Proteinase 3 and Neutrophil Apoptosis in ANCA-Associated Systemic Vasculitis

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Abstract

ANCA-Associated Systemic Vasculitis (AASV) is characterized by leukocytoclasis- dying neutrophils surrounding small blood vessels. Previous studies have shown increased plasma PR3 and increased proportion of neutrophils expressing plasma membrane-associated PR3 (mPR3+).

Neutrophils were isolated by polymorphprep and membrane expression was measured by FACS. Plasma PR3, pro-PR3 and cytokines were measured by ELISA. Neutrophil apoptosis was measured by FACS after 20h in vitro culture. Expression of pro/anti-apoptotic factors and transcription factors C/EBP-α, C/EBP-β and PU.1 was measured by real-time PCR.

Plasma PR3, pro-PR3, and proportion of mPR3+neutrophils were elevated in AASV. mPR3 and CD177 were co-expressed on a subset of neutrophils both in AASV and controls. mPR3 expression was not correlated directly to circulating PR3 or PR3 gene transcription, but was dependent on transcription of the CD177 gene. AASV neutrophils had a significantly lower rate of apoptosis than neutrophils from healthy blood donors (HBD). Lower apoptosis rate did not correlate with clinical data, plasma PR3, cytokine levels, mPR3 expression or level of pro/anti-apoptotic factors. However, C/EBP-α and C/EBP-β were significantly higher in neutrophils from AASV patients than in HBD neutrophils.

These results indicate an altered neutrophil phenotype in AASV and suggest that the rate of granulopoiesis is higher, while the rate of neutrophil apoptosis is lower. This may predispose for the presence of dying neutrophils in regions of inflammation in AASV. Additional studies are required to determine why the rate of neutrophil apoptosis is lower in AASV patients and if dysregulation of neutrophil apoptosis contributes directly to the pathogenesis.

Key words: Wegener's granulomatosis, microscopic polyangiitis, anti-neutrophil cytoplasmic antibodies (ANCA)/anti-PR3/MPO, proteinase 3, myeloperoxidase, flowcytometry/FACS.

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Cover: Scanning Electron micrograph of a neutrophil migrating through the bone marrow enothelium.

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ ، خَلَقَ الإِنسَانَ مِنْ عَلَقٍ ، اقْرَأْ
وَرَبُّكَ الأَكْرَمُ ، الَّذِي عَلَّمَ بِالْقَلَمِ ، عَلَّمَ الإِنسَانَ مَا لَمْ يَعْلَمْ

صَدَقَ اللهُ العَظِيم

In The Name of Allah The Most Gracious The Most Merciful

Read! In the name of your Lord, who created everything, Created man from a clot, Read! And your Lord is the Most Noble, He Who has taught man by the Pen, Taught man what he knew not

(From Holly Quran- Surat Al-Alaq, Verse 1-5)

This work is dedicated to my father and mother

Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV).

I. Increased neutrophil membrane expression and plasma level of proteinase 3 in systemic vasculitis are not a consequence of the -564 A/G promotor polymorphism. Abdgawad M, Hellmark T, Gunnarsson L, Westman KW, Segelmark M. Clin Exp Immunol. 2006 Jul;145(1):63-70.

II. Proteinase 3 and CD177 are expressed on the plasma membrane of the same subset of neutrophils. Bauer S, Abdgawad M, Gunnarsson L, Segelmark M, Tapper H, Hellmark T. J Leukoc Biol. 2007 Feb;81(2):458-64.

III. Elevated neutrophil membrane expression of proteinase 3 is dependent upon CD177 expression. Abdgawad M, Gunnarsson L, Bengtsson AA, Geborek P, Nilsson L, Segelmark M, Hellmark T. Clin Exp Immunol. 2010 Jul 1;161(1): 89-97.

IV. Decreased neutrophil apoptosis in ANCA-Associated Systemic Vasculitis. Abdgawad M, Gunnarsson L, Bengtsson AA, Geborek P, Nilsson L, Segelmark M, Hellmark T. *Manuscript*

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Contents

Abbreviations

ANCA	Anti-neutrophil cytoplasmic antibodies
α1-AT	Alpha1-antitrypsin
AASV	ANCA-associated systemic vasculitis
Bcl-2	B-cell lymphoma-2
BPI	Bactericidal permeability increasing protein
C/EBP-α	CCAAT-enhancer-binding protein-alpha
C/EBP-β	CCAAT-enhancer-binding protein-beta
CatG	Cathepsin G
CD177	Cluster of differentiation 177
CF	Cystic fibrosis
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CSS	Churg-Strauss syndrome
ELISA	Enzyme linked immunosorbent assay
EOPs	Eosinophil progenitors
FACS	Fluorescence-activated cell sorter
fMLP	N-formyl-metionyl-leucyl-phenylalanine
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMPs	Granulocyte-macrophage progenitors
GPI	Glycosylphosphatidylinisotol
HCS	Hematopoietic stem cells
HNA-2a	Human neutrophil antigen-2a
HLE	Human leukocyte elastase
IAP	Inhibitor of apoptosis proteins
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
IIF	Indirect immunofluorescence
IL-3	Interleukin-3
JAK-2	Janus kinase-2
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MACS	Magnetic-activated cell sorter
Mcl-1	Myeloid cell leukemia-1
MEPs	Megakaryocyte-erythrocyte progenitors
MFI	Mean fluorescence intensity
MHC-II	Major histocompatibility complex II
MPA	Microscopic polyangiitis
MPO	Myeloperoxidase
MPPs	Multipotent progenitor cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NB1	Neutrophil glycoprotein-1
NETs	Neutrophil extra cellular traps

NGAL	Neutrophil gelatinase associated lipocalin
NMPs	Neutrophil-monocyte progenitors
PAF	Platelet activating factor
PAN	Polyarteritis Nodusa
PAR-2	Proteinase activated receptor-2
PECAM-1	Platelet endothelial cell adhesion molecule-1
PGE 2	Prostaglandin-E2
PMA	phorbol-12-myristate-13-acetate
PMN	Polymorphnuclear leukocytes
PNH	Paroxysmal nocturnal hemoglobinuria
PR3	Proteinase 3
PRV-1	Polycythemia rubra vera protein-1
PTX3	Pentraxin 3
PV	Polycythemia Vera
RA	Rheumatoid Arthritis
RLV	Renal limited vasculitis
ROS	Reactive oxygen species
SHIP-1	SH2 inositol 5-tyrosine phosphatase-1
SLE	Systemic Lupus Eruthematosus
SNARE	SNAP (Soluble NSF Attachment Protein) REceptors
SNP	Single nucleotide polymorphism
SOCS	Suppressors of cytokine signaling
TGF-β	Tumor growth factor-beta
TNF-α	Tumor necrosis factor-alpha
Vamp	Vesicle associated membrane protein
WG	Wegener's granulomatosis

Introduction

ANCA-associated systemic vasculitis

Systemic vasculitides are a heterogenous group of disorders characterized by destructive inflammation of the blood vessel wall, leading to bleeding or vessel occlusion with subsequent ischemia of the surrounding tissue. Clinical manifestations vary depending on the size of the affected blood vessels, and include fever, weight loss, malaise, arthralgias and arthritis. Vasculitides can be idiopathic, primary, secondary to another disease such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Artritis (RA), or associated with infections, such as infective endocarditis, pharmaceutical drug use, such as propylthiouracil and hydralazine, or other chemical exposures[1]. Vasculitis can be isolated to one organ or vessel and be relatively insignificant clinically or can present as a systemic life-threatening illness involving several organs and vessels[2]. Examples of different types of vasculitis are depicted in Table 1.

Table 1. Classification of systemic vasculitis.

Dominant vessel involved	Primary	Secondary
Large arteries	Giant cell arteritis	Aortitis associated with RA
Medium arteries	Takayasu's arteritis	Infection (eg. Syphilis)
	Classical PAN	Infection (eg. Hepatitis B)
	Kawasaki disease	
Small vessels and medium arteries	Wegener's granulomatosis*	Vasculitis 2° to RA, SLE, Sjögren's syndrome
	Churg-Strauss syndrome*	Drugs
Small vessels (leukocytoclastic)	Microscopic polyangiitis*	Infection (e.g. HIV)
	Henoch-Shönlein purpura	Drugs**
	Essential mixed cryoglobulinaemia	Infection (e.g. Hepatitis B, C)
	Cutaneous leukocytoclastic vasculitis	

(*) Diseases most commonly associated with ANCA, pauci-immune crescentic glomerulonepghritis and which are most responsive to immunosuppression with cyclophosphamide. (**) e.g. sulphonamides, penicillins, thiazide diuretics, and many others. PAN= Polyarteritis Nodosa. RA= Rheumatoid Arthritis. SLE= Systemic Lupus Erythematosus.

Adapted from Scott and Watts with permission[16].

Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) are collectively referred to as ANCA- Associated Systemic Vasculitis (AASV), due to their similar pathological features and close relationship to Anti-neutrophil cytoplasmic antibodies (ANCA). These antibodies are directed against antigenic components of neutrophilic granules or lysosomes. Indirect immunofluorescence (IIF) of ethanol-fixed neutrophils reveals cytoplasmic (c-ANCA) or perinuclear (p-ANCA) staining. C-ANCA staining correlates with proteinase-3 (PR3) reactivity, while p-ANCA staining correlates with reactivity towards myeloperoxidase (MPO) or other antigens. c-/PR3-ANCAs are mainly detected in patients with WG, whereas p-/MPO-ANCAs are predominantly detected in patients with MPA and CSS[3].

AASV is a small-vessel vasculitis affecting arterioles, venules, capillaries, and occasionally medium-sized arteries, which commonly involves multiple organ systems. AASV is the most common primary systemic small-vessel vasculitis that occurs in adults. Although AASV was considered infrequent, recent data indicate that the incidence is increasing[4]. In the most recent studies, the annual incidence of AASV was 13.7/million in Australia[5], 13.1-18.3/million in Spain[6], 12.4/million in Germany[7] and 19.8/million in UK[8]. In two recent studies by our group, we found an incidence for AASV of 20.9/million and a point prevalence of 268/million inhabitants in southern Sweden[9, 10]. The histological lesions are called pauci-immune, because few or no immunoglobulins or complement components are detected in the vasculitic lesions. AASV is associated with significant morbidity and mortality, with almost all patients requiring aggressive immunosuppression[11].

Without treatment, patients with AASV have a very poor prognosis with a median survival time of 5 months[12]. Current treatment regimens based on cyclophosphamide and corticosteroids have dramatically improved the prognosis for these patients and increased the median survival time to 21.7 years[13]. Although this regimen achieves long-lasting remission and prolonged survival of patients with AASV, it has its drawbacks; the worst being life-threatening infections early in the course of the disease and risk of malignancy in late stages of othe disease[14, 15]. Furthermore, the disease has a high relapse rate in spite of heavy immunosuppression. Improved understanding of the mechanisms underlying AASV may help in the search for better treatment modalities for this serious and devastating illness.

Neutrophils

Circulating leukocytes (or white blood cells) are classified either as polymorphonuclear leukocytes or as mononuclear cells, based on their appearance under a light microscope. Mononuclear cells are further subdivided into lymphocytes and monocytes. Monocytes are the largest cells of the blood and are the precursors of macrophages. Lymphocytes are the smallest leukocytes and consist of natural killer cells, B- cells and T-cells. Polymorphonuclear leukocytes (PMN) have lobulated nuclei, which are variable in shape, hence their first name 'polymorphic', and chracterized by abundance of granules in their cytoplasm, hence their second

name ‘granulocytes’. Granulocytes can be subdivided into three categories based on their staining patterns: neutrophils (stain with neutral dyes), eosinophils (stain with acidic dyes) and basophils (stain with basic dyes). The granules of neutrophils typically stain pink or purple-blue following treatment with a neutral dye. Mature neutrophils are non-proliferating, non-dividing cells characterized by segmented nucleus, mixed granular populations, small golgi regions and accumulation of glycogen particles. The nucleus is segmented, usually into two to four interconnected lobes which may appear like multiple nuclei.

Neutrophils are the most abundant granulocytes, representing 60 to 70% of the total circulating leukocytes and the major phagocytes of the body’s defence against infections. Up to date, neutrophils are generally considered as a homogeneous cell population. There are few single reports of neutrophil subpopulations that have questioned this general concept[17-19].

Neutrophil Granules

On average, a neutrophil contains 200 to 300 granules, and approximately one third of them are peroxidase positive. From a functional point of view, neutrophil granules are either peroxidase-positive (azurophilic, containing MPO) or peroxidase-negative (specific and tertiary). Granules formed at the later stages of myelopoiesis are peroxidase-negative. It is thought that granules form when immature transport vesicles bud from the golgi network and aggregate[20]. According to Bainton et al., vesicles that bud from cis-Golgi form storage granules, while vesicles that bud from the trans-golgi network form specific granules[21]. Azurophilic granules are spherical, appear at the pro-myelocytic stage and contain MPO, serine proteases, and antibiotic proteins and form the microbicidal compartment of neutrophils. Specific granules emerge at the metamyelocyte stage, followed by tertiary granules containing gelatinase. Secretory vesicles, which are the most rapidly mobilizable intracellular structures, are seen in mature neutrophils, and are of endocytic origin. All granules have a phospholipid bilayer membrane and an intragranular matrix containing proteins and enzymes (Table 2). The mechanisms that sort and target proteins to specific granules are not well understood. Some proteins are constitutively-secreted, while secretion of other proteins is regulated. The “time-theory” proposes that all proteins that are synthesized at the same time localize to the same granules[22]. Thus, the window during which various proteins are translated may at least partially determine the contents of different granules. Nevertheless, it may not be possible for all proteins to co-exist in certain granules, influencing patterns of protein segregation/sorting.

The mechanisms that mediate degranulation/exocytosis are complicated and not yet fully elucidated. Granules are mobilized in an ordered hierarchical manner, with secretory vesicles the most easily and completely mobilized and azurophilic granules the most difficult to mobilize[23]. The hierarchical mobilisation of granules can be reproduced *in vitro* by gradual increase in intracellular Ca^{2+}. Several annexins, including anexin I, annexin XI and lipocortin III, appear to promote

Table 2. Selected granule contents.

Granule	Azurophil	Specific	Gelatinase	Secretory
Membrane content	CD63 CD68 V-type H^+ ATPase	CD11b, CD66, CD67, CD15 antigens, Cyt b558, Fibronectin R, Laminin R, NB1 antigen, 19-kD protein, 155-kD protein, SCAMP, TNF-R, Thrombospondin-R, Urokinase-type plasminogen activator-R, VAMP-2, Vitonectin-R	CD11b, cytochrome b558, VAMP2, Diacylglycerol-deacylating enzyme, SCAMP, Urokinase-type plasminogen activator R, V-type H^+ ATPase	CD11b, Cytochrome b558, Alkaline phosphatase, CD14, CD16, CD10, CD13, CD45, SCAMP, Urokinase-type plasminogen activator-R, VAMP2, c1q-receptor, DAF
Matrix content	Acid β-glycerophosphatse, α1-antitrypsin, α-mannosidase, CAP37, β-glycerophosphatse, β-glucuronidase, Cathepsins, Definsins, HLE, Lysozyme, PR3, MPO, Sialidase, Ubiquitin protein, BPI protein	β2-Microglobulin, Collagenase, Gelatinase, Urokinase-type plasminogen activator, lysozyme, lactoferrin, hCAP 18, NGAL, Vitamin B12 binding protein, Heparanase, Histaminase, SGP28, Sialidase, PR3, CD177	Acetyltransferase, $\beta_2$microglobulin, gelatinase, lysozyme	PR3, Plasma proteins

BPI= Bactericidal paermeability increasing. SCAMP= Secretory carrier membrane protein. DAF= Decay accelerating factor. VAMP= Vesicle associated membrane protein.

NGAL= Neutrophil gelatinase associated lipocalin. HLE= Human leukocyte elastase. PR3= Proteinase 3. MPO= Myeloperoxidase. hCAP-18= Human cationic antimicrobial protein-18. SPG-28= Specific granule protein of 28 kD.

Adapted with permission from Elsevier and N Borregaard[22].

calcium-dependent fusion events in neutrophils[24]. Ca^{2+} may also promote neutrophil degranulation by stimulating interactions between SNAP (Soluble NSF attachement protein) receptor (SNARE) proteins[25]. As per the SNAP/SNARE-hypothesis, granules and vesicles can be selectively targeted via specific interaction between v-SNAREs (on the membrane of donor organelles) with t-SNAREs (present on the target membrane). In neutrophils, the t-SNARE protein, syntaxin-4, is present on the plasma membrane, while v-SNARE, VAMP-2, is on the membrane of secretory vesicles, and gelatinase and specific granules. Guanosine triphosphate (GTP) also plays a role in the control of granule exocytosis[26].

Proteinase 3

Neutrophil azurohil granules contain three major serine proteases human leukocyte elastase (HLE), proteianse 3 (PR3) and cathepsin G (CatG). Serine proteases play important roles in facilitating leukocyte migration through the basement membrane and in digesting proteins within the phagolysosome[27-29]. Naturally occurring serine protease inhibitors include α_1-antitrypsin (α_1-AT), α_2-macroglobulin, and α_1-antichymotrypsin. Proteinase 3 (PR3), also called myeloblastin and proteinase 4, was originally identified by Ohlsson and was later characterized by Baggiolini et al[30, 31]. PR3 is a neutral serine protease found in the azurophilic granules of neutrophils and peroxidase-positive lysosomes of monocytes[32]. It is also present in specific granules and in secretory vesicles, and is expressed on the plasma membrane of normal blood neutrophils[33, 34]. Circulating PR3 is bound to α_1-AT[35]. PR3 co-localizes with MPO, HLE, and CatG in azurophilic granules[36]. It is stored as a mature and enzymatic active protein[37]. The PR3 gene maps to chromosome 19p13.3, in a cluster with HLE and azurocidin (AZU); it spans 6570 base pairs and consists of five exons and four introns[38]. Introns I and IV include regions with repeating motifs, which may cause chromosomal instability and a predisposition to genetic rearrangements and deletions[39]. A bi-allelic restriction fragment length polymorphism (RFLP) has been described in the PR3 gene[40]. Allelic variations in PR3 may be associated with quantitative/qualitative differences in the expression and/or function of PR3. Gencik et al. identified an A/G single nucleotide polymorphism (SNP) at coordinate -564 in the PR3 promoter, and suggested that it was associated with WG[41]. However, Pieters et al. showed that the -564 A/G polymorphism did not increase activity of the PR3 promoter, arguing against the possibility that the polymorphism results in an increased transcription/production of PR3 in WG patients[42].

PR3-mRNA is detected in early cells of the myeloid lineage and is down-regulated during myeloid differentiation. The mechanisms that promote high level transcription of PR3 in myeloid cells committed to granulocyte differentiation are not compleletey understood, although it is known that two transcriptional factors are needed for the expression of PR3, PU.1 and CG element[43]. Transcription is limited to the promyelocyte and promonocyte stages of differentiation, and it is down-regulated upon maturation in healthy individuals[44]. Treatment of precursors with

dimethyl sulfoxide (DMSO), 1,25-dihydroxyvitamin D3, bile acids or retinoic acid also down-regulates transcription of PR3. PR3 is synthesized as a prepro-enzyme, which is processed in four consecutive steps into a mature form consisting of 222 amino acids. Following removal of signal peptide, it is transported into the endoplasmic reticulum (ER), where it is glycosylated with high-mannose oligosaccharides. Glycosylation of PR3 may influence its subcellular localization, with certain glycosylated isoforms being designated for granular cells and others for secretion or expression on the plasma membrane. The propeptide of PR3 is removed in the post-Golgi organelle, after which a seven-amino-acid carboxy-terminal extension is removed, possibly by a trypsin-like proteinase[45]. During this process, small amounts of the pro-form of PR3 escape granular targeting and are secreted[46, 47]. These molecules may play a role in negative feedback regulation of granulopoiesis[48].

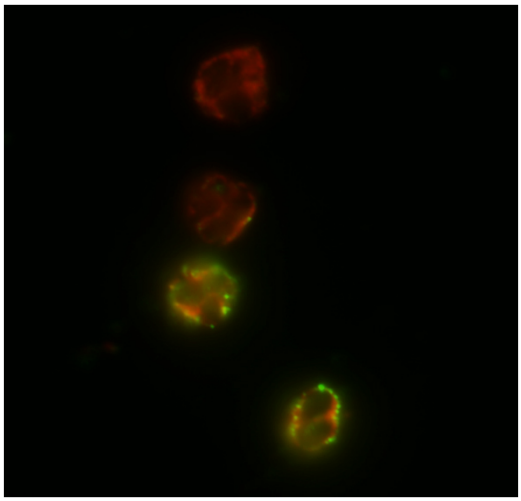

Figure1. Bimodal expression of PR3. A Fluorescent micrograph showing four neutrophils, the lower two cells express PR3 (represented with green colour, Alexa Fluor 488), while the upper two neutrophils do not express PR3 on their membrane. All neutrophils contain PR3 intracellularly shown in red (Alexa 594).

PR3 is also expressed on the plasma membrane (mPR3) of a subpopulation of resting neutrophils. Halbwachs-Mecarelli et al. noted the existence of two distinct neutrophil subpopulations, $mPR3^{+}$ and mPR3-negative, in normal healthy individuals, resulting in so-called bimodal expression of PR3, Figure 1[49]. Despite the high variability in the proportion of PR3-expressing cells among individuals, the proportion is stable in a given individual over long periods of time, suggesting genetic control of mPR3 expression. This is supported by twin studies demonstrating that the proportion of mPR3 expressing neutrophils in monozygotic twins is highly concordant[50]. The intracellular levels of PR3 do not correlate with mPR3 levels. Expression of PR3 on the membrane of neutrophils is upregulated by multiple pro-inflammatory mediators such as: TNF-α, PMA[51], IL-18[52], LPS, IL-8, PAF, fMLP[53] and GM-CSF[54]; and by one anti-inflammatory cytokine: TGF-β[55].

Membrane PR3 is active, and quite resistant to inhibition by naturally occurring proteinase inhibitors including α_1-AT, possibly due to steric hindrance of the membrane-embedded protease[53]. Campbell et al. showed that PR3 can be eluted from the membrane of PMN following cellular activation, and that ionic interactions are important in the binding of PR3 to the plasma membrane[53]. PR3 is a cationic protein with an isoelectric point of 9.1. PR3 can bind stably to anionic and neutral membranes, but binds more strongly to negatively-charged bilayers. However, hydrophobic residues in PR3 also bind to the membrane with high affinity[56]. Others suggest that PR3 membrane binding is possibly mediated by protein partners such as FcγRIIIb (CD16b), or β2 itegrin (CD11b/CD18)[57-59]. Fridlich et al. showed that cleavage of neutrophil glycosylphosphatidylinositol (GPI) anchors by phosphatidyl inositol-specific phospholipase C (PI-PLC) reduces the level of mPR3. This suggests that a GPI protein, possibly FcγRIIIb, (or another yet unidentified GPI-anchored protein) attaches PR3 to the membrane[57]. PR3 is expressed on the plasma membrane of apoptotic cells, independent of degranulation, and this is associated with phosphatidylserine (PS) externalization[60, 61]. Kantari et al. demonstrated that phospholipid scramblase- 1 (PLSCR1) interacts with PR3 and may promote its translocation to the plasma membrane during apoptosis[61].

mPR3 proteolytically degrades fibronectin, elastin, laminin, collagen type IV and heparan sulfate proteoglycans in the subendothelial matrix[53]. The soluble form of PR3 cleaves and activates cytokine precursors, including IL-8, IL-1β, and TNFα[62, 63]. PR3 also induces detachment and cytolysis of endothelial cells in vitro[64]. Yang et al. demonstrated that PR3 can trigger apoptosis in cultured endothelial cells, although the exact mechanism is not yet known[65].

A secreted proform of PR3 (retaining an amino terminal dipeptide) can down-regulate DNA synthesis in normal $CD34^+$ hematopoietic progenitor cells (S phase reduction); thus, PR3 may act as a negative feedback regulator of granulopoiesis in the bone marrow[48]. Intrestingly, this inhibitory effect of pro-PR3 is reversible and can be abrogated by G-CSF or GM-CSF.

CD 177/NB1/PRV-1

CD177, also known as Polycythemia Vera protein-1 (PRV-1), is a glycoprotein that was first discovered in 1970 in connection with studies of polycythemia vera. One year later, a protein was described in a case of neonatal neutropenia, and given the name Human Neutrophil Antigen-2a (HNA-2a or NB1)[66]. When cloning the genes encoding PRV-1 and NB1, they were found to differ only at 4 base pairs, which later has shown to be the consequence of two alleles of a single gene coding for a protein now called CD177[67]. CD177 belongs to the Leukocyte Antigen 6 (Ly-6) supergene family and is the best charecterized member of this family. The Ly-6 superfamily is a group of highly diverged proteins, first described in mice, also known as the uPAR (urukinase plasminogen activator receptor) or the snake toxin family. Mouse Ly-6 proteins play an important role in proliferation, differentiation, and homing of hematopoietic cells and lymphocytes[68]. In humans, Ly-6 genes are over-expressed in

rapidly proliferating and malignant cells[69, 70]. CD177 is a GPI-anchored, 58 to 64 kDa neutrophil-specific glycoprotein found on the plasma membrane and secondary granules of neutrophils[71]. CD177 has the unique feature of being expressed on a subset/fraction of neutrophil population. CD177 is expressed at a higher level in females than males, and is most abundant in pregnant women[72]. Neutrophils from approximately 3% of Caucasians, 5% of African Americans, and 1% -11% of Japanese are completely deficient in CD177[68]. The functions of CD177 are not known, although there is evidence that it may play a role in adhesion of neutrophils to endothelial cells[73]. CD177 can directly bind to PECAM-1 (CD31), expressed at the junctions of the enothelial cells, on the membrane of neutrophils, monocytes and platelets; thereby enhancing transendothelial migration of $CD177^+$ neutrophils[74]. Alloantibodies to CD177 have been found in individuals with adverse reactions to pulmonary transfusion or with bone marrow transplant-induced or drug-induced immune neutropenia[71, 75].

The CD177 gene is located on chromosome 19q13.2, has 9 axons, an open reading frame of 1311 base pairs, and encodes a 437 amino acid protein with an 21 amino acid N-terminal signal sequence[76]. CD177 mRNA has been reported to be higher in newborns[77]. Temerinac et al. demonstrated that CD177 is over-expressed in the peripheral blood granulocytes of patients with polycythemia vera (PV), in umbilical cord blood, in normal human bone marrow and to a lesser degree in fetal liver[78]. Also, in neutrophils that express CD177, CD177-mRNA levels are increased by exposure to G-CSF, and by inflammatory states (sepsis, burns) associated with increased neutrophil production[79, 80]. CD177 glycoprotein as well as all GPI-anchored proteins are not detected on the membrane of neutrophils from patients with paroxysmal nocturnal hemoglobinuria (PNH), who lack GPI-anchors[81]. Elevated CD177-mRNA has been observed in patients with myeloproliferative disorders, including polycythemia Vera (PV, 95-100%), essential thrombocythemia (ET, 30-50%) and idiopathic myelofibrosis (IMF, 10-30%)[68, 78]. Kralovics et al. have shown that a significant proportion of patients with myeloproliferative disorders carry a dominant gain-of-function mutation (V617F) in JAK2 (Janus Kinase). JAKs are cytoplasmic tyrosine kinases that are activated in response to hematopoietic growth factors such as erythropoietin, thrombopoietin, G-CSF and GM-CSF. The V617F mutation in JAK2 is associated with increased proliferation of hematopoietic precursors, and thus may directly contribute to disease pathology and to the elevated expression of CD177-mRNA[82]. Consistent with this notion, the V617F mutation in JAK2 is present in approximately 50% of patients with ET and IMF, and 90–95% of patients with PV[83].

CD177-mRNA is more abundant in $CD177^+$ neutrophils than in $CD177^-$ PMNs. Complete CD177-mRNA is not detected in $CD177^-$ neutrophils[75], suggesting a defect in transcription or splicing of CD177 mRNA[71]. Several polymorphisms in CD177 have been described; with the most common being a single nucleotide G to C change at bp 42[84].

Neutrophil functions

Pathogen killer and cell debris cleaner

Neutrophils are the most abundant white blood cells in the body and the first to be recruited at the site of infection or inflammation. Neutrophils contribute to immune surveillance and participate in elimination of micro-organisms and cell debris. This major function of neutrophils can be divided into 5 minor step functions; (1) adhesion. (2) trans-endothelial migration/diapedesis, (3) Interstitial migration/ locomotion, (4) phagocytosis of bacteria and/or degranulation, (5) apoptosis: this will be reviewed in detail in the forthcoming sections.

In the absence of infection, neutrophils are maintained at a resting state to ensure that their toxic contents are not released into surrounding tissues. Neutrophils become activated through two steps, priming and full activation. Multiple agents including bacterial products, cytokines such as TNF-α, GM-CSF, IL-8 and IFN-γ can prime neutrophils. Neutrophils are then mobilized to the site of infection/ inflammation by the help of chemoattractants where they encounter a second stimulus by which they become fully activated and kill bacteria or ingest cell debris.

Migration of neutrophils from the circulation to the site of infection/inflammation is controlled by interactions with the vascular endothelium. L-selectins expressed on neutrophils allow rolling and loose adhesion of neutrophils to ligands expressed on endothelial cell membrane (like E- and P-selectins). This loose adhesion leads to conformational changes in the leukocyte integrins of the β2 subfamily (CD11a, CD11b, CD11c/CD18), leading to engagement of other adhesion molecules on the membrane of endothelial cells such as intercellular adhesion molecule-1 (ICAM-1), ICAM-2, vascular cell-adhesion molecule-1 (VCAM-1) and mucosal vascular cell-adhesion molecule-1 (MDAM-1), leading to high affinity ligand binding and firm adherence[85]. Then, binding of chemoattractants such as IL-8, released from the endothelial cells, to neutrophil receptors lead to arrest of the neutrophil rolling.

At the site of infection, membrane receptors recognize and bind opsonized bacteria leading to the formation of pseudopodia, phagocytosis of the pathogen in a phagosome that fuses with protease-rich granules leading to the destruction of the pathogen within the intracellular phagosome. Neutrophil phagocytosis of bacteria and cell debris involves the Fcγ-Receptors (FcγRIIa/ CD32 and FcγRIIIb/ CD16) and the complement receptors (CR1/ CD35 and CR3 or CD11b/CD18 integrin)[86]. Neutrophils express an array of proteases, contained in their granules, and can generate reactive oxygen species (ROS) in order to rapidly kill phagocytosed bacteria[87]. Once activated, they attack the invading pathogens by a combination of three mechanisms: phagocytosis, degranulation, and extracellular traps. During phagocytosis, the neutrophils ingest the pathogen forming a phagosome, while at the same time secrete ROS and hydrolytic enzymes to destroy it. The consumption of oxygen during this process is termed as a 'respiratory burst.' Degranulation refers to the process by which various cytotoxic molecules residing in cytoplasmic granules are released. Examples include myeloperoxidase (MPO), an enzyme that is

responsible for converting hydrogen peroxide to hypochlorous acid, a highly effective bactericide[88]. Most recently, a novel extracellular mechanism of destroying pathogens has been described by Brinkmann et al[89]. Activation of neutrophils causes the release of chromatin fibers and granule proteins termed as neutrophil extracellular traps (NETs) that can trap and kill microbes extracellularly.

Expression of Major-Histo-Compatibility Molecule-II (MHC-II)

Neutrophils are capable of presenting antigens via MHC-II, thereby stimulating T-cell activation and proliferation. Expression of MHC-II molecules can be induced in *in vitro* culture by incubating neutrophils with GM-CSF, IL-3 and/or IFN-γ[90]. *In vitro* activation of neutrophils with fMLP, LPS or PMA has also been shown to induce expression of MHC-II, together with T-cell co-stimulatory molecules (CD80 and CD86)[91]. Neutrophils isolated from synovial fluid of RA patients have been shown to express MHC-II, CD80 and CD86, and are able to activate T-cell proliferation[92].

Production of inflammatory mediators

Primed neutrophils are able to actively synthesize and secrete cytokines, chemokines, leukotrienes and prostaglandins. In particular, neutrophils have been shown to synthesize and secrete IL-8, IL-1, IL-1RA, IL-6, IL-12, TGF-β, TNF-α[93, 94]. These cytokines can subsequently stimulate both neutrophils and other cells of the immune system. Neutrophils are significant source of leukotrienes and prostaglandins, especially leukotriene B4 (LTB4), which is synthesized from arachidonic acid by lipoxygenases and prostaglandin E2 (PGE2), which is synthesized from arachidonic acid by cyclo-oxygenases. LTB4 is a neutrophil chemoattractant and can promote neutrophil adherence and migration through endothelial cells while PGE2 is an anti-inflammatory molecule, inhibiting phospholipase-D activity and increasing concentrations of intracellular cyclic-adenosine monophosphate concentrations (c-AMP), which results in decreased calcium influx, loss of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase assembly and lower levels of endothelial adhesion and chemotaxis[95]. PGE2 has been reported to delay neutrophil apoptosis[96].

Neutropoiesis

General aspects of hematopoiesis

Hematopoiesis is the process by which the immature precursor cells in the bone marrow develop into mature blood cells. All mature blood-cell types develop from hematopoietic stem cells (HSCs). The most primitive HSCs are self-renewing cells with long-term reconstituting activity (LT-HSCs), which develop into short-term reconstituting cells (ST-HSCs) and subsequently to multipotent progenitor cells (MPPs), losing their self-renewal capacity along this developmental pathway[97]. HSCs represent a small number of cells in the bone marrow (~0.1%) with self-renewing capacity and ability to differentiate into all blood cell types. MPPs are

cells with high ability to differentiate into different cell types, without any significant ability to self-renew. MPPs commit either to the myeloid branch or to the lymphoid branch through a common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), respectively[98]. The CMPs are then postulated to further commit to the granulocyte-macrophage progenitors (GMPs) or the megakaryocyte-erythrocyte progenitors (MEPs)[99]. GMPs differentiate into eosinophil progenitors (EOPs) and neutrophil- monocyte progenitors (NMPs)[97]. Thus, neutrophils form a part of myeloid lineage that includes a diverse group of morphologically and functionally distinct cell types including granulocytes (neutrophils, eosinophils, basophils), monocytes, macrophages, dendritic cells, erythrocytes, megakaryocytes/platelets, and mast cells. The lymphoid lineage includes T-cells, B-cells and Natural killer cells.

Lineage commitment and differentiation of multi-potent cells (MPP) involves selective activation/silencing of specific genes; transcription factors play a key role. For example in the GM pathway, C/EBPα (CCAAT/enhancer-binding protein alpha) is upregulated during differentiation of CMPs to GMPs, whereas the expression of C/EBPβ declines, mostly at the CMP stage[100]. If uncommitted cells upregulate C/EBPα first, they differentiate to GMPs and subsequently to NMPs with further upregulation of C/EBPα. Interestingly, exposure to exogenous GM-CSF or IL-2 receptors redirects CLPs (lymphoid precursors) to the granulocytic lineage[101].

The greatest percentage of hematopoiesis is committed to the production of neutrophils; nearly 60% of all leukocytes in bone marrow are granulocyte precursors[21].

The maturation and differentiation process from HSCs into mature neutrophil is termed neutropoiesis and it takes place in the haematopoietic cords of the bone marrow[102]. After production, neutrophils have to migrate through the bone marrow sinusoidal endothelium to enter the sinusoids that drain into the central sinus and out in the general circulation[103]. The neutopoiesis takes ~6.5 days[21] and then the post-mitotic neutrophils remain in the bone marrow for a further 4-6 days, and represent the bone marrow reserve of neutrophils ($\sim 6\times10^{11}$ cells)[102, 104]. Neutrophils are normally produced from the bone marrow at the rate of 10^{11}/ day, but the rate can increase to 10^{12}/ day in response to infection, where reserve neutrophils are mobilized from their storage pool to the circulation[21]. The mature neutrophils are terminally differentiated and circulate in the blood stream with a half-life of 6-18 hours, before migrating into tissues where they survive for additional 1-2 days[105].

The first cell type in the neutrophil lineage is the myeloblast, which is characterized by a high nuclear:cytoplasmic ratio and prominent nucleoli. The myeloblast develops into a promyelocyte, which has large numbers of peroxidase-positive granules. The polymorphonuclear myelocyte has an indented nucleus, prominent Golgi complex, and a mixed population of granules, including peroxidase-negative granules, as well as large peroxidase-positive azurophil granules. The metamyelocyte band, and mature polymorphonuclear leukocytes (PMNs) in the

bone marrow are non-dividing, non-secretory cells that can be identified by their nuclear morphology, mixed granule population, inactive Golgi region and accumulation of glycogen particles. Circulating neutrophils are largely similar to the mature neutrophil in the bone marrow[21]. In the mature PMN, granules constitute the major subcellular compartment and are of three types: the azurophilic granules (containing neutral proteinases, acid hydrolases, MPO and lysozyme), the specific granules (containing collagenase, lactoferin, lysozyme and Vitamin B12 binding protein) and the tertiary granules (containing gelatinase, lysozyme, acetyltransferase).

Regulation of neutropoiesis

In the early phases of granulopoiesis, C/EBPα, PU.1/Spi-1, RAR, CBF and c-Myb are the key transcription factors, while terminal differentiation into neutrophils depends on C/EBPε, PU.1/Spi-1, CDP and Hox A10[106, 107]. C/EBPα and PU.1 are both key regulators of granulopoiesis and myelopoiesis. Neutrophil development requires co-expression of C/EBPα and low amounts of PU.1.

While GM-CSF is important for the growth of neutrophil progenitors in early stages, G-CSF is necessary for their terminal differentiation into mature neutrophilic granulocytes. G-CSF is the principal growth factor that stimulates proliferation of neutrophil progenitors, while GM-CSF also regulates macrophage, erythroid and possibly megakaryocyte development. G-CSF increases the rate of production of neutrophils by reducing their maturation time in bone marrow from 6.5 days to one day, while the half-life of circulating neutrophils is mainly unaffected. In contrast, GM-CSF markedly increases the half-life of the neutrophils in circulation, while the production rate is only slightly increased[108].

Skold et al. have shown that a secreted proform of Proteinase 3 acts as a negative feedback regulator of granulopoiesis, and counters the effect of G-CSF[48]. It is interesting that this feedback regulation by PR3 is reversible and abrogated by G-CSF and GM-CSF.

C/EBP-α:

Is the founding member of a family of related transcription factors which include C/EBP-β, C/EBP-γ, C/EBPδ, C/EBP-ε, and CHOP[109]. They share a common C-terminal region that contain a leucine-zipper dimerization motif adjacent to a basic DNA-binding region[110]. C/EBPs form a homo- or heterodimers with their leucine zipper domains and bind a common DNA element (CCAAT), via their basic DNA-binding regions[111].

C/EBP-α is expressed in multiple cell types, including adipocytes, hepatocytes and enterocytes. Withinn the hematopoietic cells, high level C/EBP-α expression is restricted to the neutrophils, monocytes and eosinophils. C/EBP-α is the predominant isoform in immature granulocytes while C/EBP-ε is the predominant isoform in maturing granulocytes[112].

C/EBP-α is abundant in early myeloid cells and its expression increases up to three fold following induction of granulocytic differentiation by retinoic acid in myeloid cell lines ; in contrast it is rapidly downregulated during monocytic differentiation.

C/EBP-α serves a nonredundant role in early granulocyte development. Absence of C/EBP-α results in a developmental block during transition from CMPs to GMPs. C/EBP-α null mice lack mature granulocytes, but retain erythrocytes, megakaryocytes, lymphocytes, and monocytes[113]. Disruption of C/EBP-α in mice resulted in an early and specific differentiation block of granulocytes, indicating its important role in early granulocytic commitment. Within the myeloid lineage, forced expression of C/EBP-α in the bipotential U937 myeloid cell line triggers granulocytic differentiation while suppressing the monocytic differentiation[112]. All these data suggest that C/EBP-α is a master regulator of steady-state granulopoiesis.

However, even in the absence of C/EBP-α, granulocytic differentiation can be restored by expression of IL-3 and GM-CSF, indicating that there is more than one pathway to maturation of granulocytes[114].

Important target genes of C/EBP-α in myeloid cells include both early and late granulocytic genes, such as G-CSF-R, myeloperoxidase (MPO), lysozyme, elastase, proteinase 3, lactoferrin, neutrophil collagenase. In addition, C/EBP-α binds and regulates promoters of other transcription factors such as C/EBP-ε and PU.1[115-117].

C/EBP-ε is required for the terminal differentiation and maturation of granulocytes[118].

C/EBP-β:

Is expressed in a variety of cells including: adipocytes, hepatocytes, keratinocytes, and epithelial cells[119]. In hematopoietic cells it is expressed in the myelomonocytic lineage[120]. Its expression is upregulated during differentiation/maturation of myeloid cells, but no defects were identified in granulopoiesis in C/EBP-β-deficient mice[121].

While C/EBP-α is the key factor in steady-state granulopoiesis, C/EBP-β is the key factor in emergency granulopoiesis[100].

Ectopic expression of C/EBP-β can induce granulocytic differentiation of primary cell lines *in vitro*. In addition, in C/EBP-α null mice, expression of C/EBP-β rescues granulopoiesis *in vivo*, suggesting that that C/EBP-β can substitute for C/EBP-α to induce granulocyric differentiation in both *in vitro* and in vivo[100].

Therefore, C/EBP-β should be able to bind to the same target promoters of the C/EBP-α, including genes encoding G-CSF-R, MPO, lysozyme, elastase, proteinase 3, lactoferrin, neutrophil collagenase.

In GMPs, C/EBP-β was upregulated after cytokine stimulation or infections. While C/EBP-α activity is inhibited by phosphorylation, C/EBP-β activity is enhanced by phosphorylation. Cytokines may phosphorylate both C/EBP-α and C/EBP-β,

thereby enhancing C/EBP-β activity and suppressing C/EBP-α activity, especially in emergency situations such as infections[100].

Taken together, C/EBP-α is required for steady-state granulopoiesis and C/EBP-β is required for emergency granulopoiesis specifically in conditions of stress, in which various factors including cytokines and pathogens play a role in this transcriptional switch.

PU.1:

Is a member of the ets (E twenty six retrovirus) transcription factor family. It is expressed by myeloid cells and B-lymphocytes, but not by T-lymphocytes[122]. In mice, it is expressed by myeloid and erythroid precursors, macrophages, and megakaryocytes, but is not found in mature granulocytes, osteocytes or vascular endothelium[123]. PU.1 expression increases during myeloid differentiation and may be required for terminal maturation of myeloid cells[124]. Treatment of monocytes with GM-CSF increases PU.1 expression and induces macrophage differentiation. Interestingly, transduction of alveolar monocytes with a PU.1-expresing retrovirus was enough to drive macrophage differentiation in the absence of GM-CSF[125]. A similar effect was seen with maturation of granulocytes. Expression of PU.1 increases as immature myeloid cells differentiate into mature granulocytes[126]. However, some studies suggest that sustained high-level expression of PU.1 drives myeloid differentiation and favors monocyte and macrophage development over granulocytic development[127]. It has been proposed that the ratios of PU.1 to C/EBP-α in uncommitted hematopoietic progenitors are important in determining cell fate decisions. Thus: high C/EBPα: PU.1 ratio favors granulocytic maturation and low C/EBPα: PU.1 ratio favors monocytic differentiation[128]. Supporting this hypothysis, PU.1 null mice lack B-cells, monocytes and have markedly reduced numbers of neutrophils[129]. These neutrophils do not express markers of terminal differentiation. Thus, PU.1 deficient cells can commit to neutrophilic lineage but cannot fully mature along this lineage[130].

Important target genes include almost all myeloid specific gene promoters including: M-CSF-R, G-CSF-R, GM-CSF-R, lysozyme, neutrophil collagenase, proteinase 3, elastase, cathepsin-G, MPO, CD45, CD11b and CD18. In addition, PU.1 binds and regulates its own promoter[131].

The role of hematopoietic cytokines in granulopoiesis:

G-CSF (in synergy with IL-3) has a role in early hematopoiesis[132]. G-CSF is the main actor on neutrophil lineage, stimulating their proliferation, survival, maturation, and functional activation[133]. G-CSF deficient mice display reduced neutrophil development but still retain some neutrophil production, possibly through alternative pathways. G-CSF is produced by a number of different cells including: monocytes, macrophages and endothelial cells[133, 134]. The serum levels of G-CSF, as well as GM-CSF and IL-3, increase during infections leading to granulocytosis[135]. In vivo administration of G-CSF causes an increase in the granulocyte numbers, and

lack of G-CSF or G-CSF receptor leads to a 70-80% decrease in circulating granulocytes[136, 137]. These data suggest a role of G-CSF in the regulation of both steady-state and emergency granulopoiesis. GM-CSF and IL-3 can also enhance granulopoiesis *in vivo*[136]. However, mice lacking GM-CSF and IL-3 signaling have normal counts of all peripheral blood cells, except eosinophils[138]. GM-CSF stimulates proliferation, survival and differentiation of myeloid progenitor cells including monocyte/macrophage, granulocyte, erythrocyte and megakaryocyte lineages[133]. GM-CSF can influence commitment choices, promoting CMP over CLP, GMP over MEP and neutrophil over monocyte fate[132]. Different concentrations of GM-CSF seem to play a role in different responses to the cytokine especially in the neutrophil versus monocyte/macrophage commitment[133]. GM-CSF is produced by an array of cell types including macrophages, eosinophils, T- and B-lymphocytes, mast cells, and a number of non-hematopoietic cells such as stromal cells, fibroblast and endothelial cells[133].

IL-3 shares common features with GM-CSF, since both shares the same beta subunit of GM-CSF receptor. The α–chain (CD123) binds specifically to IL-3 with low affinity, but complex formation with βc-chain (CD131w) either as a heterodimer or tetradimer is necessary for high affinity binding and signal transduction. The βc-chain is shared by three cytokines; GM-CSF, IL-3 and IL-5. The α–chain of IL-5 receptor is only present on eosinophils[133].

IL-3 is produced by activated T-lymphocytes, activated mast cells and perhaps other cells such as NK cells, eosinophils and stromal cells[133].

Neutrophil clearance:

In normal situations, the short-lived neutrophils die by apoptosis and are subsequently phagocytosed by macrophages. Circulating apoptotic neutrophils are suggested to be cleared from circulation by macrophages located in the liver (~29%), spleen (~31%) and the bone marrow (~32%), suggesting that these three tissues contribute equally to neutrophil clearance from the circulation[139, 140].

Tissue neutrophils, which migrate to tissues during infections, are removed by local macrophages that secrete anti-inflammatory cytokines TGF-β and IL-10 upon phagocytosis of these neutrophils[141]. For normal homeostasis to take place and in order to keep normal counts of neutrophils in the circulation (2.5-7.5 $\times 10^9$/l), neutrophil turn-over must be tightly balanced between granulopoiesis and neutrophil apoptosis/clearance. Neutrophil turn-over is estimated to be ~10^{11} cells per day in the average adult human[21]. Delayed neutrophil apoptosis has been associated with several acute and chronic inflammatory diseases[142, 143].

Neutrophil apoptosis

General aspects of apoptosis

Cell death can occur by two major distinct mechanisms – necrosis or apoptosis. Necrosis or "accidental" cell death is a pathological process that occurs when cells are subjected to severe physical or chemical attack. Apoptosis, also referred to as type I cell death or "programmed" cell death, is a physiological form of cell death characterized by cell shrinkage, nuclear and chromatin condensation, DNA fragmentation, membrane blebbing, externalization of phosphatidylserine (PS), and formation of membrane-bound apoptotic bodies[143]. It is the preferred mechanism to remove unwanted or unused cells during development and other normal biological processes[142, 144]. For example, formation of the fingers and toes of the fetus requires the removal, by apoptosis, of the tissue between them. Apoptosis is also needed to destroy cells that represent a threat to the integrity of an organism. As an example, cytotoxic T-lymphocytes kill virus-infected cells by induction of apoptosis. Another example is the induction of apoptosis of autoreactive T- and B-lymphocytes in the thymaus and the bone marrow, thereby preventing these cells from attacking self-antigens[145].

Many players are known to regulate apoptosis. Examples include caspases, cell death receptors (of the TNF family), adaptor proteins, inhibitor of apoptosis (IAP) proteins and the bcl-2 family[146, 147]. Caspases are cysteine proteases that recognize tetrapeptide motifs, and cleave at the carboxyl side of an aspartate residue. Initiator caspases like caspase 8 and 9 start a cascade of increasing caspase activity by processing and activating downstream effector caspases. These activated effector caspases cleave and inactivate vital cellular proteins, thereby inducing the characteristic morphological changes seen in apoptosis[148]. Cell death receptors are members of the tumor necrosis factor (TNF) receptor family, which are activated by structurally-related ligands. These can have pleiotropic actions depending on cell type and signals received, triggering cell proliferation, differentiation, or death. For example, CD95 contains a cytoplasmic region called the death domain (DD) that transmits signals via an adaptor protein to the caspases. Thus adaptor proteins form bridges between cell death effectors (caspases) and the cell death regulators (death receptors and Bcl-2 family members)[149]. The Bcl-2 family contains at least 20 related proteins. Family members share one or more Bcl-2 homology (BH) domains and are divided into two groups based on whether they promote or inhibit apoptosis. Anti-apoptotic members include Bcl-2-A1, Mcl-1, Bcl-xL, Bcl-w, and Boo/Diva and pro-apoptotic members include Bad, Bid, and Bax[150]. Lastly, IAP proteins help in suppressing apoptosis triggered by various stimuli. IAP proteins include cellular IAP-1 (cIAP-1), cIAP-2, X-linked IAP (XIAP), neuronal IAP (nIAP), and surviving. Most of the members are known to inhibit caspase activity[151].

Mechanisms of neutrophil apoptosis

Intrinsic pathway

This process is regulated by various proteins and molecules. Mcl-1 is a key Bcl-2 family protein in constitutive apoptosis. As neutrophils undergo apoptosis, levels of Mcl-1 fall rapidly suggesting a pro-survival role of this protein. Another anti-apoptotic protein in neutrophils is the Bcl-2-A1 (Bfl 1) gene product, which is largely cytoplasmic. There are data, which indicate that Bcl-2-A1 may function alongside Mcl-1 in neutrophils to control cell function[143]. SHIP-1 is important in limiting anti-apoptotic signals in neutrophils, via interaction, dephosphorylation and inactivation of Lyn, Tyk-2, JAK-2 and PI3K, which are known mediators of survival signals in neutrophils[152, 153].

Mitochondria play an important role in the intrinsic pathway of apoptosis. Mitochondria exerts its pivotal actions in apoptosis through three key mitochondrial proteins; cytochrome c (cyt c), Smac/DIABLO and apoptosis inducing factor (AIF). The release of cyt c from the mitochondria is recognized as an initiator of apoptosis via interaction with Apaf-1 (apoptotic protease activating factor-1). This interaction leads to activation of caspase 9, formation of the apoptosome, and triggering of the caspase cascade. At the same time, Smac/DIABLO neutralizes IAPs and allows caspase activation to proceed. The Bcl-2 family regulates mitochondrial membrane permeability and cyt c release, thus playing a central role in apoptosis. Neutrophils possess very few mitochondria and express low amounts of cyt c and Smac/DIABLO. However, these amounts are sufficient to induce apoptosis. The tendency of neutrophils towards spontaneous apoptosis is inversely correlated with Bcl-2 expression[154].

Extrinsic pathway

This pathway is initiated by an extracellular death signal. Death receptors bind extrinsic factors (FasL, TNF-α, TRAIL) leading to activation of the caspase cascade, which in turn generates intracellular death signals culminating in apoptosis. Death receptors such as Fas and the TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. Fas and Fas ligand (FasL) interaction initiates apoptosis in a caspase-dependent maner. Binding of Fas to FasL leads to trimerization of the receptor, recruitment of the Fas activated death domain and, activation of caspase 8, which in turn activates a caspase cascade. Neutrophils undergo spontaneous apoptosis more than other leukocytes, probably because they express both Fas and FasL on their plasma membrane[143, 146].

Caspase-independent pathway

Apoptosis-inducing factor (AIF) is a flavoprotein that is normally located in the inter-membrane space of mitochondria. When cells receive a signal for apoptosis, AIF is released from the mitochondria and translocates into the nucleus and causes nuclear fragmentation and cell death. The DNA destruction mediated by AIF is not blocked by caspase inhibitors and is thus considered a caspase-independent pathway.

In neutrophils, AIF does not leave the mitochondria and the caspase-independent pathway is mediated by mitochondria-derived reactive oxygen species (ROS)[143].

Regulation of neutrophil apoptosis

The mechanisms regulating spontaneous neutrophil apoptosis are not fully understood. A role for calpain as a pro-apoptotic factor in the regulation of neutrophil apoptosis has been suggested[155]. Disturbance in the normal apoptotic process can enhance survival time, leading to a persistent inflammatory response. Blood neutrophils do not express the anti-apoptotic Bcl-2 and Bcl-xL proteins, while expressing fairly high levels of a range of pro-apoptotic proteins like Bad, Bax and Bik. Several pro-inflammatory agents, including IL-1β, L-2, IL-4, IL-6, IL-15, IFN-γ, G-CSF, GM-CSF and LPS, can delay neutrophil apoptosis[142]. G-CSF induces survival of PMNs via the MEK-ERK pathway, leading to phosphorylation of Bad (inactivation); also GM-CSF induces survival via tyrosine kinase LynK-PI3K and JAK-2. Phosphorylation of JAK-2 is followed by activation of STAT proteins, leading finally to increased phosphorylation of Bad. G-CSF up-regulates the expression of Bcl-2-A1 and downregulates the expression of Bax[156]. GM-CSF upregulates the expression of Mcl-1 and down-regulates the expression of Bax[157, 158].

TNF-α has a dual action on neutrophil apoptosis, leading to accelerated apoptosis in a susceptible subpopulation and delayed apoptosis in the surviving cells. TNF-α differential effects are also dependant on its concentration and the time of exposure[159]. Adhesion of neutrophils to activated endothelial cells, inhibit their apoptosis[160]. The chemoattractant, IL-8, as well as transmigration of neutrophils through endothelial cell layer lead to delayed neutrophil apoptosis[161, 162]. Glucocorticoids also lead to delayed neutrophil apoptosis and subsequent neutrophilia[163]. The effect of glucocorticoids on neutrophils contrasts their effect on eosinophils, where they cause accelerated apoptosis[164]. In contrast to other cells, hypoxia can delay neutrophil apoptosis[165]. Neutrophils from elderly people have hyposensitivity to growth factors and their survival signals[166, 167]. This hyposensitivity is thought to be due to higher intracellular levels of SHIP-1 and SOCS proteins in the elderly[168]. SHIP-1 and SOCS proteins are known inhibitors of JAK-2 activation/phosphorylation. Patients with chronic renal failure have accelerated rate of neutrophil apoptosis[169].

IAPs regulate apoptosis by binding to TNF-receptor associated factor-1 (TRAF-1)/ TRAF-2 heterocomplex to suppress activation of caspase 8. IAPs also act via an intrinsic pathway by binding to pro-caspase 9, thereby suppressing its activation. They are capable of inhibiting the activation of caspases 3 and 7 directly[143]. G-CSF, but not GM-CSF, selectively up-regulates the expression of cIAP-2, at the protein as well as mRNA levels. Furthermore, neutrophils from patients with chronic neutrophilic leukemia show over-expression of cIAP-2 mRNA as well as prolonged survival[170].

Neutrophil apoptosis and resolution of inflammation

Resolution of inflammation requires two steps. First, cessation of recruitment of additional neutrophils and return of endothelial cells to a resting state, and secondly, apoptosis and subsequent removal by phagocytosis of the intact dying neutrophils at the site of inflammation[171]. During the inflammatory response, neutrophils produce numerous cytokines and chemokines, via up-regulation of gene expression. Once phagocytosis is complete, these functions are down-regulated in tandem with induction of apoptosis leading to a decrease in pro-inflammatory capacity. This process is tightly regulated to prevent tissue damage caused by lingering neutrophils. Walcheck et al. have shown that phagocytosis-induced neutrophil apoptosis is accompanied by increased surface expression of ADAM17, a metalloprotease that plays a role in the down-regulation of neutrophil function[172]. This is followed by ADAM17-mediated release of IL-6R from cells, which then recruits mononuclear phagocytes to the site of infection that phagocytose apoptotic neutrophils[173]. Recruitment of macrophages to sites of inflammation is also promoted by endogenous molecules called resolvins and protectins including lipoxin A4 (LXA4)[174]. Several macrophage receptors are known to play a role in phagocytosis; these include PS receptor, complement receptors, scavenger receptors and lectins, CD36/thrombospondin recognition system, CD14 and CD44 .The removal of apoptotic neutrophils is a non-phlogistic process, largely due to release of anti-inflammatory mediators[88]. There are two possible outcomes for neutrophil-bacteria interactions; phagocytosis and killing of bacteria can lead to induction of apoptosis and subsequent removal by macrophages, ultimately resulting in the resolution of infection. Or certain pathogens, such as Anaplasma phagocytophilum and Chlamydia pneumoniae, can alter/prolong neutrophil apoptosis to survive intracellularly, disseminate and cause disease[88].

Neutrophil NETosis

A novel form of PMN death named “NETosis”, characterized by the active release of chromatin, has been described recently[89]. Neutrophil extracellular traps (NETs) are extrusions of plasma membrane and nuclear material, containing granule components and histones. These structures bind gram-positive and negative bacteria, as well as fungi. In vitro, NETs have been shown to bind and kill extracellular microorganisms; in vivo, they have been documented in conditions, including appendicitis, sepsis, pre-eclampsia and experimental models of shigellosis[175]. The changes leading to NET formation follow a specific pattern, which is initiated by the loss of nuclear segregation into eu- and heterochromatin. Once the chromatin and granular components are mixed, NETs are released from the cell after cytoplasmic membrane rupture by a process distinct from necrosis or apoptosis, termed NETosis. NADPH oxidase plays a role in this process, via generation of ROS, which act as signaling molecules. Fuchs et al demonstrated that NET formation is a part of active cell death, and that NETs are released when the activated neutrophils dies[176].

ANCA:

Already in 1957 Calabrese described autoantibodies that reacted specifically with ethanol fixed leucocytes[177]. This phenomenon was for many years referred to as granulocyte specific antinuclear antibodies (GS-ANA), and was considered typical for certain variants of rheumatoid arthritis[178]. Davies reported in 1982 that sera from some patients with rapidly progressive glomerulonephritis gave rise to a more cytoplasmatic staining pattern[179]. In 1985 van der Woude and Rasmussen published a seminal article showing that such a staining pattern was highly specific for WG[180]. Today autoantibodies responsible for both these staining patterns are called anti-neutrophil cytoplasm antibodies (ANCA). The cytoplasmic version is now called c-ANCA, while the GS-ANA pattern is called p-ANCA, where p stands form perinuclear. Most targets for ANCA are proteins residing in neutrophil granules and artefacts based on redistribution during ethanol fixation cause the different staining patterns.

Indirect immunoflourescense (IIF) on ethanolfixed leucocytes is still used for the detection of ANCA, but methods based on purified antigens such as ELISA (Enzyme Linked Immunosorbant Assay) generally yield higher specificity. In systemic vasculitis the two most important ANCA antigens are PR3 and MPO. The vast majority of anti-PR3 antibodies yield a c-ANCA pattern on IIF, while most anti-MPO antibodies produce a p-ANCA pattern. However, exceptions exist and some anti-MPO antibodies do produce a c-ANCA pattern[181]. According to an international consensus document from 1999, anti-MPO and anti-PR3 antibodies should be referred to as MPO-ANCA and PR3-ANCA even if they are only detected by ELISA, The consensus statement requires that all sera be tested by IIF and any positive results be confirmed by PR3- and MPO- ELISA[3].

PR3-ANCA

PR3-ANCA are most closely associated with WG. PR3-ANCA are detected in 70-80% of patients with WG, 15-30% of patients with MPA and 15-20% of patients with CSS[182]. Few patients with WG are negative for ANCA, and these patients mostly have localized disease, and might develop ANCA after disease progression[183]. Not all patients whose sera exhibit PR3-ANCA suffer from WG, PR3-ANCA have been detected in patients with bacterial endocarditis[184].

Patients with anti-PR3-associated vasculitis present with more extensive extra-renal organ involvement than patients with anti- MPO-associated vasculitis. All PR3-ANCA$^+$ patients suffer from WG or MPA, but granulomas were found only in biopsies from PR3-ANCA$^+$ patients. PR3-ANCA$^+$ patients are further characterized by frequent relapse and faster decline of renal function than MPO-ANCA$^+$ patients[185].

PR3-ANCA are highly specific (99%), but moderately sensitive (73%) as a diagnostic marker of WG (when both IIF and ELISA methods are used)[186]. Rising titers of PR3-ANCA may indicate ongoing active disease. Moreover, increases in

PR3-ANCA during follow-up predicted disease relapse, while PR3-ANCA are often, but not always, undetectable after patients go into remission [187-189]. In one prospective study of 85 patients with PR3-ANCA associated vasculitis, a rise in PR3-ANCA titer preceded relapse in 71% of the relapsed patients, whereas a rise in PR3-ANCA was not followed relapse in 29% of vasculitis patients. [190].

MPO-ANCA

MPO-ANCA has been reported in many clinical situations. MPO-ANCA are present in 50-70% of patients with MPA, 30-40% of patients with CSS and 10-15% of patients with WG. Only a few patients with MPA are ANCA negative[182].

Interestingly, ANCA-positive patients with CSS present with small-vessel vasculitis that manifests clinically as mononuritis multiplex, purpura and glomerulonephritis, whereas ANCA-negative patients with CSS predominantly show tissue infiltration by eosinophils. This observation suggests that CSS exists as two distinct disease entities[191].

MPO-ANCA are more commonly associated with renal limited vasculitis. Respiratory tract involvement is found in only 24% of patients with MPO-ANCA, compared to 78% of PR3-ANCA^{+} patients[192]. On the other hand, almost all patients with MPO-ANCA have renal involvement (90-100%), compared to 70% in patients with PR3-ANCA[193]. Although there is little data on the relationship between MPO-ANCA and relapse, the available data suggest that MPO-ANCA predicts relapse for 90% of cases[194, 195].

Taken together, these observations suggest that PR3-ANCA and MPO-ANCA are markers for different phenotypes of AASV.

Other ANCA specificities

In classical primary AASV, ANCA are mostly directed against one antigen (PR3/MPO). ANCA directed against more than one antigen is very rare in primary AASV and should raise the suspicion of secondary vasculitis or another autoimmune disease. Although antibodies to other proteins (including lactoferrin, actin, defensin, cathepsin, catalase, enolase and elastase) have been identified in patients with inflammatory bowel disease, autoimmune liver disease, rheumatoid arthritis and drug-induced vasculitis, these antibodies have little diagnostic value[196]. Elastase is a common target for ANCA in patients with drug-induced vasculitis. In such patients, Elastase-ANCA are commonly found with other ANCA specificities and also other autoantbodies such as ANA and anti-cardiolipin antibodies[197].

Bactericidal Permeability Increasing protein (BPI)-ANCA are detected in up to 91% of cases of cystic fibrosis (CF)[198]. Interestingly, BPI-ANCA in CF strongly correlate with *p. aerogenosa* colonization in the lungs and seem to be a consequence of *p. aerogenosa* infection[199].

ANCA are predominantly of the IgG isotype[196]. Although, IgA-BPI-ANCA are described in up to 83% of cases of CF and 79% of cases of Henoch-Shönlein purpura[198, 200]. IgM-ANCA are described in few cases of vasculitis and are associated with more severe disease (Severe pulmonary hemorrhage)[201]. Combination of ANCA with anti-glomerular basement membrane (GBM) antibodies is also described in 5-30% of the cases of vasculitis and is associated with poor clinical outcome [202, 203].

Natural antibodies against PR3 and MPO have recently been described in healthy individuals. The levels of these natural anti-PR3/MPO antibodies were much lower than PR3/MPO-ANCA from patients with vasculitis. The functions and clinical significance of these natural anti-MPO, anti-PR3 antibodies is not yet known[204].

Pathophysiology of AASV

The etiology of AASV remains largely unknown. Available data suggest that neutrophils, B- and T- lymphocytes play key roles in the pathophysiology of AASV. Although the association between ANCA and pauci-immune small vessel vasculitides is well established, the exact role of ANCA in the pathogenesis of AASV is yet not fully elucidated. It is not known whether ANCA play a direct role in disease manifestations, or whether the antibodies are secondary markers of the disease process.

Genetic predisposition

Even though some case reports show clusters of WG in siblings and close relatives there are few genetic variations proven to predispose for AASV. Reports exist that point at specific HLA associations (DR1-DQw1), but not all studies have been consistent[205]. The best-established genetic risk factor for WG is the PiZ allele of the gene coding for the serine protease inhibitor α_1-AT[206-209]. If the PiZ allele also increases the risk for MPA has not been determined.

Some gene variants that seem to increase the risk for many sorts of autoimmune diseases such as CTLA-4 (affecting T cell activation) and PTPN22 (negative regulatory role in T-cell receptor signalling) have been implicated also in AASV. The percentage of neutrophils expressing PR3 on their plasma membrane ($mPR3^+$) has been reported to be increased in AASV[210]. Even though this phenomenon may be genetically determined, the mechanism remains to be elucidated. A polymorphism in the promotor region of the PR3 gene that involves a putative transcription factor binding site, has been reported to increase the risk for WG[41]. This polymorphism may lead to increased expression of PR3 and could potentially explain the high $mPR3^+$ phenotype.

Environmental factors

Clinical and epidemiological evidence demonstrate that environmental factors, including silica, asbestos, drugs (anti-thyroid medications), and various infections (bacterial endocarditis, hepatitis C visrus), correlate with circulating ANCA and development of vasculitis[211, 212]. Beaudreuil et al showed that exposure to silica is associated with a nearly seven-fold increased risk of being ANCA-positive[213]. ANCA, both PR3-ANCA and MPO-ANCA, are detected in sera of patients with protracted infections; however, in most infections, ANCA are directed against a wide repertoire of antigens. Stegeman et al. described an association between nasal S. aureus and relapses of PR3-AASV[214]. Chronic infections may prime neutrophils, which can be further activated by PR3-ANCA, leading to vasculitis. It is also possible that some exogenous non-self proteins (i.e., bacterial, viral, fungal) mimic auto-antigens, which generates ANCA and an ANCA response. For example, PR3-ANCA has been detected in sera of patients with bacterial endocarditis[184]. Another example is the BPI-ANCA that correlate with *p. aeruginosa* colonization in patients with CF. Long standing exposure of the immune system to specific antigens, may set the stage for development of ANCA and subsequent AASV. Several experimental studies support the notion of molecular mimicry as the mechanism behind AASV and ANCA generation. The mimicry may be indirect as postulated by the anti-complementary PR3 antibody theory[215] or directed against neighbouring molecules in the granules such as in the anti-LAMP (Lysosomal associated membrane protein) theory[216].

Pathogenic B-cell response and production of ANCA

B-cells are the direct precursors of antibody producing plasma cells. B-cells also produce cytokines (Interleukin IL-6, TNFα, IL-10), act as antigen presenting cells, and differentiate into long lasting memory B-cells. B-cells recognize soluble antigens via specific B-cell receptors (BCR) and co-receptor CD19 that augments BCR downstream signaling. CD19 dysregulation has been reported in patients with AASV. Culton et al. showed that CD19 expression is 20% lower in naïve B-cells from patients with AASV than from normal controls[217]. In contrast, the memory B-cells from some patients with AASV express more CD19 than normal controls. This subset of B-cells shows evidence of antigenic selection, suggesting that in AASV, mechanisms of self-tolerance may be lost leading to production of auto-reactive B-cells[217]. Experiments in transgenic mice indicate that defective B-cell regulation, specifically in pathways responsible for deletion (central and peripheral) of auto-reactive B-cells, may also play a role in generating autoantibodies in AASV[218].

Interestingly, expression of B-cell activating factor of the TNF family (BAFF) is increased in patients with WG[219]. It is postulated that BAFF may drive B-cell expansion, which then leads to ANCA production. B-cell depletion via rituximab in patients with AASV decreases ANCA levels and induces disease remission[220, 221]. Conversely, clinical relapse correlates with increased levels of B cells[222]. These data support the conclusion that B cells play a central role in ANCA production and

pathogenesis of AASV. Csernak et al. have shown that in WG patients, ANCA are produced following B-cell activation[223]. A polyclonal B-cell lymphoid infiltrate in the endonasal granulomatous lesion included PR3-ANCA-producing cells with copy number increase in three VH genes. The granulomatous lesions in WG consist of clusters of PR3 surrounded by an infiltrate consisting of maturing B-cells, antigen-presenting cells (APCs) and Th1-type $CD4^{+}CD28^{-}$ T cells. This suggests that endonasal B-cell maturation is antigen-driven, and that B-cells generate ANCA via contact with PR3 or an antigenic microbial epitope, Figure 2[224].

Aberrant T-cell response and granuloma formation

There are several lines of evidence suggesting a role for T-cells in the pathogenesis of AASV. The skewed genetic balance for genes regulating T-cell function (CTLA-4 and PTPN22) has already been mentioned. The IgG subclass distribution of ANCA, with predominance for IgG1 and IgG4, also argue for T-cell involvement since switch to these subclasses require T-cell help[225]. Paucity of immunoglobulins in the vasculitic lesions and the finding that treatment targeting T-cells produce remissions in AASV patients are also consistent with a role for T-cells[226, 227].

In patients with active WG, higher proportion of activated T-cells and higher concentration of soluble T cell activation markers (including soluble IL-2 receptor or CD30) are reported to correlate with disease activity[228]. High levels of activation markers also correlate with ANCA-positivity, which suggests persistent T-cell activation. This is consistent with reports of persistent expansion of $CD4^{+}$ effector memory T-cells (Tem) combined with a decrease in naïve T-cells in patients with AASV[229, 230]. A polarization of Th1 and Th2 response has also been reported in AASV. In particular, a Th2-type response is predominant in patients with active generalized WG or CSS, while a Th1 response is dominant in patients with localized WG or MPA, indicating that aberrant T cell response plays a role in the disease process[231, 232]. CCR5 is also expressed on T-cells in early, localized WG, which might also favor recruitment of Th1-type cytokine secreting cells into inflammatory lesions in localized WG[233]. Conversion from Th1 to Th2 type response could underlie progression from localized to generalized WG. This shift could reflect B-cell expansion and T-cell-dependent PR3-ANCA production, secondary to interaction between neutrophils and auto-reactive T- and B-cells in inflammatory lesions, Figure 2.

The granulomas in AASV resemble a germinal centre, with a cluster of primed neutrophils surrounded by dendritic cells, T- and B-cells. $CD4^{+}$ T cells are likely to play an important role in the granulomatous response in WG and CSS. The decrease in $CD4^{+}CD28^{--}$ Tem subset of T-cells during active disease, in patients with WG, indicates an increased migration of these cells to sites of inflammation[229]. In an experimental model of autoimmune, anti-MPO-associated glomerulonephritis, it was noted that mice depleted of $CD4^{+}$ T cells, at the time of administration of anti-mouse anti-GBM antibodies, developed significantly less crescent formation and cell response, compared to controls[234]. In patients with ANCA-associated

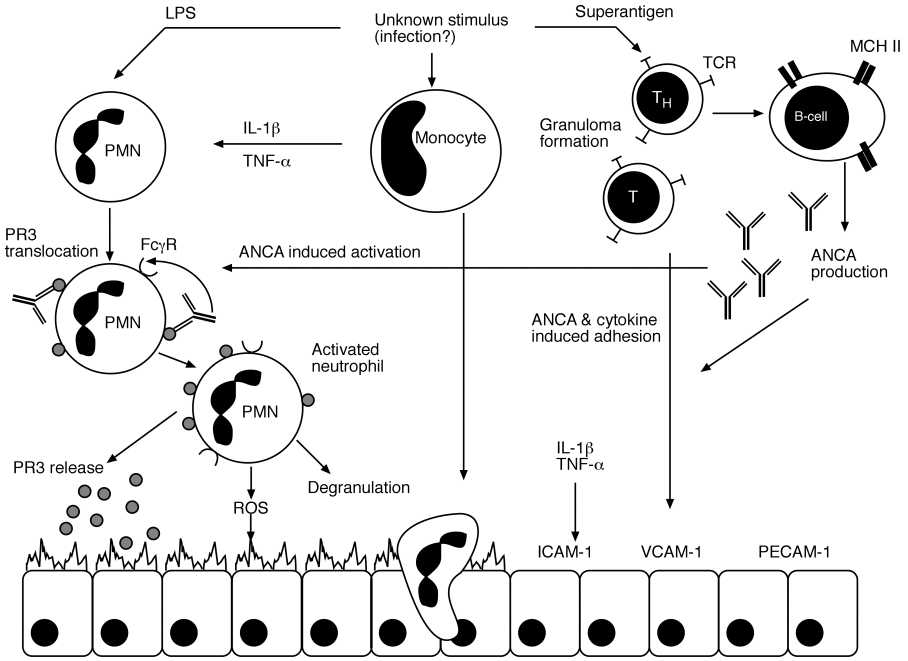

Figure 2. Pathophysiology of AASV. The stimulation of neutrophils by TNF-α or IL-1β (priming), e.g. during a preceding infection, leads to the translocation of the ANCA-antigens, PR3 and MPO, from the cytoplasmic granules (specific granules and secretory vesicels) to the cell surface, where they are accessible for ANCA, which leads to a further activation of the cell. ANCA-induced neutrophil activation initiates production of ROS, neutrophil degranulation with release of inflammatory cytokines and granule contents (e.g., PR3 and HLE) from azurophilic granules, leading to endothelial cell detachment and lysis. Furthermore, neutrophil activation leads to leukocyte adhesion (via ICAM-1, VCAM-1) and transmigration through endothelium (via PECAM-1), and release of ROS and proteases into tissues. Superantigen (e.g., Staphylococcal exotoxins) or PR3 presented to the T-cells directly or via dendritic cells, are capable of stimulating the proliferation of T-cells, leading to granuloma formation and finally to maturation of PR3-specific autoreactive B-cells, culminating in ANCA production. ROS= Reactive oxygen species. PR3= Proteinase 3, MPO= Myeloperoxidase, HLE= Human Leukocyte elastase, ICAM= Intercellular adhesion molecule-1, VCAM-1=Vascular cell adhesion molecule-1, PECAM-1= Platelet endothelial cell adhesion molecule-1, TCR= T-cell receptor, MHC-II= Major Histocompatibility complex-II, TNF-α=Tumor necrosis factor-alpha, IL-1β=Interleukin-1 Beta. The figure is reproduced, with kind permission of Springer Science+Business Media, and kind approval from the authors[238].

glomerulonephritis, Tem cells are the predominant T-cell subtype in the glomerular infiltrate[235]. Together, these observations suggest that a cell-mediated immune response contributes to the pathogenesis of renal lesions. Indeed, $CD4^+$ Tem cells from WG patients lack NKG2A (inhibitory receptor) and demonstrate increased expression of NKG2D, which is a member of the killer immunoglobulin-like

receptor family[236]. A significant increase in the proportion of IL-17 producing $CD4^+$ T cells (Th17 cells) in *in vitro* stimulated peripheral blood cells from WG patients has also been reported[237]. IL-17 induces secretion of neutrophil-attracting chemokines, and release of pro-inflammatory cytokines (IL-1β, TNF-α) capable of increasing expression of PR3 on the membrane of neutrophils. Patients with ANCA-positive WG are reported to have more PR3-specific Th17 cells than ANCA-negative WG patients and healthy controls[237]. It is, therefore, likely that Th1, Th2 and Th17 responses play an important role in antibody production and granuloma formation in AASV.

Monocyte activation and production of pro-inflammatory cytokines

Wickman et al compared monocytes and cytokine profiles in patients with acute anti-PR3 vasculitis and normal controls; monocytes from patients were reported to have a reduced capacity to produce oxygen radicals[239]. Ohlsson et al., from our group, reported a positive correlation between circulating levels of IL-8 and monocyte IL-8 mRNA in patients with AASV, suggesting prolonged immune activation[240]. Pathological analysis of renal tissue from patients with AASV revealed the presence of monocytes in the glomerular crescents and granulomas[241]. *In vitro* studies demonstrated that ANCA are capable of stimulating monocytes, leading to release of cytokines including IL-8, monocyte chemotactic protein-1 (MCP-1), TNF-α, IL-1β, IL-6 and thromboxane A2, Figure 2[242, 243]. On the other hand, membrane PR3 expression on monocytes does not correlate with disease activity. There are many possible explanations for the presence of activated monocytes in glomerular crescents. For example, it is possible that monocytes are activated by direct physical interaction with components of glomerular lesions once they reach site of lesion; alternatively, dysfunctional apoptosis may stimulate monocyte activation[244].

Endothelial cell activation and enhanced expression of adhesion molecules

Endothelial damage, neutrophil invasion and necrosis are histopathological features of AASV[245]. Activated endothelial cells (ECs) express high levels of adhesion molecules. Increased circulating levels of endothelial proteins (thrombomodulin, vWF), and adhesion molecules (soluble intercellular adhesion molecule (sICAM)-1 and the soluble endothelial cell leukocyte adhesion molecule (sELAM)-1) have been reported in vasculitis[246]. Woywodt et al. reported the presence of significant numbers of circulating ECs and necrotic endothelial cell fragments in patients with active AASV[247]. A significant proportion of the circulating ECs stain positive for tissue factor (TF), which links proinflammatory mechanisms with thrombosis[247]. Interestingly, TF expression can be induced in ECs by the release of PR3 and elastase from neutrophils; this may be mediated via PR3 receptors on the endothelial cell surface[248]. Endothelial cell necrosis, and release of TF, may play a role in development of vasculitic lesions. The mechanism of endothelial cell necrosis is not

yet fully elucidated. Although anti-endothelial cell antibodies have been detected in AASV, their significance in this regard is not clear[249]. ANCA antigens, PR3 and MPO, can bind to endothelial cells via endothelial cell receptors[250, 251]. ANCA can bind to endothelial cell bound antigens, leading to EC activation. It is possible that ANCA-induced neutrophil activation induces release of cytotoxic enzymes that damage endothelial cells, Figure 2. In AASV patients with renal involvement, the levels of circulating angiopoietin-2 (Ang-2) correlate with the increased number of circulating ECs. *In vitro* studies suggest that the endothelial-specific angiopoietin (Ang)-Tie ligand-receptor system regulates endothelial cell detachment. By analogy, Ang-2 might regulate endothelial cell detachment in AASV[252]. Recently, it has been shown that PR3 has the ability to cleave high molecular weight kininogen, liberating a novel vasoactive kinin, termed PR3-kinin, thereby initiating kinin system activation. PR3-kinin binds to and activates kinin B_1-receptor, expressed by endothelial cells and leukocytes, and thereby enhances inflammation and capillary leakage[253]. Kinin B1-receptor activation enhances neutrophil migration and chemotaxis. PR3 may also lead to activation of complement system, through inactivation of C1 inhibitor, leading to complement-mediated endothelial cell injury[250].

Role of neutrophils

Neutrophils are present and activated at sites of injury in vasculitis lesions, both in lung infiltrates and renal biopsies[254, 255]. The number of activated neutrophils in renal biopsies correlated with renal tissue damage. In a mouse model of MPO–ANCA-associated vasculitis, neutrophil depletion reduces the number of vasculitic lesions[256]. In another Brown-Norway rat model of systemic necrotizing leukocytoclastic vasculitis induced by mercuric chloride and characterized by development of MPO-ANCA, a monoclonal antibody that depletes neutrophils could ameliorate vasculitis lesions[257]. All these observations provide indirect evidence that neutrophils play a key role in the pathogenesis of AASV. To reach to the site of injury/inflammation, neutrophils must be activated first.

The classical pathophysiological model of neutrophil activation could be formulated as follows: first, an initial event (antigenic stimulus) primes neutrophils via low concentrations of cytokines resulting from local infection, such as TNFα/IL-18/LPS, which subsequently induces membrane expression of PR3 and MPO. Second, priming induces clustering of FcγRIIa and β2-integrins and the formation of NADPH oxidase complex. Moreover, neutrophil priming results in increased expression of CD11b/CD18 and loose adherence of neutrophils to the endothelial cells. This is followed by binding of circulating ANCA to MPO and PR3 expressed on the neutrophil membrane. In the activation process, F(ab)′2 fragments of ANCA bind to their autoantigens, while Fc fragments bind to neutrophil Fcγ-receptors (FcγRIIa and FcγRIIIb) resulting in full activation of neutrophils[51]. There are many evidences for the activated state of neutrophils in AASV. For example, in patients with active WG, neutrophils show increased expression of β1-(CD29), β2-(CD18)

and the α-(CD11b) integrin subunits[258]. Neutrophils from patients with AASV also have increased mPR3 expression and higher basal production of superoxide radicals[259]. Alcorta et al have studied the leukocyte gene expression in ANCA-positive vasculitis by microarrays and revealed >200 upregulated genes. Most of these genes were expressed in neutrophils and correlated with disease activity. Among these genes were annexin-3, arginase-I, IL-1 receptor, and LTB4 receptor[260]. All these studies confirm that circulating neutrophils are activated in AASV. ANCA-induced neutrophil activation initiates multiple pathological processes, including production of reactive oxygen species (ROS), neutrophil degranulation with release of pro-inflammatory cytokines and granule contents (serine proteases, such as proteinase 3, and elastase), leading to EC detachment and lysis, Figure 2[250, 261]. Activated neutrophils also release factors that activate the alternative complement pathway, contributing to tissue injury[262]. Furthermore, neutrophil activation leads to leukocyte adhesion, transmigration across endothelium, and release of proteases and oxygen radicals into tissues that ultimately culminate in formation of vasculitic lesions[263].

Role of neutrophil apoptosis

Increased neutrophil apoptosis has been observed in AASV. Pathological specimens from patients of WG show clear presence of apoptotic and necrotic neutrophils[254, 264]. Leucocytes, with degraded nuclear material, undergoing disintegration and apoptotic cells have been observed in tissue specimens from ANCA-positive renal vasculitis[265]. Histologically, AASV is characterized by leukocytoclasis, with infiltration and accumulation of unscavenged apoptotic and necrotic neutrophils in tissues around blood vessels, and fibrinoid necrosis of the blood vessel walls[266]. E/M studies of the leukocytoclastic lesions, have suggested that there may be a defect in the clearance of apoptotic neutrophils. The minority of neutrophils in this study showed typical apoptotic changes of the condensed and marginated nuclei, while the majority showed intact nuclei with disintegrated cytoplasmic organelles and plasma membranes[267]. Apoptotic neutrophils may, in fact, be a source of immunologically exposed neutrophil antigens that promote the production of ANCAs. It has been speculated that the development of ANCA-positive vasculitis is a three-step pathological process. The first step involves an exogenous stimulus that increases neutrophil and macrophage apoptosis. An example is exposure to an inhaled substance like silica, which is known to induce apoptosis in human peripheral blood lymphocytes and to also induce Fas-ligand expression in lung macrophages (*in vitro* and *in vivo*), promoting Fas-dependent macrophage apoptosis in a murine model of silicosis[268, 269]. Similarly, other postulated etiological agents for AASV (propylthiouracil, Streptococcus Pneumoniae) have also been shown to induce/ accelerate apoptosis[270, 271]. In the second step the increased exposure to apoptotic neutrophils/defective clearance of these neutrophils may be the initiating factor for ANCA production and development of AASV. Finally, environmental and genetic factors may contribute to and trigger disease expression[171].

Gilligan et al. showed that aging neutrophils (unprimed) were capable of translocating PR3 and MPO to the membrane during apoptosis, as assessed by increased ANCA binding[272]. Another study showed that a small sub-fraction of TNFα-accelerated apoptotic neutrophils expressed higher levels of PR3 and MPO than TNFα-primed live neutrophils[273]. Also, Kantari et al have shown that scramblase-1 translocates PR3 to the plasma membrane in a flip-flop manner during apoptosis[61]. In contrast to all that, Yang et al demonstrated that the level of mPR3 is similar between apoptotic and non-apoptotic primed neutrophils[274]. Thus, although evidence for increased membrane expression of auto-antigen in apoptotic neutrophils is inconclusive, it can be concluded that MPO and PR3 remain accessible for ANCA on the membrane of apoptotic neutrophils.

Interestingly, Patry et al showed that injection of syngenic apoptotic neutrophils, but not freshly isolated neutrophils, into Brown Norway rats resulted in development of p-ANCA, with the majority being specific for elastase, again indicating that apoptotic neutrophils may boost an autoimmune response[275]. In another study, intraperitoneal infusion of live or apoptotic human neutrophils (but not formaline fixed or lysed neutrophils) into C57BL/6J mice resulted in development of ANCA specific for lactoferrin or myeloperoxidase. A second intravenous infusion of apoptotic neutrophils resulted in the development of PR3-specific ANCA. Again no vasculitic lesions were found in those mice developing ANCA[276].

ANCA may also dysregulate the process of neutrophil apoptosis. In an *in vitro* study conducted by Harper et al., ANCAs accelerated apoptosis of TNF-α-primed neutrophils by a mechanism dependent on NADPH oxidase and the generation of ROS. This was accompanied by uncoupling of the nuclear and cytoplasmic changes from the surface membrane changes. That is, while apoptosis progressed more rapidly, there was no corresponding change in the rate of externalization of PS (phosphatidyl serine) following activation of neutrophils by ANCAs. This dysregulation created a 'reduced window of opportunity' for phagocyte clearance by macrophages, leading to a more pro-inflammatory environment[144]. It must be noted here that ANCAs were unable to accelerate apoptosis in unprimed neutrophils. Additionally, although there was increased expression of PR3 and MPO as apoptosis progressed, ANCAs were unable to activate these neutrophils. In fact, there was a time-dependent decrease in ROS generation as these neutrophils aged[144]. ANCA accelerates neutrophil apoptosis via generation of ROS, which act as amplifying factors for apoptosis. ROS are critical since neutrophils isolated from patients with chronic granulomatous disease (causing a defect in ROS production) do not show accelerated apoptosis after ANCA activation[144]. The same authors, in a later study, as well as another independent group showed that ANCA binding to apoptotic neutrophils enhanced phagocytosis by human monocyte-derived macrophages but also increased the secretion of pro-inflammatory cytokines like IL-1, IL-8 and TNF-α[259, 277]. IL-1 and IL-8 are capable of retarding apoptosis and are powerful chemo-attractants. The pro-inflammatory neutrophil clearance will result in further cell recruitment and perpetuation of inflammation.

In the same study, it has been shown that neutrophils from AASV patients with active disease express elevated levels of mPR3 and increased apoptosis when incubated *in vitro*[259]. In a recent study, it has been shown that that anti-PR3 antibodies can also penetrate into human neutrophils (*in vitro*) and lead to enhancement of the apoptotic process[278]. These data demonstrate that apoptotic neutrophils are capable of presenting immuno-reactive surface autoantigens, and ANCA are capable of inducing neutrophil apoptosis in a vicious cycle. The autoimmune response may be promoted by aberrant phagocytosis of apoptotic neutrophils by dendritic cells[279].

Clearance of neutrophils from the site of inflammation

Approximately 50% of WG patients have leukocytoclastic vasculitis, characterized histopathologically by infiltration and accumulation of unscavenged apoptotic or necrotic neutrophils or fragmented nuclei of neutrophils in the tissues around the vessels (leukocytoclasis)[266]. This suggests defective/incomplete removal of apoptotic or necrotic cells. This sugestion was reinforced by observations from E/M study of leukocytoclastic lesions[267].

In vasculitic patients, levels of PTX3 (pentraxin3) correlate with disease activity[280, 281]. PTX3, which is produced locally at sites of inflammation by endotelial cells and fibroblasts, binds to late apoptotic neutrophils and inhibits their clearance by macrophages. PTX3 may thus decrease clearance and persistence of these cells at the site of inflammation. Interestingly, membrane expression of PR3 on apoptotic neutrophils also inhibits their uptake by macrophages, thus decreasing neutrophil clearance[61]. ANCA opsonize apoptotic neutrophils, that express membrane PR3, enhancing their phagocytosis by macrophages, but simultaneously enhance the production of proinflammatory cytokines by these macrophages, thus stimulating a pro-inflammatory milieu, which may in turn stimulate more neutrophil recruitment[277]. Therefore in AASV, it is likely that PR3, ANCA and PTX3 are able to disturb normal clearance of neutrophils, which evokes a pro-inflammatory response, enhanced neutrophil recruitment and persistent inflammation.

Netting Neutrophils in ANCA associated systemic vasculitis

Based on recent studies on activated neutrophils in patients with AASV, ANCA-induced neutrophil activation also generates NETs (neutrophil extracellular traps), which contain PR3 and MPO, DNA–histone complexes and neutrophil granule proteins. Kessenbrock et al showed that NETs were prominent in specimens with strong neutrophilic infiltrate, correlating NETs with active disease[282]. Neutrophil proteins on NETs include LL37, which binds endogenous DNA and activates auto-reactive B-cells and plasmacytoid dendritic cells. This in turn, likely promotes secretion of INF-α and formation of ANCA, thus promoting an autoimmune response. *In vivo* presence of NETs was shown in tissues (kidney biopsies from patients with small vessel vasculitis), with maximal concentration in areas showing neutrophilic infiltration, which suggests that NET formation occurs predominantly during active disease[282]. In patients of AASV, increased levels of circulating

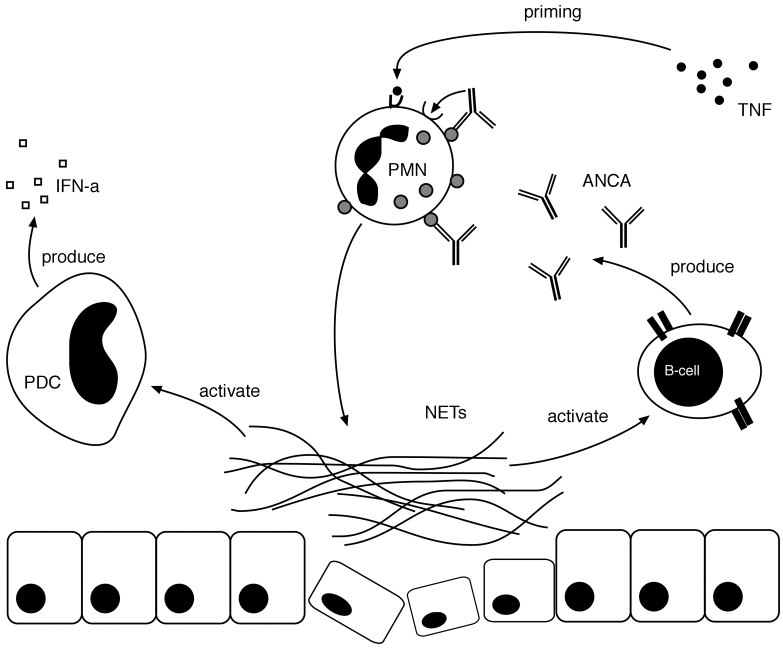

Figure 3. Pathophysiological model of neutrophil extracellular traps (NETs) in ANCA-associated vasculitis. ANCA can induce TNF-α-primed neutrophils to produce NETs. The deposition of NETs may activate plasmacytoid dendritic cells that produce large amounts of interferon-α driving the autoimmune response. In this context, NETs may activate autoreactive B cells to the production of ANCA, which results in a vicious circle of NET production that maintains the delivery of antigen–chromatin complexes to the immune system. Moreover, NETs may also stick to the endothelium and cause endothelial damage. Adapted from Oxford University press with kind permission[284].

nucleosomes has been reported[283]. It is likely that these may, in fact, be derived from and reflect NET formation in AASV. NETs may incite production of ANCA, via presentation of antigen-chromatin complexes to the immune system, and ANCA may incite production of NETs, which then could aggravate the immune response, leading to perpetuation of the auto-immune response, Figure 3.

Role of the enzymatic function of PR3 and MPO

The fact that heterozygocity for deficiency alleles of α_1-AT are associated with WG suggests that defects in the enzymatic function of PR3 may have functional effects. It has also been reported that presence of the PiZ allele correlates with poor prognosis[285]. PR3 can degrade the primary components of the extracellular matrix such as elastin, collagen and proteoglycans. PR3 is also enzymatically active against many cytokines and cytokine inhibitors, most often eliciting a pro-inflammatory response[45]. All these actions are inhibited by α_1-AT. MPO protects the enzymatic activity of PR3 by oxidizing a histidine residue on α_1-AT, which tilts the protease anti-protease balance at sites of inflammation[286].

Patients with systemic small vessel vasculitis exhibit higher plasma levels of PR3 than healthy persons and disease controls[287, 288]. This is true also during stable remission and is not related to general inflammation, medical treatment or decreased renal function. Circulating levels of MPO are also increased but not to the same extent, and levels of PR3 and MPO are elevated in patients with PR3-ANCA as well as MPO-ANCA. The origin of these increased plasma PR3 levels remains obscure. Circulating neutrophils and monocytes from patients with AASV display up-regulated transcription of the PR3 gene[289, 290], but it is not clear if these findings are related. As mentioned above, the secreted proform of PR3 exhibit a negative feed-back inhibition on neutropoiesis[48]. However, it is not been clarified whether the increased levels consist of mature PR3 or some proform.

Another possible origin of high plasma levels is shedding of membrane PR3. Witko-Sarasat et al. reported that the $mPR3^{high}$ phenotype was more frequent in vasculitis patients than in controls, independent of the ANCA antigen specificity[210]. However, this was not a vasculitis-specific effect, because the $mPR3^{high}$ phenotype was also reported to be increased in patients with rheumatoid arthritis and spodyloarthropathy. Rarok et al. found that the length of time between diagnosis and relapse was significantly shorter in WG patients with high mPR3 expression (total level of mPR3 expression), and that individuals with high total mPR3 expression were almost twice as likely to have a relapse than patients with low mPR3[291].

Csernok et al. showed that PR3 induces maturation of a fraction of blood monocyte-derived dendritic cells (DC) *in vitro*. In this context, they also observed that PR3 activates PAR-2 receptor-dependent signaling, which in turn up-regulates HLA-DR, CD80, CD83 and CD86 and down-regulates CD14. These PR3-activated DCs stimulate autoreactive Th1-type PR3-specific $CD4^+$T cells[292].

A recombinant cellular model was used to demonstrate that PR3 plays a role in neutrophil survival[293]. In particular, PR3 activates procaspase-3 into a specific 22-kDa fragment localized to the membrane compartment of neutrophils, but lacking from apoptotic neutrophils. This PR3-activated caspase-3 is restricted to the plasma membrane-enriched compartment, and segregated from its target proteins that mediate apoptosis from downstream components of the caspase-3 cleavage cascade. Thus in this model, PR3 can cause activation of caspase 3, but not apoptosis. Vong et al. devised a novel assay for PR3-protease activity using double-tagged recombinant annexin A1 (AnxA1) as substrate. This substrate was cleaved by recombinant PR3 or the membrane fraction of cells stably-transfected with PR3 *in vitro* and *in vivo*, suggesting that AnxA1 may be a physiologically-relevant substrate for PR3[294]. AnxA1 has counter-regulatory inhibitory properties, and functions as an anti-inflammatory protein as well as inducer of neutrophil apoptosis[295]. In activated neutrophils, AnxA1 translocates to the membrane, and becomes available for PR3[296]. It is likely that cleavage of AnxA1 by PR3 decreases its innate inhibitory function, and promotes a pro-inflammatory response.

All these studies, together with the observation of high levels of PR3 within fibrinoid necrotic lesions in vasculitis[297], provide strong evidence that PR3 promotes a pro-inflammatory response, and plays an important role in the pathogenesis of AASV.

Role of ANCA

Interaction between neutrophils and ANCA

ANCA are capable of activating neutrophils *in vitro*; PR3-ANCA and MPO-ANCA can activate TNF-α-primed neutrophils leading to respiratory burst and degranulation. Abdel-Salam et al tested neutrophils from patients with active WG, after stimulation with PMA, which upregulates PR3 expression, for autoantibody binding. They reported that, despite membrane PR3 expression and high ANCA titres, the autoantibodies to PR3 had a low affinity for membrane-associated PR3 on intact neutrophils[298]. Subsequently, Van Rosum et al observed significant binding of IgG in serum and plasma from PR3-ANCA-positive WG patients to PMA-stimulated neutrophils and to TNFα-primed neutophils. This binding was restricted to mPR3-expressing neutrophils[299]. Thus; membrane antigen expression is a prerequisite for neutrophil activation by ANCA. In another study Schreiber et al. separated the neutrophils of one individual into two populations, $mPR3^{high}$ neutrophils and $mPR3^{low}$ neutrophils (by magnetic cell sorting, MACS). Results showed that stimulation of $mPR3^{high}$ neutrophils resulted in significantly higher amounts of superoxide generation, compared to $mPR3^{low}$ cells[300].

Once ANCA bind to the plasma membrane, full activation of neutrophils seems to depend on the binding of ANCA to a second or the same neutrophil, via interaction of ANCA Fc regions with FcγRIIa (CD32). β2 integrins seem to play an important role in neutrophil-ANCA interaction[301]. β2-integrins cooperate with Fcγ-receptors and likely contribute to antigen stabilization on the plasma membrane. ANCA possibly interact with neutrophils by recognizing and binding PR3 or MPO through the Fab portion of the IgG molecule. ANCA also modulate β2-integrin function through inside-out signaling which is needed for firm adhesion of neutrophils to endothelium[302]. ANCA may also play a role in directing neutrophil adhesion and transmigration. FcγRIIa, Lymphocyte Function associated antigen-1 (LFA-1), Macrophage antigen-1 (Mac-1) and the chemokine receptors (CXCR) may be important in this process[303, 304]. While the activation of neutrophils by ANCA is largely mediated by FcγRIIa receptors, there is evidence that the F(ab)´2 fragments of the ANCA antibodies also activate neutrophils[305]. Anti-PR3 and Anti-MPO differ in neutrophil-activating capacity, with anti-PR3 being the more potent activator[306]. This may be due to variation in antigen affinity or differences in signal transduction pathways between these two specificities of ANCA.

Activated neutrophils activate the NADPH oxidase complex, which generates superoxide anion. Following ligation of ANCA to the Fc-γ-receptor, p38-MAPK-mediated tyrosine phosphorylation contributes to Fc-γ-receptor activation and

mobilization of TNFα- induced granules[307]. p38-MAPK and ERK (mitogen-activated kinases) also contribute to NADPH oxidase activation[308]. Phosphatidylinositol-3- kinase (PI3-kinase), and its downstream mediator Akt, also play a role in ANCA-induced activation of NADPH oxidase. ANCA-F(ab)´2 can stimulate pertussis sensitive Gi/0 GTPase activity, which contributes to ANCA-induced activation of the small GTPase p21 Ras, which in turn is involved in NADPH oxidase activation[309]. Yang et al, have shown that ANCA-IgG and ANCA-$F(ab)^2$ can up-regulate the transcription of specific genes, including IL-8 (neutrophil chemoattractant)[310]. Additionally, once FcγRIIIb is activated on the neutrophil surface by IgG ANCA (through cross linking of FcγRIIIb and FcγRIIa), L-selection expression is maintained, allowing the activated neutrophil to adhere to the endothelium[311].

"Priming" may not be the sole mechanism contributing to the ANCA-neutrophil interaction. Gilligan et al showed that ANCA antigens (PR3 and MPO) are expressed on the membrane of apoptotic neutrophils, and that translocation of primary granules may occur as a natural consequence of apoptosis[272]. After granule contents are expressed on the membrane of apoptotic neutrophils, they can interact with circulating ANCA. After ANCA binds to the membrane of an apoptotic neutrophil, ANCA may enhance phagocytosis of that neutrophil, but in the same time may stimulate the production of pro-inflammatory cytokines by the phagocytosing macrophage[277]. Interestingly, Deutsch et al showed that PR3-ANCA may penetrate neutrophils and enhance apoptosis[278].

Are ANCA pathogenic?

The subject of pathogenicity of ANCA is controversial. ANCA are absent in some patients with small vessel vasculitis, while especially MPO-ANCA can be detected in some patients with other disease such rheumatoid arthritis and inflammatory bowel disease[312]. Also, the paucity of immune complexes at sites of pathological lesions has been used as an arguement against a direct role for ANCA. ANCA have the ability to activate primed neutrophils *in vitro*[51], but this evidence has been questioned because of two reason; first, there is a controversy whether ANCA bind or do not bind to neutrophils *in vivo*[298, 299]; and second, ANCA-negative sera from systemic vasculitis patients may also be able to activate neutrophils *in vitro*[313]. However, animal models of small vessel vasculitis have been developed that provide convincing evidence that ANCA are pathogenic. Xiao et al demonstrated that $Rag2^{-/-}$ mice, which are completely deficient in T- and B-lymphocytes with antigen receptors, developed a severe necrotizing glomerulonephritis and small vessel vasculitis when they were injected with anti-MPO splenocytes, while mice that received anti-Bovine Serum Albumin (BSA) or normal splenocytes remained disease-free. Similarly, $Rag2^{-/-}$ and wild type WT B6-mice injected with anti-MPO IgG developed focal glomerular necrosis and crescent formation, clearly indicating that the antibodies were pathogenic[314].

Neumann et al demonstrated excessive immune deposits in the early stages of life of SCG/Kinjoh mice (that spontaneously develop small vessel vasculitis and p-ANCA), and suggested that immune complex deposition leads to an inflammatory state, which when amplified by ANCA, likely leads to severe vasculitis[315]. In renal biopsies from AASV patients with renal involvement, Bajema et al showed that PR3, MPO, elastase and lactoferrin localized within or around fibrinoid necrotic lesions, and the lesions contained high levels of PR3 and elastase, which were also enriched inside the lesions[297]. Schlieben et al described a case of pulmonary renal syndrome in a newborn who received MPO-ANCA via passive transfer from the mother, supporting the idea that ANCA are pathogenic[316]. Animal models proofing the pathogenicity of PR3-ANCA have not been developed, may be because human and murine PR3 share a low level of homology. However, an animal model of vasculitis and severe segmental and necrotizing glomerulonephritis, similar to WG, was recently developed in non-obese diabetic-severe combined immune deficiency (NOD-SCID) mice. In this model, splenocytes were isolated from NOD mice immunized with recombinant mouse PR3 and transferred into NOD-SCID mice, who developed disease pathology. Transfer of the splenocytes into wild type NOD mice did not produce the disease pathology. These findings suggest that PR3-ANCA may play a direct role in PR3-ANCA-associated renal disease; however, in this model, a specific genetic background and autoimmune predisposition for kidney pathology are pre-requisites for disease manifestation[317].

Aims of the study:

ANCA-Associated Systemic Vasculitis (AASV) is characterized histologically by leukocytoclasis, infiltration and accumulation of apoptotic and necrotic neutrophils in tissues, and fibrinoid necrosis of the vessel walls. Increased expression of PR3 protein, especially plasma membrane-associated PR3 (mPR3) is also a characteristic feature of neutrophils from AASV patients. It has been proposed that aberrant expression of PR3 reflects and could be causally related to a fundamental functional defect in AASV neutrophils.

The objectives of this research were to:

1 Characterize the mPR3 expression in AASV neutrophils.

2 Analyze the relationship between expression of mPR3, plasma PR3 and PR3 gene in AASV patients.

3 Elucidate the nature and mechanism by which PR3 associates with the plasma membrane.

4 Examine the CD177 as a possible mediator or co-factor for mPR3 expression on the plasma membrane of human neutrophils.

5 Identify molecular mechanisms underlying the elevated plasma and mPR3 levels.

6 Investigate whether dysregulation of neutrophil apoptosis plays a role in the pathogenesis of AASV.

Methods:

Patients

Paper I

In the initial stage, the plasma PR3 levels, membrane PR3 (mPR3) expression and the PR3 gene were analyzed in patients with AASV and healthy blood donors (HBD). Patients with microscopic polyangiitis (MPA; 56) and Wegener's granulomatosis (WG; 76) were recruited from the Departments of Nephrology at the Lund University Hospital and Malmö University Hospital. Most patients were in stable remission at time of recruitment. Healthy blood donors (HBD; 189) from local blood bank were included as a control group. Samples were collected for DNA analysis between 1996 and 2004 while samples for membrane expression and plasma concentrations were drawn during 2004. In all phases of this study, patients diagnosed with AASV were classified as WG or MPA using the European Medicines Agency (EMEA) algorithm[318]. The Birmingham Vasculitis Activity Score (BVAS) was used to determine the activity of vasculitis[319].

Paper II

For analyzing whether CD177 and PR3 are expressed on the plasma membrane of the same subpopulation of neutrophils, polymorphonuclear leukocytes were isolated from blood provided by 10 healthy volunteers.

Paper III

To investigate the mechanisms underlying elevated mPR3 and the co-expression of mPR3 and CD177 in AASV, 55 AASV patients (WG or MPA) were recruited from Department of Nephrology, Lund University (2006–08). The controls included 93 healthy blood donors (HBD; local blood bank), 20 renal transplant (TP) recipients (Department of Nephrology) and disease controls (17 polycythemia vera (PV), 1 paroxysmal nocturnal haemoglobinuria (PNH), 21 systemic lupus erythematosus (SLE), 21 rheumatoid arthritis (RA); Departments of Rheumatology and Hematology). At the time of sampling, 40 patients were in stable remission (BVAS 0-1), 13 moderately active in their disease (BVAS 2-5) and two patients highly active in their disease (BVAS > 5). Twenty-three patients were treated with cytotoxic drugs and steroids, 13 with cytotoxic drugs only; seven with steroids only and 12 patients did not have any form of immunosuppressive treatment.

Paper IV

To study the rates of spontaneous *in vitro* apoptosis in AASV in relation to clinical parameters and apoptotic and growth factors, 44 AASV patients (WG or MPA; Department of Nephrology, Lund University Hospital) were recruited between September 2006 and February 2008. AASV patients were receiving the following

treatments at the time of sampling: 21 patients, cytotoxic drugs and steroids; 10 patients, cytotoxic drugs; 5 patients, steroids; 8 patients, no treatment. Controls included 93 HBD (from local blood bank), 20 TP recipients (Department of Nephrology), 17 PV patients (Department of Hematology), 21 SLE and 21 RA patients (Department of Rheumatology), all at Lund University Hospital.

Blood Sampling, Separation, Sampling, Neutrophil Isolation and DNA extraction:

Peripheral blood was collected from each patient/control in Ethylene Diamine Tetraacetic Acid- (EDTA)-anti-coagulated tubes. Leucocytes were freshly isolated from whole blood by centrifugation on Polymorphprep™ (Axis-Shield, Oslo, Norway). For neutrophil isolation, samples were centrifuged and the neutrophil layer was recovered. Contaminating red blood cells among polymorphonuclear bands were lysed. The percentage of neutrophils was 95–99%, as determined by Türk staining. Viability, checked using Trypan blue staining, was >95%.

Experiments were performed in RPMI medium within 1 h after isolation of the cells. The plasma layer, on top of the mononuclear band, was saved used to measure plasma PR3, pro-PR3, G-CSF, GM-CSF, IL-3, other cytokines and ANCA levels. The polymorphonuclear band was used to study membrane PR3 expression, neutrophil survival, apoptosis and necrosis by FACS. The PMN band was also used to extract mRNA to measure mRNA-expression of: PR3, CD177, MPO, IL-8, pro-apoptotic factor (Bax), anti-apoptotic factors (cIAP-2, Bcl-2-A1, Mcl-1) and transcription factors (C/EBP-α, C/EBP-β and PU.1). DNA was extracted from the mononuclear band for studies on the PR3 gene polymorphism. DNA was extracted as per methods described by Miller et al, and it was quantified by spectrophotometrically[320].

Genotyping of PR3 polymorphism:

Using Applied Biosystems' guidelines, SNP-specific polymerase chain reaction (PCR) primers and fluorogenic probes were designed using Primer Express; -564-Forward Primer: 5'- GGCCTCCACCCACTCCAT -3', -564-Reverse Primer: 5'-AGGATTCTCAATCAAGAGGTGATTCT -3'. The probes were labelled with a reporter dye (either FAM or VIC) and are specific for one of the two possible bases (-564 G or A) in the PR3 promoter region; -564A-Taqman Probe: FAM-AGACCTCACCCAGGGT-MGB; 564G-Taqman Probe: VIC-ACCTCGCCCAGGGT-MGB. The PCR was run according to the manufacturers recommendations. Controls (no DNA template) and three reference DNA (AA, GG and AG genotype) were run in each 96 well plate to ensure there was no amplification of contaminating DNA and that signals from both probes was achieved. All PCR reactions were made in triplicates using the ABI Prism 7000 Sequence Detection System with two initial hold steps (50°C for 2 min., followed by 95°C for 10 min.) and 40 cycles of a two step PCR (95°C for 15 sec., 60°C for 1

min.). The -564 nucleotide was determined by the fluorescence ratio of the two SNP-specific fluorogenic probes.

Quantitative polymerase chain reaction (Q-PCR) assay

Total RNA from neutrophils was isolated by RNeasy Mini kit and reverse transcription was performed using the TaqMan Reverse Transcription Reagents kit according to the manufacturer's instructions. RNA purity was evaluated by spectrophotometric analysis using a NanoDrop equipment. The gene expression of PR3, CD177, MPO, interleukin (IL)-8 Bax, Mcl-1, Bcl-2A1, c-IAP2, C/EBP-α, C/EBP-β and PU.1 was determined by Assays on demand from Applied Biosystems using Q-PCR assays on an ABI PRISM 7000 Sequence Detector. Q-PCR data were analyzed using the ΔΔCt method with normalization to Cyclophilin A and standard $2(^{-\Delta\Delta}\,Ct)$ calculations[321].

Analytical flow cytometry

Flow cytometry was used to measure the membrane expression of surface markers on neutrophils and for quatification of viable, apoptotic and necrotic cells.

In paper I, isolated neutrophils were blocked with heat-aggregated rabbit immunoglobulin, washed and then incubated with murine monoclonal anti-human PR3 antibodies or with an isotype-matched murine control antibody followed by a 30-min incubation with fluorescein isothiocyanate (FITC)- conjugated (Fab)2 fragments of rabbit anti-mouse antibodies. Labelled neutrophils were then fixed with 1% paraformaldehyde and fluorescence was analysed by FACS. The percentage of $mPR3^{+}$neutrophils as well as mean fluorescence intensity (MFI) were measured. Expression index was calculated after correction of the MFI for non-specific binding by an isotype matched irrelevant antibody (NSB) and multiplied by the percentage of $mPR3^{+}$ neutrophils; The expression index = (MFI-NSB)×% $mPR3^{+}$ neutrophils, and expressed in Arbitrary Units (AU)[291]. Individuals were categorized into three mPR3 phenotypes, as described by Witko-Sarsat et al, according to their percentage of $mPR3^{+}$neutrophils; 0–20% of $mPR3^{+}$neutrophils ($mPR3^{low}$ phenotype), 21-58% of $mPR3^{+}$neutrophils ($mPR3^{intermediate}$ phenotype) and 59–100% of $mPR3^{+}$neutrophils ($mPR3^{high}$ phenotype)[210].

In paper II, rabbit anti-PR3, mouse anti-PR3, mouse anti-CD177, mouse anti-CD18, or mouse anti-CD16 were incubated with the purified neutrophils for 15 min on ice. The samples were washed and stained with the secondary antibodies; anti-rabbit Alexa488 or anti-mouse R-PE/Cy5.

For detection of pro-PR3 on the neutrophil membrane (paper III), a new antibody was made by immunization of rabbits with a keyhole limpet haemocyanin (KLH)-conjugated peptide from the C-terminal end of the pro-PR3 (CRRVEAKGRP). Bound rabbit anti-proPR3 antibodies were visualized by goat anti-rabbit Alexa488 antibodies.

Apoptotic and necrotic cells were demonstrated by double-staining using AnnexinV-Alexa Fluor 488 and BD Via-Probe. Unstained cells were regarded as alive, Annexin V binding cells as apoptotic, and double labeled cells as necrotic.

Cell sorting:

Neutrophils were isolated from three donors and three patients, labelled with anti-PR3 (4A5-Alexa647). For separation of mPR3-positive and mPR3-negative neutrophils a FACS Aria flow cytometer equipped with automatic cell deposition unit was utilized. After sorting, RNA was isolated and CD177- as well as the PR3-specific cDNA was measured in each subpopulation by Q-PCR

Fluorescence microscopy:

Samples were fixed using 2% PFA and seeded on poly L-lysine. Visual inspection and recording of images were performed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled charged-coupled device camera, using a Plan Apochromat 100x oil immersion objective. Images were acquired and handled using Image Pro Plus and Adobe Photoshop 7.0.

In membrane expression of PR3 and co-localization experiments (CD177 and PR3) in paper II anti-mouse or anti-rabbit Alexa594 or Alexa 488 were used for fluorescence microscopy.

ELISA:

For measuring plasma PR3, a microtitre plate was coated overnight with a mixture of two monoclonal PR3 antibodies (4A3 and 4A5). Plasma samples diluted to 1/20 and 1/40 in sample buffer were added, followed by incubation for 2 hrs. After washing, samples were incubated for 2 h with affinity-purified rabbit anti-PR3 antibodies. The plates were washed and followed by addition of alkaline phosphatase-labelled swine anti-rabbit IgG. P-nitrophenyl-phosphate disodium was used as substrate and incubated with the samples. Optical densities were read at 405 nm. A standard curve was produced by incubation of a twofold dilution series of purified neutrophil PR3.

For detection of plasma Pro-PR3, a new sandwich ELISA was developed. A microtitre plate was coated overnight with affinity purified anti-pro-PR3 antibody. Plasma samples were added and plates were incubated for 2 h. After washing, bound pro-PR3 was detected by incubation with monoclonal murine anti-PR3 (4A3). After washing, a conjugated anti-mouse antibody, alkaline phosphatase-labelled rabbit antimouse was added and then developed as described above. A standard curve was produced by incubation of a twofold dilution series of recombinant PR3 containing the C-terminal pro-peptide.

ANCA was detected by standard direct ELISA using the Wielisa® PR3–ANCA kit (Wieslab AB, Lund, Sweden). Plasma G-CSF, GM-CSF and IL-3 were measured using Quantikine® ELISA Development kits (R&D systems, Abingdon, UK).

Measurement of neutrophil survival factors in plasma by Cytometric Bead Array (CBA)

G-CSF, GM-CSF, IL-3, TNF-α, IFN-γ, IL-1β, IL-2, Il-4, IL-6 and IL-8 were analyzed in 50 μl plasma by flow cytometry using the BD CBA Human Soluble Protein Flex Set system according to the manufactures instructions.

Neutrophil in vitro culture:

Isolated neutrophils were incubated at 37° C in DMEM (paper II) or AIM-V medium (paper III and IV) for 20 h. An aliquot (10^6 neutrophils) was taken labelled with Annexin-V and 7-AAD analyzed by flow cytometry to report % of apoptotic, necrotic or alive cells.

In paper IV, neutrophils isolated from AASV patients or HBD were incubated at 37° C in AIM-V medium for 20 h, with/without plasma (20%) derived from other AASV patients or other HBD. Neutrophils isolated from AASV patients or HBD were also incubated under similar conditions with or without neutrophil growth factors (recombinant G-CSF, GM-CSF and IL-3) at a concentration of 0.2 ng/ml. Survival was analysed by FACS.

Membrane Expression (PR3) and Co-localization experiments (CD177 and PR3): or anti-mouse Alexa594 (1:1000; Molecular Probes) for fluorescence microscopy for another 15 min on ice. Some samples were stained with cholera toxin subunit B conjugated with Alexa Fluor594 (CT-B; Molecular Probes). These samples were prefixed with 0.1% paraformaldehyde (PFA), washed, and then incubated in the dark with CT-B for 60 min on ice.

Stimulation, Apoptosis and Necrosis investigation:

Neutrophils were stimulated with TNF-α (20 ng/ml, 15 min), PMA (100 nM, 15min), fMLP (1 μM, 5 min), or cytochalasin B (CyB; 10 μM) followed by addition of fMLP (1 μM) All incubations were performed at 37°C. The samples were stained with antibodies against PR3 and CD177. Apoptotic and necrotic cells were demonstrated as described above and the samples were then analyzed by FACS.

Internalization and time-course experiments:

Neutrophil samples were stained with primary antibodies (monoclonal anti-PR3, anti-CD177, or anti-CD18) for 15 min on ice. After washing, the secondary antibody Alexa488 anti-mouse was added for 15 min on ice. The samples were put on ice or incubated for 15, 30, or 60 min, rotating at 37°C, 8 rpm. Incubation with the primary

antibodies, as described above, was repeated followed by staining with the secondary antibody Alexa594 anti-mouse for 15 min (or anti-mouse PE-Cy5 for FACS experiments) on ice. The samples were fixed using 2% PFA and mounted for fluorescence microscopy evaluation.

Blockage of protein synthesis:

Neutrophils were incubated with cycloheximide (100 μg/ml) for 0, 30, 60, 90, and 120 min. After washing, cells were stained with monoclonal anti-PR3 and CD177 for 15 min on ice and then washed again. The secondary anti-mouse PE-Cy5 antibody was added for 15 min on ice . The cells were stained with Annexin V-Alexa Fluor 488 for 5 min in the dark to monitor the viability of the cells. The samples were evaluated by FACS.

CD177 and PR3 interactions in vitro

Human histiocytic lymphoma cells (U937) were stably transfected with CD177–cDNA in a pcDNA vector 3·1 or with a negative control vector by electroporation. Two clones transfected with CD177–cDNA were selected, one positive for CD177 expression and one negative for CD177 expression. Both clones were then incubated with exogenous PR3 for 2 h on ice, and labelled with anti-PR3 and anti CD177 antibodies and the fluorescence was measured by FACS.

Statistical analysis:

Differences in continuous variables between two groups were analysed using the Mann–Whitney U-test; some data sets that did not follow Gaussian distribution were transformed and then analyzed by unpaired t-test. For data sets that follow Gaussian distribution, a t-test was used and results are given as mean ± standard deviation (s.d.). All other results are given as median, range or interquartile range (IQR). One-way ANOVA with Bonferroni's post-test was used for comparisons between more than two groups. Correlations were analysed using Pearson's rank test and for non-parametric data Spearman's rank test was used. Proportions between groups were compared with the $\chi 2$ test. A two-sided $p<0.05$ was considered to be statistically significant.

Results:

Paper I

Plasma PR3 and ANCA

Plasma PR3 was significantly higher in patients (224 ± 128 µg/l: mean± s.d.; n= 63) than in healthy blood donors (155± 52 µg/l, n= 130) (unpaired t-test, p< 0.0001). MPA patients had significantly higher PR3 concentrations (256 ± 162 µg/l, n = 26) than healthy blood donors (p< 0.0001). WG patients also had higher PR3 concentration (202 ± 95 µg/l, n = 37) than healthy blood donors (p= 0.0029). AASV patients were divided into two subgroups based on their ANCA serology, and similar results were obtained in both subgroups. ANCA level in the patients' plasma was also evaluated; 13 patients were PR3–ANCA-positive at the time of sampling of the 35 who were PR3–ANCA-positive at the time of diagnosis. In addition, 10 patients were MPO–ANCA positive at the time of sampling of the 26 who were MPO–ANCA-positive at the time of diagnosis.

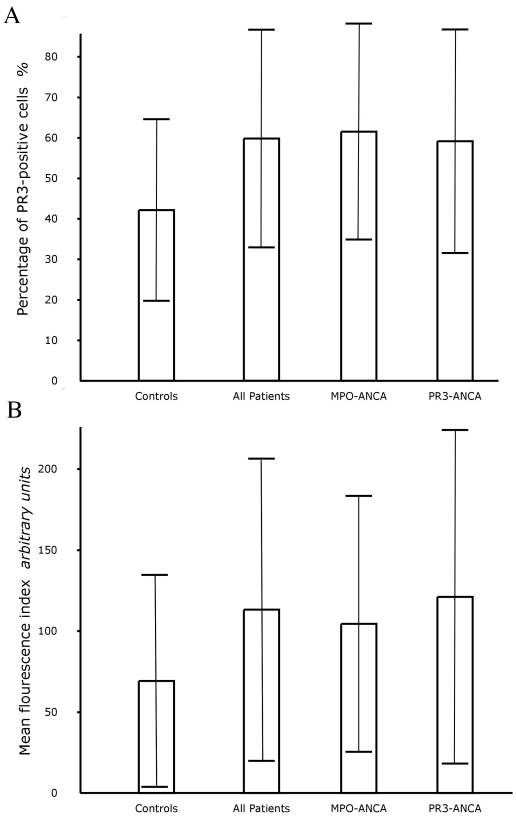

Figure 4. Membrane expression of PR3 on neutrophils. Evaluated by flow cytometry analysis, mPR3 was stained with anti-PR3 monoclonal antibodies and revealed by FITC-conjugated antibodies to visualize PR3. (A) The percentage of mPR3 positive neutrophils in 107 healthy controls, 58 patients with AASV and two subsets of the same patients (23 having MPO-ANCA and 34 having PR3-ANCA). (B) The expression index of mPR3 on neutrophils measured by arbitrary units (AU) for the same groups as in (A). All results are given as mean values ± SD.

Membrane PR3

Flow cytometry analysis of mPR3 on isolated neutrophils was performed on 107 healthy blood donors and 58 patients with AASV (31 men, 27 women). Their mean age (at sampling) was 63.2 ±16.4 years (range 17.8 to 86.5 years). A clinical diagnosis of WG was made for 35 patients (31 were PR3–ANCA positive, three MPO–ANCA positive and one ANCA-negative) and 23 patients were diagnosed as having MPA (20 MPO–ANCA-positive, three PR3–ANCA positive). The percentage of $mPR3^+$ neutrophils (Figure 4a) was significantly higher in vasculitis patients (60±27%) than in healthy blood donors (42±22%; $p<0.0001$). The difference was independent of diagnosis; MPA group had 62±24% $PR3^+$ cells and the WG group had 58±29% ($p= 0.0002$ and $p= 0.0007$, respectively). Similar results were obtained when the patients were stratified according to ANCA serology.

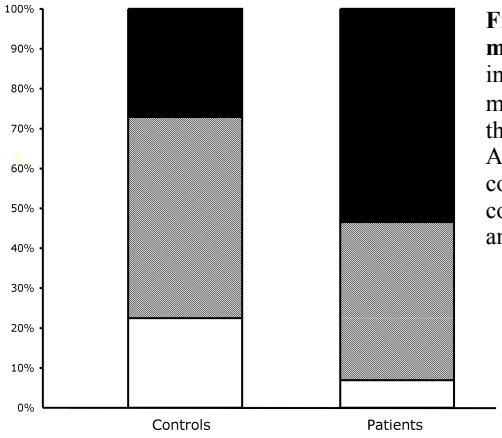

Figure 5. Phenotype distribution of mPR3 on neutrophils. Individuals divided into 3 groups according to their % of $mPR3^+$ neutrophils[210]. The distribution of these 3 groups is shown in 58 patients with AASV compared to 107 HBD. Black colour represents high phenotype, grey colour represents intermediate phenotype and white colour represents low phenotype.

Vasculitis patients and controls were stratified by level of mPR3 into three phenotypes; $mPR3^{high}$, $mPR3^{intermediate}$ and $mPR3^{low}$ as previously described[210]. For vasculitis patients, the distribution was skewed toward the $mPR3^{high}$ phenotype (Figure 5), while the control group exhibited a normal distribution (27% high, 50% intermediate, and 23% low). This difference was significant ($\chi 2 = 13.47$, $p= 0.0012$). mPR3-MFI was higher in vasculitis patients (median 1.43, IQR 1.03– 2.12) than in HBD (1.19, IQR 0.915–1.58). However, the differences were relatively small and only statistically significant, according to the Mann–Whitney U-test, when all patients or the PR3–ANCA-positive subgroup were compared to controls ($p= 0.04$ and 0.03, respectively). On comparing the expression index (EI) of mPR3, higher values for the AASV patients (median 89.3, IQR 43.6–163 units) than for controls (53.1, 25.2–87.8 units, $p= 0.001$, Figure 4b). The median expression index was significantly higher for all subgroups than for HBD. There were no significant differences between the subgroups, either based on diagnosis or on serology.

Genotype

Looking for the -564 A/G polymorphism in the PR3 gene, DNA genotype was determined by allelic discrimination-real time PCR using DNA from peripheral blood mononuclear cells from AASV (WG and MPA) patients and HBD. No significant differences in gene or allele frequencies among patient groups were observed. The allele frequencies were: all patients 36% A allele and 64% G allele; HBD 31% A and 69% G (Figure 6). In patients with WG were, 40% A allele and 60% G allele; the corresponding figures for MPA patients were 30% A and 70% G. Thus, previous findings that the frequency of the G allele was higher in WG patients, were not verified.

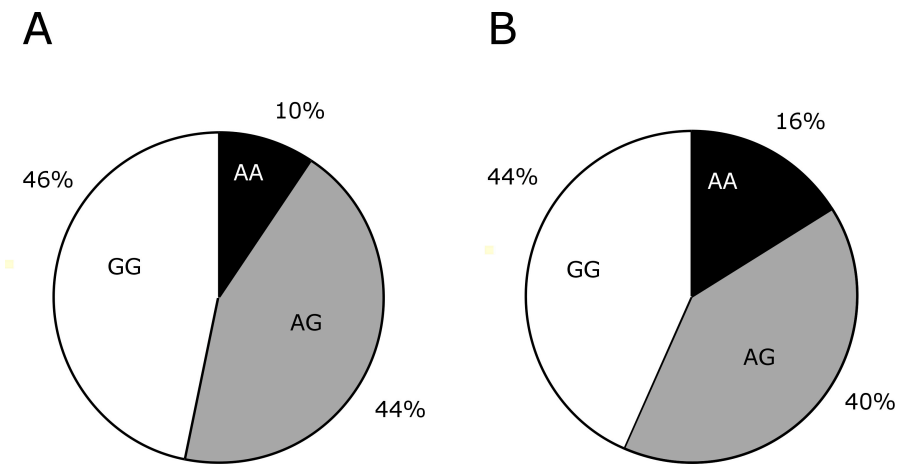

Figure 6. Distribution of the –564 A/G polymorphism in the promoter region of the PR3 gene. Black colour represents individuals homozygous for the A allele, white colour represents individuals with the G allele and grey colour represents heterozygotes. (A) Shows the results of 189 healthy blood donors. (B) Shows the results of 136 patients with AASV.

Genotype-phenotype correlation

Promoter genotype (AA, AG, GG) did not correlate with mPR3 parameters (% of $mPR3^+$ cells, MFI and EI) in HBD (χ test) or AASV patients. However, the GG genotype showed statistically significant inverse correlation with plasma PR3 concentration in HBD ($p= 0.0002$), but not in patients ($p= 0.08$) Table 3.

Phenotype–phenotype correlation

There was no correlation between plasma PR3 and percentage of $mPR3^+$ neutrophils in HBD or AASV patients. There was a weak but significant correlation between plasma PR3 and MFI in HBD ($r= 0.24$, $p= 0.015$). A similar correlation was seen in the MPO–ANCA-positive subgroup of AASV patients ($r= 0.52$, $p= 0.011$), while no correlation was observed in the PR3–ANCA subgroup. On the other hand, a

Table 3. Genotype-phenotype correlation. Comparison table showing the correlation between the three genotypes of the -564 A/G promotor polymorphism in the PR3 gene and the four PR3 phenotypes, % of mPR3 positive cells, MFI, E.I. and Plasma PR3 (median). (A) Shows the results of healthy blood donors. (B) Shows the results of patients with AASV.

A

Genotype	% $mPR3^+$cells	MFI	E.I.	Plasma PR3
AA	45	1.61	54.8	185
AG	40	1.13	53.3	167
GG	45	1.13	50.3	130
p	0.98	0.57	0.79	0.0002*
n	83	83	83	110

B

Genotype	% $mPR3^+$cells	MFI	E.I.	Plasma PR3
AA	71	1.52	100	223
AG	65	1.42	109	199
GG	52	1.01	64.8	154
p	0.28	0.07	0.17	0.082
n	52	52	52	57

All results are expressed as median. MFI= Mean fluorescence intensity. EI= Expression index = (MFI-NSB)×% $mPR3^+$ neutrophils. NSB= Non-specific bindning. (*) P value <0.05

significant negative correlation between plasma ANCA levels and plasma PR3 level was evident in PR3–ANCA patients (r = -0.486, p= 0.0031), while MPO–ANCA levels did not correlate with plasma PR3.

Paper II

PR3 and CD177 membrane expression:

Cells were co-stained with antibodies against CD18/CD16/ CD177 and PR3, and samples were evaluated by FACS and Fluorescence microscopy. Anti-CD18, anti-CD16, and CT-B stained virtually all cells, and no correlation was seen with mPR3 staining. CD18 and CD16 were found on the majority of the neutrophils, including those without mPR3. A strong correlation was observed between PR3 and CD177 surface expression. The expression of CD177 was heterogeneous and coincided with the expression of PR3. The mPR3-positive cells were also positive for CD177, and mPR3-negative cells were negative for CD177. Fluorescence microscopy verified

plasma membrane co-localization of PR3 and CD177. In 100 cells, 96% were double-positive or double-negative, and only 4% displayed expression of one marker but not the other, Figure 7.

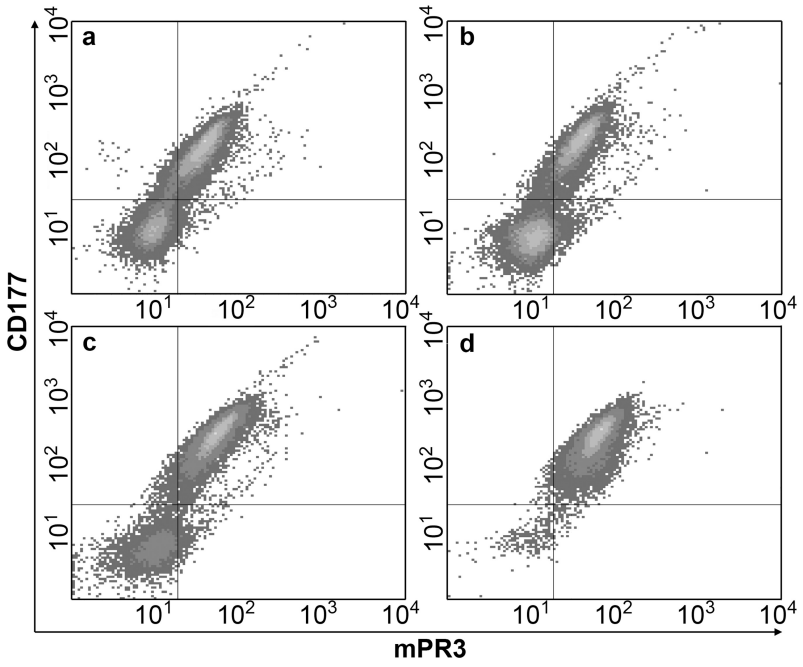

Figure 7. Co-localization of CD177 and PR3. Neutrophils were stained with anti-CD177 and anti-PR3 antibodies and the surface expression was then evaluated by FACS analysis. Fig.7a-d represents four different individuals with different proportions of mPR3/CD177 expressing neutrophils.

Effect of stimulation on surface expression:

The effect of stimulation on surface expression was evaluated. Cells were treated with TNFα, PMA, fMLP, or fMLP in combination with CyB and analyzed by FACS. Stimulation with TNFα or fMLP caused a moderate increase (1.7 and 2.0 fold, respectively) in fluorescence intensity for mPR3- and CD177-positive cells, Figure 8. PMA stimulation caused a marked shift in fluorescence intensity. The mPR3- and CD177-positive cell population was shifted further to the right (4.9 fold). Also, stimulation with PMA converted mPR3- and CD177-negative cells into mPR3- and CD177-expressing cells. The distribution remained bimodal, but instead of a positive/negative population, the cells divided into mPR3 and CD177 high and low subgroups. The proportion of mPR3 high-expressing cells remained constant and independent of stimulation. Stimulation with fMLP and CyB had a similar effect.

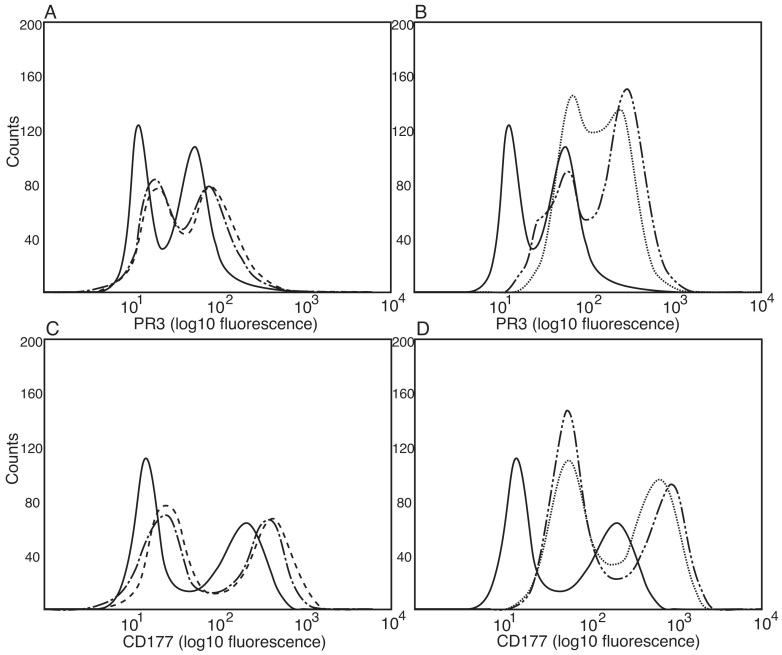

Figure 8. Cell membrane PR3 and CD177 expression in stimulation experiments. The histogram shows the fluorescence intensity of unstimulated neutrophils (——) and neutrophils were stimulated with TNF-α (– – –), PMA (–..–..–), fMLP (……) or CyB in combination with fMLP (–.–.–) at 37˚C. The samples were then blocked using Heat-inactivated-IgG, incubated with the primary antibodies, rabbit anti-PR3 (A, B) and mouse anti-CD177 (C, D), washed and then dyed with the secondary antibodies anti-rabbit ALEXA 488 and anti-mouse PE-Cy5. Analyses were done by FACS.

Effect of apoptosis on surface PR3 and CD177:

mPR3 is thought to be a pre-apoptotic marker for aging neutrophils. To explore the effect of mPR3 on neutrophil apoptosis *in vitro*, purified neutrophils were incubated at 37°C for up to 22 h, and were analyzed every 3 h for mPR3 expression and for viability. The percentage of mPR3-positive cells remained constant over time, whereas the MFI slowly decreased with increasing age of the live neutrophils (Annexin V-negative cells), Figure 9A. Similar results were obtained with the total neutrophil population. In three experiments, membrane-associated CD177 was also measured at the zero time-point and after incubation for 22 h. The percentage of CD177-positive cells decreased, but this decrease was not significant. However, a significant decrease in MFI was observed for both CD177 and mPR3 after 22 h (60% mPR3; 40% CD177; Figure 9B). This loss of CD177 and mPR3 could be a result of shedding into the surrounding media or internalization. To distinguish between these possibilities, internalization and the dynamics of membrane trafficking of mPR3 and CD177 were examined further.

Internalization experiments:

CD177 was transported into the cells from the surface, and after a 15 min incubation, intracellular CD177 was observed, with only weak staining on the cell surface. After 30 min, the majority of the surface-bound CD177 had been internalized. For PR3, the majority of the surface staining disappeared after 15 min. CD18, used as a control, was retained on the cell surface throughout the study, and only a small proportion was internalized and detected intracellularly. After incubation, the cells were stained with the same primary antibodies but a different secondary antibody labeled with another fluorochrome. All cells displayed strong surface staining, indicating that new proteins had been transported to the plasma membrane. Similar results were obtained when samples were incubated for 60 min instead of 30 min. FACS analysis showed that the MFI for the green and red probes did not change throughout the experiment. To investigate whether de novo synthesis is a major factor contributing to the mPR3 and CD177, which reappeared at the cell surface, and protein synthesis was inhibited with cycloheximide; cycloheximide treatment did not cause any major difference in the number or the staining of the positive cells.

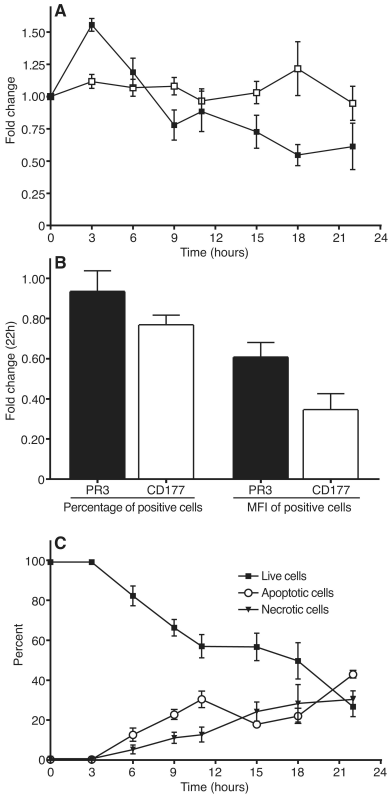

Figure 9. Time-course study with respect of PR3, CD177 and apoptosis. Primary antibody staining with anti-PR3 or anti-CD177 was performed for 15 min on ice followed by the secondary antibody anti-mouse PE-Cy5 (1:200) for another 15 min on ice. AnnexinV was then added for 5 min in the dark at RT. One sample was also double-stained with annexin-V and 7-AAD in order to check the viability. The samples were evaluated by FACS analysis. (□) % $mPR3^{+}$ cells. (■) MFI-PR3. MFI= mean fluorescence intensity.

Paper III

Membrane expression of PR3 and CD177:

Neutrophils from 223 individuals were analyzed for membrane expression of PR3 and CD177. A strong correlation was seen between the percentage of $mPR3^+$ subpopulation and the percentage of $CD177^+$ subpopulation ($r= 0.93$, $p< 0.0001$, $n = 223$). In the patient with PNH, which is characterized by blood cells lacking GPI-anchors, there were fewer than 1% cells positive for CD177 and fewer than 1% cells positive for mPR3, Figure 10. Single positive cells were insubstantial in all disease conditions. Therefore, $mPR3^+$ cells were identical to $CD177^+$ cells, and this subpopulation was defined as double-positive for PR3 and CD177.

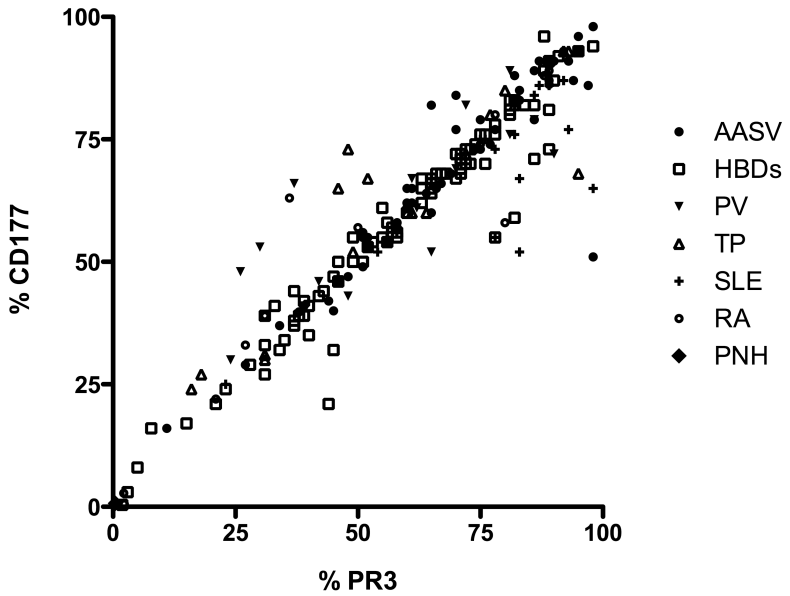

Figure 10. Correlation between mPR3 and CD177 among all the groups. Shows the results of 91 HBDs, 52 AASV patients, 17 PV patients, 20 TP, 21 SLE patients, and 17 RA patients and one patient with PNH. There was a strong correlation between % of mPR3-positive neutrophils and % of CD177-positive neutrophils among all the groups, i.e. they define the same population of neutrophils (mPR3- and CD177-positive population). AASV= ANCA-associated Systemic Vasculitis. HBD= healthy blood donors. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis. PNH= Paroxysmal Nocturnal Hemoglobinuria.

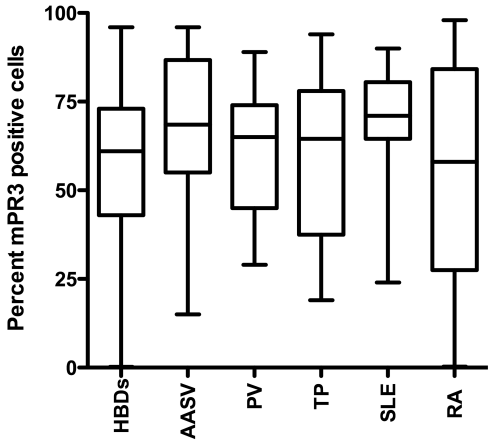

Figure 11. Double membrane expression. Compares the percentage of the double-positive population of neutrophils (mPR3- and CD177-positive population) among all groups of patients and healthy controls. The percentage of double-positive neutrophils was significantly higher in AASV patients and SLE patients compared to HBD. AASV= ANCA-associated Systemic Vasculitis. HBD= healthy blood donors. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis.

This $mPR3^+/CD177^+$ subpopulation was used as the standard for subsequent comparisons and correlations. It was found that the percentage of $mPR3^+/CD177^+$ neutrophils was significantly higher in AASV patients (69%, p= 0.0042) and SLE patients (70%, p= 0.022) than in HBD (58%). Patients of PV, TP and RA did not show any significant difference in the percentage of $mPR3^+/CD177^+$ neutrophils compared to HBD (61%, 60% and 53% versus 58%, respectively), Figure 11.

Correlation between membrane expression and clinical data

The PR3–ANCA-positive AASV patients had a significantly higher percentage of $mPR3^+/CD177^+$ neutrophils than HBD (71.2% versus 58.4%, p= 0.0044). MPO–ANCA-positive patients had non-significantly higher $mPR3^+/CD177^+$ cells than HBD (66.5% versus 58.4%, p= 0.142). No correlation was observed between the percentage of $mPR3^+/CD177^+$ neutrophils and clinical data. There was no correlation between the percentage of $mPR3^+/CD177^+$ neutrophils and CRP, the estimated GFR, cytotoxic drug treatment, steroid dose or BVAS.

Correlation between membrane expression and gene expression

In 115 samples, mRNA levels of PR3, CD177, MPO and IL-8 were measured. Data was expressed as calibrated fold-change of mRNA, setting mRNA expression of the HBD equal to 1. PR3-mRNA expression was significantly higher in AASV and SLE patients (x 2.5 and x 5.4, respectively) than in HBD. The CD177-mRNA expression was significantly higher in AASV, SLE and RA patients (4.5, 6.0 and 5.0, respectively) than in HBD and marked elevation was seen in PV patients (x26.7) relative to HBD, Table 4. There was a weak positive correlation between the CD177–mRNA expression and the percentage of $mPR3^+/CD177^+$ neutrophils, which was statistically significant only when all samples were pooled together (Spearman's $r = 0.37$, $p < 0.0001$, $n = 115$).

Table 4. Gene expression of PR3 and CD177.

	HBD	AASV	PV	TP	SLE	RA
n (RNA data)	32	26	13	16	17	21
PR3-mRNA	1	2.5*	1.5	2.0	5.4**	1.3
CD177-mRNA	1	4.5**	26.7***	1.8	6.0***	5.0**
MPO-mRNA	1	2.1**	1.2	1.2	3.2**	0.5*
IL-8-mRNA	1	0.6	0.1**	0.6	1.2	0.8

All results are expressed as mean. (*) P value <0.05, (**) P value <0.01, and (***) P value <0.001. AASV= ANCA-associated Systemic Vasculitis. HBD= healthy blood donors. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis.

Gene expression of sorted cells

The relationship between PR3- and CD177-mRNA levels and membrane PR3 expression was examined in mature neutrophils. Neutrophils were sorted based on their mPR3 expression, and PR3- and CD177-mRNA levels were measured. PR3-mRNA levels did not differ between mPR3-positive and mPR3-negative cells. On the other hand, the median level of CD177-mRNA was 13 times higher in $PR3^+$ cells than in PR3-negative cells.

U937 cells and exogenous PR3 binding

To correlate CD177 to mPR3-membrane binding, U937 cells (low mPR3, high PR3–mRNA) were used. These cells were stably transfected with CD177-cDNA. Of 20 clones, eight clones became stably positive for CD177 surface expression. All 20 clones shut down their PR3-mRNA expression and neither the protein (as measured by immunoblotting and FACS) nor the mRNA expression (measured by real-time PCR) could be detected. Sixteen of the 20 mock-transfected clones continued to express PR3. Further, two clones transfected with CD177–cDNA were selected, one positive for CD177-membrane expression and one negative. These clones were incubated with exogenous PR3; only cells expressing membrane-associated CD177 bound exogenous PR3, as shown in Figure 12.

Pro-PR3 and PR3

Looking for the source of the circulating PR3, mature form of PR3, as well as the pro-PR3 was measured in plasma. Total plasma PR3 was elevated significantly in AASV patients (median 148, range 30–2553 µg/l, n = 49) relative to HBD (84, 38–246 µg/l, n = 63) (Mann–Whitney U-test, $p < 0.0001$). A pro-PR3 specific anti-serum reacted with only a small fraction of the total plasma PR3 in both AASV patients and in HBD (10% each). Pro-PR3 levels were higher in AASV patients

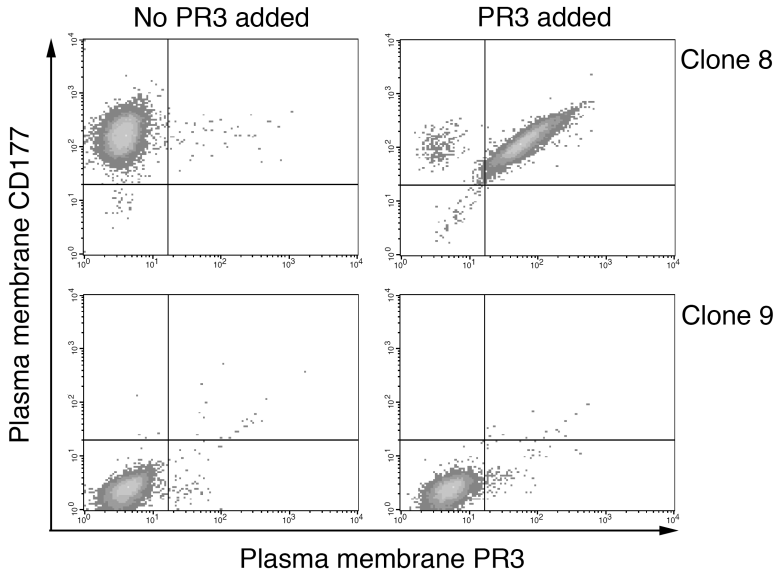

Figure 12. U937 and exogenous PR3 binding. The left panel shows the membrane expression of U937-clone 8-cells (express CD177 but not PR3 on their plasma membrane), and U937-clone 9-cells (do not express PR3 or CD177 on their plasma membrane), measured by FACS. In the right panel, membrane expression of PR3 and CD177 was measured again on the same cells after incubation with mature PR3 for two hours. Clone 8 cells expressed the PR3 on their plasma membrane (upper right), while clone 9 cells did not express any PR3 or CD177 on their membranes (lower right).

(12.6–184 μg/l) than in HBD (7.4–28 μg/l) ($p < 0.0001$). No significant correlation was found between levels of total PR3, mature PR3 and pro-PR3 and the percentage of $mPR3^+/CD177^+$ neutrophils. The new pro-PR3 specific anti-serum did not bind to mPR3, indicating that the mPR3 is mature PR3.

Paper IV

Neutrophil apoptosis and necrosis (in vitro)

Apoptosis, necrosis and survival was quantified in neutrophils from 44 patients with AASV, 93 HBD, 20 TP recipients, 17 PV, 21 SLE and 21 RA patients after 20 h in culture as described in the method section. Significantly higher rate of survival (mean ±SD 34±13% vs. 23± 9%, $p < 0.0001$, Figure 13) and lower rate of apoptosis (50±14 % vs. 64±11 %, $p < 0.0001$) was observed in AASV neutrophils than in neutrophils from HBD. Similar results were obtained in neutrophils from RA and PV patients, with survival rates of 31±13 % and 49±15 % ($p= 0.015$ and $p< 0.0001$), and apoptosis rates of 57± 12 % and 41± 14 % ($p = 0.027$ and $p< 0.0001$).

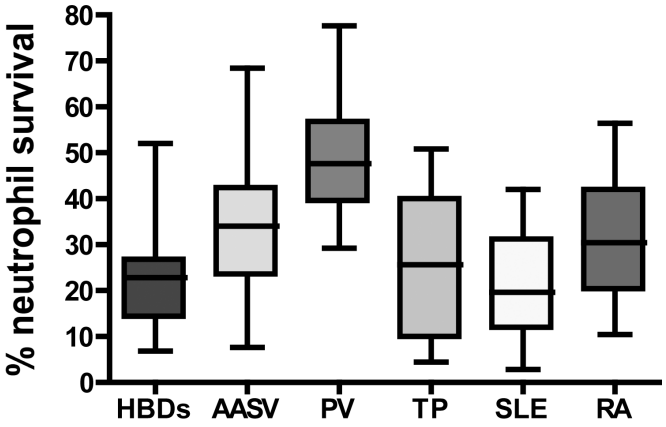

Figure 13. Rate of neutrophil survival. Neutrophils isolated from 60 HBD, 44 AASV patients, 8 PV patients, 18 TP, 21 SLE patients, and 20 RA patients were cultured *in vitro* in AIM-V medium. The percentage of surviving neutrophils and apoptotic neutrophils was measured after 20 hours. HBD= healthy blood donors. AASV= ANCA-Associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis.

No significant difference for necrosis was seen between neutrophils from AASV, PV and RA neutrophils compared to HBD neutrophils. Rates of survival, apoptosis and necrosis were similar in neutrophils from HBD and SLE and TP patients.

Relation between neutrophil apoptosis and clinical parameters

The rate of neutrophil apoptosis was analyzed and correlated with gender, age, renal function, general inflammation and treatment status of patients (or HBD). No significant correlation was observed with gender (men vs women: 49% vs. 51%; p=0.5), age (r=0.06, p=0.7), serum creatinine concentrations (r=-0.13, p=0.4) or estimated GFR (r=0.1, p=0.5). No specific effect of disease activity was observed. Patients in remission had comparable rates as patients with moderate (BVAS 2-5) or high vasculitic activity (BVAS >5), (51% and 52% vs. 28%; p=0.09). Also, there was no correlation with CRP (r=-0.11, p=0.5). Similar results were observed for patients with WG or MPA (49% vs. 53%; p=0.4). There was no correlation with the dose of prednisolone (r=-0.11, p=0.5). For anti-proliferative drugs the situation was somewhat complex; the 27 AASV patients who were being treated with immunosuppressive drugs tended to have lower neutrophil apoptosis rates than the 8 patients who were not be treated with immunosuppressive drugs (47% vs. 57%; p=0.02); there was no obvious difference when donor patients were being treated with different immunosuppressive drugs. The 10 methotrexate-treated patients had a mean neutrophil apoptosis rate of 46%, compared with 47% for 10 patients on azathioprine and 47% for 7 patients on mycophenolate mofetil (p=0.94); in contrast, 11 renal transplant recipients on mycophenolate mofetil had a mean neutrophil apoptosis rate of 57%.

Response of neutrophils to plasma

In order to explore the mechanism that down-regulates apoptosis in neutrophils from AASV patients, the effect of plasma on neutrophil survival was analyzed. Neutrophils from HBD and AASV patients were incubated with plasma from HBD, RA or AASV patients prior to FACS analysis to determine cell survival. After incubating neutrophils from 6 AASV patients and 4 HBD with plasma from HBD, AASV patients still had more surviving cells after 20 h (66% vs 56%). When the neutrophils were incubated with plasma from AASV or RA patients, neutrophil survival was enhanced, and there was no longer any difference between cells from AASV patients and HBD (72% vs 71% in AASV-plasma and 72% vs 70% in RA-plasma). Thus, incubation in plasma from AASV/RA increased neutrophil survival, and decreased differences between HBD and AASV neutrophils.

Measurement of neutrophil growth factors in plasma

The plasma level of an array of cytokines, known to influence neutrophil survival, was compared in HBD and AASV patients. G-CSF, GM-CSF, IL-3, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6 and IL-8 were measured in plasma from 40 AASV and 40 HBD individuals using Cytometric Bead Analysis (CBA). IFN-γ levels were undetectable in plasma from HBD, while 5 AASV patients had elevated levels of IFN-γ in their plasma (range 8-25 pg/ml, median 19 pg/ml). G-CSF and GM-CSF were also elevated in some AASV plasma samples. For all other cytokines examined, no significant difference between HBD and AASV was noted.

G-CSF, GM-CSF and IL-3 were also quantified using ELISA. GM-CSF was measured in the plasma from all patients and controls. It was < 2 pg/ml in the majority of samples; it was elevated in plasma samples from one PV patient (23 pg/ml), one SLE patient (42.7 pg/ml), 8 RA patients (median 54.7, range 11-178 pg/ml) and 4 AASV patients (484.2, 7.7-3135 pg/ml). G-CSF was measured in plasma from 10 HBD, 10 AASV, 5 PV, 5 TP, 5 SLE and 5 RA patients. Although G-CSF was higher in AASV patients than in HBD (37 pg/ml vs 30, p=0.29), there was no correlation between the plasma levels of G-CSF and the rates of neutrophil survival, apoptosis and necrosis in these patients. IL-3 was below the detection limit in all tested samples.

When results from all groups were pooled, there was no significant correlation between the plasma levels of G-CSF, GM-CSF or IL-3 and the rates of neutrophil survival, apoptosis or necrosis. Additional analysis was performed after stratifying RA and AASV patients according to relative plasma GM-CSF levels (i.e., high and low GM-CSF groups). In RA patients, neutrophils from the 8 patients with high GM-CSF had higher survival rate and significantly lower apoptosis rate than those from RA patients with low GM-CSF (37% vs 27% survival rate, p=0.09 and 51% vs 61% apoptosis rate, p=0.034). For neutrophils from AASV patients with high GM-CSF (n=4 out of 44), the survival rate was only marginally higher than the AASV

group with low plasma GM-CSF; the difference was not statistically significant (38% ss 34%, p=0.5).

Sensitivity of neutrophils to growth factors

A possible explanation for prolonged neutrophil survival in AASV patients, in the absence of increased levels of growth factors in the plasma, could be that neutrophils are hypersensitive to low or normal levels of growth factors. This postulate was tested by incubating neutrophils in culture medium with or without exogenous growth factors (G-CSF, GM-CSF or IL-3).

Neutrophils from 6 AASV and 4 HBD individuals were tested. The experiment was done with 0.2 ng/ml of GM-CSF, IL-3 or G-CSF. Generally, there was no significant elevation in average neutrophil survival, after incubation with G-CSF, GM-CSF or IL-3. However, when individual results were examined, exogenous GM-CSF or IL-3 increased survival of neutrophils from 3 AASV patients, but did not enhance survival of neutrophils from 3 other AASV patients.

Apoptosis and proportion of $PR3^+/CD177^+$ neutrophils

An increased fraction of neutrophils double-positive for membrane PR3 expression and the surface marker CD177 (69% for AASV, 58% for HBD; p=0.004) was found in AASV. There was, however, no correlation between the percentage of double-positive neutrophils and the rate of apoptosis (r= -0.02, p=0.7).

Transcription of pro-/anti-apoptotic factors and transcription factors

In order to further elucidate the mechanisms of delayed apoptosis, the expression of three anti-apoptotic factors (c-IAP2, Bcl2-A1 and Mcl-1), one pro-apoptotic factor (Bax) and three transcription factors (C/EBP-α, C/EBP-β and PU.1) was measured by quantitative PCR for their respective mRNA transcripts. These factors were quantified in neutrophils from patients with AASV, PV and RA (all of which showed a lower rate of apoptosis than HBD), and in neutrophils from TP and HBD.

Table 5. Gene expression of pro-/anti-apoptotic factors in neutrophils.

	HBD	AASV	PV	TP	RA
n	19	20	10	12	21
cIAP2-mRNA	1	1.05	1.16	1.03	1.33
Bcl2-A1-mRNA	1	1.45	0.58	1.28	2.25*
Bax-mRNA	1	1.56	0.60	0.86	1.14
Mcl-1-mRNA	1	1.78	0.64	1	2.09*

All results are expressed as mean. (*) p value <0.05. HBD= healthy blood donors. AASV= ANCA-associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. RA= Rheumatoid Arthritis.

As shown in Table 5, the results showed slightly higher expression of Bcl-2A1 (1.45), Mcl-1 (1.78) and Bax (1.56) in AASV neutrophils than in HBD neutrophils; however, these differences were not statistically significant. No significant correlation was seen between the rates of neutrophil apoptosis or necrosis in neutrophils from AASV patients and relative expression of pro-/anti-apoptotic factors. Expression of Bcl-2A1 (2.25, p=0.014) and Mcl-1 (2.09, p=0.015) was significantly higher in RA neutrophils than in HBD neutrophils. Expression of pro and anti-apototic factors was not higher in neutrophils from PV patients and TP recipients than in HBD, Table 5.

Table 6. Gene expression of transcription factors in neutrophils.

	HBD	AASV	PV	TP	RA
n	22	25	10	11	10
C/EBP-α-mRNA	1	4.39**	0.11*	0.41	0.45
C/EBP-β-mRNA	1	3.53***	0.08**	2.1	1.58
PU-1-mRNA	1	1.64	0.29**	1.06	0.77

All results are expressed as mean. (*) p value <0.01, (**) p value <0.001, and (***) p value <0.0001 as compared to HBD. HBD= healthy blood donors. AASV= ANCA-associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. RA= Rheumatoid Arthritis.

Transcription of pro-/anti-apoptotic factors and transcription factors

Transcription factors involved in the process of granulopoiesis were quantified in neutrophils from HBD (n=22), AASV (n=25), RA (n=10), PV (n=10) and TP (n=12) individuals. Results showed significantly higher mRNA encoding C/EBP-α and C/EBP-β in AASV patients than in HBD, as shown in Table 6. Neutrophils from PV patients had significantly lower levels of C/EBP-β and PU.1 than neutrophils from HBD. There was no significant correlation between mRNA levels of any of the transcription factors and the rate of neutrophil survival/apoptosis. On the other hand, there was a significant positive correlation between C/EBP-α and G-CSF levels in plasma (n=9, r=0.7, p=0.03) in AASV patients.

Discussion:

Although the exact role of PR3 in the pathogenesis of AASV remains unknown, the data presented in this work confirm that levels of plasma PR3, mPR3 and pro-PR3 are all elevated in patients with AASV. The fact that all three are increased suggested a common mechanism. Thus, one of the hypotheses explored here is that aberrant PR3/mPR3 expression may reflect, or be a marker of a specific functional defect in neutrophils. Consistent with an altered neutrophil phenotype, decreased apoptosis and increased expression of transcription factors such as PU.1/ C/EBP-α/ C/EBP-β were observed in neutrophils from AASV patients. In fact, significantly higher levels of C/EBP-α mRNA in AASV neutrophils correlated positively with higher plasma G-CSF in AASV patients. No significant correlation between the rate of apoptosis and C/EBP-α and C/EBP-β mRNAs were observed in AASV neutrophils; however, this may be due to limitations in sample size in the current experiments. It is possible that altered transcription factor expression in AASV neutrophils stimulates expression/secretion of additional as yet unidentified growth factors, or stimulates hypersensitivity to growth factors, or they by themselves may work as independent endogenous growth factors that promote granulopoiesis and prevent apoptosis in AASV neutrophils. Overall, as discussed in greater detail below, the evidence of this study supports the hypothesis that an altered neutrophil phenotype is associated with and plays a role in the pathogenesis of AASV.

As mentioned, vasculitis patients have higher plasma levels of PR3 than healthy controls (224 versus 155, $p<0.0001$) and $mPR3^+$ neutrophils are more abundant in AASV than in HBD, which agrees with previous studies suggesting that a high percentage of $mPR3^+$ cells is a risk factor for vasculitis[210, 288]. AASV patients also had slightly higher mPR3-MFI than healthy controls. This suggests that mPR3-MFI may correlate with and be a marker of disease activity. However, the mild elevation in MFI may be secondary to low-grade inflammation. In this regard, circulating cytokines are lower in AASV patients who are in remission[240]. mPR3 expression ($mPR3^+$ proportion or MFI) did not vary according to ANCA status of vasculitis patients.

A weak but significant correlation between plasma PR3 and mPR3–MFI was observed in controls and MPO–ANCA-positive patients. This suggests that shedding of PR3 from the membrane may be at least partly responsible for increasing the plasma level of PR3. However, this correlation was not seen in PR3–ANCA patients; this could indicate that PR3–ANCA either enhances clearance of plasma PR3 from the circulation, or that it sterically interferes with detection of PR3 by ELISA. In support of this hypothesis, a significant negative correlation between plasma ANCA levels and plasma PR3 levels in the subgroup of PR3–ANCA patients was observed, while this was not seen in the MPO–ANCA patients. Though shedding of PR3 from the membrane may partly explain high plasma PR3, it is unlikely to be the only mechanism.

A significant finding of this study is that mPR3 and CD177 are co-expressed on the same subset of circulating neutrophils in AASV patients. Interestingly, both CD177 and mPR3 are up-regulated in parallel, and to a similar extent, in this neutrophil subset. Following stimulation of cells with PMA or with CyB/fMLP, mPR3 and CD177 expression are co-induced approximately five-fold on the membrane of $mPR3^{+}/CD177^{+}$ cells and further converted the mPR3-negative/CD177-negative cells to mPR3/CD177-expressing cells. Intracellular PR3 is stored primarily in the primary granules, and smaller amounts are found in other granule types. CD177, on the other hand, is found primarily in secondary granules. The fact that both proteins are up-regulated to the same extent by fMLP / CyB suggests that only PR3 stored in secondary granules and secretory vesicles can be expressed on the plasma membrane. Recent evidence shows that mPR3 can be co-precipitated with CD18 and CD16, and that mPR3 is localized in lipid rafts on neutrophils[58, 59]. The data from this study does not contradict this; however, mPR3 is also expressed outside of the lipid rafts, indicating that other proteins play a role in membrane localization of PR3. The bimodal expression of mPR3 in neutrophils is not explained by binding of mPR3 to CD16 and CD18 only, because CD16 and CD18 are expressed on all neutrophils. Therefore, it is possible that adaptor/transport proteins, possibly CD177 itself, that are expressed primarily in mPR3-positive cells, play a role in the expression of mPR3 on a subset of neutrophils. Because the concentration of intracellular PR3 is similar in all cells, these putative adaptor proteins would be required to selectively facilitate PR3 localization to the plasma membrane in CD177-positive cells. Another possibility is that a subset of cells in which large amounts of PR3 and CD177 are stored in secondary and secretory vesicles during granulopoiesis are precursors to the $mPR3^{+}/CD177^{+}$ circulating neutrophils. If true, this suggests a genetic mechanism, whereby the genes encoding PR3 and CD177 are co-regulated during the later stages of granulopoiesis. The fact that only 4% of cells express only one of the two markers favors this hypothesis, and suggests that a similar mechanism is involved in mobilizing PR3 and CD177 from a common intracellular storage site to the plasma membrane.

Dynamic plasma membrane expression with rapid internalization of PR3 and ANCA from the cell surface has been reported previously[180]. This study provides evidence that mPR3 is released from and recruited to the plasma membrane on a continuous ongoing basis, such that the amount of mPR3 on the surface of $mPR3^{+}$ neutrophils remains relatively constant. However, due to methodological limitations, it was not possible to determine whether internalized mPR3 is recycled and returned to the cell surface, or whether mPR3 is replenished, and the steady-state level of mPR3 maintained, by recruiting new PR3 from granular structures. While the internalization of mPR3 and CD177 could be antibody-dependent, the fact that internalization of CD18 occurs with different kinetics argues strongly against this possibility. Most of the CD18 was still present on the surface one hour after incubation with antibody. A slow decrease in MFI of mPR3 was noted as the neutrophils aged. This, when considered in tandem with dynamic trafficking of PR3 despite inhibition of de-novo synthesis may indicate that mPR3 is re-circulating

constantly. Part of the re-circulating pool of PR3 may be lost via storage in granules or by degradation, Figure 14. Rapid internalization of mPR3 could explain the variable results following *in vitro* binding of PR3-ANCA to mPR3[298, 299, 322]. Even if ANCA binds to mPR3 on the neutrophil plasma membrane *in vivo*, this would be difficult to detect *in vitro*, because, even a short incubation at RT (or a centrifugation step), will allow internalization of ANCA. Although it is unclear how ANCA affects the function of the neutrophil after internalization; previous evidence indicates that internalized PR3-ANCA can induce neutrophil apoptosis[278].

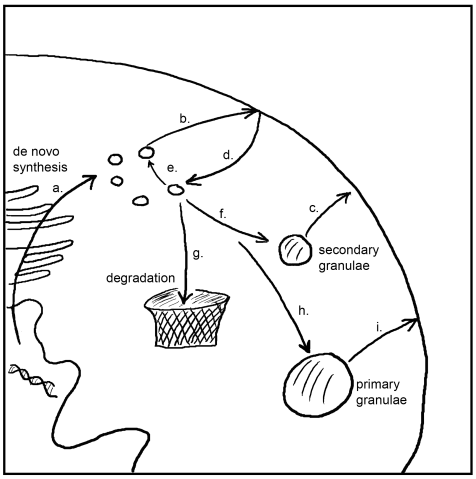

Figure 14. Membrane traffic of PR3. The mPR3 comes from either secretory vesicles (b) or seconday granules (c). The mPR3 is being rapidly internalized (d) and replaced (b) continuously, leaving its amount on the membrane constant. The recirculation circle consists of the plasma membrane and the secretory vesicles (b,d and e). Recirculating membrane PR3 is not coming from de novo synthesis (a) or from primary granules (i). Part of the recirculating pool of PR3 is lost by storage in secondary granules (f) and primary granules (h), or by degradation by other proteolytic enzymes in the cytoplasm (g). mPR3=Membrane-associated PR3.

CD177 is elevated in patients with diseases characterized by aberrant myelopoiesis. G-CSF and/or GM-CSF stimulate expression of PR3 and CD177 mRNA and plasma membrane levels of PR3 and CD177 proteins. Consistent with this, CD177 is thought to be a marker of increased granulopoiesis. It is possible that the increased percentage of mPR3-positive cells in patients with AASV could reflect increased granulopoiesis and associated increased expression of G-CSF and/or GM-CSF. This would suppress the rate of apoptosis rate, maintain the overall number of neutrophils, stimulate expression of PR3 and CD177 mRNA, and increase the fraction of $mPR3^+/CD177^+$ neutrophils in AASV individuals. However, GM-CSF was only elevated in four of 51 AASV patients; therefore, the level of G-CSF/GM-CSF is not or is not the only factor stimulating expression of PR3 and CD177 mRNA and increasing the fraction of $mPR3^+/CD177^+$ neutrophils in AASV individuals.

mPR3 and CD177 were co-expressed on the plasma membrane and the number of $mPR3^+/CD177^-$ or $mPR3^-/CD177^+$ neutrophils was negligible in all patient subgroups (AASV, PV, SLE, RA and TP patients). The $mPR3^+/CD177^+$ neutrophil subpopulation was larger in AASV and SLE patients than in other patient subgroups and healthy controls, which suggests a distinct pathophysiological basis. The number/proportion of $mPR3^+/CD177^+$ neutrophils did not correlate with treatment,

general inflammation or renal failure in AASV patients. However, this does not eliminate the possibility that inflammation could contribute to a higher number of mPR3^{+}/CD177^{+} cells, for example, by enhancing expression of a specific set of cytokines.

The level of PR3-mRNA was higher in AASV patients than in HBD, as described previously[289, 290], and the level of CD 177 mRNA was higher in AASV, PV, SLE and RA patients than in HBD. Furthermore, higher CD177–mRNA, but not PR3–mRNA correlated with a higher proportion of mPR3^{+}/CD177^{+} cells, suggesting that over-production of CD177 could lead to an increase in the proportion of mPR3^{+}/CD177^{+} neutrophils. Consistent with this, CD177 mRNA was significantly higher in mPR3-positive than in mPR3-negative human neutrophils, while PR3 mRNA was not. However, in PV patients, a high level of CD177 mRNA did not correlate with a larger proportion of mPR3^{+}/CD177^{+} neutrophils than HBD.

When U937 cells were transfected with CD177 cDNA, only cells expressing CD177 on the membrane bound exogenous PR3. This suggests that plasma membrane-associated CD177 correlates with and may be required for co-expression of mPR3 and CD177 on mPR3^{+}/CD177^{+} neutrophils. Though von Vietinghoff et al. provided evidence of direct binding between PR3 and CD177[323], their observation was not reproduced in this study. It is possible that CD177 promotes localization of PR3 to the plasma membrane or to another plasma membrane-associated protein. Hajjar et al. showed that electrostatic and hydrophobic interactions facilitate stable association of PR3 into the plasma membrane lipid bilayer[56]. However, without CD177 these interactions do not explain the expression of mPR3 on a specific subset of neutrophils.

Although plasma pro-PR3 was also detected at a higher concentration in AASV patients than in HBD, the ratio of pro-PR3 to total PR3 (approximately 10%) was relatively constant. Therefore the relationship between pro-PR3 and mPR3 is not clear. The elevated levels of pro-PR3 could indicate increased production of neutrophils in the bone marrow or increased synthesis of PR3 in mature cells. It is possible that the elevated levels of mature PR3 reflect increased degranulation upon neutrophil activation, or release of granular content as a consequence of necrosis. However, because increased plasma PR3 did not correlate with increased plasma NGAL, the degranulation theory also seems unlikely[288]. Thus, although the determinants that increase the proportion of mPR3^{+}/CD177^{+} neutrophils in AASV patients remain unclear, the most likely factors are: external stimuli, genetic factors associated with increased risk of AASV, or a specific functional defect in AASV neutrophils, as discussed above.

The G allele of the -564 A/G polymorphism in the promoter of PR3 was not associated with WG (60% WG versus 69% HBD) or the mPR3^{high} phenotype in AASV patients. Consistent with this, previous studies indicate that the -564 polymorphism does not affect PR3 promoter activity *in vitro*[42]. Although it is reasonable to conclude that this SNP is not associated with increased mPR3 in

AASV patients, a significant correlation was seen between high plasma PR3 and the A allele of the -564 A/G polymorphism in HBD (185 for AA versus 130 for GG; $p<0.0002$) and in AASV patients (223 for AA versus 154 for GG). This suggests that the -564 A/G polymorphism might influence plasma PR3 levels. Because high plasma PR3 was associated with the AA genotype, this effect can not involve binding of SP1 transcription factor to the PR3 promoter. It is possible that the A allele is in linkage disequilibrium with an allele at another locus that influences plasma level of PR3. This would still not explain the difference in plasma levels between AASV patients and controls.

This study analyzed apoptosis in neutrophils from patients with AASV. The results showed that neutrophils from AASV had a lower rate of spontaneous apoptosis, and longer *in vitro* survival, than neutrophils from HBD, SLE patients, and TP recipients. According to a study by Harper et al., neutrophils from AASV patients with active systemic vasculitis (n=8) had an accelerated rate of spontaneous apoptosis; this was correlated with high mPR3 expression and high intracellular superoxide[259]. Accelerated apoptosis was not noted in patients who were in remission (n=17). This result does not agree with the findings of the study. The number of patients in both studies is relatively small; however, it is unlikely that the difference could be explained by random variation. In this study, decreased apoptosis was also observed in neutrophils from PV and RA patients, similar to previous reports[324-329]. Delayed neutrophil apoptosis has also been associated with several other clinical conditions including sepsis, sleep apnea, cystic fibrosis, pneumonia, idiopathic pulmonary fibrosis, systemic inflammatory response syndrome after major trauma, inflammatory bowel disease, and Kawasaki disease[330-335]. Thus, reduced apoptosis in AASV might be secondary to chronic inflammation. However, the rate of apoptosis did not correlate with clinical parameters such as CRP concentration, BVAS score or reduced GFR. Although exposure to immunosuppressive drugs could influence these results, the difference between AASV and TP patients suggests this would not be significant. Apoptosis plays a crucial role in resolution of inflammation and maintaining self-tolerance. Defects in apoptotic pathways could potentially lead to the persistence of autoreactive T- or B-cells and contribute to development of autoimmune disease[145].

Apoptosis can be influenced by circulating factors in the plasma. Previously, Christensson et al. showed that AASV patients in remission had higher circulating levels of soluble Fas than HBD and other disease controls[336]. No data from functional tests was available, and the effect of soluble Fas on Fas-mediated neutrophil apoptosis is not known. As discussed above, G-CSF, GM-CSF and IL-3 enhance neutrophil survival, and delay or prevent neutrophil apoptosis[337-339]. G-CSF and IL-3 levels were normal in plasma from all vasculitis patient subgroups examined in this study. As mentioned above, GM-CSF level was higher than normal in four of 44 AASV patients. GM-CSF was also higher in 8 of 20 RA patients. Interestingly, these 8 patients exhibited delayed neutrophil apoptosis, relative to the

other RA patients, indicating that the mechanisms underlying delayed apoptosis in AASV may be different from that in RA.

Although the levels of GM-CSF and IL-3 were not elevated in AASV, they could still be related to delayed apoptosis, if neutrophils exhibit increased sensitivity to these cytokines. This hypothesis was tested by culturing neutrophils with the growth factors. Increased sensitivity was not observed, except for three patients, who were more sensitive to GM-CSF/IL-3 than HBD. Growth factors are known to prolong survival by up-regulating anti-apoptotic factors and down-regulating pro-apoptotic factors. G-CSF, GM-CSF and LPS up-regulate expression of anti-apoptotic Bcl-2A1 and promote neutrophil survival, while Mcl-1 is up-regulated by GM-CSF, IL-1β and LPS[156, 157, 340]. G-CSF up-regulates c-IAP2 (Inhibitor of Apoptosis Protein, IAP). IAP-2 is strongly up-regulated in mature neutrophils from patients with chronic neutrophilic leukemia, which also show prolonged *in vitro* survival[170]. The pro-apoptotic factor Bax is down-regulated in response to G-CSF, GM-CSF, IL-3 and IFN-γ[332]. The reduced rate of apoptosis or necrosis in AASV and PV patients in this study did not correlate with higher levels of mRNA encoding these factors. However, expression of Bcl-2A1 and Mcl-1 was significantly higher in neutrophils from RA patients than from HBD. The increased $mPR3^+/CD177^+$ neutrophil subpopulation did not correlate with reduced apoptosis in AASV patients, indicating that these phenomena likely have different origins.

Another possible mechanism of reduced apoptosis is alteration in neutrophil growth factor signaling. The level of mRNA encoding three transcription factors involved in steady-state and emergency granulopoiesis (C/EBP-α, C/EBP-β and PU.1[100, 131]) were significantly higher in AASV than in HBD, lower in PV patients, and unchanged in RA patients and TP recipients. The target genes of these transcription factors include important neutrophil proteins including G-CSF receptor, GM-CSF receptor, myeloperoxidase, PR3, elastase, lysozyme and lactoferrin[43, 341, 342]. Importantly, elevated expression of C/EBP-α, C/EBP-β and PU.1 in AASV neutrophils could lead to enhanced sensitivity to cytokines. In fact, these transcription factors may have a direct positive influence on neutrophil survival and granulopoiesis, independent of G-CSF and GM-CSF and their respective receptors[343, 344].

Normally, the transcription factors arise as an end product of the stimulated signaling pathway of immature neutrophils by G-CSF/GM-CSF/IL-3 to help these cells to differentiate into mature neutrophils. Conferred maturation of these cells gradually down-regulates these transcription factors with time; they should not be present in high amounts in mature neutrophils any longer after maturation. If this holds true, it means that there is a feedback inhibitory mechanism to control the production of these transcription factors, which is functional in PV, RA patients as well as TP recipients and HBD, while non-functional in AASV patients.

Hence the speculated defective feedback inhibitory mechanism of G-CSF/GM-CSF/IL-3 signaling may explain the increased production of transcription factors,

increased granulopoiesis, decreased apoptosis, increased PR3 (as the PR3 is one of the targets of these transcription factors) in AASV.

SHIP-1, SOCS-1 and SOCS-3 are the most important inhibitory signals to the survival signals initiated by G-CSF, GM-CSF and IL-3 at different levels of the signaling pathway. Defect/deficiency of these inhibitory factors may lead to perpetuation and exaggeration of survival signals and increased transcription factors[152, 168].

In summary, this study showed increased levels of plasma PR3, mPR3 and pro-PR3 in patients with AASV, and co-expression of CD177 and PR3 on the plasma membrane, increased expression of transcription factors PU.1/ C/EBP-α/ C/EBP-β and decreased and/or delayed apoptosis in neutrophils from AASV patients. The results also show that mPR3 is released from, internalized, and recruited to the plasma membrane on a continuous ongoing basis, such that the amount of mPR3 on the membrane of $mPR3^+$ neutrophils remains relatively constant. mPR3 and CD177 are co-expressed on the same subset of circulating neutrophils and the $mPR3^+$/ $CD177^+$ neutrophil subpopulation is increased in AASV patients. Thus, this study provides evidence for an altered neutrophil phenotype associated with AASV. Further research into the mechanisms by which neutrophil survival and apoptosis are regulated will help us understand the pathophysiology of AASV and design advanced diagnostic and treatment modules for this disease.

Conclusions:

1. Higher plasma PR3 levels, higher proportion of $mPR3^+$ neutrophils and higher PR3-mRNA expression were found in patients with AASV as compared to healthy controls.
2. 10% of the total plasma PR3 is pro-PR3 and 90% is mature PR3 while all the membrane-bound PR3 is in mature form.
3. mPR3 and CD177 are co-expressed on the same subset of neutrophils in all individuals and mPR3 depends on CD177 for its membrane expression.
4. Neutrophils derived from AASV, PV and RA patients have a lower rate of apoptosis and a higher rate of survival after 20 hours of *in vitro* culture as compared to neutrophils derived from healthy controls.
5. Mature neutrophils from AASV patients exhibit upregulated mRNA expression of the transcription factors involved in steady-state and emergency granulopoiesis (C/EBP-α, C/EBP-β and PU.1).
6. The elevated/upregulated transcripton factors, may potentially explain the increased PR3 mRNA expression and the decreased neutrophil apoptosis (PR3, G-CSF receptors and GM-CSF receptors are known targets of these transcription factors).
7. The elevated transcription factors as well as other observations of increased proportion of $mPR3^+$ neutrophils, decreased apoptosis rate, increased transcription of the PR3 gene provide evidence for altered neutrophil phenotype in AASV.
8. Further studies are needed to explore the mechanisms underlying the decreased neutrophil apoptosis in AASV, looking for factors inside neutrophils that affect survival signaling pathways or outside neutrophils looking for other survival factors in the plasma.

Popularized scientific summary in Swedish

Populärvetenskaplig sammanfattning

Proteinas 3 och neutrofil apoptos i ANCA-associerad systemisk vaskulit

Ordet vaskulit betyder kärlinflammation och med systemisk vaskulit menas inflammation i blodkärl på flera olika ställen i kroppen. Vaskulit kan drabba alla typer av kärl i kroppen, både stora och små, artärer, vener och kapillärer. Inflammationen i kärlväggen kan leda till att kärlet skadas och att blodförsörjningen till olika organ då kan bli bristfällig. Symtomen vid vaskulit beror helt på vilket/vilka organ som påverkas.

ANCA står för anti-neutrofila cytoplasmiska antikroppar. Detta är antikroppar riktade mot proteiner som finns inuti vita blodkroppar. Antikroppar känner alltså igen kroppens egna proteiner och kallas därför auto-antikroppar. De vanligaste målen för ANCA är proteinas 3 (PR3) och myeloperoxidas (MPO). PR3 är ett protein med kapacitet att bryta ner andra proteiner, men PR3 har dessutom en rad andra egenskaper som påverkar immunförsvaret.

ANCA-associerad systemisk vaskulit (AASV) är en grupp inflammationssjukdomar som drabbar framförallt små kärl och kännetecknas av ANCA i blodet. Genom att mäta ANCA i blodet hos patienterna kan man lättare ställa diagnos vid dessa sjukdomar. AASV påverkar många organ i kroppen men framförallt njurarna och lungorna. Njurarna har en riklig blodförsörjning med många små kärl. Skada på dessa kärl leder till att njurfunktionen blir sämre och i värsta fall i sådan utsträckning att patienten behöver dialysbehandling. Tidigare studier visar att ca 5 procent av alla patienter som får dialysbehandling har AASV som grundsjukdom. AASV kännetecknas av ansamlingar av döende neutrofiler i regioner med inflammation.

Neutrofiler är den vanligaste typen av vita blodkroppar (ca 60 % av alla vita blodkroppar) och representerar den första linjens försvar mot bakteriell invasion. Varje dag bildas 100 miljarder neutrofiler i benmärgen innan de släpps ut i blodbanan. Deras livslängd i blodbanan är kort, mellan 10 och 24 timmar. Vid infektion bildas tio gånger fler neutrofiler per dag, och de överlever ett par dagar längre. Vid bakteriell infektion, vandrar stora mängder neutrofiler ut ur blodkärlen till infektionshärden och bekämpar invasionen. Neutrofilers huvudfunktion är att äta upp mikroorganismer och döda dem. Neutrofiler dödar bakterier med hjälp av bakteriedödande substanser, bland annat PR3 och MPO, som lagras i små korn (granula). När bakterierna är bekämpade elimineras överflödet av neutrofiler annars kan deras bakteriedödande substanser skada omkringliggande vävnad. Detta kan ge en efterföljande inflammation, som kan ge upphov till allvarlig vävnadsskada och inflammatorisk sjukdom. Neutrofiler elimineras/äts upp av andra större celler, en annan typ av vita blodkroppar som kallas “makrofager”. För att kunna ätas upp av makrofager, måste neutrofilerna först begå spontant självmord, en process som kallas apoptos eller programmerad celldöd.

PR3 representerar en av de bakteriedödande substanser som lagras i neutrofilens granula. PR3 kan frisättas ur granula för att underlätta för neutrofilen att ta sig ut till den infekterade vävnaden och för att döda bakterier utanför neutrofilen. PR3 kan dessutom hittas på ytan (mPR3) hos en del av alla neutrofiler. Förekomsten av två olika delpopulationer, mPR3 negativa och mPR3 positiva neutrofiler, inom en individ kallas bimodalt membranuttryck. Andelen mPR3 positiva neutrofiler varierar från 0 till 100% hos olika individer, men är mycket stabilt i en viss person under längre perioder. Detta tyder på en genetisk bakgrund till fenomenet, och stöds bland annat av iakttagelser i familje- och tvillingstudier. Hur och varför PR3 associerar med membranet är fortfarande oklart. Vissa forskare föreslår att bindningen mellan PR3 och membranet är direkt d.v.s. att PR3 kan binda direkt till membranet, medan andra har funnit bevis på en receptor-medierad bindning. Olika membranbundna proteiner, receptorer, för mPR3 i neutrofiler har föreslagits, till exempel β2 integrin (CD11b/CD18), CD16b (FcγRIIIb) och scramblase1. Ingen av dessa molekyler uttrycks på alla neutrofiler och kan således inte förklara det bimodala membran-uttrycket för mPR3.

I det första delarbetet kunde vi visa att patienter med AASV har förhöjda nivåer av PR3 i plasma och högre nivåer av mPR3. Det faktum att både cirkulerande och membranbundet PR3 är förhöjt tyder på en gemensam mekanism. Vi har därför arbetat efter hypotesen att avvikande PR3/mPR3 nivåer kan vara en markör för en specifik funktionell defekt hos neutrofiler. Vi kunde också visa att denna defekt inte var en tidigare genetisk förändring i genen som kodar för PR3. Ett svagt men signifikant samband mellan plasma PR3 och mPR3 observerades i kontrollgruppen och MPO-ANCA-positiva patienter. Detta tyder på att avlägsnande av PR3 från membranet, åtminstone delvis, kan förklara ökningen av plasma nivåer av PR3 hos AASV patienter, men det är osannolikt att det är den enda mekanismen.

I det andra arbetet studerades varför PR3 endast hittas på cellytan hos en del av neutrofilerna. CD177 är den enda molekyl, förutom PR3, som är känd för att ha ett bimodalt membran-uttryck i neutrofiler. Vi kunde visa att mPR3 finns på samma undergrupp av neutrofiler som också är positiva för CD177. Dessutom visades att både CD177 och mPR3 regleras på samma sätt och att mPR3 frigörs från och rekryteras till membranet löpande, så att mängden av mPR3 på ytan av dessa mPR3 positiva neutrofiler är relativt konstant.

I delarbete tre visar vi att CD177 och mPR3 alltid hittas på samma neutrofiler hos alla individer, sjuka som friska. Den membran-PR3/CD177-positiva neutrofila subpopulationen var större hos AASV och Systemisk Lupus Erythematosus (SLE)–patienter än hos andra subgrupper av patienter och friska kontroller, vilket tyder på ett specifikt samband. CD177 är en markör för ökad bildning av neutrofiler i benmärgen och är förhöjd hos patienter med sjukdomar som kännetecknas av ökad neutrofilbildning. G-CSF eller GM-CSF är två signalproteiner (cytokiner) som stimulerar uttrycket av PR3 och CD177 på plasma membranet. Förhöjda nivåer av dessa signalproteiner skulle kunna förklara de förhöjda nivåerna av mPR3, men vi kunde bara hitta höga nivåer av dem i 4 av 51 AASV patienter.

Sambandet mellan mPR3 och CD177 undersöktes ytterligare genom att mäta avskrivningen av deras respektive gener. Avskrivningen av PR3 genen var högre hos AASV patienter än hos friska individer, men inget samband kunde visas till mängden mPR3. Däremot hittades ett tydligt samband mellan avskrift av CD177 genen och mPR3 nivåer. Genom att först sortera de mPR3 positiva och mPR3 negativa cellerna i två populationer och sedan mäta genavskriften kunde vi också visa att CD177 genen hade en förhöjd avskrivning i de mPR3 positiva cellerna medan PR3 genen var lika aktiv.

För att ytterligare karakterisera förhållandet mellan de två molekylerna gjordes ett försök där CD177 genen sattes in i odlade humana celler som normalt inte har CD177 på sin yta. Vi kunde då visa att endast de celler som uttryckte CD177 på cellytan kunde binda in PR3 till sin cellyta.

I det fjärde delarbetet undersöktes apoptoshastigheten i neutrofiler från patienter med AASV. Resultaten visade att neutrofiler från AASV hade en lägre spontanapoptoshastighet och längre överlevnad jämfört med neutrofiler från friska individer, SLE-patienter, och njurtransplanterade individer. En längre överlevnad observerades också hos neutrofiler från Polycytemia Vera (PV) patienter och reumatid artrit (RA) patienter. Fördröjd neutrofil apoptos har i andra studier associerats med andra kliniska tillstånd såsom sepsis, sömnapné, cystisk fibros, lunginflammation, idiopatisk lungfibros, systemisk inflammatorisk respons syndrom efter större trauma, inflammatorisk tarmsjukdom, och Kawasakis sjukdom. Således kan minskad apoptos i AASV vara en följd av kronisk inflammation. Vi kunde dock inte finna några samband mellan apoptoshastighet och andra kliniska parametrar för inflammation som till exempel sänkan.

Apoptoshastigheten kan påverkas av faktorer i plasman. Som nämnts ovan, kan G-CSF, GM-CSF men även signalmolekylen IL-3 öka neutrofil-överlevnaden och fördröja eller förhindra neutrofil-apoptos. G-CSF och IL-3 nivåerna var normala i plasma hos alla subgrupper i denna studie. Som nämnts ovan, var GM-CSF nivåerna högre än normalt i fyra av 44 AASV patienter. GM-CSF var också högre i 8 av 20 RA-patienter, vilket är intressant, då dessa åtta patienter hade en fördröjd neutrofil-apoptos, i förhållande till andra RA-patienter. Detta visar att mekanismerna bakom den långsamma apoptoshastigheten i AASV kan vara annorlunda än i RA.

Om neutrofiler uppvisar ökad känslighet för dessa cytokiner, skulle det ändå kunna förklara minskad neutrofil-apoptos. Denna hypotes har testats genom odling av neutrofiler med tillväxtfaktorer. Endast tre patienter hade en högre känslighet för GM-CSF/IL-3 än friska individer.

Tillväxtfaktorer är kända för att förlänga överlevnaden genom uppreglering av antiapoptotiska faktorer och nedreglering av proapoptotiska faktorer. Den minskade apoptosen i AASV och PV patienter i denna studie visade inte ha något samband med högre genavskrift av dessa faktorer. Den ökade mPR3/ CD177 positiva neutrofila subpopulation visade inte heller ha något samband med den långsammare

apoptoshastigheten i AASV patienter, vilket tyder på att dessa fenomen kan ha olika ursprung.

En annan möjlig mekanism som kan bidra till en långsammare apoptoshastighet är förändringar i olika tillväxtfaktorers signalering. Nivån av genavskrift utav tre transkriptionsfaktorer som deltar i neutrofilbildningen (C/EBP-α, C/EBP-β och PU. 1) var avsevärt högre hos AASV-patienter än hos friska individer, lägre i PV patienter, och oförändrad hos RA patienter och njurtransplanterade individer. Ett förhöjt uttryck av C/EBP-α, C/EBP-β och PU.1 i AASV neutrofiler kan leda till ökad känslighet för cytokiner. I själva verket kan dessa transkriptionsfaktorer ha en direkt positiv inverkan på neutrofilers överlevnad och nybildning, oberoende av G-CSF och GM-CSF och deras respektive receptorer.

Sammanfattningsvis ger denna avhandling bevis för en förändrad neutrofilfenotyp i samband med AASV. Ytterligare forskning om de mekanismer genom vilka neutrofiler överlever och hur apoptos regleras kommer att hjälpa oss att förstå patofysiologin vid AASV och vara till hjälp för design av ny avancerad diagnostik och behandling.

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

سَنُرِيهِمْ آيَاتِنَا فِي الْآفَاقِ وَفِي أَنفُسِهِمْ حَتَّىٰ يَتَبَيَّنَ لَهُمْ
أَنَّهُ الْحَقُّ أَوَلَمْ يَكْفِ بِرَبِّكَ أَنَّهُ عَلَىٰ كُلِّ شَيْءٍ شَهِيدٌ

صَدَقَ اللهُ العَظِيم

In The Name of Allah The Most Gracious The Most Merciful

Soon will We show them Our Signs in the horizons, and in their own selves until it becomes manifest to them that this is the Truth , Is it not sufficient enough that your Lord is a witness over all things?

(From Holly Quran- Surat Fussilat, Verse 53)

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Increased neutrophil membrane expression and plasma level of proteinase 3 in systemic vasculitis are not a consequence of the −564 A/G promotor polymorphism

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Summary

Several findings link proteinase 3 (PR3) to small vessel vasculitis. Besides being a major target of anti-neutrophil cytoplasm antibodies (ANCA), previous findings have shown increased circulating levels of PR3 in vasculitis patients, increased levels of neutrophil membrane-PR3 (mPR3) expression and a skewed distribution of the −564 A/G polymorphism in the promotor region of the PR3 gene. In this study we elucidate how these three findings relate to each other. The plasma concentration of PR3 was measured by enzyme-linked immunosorbent assay (ELISA), mPR3 expression by fluorescence activated cell sorter (FACS) and the gene polymorphism by real-time polymerase chain reaction (PCR). We compared results from 63 patients with ANCA-associated systemic vasculitis (AASV) with 107 healthy blood donors. In accordance with previous reports, AASV patients had increased plasma concentrations of PR3 compared to healthy controls (mean 224 μg/l *versus* 155 μg/l, $P < 0{\cdot}0001$). They also showed an increased number of mPR3-positive neutrophils (60% *versus* 42%, $P < 0{\cdot}001$). However, contrary to a previous report, we found no skewed distribution of the polymorphism in PR3 gene. There was a weak correlation between mPR3 mean fluorescence intensity (MFI) and plasma PR3 among healthy controls and myeloperoxidase–ANCA (MPO–ANCA)-positive patients ($r = 0{\cdot}24$, $P = 0{\cdot}015$ and $r = 0{\cdot}52$, $P = 0{\cdot}011$, respectively). In conclusion, increased plasma PR3 and high expression of mPR3 are associated with small vessel vasculitis, but neither of them is a consequence of the −564 A/G polymorphism of the PR3 gene promotor.

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Introduction

The most common forms of small vessel vasculitis are Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), and these diseases are associated strongly with anti-neutrophil cytoplasm antibodies (ANCA). The main target of ANCA in WG is proteinase 3 (PR3). PR3 is an intracellular serine protease produced during the development of neutrophils and monocytes. It was described originally as an enzyme which degrades elastin and belongs to the family of neutrophil serine proteases [1–4].

Intriguingly, there are also several observations linking PR3 to ANCA-associated systemic vasculitis (AASV); (i) AASV patients have increased levels of circulating PR3 in the plasma [5]; (ii) the proportion of neutrophils expressing PR3 on their plasma membrane is increased among AASV patients [6]; (iii) circulating leucocytes from patients with AASV display up-regulated transcription of the PR3 gene [7,8]; (iv) deficiency of alpha1-antitrypsin, the main inhibitor of PR3, seems to predispose for PR3–AASV [9,10]; and (v) a single nucleotide polymorphism in the promotor region of the PR3 gene have been found to be associated with WG [11].

Increased circulating levels of PR3 was first described by Henshaw *et al.* [12] and was later explored in our group by Baslund *et al.* [13] and in more detail by Ohlsson and coworkers [5]. It was shown that AASV patients had significantly higher levels of plasma PR3 compared to healthy blood donors as well as disease controls; this difference was independent of disease activity or ANCA type. The high plasma PR3 levels could not be explained by increased general inflammatory activity or decreased renal function and could not be connected to neutrophil degranulation [5].

In addition to azurophilic granules, PR3 is localized in specific granules and secretory vesicles [14]. It has also been found on the membrane of resting neutrophils [15]. Intriguingly, PR3 can be detected on the membrane of either the total neutrophil population or on a subset of neutrophils

(bimodal expression) [16]. The proportion of mPR3-positive (mPR3$^+$) neutrophils varies between individuals, but is highly stable over prolonged periods of time in a given individual, suggesting a genetic background for this phenomenon [16]. This suggestion was reinforced by observations in two families and twin studies [6,17].

Witko-Sarsat *et al.* reported that the vasculitis patients were skewed toward the mPR3^{high} phenotype. Considering the stability over time and the absence of relation to disease activity, they proposed the mPR3^{high} phenotype to be a genetic risk factor for vasculitis [6]. In contrast to these findings, Harper *et al.* reported that the proportion of mPR3$^+$ neutrophils is higher in patients with acute vasculitis and sepsis and lower in patients with vasculitis in remission and healthy controls [18]. Results in the same direction were presented by Muller Kobold *et al.*, who observed that the total level of mPR3 expression on neutrophils, measured as an expression index, was increased during active WG, while during remission the values decrease to levels comparable to those of healthy controls [19]. Supporting the French results, Rarok *et al.* showed that the mPR3$^+$ proportion indeed is a stable feature in a given individual but, on the other hand, they reported that the total level of mPR3 expression is significantly associated with the incidence and rate of relapse in WG [20]. Taken together, mPR3 seems to be associated with AASV, although the mechanisms underlying this association have not yet been completely elucidated.

Studies by Gencik *et al.* revealed 10 polymorphisms in the PR3 gene but only one of them was found to be associated with WG. This was a single nucleotide polymorphism (SNP) in the promotor region of the PR3 gene (− 564 A/G). Allele frequency revealed a strong preponderance for the G allele in WG patients (49·2%) compared with the control group (34·4%, $P < 0{\cdot}01$, odds ratio = 0·5) [11]. The −564 G allele of this polymorphism results in a GC-box element, which is the potential binding site for the transcription factor SP1 that could result in increased transcription of the gene and subsequently increased expression of the protein. Recent *in vitro* experiments did not exhibit any differences in promotor activity between G and A allele-containing reporter constructs [21].

In this study, we have investigated whether the level of mPR3 on isolated neutrophils and the level of PR3 in the plasma have a common origin and if that is the described polymorphism in the promotor region of the PR3 gene. This has been conducted by measuring the mPR3, plasma PR3 levels and ANCA in the same sample and correlating these parameters to the genotype as well as to each other.

Materials and methods

Patients

Patients with systemic vasculitis were recruited from the departments of nephrology at Lund University Hospital and Malmö University Hospital. The distinction between WG and MPA was made according to Chapel Hill Consensus Conference nomenclature [22]. Samples for DNA analysis were collected between 1996 and 2004, while all samples for surface expression and plasma concentrations were drawn during 2004. Most patients were in stable remission at the time of sampling. Healthy blood donors from the Blood Center Skåne were included as a control group. The study was approved by the local ethical committee at the Faculty of Medicine, Lund University and informed consent was obtained from all subjects participated in the study.

Blood sampling and separation

Approximately 6 ml of peripheral blood was collected from each patient or donor in ethylenediamine tetraacetic acid (EDTA)-anti-coagulated tubes and then leucocytes were freshly isolated from whole blood by centrifugation on Polymorphprep™ according to the manufacturer's instructions (Axis-Shield, Oslo, Norway). After centrifugation, two bands were formed; the upper band contains mononuclear cells and the lower band contains polymorphonuclear cells. The contaminating red blood cells among polymorphonuclear bands were lysed using 0·83% NH_4 Cl 10 mM Hepes pH 7·0 for 7 min at 37°C. The plasma layer, on top of the mononuclear band, was used to measure PR3 level in the plasma and ANCA level. The mononuclear band was used to extract DNA for studies on the PR3 gene and the polymorphonuclear band was used to study membrane PR3.

DNA isolation

DNA was extracted as described by Miller *et al.* [23]. The DNA was quantified by spectrophotometer; the 260/280 ratios were consistently 1·8–2·0.

Gene typing

The SNP-specific polymerase chain reaction (PCR) primers and fluorogenic probes were designed using Primer Express (version 1·5; Applied Biosystems, Foster City, CA, USA) following Applied Biosystems' guidelines as the following; −564-forward primer: 5′-GGCCTCCACCCACTCCAT-3′, −564-reverse primer: 5′-AGGATTCTCAATCAAGAGGTGA TTCT-3′. The fluorogenic probes were labelled with a reporter dye (either FAM or VIC) and are specific for one of the two possible bases (− 564 G or A) in the PR3 promoter region; −564 A-*Taq*man probe: FAM- AGACCTCACCC AGGGT-MGB, −564G-*Taq*man probe: VIC- ACCTCGCCC AGGGT-MGB. A MGB quencher probe was utilized on the 3′ end by a linker arm. *Taq*Man Universal PCR Master Mix (Applied Biosystems) was used to prepare the PCR. Primers, probes and genomic DNA were added to the final concentrations of 300 nM, 100 nM and 0·5–2·5 ng/μl, respectively. Controls (no DNA template) and reference control DNA

were run in each 96-well plate to ensure that there was no amplification of contaminating DNA and that signals from both probes was achieved. All PCR reactions were made in triplicate (25 µl per reaction) in optical 96-well plates using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with two initial hold steps (50°C for 2 min, followed by 95°C for 10 min) and 40 cycles of a two-step PCR (95°C for 15 s, 60°C for 1 min). The −564 nucleotide was determined by the fluorescence ratio of the two SNP-specific fluorogenic probes.

Determination of surface PR3 expression by fluorescence activated cell sorter (FACS) analysis

Membrane PR3 expression on neutrophils was measured using flow cytometry as described previously [6]. In brief, isolated cells were treated with 0·5 mg/ml heat-aggregated rabbit immunoglobulin (IgG; Sigma, St Louis, MO, USA) for 30 min. After washing, cells were incubated with murine monoclonal anti-human PR3 antibodies (4A5; Wieslab, Lund, Sweden) or with an isotype-matched murine control antibody (IgG1; Sigma Biosciences) for 30 min followed by a 30-min incubation with fluorescein isothiocyanate (FITC)-conjugated $(Fab')^2$ fragments of rabbit anti-mouse antibodies (Dako Cytomation, Glostrup, Denmark). Labelled neutrophils were fixed with 1% paraformaldehyde for 10 min.

Fluorescence was analysed on an EPICS XL-MCL flow cytometer (Beckman Coulter Inc., Miami, FL, USA), and fluorescence intensity was standardized using Flow-Set Fluorospheres (Beckman Coulter Inc., Fullerton, CA, USA). The cytometer can show the percentage of $mPR3^+$ neutrophils as well as mean fluorescence intensity (MFI).

The expression index of mPR3 was calculated as described previously by Rarok *et al.* the MFI was corrected for the non-specific binding (NSB) of an isotype-matched irrelevant antibody and multiplied by the percentage of $mPR3^+$ neutrophils; expression index = (MFI–NSB) × % $mPR3^+$ neutrophils, and expressed in arbitrary units (AU) [20]. The individuals were divided into three mPR3 phenotypes, as described previously by Witko-Sarsat *et al.* according to their percentage of $mPR3^+$ neutrophils corresponding, respectively, to 0–20% of $mPR3^+$ neutrophils ($mPR3^{low}$ phenotype), 21–58% of $mPR3^+$ neutrophils ($mPR3^{intermediate}$ phenotype) and 59–100% of $mPR3^+$ neutrophils ($mPR3^{high}$ phenotype) [6].

Enzyme-linked immunosorbent assays (ELISAs) of plasma PR3 and ANCA

Plasma PR3 level was detected as described previously [5]. Briefly, a microtitre plate was coated overnight with a purified mixture of two monoclonal PR3 antibodies (4A3 and 4A5, 3 µg/ml each). Plasma samples diluted to 1/20 and 1/40 in sample buffer were added and the plates were incubated for 2 h. After washing, bound PR3 was detected by incubation for 2 h with affinity-purified rabbit anti-PR3 diluted to 1/300 in sample buffer. Washing was followed by the addition of the conjugate (alkaline phosphatase-labelled swine anti-rabbit IgG from Dako Cytomation), diluted to 1/1000 in sample buffer and then incubated for 1 h. ANCA was detected by standard direct ELISA using the Wielisa® PR3–ANCA kit.

Statistical analyses

Differences in continuous variables between two groups were analysed using the Mann–Whitney *U*-test; some data sets that did not follow Gaussian distribution were transformed and then analysed by unpaired *t*-test. For data sets that follow Gaussian distribution, a *t*-test was used and results are given as mean ± standard deviation (s.d.). All other results are given as median, interquartile range (IQR).

One-way ANOVA with Bonferroni's post-test was used for comparisons between more than two groups. Correlations were analysed using Pearson's rank test. Proportions between groups were compared with the χ^2 test. A two-sided $P < 0{\cdot}05$ was considered to be statistically significant.

Results

Genotype

DNA purified from peripheral blood of 189 blood donors and 132 patients with AASV (76 with WG and 56 with MPA) was subjected to genotyping using allelic discrimination-real time PCR. No significant differences were seen between any group of patients regarding gene or allele frequencies. Figure 1 shows the gene frequencies of patients and controls of the −564 A/G polymorphism in the promotor region of the PR3 gene. The allele frequencies in the patients were 36% for the A allele and 64% for the G allele compared to 31% and 69% for healthy controls. When limiting the analysis to patients with a clinical diagnosis of WG, we found an allele frequency of 40% for the A allele and 60% for the G allele; the corresponding figures for MPA patients were 30% and 70%. Thus, we could not verify the previous findings of an increased frequency of the G allele among WG patients.

Membrane PR3

Flow cytometry analysis of mPR3 on isolated neutrophils was performed on 107 healthy blood donors and 58 patients with AASV. Thirty-one of these patients were men and 27 were women. Their mean age (at sampling) was 63·2 ± 16·4 years ranging from 17·8 to 86·5 years. A clinical diagnosis of WG was made for 35 patients (31 were PR3–ANCA-positive, three MPO–ANCA-positive and one ANCA-negative) and 23 patients were diagnosed as having MPA (20 MPO–ANCA-positive, three PR3–ANCA-positive). In total, 34 patients were PR3–ANCA-positive, 23

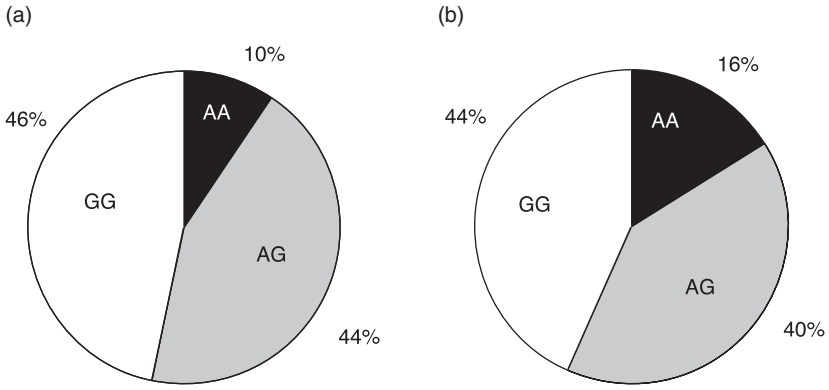

Fig. 1. Distribution of the −564 A/G polymorphism in the promotor region of the proteinase 3 gene. Black colour represents individuals homozygous for the A allele, white colour represents individuals homozygous for the G allele and grey colour represents heterozygotes. The results of 189 healthy blood donors are shown in (a) and 136 anti-neutrophil cytoplasm antibodies-associated systemic vasculitis patients are shown in (b).

patients were MPO–ANCA-positive at diagnosis and one patient never exhibited any positive ANCA test.

The percentage of mPR3$^+$ neutrophils as shown in Fig. 2a was significantly higher in vasculitis patients (60 ± 27%) compared to healthy blood donors (42 ± 22; $P < 0{\cdot}0001$). This difference was independent of diagnosis as the MPA group had 62 ± 24% PR3$^+$ cells and the WG group had 58 ± 29% ($P = 0{\cdot}0002$ and $P = 0{\cdot}0007$, respectively). Similar results were observed when the patients were divided according to ANCA serology.

When the patients were divided into the three predefined mPR3 phenotypes [6], the distribution of the vasculitis patients were skewed toward the mPR3^{high} phenotype, as shown in Fig. 3. This stands in contrast to the control group that exhibited an equal distribution between the high and low phenotype groups (27% high, 50% intermediate, and 23% low). This difference between the two distributions was significant ($\chi^2 = 13{\cdot}47$, $P = 0{\cdot}0012$).

Also when comparing the MFI of mPR3, we found higher values for the vasculitis patients (median 1·43, IQR 1·03–2·12) compared to healthy blood donors (1·19, 0·915–1·58). However, the differences were relatively small and only statistically significant, according to the Mann–Whitney *U*-test, when comparing all patients or the PR3–ANCA-positive subgroup with the controls ($P = 0{\cdot}04$ and 0·03, respectively).

Consequently, when comparing the expression index (EI) of mPR3, we found higher values for the AASV patients (median 89·3, IQR 43·6–163 units) compared to the group of controls (53·1, 25·2–87·8 units, $P = 0{\cdot}001$, Fig. 2b). The median expression index was significantly higher for all subgroups compared to healthy blood donors. There were no significant differences between the subgroups, either based on diagnosis or on serology.

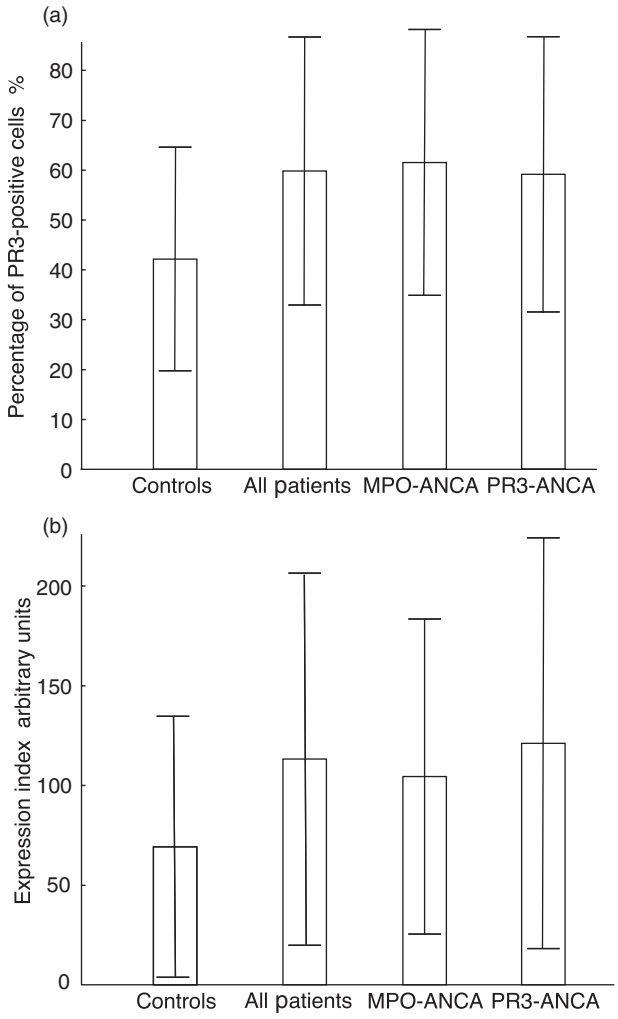

Fig. 2. Membrane expression of proteinase 3 (PR3) on neutrophils. Neutrophils stained with anti-PR3 murine monoclonal antibodies and followed by fluorescein isothiocyanate-conjugated anti-mouse antibodies were analysed by flow cytometry. (a) Shows the percentage of membrane-PR3$^+$ neutrophils in 107 healthy blood donors, 58 patients with anti-neutrophil cytoplasm antibodies (ANCA)-associated systemic vasculitis (AASV) and two subsets of the same patients (23 having myeloperoxidase–ANCA and 34 having PR3–ANCA). (b) Shows the expression index of mPR3 on neutrophils measured by arbitrary units (AU) for the same groups as in (a). All results are given as mean values ± s.d.

Plasma PR3 and ANCA

Plasma PR3 was significantly elevated in the patients (mean ± s.d. 224 ± 128 μg/l, $n = 63$) compared to healthy blood donors (155 ± 52 μg/l, $n = 130$) (unpaired *t*-test, $P < 0{\cdot}0001$). When dividing the AASV patients based on their clinical diagnosis, we found that the MPA patients had

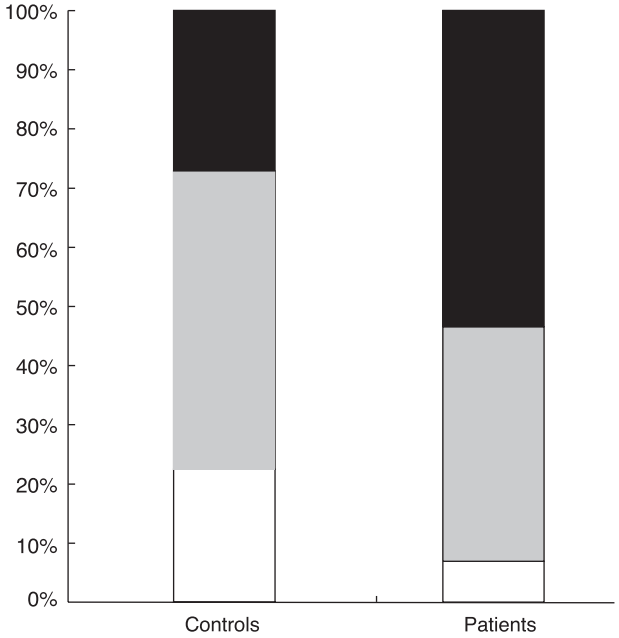

Fig. 3. Phenotype distribution of membrane-proteinase 3 (mPR3) on neutrophils. Individuals divided into three predefined groups, high (black)/intermediate (grey)/low (white), according to their percentage of mPR3$^+$ neutrophils [6]. The distribution of these three groups is shown in 58 patients with anti-neutrophil cytoplasm antibodies-associated systemic vasculitis compared to 107 healthy blood donors.

significantly higher PR3 concentrations (256 ± 162 μg/l, $n = 26$) than healthy blood donors ($P < 0{\cdot}0001$). Similarly, but to a lesser extent, WG patients had higher PR3 concentrations (202 ± 95 μg/l, $n = 37$) compared to healthy blood donors ($P = 0{\cdot}0029$), as shown in Fig. 4. Similar results were found when we divided the AASV patients into two subgroups based on their ANCA serology.

ANCA level in the patient plasma was also tested; 13 patients were PR3–ANCA-positive at the time of sampling of the 35 who were PR3–ANCA-positive at the time of diagnosis. Regarding MPO–ANCA, 10 patients were MPO–ANCA-positive at the time of sampling of the 26 who were MPO–ANCA-positive at the time of diagnosis.

Genotype–phenotype correlations

There was no significant difference between the three promotor genotypes regarding any of the three mPR3 parameters (% of mPR3$^+$ cells, MFI and EI) in the healthy blood donors (χ^2 test). Similarly, there was no difference between the three promotor genotypes regarding their mPR3 expression in the patients (Table 1).

When correlating the three promotor genotypes with the plasma PR3 in the healthy blood donors, there was a significant difference between the three genotypes ($P = 0{\cdot}0002$). The GG genotype had lower levels, which is opposite to the expected. A similar difference was observed in the patient group, but it was not significant ($P = 0{\cdot}08$), Table 1.

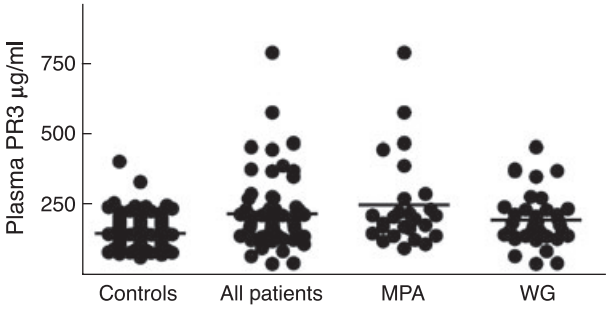

Fig. 4. Concentration of proteinase 3 in the plasma measured by enzyme-linked immunosorbent assay; 130 healthy blood donors are compared with 63 patients with anti-neutrophil cytoplasm antibodies-associated systemic vasculitis and two subsets of the same patients (26 with myeloperoxidase and 37 with Wegener's granulomatosis.

Phenotype–phenotype correlations

There was no correlation between plasma PR3 and percentage of mPR3$^+$ neutrophils in either controls or AASV patients. There was, however, a weak but significant correlation between plasma PR3 and MFI in healthy controls ($r = 0{\cdot}24$, $P = 0{\cdot}015$). A similar correlation was found in the MPO–ANCA-positive subgroup of AASV patients ($r = 0{\cdot}52$, $P = 0{\cdot}011$), while no correlation was found in the PR3–ANCA subgroup (Fig. 5). On the other hand, we did observe a significant negative correlation between plasma ANCA levels and plasma PR3 levels in the subgroup of PR3–ANCA

Table 1. Genotype–phenotype correlation. Comparison table showing the correlation between the three genotypes of the −564 A/G promotor polymorphism in the proteinase 3 (PR3) gene and the four PR3 parameters, % of membrane-PR3 (mPR3)-positive cells, mean fluorescence intensity (MFI), expression index (EI) and plasma PR3 (median). (a) Shows the results of healthy blood donors. (b) Shows the results of patients with anti-neutrophil cytoplasm antibodies-associated systemic vasculitis.

Genotype	% of positive cells	MFI	EI	Plasma PR3
(a)				
AA	45	1·61	54·8	185
AG	40	1·13	53·3	167
GG	45	1·13	50·3	130
P	0·98	0·57	0·79	0·0002
n	83	83	83	110
(b)				
AA	71	1·52	100	223
AG	65	1·42	109	199
GG	52	1·01	64·8	154
P	0·28	0·07	0·17	0·082
n	52	52	52	57

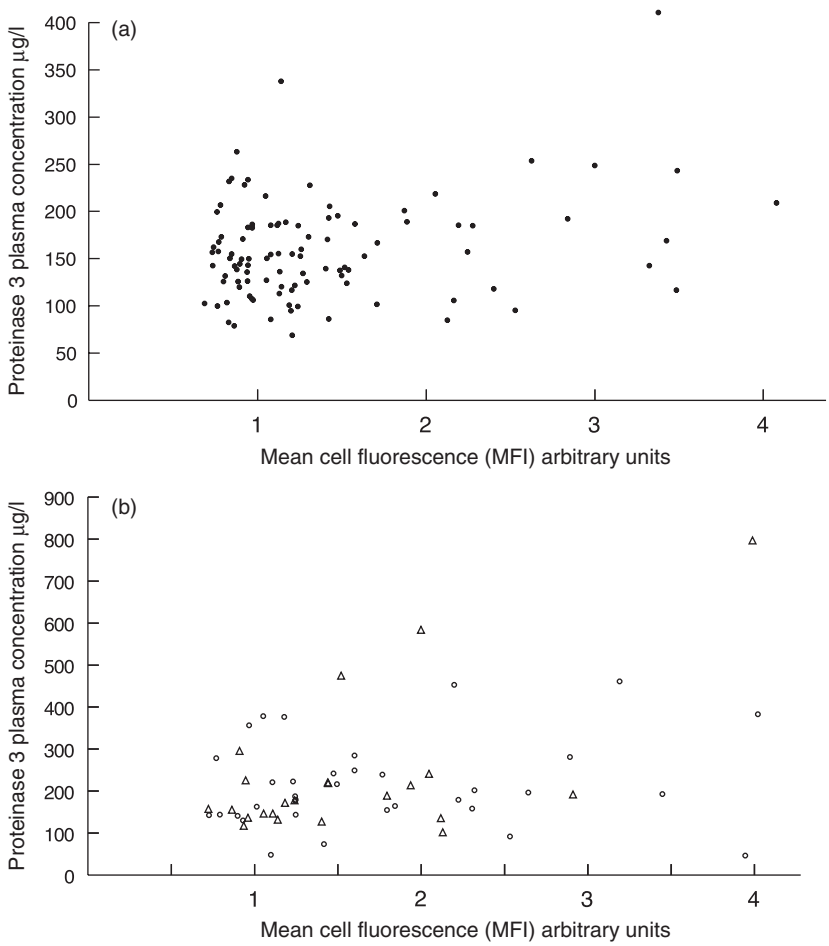

Fig. 5. Scatter diagrams showing the correlation between the mean fluorescence intensity of membrane proteinase 3 (mPR3) on neutrophils and plasma PR3 concentrations. (a) Shows the results of 102 healthy blood donors. (b) Shows the results of 58 patients with anti-neutrophil cytoplasm antibodies (ANCA)-associated systemic vasculitis. Circles indicate 23 myeloperoxidase–ANCA-positive patients and triangles 34 PR3–ANCA-positive patients.

patients ($r = -0{\cdot}486$, $P = 0{\cdot}0031$), while MPO–ANCA levels did not correlate with plasma concentrations of PR3.

Discussion

The aim of this study was to shed light on the possible relationship between different features of the PR3 protein linked previously to AASV. To this end, we have analysed all these parameters in the same sample from a new cohort of AASV patients. In this study, we confirm that AASV patients have more PR3 on the surface of isolated neutrophils compared to healthy blood donors. This was due primarily to an increased number of mPR3$^+$ cells, which is in accordance with studies suggesting that a high percentage of mPR3$^+$ cells is a risk factor for vasculitis [6]. In addition to the high percentage, we also observed that our AASV patients had slightly increased mPR3 expression per cell (MFI) compared to healthy controls. This observation is compatible with the notion that MFI is a variable feature that correlates with disease activity and a possible marker of disease activity [19]. The slightly elevated MFI levels could be caused by low-grade inflammation, and our earlier studies have shown that AASV patients also have increased levels of circulating cytokines during remission [24]. Moreover, we found no significant differences regarding mPR3 expression (mPR3$^+$ proportion or MFI) in vasculitis patients according to ANCA status.

In contrast to the mPR3 case, we were not able to confirm the findings from Gencik *et al*. regarding the −564 A/G polymorphism. In our cohort, there was no skewing of this polymorphism towards the G allele (60% for WG patients *versus* 69% for healthy controls). The main discrepancy between our results and theirs was a lower percentage among their healthy controls (34%), a difference that could be explained by a genetic heterogeneity in the background populations [11]. As no skewing was observed, it is not surprising that the mPR3$^+$ high phenotype did not correlate to the GG genotype. This, together with our previous finding that the −564 A/G SNP in the promoter region of proteinase 3 does not affect the promoter activity *in vitro*, we conclude that this SNP is not associated with increased mPR3 expression [21].

In this study, we confirm our previous finding that vasculitis patients have elevated plasma levels of PR3 compared to healthy controls (224 *versus* 155, $P < 0{\cdot}0001$) [5]. We also found a significant correlation between plasma PR3 and the −564 A/G polymorphism in the healthy controls ($P < 0{\cdot}0002$). This argues for a genetic component of plasma PR3 levels. However, the high plasma PR3 cannot be explained by the binding of the SP1 transcription factor to

the G allele, as the highest levels were found among those with the AA genotype (185 for AA *versus* 130 for GG). A more plausible explanation is that the A allele is in linkage disequilibrium with an allele at another locus that is actually associated with the high plasma phenotype. However, the existence of such gene locus could not explain the difference in plasma levels between patients and controls, as patients exhibited a similar difference in plasma levels with regard to genotypes (223 for AA *versus* 154 for GG).

Our earlier studies did not show any correlation between plasma PR3 and NGAL, a marker of secondary granules, arguing strongly against increased degranulation being the source for high plasma PR3 levels [5]. The present study shows a weak but significant correlation between plasma PR3 and mPR3–MFI among controls and among the MPO–ANCA-positive patients. The existence of such a correlation leads us to suggest that at least part of the plasma PR3 comes from the shedding of PR3 from the membrane of neutrophils. The absence of correlation in PR3–ANCA patients could be explained by interference of the autoantibodies. PR3–ANCA may enhance the clearance of plasma PR3 from the circulation or may act as a blocking agent for PR3, preventing its detection by ELISA. Supporting this notion, we found a significant negative correlation between plasma ANCA levels and plasma PR3 levels in the subgroup of PR3–ANCA patients, but not among those with MPO–ANCA.

This study shows increased mPR3 and plasma PR3 levels among AASV patients but fails to reveal which precedes the other; nor can we answer if either of them is directly implicated in the pathogenesis of vasculitis as a risk factor or activity marker. As these questions might be fundamental for the understanding of the underlying mechanisms of the disease, they deserve further study. In order to resolve these questions, more knowledge is needed regarding the origin of the plasma PR3 and the nature of the association of PR3 with the plasma membrane.

In conclusion, this study demonstrates that the elevated mPR3 expression on the membrane of neutrophils and the elevated plasma PR3 are associated with vasculitis. In addition, our data show that the elevated mPR3 expression and elevated plasma PR3 are correlated to each other. According to our results, these elevated PR3 levels found in AASV patients are not a consequence of the −564 A/G polymorphism in the PR3 gene and further studies are needed to reveal their origin, as it may be a fundamental predisposing factor for the generation of PR3–ANCA.

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II

Proteinase 3 and CD177 are expressed on the plasma membrane of the same subset of neutrophils

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Abstract: Proteinase 3 (PR3) is found in granules of all neutrophils but also on the plasma membrane of a subset of neutrophils (mPR3). CD177, another neutrophil protein, also displays a bimodal surface expression. In this study, we have investigated the coexpression of these two molecules, as well as the effect of cell activation on their surface expression. We can show that CD177 is expressed on the same subset of neutrophils as mPR3. Experiments show that the expression of mPR3 and CD177 on the plasma membrane is increased or decreased in parallel during cell stimulation or spontaneous apoptosis. Furthermore, we observed a rapid internalization and recirculation of mPR3 and plasma membrane CD177, where all mPR3 is replaced within 30 min. Our findings suggest that the PR3 found on the plasma membrane has its origin in the same intracellular storage as CD177, i.e., secondary granules and secretory vesicles and not primary granules. PR3- and CD177-expressing neutrophils constitute a subpopulation of neutrophils with an unknown role in the innate immune system, which may play an important role in diseases such as Wegener's granulomatosis and polycythemia vera. *J. Leukoc. Biol.* 81: 458–464; 2007.

Key Words: *trafficking · colocalization · serine proteases · Wegener's granulomatosis · polycythemia vera*

INTRODUCTION

Proteinase 3 (PR3) was first described in 1973 [1] and is a member of the neutrophil serine protease family, which also includes human neutrophil elastase (HNE), cathepsin G, and enzymatically inactive azurocidin. These proteins are stored in primary granules [2]. However, PR3 has also been found in secondary granules, in secretory vesicles [3], and on the plasma membrane (mPR3) of a subset of neutrophils [4]. This nonuniform, bimodal expression of mPR3 on neutrophils is specific to PR3. Other serine proteinases are found on the plasma membrane of activated neutrophils but do not exhibit a bimodal distribution [5]. Furthermore, PR3 has been demonstrated to have specific biological functions such as processing of cytokines [6–8] and induction of IL-8 synthesis by endothelial cells [9]. It has been shown that PR3 can promote platelet aggregation [10] and that it interferes with the apoptosis machinery [11–14]. Secreted pro-forms of PR3 as well as azurocidin and granzymes A, B, H, K, and M, but not cathepsin G or HNE, have been shown to reduce the fraction of granulopoietic progenitors in S-phase [15, 16]. In summary, PR3 is considered to be essential for the regulation of proliferation, maturation, and apoptosis of granulocytes.

Several investigations have shown that the proportion of neutrophils expressing mPR3 varies between individuals but is remarkably stable over time in a given individual [17–19]. The percentage of mPR3-positive cells seems to be determined genetically and regulated by two co-dominant alleles [17, 19]. Patients with Wegener's granulomatosis but also with rheumatoid arthritis have shown an increased proportion of mPR3-positive cells [17]. A high percentage of mPR3-positive neutrophils has been linked to an increased risk of developing vasculitis [17, 19], increased relapse rate [18], and adverse renal outcome [20]. PR3 is important in Wegener's granulomatosis, as these patients develop autoantibodies against PR3 {PR3-antineutrophil cytoplasmic antibody (ANCA) [21–24]}.

Different receptors for mPR3 have been proposed, and most of the evidence points toward a β2 integrin, Cd11b/CD18 [25], but it has also been shown that mPR3 colocalizes with CD16 (FcγRIIIb) [26, 27]. However, these molecules are expressed on all neutrophils and can thus not explain why only a subset of the cells is mPR3-positive.

CD177 is the only other cell surface molecule known to have a bimodal plasma membrane expression pattern in neutrophils. It is a GPI-anchored glycoprotein, first described in 1971 as the NB1 antigen [28] and a member of the leukocyte antigen 6 superfamily [29]. The gene coding for CD177 is localized to chromosome 19q13.2 and has at least two known alleles, NB1 and polycythemia rubra vera (PRV)-1 [30]. The plasma membrane expression of CD177 can be increased by administration of G-CSF [31], regarding the percentage of positive cells and the number of molecules per cell. Furthermore, it is increased during pregnancy and is probably affected by estrogen and progesterone. Several studies have shown that 95–100% of

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patients with PRV have elevated levels of CD177 mRNA [29, 32–34]. A similar increase, but in smaller proportion, has been found in essential thrombocythemia and idiopathic myelofibrosis [32–34], whereas chronic myelogenous leukemia is not associated with an increased CD177 expression [29]. Recently, a dominant gain of function mutation in the JAK2 was found in a high proportion of patients with myeloproliferative disorders [35].

In this study, we investigate if CD177 and PR3 are expressed on the plasma membrane of the same subpopulation of neutrophils. We also study the effect of cell activation and spontaneous apoptosis on the plasma membrane expression of these two molecules.

MATERIALS AND METHODS

Neutrophil isolation

Polymorphonuclear leukocytes were isolated from human blood, kindly provided by healthy volunteers. In total, 10 different donors have been used, and in each experiment, we have used neutrophils from at least three donors. As described by the manufacturer, whole blood from EDTA-anticoagulated tubes was layered carefully on Polymorphprep™ (Axis-Shield PoC AS, Oslo, Norway) and centrifuged at 625 g for 1 h at room temperature (RT). The neutrophil layer was recovered and suspended in PBS before pelleting the cells at 250 g for 10 min at RT. Contaminating erythrocytes were removed by hypotonic lysis during 7 min at 37°C using 0.83% NH_4Cl in 10 mM Hepes, pH 7.0. The cells were then pelleted at 250 g for 5 min at RT and resuspended in RPMI (PAA Labs, Göteborg, Sweden). The percentage of neutrophils was 95–99%, as determined by Türk staining. Viability, checked using Trypan blue staining, was >95%. All experiments were performed in RPMI medium within 1 h after isolation of the cells.

Colocalization experiments

The neutrophil-containing samples were blocked using human IgG (0.5 mg/ml in PBS containing 5% goat serum and 1% BSA; Sigma Chemical Co., St. Louis, MO) for 20 min on ice, followed by addition of the primary antibodies, rabbit anti-PR3 (3 μg/ml; Wieslab AB, Lund, Sweden), mouse anti-CD177 MCA2045 (1:200–1000; Serotec, Oxford, UK), mouse anti-CD18 (1:1000), or mouse anti-CD16 (1:1000; both from BD PharMingen Biosciences, Palo Alto, CA) for 15 min on ice. The samples were washed and then stained with the secondary antibodies antirabbit ALEXA 488 (1:1000; Molecular Probes, Eugene, OR) and antimouse R-PE-cytochrome 5 (RPE-Cy5; 1:200; Dako Cytomation, Glostrup, Denmark) for flow cytometry (FACS) analysis or antimouse ALEXA 594 (1:1000; Molecular Probes) for fluorescence microscopy for another 15 min on ice. Some samples were stained with cholera toxin subunit B conjugated with Alexa Fluor 594 (CT-B; Molecular Probes). These samples were prefixed with 0.1% paraformaldehyde (PFA), washed, and then incubated in the dark with CT-B for 60 min on ice.

Stimulation experiments

Neutrophils were stimulated with TNF-α (20 ng/ml, 15 min), PMA (100 nM, 15 min), fMLP (1 μM, 5 min), or cytochalasin B (CyB; 10 μM, 15 min), followed by addition of fMLP (1 μM, 5 min; all from Sigma Chemical Co.). All incubations were performed at 37°C. The samples were prepared as described in the colocalization experiment section and stained with antibodies against PR3 and CD177.

Apoptosis and necrosis investigation

Apoptotic and necrotic cells were demonstrated by double-staining using 1 μl AnnexinV-Alexa Fluor 488 (Molecular Probes) and 20 μl BD Via-Probe (BD PharMingen Biosciences) for 5 min in the dark. Samples were then investigated by FACS.

Internalization experiments

Neutrophil samples were stained with primary antibodies, the monoclonal anti-PR3 4A5 (1:100; Wieslab AB), anti-CD177 (1:1000), or anti-CD18 (1:1500) for 15 min on ice. After washing, the secondary antibody ALEXA 488 antimouse (1:1000) was added for another 15 min on ice. The samples were then put on ice or incubated for 15, 30, or 60 min, rotating at 37°C, 8 rpm. Incubation with the primary antibodies, as described above, was repeated followed by staining with the secondary antibody ALEXA 594 antimouse (1:1000) for 15 min on ice. The samples were fixed using 2% PFA and mounted for fluorescence microscopy evaluation.

Blockage of protein synthesis

Neutrophils were incubated with cycloheximide (100 μg/ml) for 0, 30, 60, 90, and 120 min. After washing, the cells were stained with mAb against PR3 (1:100) and CD177 (1:300) for 15 min on ice and then washed again. The secondary antimouse PE-Cy5 antibody was added for 15 min on ice (1:200). Finally, the cells were stained with Annexin V-Alexa Fluor 488 for 5 min in the dark to monitor the viability of the cells. The samples were evaluated by FACS.

Time-course study

Staining with the primary antibodies, 4A5 (1:300), anti-CD177 (1:200), or anti-CD18 (1:1500), was performed for 15 min on ice followed by antimouse PE-Cy5 (1:200) for another 15 min on ice. Fluorescent Annexin V was then added for 5 min in the dark at RT. Viability was also checked by double-staining with Annexin V and BD Via-Probe. During incubation, the samples were rotated at 37°C, 8 rpm. The samples were evaluated by FACS analysis.

FACS analysis

Flow cytometry analysis was performed using a FACSCalibur flow cytometer, equipped with a 15-mW argon laser tuned at 488 nm (Becton Dickinson, Franklin Lakes, NJ). The neutrophil population was selected by gating with appropriate settings for forward scatter and sideward scatter. The FL1 fluorescence channel was used to record the emitted fluorescence from ALEXA 488, and the FL3 fluorescence channel was used for PE-Cy5. For each condition, 20,000 gated events were acquired. Acquisition and analysis of the data were performed using the CellQuest Pro software.

Fluorescence microscopy

Samples were fixed using 2% PFA for 15 min at 4°C, followed by overnight incubation at 8°C. Glass coverslips were washed with methanol and overlaid with 0.25 ml poly L-lysine (0.2 mg ml^{-1} in water; Sigma Chemical Co.). After evaporating the added fluid at 50–65°C, the coverslips were washed twice with distilled water. Following a final wash with PBS, the fixed and stained cells were adhered to the poly L-lysine-coated coverslips, and the samples were mounted using Dako mounting medium (Dako, Carpinteria, CA).

Visual inspection and recording of images were performed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled charged-coupled device camera, using a Plan Apochromat 100× oil immersion objective. Images were acquired and handled using Image Pro Plus and Adobe Photoshop 7.0.

RESULTS

Cells were costained with antibodies against CD18/CD16/CD177 and PR3. The samples were evaluated by FACS and fluorescence microscopy. A strong correlation between PR3 and CD177 surface expression was found, as shown in **Figure 1**. Anti-CD18, anti-CD16, and CT-B stained virtually all cells, and no correlation to mPR3 staining was seen (data not shown). Representative fluorescence microscopy data are displayed in **Figure 2**. CD18 (Fig. 2B) and CD16 (Fig. 2E) are found on the majority of the neutrophils and not only on cells expressing

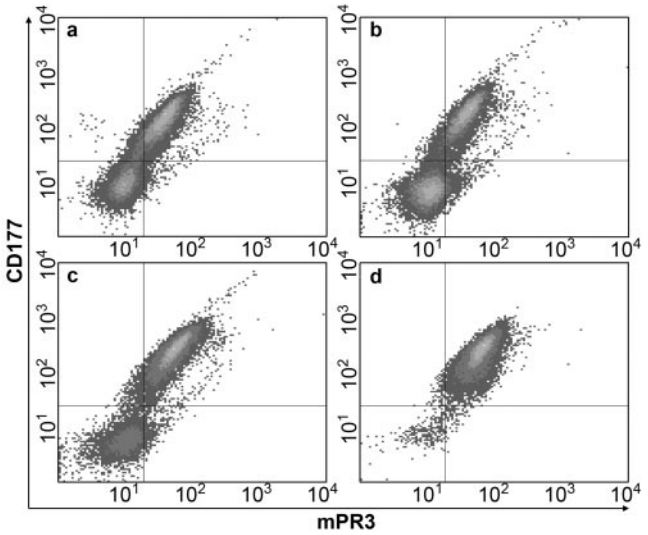

Fig. 1. Coexpression of CD177 and PR3. Neutrophils were stained with anti-CD177 and anti-PR3 antibodies. Figure 1, a–d, represents four different individuals with different proportions of mPR3/CD177 expressing neutrophils (in total, 10 different individuals were studied). As can be seen, PR3 and CD177 are expressed on the plasma membrane of the same subset of neutrophils.

mPR3. However, the expression of CD177 (Fig. 2H) is heterogeneous and coincides with the expression of PR3 (Fig. 2I). Cells that are mPR3-positive are also positive for CD177, and cells that are mPR3-negative are also negative for CD177. When examining 100 cells, 96% were double-positive or double-negative, and only 4% displayed an expression of one of the markers and not the other.

Next, we investigated how the surface expression was affected by stimulation of the neutrophils and whether mPR3- and CD177-negative cells could be stimulated into becoming mPR3- and CD177-positive. Cells were treated with TNF-α, PMA, fMLP, or fMLP in combination with CyB and analyzed by FACS. Stimulation with TNF-α or fMLP caused a moderate increase (a factor 1.7 and 2.0, respectively) in fluorescence intensity for mPR3- and CD177-positive cells (**Fig. 3**). However, stimulating the neutrophils with PMA caused a major shift in fluorescence intensity. The mPR3- and CD177-positive cell population was shifted further to the right; i.e., the expression of mPR3 and CD177 increased by a factor 4.9. Furthermore, the mPR3- and CD177-negative population was converted into mPR3- and CD177-expressing cells. The distribution remained bimodal, but instead of a positive/negative population, the cells divided into a mPR3 and CD177 high and low subgroup. The proportion of mPR3 high-expressing cells was constant and independent of stimulation. When stimulating with the combination of fMLP and CyB, a similar effect was observed. Fluorescence microscopy verified a plasma membrane localization of the staining (not shown). Our results indicate that PR3 and CD177 are coregulated, as their surface expression is increased in parallel. Moreover, PR3 and CD177 surface-negative cells have an intracellular pool of these markers, which can be transported to the plasma membrane upon stimulation.

mPR3 has been suggested to be a preapoptotic marker for aging neutrophils. To study this, we incubated purified neutrophils at 37°C for various times, up to 22 h. The neutrophils were analyzed every 3 h for mPR3 expression (**Fig. 4A**) and for viability (Fig. 4C). The percentage of mPR3-positive cells remained constant over time, whereas the MFI slowly decreased with increasing age of the live neutrophils (Annexin V-negative cells). When analyzing the total neutrophil population, similar results were obtained (data not shown). CD177 plasma membrane expression was also evaluated at time-point 0 and after 22 h incubation in three experiments (Fig. 4B). The results show that the percentage of positive cells tended to diminish (not significant); however, the MFI was reduced, and after 22 h, only 40% of the plasma mPR3 remained. This loss of CD177 and mPR3 could be a result of shedding into the surrounding media or internalization.

The dynamics of the membrane traffic of mPR3 and CD177 was studied by internalization experiments. CD177 was transported into the cells from the surface, and after 15 min of incubation, intracellular CD177 was observed (**Fig. 5E**, II), with only weak staining on the cell surface. After 30 min, the majority of the surface-bound CD177 had been internalized (Fig. 5F, II). For PR3, the majority of the surface staining had been internalized already after 15 min (Fig. 5H, II). CD18, used as a control, displayed a cell surface staining throughout the study, and only a smaller proportion was transported intracellularly (Fig. 5, A–C, II). After this incubation, the cells were stained again using the same primary antibodies but a different secondary antibody, labeled with another fluorochrome. All cells displayed a strong surface staining (Fig. 5,

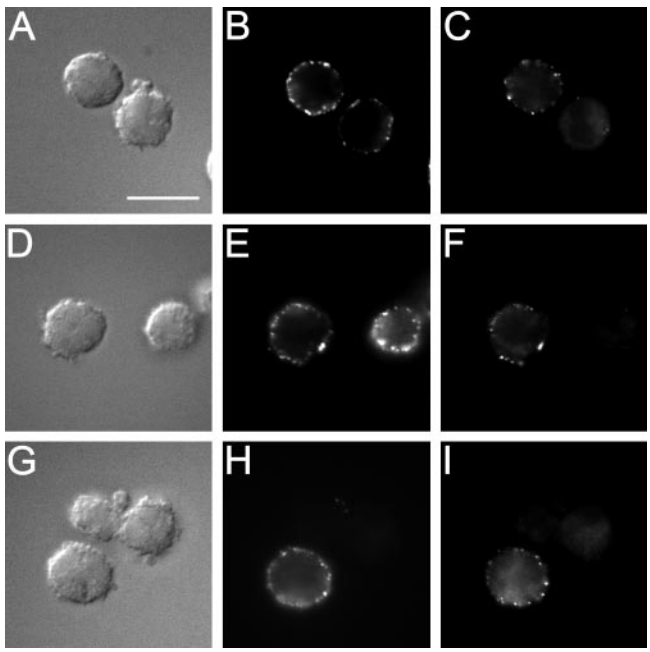

Fig. 2. Plasma membrane expression of CD18, CD16, or CD177 and mPR3. Neutrophils were stained using antibodies directed against CD18 (B), CD16 (E), or CD177 (H) and PR3 (C, F, I). CD18 and CD16 are expressed on the majority of neutrophils; however, the CD177 surface staining is bimodal and coincides with the mPR3 expression. The images are a representative of five experiments. Original size bar = 10 μm.

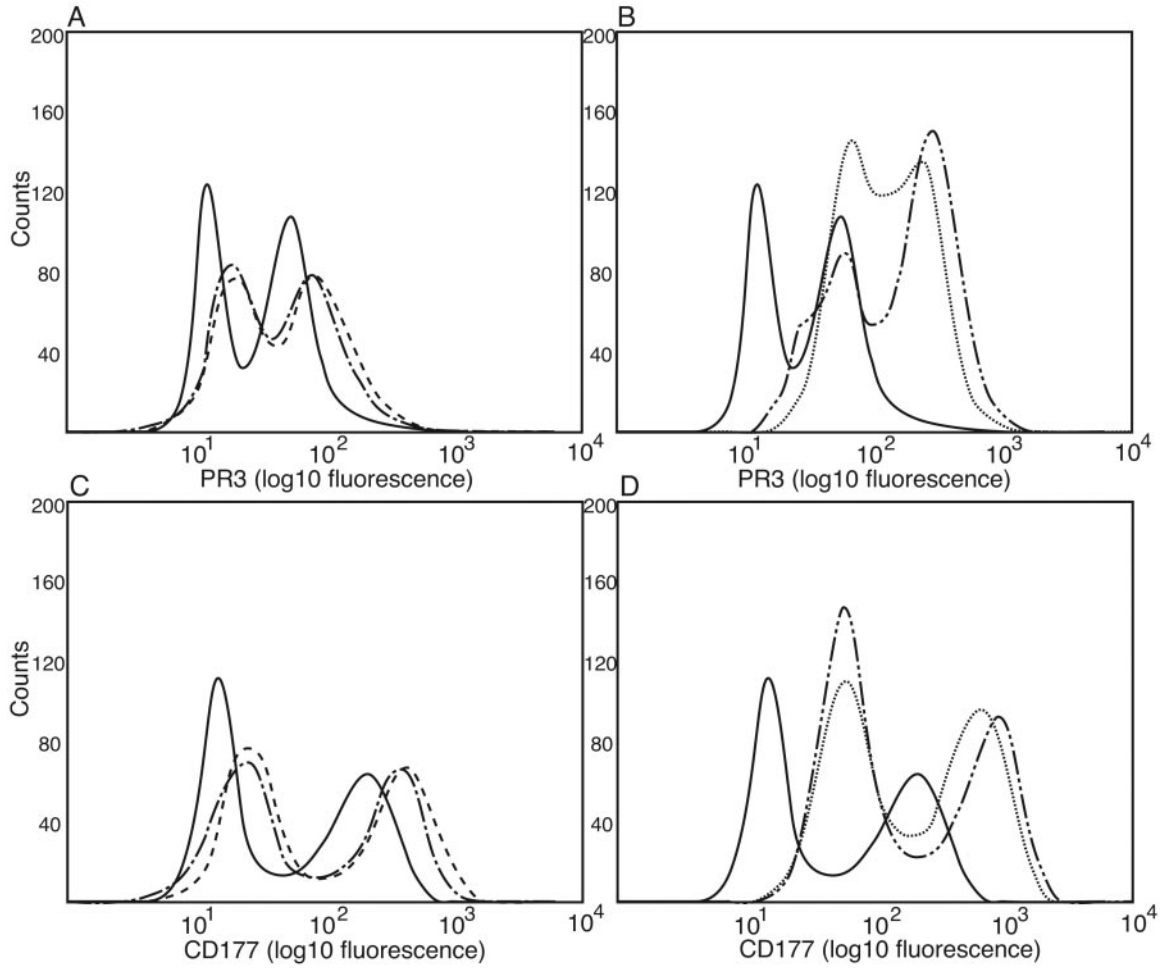

Fig. 3. Up-regulation of mPR3 and plasma membrane CD177. The histograms show the fluorescence intensity of unstimulated neutrophils (——) and neutrophils stimulated with TNF-α (—·—·—), fMLP (— — —), PMA (—··—··—), or CyB in combination with fMLP (·····) at 37°C. (A and B) The mPR3 is shown; (C and D) CD177 staining is shown. This demonstrates one experiment out of four.

III), indicating that new proteins had been transported to the plasma membrane. Samples incubated for 60 min were similar to those incubated for 30 min. FACS analysis of the cells showed that the MFI for the green and red probes was unchanged throughout the experiment (data not shown). This shows that the plasma membrane-bound proteins were internalized and that the amount of proteins on the plasma membrane was constant. Our results demonstrate an active uptake of surface PR3 and CD177 and that new PR3 and CD177 molecules are transported to the surface. These proteins could originate from granules, a recycling compartment, or from de novo synthesis.

To investigate whether de novo synthesis is a major factor contributing to the mPR3 and CD177, which reappeared at the cell surface, as seen in Figure 5, III, we inhibited protein synthesis with cycloheximide, and the cycloheximide treatment did not cause any major difference in the number or the staining of the positive cells.

DISCUSSION

It is well established that circulating human neutrophils have a bimodal distribution of PR3 and CD177 on their plasma membrane. In this study, we show that the two markers are expressed on the same subset of neutrophils. Furthermore, both proteins can be up-regulated in parallel, indicating that they share mechanisms of trafficking and that they originate from the same intracellular storage. When stimulating the cells with PMA or with CyB in combination with fMLP, we show that mPR3- and CD177-negative cells become positive. The increased expression of plasma membrane CD177 follows that of mPR3, and levels of both proteins are increased about five times. The majority of the intracellular PR3 is stored in primary granules, and only smaller amounts are found in other granule types. CD177 is not present in primary granules but is found mainly in secondary granules. Still, both proteins are up-regulated to the same degree after potent stimulation using fMLP in combination with CyB, causing a total degranulation. This suggests that only PR3 stored in secretory vesicles and secondary granules can be expressed on the plasma membrane.

Recently, it has been shown that mPR3 can be coprecipitated with CD18 and CD16 and that mPR3 is localized in lipid rafts on neutrophils [25, 26]. Our data do not exclude that mPR3 colocalizes with CD16 and CD18 and that it, to some degree, is found in lipid rafts (colocalization experiments with CT-B, data not shown). However, mPR3 is also expressed in

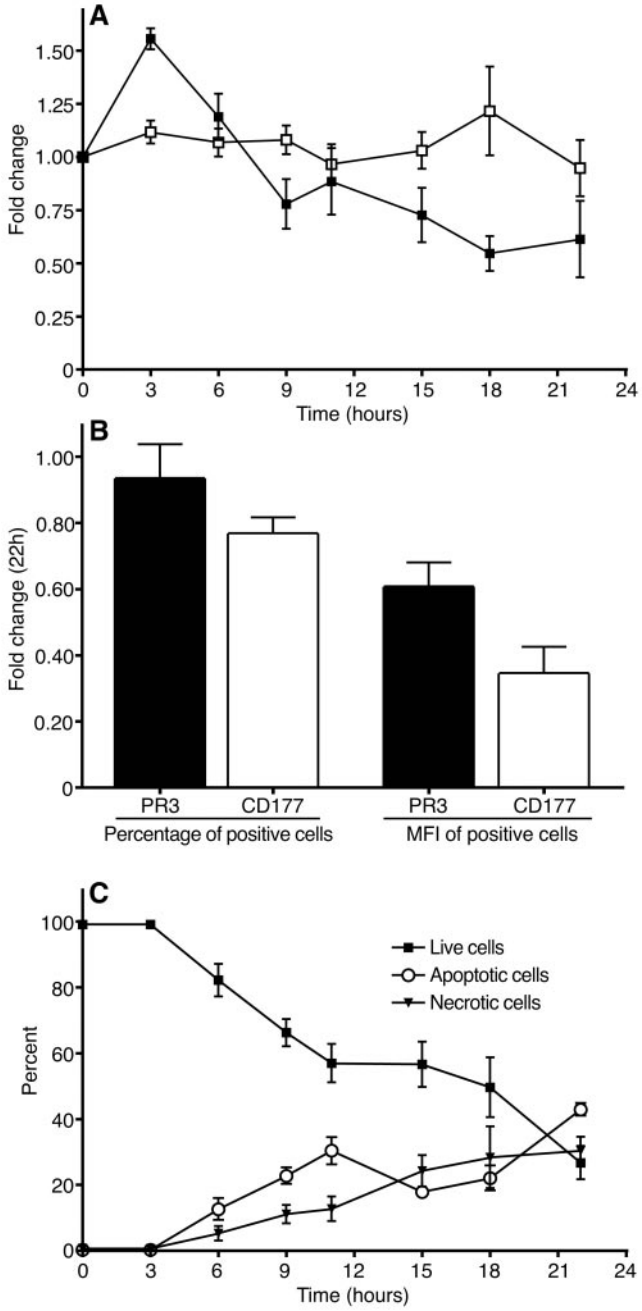

Fig. 4. Apoptosis and plasma membrane expression of CD177 and mPR3. Neutrophils were incubated for 22 h, and mPR3 was measured every 3 h. Plasma membrane CD177 was measured at time-point 0 and after 22 h. (A) The relative percentage of mPR3-positive cells compared with the baseline is indicated (□), and the mean fluorescence intensity (MFI) of the positive cells (■). (B) The relative expression of mPR3 and CD177 is shown. (C) The percentage of live (■), apoptotic (○), and necrotic (▲) cells is shown. All values are given as mean ± SEM. (A and C) Based on eight experiments; (B) based on three experiments.

other areas of the plasma membrane, indicating that there may be other proteins linking PR3 to the plasma membrane. Binding of mPR3 to CD16 and CD18 cannot explain the bimodal expression of mPR3, as they are present on all neutrophils. This leaves us with two possible explanations to the bimodal expression. The first is adaptor proteins, which transport proteins, possibly CD177, and are expressed primarily in mPR3-positive cells. The adaptor proteins are the limiting factor, and the amount of PR3 stored intracellularly is the same in all cells. In the second explanation, the positive cells have stored much more PR3 and CD177 in their secondary and secretory vesicles during the granulopoiesis compared with the negative ones. This indicates common signals regulating the gene expression of the two genes during the later stages of the granulopoiesis. The 4% of the cells expressing only one of the two molecules favors this latter explanation.

Our results show a rapid internalization of PR3 from the surface (Fig. 5), and similar results were obtained in 1985, when it was shown that ANCA was internalized [24]. We now show that mPR3 is replaced constantly and that the amount of mPR3 is constant. As a result of limitations in our methodology, we cannot conclude whether the mPR3 that appears on the cell surface after internalization is recirculated or replaced by PR3 stored in granular structures, e.g., secondary granules. The internalization of mPR3 and CD177 could be antibody-dependent. However, the different kinetics of the internalization of CD18 strongly argues against this. One hour after incubation with antibody, most of the CD18 is still present on the surface. The dynamic trafficking of PR3 in combination with the finding that the MFI of mPR3 is decreasing slowly with the age of the neutrophil (Fig. 4) and the lack of de novo synthesis lead us to propose that mPR3 is recirculating constantly. Part of the recirculating pool of PR3 is lost by storage in granules or by degradation.

The rapid internalization of mPR3 could explain the different results seen when trying to investigate whether PR3-ANCA can bind to mPR3 [36–38]. Even if ANCA does bind to the neutrophils in the circulation, it will not be found on the surface when analyzing the cells but rather, inside the cells. In fact, if the primary and secondary antibodies are not added simultaneously, even a short incubation in RT, e.g., a centrifugation step, will hide the primary antibody inside the cell. Another interesting question that we have not addressed in this investigation is how ANCA affects the function of the neutrophil once internalized. Previous experiments indicate that internalized PR3-ANCA induces apoptosis [39], and further studies about this subject may be crucial to fully understand the pathogenic mechanisms of ANCA on neutrophils.

Elevated levels of the percentage of PR3-positive cells and the MFI of PR3 have been found for neutrophils in patients with ANCA-associated vasculitis. Other results have shown an up-regulation of CD177 in severe bacterial infections [40] and diseases such as PRV. G-CSF and/or GM-CSF are a potential link between PR3 and CD177 expression, as they have been shown to increase the level of expressed mRNA and protein on the plasma membrane for PR3 and CD177 [31, 41], and CD177 is considered to be a marker of increased granulopoiesis, and we speculate that the increased percentage of mPR3-positive cells found in patients with ANCA-associated, systemic vasculitis could be a result of similar mechanisms, i.e., an increased granulopoiesis. Potentially, an increased apoptosis rate could be compensated for by increased G/GM-CSF levels, resulting in normal neutrophil counts but increased levels of mPR3 and CD177 and a larger percentage of positive cells. Another

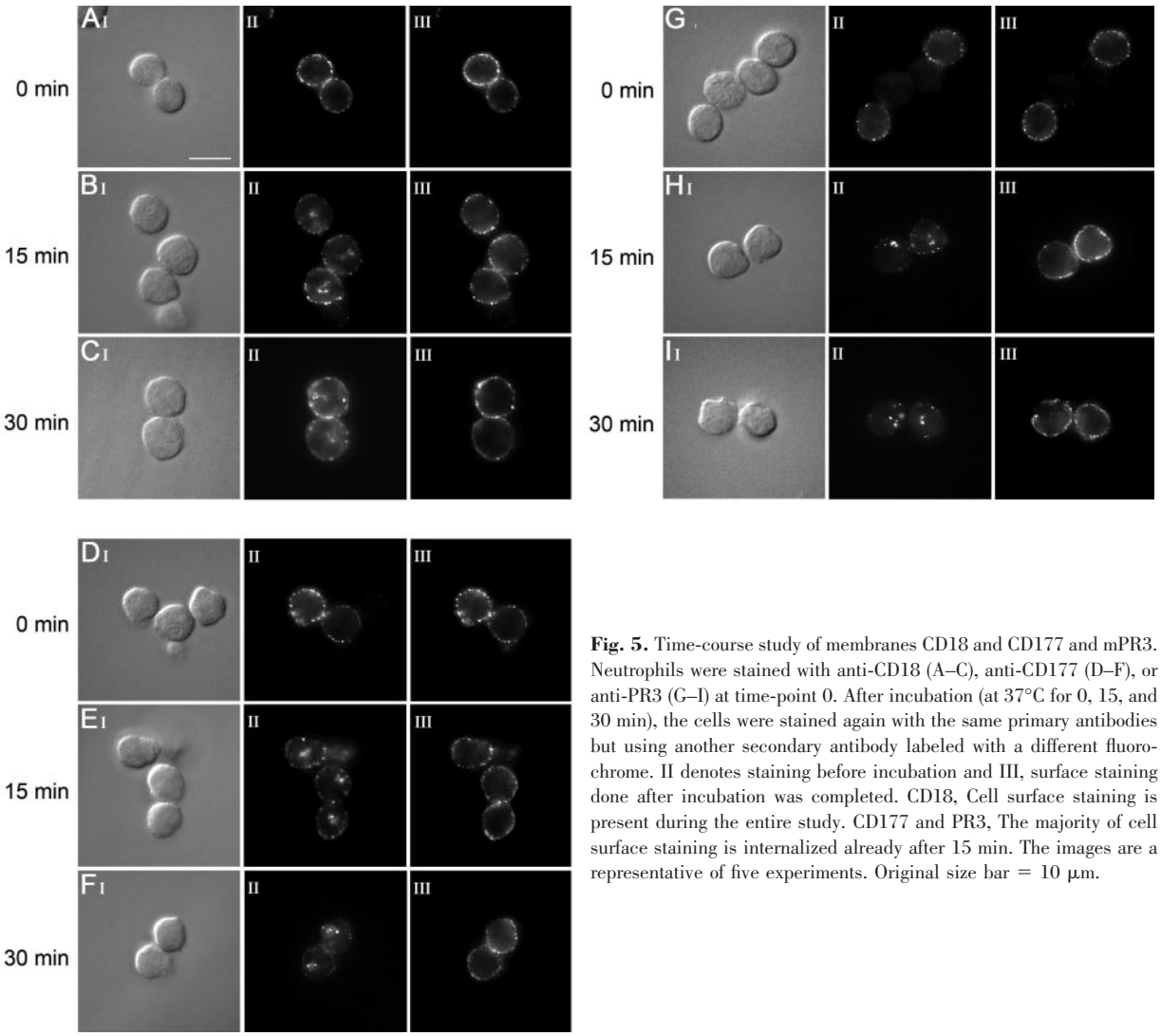

Fig. 5. Time-course study of membranes CD18 and CD177 and mPR3. Neutrophils were stained with anti-CD18 (A–C), anti-CD177 (D–F), or anti-PR3 (G–I) at time-point 0. After incubation (at 37°C for 0, 15, and 30 min), the cells were stained again with the same primary antibodies but using another secondary antibody labeled with a different fluorochrome. II denotes staining before incubation and III, surface staining done after incubation was completed. CD18, Cell surface staining is present during the entire study. CD177 and PR3, The majority of cell surface staining is internalized already after 15 min. The images are a representative of five experiments. Original size bar = 10 μm.

explanation could be a defective signaling pathway in analogy with the JAK2 mutation found in patients with PRV [35].

In this study, we show that PR3 and CD177 are coexpressed on a subset of neutrophils. Furthermore, both proteins are increased in parallel and exhibit a dynamic plasma membrane expression with rapid internalization and re-expression. Elevated levels of plasma mPR3 are linked to ANCA-associated small vessel vasculitis, whereas elevated levels of CD177 are found in diseases with disturbed myelopoiesis. We believe that future studies in this area will lead to a better understanding of the mechanisms underlying ANCA-associated systemic vasculitis and PRV.

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III

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Elevated neutrophil membrane expression of proteinase 3 is dependent upon CD177 expression

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Summary

Proteinase 3 (PR3) is a major autoantigen in anti-neutrophil cytoplasmic antibodies (ANCA)-associated systemic vasculitis (AASV), and the proportion of neutrophils expressing PR3 on their membrane (mPR3$^+$) is increased in AASV. We have shown recently that mPR3 and CD177 are expressed on the same cells in healthy individuals. In this study we try to elucidate mechanisms behind the increased mPR3 expression in AASV and its relationship to CD177. All neutrophils in all individuals were either double-positive or double-negative for mPR3 and CD177. The proportion of double-positive neutrophils was increased significantly in AASV and systemic lupus erythematosus patients. The proportion of mPR3$^+$/CD177$^+$ cells was not correlated to general inflammation, renal function, age, sex, drug treatment and levels of circulating PR3. AASV patients had normal levels of granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor. Pro-PR3 was found to constitute 10% of circulating PR3 but none of the mPR3. We found increased mRNA levels of both PR3 and CD177 in AASV, but they did not correlate with the proportion of double-positive cells. In cells sorted based on membrane expression, CD177–mRNA was several-fold higher in mPR3$^+$ cells. When exogenous PR3 was added to CD177-transfected U937 cells, only CD177$^+$ cells bound PR3 to their membrane. In conclusion, the increased membrane expression of PR3 found in AASV is not linked directly to circulating PR3 or PR3 gene transcription, but is dependent upon CD177 expression and correlated with the transcription of the CD177 gene.

Keywords: anti-neutrophil cytoplasmic antibody (ANCA)/anti-PR3/MPO, flow cytometry/FACS, glomerulonephritis, vasculitis, Wegener's granulomatosis

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Introduction

PR3 was first described in 1973 as an intracellular protein [1], but it is also found in the circulation in complex with its natural inhibitor, α1-anti-trypsin [2]. PR3 belongs to the microbicidal serine proteases, and is stored in azurophilic granules of neutrophils in a mature form. Early during synthesis, some PR3 molecules escape targeting into granules and become secreted as pro-PR3, which has a negative regulatory effect on haematopoiesis [3]. PR3 is also found in secondary granules, secretory vesicles [4] and on the plasma membrane of neutrophils (mPR3), suggesting additional functions for this protein [5]. The mPR3 is accessible for interaction with the immune system and is not inhibited by α1-anti-trypsin [6]. Moreover, mPR3 has a peculiar feature of being expressed on the plasma membrane of only a subset of neutrophils. The existence of two distinct neutrophil subpopulations within one individual is called bimodal membrane expression [7]. For both membrane-bound and circulating PR3, its functional significance as well as its origin remains unexplained. It is unclear to what extent circulating PR3 emanates from pro-PR3, secreted by proliferating neutrophils in the bone marrow, from the granule-stored mature PR3 released by circulating mature neutrophils or if it is released during apoptosis.

CD177 is the only other molecule known to have a bimodal membrane expression on neutrophils. It is a glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein, first described in 1971 as the NB1 antigen and a member of the leucocyte antigen 6 superfamily [8]. The membrane

expression of CD177 is increased during pregnancy [9] and in situations of increased granulopoiesis, such as bacterial infections and burns [10,11]. Several studies have shown that 95–100% of patients with polycythaemia vera (PV) have elevated levels of CD177mRNA [12–14] due to a dominant gain-of-function mutation in the JAK2 gene [15]. CD177mRNA expression in neutrophils is up-regulated in response to administration of granulocyte colony-stimulating factor (G-CSF) to healthy subjects or by stimulation by G-CSF or granulocyte–macrophage colony-stimulating factor (GM-CSF) *in vitro* [12]. It has also been shown that GM-CSF could increase significantly the PR3 membrane expression on neutrophils *in vitro* [16].

Anti-neutrophil cytoplasmic antibodies (ANCA)-associated systemic vasculitis (AASV) is a group of diseases characterized histologically by necrotizing vasculitis affecting small blood vessels and which is often associated with pauci-immune necrotizing crescentic glomerulonephritis [17,18]. Serologically, they are characterized by autoantibodies directed against constituents of neutrophil granules (ANCA) [19–21]. The Chapel Hill international consensus conference defined three major categories of AASV: Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg–Strauss syndrome (CSS) [22]. WG is differentiated from MPA by the presence of necrotizing granulomatous inflammation of the respiratory tract. CSS is differentiated from WG and MPA by the presence of asthma and eosinophilia [23]. In WG, most patients have ANCA with specificity against proteinase 3 (PR3–ANCA), while in MPA and CSS, ANCA is directed most often against myeloperoxidase (MPO–ANCA). Several observations suggest a pathophysiological role of ANCA in AASV [24,25]. However, the mechanisms leading to the production of ANCA are still unknown.

Previous studies have shown higher membrane expression of PR3 and higher plasma PR3 levels in patients with AASV compared to healthy controls [26,27]. In addition, mPR3 has been shown to be co-expressed with CD177 on neutrophils in healthy individuals [28].

In this study, we investigate the mechanisms underlying the elevated plasma PR3 levels, elevated membrane expression of PR3 and the co-expression of mPR3 and CD177 in AASV.

Patients and methods

Patients and controls

Fifty-five AASV were recruited from Department of Nephrology, Lund University Hospital in the period 2006–08. As controls, we recruited 93 healthy blood donors (HBD) from the local blood bank, 20 renal transplant (TP) recipients from the Department of Nephrology, 17 PV patients and one paroxysmal nocturnal haemoglobinuria (PNH) patient from the Department of Haematology, 21 systemic lupus erythematosus (SLE) and 21 rheumatoid arthritis (RA) patients as disease controls from the Department of Rheumatology (Table 1).

Patients were diagnosed as vasculitis and classified into WG or MPA according to the European Medicines Agency (EMEA) algorithm [23]. The Birmingham Vasculitis Activity Score (BVAS) was used to determine the activity of vasculitis [29].

The study was approved by the Regional Ethical Review Board and informed signed consent was obtained from all individuals participated in the study.

Blood sampling and separation

Leucocytes were isolated by centrifugation on Polymorphprep (Axis-Shield, Oslo, Norway). Plasma band was used to measure PR3, pro-PR3, G-CSF and GM-CSF levels. Neutrophil band was used to study membrane and RNA expression.

Membrane expression and flow cytometry

The neutrophil-containing samples were blocked using human immunoglobulin (Ig)G (0·5 mg/ml; European Institute of Science AB, Lund, Sweden) for 20 min on ice. Neutrophils (1×10^6) were single-labelled with a primary antibody, affinity-purified rabbit anti-PR3 (3·3 μg/ml) purified at our nephrology laboratory as described previously [27], mouse-anti-PR3 (4A5, 5 μg/ml; Wieslab, Lund, Sweden), mouse anti-CD177 (1:200; Serotec, Oxford, UK). In addition, 1×10^6 cells were double-labelled with two primary antibodies: rabbit anti-PR3 and mouse anti-CD177.

Table 1. Demographic data and membrane expression of PR3 and CD177.

		AASV						
	HBD	PR3-ANCA	MPO-ANCA	ANCA-neg	PV	TP	SLE	RA
Total no.	93	33	18	4	17	20	21	21
Age years	41 ± 13	61 ± 18	64 ± 13	57 ± 17	61 ± 12	51 ± 11	44 ± 13	63 ± 13
F/M ratio (*n*)	33/57	15/18	11/7	2/2	7/10	7/13	20/1	13/8
Double-membrane expression	58·4%	71·2%**	66·5%	63·5%	60·6%	60·4%	69·9%*	53·2%

*P-value < 0·05; **P-value < 0·01, compared to HBD. All results are expressed as mean ± standard deviation of the mean. AASV, anti-neutrophil cytoplasmic antibodies (ANCA)-associated systemic vasculitis; ANCA-neg, ANCA-negative patients; F, female; HBD, healthy blood donors; M, male; MPO-ANCA, myeloperoxidase-ANCA-positive patients; PR3-ANCA, proteinase 3-ANCA-positive patients; PV, polycythaemia vera; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TP, renal transplant recipients.

For detection of pro-PR3 on the neutrophil membrane, a new antibody was made by immunization of rabbits with a keyhole limpet haemocyanin (KLH)-conjugated peptide from the C-terminal end of the pro-PR3 (CRRVEAKGRP). Peptide synthesis, conjugation, immunization and antibody purification using immobilized peptides was performed by Innovagen AB (Lund, Sweden). After washing, the samples incubated with secondary antibodies goat-anti-rabbit Alexa 488 (1 : 600; Molecular Probes, Eugene, OR, USA) and goat-anti-mouse Alexa647-RPE (1:600; Molecular Probes) for 15 min on ice. These samples were fixed using 2% paraformaldehyde. Fluorescence was measured by fluorescence activated cell sorter (FACS) and neutrophil population was selected by gating for appropriate forward- and side-scatter.

Quantitative polymerase chain reaction (PCR) assay

Total RNA was isolated by RNeasy Mini kit (Qiagen, Gaithersburg, MD, USA), and reverse transcription was performed using the *Taq*Man Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA). The gene expression of PR3, CD177, MPO and interleukin (IL)-8 was determined using quantitative PCR assays on an ABI PRISM 7000 Sequence Detector (Applied Biosystems) with *Taq*Man Universal Master Mix UNG, as described previously [30]. Relative expression was determined by the difference in the Ct values for the target genes after normalization to RNA input level, using Cyclophilin A Ct values. Relative quantification was determined by standard $2^{(-\Delta\Delta\ Ct)}$ calculations [31].

Separation of mPR3-positive and mPR3-negative cells

Neutrophils were isolated from three donors and three patients, labelled with anti-PR3 (monoclonal mouse anti-PR3, 4A5, Wieslab; conjugated with Alexa647, Molecular Probes). For separation of the two subpopulations, mPR3-positive and mPR3-negative, we used FACS Aria flow cytometer equipped with automatic cell deposition unit (BD Biosciences, Immunocytometry Systems, San Jose, CA, USA). After sorting, RNA was isolated and CD177- as well as the PR3-specific cDNA was measured in each subpopulation by real time-PCR.

Cell culture, transfection and addition of exogenous PR3

Human histiocytic lymphoma cells (U937) were stably transfected with CD177–cDNA in a pcDNAvector$_{3\text{-}1}$ or with a negative control vector by electroporation [Gene Pulser II (Bio-Rad); 0·4 cm cuvette; 0·2 kV, 950 μF]. Two clones transfected with CD177–cDNA were selected, one positive for CD177 expression and one negative for CD177 expression. They were cultured for 1 week in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Both clones were then incubated with exogenous PR3 for 2 h on ice (1 μg/ml in each tube). The cells were labelled with anti-PR3 and anti-CD177 antibodies and the fluorescence was measured by FACS.

Measurement of plasma G-CSF by enzyme-linked immunosorbent assay (ELISA)

Plasma G-CSF and GM-CSF were measured using Quantikine® (R&D systems, Abingdon, UK) ELISA Development kits from R&D Systems.

PR3 and pro-PR3

Plasma PR3 level was detected by sandwich ELISA, as described previously [26,27]. For detection of plasma Pro-PR3, a new sandwich ELISA was developed. Briefly, a microtitre plate was coated overnight with new affinity-purified anti-pro-PR3 antibody (2 μg/ml). Plasma samples were added and the plates were incubated for 2 h. After washing, bound pro-PR3 was detected by incubation for 2 h with monoclonal murine anti-PR3 (4A3, 0·5 μg/ml) in sample buffer. After washing, a conjugated anti-mouse antibody (1:2000, alkaline phosphatase-labelled rabbit-anti-mouse IgG; Dako, Glostrup, Denmark) was added and incubated for 1 h. P-nitrophenyl-phosphate disodium (Sigma, St Louis, MO, USA) 1 mg/ml in substrate buffer was used as substrate and incubated with the samples for 30 min. Optical densities were read at 405 nm. A standard curve was produced by incubation of a twofold dilution series of recombinant PR3 containing the C-terminal pro-peptide, starting with 0·25 ng/ml and using the sample buffer as a blank.

Statistical analyses

Differences in continuous variables between two groups were analysed using the unpaired *t*-test and results are given as mean ± standard deviation (s.d.). For data sets that did not follow Gaussian distribution, the Mann–Whitney *U*-test was used and results were given as median and range.

Correlations were analysed using Pearson's rank test and for non-parametric data Spearman's rank test was used. A two-sided $P < 0{\cdot}05$ was considered to be statistically significant.

Results

Demographic data

Fifty-five patients with AASV were included in this study (Table 1). At the time of sampling, 40 patients were in stable remission (BVAS 0-1), 13 moderately active in their disease (BVAS 2-5) and two patients highly active in their disease (BVAS > 5). Twenty-three patients were treated with cyto-

toxic drugs together with steroids, 13 with cytotoxic drugs only, seven with steroids only and 12 patients did not have any form of immunosuppressive treatment.

Membrane expression results

Neutrophils from 223 individuals were analysed for membrane expression of PR3 and CD177. A strong correlation between the percentage of mPR3$^+$ subpopulation and the percentage of CD177$^+$ subpopulation ($r = 0{\cdot}93$, $P < 0{\cdot}0001$, $n = 223$) was observed (Fig. 1). In the patient with PNH, which is characterized by blood cells lacking GPI-anchors, there were fewer than 1% cells positive for CD177 and fewer than 1% cells positive for mPR3. Neither in any specific disease condition nor in any single individual did we find a substantial number of single-positive cells. We concluded that mPR3$^+$ cells are identical to CD177$^+$ cells and defined this subpopulation as double-positive for PR3 and CD177. The mPR3$^+$/CD177$^+$ subpopulation was used as the standard tool for subsequent comparisons and correlations.

We found that the percentage of mPR3$^+$/CD177$^+$ neutrophils was significantly higher in AASV patients (69%, $P = 0{\cdot}0042$) and SLE patients (70%, $P = 0{\cdot}022$) compared to healthy blood donors (HBD, 58%). Meanwhile, PV patients, renal transplant (TP) recipients and rheumatoid arthritis (RA) patients did not show any significant difference in the percentage of mPR3$^+$/CD177$^+$ neutrophils compared to healthy controls (61%, 60% and 53% *versus* 58%, respectively), Fig. 2.

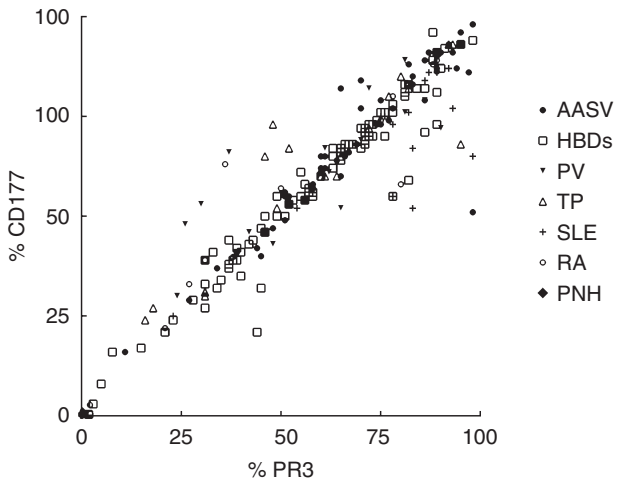

Fig. 1. Correlation between mPR3 and CD177 among all the groups. Shows the results of 91 HBDs, 52 AASV patients, 17 PV patients, 20 TP, 21 SLE patients and 17 RA patients and one patient with PNH. There was a strong correlation between % of mPR3-positive neutrophils and % of CD177-positive neutrophils among all the groups, i.e. they define the same population of neutrophils (mPR3- and CD177-positive population). AASV, anti-neutrophil cytoplasmic antibodies (ANCA)-associated systemic vasculitis; HBD, healthy blood donors; PNH, paroxysmal nocturnal haemoglobinuria; PV, polycythaemia vera; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TP, renal transplant recipients.

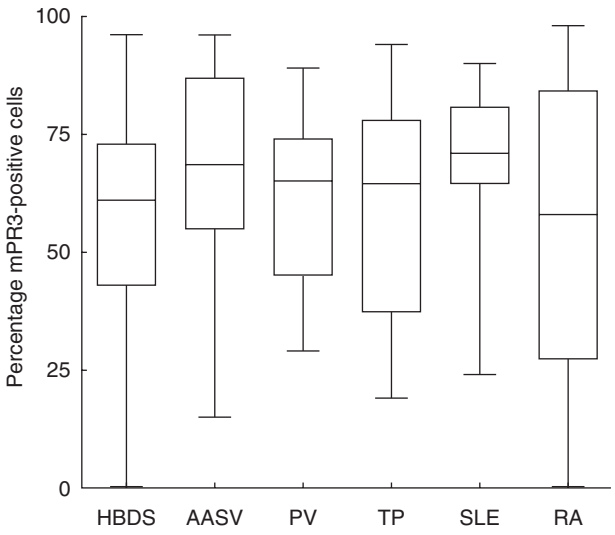

Fig. 2. Double membrane expression. Compares the percentage of the double-positive population of neutrophils (mPR3- and CD177-positive population) among all groups of patients and healthy controls. The percentage of double-positive neutrophils was significantly higher in AASV patients and SLE patients compared to HBD. AASV, anti-neutrophil cytoplasmic antibodies (ANCA)-associated systemic vasculitis; HBD, healthy blood donors; PV, polycythaemia vera; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TP, renal transplant recipients.

Correlation between membrane expression and clinical data

In AASV patients, PR3–ANCA-positive patients had a significantly higher percentage of mPR3$^+$/CD177$^+$ neutrophils compared to HBD (71·2% *versus* 58·4%, $P = 0{\cdot}0044$), while MPO–ANCA-positive patients had non-significantly higher mPR3$^+$/CD177$^+$ cells compared to HBD (66·5% *versus* 58·4%, $P = 0{\cdot}142$). Otherwise, no correlation was found between the percentage of mPR3$^+$/CD177$^+$ neutrophils and clinical data.

Similarly, no difference in percentage of mPR3$^+$/CD177$^+$ neutrophils and current disease status or treatment was observed. There was no correlation with CRP, estimated GFR, cytotoxic drug treatment, steroid dose or BVAS.

Correlation between membrane expression and gene expression

In 115 samples, we measured mRNA levels of PR3, CD177, MPO and IL-8 (Table 2). Data are expressed as calibrated fold change of mRNA; setting mRNA expression of the healthy controls equal to 1.

PR3-mRNA expression was significantly higher in AASV and SLE patients (×2·5 and ×5·4, respectively) compared to healthy controls. The CD177-mRNA expression was signifi-

Table 2. Gene expression of PR3 and CD177.

	HBD	AASV	PV	TP	SLE	RA
n (RNA data)	32	26	13	16	17	21
PR3-mRNA	1	2·5*	1·5	2·0	5·4**	1·3
CD177-mRNA	1	4·5**	26·7***	1·8	6·0***	5·0**
MPO-mRNA	1	2·1**	1·2	1·2	3·2**	0·5*
IL-8-mRNA	1	0·6	0·1**	0·6	1·2	0·8

*P-value < 0·05; **P-value < 0·01; ***P-value < 0·001. All results are expressed as mean. AASV, anti-neutrophil cytoplasmic antibodies (ANCA)-associated systemic vasculitis; HBD, healthy blood donors; PV, polycythaemia vera; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TP, renal transplant recipients.

cantly higher among AASV, SLE and RA patients (4·5, 6·0 and 5·0, respectively) compared to healthy controls and even more elevated among PV patients (×26·7) compared to healthy controls (Table 2).

There was a weak positive correlation between the CD177–mRNA expression and the percentage of mPR3$^+$/CD177$^+$ neutrophils, which was statistically significant only when all samples were pooled together (Spearman's $r = 0·37$, $P < 0·0001$, $n = 115$).

Gene expression of sorted cells

To explore further the relationship between gene transcription in mature neutrophils and membrane PR3 expression, neutrophils were sorted based on their mPR3 expression and their mRNA levels of PR3 and CD177 were measured. We found that PR3-mRNA expression did not differ between mPR3-positive and -negative cells. On the other hand, the median mRNA levels of CD177 was 13 times higher in the mPR3$^+$ cells compared to the negative ones, as shown in Table 3.

U937 cells and exogenous PR3 binding

To correlate CD177 to mPR3-membrane binding, we used U937 cells that normally express low mPR3 but high PR3–mRNA levels. These cells were stably transfected with CD177-cDNA. Of 20 clones, eight clones became stably positive for CD177 surface expression. Surprisingly, all the 20 clones shut down their PR3-mRNA expression and neither the protein (measured by immunoblotting and FACS) nor the mRNA expression (measured by real-time PCR) could be detected. Sixteen of the 20 mock-transfected clones continued to express PR3.

Two clones transfected with CD177–cDNA were selected, one positive for CD177-membrane expression and one negative. These two clones were incubated with exogenous PR3. As shown in Fig. 3, only cells expressing CD177 on their membrane bound PR3.

G-CSF and GM-CSF in the plasma

We measured the plasma levels G-CSF and GM-CSF because they have been shown to increase the membrane expression of mPR3 and the mRNA expression of CD177 [12,16].

There was no significant difference between the groups regarding their plasma levels of G-CSF, and most of the samples were within normal or slightly higher than the normal plasma levels (normal range 2·2–30·9 pg/ml).

GM-CSF levels were within the normal range (< 2 pg/ml) for the majority of the samples. Elevated levels were found in four AASV patients (median 484·2, range 7·7–3135 pg/ml), eight RA patients (54·7, 11–178 pg/ml), one PV patient (23 pg/ml) and one SLE patient (42·7 pg/ml). However, there was no correlation between plasma levels of G-CSF or GM-CSF and size of the mPR3$^+$/CD177$^+$ subpopulation.

Table 3. Gene expression of sorted cells.

	PR3-RNA levels			CD177-RNA levels		
	mPR3$^+$ cells	mPR3$^-$ cells	PR3-index	mPR3$^+$ cells	mPR3$^-$ cells	CD177-index
Patient no 1	0·22	0·23	0·96	57·81	0·8	72·3
Patient no 2	0·01	1·34	0·007	24·2	9·19	2·63
Patient no 3	0·03	0·39	0·077	308	15·42	19·9
HC no 1	0·11	0·01	11	37·44	0·26	141
HC no 2	0·01	0·01	1	0·2	0·08	2·5
HC no 3	0·02	0·04	0·5	6·08	0·92	6·6
Median	0·025	0·13	0·73	30·8	0·86	13·3

All results are expressed as calibrated fold change compared to standard RNA; HC, healthy control; PR3-index, the index between PR3-RNA levels in mPR3-positive cells and PR3-RNA levels in mPR3-negative cells; CD177-index, the index between CD177-RNA levels in mPR3-positive cells and CD177-RNA levels in mPR3-negative cells.

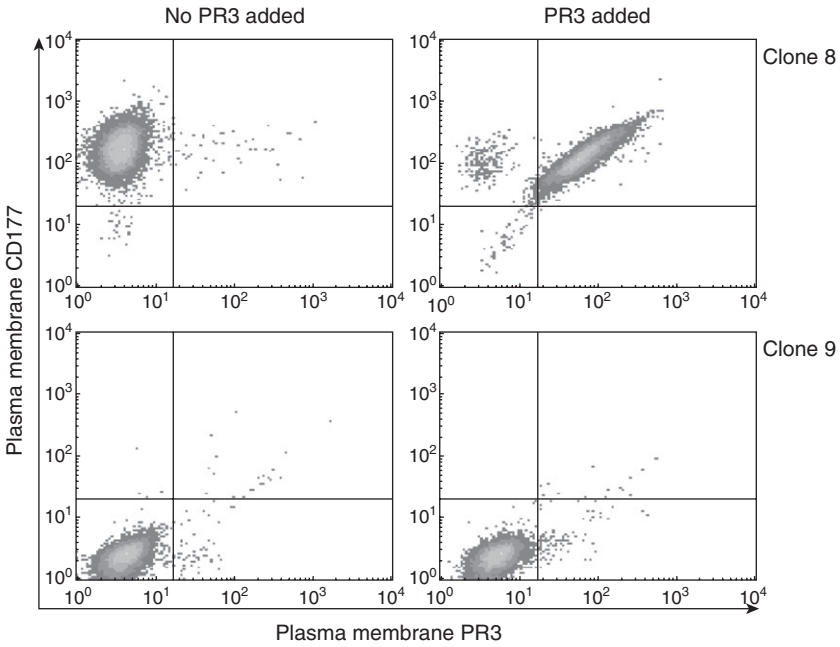

Fig. 3. U937 and exogenous PR3 binding. The left panel shows the membrane expression of U937 clone 8 cells (express CD177 but not proteinase 3 (PR3) on their plasma membrane), and U937 clone 9 cells clone (do not express PR3 or CD177 on their plasma membrane), measured by fluorescence activated cell sorter analysis. In the right panel, membrane expression of PR3 and CD177 was measured again on the same cells after incubation with mature PR3 for 2 h. Clone 8 cells expressed the PR3 on their plasma membrane (upper right), while clone 9 cells did not express any PR3 or CD177 on their membranes (lower right).

Pro-PR3 and PR3

Looking for the source of the circulating PR3, we measured the mature form of PR3 as well as the pro-PR3 in plasma. Total plasma PR3 was elevated significantly in AASV patients (median 148, range 30–2553 μg/l, $n = 49$) compared to healthy controls (84, 38–246 μg/l, $n = 63$) (Mann–Whitney *U*-test, $P < 0{\cdot}0001$). Using a pro-PR3 specific anti-serum we found that plasma pro-PR3 constitute only a small fraction of the total plasma PR3, in both AASV patients and in healthy controls (10% each). Accordingly, pro-PR3 levels were higher in AASV patients (12, 6–184 μg/l) compared to healthy controls (7, 4–28 μg/l) ($P < 0{\cdot}0001$). When correlating levels of total PR3, mature PR3 and pro-PR3 to the percentage of mPR3$^+$/CD177$^+$ neutrophils, no significant correlation was found. The new pro-PR3 specific anti-serum did not bind to mPR3, indicating that the mPR3 is mature PR3 (data not shown).

Discussion

In this study, we show that mPR3 and CD177 are also co-expressed on the plasma membrane of neutrophils in all individuals under pathological conditions. In no subgroup including AASV, PV, SLE and RA patients, as well as renal transplant recipients, did we find evidence for any significant amounts of single-positive cells (Fig. 1). Moreover, the mPR3$^+$/CD177$^+$ neutrophil subpopulation was larger in AASV and SLE patients compared to other diseases and healthy controls, suggesting a specific distinct cause of this phenomenon and raising several questions regarding the pathophysiological significance and origin of this subpopulation. Is this a result of increased plasma levels of specific cytokines, or could it be due to increased production of one of the two proteins on mRNA level in mature neutrophils?

First, there could have been several relatively trivial reasons for the increase of mPR3$^+$/CD177$^+$ cells, such as general inflammatory activity, reduced renal function or specific drug therapy. To address these possibilities, we correlated the mPR3$^+$/CD177$^+$ cells with clinical data from AASV patients. According to our results, the elevated percentage of mPR3$^+$/CD177$^+$ cells found in AASV patients does not seem to be due to treatment, general inflammation or renal failure.

Even though there was no correlation with either CRP or BVAS, ruling out that the elevation of mPR3$^+$/CD177$^+$ cells could be caused by general inflammation, it could still be mediated by some specific set of cytokines. Human blood plasma comprises a very effector-enriched environment for the neutrophils [32]. The search for external factors that are responsible for the elevated mPR3$^+$/CD177$^+$ cells in vasculitis patients is not easy. We focused upon two cytokines in the plasma, G-CSF and GM-CSF. We found that only GM-CSF levels were elevated in four AASV patients. Although this may explain the increased percentage of mPR3$^+$/CD177$^+$ neutrophils in these four patients, it does not explain it in the remaining 51 AASV patients. Thus, these experiments do not explain if the elevated mPR3$^+$/CD177$^+$ cells are due to external stimuli, a genetic predisposition to develop the disease, or if it is a reflection of a disease-specific defect in the neutrophils.

One explanation that could account for an increased number of mPR3$^+$/CD177$^+$ neutrophils is a continued production of PR3 and/or CD177 on a gene transcription level in mature neutrophils. To determine if this was the case, we measured the mRNA expression by *Taq*Man real-time PCR in neutrophils. The up-regulated PR3-mRNA levels

described previously in AASV patients [33] was reconfirmed. Furthermore, we found significantly higher mRNA expression of CD177 among AASV, PV, SLE and RA patients compared to HBD, as shown in Table 2.

From the finding that CD177–mRNA expression, but not PR3–mRNA expression, correlates with the percentage of $mPR3^+/CD177^+$ cells we concluded that the underlying mechanism behind the shift of the neutrophil subpopulation towards an $mPR3^+/CD177^+$ phenotype could be linked to an over-production of CD177 by mature human neutrophils. However, increases only in CD177 gene transcription is not sufficient to achieve an increase in the percentage of double-positive cells, as the PV patients who had a very high CD177–mRNA expression ($\times 26{\cdot}7$) did not exhibit a significantly increased proportion of $mPR3^+/CD177^+$ cells compared to healthy controls.

When we sorted the human neutrophils into two groups according to their mPR3 expression, the PR3 mRNA level did not differ between the mPR3-positive and -negative cells. However, the mRNA level of CD177 was significantly higher ($\times 13$) in the positive group compared to the negative one. Similar results for CD177 have been shown previously [34]. In order to show the importance of CD177 on protein level for the mPR3 expression we transfected U937 cells with CD177 cDNA. The results showed that only cells expressing CD177 on their membranes were able to bind exogenously added PR3. These two experiments, together with the correlations discussed above, suggest clearly that CD177 expression on the plasma membrane is responsible for the bimodal expression pattern of mPR3. In other words, CD177 seems to be a prerequisite for PR3 to be expressed on the plasma membrane of neutrophils.

von Vietinghoff *et al*. has shown a direct physical binding between PR3 and CD177 (NB-1) [35]. Despite several attempts, we have not been able to reproduce their results at our laboratory, and hence cannot share their conclusion that CD177 is a neutrophil membrane receptor for PR3. Saying that CD177 is a prerequisite for mPR3 expression with no evidence for direct physical binding is contradictory, but could be explained if CD177 assist PR3 to bind directly to the membrane or to another membrane protein, but it does not stay attached afterwards. A similar theory has been postulated previously for the relation between the soluble endothelial protein C receptor (sEPCR) and PR3, where sEPCR attaches more effectively to the neutrophil membrane in the presence of mPR3 [36]. Another example is the relationship between klotho and FGF23 in renal cells, where klotho is essential for the binding of FGF23 to its receptor in a specific and high-affinity manner [37].

Recently, it has been shown by Hajjar *et al*., via computational simulation, that PR3 binding to the membrane depends partly upon electrostatic and hydrophobic interactions that keep the PR3 stably inserted into the lipid bilayer structure of the membrane, without the need of another molecule [38]. This theory does not explain why PR3 is expressed on only a subset of neutrophils, even though they have similar membrane structure and contain equal amounts of intracellular PR3 protein [39].

A recent study by Hu *et al*. has shown similar results to ours. However, they show that ANCA-induced activation of neutrophils is independent of CD177/mPR3 positivity. Cells that are CD177-negative become mPR3-positive after tumour necrosis factor (TNF)-α priming and are equally stimulated with ANCA as CD177-positive cells [40].

The origin of the mPR3 and the PR3 found in plasma is not known; nor is it known if the mPR3 or the plasma PR3 is in a pro-form or mature protein. We have measured the amounts of circulating plasma PR3 previously and found it to be elevated in AASV patients compared to healthy controls [27]. This finding was verified in this study. To be able to measure the pro-PR3 we had to develop a new antibody recognizing the pro-form only. The new ELISA based on this new anti-pro-PR3 antibody showed that the pro-PR3 was also elevated in our AASV patients compared to healthy blood donors. However, the proportion between the pro-PR3 and total PR3 was, on average, 10% and did not differ significantly between AASV patients and HBD. There could be several different explanations for these findings: the elevated levels of the pro-PR3 could indicate an increased production of neutrophils in the bone marrow or possibly reflect an increased synthesis of PR3 in mature cells; or the elevated levels of mature PR3 could reflect an increased degranulation upon activation or release of granular content as a consequence of necrosis. Arguing against the degranulation theory is the fact that there is no correlation between plasma PR3 and neutrophil gelatinase-associated lipocalin (NGAL), a marker of secondary granules [27]. None the less, the anti-pro-PR3 antibody did not recognize the plasma membrane-bound PR3, indicating that mPR3 is not the pro-form of PR3 but mature PR3.

To conclude, in this study we show that mature PR3 and CD177 are co-expressed on the plasma membrane of neutrophils in all individuals including AASV patients. The $mPR3^+/CD177^+$ phenotype as well as mRNA expression of PR3 and CD177 are increased in neutrophils from patients with AASV and SLE, while in PV patients only the mRNA expression of CD177 is increased. The increased levels of PR3 and CD177 are not related to plasma levels of G-CSF or GM-CSF. We also show that PR3 depends upon CD177 for its membrane expression. Thus, further studies are needed to reveal the potential factors leading to this over-expression of PR3 and CD177 in AASV patients. Understanding the PR3–CD177 interaction may improve our knowledge of the pathophysiology of AASV and thereby facilitate the search for better treatment modalities for this serious and devastating illness.

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Disclosure

None.

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IV

Decreased neutrophil apoptosis in ANCA-Associated Systemic Vasculitis

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Abstract

Background ANCA-Associated Systemic Vasculitis (AASV) is characterized by leukocytoclasis with infiltration and accumulation of unscavenged apoptotic and necrotic neutrophils in tissues surrounding vessels and fibrinoid necrosis of the vessel walls. Dysregulation of neutrophil apoptosis may contribute directly to the pathogenesis of AASV.

Methods Neutrophils from Healthy Blood Donors (HBD) and patients with AASV, Polycythemia Vera (PV), Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA) and renal transplant recipients (TP) were incubated *in vitro*, and the rate of spontaneous apoptosis was measured by FACS. Plasma levels of cytokines were measured with cytometric bead array and ELISA. Expression of pro/anti-apoptotic factors as well as transcription factors C/EBP-α, C/EBP-β and PU.1 was measured by real-time PCR.

Results AASV, PV and RA neutrophils had a significantly lower rate of apoptosis compared to HBD neutrophils (AASV 50±14% vs. HBD 64±11%, $p < 0.0001$). In RA but not in AASV and PV, low apoptosis rate was correlated with increased plasma levels of GM-CSF and high mRNA levels of the anti-apoptotic factors Bcl-2A1 and Mcl-1. The majority of AASV patients had normal plasma levels of G-CSF, GM-CSF and IL-3. However, both C/EBP-α, C/EBP-β were significantly higher in neutrophils from AASV patients than in neutrophils from HBD, while in PV neutrophils the levels were significantly decreased.

Conclusion Spontaneous neutrophil apoptosis rates in vitro are decreased in AASV, RA and PV but mechanisms seems to differ. Only in AASV patients we found increased mRNA levels of the granulopoiesis associated transcription factors C/EBP-α, C/EBP-β and PU.1. Additional studies are required to define the mechanisms behind decreased apoptosis rates in AASV, and to elucidate the possible connection with the accumulation of dying neutrophils in regions of inflammation in AASV patients.

Introduction

Neutrophil apoptosis may play a central role in the pathogenesis of Anti-Neutrophil Cytoplasmic Antibody (ANCA)-Associated Systemic Vasculitis (AASV) and production of ANCA[1-6]. Activated, apoptotic and necrotic neutrophils are present in histological samples from patients with Wegener's granulomatosis (WG) with respiratory disease[7]. Pathologically, AASV is characterized by leukocytoclasis with infiltration and accumulation of unscavenged apoptotic or necrotic neutrophils in tissues surrounding vessels and fibrinoid necrosis of the vessel walls[8, 9]. Moreover, injection of brown Norway rats with syngenic apoptotic neutrophils induced ANCA but not AASV, suggesting that additional environmental and/or genetic factors contribute to AASV and are required for disease onset[10].

Previous studies have shown that plasma PR3 is present at higher levels in patients with AASV than in Healthy Blood Donors (HBD)[11, 12]. Furthermore, membrane PR3-positive (mPR3$^+$) neutrophils are more abundant in individuals with AASV, Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA)[13-16].

Although previous studies suggest that PR3 plays a role in neutrophil apoptosis, the nature and mechanism of this putative role is not known. PR3 may be mobilized to the plasma membrane during neutrophil apoptosis in the absence of prior neutrophil priming and independent of degranulation[17]. Kantari et al demonstrated that Phospho-Lipid scramblase-1 (PLSCR-1) interacts with PR3 and promotes its translocation to the plasma membrane in a flip-flop manner during apoptosis[18]. However, the level of mPR3 is similar in apoptotic and non-apoptotic primed neutrophils, implying that the mPR3 on apoptotic neutrophils may be a result of minor trauma during neutrophil isolation[19]. Our group also found that the level of mPR3 was similar before and after apoptosis[20].

Yang et al demonstrated that PR3 can trigger cultured endothelial cell apoptosis, although the exact mechanism was not defined[21]. PR3 activates procaspase-3 into a specific 22-kDa fragment localized to the plasma membrane-enriched compartment, and segregated from its target cytosolic proteins that promote apoptosis, thus causing activation but not apoptosis[22].

Vong et al showed that recombinant PR3 or the membrane fraction of cells stably-transfected with PR3 can cleave Annexin-A1 (AnxA1), suggesting that AnxA1 may be a physiologically relevant substrate for PR3[23]. AnxA1, though originally identified as an enodogenous anti-inflammatory protein, was recently recognized as an important inducer or promotor of neutrophil apoptosis. This effect of AnxA1 is receptor-mediated, is specific for AnxA1, and is not shared by Annexin-V[24]. While the former studies suggest the PR3 as a pro-apoptotic factor, the latter suggests it as an anti-apoptotic factor.

Harper et al examined the kinetics of neutrophil apoptosis in AASV during 18 h *in vitro* culture, and observed faster apoptosis in neutrophils from patients with active vasculitis than in neutrophils from patients with quiescent vasculitis or from HBD. Neutrophils from

patients with active vasculitis also had higher levels of mPR3 and higher levels of superoxide, suggesting that superoxide may promote accelerated apoptosis in these cells[25].

Because PR3 is elevated in AASV patients and linked to neutrophil apoptosis, we hypothesize that the neutrophil apoptosis in AASV might be dysregulated.

In this study, the rates of spontaneous *in vitro* apoptosis were compared in neutrophils from HBD, transplant recipients (TP) and individuals with AASV, Polycythemia Vera (PV), SLE and RA. The relationship between these data and clinical data for AASV patients was examined, and expression of anti-apoptotic and pro-apoptotic factors as well as transcription factors was quantified in neutrophils from AASV patients. Plasma levels of neutrophil growth factors, G-CSF, GM-CSF and IL-3 were also quantified in HBD and individuals with AASV, PV, SLE and RA.

Patients and Methods

Patients

During the period between September 2006 and February 2008, 44 AASV patients from the Department of Nephrology, Lund University Hospital were recruited into the current study. Patients diagnosed with AASV were classified as WG or MPA using the European Medicines Agency (EMEA) algorithm[26]. The vasculitis activity status of all patients was determined using the Birmingham Vasculitis Activity Score (BVAS)[27]. AASV patients were receiving the following treatments at the time of sampling: 21 patients, cytotoxic drugs and steroids; 10 patients, cytotoxic drugs; 5 patients, steroids; 8 patients, no treatment (Table 1).

Additional study participants included HBD from the local blood bank, TP recipients from the Department of Nephrology, PV patients from the Department of Haematology, SLE and RA patients from the Department of Rheumatology, all at Lund University Hospital (Table 2).

This study was approved by the Regional Ethical Review Board and performed in accordance with the Declaration of Helsinki. Informed signed consent was obtained from all study participants.

Blood Sampling and separation

Leukocytes were isolated by centrifugation on Polymorphprep (Axis- Shield, Oslo, Norway). Plasma band was used to measure levels of different cytokines. The neutrophil band was used to study neutrophil survival, apoptosis and necrosis by FACS and to extract RNA for real time PCR.

Neutrophil *in vitro* culture and FACS

Isolated neutrophils were incubated at 37° C in AIM-V medium for 20 h. An aliquot (10^6 neutrophils) was taken and incubated for 5 min in the dark with 1 μl Annexin-V (marker of apoptosis from Invitrogen, Molecular probes, Oregon, USA) and 10 μl 7-AAD (marker of necrosis from BD-Biosciences, San Jose, CA, USA). Neutrophils were then analyzed by

flow cytometry using BD FACSCanto II (BD Pharmingen, CA, USA) to report % of apoptotic, necrotic or alive cells after 20 h of *in vitro* culture.

RNA extraction

Total RNA was extracted with RNeasy Mini kit (Qiagen, VWR International, West Chester, PA, USA) according to the manufacturer's protocol. RNA purity was evaluated by spectrophotometric analysis using NanoDrop (Saveen& Werner, Malmö, Sweden).

Quantitative PCR assay

cDNA was prepared from total RNA using *Taq*Man Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, reverse transcription was performed using random hexamers, MultiScribe reverse transcriptase, RNase inhibitor, dNTPs, 5.5 mM $MgCl_2$, reverse transcription buffer, and 200 ng total RNA in a volume of 50 μl. The reaction cycle was 25°/10 min, 48°/30 min and 95°/5 min. Quantitative PCR assays were performed in an ABI PRISM 7000 Sequence Detector (Applied Biosystems, CA, USA) with *Taq*Man Universal Master Mix UNG under standard conditions. Assay on Demand provided a unique combination of forward and reverse primers and fluorescent MGB-probes for each target gene (Bax, Mcl-1, Bcl-2A1, c-IAP2, C/EBP-α, C/EBP-β and PU.1). Cyclophilin A expression was used as an internal control for data normalization. Each 25 μl reaction contained the amount of cDNA produced from 10 ng RNA. All reactions were performed in triplicates. Q-PCR data were analyzed using the ΔΔCt method with normalization to Cyclophilin A and standard $2^{(-\Delta\Delta Ct)}$ calculations[28].

Measurement of neutrophil growth factors in plasma by ELISA.

Plasma G-CSF, GM-CSF and IL-3 were measured with the Quantikine® ELISA Development Kit (R&D systems, Abingdon, UK), which was used according to the manufacturer's protocol.

Neutrophil hypersensitivity to plasma and growth factors

Neutrophils isolated from AASV patients or HBD were incubated at 37° C in AIM-V medium for 20 h, with/without plasma (20%) derived from other AASV patients or other HBD. Neutrophils isolated from AASV patients or HBD were also incubated under similar conditions with or without neutrophil growth factors (recombinant G-CSF, GM-CSF and IL-3 from R&D systems, Abingdon, UK) at a concentration of 0.2 ng/ml.

Measurement of neutrophil survival factors in plasma by Cytometric Bead Array (CBA)

G-CSF, GM-CSF, IL-3, TNF-α, IFN-γ, IL-1β, IL-2, Il-4, IL-6 and IL-8 were simultaneously analyzed in 50 μl plasma by flow cytometry using the BD CBA Human Soluble Protein Flex Set system (BD Pharmingen, CA, USA) according to the manufactures instructions.

Statistical Analysis

For continuous variables, an unpaired t-test was used to measure statistical significance of differences between two groups. Results are presented as mean± SD. For data sets that follow a non-Gaussian distribution, statistical significance was measured using a Mann Whitney test. Results are presented as median and range.

One-way ANOVA with Bonferroni's post-test was used to compare data from more than two groups. Correlations were analyzed using Pearson rank test. Spearman rank test was used for non-parametric data. A two-sided $p<0.05$ was considered to be statistically significant.

Results

Neutrophil apoptosis and necrosis in vitro

Apoptosis, necrosis and survival of neutrophils from 44 patients with AASV, 93 HBD, 20 TP recipients, 17 PV, 21 SLE and 21 RA patients was quantified after 20 h in culture as described in the method section. The results showed a significantly higher rate of survival (mean ±SD 34±13% vs. 23± 9%, $p< 0.0001$; Figure 1a) and lower rate of apoptosis (50±14 % vs. 64±11 %, $p< 0.0001$; Figure 1b) in AASV neutrophils compared to neutrophils from HBD. Similar results were obtained when examining neutrophils from RA and PV patients, with survival rates of 31±13 % and 49±15 % ($p= 0.015$ and $p< 0.0001$), and apoptosis rates of 57± 12 % and 41± 14 % ($p= 0.027$ and $p< 0.0001$). For necrosis there was no significant difference between neutrophils from AASV, PV and RA neutrophils compared to HBD neutrophils (data not shown).

No significant difference in rates of survival, apoptosis or necrosis was found when comparing neutrophils from SLE and TP patients with HBD neutrophils.

Correlation between neutrophil apoptosis and clinical data

To ascertain that the reduced rate of apoptosis was not a consequence of gender, age, renal function, general inflammation or treatment, apoptosis rates were correlated with clinical data for the AASV patients. No significant correlations were found. Men and women had similar rates (49% vs. 51%; $p=0.5$) and apoptosis rates did not increase with age ($r=0.06$, $p=0.7$). There was no correlation with serum creatinine concentrations ($r=-0.13$, $p=0.4$) or estimated GFR ($r=0.1$, $p=0.5$). In a similar fashion we found no specific effect of disease activity; patients in remission had comparable rates as patients with moderate (BVAS 2-5) or high vasculitic activity (BVAS >5), (51% and 52% vs. 28%; $p=0.09$), and there was no correlation with CRP ($r=-0.11$, $p=0.5$). Similar results were observed for patients with WG or MPA (49% vs. 53%; $p=0.4$).

Ongoing treatment with steroids did not seem to influence the measured rate of apoptosis, as there was no correlation with the dose of prednisolone ($r=-0.11$, $p=0.5$). For antiproliferative drugs the situation was somewhat more complex, the 27 AASV patient who were on treatment tended to have lower apoptosis rates as compared to the 8 patients who

were off all kinds immunosuppressive treatment (47% vs. 57%; p=0.02), with no obvious difference between drugs. The 10 methotrexate treated patients had a mean rate of 46%, compared with 47% for 10 patients on azathioprine and 47% for 7 patients on mycophenolate mofetil (p=0.94); contrasting to this 11 renal transplant recipients on mycophenolate mofetil had a mean apoptosis rate of 57%.

Response of neutrophils to plasma

In order to explore the mechanism that lowers the apoptosis rate in neutrophils from AASV patients, we studied the effect of plasma on neutrophil survival. Neutrophils from HBD and AASV patients were incubated with plasma from HBD, RA or AASV patients prior to FACS analysis to determine cell survival. Incubation in plasma increased neutrophil survival, and decreased differences between HBD and AASV neutrophils. After incubating neutrophils from 6 AASV and 4 HBD patients with plasma from HBD, there was a difference in outcome; AASV patients still had more surviving cells after 20h (66% vs 56%). However, when the neutrophils were incubated with plasma from AASV or RA patients, neutrophil survival was enhanced, and there was no longer any difference between cells from AASV patients and HBD (72% vs 71% in AASV-plasma and 72% vs 70% in RA-plasma), Figure 2.

Measurement of neutrophil growth factors in plasma

Because cytokines can prolong neutrophil survival, the plasma level of an array of cytokines was compared in HBD and AASV patients. G-CSF, GM-CSF, IL-3, TNF-α, IFN-γ, IL-1β, IL-2, Il-4, IL-6 and IL-8 were measured in plasma from 40 AASV and 40 HBD individuals using Cytometric Bead Analysis (CBA).

IFN-γ levels were undetectable in plasma of HBD, while 5 AASV patients had elevated levels of IFN-γ in their plasma (range 8-25 pg/ml, median 19 pg/ml). G-CSF and GM-CSF were also elevated in some AASV plasma samples. For all other cytokines examined here, there were no significant difference between HBD and AASV.

As G-CSF, GM-CSF and IL-3 are the main cytokines in human plasma that are known to promote survival of human neutrophils, they were also quantified using ELISA. GM-CSF was measured in the plasma from all patients and controls. It was < 2 pg/ml in the majority of samples, which is within the expected normal range for healthy individuals. GM-CSF was elevated in plasma samples from one PV patient (23 pg/ml), one SLE patient (42.7 pg/ml), 8 RA patients (median 54.7, range 11-178 pg/ml) and 4 AASV patients (484.2, 7.7-3135 pg/ml).

G-CSF was measured in plasma from 10 HBD, 10 AASV, 5 PV, 5 TP, 5 SLE and 5 RA patients. The results showed that G-CSF was higher in AASV patients than in HBD (37 pg/ml vs 30, p=0.29). However, there was no correlation between the plasma levels of G-CSF and the rates of neutrophil survival, apoptosis and necrosis in these patients (Figure 3).

IL-3 was below the detection limit in all tested samples.

When pooling results from all groups of patients and controls there was no significant correlation between the plasma levels of G-CSF, GM-CSF or IL-3 and the rates of neutrophil survival, apoptosis or necrosis. Additional analysis was performed after stratifying RA and AASV patients according to relative plasma GM-CSF levels (i.e., high and low GM-CSF groups). For RA patients, the 8 patients with high GM-CSF had higher survival rate and significantly lower apoptosis rate than RA patients with low GM-CSF (37% vs 27% survival rate, p=0.09 and 51% vs 61% apoptosis rate, p=0.034) (Figure 4). For AASV patients with high GM-CSF (n=4 out of 44), the survival rate was only marginally higher than the AASV group with low plasma GM-CSF and the difference was not statistically significant (38% ss 34%, p=0.5).

Sensitivity of neutrophils to growth factors

A possible explanation for prolonged neutrophil survival in AASV patients in the absence of increased levels of growth factors in the plasma is that neutrophils are hypersensitive to low or normal levels of growth factors. This notion was tested by incubating neutrophils in culture medium with or without exogenous growth factors (G-CSF, GM-CSF or IL-3).

Neutrophils from 6 AASV and 4 HBD individuals were tested. The experiment was done with 0.2 ng/ml of GM-CSF, IL-3 or G-CSF. Generally, there was no significant elevation in average neutrophil survival, after incubation with G-CSF, GM-CSF or IL-3, as shown in Table 3. However, when individual results were examined, exogenous GM-CSF or IL-3 increased survival of neutrophils from 3 AASV patients, but did not enhance survival of neutrophils from 3 other AASV patients (data not shown).

Apoptosis and proportion of $PR3^+/CD177^+$ neutrophils

In accordance with previous studies we found an increased fraction of neutrophils double positive for membrane PR3 expression and the surface marker CD177 (69% for AASV, 58% for HBD; p=0.004). There was, however, no correlation between the percentage of double-positive neutrophils and the rate of apoptosis (r=-0.02, p=0.7).

Transcription of pro-/anti-apoptotic factors and transcription factors

In order to further elucidate the mechanisms of delayed apoptosis we measured the expression of three anti-apoptotic factors (c-IAP2, Bcl2-A1 and Mcl-1), one pro-apoptotic factor (Bax) and three transcription factors (C/EBP-α, C/EBP-β and PU.1) by quantitative PCR for their respective mRNA transcripts. These factors were quantified in neutrophils from patients with AASV, PV and RA (all of which showed a lower rate of apoptosis than HBD), and in neutrophils from TP and HBD.

The results showed slightly higher expression of Bcl-2A1 (1.45), Mcl-1 (1.78) and Bax (1.56) in AASV neutrophils than in HBD neutrophils; however, these differences were not statistically significant (Table 4). No significant correlation was observed between the rates of neutrophil apoptosis or necrosis in neutrophils from AASV patients and relative expression of pro-/anti-apoptotic factors. However, expression of Bcl-2A1 (2.25, p=0.014) and Mcl-1 (2.09, p=0.015) was significantly higher in RA neutrophils than in HBD neutrophils

(Table 4). Expression of pro and anti-apototic factors was not higher in neutrophils from PV patients and TP recipients than in HBD (Table 4).

Transcription factors involved in the process of granulopoiesis were quantified in neutrophils from HBD (n=22), AASV (n=25), RA (n=10), PV (n=10) and TP (n=12). Results showed significantly higher mRNA encoding C/EBP-α and C/EBP-β in AASV patients than in healthy controls (Table 5). Neutrophils from PV patients had significantly lower levels of C/EBP-β and PU.1 than neutrophils from HBD.

There was no significant correlation between mRNA levels of any of the transcription factors and the rate of neutrophil survival/apoptosis. On the other hand, there was a significant positive correlation between C/EBP-α and G-CSF levels in plasma (n=9, r=0.7, p=0.03) among AASV patients.

Discussion

Neutrophils are potentially toxic because of their ability to release reactive oxygen species and proteases into the tissue microenvironment. Neutrophils can be removed from tissues via necrosis or apoptosis followed by phagocytosis by macrophages[29-34]. The latter process is important for resolving inflammation and maintaining self-tolerance. Defects in apoptotic pathways could lead to the persistence of autoreactive T- or B-cells and contribute to development of autoimmune disease[35].

Neutrophils undergo spontaneous apoptosis when cultured in vitro in the absence or presence of insufficient concentrations of neutrophil survival factors[36]. This is the first study to show that neutrophils from AASV have a lower rate of spontaneous apoptosis and longer *in vitro* survival than neutrophils from HBD, SLE patients, and TP. In accordance with previous studies, we found decreased apoptosis in neutrophils from PV and RA patients[37-44]. Previous studies have shown accelerated apoptosis and decreased phagocytosis by macrophages for neutrophils from SLE patients[45-47].

In contrast to the results presented here, Harper et al. showed that neutrophils from AASV patients with active systemic vasculitis, but not in neutrophils from AASV patients with quiescent disease, have an accelerated rate of spontaneous apoptosis, which they correlated with high mPR3 expression and high intracellular superoxide[25]. Their study involved 8 patients with active systemic vasculitis and 17 patients in remission. We see no obvious explanation for this discrepancy. Even though there is a high degree of interindividual variation, and numbers in both studies are relatively small, it is unlikely that difference could be explained by random variation.

Delayed neutrophil apoptosis has also been associated with other clinical syndromes and human diseases including sepsis[48], sleep apnea[49], cystic fibrosis, pneumonia, idiopathic pulmonary fibrosis[50], Behçet disease in the remission phase of uveitis[51], inflammatory bowel disease[52], systemic inflammatory response syndrome after major trauma[53] and in Kawasaki disease[54]. The fact that delayed neutrophil apoptosis occurs in many disease states suggest that reduced apoptosis in AASV might be secondary to a

common condition such as chronic inflammation. We found, however, no correlation with basic clinical parameters such as CRP concentrations, BVAS score or reduced GFR. We cannot completely role out that immunosuppressive drugs do not influence results in our assay, but the difference between AASV and TP patients suggests that drugs could at most account for a minor part of the prolonged neutrophil survival.

Our results with plasma are compatible with a circulating factor causing our findings.

Plasma contains a multitude of neutrophil growth factors [55] but experiments, involving CBA technique measuring different cytokines, previously described to increase neutrophil survival, failed to show any conclusive results[56]. Christensson et al. showed that AASV patients, also in remission, had higher circulating levels of soluble Fas than HBD and other disease controls[57]. Soluble Fas may antagonize Fas-mediated neutrophil apoptosis, but they did not present any data from functional tests. G-CSF, GM-CSF and IL-3, enhance neutrophil survival and delay or prevent neutrophil apoptosis[50, 58-60]. G-CSF and IL-3 levels were normal in plasma from all vasculitis patient subgroups examined in this study. G-CSF and IL-3 levels were normal in plasma from all vasculitis patient subgroups examined in this study. GM-CSF level was higher than normal in four AASV patients, but was undetectable in 40 other AASV patients. Increased GM-CSF was also found in 8 of 20 RA patients. Interestingly, these 8 patients exhibited delayed neutrophil apoptosis as compared to the other RA patients (Figure 4). This indicates different mechanisms for delayed apoptosis in RA and AASV.

Even though levels of G-CSF, GM-CSF and IL-3, were not elevated in AASV, they could still cause delayed apoptosis if AASV neutrohils exhibit increased sensitivity to these cytokines. This hypothesis was examined by culturing neutrophils with the growth factors. This was not generally the case, but three patients responded more to GM-CSF/IL-3 than HBD.

An increased proportion of the $CD177^{+}/PR3^{+}$ subpopulation of neutrophils is a feature seen in AASV and SLE as well as in states associated with increased granulupoiesis such as sepsis. We found no correlation between these two phenomena, indicating that they have different origins.

Growth factor signalling prolongs survival through production of anti-apoptotic factors and down regulation of pro-apoptotic factors. Expression of anti-apoptotic Bcl-2A1 is up-regulated by G-CSF, GM-CSF and LPS, which also promote neutrophil survival[61, 62], while Mcl-1 is upregulated by GM-CSF, IL-1β and LPS[63]. c-IAP2, which is an Inhibitor of Apoptosis Protein (IAP), is selectively up-regulated by G-CSF, but not by GM-CSF and is strongly upregulated in mature neutrophils from patients with chronic neutrophilic leukemia, which also show prolonged *in vitro* survival [64]. The pro-apoptotic factor Bax is down-regulated in response to G-CSF, GM-CSF, IL-3 and IFN-γ[50]. In this study, the levels of mRNA of these factors did not correlate with reduced apoptosis or necrosis in neutrophils from AASV or PV patients. However, expression of Bcl-2A1 and Mcl-1 was significantly higher in neutrophils from RA patients than in HBD.

Looking for altered neutrophil growth factor signaling, we measured expression of three transcription factors involved in steady-state and emergency granulopoiesis[65, 66]. The mRNA levels of all three factors were significantly higher in AASV patients than in HBD, but were not elevated in patients with RA or TP recipients. In PV patients they were significantly decreased. The target genes of these transcription factors include many important neutrophil proteins; including G-CSF receptor, GM-CSF receptor, myeloperoxidase, PR3, elastase, lysozyme and lactoferrin[67-70]. This suggests that elevated expression of these proteins in AASV neutrophils might enhance their susceptibility/sensitivity to cytokines. Interestingly, these transcription factors may stimulate neutrophil survival and granulopoiesis directly, independent of G-CSF and GM-CSF and their respective receptors[71-73].

In summary, this study shows that neutrophils from AASV, RA and PV patients have longer survival and decreased rate of apoptosis during *in vitro* culture than neutrophils from HBD, but mechanisms seems to differ. In RA, increased survival is associated with high plasma levels of GM-CSF and increased transcription of anti-apoptotic genes. In PV levels of growth factors are low and anti-apoptotic genes close to normal. In AASV we could not define a general mechanism, but high GM-CSF and increased sensitivity to GM-CSF might explain the results for some patients. Our data on decreased apoptosis and increased mRNA levels of the C/EBP-α and C/EBP-β transcription factors as well as previous findings of increased proportion double positive $CD177^{+}/PR3^{+}$ cells and increased transcription of the PR3 gene provides evidence for an altered neutrophil phenotype in AASV[16, 74, 75]. Improved understanding of mechanisms by which neutrophil survival and apoptosis are regulated will undoubtedly help explain the pathophysiology of AASV and may have implications for the diagnosis and treatment of AASV and related diseases.

Tables:

Table 1- Demographic data for the AASV patients.

	AASV		
	WG	MPA	All
n	31	13	44
PR3-ANCA	26	1	27
MPO-ANCA	3	12	15
ANCA-negative	2	0	2
Age years (range)	61 (18-86)	64 (37-87)	62 (18-87)
F/M ratio (n)	15/16	7/6	22/22
BVAS 0-1	23	8	31
BVAS 2-5	6	5	11
BVAS > 5	2	0	2

AASV= ANCA-associated Systemic Vasculitis. WG= Wegener's granulomatosis. MPA=Microscopic polyangiitis. F= Female. M= Male. BVAS= Birmingham Vasculitis Activity Score.

Table 2- Demographic data for the controls.

	HBD	PV	TP	SLE	RA
n	93	17	20	21	21
Age years (range)	41(21-68)	61(37-81)	51(29-71)	44(22-68)	63(32-86)
F/M ratio (n)	33/57	7/10	7/13	20/1	13/8

HBD= healthy blood donors. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis. F= Female. M= Male.

Table 3- Hypersensitivity of neutrophils to growth factors after 20h of *in vitro* incubation.

	Spontaneous	+G-CSF 0.2ng/ml	+GM-CSF 0.2 ng/ml	+IL-3 0.2 ng/ml
Alive PMNs after 20h (From 4 HBD)	28%	26%	31%	25%
Alive PMNs after 20h (From 6 AASV pts)	31%	28%	35%	38%

All results are expressed as mean%. PMN= Pomymorphneutrophils. AASV= ANCA-Associated Systemic Vasculitis. HBD= healthy blood donors

Table 4- Gene expression of pro-/anti-apoptotic factors in neutrophils.

	HBD	AASV	PV	TP	RA
n	19	20	10	12	21
cIAP2-mRNA	1	1.05	1.16	1.03	1.33
Bcl2-A1-mRNA	1	1.45	0.58	1.28	2.25*
Bax-mRNA	1	1.56	0.60	0.86	1.14
Mcl-1-mRNA	1	1.78	0.64	1	2.09*

All results are expressed as mean. (*) p value <0.05. HBD= healthy blood donors. AASV= ANCA-associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. RA= Rheumatoid Arthritis.

Table 5- Gene expression of transcription factors in neutrophils.

	HBD	AASV	PV	TP	RA
n	22	25	10	11	10
C/EBP-α-mRNA	1	4.39**	0.11*	0.41	0.45
C/EBP-β-mRNA	1	3.53***	0.08**	2.1	1.58
PU-1-mRNA	1	1.64	0.29**	1.06	0.77

All results are expressed as mean. (*) p value <0.01, (**) p value <0.001, and (***) p value <0.0001 as compared to HBD. HBD= healthy blood donors. AASV= ANCA-associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. RA= Rheumatoid Arthritis.

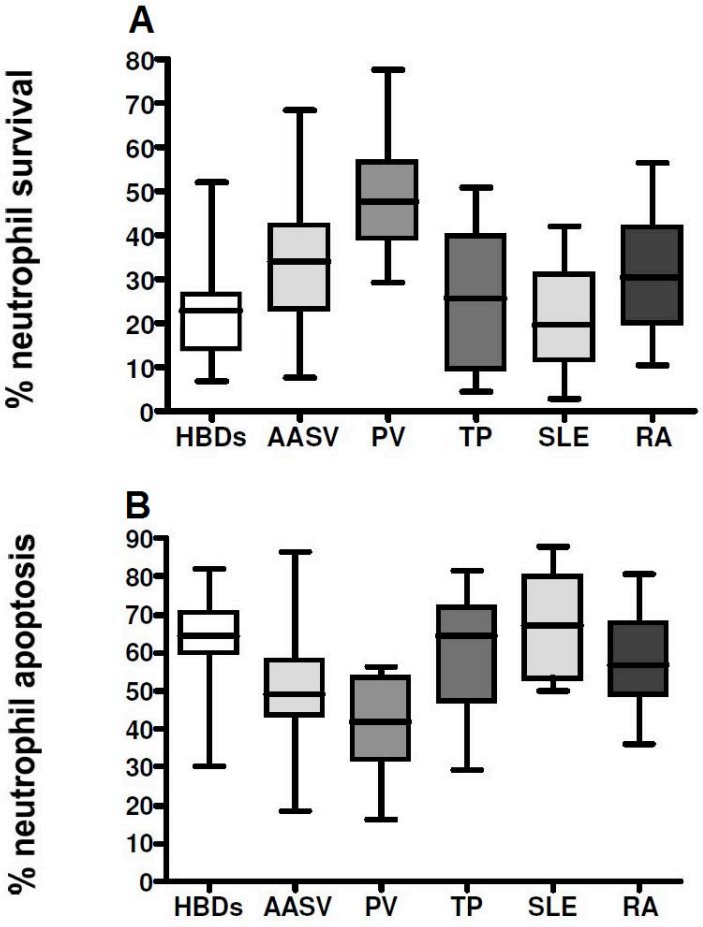

Figure 1- Rate of neutrophil survival and apoptosis. Neutrophils isolated from 60 HBD, 44 AASV patients, 8 PV patients, 18 TP, 21 SLE patients, and 20 RA patients were cultured in vitro in AIM-V medium. The percentage of surviving neutrophils **(1a)** and apoptotic neutrophils **(1b)** was measured after 20 hours. HBD= healthy blood donors. AASV= ANCA-Associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis.

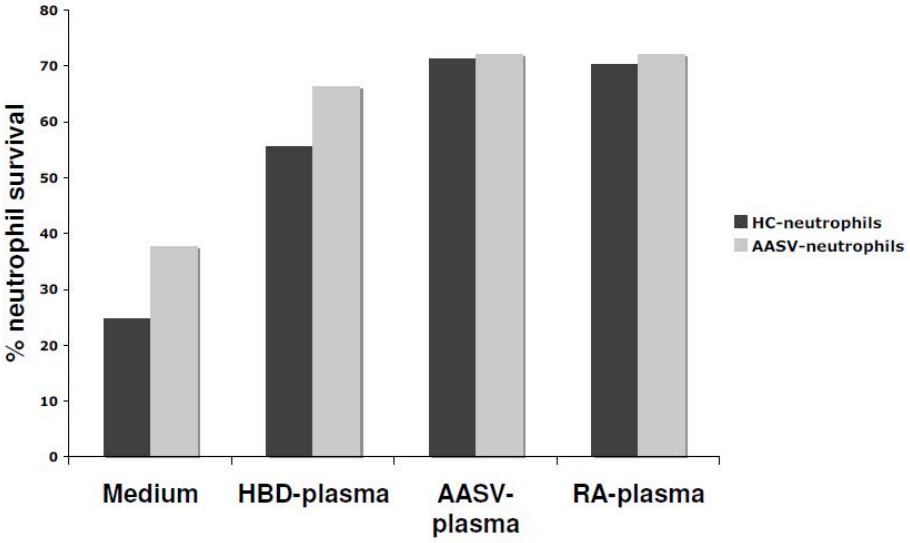

Figure 2- response to plasma. Neutrophils isolated from 6 AASV patients and 4 HBD were incubated with AIM-V medium only, with plasma from HBD, with plasma from AASV patients or with plasma from RA patients. The percentage of neutrophil survival was then measured after 20 hours. HBD= healthy blood donors. AASV= ANCA-Associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis.

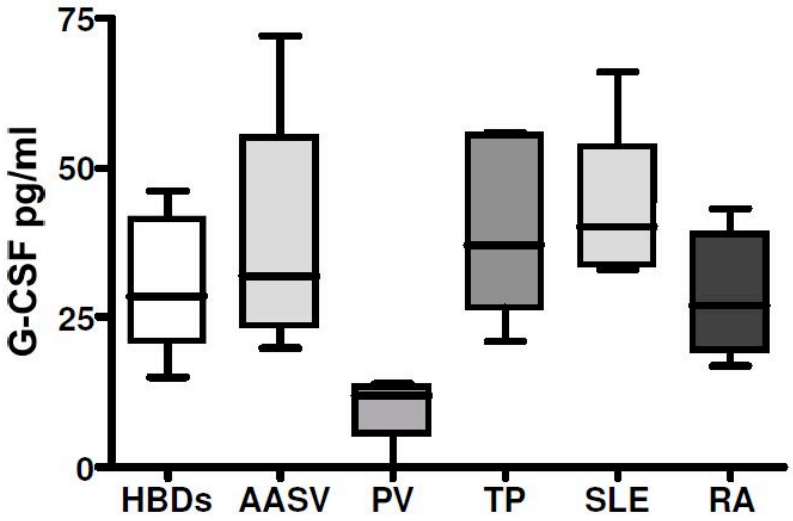

Figure 3. Plasma G-CSF levels measured by ELISA. Compares the results of ELISA measurements of G-CSF in the plasma from 10 HBD, 10 AASV, 5 PV, 5 TP, 5 SLE and 5 RA patients. HBD= healthy blood donors. AASV= ANCA-Associated Systemic Vasculitis. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis.

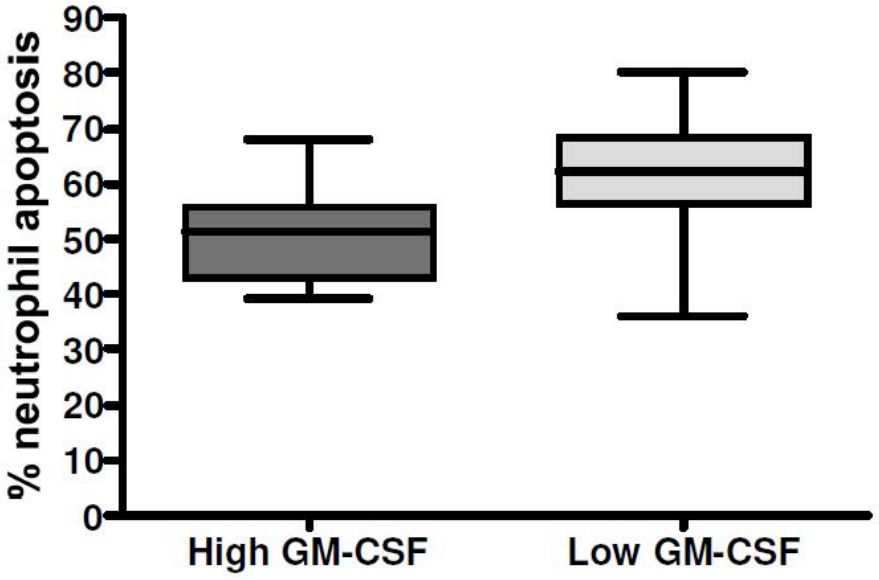

Figure 4. Rate of neutrophil apoptosis among GM-CSF-subgroups of RA patients. Comparing the percentage of neutrophil apoptosis rates after dividing RA patients into two subgroups; First group with high GM-CSF levels (n=8) and the second group with low GM-CSF levels (n=12). Neutrophils from high GM-CSF subgroup showed significantly lower rate of apoptosis compared to neutrophils from the second subgroup. RA= Rheumatoid Arthritis.

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