation induced by anti-PR3 antibody was not correlated with the percentage of CD177$^+$/mPR3$^{\text{high}}$ neutrophils and that CD177$^-$ neutrophils could also be activated by anti-PR3 antibody. Additionally, in agreement with previous studies,\textsuperscript{26,27} priming with TNF-\(\alpha\) was required in this process, regardless of high levels of CD177-dependent mPR3 expression on resting neutrophils. These results suggest that colocalization of CD177 and mPR3 on the membrane is neither sufficient nor necessary for neutrophil activation mediated by PR3-ANCA.

In conclusion, the proportions of CD177 expressing neutrophils are increased in AAV and SLE patients, while CD177 negative neutrophils also express mPR3 on their membrane and are susceptible to anti-PR3-antibody-induced oxidative burst. The results indicate that CD177$^+$/mPR3$^{\text{high}}$ and CD177$^-$/mPR3$^{\text{low}}$ expressing neutrophils may be equally involved in the pathogenesis of PR3-ANCA associated
vasculitis and suggest different mechanisms of membrane-binding of PR3 other than to CD177.

References

Chapter

Differential expression of granulopoiesis related genes in neutrophil subsets distinguished by membrane expression of CD177
the receptor of the autoantigen Proteinase 3 in granulomatosis with polyangiitis (Wegener’s Granulomatosis)

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Submitted for publication
Abstract

Objectives. Recent studies have shown that membrane-bound PR3 (mPR3) is differentially expressed and colocalizes with CD177 on circulating neutrophils, and that the percentage of mPR3/CD177-expressing neutrophils is elevated in antineutrophil cytoplasmic autoantibody (ANCA)-associated systemic vasculitis (AAV). We investigated differential gene expression in CD177+ and CD177-neutrophils in order to detect possible differences in neutrophil function between both subsets which could be related to the pathogenesis of AAV.

Methods. Neutrophils were isolated from healthy donors (HC) with moderate and high levels of CD177 expression and without CD177 expression. For donors with moderate expression, neutrophils were also sorted into CD177+ and CD177-subpopulations. Total RNA was isolated from all of the above mentioned neutrophil populations and expression profiles of, in total, 24,000 probes were screened with Illumina Ref 8 Expression Beadchips. Expression of genes showing differential expression between CD177+ and CD177- subsets in microarray was re-assessed with additionally sorted CD177+/− neutrophils and compared between HC and patients with quiescent AAV using quantitative PCR. Expression of granule proteins (GP) in neutrophil subsets was measured by quantitative Western Blot. In order to assess the relation between CD177 expression and neutrophil maturation, CD177 expression on neutrophil precursors in bone marrow or in peripheral blood was analyzed using flow cytometry.

Results. A number of neutrophil granule proteins (GP), such as defensin α1 and α3, defensin α4, NGAL, BPI or cathepsin G, showed higher mRNA expression levels in the CD177- neutrophil subset in healthy donors, while the amounts of these granule proteins stored in neutrophils were comparable between CD177+ and CD177- subsets. The proportion of CD177+ cells increased during neutrophil maturation in the bone marrow. Upregulation of these GP genes was also observed in AAV patients and in neutrophils activated by PMA or LPS.

Conclusions. The neutrophil population is not homogeneous and can be distinguished by membrane expression of CD177 into subsets that are different in expression of GP-related genes but not in GP production. GP gene expression is also elevated in AAV patients, which is not explained by skewed distribution of CD177+ and CD177- subsets, but may be associated with neutrophil activation during on-going inflammation.
**Introduction**

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) comprise granulomatosis with polyangiitis (GPA, formerly Wegener’s Granulomatosis), microscopic polyangiitis (MPA) and Churg Strauss syndrome (CSS), which share a spectrum of clinical manifestations reflecting necrotizing damage to small- and medium-sized vessels.\(^1\)\(^2\) The pathogenic role of ANCA in AAV is supported by a large body of *in vitro* and *in vivo* evidence, and the presence of ANCA in the circulation is an important serologic marker for diagnosis and follow-up of AAV.\(^3\)\(^4\) Proteinase 3 (PR3) and myeloperoxidase (MPO), both of which are mainly stored in primary granules of neutrophils, have been identified as ANCA antigens.\(^5\)\(^7\) Although either specificity can occur with any AAV phenotype, PR3-ANCA are most frequently detected in sera of GPA patients.\(^8\)

In resting neutrophils, PR3 is mainly contained in azurophilic and specific granules. However, in many individuals a membrane bound form of PR3 (mPR3) can also be detected in a subset of neutrophils making it accessible for ANCA binding. In the general population, the percentage of mPR3 expressing neutrophils ranges from 0 to 100%.\(^9\) Within a given individual, the percentage of mPR3\(^{\text{high}}\) neutrophils is constant in time and is not affected by neutrophil activation, disease activity or therapy.\(^9\)\(^\text{a}\)\(^\text{b}\)

CD177 is a neutrophil specific, GPI-anchored glycoprotein, compartmentalized in specific granules.\(^12\) Concurrent with mPR3, CD177 also shows differential expression on the neutrophil surface, with percentages of CD177\(^+\) neutrophils ranging from 0 to 100%.\(^13\) It has also been observed that mPR3 co-localizes with CD177 on the neutrophil membrane, and the subpopulation of neutrophils expressing CD177 is identical to that expressing mPR3.\(^14\) Although the mechanism of mPR3-CD177 interaction has not been clearly demonstrated, CD177 is currently proposed as a receptor of mPR3 on the neutrophil surface.\(^14\)\(^\text{a}\)\(^\text{b}\)\(^\text{c}\)

In our previous studies, we have reported that proportions of both mPR3- and CD177-expressing neutrophils are increased in AAV patients and a high percentage of mPR3\(^{\text{high}}\) neutrophils is a risk factor for relapse in GPA.\(^16\)\(^\text{a}\)\(^\text{b}\)\(^\text{c}\) These observations indicate that two subsets of neutrophils exist according to CD177 expression and skewed distribution of these two subpopulations may play a role in the pathogenesis of AAV. However, CD177\(^+\) and CD177\(^-\) neutrophils can be equally activated by PR3-ANCA and any functional differences cannot be easily predicted due to lacking knowledge on the biological functions of the CD177 molecule.\(^16\) Therefore, we performed a gene microarray-based study to investigate differences between CD177\(^+\) and CD177\(^-\) neutrophils, in order to clarify the
pathophysiologic significance of an increased CD177+/mPR3-high neutrophil subset for the pathogenesis of AAV.

Materials and Methods
Study populations
Neutrophils were isolated from healthy donors (HC) with low (0%, CD177-low, n=3), moderate (40–70%, CD177-moderate, n=3) and high (>70%, CD177-high, n=3) levels of CD177 expression. For the CD177-moderate donors (n=3), neutrophils were also sorted into CD177+ and CD177- subpopulations. For the Illumina microarray study, total RNA was isolated from all of the above-mentioned populations of neutrophils.

Genes showing differential expression between CD177+ and CD177- subsets in microarray were re-assessed with additionally sorted CD177+-/- neutrophils from healthy CD177-moderate donors (n=7) using quantitative RT-PCR. Whether imbalanced expression distribution of CD177+ and CD177- neutrophil subsets in AAV leads to altered expression levels of these genes was assessed within the total population of neutrophils from HC (n=19) and AAV patients in remission (n=8). Characteristics of AAV patients and HC are listed in Table 1. AAV patients included in the current study were not receiving treatment when blood samples were drawn for analysis.

Table 1. Characteristics of patients and healthy controls included in the analysis of granule proteins-related gene expression by RT-PCR.

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HC: healthy controls; AAV: patients with ANCA-associated vasculitis

To evaluate CD177 expression during neutrophil maturation, bone marrow (BM) samples were collected from one healthy donor, one donor with mantel cell lymphoma and one suspected of hypereosinophilic syndrome, all considered normal by histopathology.

All patients and controls gave informed consent and the study was approved by the Medical Ethical Committee of the hospital.
Neutrophil isolation and stimulation

Neutrophils were isolated from peripheral blood according to routine procedures as described previously. Briefly, heparinized venous blood was centrifuged on Lymphoprep (Axis-Shield, Oslo, Norway). Contaminating erythrocytes were lysed with ice-cold ammonium chloride buffer. Afterwards, cells were washed with cold Hanks’ balanced salt solution (HBSS) without Ca$^{2+}$/Mg$^{2+}$ (HBSS$^{-/-}$; GIBCO/Life Technologies, Breda, The Netherlands) and resuspended in HBSS$^{-/-}$ to obtain $10^8$ cells/ml.

Where indicated, cells were resuspended in RPMI 1640 (Cambrex BioScience, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS; BioWhittaker Europe, Verviers, Belgium) and 50 μg/ml of gentamycin (Gibco, Paisley, UK), to obtain $10^6$ cells/ml. Afterwards, the cell suspension was transferred to 6-well plates and stimulated with 1 μg/ml lipopolysaccharide (LPS; *Escherichia coli* O26:B6, Sigma, St Louis, MO, USA) or 100 ng/ml phorbol-myristate acetate (PMA; Sigma, Zwijndrecht, The Netherlands) at 37°C for 4 hours. Cells incubated with normal medium under the same conditions were included as control.

Membrane staining and sorting of neutrophils

Isolated neutrophils from healthy volunteers were labeled with a monoclonal antibody against human CD177 (NB1, MEM166; Abcam, Cambridge, UK) on ice for 30 min following manufacturer’s instructions. After washing steps, CD177$^+$ and CD177$^-$ neutrophils were sorted and collected in ice-cold HBSS$^{-/-}$ by a MoFlo high-speed cell sorter (DakoCytomation). Contaminated erythrocytes and dead cells were excluded from analysis by gating on forward and sideward light scatter. For RNA extraction, at least $1 \times 10^7$ cells of each subset were collected.

RNA isolation and microarray hybridization

Total RNA was isolated from neutrophils using a commercially available kit followed by a DNase digestion step (Qiagen RNeasy mini kit and Qiagen RNase free DNase set, respectively, Qiagen Benelux, The Netherlands). Quality and concentration of RNA samples was assessed with the Experion™ Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA). The RNA samples with quality indicator (RQI) number $>7.0$ were used for further analysis on expression arrays.

Starting with 200 ng of RNA, the Ambion Illumina TotalPrep Amplification Kit was used for anti-sense RNA synthesis, amplification, and purification, according to the manufacturer’s protocol (Applied Biosystems/Ambion, USA). Afterwards, 750 ng of complementary RNA per sample was hybridized to Illumina
HumanRef8 Bead-Chips (Illumina, San Diego, CA, USA) and scanned on the Illumina BeadArray Reader. These microarrays contain 24,000 different probes representing 16,238 different genes; some genes are targeted by more than one probe.

**Microarray data analysis**

The initial analysis of processing was performed in the Illumina BeadStudio Gene Expression module v3.2. Quantile normalization and data analysis was done by GeneSpring package version 10.0.0 (Agilent Technologies, Santa Clara, CA, USA). Only samples were included that passed quality control filtering, which was based on the median probe intensity, the correlation with all other samples and the principal component analysis over the samples. The probes were filtered for further analysis with the criterion that the expression value was present in the upper 75% range of all entities in all of the samples from at least one of the compared groups. Differences in gene expression between the compared groups were considered significant based on a fold change (fc) >2.0 in gene expression, and a t-test or ANOVA p-value <0.05 after correction for multiple testing by the Benjamini-Hochberg method.

Functional annotation and pathway enrichment of genes was analyzed using the Kyoto encyclopedia of genes and genome (KEGG) pathways with the GeneCodis functional annotation web based tool.\(^{19,20}\)

**Quantitative RT-PCR (q-PCR)**

RNA was extracted from isolated neutrophils. cDNA was synthesized from 1.0 µg of total RNA. To measure mRNA of CD177, MPO, PR3, lipocalin-2 (NGAL), defensin α, defensin α3, defensin α4, bactericidal/permeability-increasing protein (BPI), cathepsin G and β-actin, 1 µl of cDNA of each sample in triplicate was used for amplification by the Taqman real-time PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA) with specific Taqman primers/probes (Applied Biosystems). Amplification was performed using standard conditions and the amount of target transcript was presented as relative expression (2^\(-ΔCT\)) or fold induction (2^\(ΔΔCT\)) in comparison to unstimulated conditions, after being normalized to the expression of β-actin as an endogenous reference.

**FACS staining of immature neutrophils**

Neutrophil precursors in the bone marrow (BM) or peripheral blood were labeled and gated into different maturation stages as described previously.\(^{21}\) Briefly,
heparinized blood or BM samples were stained with PerCP-labeled mouse monoclonal antibody against CD45 (Becton Dickinson, The Netherlands), APC-conjugated anti-human CD1b (Becton Dickinson), FITC-conjugated anti-human CD16 (Becton Dickinson) and PE-conjugated anti-human CD177 (MEM166; Abcam, Cambridge, UK). All neutrophil precursors displayed a high level of side scatter (SSC) and moderate expression of CD45. Increasing surface expression of both CD1b and CD16 is correlated with granulocyte differentiation and maturation in the BM, and three subpopulations within SSC\textsuperscript{high}CD45\textsuperscript{mod} cells could be distinguished based on CD1b and CD16 expression. Morphological characteristics of each subpopulation revealed that myeloblasts (MBs)/promyelocytes (PMs) are enriched in the CD16\textsuperscript{low}CD1b\textsuperscript{low} subpopulation; CD16\textsuperscript{low}CD1b\textsuperscript{high} cells are mainly metamyelocytes (MYs); the majority of CD16\textsuperscript{high}CD1b\textsuperscript{high} cells are bone marrow neutrophils (BM-PMNs), which are genetically similar to blood neutrophils.\textsuperscript{21,22}

**Measurement of granule proteins by quantitative Western Blot**

Cell pellets of CD177\textsuperscript{+} and CD177\textsuperscript{−} neutrophils sorted from healthy donors were suspended with Cell Lysis Buffer (BIOKE, Cell Signaling, The Netherlands) supplemented with 10% of a protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, Netherlands) containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin, and phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, Zwijndrecht, Netherlands). To lyze neutrophil granules and vesicles completely, cell lysates were snap-frozen in liquid nitrogen and thawed 3 times, followed by 3 cycles of sonification for 20 seconds on ice with 20 seconds interval.

Cell lysates were afterwards mixed with 2× sampling buffer under non-reducing conditions. After boiling, protein bands were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes with 10% Tris-HCL precast gels or 10–20% gradient Tris-HCL precast gels (for detection of defensin α3) using Criterion electrophoresis and blotting systems (All the precast gels and facilities were purchased from Bio-Rad laboratories, The Netherlands). Protein expression of CD177, PR3, MPO, cathepsin G, BPI, defensin α3 and β-actin (as loading control) was detected and quantified with Odyssey infrared imaging system (Li-COR biosciences, Germany) following the manufacturer’s instructions. Briefly, membranes, after blocking, were incubated with specific antibodies against CD177 (MEM166; abCAM, Cambridge, UK), PR3 (PR3.G3, house-made), MPO (4A4, Santa Cruz Biotechnology, Germany), cathepsin G (#H00001511-B01, Abnova GmbH,
Germany), BPI (H-130, Santa Cruz Biotechnology, Germany) or defensin α3 (#MA1-35495, Pierce Biotechnology, Rockford, USA) together with anti-β-actin (#A5060, Sigma-Aldrich, Zwijndrecht, Netherlands), followed by detection with IRDye secondary antibodies (800CW and 680LT, Li-COR bioscience, Germany). Membranes were scanned and analyzed using an Odyssey IR scanner using Odyssey imaging software 3.0.

Antibody signals were analyzed as integrated intensities of regions defined around the bands of interest in either 800- or 700-channel. The protein of interest was expressed as percentage related to the integrated intensity of the loading control.

**Statistical analysis**

In microarray data analysis, a t-test or ANOVA p-value <0.05, which was corrected for multiple testing by the Benjamini-Hochberg method, was considered significant. Results of quantitative RT-PCR and western blotting were presented as means and analyzed for statistical differences using Wilcoxon matched pairs test, Spearman correlation and Mann-Whitney U-test, performed with GraphPad Prism 4.03 (GraphPad Software, San Diego, CA). Two-tailed p values of <0.05 were considered significant.

**Results**

**Microarray analysis**

Gene expression of circulating neutrophils from healthy donors was profiled with Illumina HumanRef-8 beadchips. As mentioned before, 5 groups were analyzed separately for two sets of comparison, namely analysis of the total neutrophil population from CD177\(^\text{low}\), CD177\(^\text{moderate}\) and CD177\(^\text{high}\) donors and comparison between two sorted neutrophil subsets, CD177\(^+\) and CD177\(^-\), from CD177\(^\text{moderate}\) donors. After initial quality control testing and filtering based on expression levels, one sample from a CD177\(^\text{low}\) donor was excluded, and 18,448 probes for comparison between groups with total neutrophil populations and 15,774 probes screened on sorted neutrophil subsets were subjected to further analysis. Microarray data were further confirmed for low frequency or absence of non-granulocytic cells by undetectable levels of lineage-specific genes highly expressed in T- and B-cells (CD3, CD19), monocytes (M-CSFR), and erythroid cells (glycophorin-A).

When analyzing differentially expressed genes among CD177\(^\text{low}\), CD177\(^\text{moderate}\) and CD177\(^\text{high}\) donors of the analyzed transcripts, 472 gene probes showed an \(\text{fc} > 2.0\) difference in expression level between CD177\(^\text{low}\) and CD177\(^\text{high}\) donors; 565 showed
an fc >2.0 expression difference between CD177<sub>low</sub> and CD177<sub>moderate</sub> donors; and 284 transcripts displayed an fc >2.0 difference between CD177<sub>high</sub> and CD177<sub>moderate</sub> groups. Among these gene probes, 17 transcripts were significantly different in single gene expression corrected for multiple testing (p<0.05, Table 2). Notably, the number of genes differentially expressed between CD177<sub>high</sub> and CD177<sub>moderate</sub> donors was much smaller than that resulting from the comparisons between CD177<sub>high</sub> and CD177<sub>low</sub>, or between CD177<sub>mod</sub> and CD177<sub>low</sub> (Figure 1). It seemed that neutrophils from CD177<sub>high</sub> and CD177<sub>moderate</sub> donors were similar in gene expression profile, but different from CD177<sub>low</sub> donors, although the correlation coefficients of all measured probes between any two samples were comparably high and ranged from 0.96~0.99. Therefore, we combined CD177<sub>high</sub> and CD177<sub>moderate</sub> groups and compared these to CD177<sub>low</sub> donors in differential gene expression. We found 107 genes with fc>2.0 differences that were significantly different in single gene expression corrected for multiple testing (p<0.05) between CD177<sub>low</sub> and CD177<sub>high+moderate</sub> populations. However, no functional pathways or annotation clusters were significantly enriched within these gene entities (Supplementary data).

**Figure 1.** Genes differentially expressed in neutrophils from donors with high, moderate or low levels of CD177 expression.

Microarray data were analyzed with GeneSpring GX 10.0. After filtering with the criterion that expression values are present in the upper 75% range of all entities in at least one group of samples, the numbers of genes with fold change>2.0 differences in expression levels between either two groups are depicted. In the Venn diagram, each circle represents a gene list generated from a comparison between either two groups of CD177<sub>high</sub>, CD177<sub>low</sub> and CD177<sub>moderate</sub> expressing neutrophils. Overlapping areas of two or three circles show the genes shared by the two or three sets of comparisons, in which the numbers show the amount of genes.
Table 2: Genes differentially expressed (FDR-corrected) with high, moderate, and low levels of CD71 expression.

<table>
<thead>
<tr>
<th>Fold change</th>
<th>Gene Symbol</th>
<th>Gene Name and Annotation</th>
<th>Gene Name and Annotation</th>
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<td>3.97</td>
<td>CD71</td>
<td>Intracellular superoxide dismutase 1</td>
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</tr>
<tr>
<td>2.96</td>
<td>CD71</td>
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<tr>
<td>1.45</td>
<td>CD71</td>
<td>Mitochondrial matrix protein</td>
<td></td>
</tr>
<tr>
<td>-2.95</td>
<td>CD71</td>
<td>Intracellular superoxide dismutase 1</td>
<td></td>
</tr>
<tr>
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<td>CD71</td>
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<tr>
<td>-1.44</td>
<td>CD71</td>
<td>Mitochondrial matrix protein</td>
<td></td>
</tr>
</tbody>
</table>

Method: Genes are grouped with high, moderate, and low levels of CD71 expression.
Subsequently, we analyzed differential gene expression between sorted CD177⁺ and CD177⁻ neutrophil subsets. Among analyzed transcripts, CTSG (a protease participating in killing and digestion of engulfed pathogens), ADAM28 (with implicated functions in cell-cell and cell-matrix interactions) and EIF4E (the rate-limiting component of the eukaryotic translation apparatus involved in the mRNA-ribosome binding step of eukaryotic protein synthesis) showed significant increases in the CD177⁻ subset while ADAMTSL4 (a member of the ADAMTS [a disintegrin and metalloproteinase with thrombospondin motifs]-like gene family) expression was significantly lower in CD177⁻ cells than in CD177⁺ cells. To gain a better overview of gene expression profile of these neutrophil subsets, fc analysis was also performed. This resulted in 14 genes with fc>3.0 differences in expression between the two subsets (Table 3). Interestingly, 10 of these genes (71%) have been reported to change significantly in expression during neutrophil maturation, and most of them were granule protein (GP) coding genes. Moreover, all 10 genes, displaying up- or down-regulation in BM-neutrophils compared to early-staged neutrophil precursors, showed accordingly higher or lower expression levels in the CD177⁺ subset as compared to the CD177⁻ subset. We speculated that differential expression of GP-related genes, which also represent different stages in neutrophil maturation, might be one of the features distinguishing the CD177⁻ neutrophil subset from the positive subset. Therefore, expression of 43 GP-related genes was compared between the two subsets. Strikingly, 35 (81%) genes showed higher expression levels in CD177⁻ neutrophils as compared to the CD177⁺ subset. Genes with fc>1.5 differences in expression levels are listed in Table 4.

**Validation of differential expression of granule proteins by q-PCR and Western Blot**

To further confirm the findings, we analyzed gene expression of 8 granule proteins, most expressed in azurophilic or specific granules, including MPO, PR3, defensin α1, α3, and α4, cathepsin G, BPI and NGAL by q-PCR. Expression of CD177 and β-actin was also tested as sorting control or endogenous reference. Confirming proper sorting, CD177-mRNA was low/absent from CD177⁻ neutrophils, but expressed at significantly higher level in the CD177⁺ subset. Levels of CD177-mRNA in the total population of neutrophils positively correlated with the proportions of CD177⁺ neutrophils (r=0.6257, p=0.0042, Figure 2B). Results showed that cathepsin G-mRNA was undetectable and MPO expression was low and showed no difference between the two subsets. All the other granule proteins, PR3, defensin α1, α3 and α4, BPI and NGAL, showed a significantly higher mRNA level in CD177⁻ neutrophils than in CD177⁺ cells (Figure 2A).
Moreover, the percentage of CD177⁺ neutrophils negatively correlated with the mRNA level of MPO, BPI, elastase and defensin α4 (Figure 2B).

Figure 2. Expression of GP-related genes measured by taqman RT-PCR.
Expression levels of mRNA of CD177, PR3 (PRTN3), MPO, defensin α1, α3 and α4 (DEFA1,3 and 4), cathepsin G (CTSG), BPI, elastase (ELA2) and lipocalin 2 (LCN2) were measured by q-PCR. (A) Expression levels of GP-related genes in CD177⁺ and CD177⁻ subsets sorted from healthy donors (n=7) are depicted. Horizontal lines denote the medians. *, P<0.05. (B) Relative expression of GP-mRNA in the total neutrophil population isolated from healthy donors (n=19) significantly correlates with the percentages of CD177-expressing neutrophils.
Table 3. Genes with fold change>3.0 up/down regulated in the CD177+ subset as compared to the CD177- subset of neutrophils from HC.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>FC/Regulation</th>
<th>Synonym</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>DEFA4*#</td>
<td>8.81/down</td>
<td>HNP-4; HP4; HP-4; DEF4</td>
<td>Defensin, alpha 4, corticostatin.</td>
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<tr>
<td>LOC728358</td>
<td>8.16/down</td>
<td></td>
<td>Defensin, alpha 1.</td>
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<tr>
<td>DEFA3#</td>
<td>6.74/down</td>
<td>HNP3; HNP-3; DEF3; HP-3</td>
<td>Defensin, alpha 3, neutrophil-specific.</td>
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<tr>
<td>DEFA1*#</td>
<td>6.35/down</td>
<td>DEF1; HP-1; HNP-1</td>
<td>Defensin, alpha 1.</td>
</tr>
<tr>
<td>MS4A3*</td>
<td>5.20/down</td>
<td>CD201L; HTM4</td>
<td>Membrane-spanning 4-domains, subfamily A, member 3.</td>
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<td>CEACAM8*#</td>
<td>4.65/down</td>
<td>NCA-95; CD67; CD66b</td>
<td>Carinoembryonic antigen-related cell adhesion molecule 8.</td>
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<tr>
<td>CYBB*#</td>
<td>4.12/down</td>
<td>CGD; NOX2; GP91-1</td>
<td>Cytochrome b-245, beta polypeptide (chronic granulomatous disease).</td>
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<td>3.98/down</td>
<td>OflD; GW112; GC1</td>
<td>Olfactomedin 4.</td>
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<td>CEACAM6*#</td>
<td>3.95/down</td>
<td>CD66c; NCA; CEAL</td>
<td>Carinoembryonic antigen-related cell adhesion molecule 6.</td>
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<td>RNASE3*#</td>
<td>3.73/down</td>
<td>RNS3; ECP</td>
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<td>JTVI*</td>
<td>3.28/down</td>
<td>AIMP2; PRO0992; P38</td>
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<td>LCN2*#</td>
<td>3.24/down</td>
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<td>Lipocalin 2.</td>
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<td>ZCCHC11</td>
<td>3.22/up</td>
<td>PAPD3</td>
<td>Zinc finger, CCHC domain containing 11.</td>
</tr>
<tr>
<td>CPVL*</td>
<td>3.18/down</td>
<td>HVLP; MGC10029</td>
<td>Carboxypeptidase, vitellogenic-like.</td>
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</table>

N=3. *, Genes showing higher levels of expression in earlier stages of neutrophil maturation. #, genes related to neutrophil granule proteins.
Figure 3. Expression of granule proteins measured by quantitative Western Blot. Expression of CD177, PR3, MPO, defensin α3, cathepsin G and BPI in CD177⁺ and CD177⁻ subsets sorted from healthy donors (n=5) was measured and quantified by Western Blot (Odyssey). Expression of β-actin was included as loading control for each sample. Protein bands of granule proteins from 2 donors are shown (A). Results are presented as relative expression, that is percentage as compared to the signal intensity of the loading control (B).

To assess the differentially expressed GP-related genes at the protein level, we compared the amounts of granule proteins between CD177⁺ and CD177⁻ neutrophil subsets by quantitative Western Blot. CD177 protein (NB1) was absent in the lysates of membrane-bound CD177 (mCD177) negative neutrophils, but present in the positives. The amounts of GPs, taking PR3, cathepsin G, defensin α3 and BPI as representatives, were comparable between the two neutrophil subsets (Figure 3).

Membrane CD177 emerges gradually during neutrophil differentiation
As shown before, GP-related genes, which are supposed to be actively expressed in neutrophil precursors but remarkably down-regulated in mature neutrophils,⁲²
Figure 4. CD177 expression on neutrophil precursors in the bone marrow.
(A) Neutrophil precursors in the bone marrow were gated into myeloblasts/promyelocytes (MBs/PMs, SSC<sup>high</sup>CD<sub>45</sub><sup>mod</sup>CD<sub>16</sub><sup>low</sup>CD<sub>11b</sub><sup>low</sup>), metamyelocytes (MYs, SSC<sup>high</sup>CD<sub>45</sub><sup>mod</sup>CD<sub>16</sub><sup>low</sup>CD<sub>11b</sub><sup>high</sup>) and bone marrow neutrophils (BM-neutrophils, SSC<sup>high</sup>CD<sub>45</sub><sup>mod</sup>CD<sub>16</sub><sup>high</sup>CD<sub>11b</sub><sup>high</sup>). (B) CD177 expression was measured on the membrane of peripheral blood neutrophils and neutrophil precursors from each maturation stage, and expressed as mean fluorescence intensity in the histograms as representative samples. (C) Bone marrow samples from 4 donors were assessed for CD177 expression on neutrophil precursors. Each line represents one donor; dots denote the percentages of CD177<sup>+</sup> neutrophils in neutrophil precursors of the various differentiation stages.

were significantly increased in CD177<sup>+</sup> neutrophils in a healthy population, suggesting a link between lack of CD177 expression and immature neutrophils. Therefore, CD177 expression on the membrane of neutrophil precursors was measured in bone marrow (BM). Neutrophil maturation was divided into 3 stages according to SSC and membrane expression of CD45, CD11b and CD16, namely the myeloblast/promyelocyte stage (MBs/PMs), the metamyelocyte stage (MYs) and the stage of bone marrow neutrophils (BM-PMNs) (Figure 4A). In general, the percentage of CD177<sup>+</sup> neutrophils in BM increased towards the stage of BM-PMNs which are comparable with blood neutrophils (Figure 4B). In all of the 4 BM samples, CD177 expression was hardly detected on MBs/PMs and emerged on MYs. The percentages of CD177<sup>+</sup> MYs ranged from 19% to 43% and further increased to 60% in BM-PMNs in three donors and remained stable in the other donor (Figure 4C).
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Definition</th>
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<tr>
<td>NCG1</td>
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The C177 subset included 4 C177-related genes with fold change > 1.5 up-regulated expression in the C177 neutrophil subset as compared to...
Increased GP gene expression in AAV

As mentioned before, percentages of CD177\(^+\) neutrophils are elevated in patients with AAV. This subset shows lower transcription of GP genes than the CD177\(^-\) subset. It is, therefore, reasonable to assume that circulating neutrophils from AAV patients show a decreased expression of GP genes than HC. Total neutrophils were isolated from the peripheral blood of 8 patients with quiescent AAV and 19 healthy donors. Expression of CD177, MPO, PR3, defensin α1, α3, and α4, cathepsin G, BPI and NGAL was tested and compared between AAV patients and HC. Although most of these genes showed comparable or slightly higher levels of GP gene expression compared to HC, CD177\(^+\), DEFA3- and MPO-mRNA showed significantly increased expression levels in AAV patients (Figure 5A).

The influence of immature neutrophils on GP gene expression was tested and excluded by FACS analysis of peripheral blood, which showed that median percentages of CD16\(^\text{low}\) neutrophils, representing neutrophil maturation stages earlier than BM-PMNs, were low and comparable between AAV patients and controls (data not shown). However, one patient showed an increased percentage of immature neutrophils, which was further analyzed for CD177 expression. Percentage of CD177\(^+\) neutrophils was 43% in CD16\(^\text{low}\)CD16\(^\text{low}\) neutrophils and increased to 89% in CD16\(^\text{high}\)CD16\(^\text{low}\) cells. The percentage remained around 90% in the mature population, while the mean fluorescent intensity (MFI) was higher on mature neutrophils than on CD16\(^\text{high}\)CD16\(^\text{low}\) (immature) cells (Figure 5B).

Elevated levels of circulating proinflammatory cytokines have been reported in patients with AAV, which may cause in vivo activation of neutrophils.\(^{23}\) Whether GP gene expression in AAV is influenced by neutrophil activation was subsequently investigated. Neutrophils from healthy donors were ex vivo stimulated with PMA or LPS for 4 hours and total RNA was extracted for RT-PCR. Expression of CD177- and cathepsin G-mRNA was not influenced during activation, while transcription of NGAL, defensins, MPO, PR3 and BPI were all significantly induced by either stimuli (Figure 5C).

Discussion

In this microarray-based study, we investigated differences in gene expression profile between neutrophils from donors with varying levels of mCD177 and between CD177\(^+\) and CD177\(^-\) neutrophil subsets from the same individual, in order to clarify the pathophysiologic significance of an increased CD177\(^+\)/mPR3\(^{\text{high}}\) neutrophil subset for the pathogenesis of AAV.
Figure 5. Expression of GP-related genes in AAV.
(A) Expression levels of mRNA of CD177, PR3 (PRTN3), MPO, defensin α1, α3 and α4 (DEFA1, 3 and 4), cathepsin G (CTSG), BPI and lipocalin 2 (LCN2) were measured by q-PCR. Relative expression of GP-mRNA in the total neutrophil population was compared between patients with AAV (n=8) and healthy donors (n=19). Horizontal lines denote the medians. *, P<0.05; **, P<0.01. (B) Whole blood from an AAV patient was analyzed for the frequency of immature neutrophils. Three populations of neutrophils in different stages of maturation are observed. MFI of CD177 expression in these three populations is shown in the histogram. (C) Isolated neutrophils were stimulated with PMA or LPS for 4 hours. Total RNA was extracted and quantified for GP gene expression by RT-PCR. Results are presented as mean levels of fold induction related to unstimulated conditions. N=3; *, P<0.05.
Based on the data from this microarray analysis, neutrophils from donors positive in mCD177 expression, regardless of their expression level, are likely to share a comparable expression profile, being different from neutrophils of CD177 negative donors. However, by KEGG and GeneCodis pathway analysis, no pathways or annotations of biological functions were significantly enriched when analyzing the genes differentially expressed between donors with and without CD177-expressing neutrophils. Granule protein (GP)-related genes, at the mRNA level, were expressed at higher levels in the CD177+ neutrophil subset than in the CD177- subset, the underlying mechanism of which is not clear but may be related to the maturation state of neutrophils. Patients with AAV also displayed increased expression of GP-related genes in their neutrophils, which mismatches their higher percentage of CD177- neutrophils compared to HC. On-going inflammation in AAV might explain this observation, since mRNA expression of these GP-related genes could be induced during neutrophil activation.

Mechanisms underlying differential expression of CD177 on neutrophils are not fully understood. Polymorphisms in DNA sequence of CD177 that is, C34G, A778C and G1069A, or methylation in the CpG islands close to the promoter region have been suggested to be associated with a low percentage of CD177+ neutrophils in donors with bimodal expression of CD177.24-26 Total CD177-deficiency in healthy donors is probably due to other mechanisms, because it has been found that CD177-mRNA is detectable in CD177- donors with an abnormal insertion containing stop codon, but not in CD177- neutrophils from donors with bimodal expression of CD177.25-27 Donors without CD177 expression also displayed different properties compared to CD177-expressing donors in our microarray analysis, which showed 107 gene probes differentially expressed between total neutrophils from CD177-positive donors and donors with CD177 deficiency. However, these genes were described to be associated with various biological functions and no specific biological processes were indicated by the pathway analysis performed with KEGG pathways or GeneCodis functional annotation web-based tool. Besides, no studies have demonstrated abnormal neutrophil function in CD177-deficient healthy donors. Whether these genes are related to the regulation of CD177 expression or to distinct cellular functions between donors with and without CD177 expression needs to be answered by further investigation.

When analyzing the differences between the CD177+ and CD177- neutrophil subsets, GP-related genes showed higher expression levels in the CD177- subset than in CD177+ neutrophils from the same individual. As a result, GP gene expression negatively correlated with the proportion of CD177-expressing
neutrophils in a healthy population. These GP genes are variably expressed during neutrophil differentiation in the bone marrow and highly expressed in neutrophil precursors compared to mature neutrophils. Although circulating immature neutrophils are rarely seen in the normal situation, enriched expression of GP-related genes in both immature neutrophils and the CD177− subset suggests a relationship between CD177− and immature neutrophils. FACS analysis supported this assumption and revealed gradual emergence of CD177 on the membrane of neutrophils during maturation in the bone marrow. CD177+ cells appear from the stage of PMs/MBs on and their percentage increases towards the stage of BM-PMNs, which are comparable with circulating neutrophils. These data suggest that neutrophils are probably not a homogenous population and differences in expression of CD177 and GP-related genes might reflect a different state of maturation. On the other hand, neutrophil precursors in the bone marrow comprise lower proportions of CD177+ cells than blood neutrophils. When migrated into the blood, these immature neutrophils will be higher enriched in the CD177− subset than in the CD177+ neutrophil subset, thus, to some extent, also explain higher levels of GP gene expression in CD177− neutrophils. In any case, the underlying mechanism should be further investigated.

Cell functions are mostly dependent on proteins stored or produced within the cell. Although mRNA of GP-related genes are higher expressed in CD177− neutrophils, the amounts of granule proteins, except for CD177, were not significantly different between the two subsets. It has also been reported that changes of granule proteins and mRNA expression are not always identical, especially for the early produced proteins during neutrophil maturation, such as proteins stored in azurophilic granules, which are synthesized transiently at the promyelocyte or metamyelocyte stages, and remain stored in granules throughout the terminal granulocytic differentiation. However, not all of the genes showing differential expression between CD177+ and CD177− neutrophils were assessed for expression at the protein level. CYBB, encoding for the cytochrome-245 subunit, and NCF-1, neutrophil cytosolic factor 1, are both involved in NADPH oxidation and superoxide anion generation, suggesting their potential role in granulomatous diseases. To verify and compare the expression levels of these proteins between the CD177− and the CD177+ neutrophil subsets and between AAV patients and HC would also be of interest. However, based on the present data, the functional differences between the CD177+ and CD177− neutrophil subsets remain obscure.
Considering the situation in AAV, patients have an expanded subset of CD177+ neutrophils, which is supposed to be associated with decreased levels of GP gene expression, as based on the findings in healthy donors. However, our cohort of patients with quiescent AAV showed, conversely, increased expression of GP genes, and DEFA4 and MPO expression was significantly higher in these patients compared to controls. This is in line with an earlier study by Yang et al. The authors also observed significantly increased expression of GP genes in neutrophils from AAV patients, which was correlated with disease activity and absolute neutrophil number. However, the underlying mechanism was not delineated.\textsuperscript{28} In another autoimmune disease, that is systemic lupus erythematosus (SLE), a microarray study conducted by Bennett et al. showed increased expression of GP-related genes in patients compared to healthy controls. Meanwhile, they detected immature neutrophils in the peripheral blood of those patients, numbers of which correlated with increased expression of GP genes.\textsuperscript{29} However, this is unlikely the case in AAV, since comparable amounts of immature neutrophils were detected in patients and controls. Yang et al. have also shown that patients with acute infection, showing a so-called “left shift” of neutrophils, did not display higher levels of GP gene expression compared to HC.\textsuperscript{28} Otherwise, it has been suggested that low levels of immune activation exist in AAV patients even in remission.\textsuperscript{30} Thus, on-going inflammation in AAV patients may be responsible for GP gene activation. Indeed, upregulated expression of these GP genes could be induced during neutrophil activation. Furthermore, it has to be considered that most neutrophils are not in the circulation but sequestered in the marginalizing pool, which has been poorly described with respect to phenotype and function.\textsuperscript{31} Better understanding of margined neutrophils, concerning mCD177 and GP gene expression, and regulation of the balance between margined and circulating neutrophils will give more insight into this issue.

In summary, the neutrophil population is not homogenous and can be discriminated by membrane expression of CD177 into subsets which are different in expression of GP-related genes but, probably, comparable at the protein level. GP gene expression is also elevated in AAV patients, which is not explained by skewed distribution of CD177\textsuperscript{+} and CD177\textsuperscript{−} subsets, but may be associated with neutrophil activation during on-going inflammation.
References

Summary and general discussion
Summary
In this thesis, the interaction between neutrophils and endothelial cells in AAV was investigated, in particular the mechanisms containing neutrophils within the microvascular compartment in connection with the expression of PR3 on the neutrophil membrane.

The pathogenesis of AAV has not been fully understood, and it is intriguing that ANCA-associated vascular damage has a predilection for small-sized vessels. Capillaries and venules are the loci where leukocyte trafficking takes place during inflammation, and the endothelium of these vessels is particularly responsive to proinflammatory signals. There is no direct evidence that ANCA from AAV patients bind to and activate their own neutrophils in the circulation. In addition, ROS or degranulates released into the circulation by activated neutrophils will be rapidly diluted via blood flow or blocked by circulating inhibitors. So, these non-specific reagents may only damage the blood vessels when they get into close contact with vascular ECs. In Chapter 2, the interaction between neutrophils and endothelial cells in AAV and the effector mechanisms causing vascular damage were reviewed. We hypothesize that the synapse-like interaction formed between the neutrophil and the endothelial surface during adhesion or transmigration is the real battlefield for ANCA-activated neutrophils and the endothelium, and a prerequisite for the persistent inflammation in the vessel wall. Several factors, such as ANCA, proinflammatory and chemotactic cytokines are involved in this process. ANCA further activate neutrophils and cause release of ROS and proteolytic enzymes, which are concentrated close to adjacent endothelial cells by NETs and directly attack the vessel wall or activate endothelial cells resulting in more leukocyte recruitment. Activated endothelial cells are also actively involved in this sticky neutrophil-endothelial interaction by undergoing a protective mechanism against ROS and producing chemokines and cytokines. It is a complex process and involves many adhesion molecules, chemoattractants and their receptors, and immune modulators, which could offer plenty of opportunities for disease intervention.

We first tested the hypothesis that down-regulated expression of CXCR1/2 retains neutrophils within the vessel wall and, consequently, leads to persistence of neutrophils within the microvasculature. In Chapter 3, membrane expression of CXCR1 and CXCR2 on neutrophils was measured in a group of AAV patients in remission in comparison to HC. Serum levels of IL-8, TNF-α, ANGPT-1 and ANGPT-2 from quiescent and active AAV patients and HC were quantified.
Adhesion and transendothelial migration of isolated neutrophils was analyzed, with and without blockade of CXCR1 and CXCR2. Expression of CXCR1 and CXCR2 on neutrophils was significantly decreased in AAV compared to HC. Levels of IL-8, which dose-dependently down-regulated CXCR1 and CXCR2 expression on neutrophils in vitro, were significantly increased in the serum of patients with active AAV and correlated negatively with CXCR1/CXCR2 expression on neutrophils, even in quiescent patients. Blocking CXCR1 and CXCR2 with repertaxin, a specific inhibitor of these chemokine receptors, increased neutrophil adhesion and inhibited migration through a glomerular endothelial cell layer. It can be speculated that, in vivo, circulating IL-8 produced by activated endothelial cells or ANCA-activated neutrophils leads to decreased CXCR1 and CXCR2 expression on neutrophils, which, in turn, show increased adhesion and deficiency in transendothelial migration. Neutrophils accumulating in the microvascular compartment are subsequently activated by ANCA, and released ROS and proteolytic enzymes cause vessel damage.

As for the effector mechanisms causing damage to the vessel wall in AAV, the role of AECA is interesting but has been poorly analyzed. The presence of AECA in AAV has been reported by several groups with conflicting data regarding their prevalence ranging from 8% to 100% in AAV patients. Increased binding of AECA to endothelial cells isolated from nose, kidney and lungs, which are the most frequently involved organs in AAV, was demonstrated. These results suggest that AECA in AAV patients are organ-specific and could imply that, as substrates, endothelial cells from relevant organs should be used in AECA detection. Therefore, in Chapter 4, we investigated the prevalence of AECA in AAV using a human glomerular endothelial cell (GenC) line in comparison with primary human umbilical vein endothelial cells (HUVEC), which are frequently used for AECA detection. As AECA might induce endothelial activation, serum levels of adhesion molecules, markers of endothelial activation, were also analyzed in a group of AAV patients. Generally, AECA had low frequency in AAV patients. AECA were detected in 4 of 29 WG patients (14%) and in none of 14 MPA patients using conditionally immortalized GenCs as substrate, whereas AECA were positive in 10% of WG patients and 14% of MPA patients on HUVEC. No significant difference in OD value was found between AAV patients and controls in AECA testing. Serum levels of soluble VCAM-1 and ICAM-1 in AAV patients were significantly higher than in controls. However, no correlation was found between AECA titers and levels of soluble adhesion molecules and there were no differences between AECA-positive and -negative patients for both activation
markers. Theoretically, it could be helpful to use more than one type of substrate cells for AECA testing in order to increase their detection rate. However, the pathogenic relevance of AECA is doubtful, and elucidating the antigens of AECA in AAV is a prerequisite for further assessing their diagnostic and pathogenic role.

Membrane expression of ANCA-antigens, such as PR3, allows ANCA binding, is a crucial step in ANCA-mediated neutrophil activation, and has been shown to be significantly up-regulated during neutrophil adhesion. The PR3 molecule does not contain a transmembrane domain in its sequence, The mechanisms of membrane expression of PR3 and the signal transduction events following ANCA binding, therefore, become interesting and are reviewed in Chapter 5. PR3 is differentially expressed on the neutrophil membrane. The percentage of neutrophils with high levels of mPR3 expression ranges from 0 to 100%, and is rather constant within a given individual. CD177 is the receptor of mPR3 accounting for a substantial expression of mPR3 on the neutrophil membrane. On this CD177+ subset of neutrophils, a complex of molecules colocalizes with PR3 on the neutrophil membrane and appears to be involved in signal transduction, including CD177, FcγRIIIb and β2-integrins. This complex probably functions in neutrophil recruitment, bringing these effector cells close to the endothelium and further mediates PR3-ANCA induced neutrophil activation. However, slight expression of mPR3 on CD177− neutrophils can also be detected, suggesting that CD177 is not an exclusive binding partner of mPR3. Other possible binding site(s) for PR3 and mechanism(s) involved in signal transduction, such as chondroitin sulfate- and heparin sulfate- containing proteoglycans, PLSCR1 and hydrophobic insertion, need further investigation. Possibly, these various molecules allow the two subsets of neutrophils to be equally involved in the pathophysiology of PR3-ANCA associated vasculitis regardless of CD177 expression.

The percentage of mPR3high neutrophils is increased in AAV patients and is a risk factor for relapse of GPA. Whether CD177 expression is also increased in AAV and responsible for mPR3 up-regulation, and what the role of CD177 is in PR3-ANCA-mediated neutrophil activation in AAV was investigated in Chapter 6. Expression of CD177 and mPR3 was analyzed in parallel on isolated neutrophils from patients with AAV, SLE, or RA, and healthy controls. Neutrophil activation mediated by anti-PR3 antibodies was assessed by measuring the oxidative burst. Percentages of CD177+ neutrophils were significantly higher in patients with AAV and SLE compared to healthy controls. In 3 healthy donors, CD177 expression was not
detected. After priming with TNF-α, neutrophils from these 3 donors remained negative for CD177 while mPR3 expression was induced. Neutrophils from CD177+ donors or CD177− neutrophils sorted from donors with bimodal expression were susceptible to anti-PR3-mediated oxidative burst. Variation in the extent of anti-PR3-mediated neutrophil activation among different donors occurred independent of the percentage of CD177-expressing neutrophils. These data confirmed our hypothesis that CD177-independent mPR3 expression does exist and both CD177+ and CD177− neutrophils are susceptible for PR3-ANCA-mediated neutrophil activation.

Next, we studied differences between neutrophil subsets with and without CD177 expression. The molecular function of CD177 is largely unknown, except that it is a counterpart of PECAM-1 on endothelial cells, suggesting a role in neutrophil recruitment to the microvasculature. However, we did not observe significant differences in neutrophil adhesion or migration through GenC monolayers between these two subsets (data not included in this thesis). Therefore, a microarray-based study was performed (Chapter 7). Differentially expressed genes between CD177+ and CD177− neutrophil subsets might indicate differences in function. Gene expression in neutrophils was compared among donors with varying levels of CD177 expression, and between CD177+ and CD177− subpopulations from donors with bimodal expression of CD177. A number of neutrophil granule proteins (GP), such as defensin α1, α3 and α4, NGAL, BPI or cathepsin G, which decline during neutrophil maturation, showed higher mRNA expression levels in the CD177− neutrophil subset in healthy donors, suggesting a link between CD177− and immature neutrophils. Indeed, by FACS analysis, we observed that CD177 expression emerges during neutrophil development. Therefore, differential expression of GP-related genes between CD177+ and CD177− neutrophil subsets might reflect a different state of neutrophil maturation or different levels of enrichment with immature neutrophils. The amounts of these granule proteins stored in neutrophils, however, were comparable between CD177+ and CD177− subsets; thus, functional differences at the protein level were not observed. Interestingly, up-regulation of these GP genes was also observed in AAV patients, which was unlikely to be related to a disturbed balance of the CD177+ and CD177− subsets or an enrichment of immature neutrophils. Induction of GP gene expression in neutrophils by PMA or LPS suggests that on-going inflammation in AAV patients might be the reason for up-regulation of genes encoding granule proteins.
General discussion

It is an important feature of AAV that necrotic lesions, with paucity of immune complex deposition, occur preferentially in small-sized vessels. Neutrophils are the cells expressing ANCA antigens and also the cells producing ROS and proteolytic enzymes, which induce necrosis of endothelial cells. Early infiltration of large amounts of neutrophils in the target vasculature suggests that neutrophil accumulation is a sign of an upcoming vascular lesion and a prerequisite of ANCA-mediated vascular damage. It has been suggested that circulating neutrophils are unlikely to be activated by ANCA and to undergo the respiratory burst and degranulation, while adherent neutrophils attack endothelial cells. Neutrophil recruitment is a cascade process. Once triggered, rolling neutrophils will adhere to and subsequently migrate through the endothelium. Following these cascades, one would expect ANCA-mediated tissue damage to occur not only in the vessel wall but also in the interstitial tissues, which is obviously not the case. Careful study of renal biopsies from AAV patients revealed that neutrophils are accumulated in the glomeruli but poorly penetrate into the interstitial tissue where neutrophil chemoattractants were detected. Taken together, it is likely that neutrophil trafficking is triggered in AAV but it is stuck in the middle of the process by certain mechanisms. Therefore, answering the questions which mechanisms hamper neutrophil recruitment and what exactly happens during neutrophil-endothelial cell interaction are essential for understanding disease pathogenesis and for the development of novel therapeutic strategies.

Dysregulated neutrophil-endothelial interaction in AAV involves ANCA and proinflammatory cytokines in the circulation, which stimulate both neutrophils and endothelial cells leading to up-regulation or activation of adhesion molecules. In the current thesis, a chemokine-based mechanism is highlighted. CXCR1 and CXCR2 expression is significantly decreased on neutrophils from AAV patients and this decrease seems to be correlating with disease activity. Circulating IL-8, which shows increased levels in AAV, may be one of the factors inducing decreased expression of these chemokine receptors. As a consequence, neutrophils with decreased expression of CXCR1 and CXCR2 display increased adhesion and decreased migration through glomerular endothelium. These data suggest a relationship between circulating IL-8, chemokine receptors and neutrophil accumulation in the vessel wall. However, the role of CXCR1/2 in neutrophil adhesion has not been fully explained, as it has been shown that CXCR2 activation transduces a signal leading to firm adhesion. Therefore, the
detailed mechanisms of increased adhesion of CXCR1/2 deficient neutrophils deserves further investigation. Particularly, the adhesion molecules involved in this process and the structural or functional features of glomerular endothelial cells leading to these consequences are of great interest.

The effector mechanisms causing vascular damage are mainly ANCA-associated events. ANCA activate primed neutrophils leading to release of ROS and proteolytic enzymes, which directly attack the vessel wall and stimulate endothelial cells to recruit more inflammatory cells. NETs are formed by activated neutrophils. This structure may concentrate PR3 and MPO on the neutrophil surface for ANCA recognition and attacking of endothelial cells. AECA have been documented in AAV. However, their specific antigen(s) on endothelial cells have not been clarified so far. Our study on AECA in this thesis does not support a pathogenic role for AECA, but lets the possibility open that planted ANCA antigens on endothelial cells mediate ANCA-induced necrotic damage.

ANCA recognition of PR3 or MPO on the neutrophil membrane is a prerequisite for neutrophil activation. Membrane expression of these ANCA antigens is inducible by proinflammatory cytokines, such as TNF-α and IL-1β, and mPR3 expression is remarkably up-regulated during neutrophil adhesion. CD177 has been demonstrated to be a receptor of mPR3 on neutrophils. However, the detailed mechanisms of the physical interaction of these two molecules are not clear. As CD177 is possibly an adhesion molecule, and β2-integrins, key adhesion molecules on neutrophils, are colocalized with mPR3 in lipid rafts and mediate signals into the cell upon PR3-ANCA ligation, membrane expression of PR3 is no longer an event independent from neutrophil-endothelial interaction. In this context, a complex of molecules that colocalize with PR3, including CD177 and β2-integrins, on the neutrophil membrane appears to be involved. This complex probably functions in neutrophil recruitment by bringing these effector cells close to the endothelium and further mediating PR3-ANCA induced neutrophil activation and vessel damage.

We showed that the percentage of CD177⁺ neutrophils is increased in AAV, which accounts for an enlarged subset of neutrophils with mPR3high expression. This skewed distribution could be genetically determined. However, increased CD177⁺ neutrophil proportions have been observed in some other pathological conditions, such as severe infection, G-CSF treatment or pregnancy, suggesting that other mechanism(s) exist modulating the balance between these neutrophil subsets. This mechanism may become self-evident when the basic question is answered.
why CD177 is differentially expressed in neutrophils. Functional consequences of increased CD177/mPR3 expression in AAV depend on the molecular function of CD177 and its implication on differences in function, if any, between CD177+ and CD177− neutrophil subsets. The ability of CD177 to bind to PECAM-1 does not result in differences in adhesion or migration between these two subsets. Neutrophils are equally activated by PR3-ANCA, regardless of CD177 expression, suggesting that CD177 is not involved in the signaling pathway upon PR3-ANCA ligation. Finally, whole genome-wide analysis of gene expression revealed that granule protein-related genes are expressed at higher levels in the CD177+ than in the CD177− neutrophil subset. This observation can be a departure point for further disclosure of the characteristics of these neutrophil subsets and the pathological role of this molecule in AAV.

In conclusion, the interaction between neutrophils and endothelial cells is crucial for disease development and intervention. Decreased expression of CXCR1 and CXCR2 on neutrophils in AAV is instrumental in neutrophil accumulation in the microvascular compartment. Effector mechanisms become operational in this process, but the role of AECA is minor. Membrane expression of ANCA-antigens, such as PR3, is induced during neutrophil recruitment. CD177 is responsible for mPR3 expression, but it is not essential for PR3-ANCA-mediated neutrophil activation. The underlying mechanism of the increased percentage of CD177+/PR3high neutrophils in AAV is not clear. Differences in gene expression between CD177+ and CD177− subsets cannot easily be translated into functional differences, but indicate heterogeneity of neutrophil populations. This heterogeneity is dysbalanced in AAV, but its exact pathophysiological role awaits further studies.
Nederlandse Samenvatting
In dit proefschrift is de interactie tussen granulocyten en endotheelcellen in AAV (ANCA-associated vasculitis) onderzocht, vooral de mechanismen waardoor granulocyten achterblijven in de wand van bloedvaten, en de rol die expressie van het eiwit proteinase 3 (PR3) op de membraan van granulocyten hierin speelt.

De pathogenese van AAV is nog niet helemaal ontrafeld, maar het is intrigerend dat de vaatschade, die geassocieerd is met de aanwezigheid van anti-neutrofiel cytoplasmatische autoantistoffen (ANCA), bij voorkeur voorkomt in kleine vaten. Haarvaten en venulen zijn met name de plaatsen waar het uittreden van witte bloedcellen tijdens ontstekingsplaatsvindt, en de endotheelcellen zijn daar erg gevoelig voor ontstekingssignalen. Er is geen bewijs dat ANCA bij AAV patiënten in de bloedcirculatie aan granulocyten binden en ze activeren. Bovendien zouden de eiwitten en enzymen die vrijkomen uit de geactiveerde granulocyten in de circulatie snel verdund worden in de bloedstroom of geblokkeerd worden door remmers, en zo geen schade aan de wand van bloedvaten veroorzaken. Dus kunnen deze producten van granulocyt alleen schade toebrengen aan de vaten als ze rechtstreeks in contact komen met de endotheelcellen die de wand van bloedvaten bekleden. In Hoofdstuk 2 is de interactie tussen granulocyten en endotheelcellen bij AAV besproken en de mechanismen die de vaatschade veroorzaken. Wij veronderstellen dat de synapse-achtige interactie die gevormd wordt tussen het granulocyt- en endotheelooppervlak tijdens adhesie of transmigratie van endotheelcellen een soort slagveld is, en noodzakelijk voor de voortdurende ontsteking in de vaatwand. Vele factoren, zoals ANCA, ontstekings- en chemotactische cytokines zijn betrokken bij dit proces. ANCA activeren de granulocyten en veroorzaken het vrijkomen van reaktieve zuurstof metabolieten (ROS - reactive oxygen species) en proteolytische enzymen. Deze worden door NETs (neutrophil extracellular traps) geconcentreerd vlakbij de endotheelcellen, waardoor de vaatwand beschadigd wordt of waardoor de endotheelcellen geactiveerd worden wat leidt tot nog meer influx van leukocyten. Het is een gecompliceerd proces waarbij vele adhesiemoleculen, chemo-attractanten en hun receptoren, en immuun-modulatoren betrokken zijn, die daardoor ook allemaal de mogelijkheid bieden voor interventie in de ziekte.

We hebben eerst de hypothese getest dat verlaagde expressie van de chemokine-receptoren CXCR1 en CXCR2 de granulocyten bij de vaatwand houdt, waardoor de granulocyten in de kleine vaten blijven steken. In Hoofdstuk 3 is de expressie van CXCR1 en CXCR2 op de membraan van granulocyten gemeten bij AAV patiënten in remissie in vergelijking tot gezonde controles. Serumwaarden van
IL-8, TNF-α, Angiopoietin-1 en -2 van patiënten met rustige en actieve ziekte en controles zijn gemeten. Vervolgens is de adhesie en de trans-endotheliale migratie van granulocyten bepaald, met en zonder blokkering van CXCR1 en CXCR2. De expressie van CXCR1 en CXCR2 was significant verlaagd bij AAV patiënten in vergelijking met de controles. In vitro vonden we dat IL-8 de CXCR1 en CXCR2 expressie op granulocyten dosis afhankelijk verlaagd en bovendien dat de serum IL-8 spiegels bij patiënten met actieve AAV verhoogd waren. Verder waren de IL-8 spiegels negatief gecorreleerd met de CXCR1/CXCR2 expressie op granulocyten, ook bij patiënten met rustige ziekte. Het blokkeren van CXCR1 en CXCR2 met repertaxin, een specifieke remmer van deze chemokine receptoren, verhoogde de adhesie van granulocyten, maar verhinderde de migratie door een laag van glomerulaire endotheelcellen. Men zou kunnen speculeren dat, in vivo, circulerend IL-8 geproduceerd door geactiveerde endotheelcellen of granulocyten, de CXCR1 en CXCR2 expressie op granulocyten verlaagd, waardoor deze vervolgens een verhoogde adhesie en verlaagde trans-endotheliale migratie laten zien. De granulocyten, die hierdoor accumuleren in het micro-vasculaire compartiment worden vervolgens geactiveerd door ANCA, en de ROS en enzymen die hierdoor vrijkomen veroorzaken de schade aan de vaatwand.

Wat betreft de mechanismen die vaatschade veroorzaken bij AAV is de rol van andere autoantistoffen, de AECA (anti-endothelial cell autoantibodies) interessant maar nog slecht onderzocht. De aanwezigheid van AECA bij patiënten met AAV is door verschillende groepen gerapporteerd maar met tegenstrijdige data wat betreft het voorkomen, namelijk variërend van 8% tot 100%. Verhoogde binding van AECA werd aangetoond aan endotheliale cellen geïsoleerd uit de neus-, de nier- of longweefsel, de organen die het meest betrokken zijn bij AAV. Deze resultaten doen vermoeden dat AECA bij AAV orgaanspecifiek zijn en dat zou kunnen betekenen dat als substraat voor AECA detectie endotheliale cellen van relevante organen gebruikt zouden moeten worden. Daarom hebben we in Hoofdstuk 4 de prevalentie van AECA in AAV onderzocht en daarbij een humane glomerulaire (nier) endotheliale (GENC) cellijn gebruikt in vergelijking met primaire HUVEC (human umbilical vein endothelial cells), die vaak gebruikt worden voor detectie van AECA. Aangezien AECA endotheelcellen kunnen activeren, zijn ook serum spiegels gemeten van adhesie moleculen, als zijnde merkers van endotheel activatie. In het algemeen was er een lage frequentie van AECA bij patiënten met AAV. AECA werden gevonden in 4 van de 29 patiënten met de ziekte van Wegener (14%) en bij geen van de MPA patiënten wanneer de GENCs als substraat werden gebruikt, terwijl AECA positief waren in 10% van de
Wegener en in 14% van de MPA patiënten wanneer HUVEC als substraat gebruikt werden. Er werd geen significant verschil gevonden in de hoogte van de AECA test waarden tussen AAV patiënten en controles. De serum waarden van circulerend VCAM-1 en ICAM-1, endotheliale adhesiemoleculen, bij AAV patiënten waren significant verhoogd ten opzichte van controles. Er werd echter geen correlatie gevonden tussen AECA titers en de spiegels van de circulerende adhesiemoleculen. Ook was er geen verschil tussen AECA-positieve en -negatieve patiënten wat betreft de endotheliale activatiemerkers. Theoretisch zou het van belang kunnen zijn om meer dan één type substraatcellen te gebruiken om de kans om AECA te detecteren te vergroten. De pathogenetische betekenis van AECA is echter nog onzeker, en het zal eerst nodig zijn om de antigenen waartegen AECA reageren te ontmaskeren alvorens de diagnostische en pathogenetische betekenis vast te kunnen stellen.

Membraanexpressie van ANCA-antigenen, zoals proteinase 3 (PR3), is nodig voor binding van ANCA en is een cruciaal onderdeel van de ANCA-gemediëerde granulocyt activatie. PR3 expressie is sterk verhoogd tijdens de adhesie van granulocyten. Het PR3 molecuul heeft geen transmembraan domein in zijn sequentie. De mechanismen hoe PR3 tot expressie komt en de signaal transductie na binding van ANCA zijn daarom interessant en worden besproken in Hoofdstuk 5. PR3 komt verschillend tot expressie op de membraan van granulocyten, de percentages variëren van 0 tot 100% tussen individuen, en zijn min of meer constant in een persoon. CD177 is de receptor van PR3 en verantwoordelijk voor het grootste deel van de expressie van PR3 op de membraan van de granulocyt. Bij de CD177* subset van de granulocyten vindt co-localisatie plaats van een aantal moleculen samen met PR3 op de membraan en dit complex bestaande uit CD177, PR3, FcyRIIib en β2-integrines is betrokken bij de signaal transductie. Dit complex heeft waarschijnlijk een rol in het rekruteren van granulocyten, het leiden van de cellen naar het endotheel en vervolgens bij de via binding aan PR3 verlopende activatie door ANCA gericht tegen PR3. Echter, ook een lage expressie van CD177 op granulocyten wordt gevonden en ook deze granulocyten kunnen geactiveerd worden door PR3-ANCA, wat doet vermoeden dat CD177 niet de enige receptor van PR3 is. Andere mogelijke receptoren en mechanismen betrokken bij signaal transductie zullen nader onderzocht moeten worden, zoals bijvoorbeeld chondroitine sulfaat- en heparansulfaat- bevattende proteoglycanen, PLSCR1 (phosholipid scramblase 1), en hydrofobe insertie. Het is mogelijk dat deze verschillende moleculen het mogelijk maken dat zowel granulocyten met lage als met hoge PR3 expressie op
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de membraan betrokken zijn bij de pathofysiologie van PR3-ANCA geassocieerde vasculitis onafhankelijk van CD177 expressie.

Het percentage van granulocytten met hoge membraan expressie van PR3 (mPR3) is verhoogd bij AAV patiënten en is een risicofactor voor het optreden van een recidief van de ziekte van Wegener. In Hoofdstuk 6 is onderzocht of CD177 expressie ook verhoogd is bij AAV en verantwoordelijk is voor mPR3 op-regulatie, en wat de rol is van CD177 bij PR3-ANCA gemediëerde granulocyt activatie. De expressie van CD177 en PR3 is geanalyseerd op geïsoleerde granulocytten van patiënten met AAV, SLE, reumatoïde artritis en gezonde controles. Granulocyt activatie ten gevolge van anti-PR3 antilichamen werd bepaald door het meten van uitstoot van zuurstofradicalen. Het percentage van CD177+ granulocytten was significant hoger bij AAV en SLE patiënten vergeleken met controles. Bij 3 controles werd helemaal geen CD177 expressie gemeten. Voorincubatie van de granulocytten van deze donoren met TNF-α leidde niet tot CD177 expressie, maar wel tot PR3 expressie. Granulocytten van CD177+ donoren of de fractie CD177- granulocytten van donoren met een bimodale expressie van CD177 waren echter wel in staat tot zuurstof radicalen productie na stimulatie met anti-PR3. De variatie in hoogte van de anti-PR3 gemediëerde granulocyt activatie was onafhankelijk van het percentage CD177 positieve granulocytten. Deze data bevestigen onze hypothese dat CD177-onafhankelijke mPR3 expressie voorkomt en dat ook CD177- granulocytten geactiveerd kunnen worden door PR3-ANCA.

Vervolgens hebben we de verschillen bestudeerd tussen granulocytten subsets met en zonder CD177 expressie. De functie van CD177 is onbekend, behalve dat het een ligand is van PECAM-1, een adhesie molecuul op endotheliale cellen, en dit doet vermoeden dat CD177 een rol zou kunnen spelen in granulocyt migratie naar de micro-vasculatuur. Wij zagen echter geen significant verschil tussen de subsets wat betreft granulocyt adhesie of migratie door een GEnC cellaag (data niet geïncludeerd in dit proefschrift). Daarom is een micro-array studie uitgevoerd in Hoofdstuk 7, omdat verschillen in genen in CD177 subsets zouden kunnen duiden op verschillen in functie. Gen expressie in granulocytten van donoren met verschillende CD177 expressie werd vergeleken, en ook tussen CD177+ en CD177- subpopulaties van donoren met bimodale expressie. Een aantal granule eiwitten, zoals defensin α1, α3, en α4, NGAL, BPI, en cathepsin G, die in hoeveelheid afnemen tijdens rijping van de granulocyt, hadden een hoge mRNA expressie in CD177- granulocytten. Dit zou kunnen betekenen dat er een relatie is tussen onrijpe granulocytten en de CD177- populatie, en inderdaad vonden we met
Behulp van FACS analyse dat CD177 expressie opkomt tijdens granulocyt differentiatie. Verschillen in expressie van genen van granule eiwitten in de CD177 subsets zouden een verschillende differentiatie status van granulocyten kunnen weergeven. Echter de hoeveelheden van de granule eiwitten, opgeslagen in de granulocyten, waren niet verschillend tussen de CD177⁺ en CD177⁻ subsets; dus functionele verschillen op eiwitniveau werden niet gevonden. Deze verhoogde expressie van genen coderend voor granule eiwitten was al eerder gevonden bij AAV patiënten, maar het is onwaarschijnlijk dat dit het gevolg is van een verstoorde balans van CD177⁺ en CD177⁻ subsets of een verrijking met onrijpe granulocyten. De inductie van gen expressie van granule eiwitten die wordt gevonden na stimulatie van granulocyten met PMA of LPS, zou kunnen betekenen dat de voortdurende ontsteking bij AAV patiënten de werkelijke oorzaak is van de verhoogde genexpressie van granule eiwitten.

**Algemene discussie**

Een belangrijk kenmerk van AAV is dat de necrotische lesies, die weinig deposities van immuuncomplexen laten zien, met name voorkomen in de kleine vaten. Granulocyten zijn de cellen die ANCA antigenen dragen en de producenten van ROS en proteolytische enzymen, wat leidt tot necrose van de endotheel cellen. Vroege adherentie van grote hoeveelheden granulocyten in de vaten suggereert dat de ophoping van granulocyten een teken is van opkomende vaatschade en een vereiste voor ANCA-gemediaerde vaatschade. Men neemt aan dat circulerende granulocyten niet gemakkelijk geactiveerd worden door ANCA, maar dat adherente granulocyten na activatie door ANCA endotheel cellen beschadigen. De rekrutering van granulocyten is een proces van opeenvolgende stappen. Wanneer de granulocyten rollen over het endotheel zullen ze daarna adhereren en vervolgens migreren. Men zou daarom verwachten dat de ANCA-gemediëerde weefsel schade niet alleen in de vaatwand optreedt, maar ook in het onderliggende weefsel, wat echter niet het geval is. Studies van nier-biopaten van AAV patiënten hebben laten zien dat de granulocyten, die ophopen in de glomeruli slecht doordringen in het interstitiële weefsel, hoewel daar chemo-attractanten voor granulocyten aanwezig zijn. Het lijkt erop dat de mobilisatie van granulocyten op gang is gebracht bij AAV, maar dat het stagneert door bepaalde mechanismen. Om het ziekte proces te begrijpen en nieuwe geneesmiddelen te ontwikkelen is het daarom heel belangrijk om te onderzoeken welke mechanismen de rekrutering van granulocyten tot in de weefsels belemmeren en wat er gebeurt tijdens granulocyt-endotheelcel interactie.
ANCA en cytokines in de bloedbaan zijn betrokken bij de verstoorde granulocyt-endothel interactie bij AAV, en beiden stimuleren granulocyt en endothelcellen wat leidt tot expressie van adhesie moleculen. In dit proefschrift is een mechanisme gebaseerd op chemokines toegelicht. Expressie van CXCR1 en CXCR2 is significant verlaagd op granulocyten van AAV patiënten, en deze verlaging is gecorreleerd met ziekteactiviteit. Circulerend IL-8, wat verhoogd voorkomt bij AAV, kan één van de factoren zijn waardoor de chemokine receptoren verlaagd zijn. Ten gevolge hiervan hebben deze granulocyt met verlaagde CXCR1 en CXCR2 expressie een verhoogde adhesie en verlaagde migratie door het glomerulaire endothel. Deze data suggereren een relatie tussen circulerend IL-8, chemokine receptoren en ophoping van granulocyten in de vaatwand. Echter, de rol van CXCR1/2 in granulocyt adhesie is hiermee niet verklaard, aangezien ook eerder is aangetoond dat activatie van CXCR2 een signaal voor sterke adhesie geeft. Daarom is het nodig dat het mechanisme achter de verhoogde adhesie van granulocyten zonder CXCR1/2 expressie verder onderzocht wordt. In het bijzonder de betrokken adhesie moleculen en de eigenschappen en de structuur en functie van glomerulaire endothelcellen zijn zeer interessant.

De mechanismen, die vaatschade veroorzaken zijn vooral gerelateerd aan ANCA. ANCA activeren de granulocyten wat leidt tot uitstoting van ROS en proteolytische enzymen, die direct de vaatwand beschadigen en de endothelcellen aanzetten tot het rekruteren van nog meer ontstekingscellen. NETs worden gevormd door geactiveerde granulocyten. Deze structuren concentreren PR3 en MPO op het granulocyt oppervlak, zodat ANCA ze kunnen herkennen. Andere autoantistoffen, AECA, komen ook voor bij AAV. Echter, hun specifieke antigenen op endothelcellen zijn nog niet gevonden. Wij hebben geen pathogene rol voor AECA in onze studie in dit proefschrift gevonden, maar kunnen niet uitsluiten dat ANCA antigenen op endothelcellen ANCA-geïnduceerde schade kunnen mediëren.

Herkennen van PR3 of MPO door ANCA is een vereiste voor activatie van granulocyten. Expressie van deze ANCA-antigenen op de membraan wordt geïnduceerd door pro-inflammatoire cytokines, zoals TNF-α en IL-1β, en verder wordt mPR3 expressie sterk verhoogd tijdens adhesie van de granulocyt. CD177 is een receptor voor PR3 op granulocyten. De fysieke interactie tussen deze twee moleculen is echter nog niet duidelijk. CD177 is waarschijnlijk een adhesiemolecuul, en β2-integrines, belangrijke adhesiemoleculen op granulocyten, co-
lokaliseren met PR3 in lipid-rafts en geven signalen door in de cel als ANCA binden aan PR3. Daarom kan membraan PR3 expressie niet langer beschouwd worden als een gebeurtenis onafhankelijk van granulocyt-endotheel interactie. In deze context is het complex van moleculen van belang, dat co-lokalisert op de membraam met PR3, zoals CD177 en β2-integrines. De functie van dit complex is waarschijnlijk om de granulocytten te rekruteren en dicht bij het endotheel te brengen. Verder speelt het bij AAV een rol in de PR3-ANCA gemedieerde granulocyt activatie en daaruit resulterende vaatschade.

We hebben laten zien dat het percentage CD177+ granulocytten verhoogd is bij AAV, en dit is de reden voor een verhoogd percentage granulocytten met hoge mPR3 expressie. Deze scheve verdeling zou genetisch bepaald kunnen zijn. Echter, verhoogde CD177+ zijn ook gevonden bij andere ziektebeelden, zoals bij ernstige infectie, behandeling met G-CSF of zwangerschap, suggererend dat er een ander mechanisme bestaat wat de balans tussen granulocyt subsets bepaalt. Wanneer bekend is waarom CD177 verschillend tot expressie komt in granulocytten zou dit mechanisme duidelijk kunnen worden. Functionele gevolgen van verhoogde CD177/mPR3 expressie bij AAV zijn afhankelijk van de moleculaire functie van CD177, of zou tot uiting kunnen komen in verschillen in functie tussen CD177+ en CD177- granulocyt subsets. Het feit dat CD177 kan binden aan PECAM-1 leidt niet tot verschillen in adhesie of migratie tussen de subsets. Granulocytten worden gelijkwaardig geactiveerd door PR3-ANCA, onafhankelijk van CD177 expressie, suggererend dat CD177 niet betrokken is bij de signaal transductie route na PR3-ANCA binding. Tenslotte, analyse van genexpressie van het hele genoom onthulde dat genen van granule eiwitten verhoogd tot expressie komen in CD177 granulocytten ten opzichte van CD177- granulocytten. Deze bevinding kan een uitgangspunt vormen voor het ontrafelen van de karakteristieken van deze granulocyt subsets en de pathogene rol van dit molecule in AAV.

Concluderend kunnen we stellen dat de interactie tussen granulocytten en endotheelcellen van groot belang is voor de ontwikkeling van de ziekte en voor interventie. Verlaagde expressie van CXCR1 en CXCR2 is bevorderlijk voor de ophoping van granulocytten in de kleine vaten. De rol van AECA in dit proces is niet erg belangrijk. Membraan expressie van ANCA-antigenen zoals PR3 wordt op-gereguleerd tijdens rekrutering van granulocytten. CD177 is verantwoordelijk voor PR3 expressie, maar niet essentieel voor PR3-ANCA gemedieerde activatie van granulocytten. Het onderliggende mechanisme voor de verhoogde
percentages CD177⁺/PR3⁺ granulocyten bij AAV is niet duidelijk. Verschillen in gen expressie tussen CD177⁺ en CD177⁻ subsets laten zich niet eenvoudig vertalen naar verschillen in functie, maar duiden eerder op verschillen in rijping of aktivatie van granulocyten. Deze populaties zijn uit balans in AAV, maar verdere studies zullen moeten uitwijzen wat de pathofysiologische betekenis hiervan is.
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