Neutrophil-endothelial interaction in ANCA associated vasculitis

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Chapter

6

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

anti-PR3 mediated neutrophil activation is independent of CD177 expression

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Abstract

Objectives. Wegener's Granulomatosis (WG) is strongly associated with anti-neutrophil cytoplasmic autoantibodies (ANCAs) directed against Proteinase 3 (PR3). Recent studies have shown that membrane-bound PR3 (mPR3) is differentially expressed and colocalizes with CD177/NB1 on circulating neutrophils. We undertook this study to assess differential expression of CD177 on neutrophils from ANCA-associated systemic vasculitides (AAV) patients in comparison to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients and healthy individuals, and to investigate whether colocalization of mPR3 and CD177 affects anti-PR3-mediated neutrophil activation.

Methods. CD177 and mPR3 expression on isolated neutrophils from patients with AAV (n=53), SLE (n=30), RA (n=26), and healthy controls (n=31), was analyzed by flow cytometry. Neutrophil activation mediated by anti-PR3 antibodies was assessed by measuring the oxidative burst using dihydrorhodamine assay.

Results. Percentages of CD177-expressing neutrophils were significantly higher in patients with AAV and SLE than in healthy controls. In three healthy donors, CD177 expression was not detected and neutrophils remained negative after priming while mPR3 expression was induced. Neutrophils from CD177 negative donors or CD177 negative neutrophils sorted from donors with bimodal expression were susceptible to anti-PR3-mediated oxidative burst. Variation in the extent of anti-PR3 mediated neutrophil activation among different donors appeared independent of the percentage of CD177-expressing neutrophils.

Conclusion. Membrane expression of CD177 on circulating neutrophils is increased in AAV and SLE patients but not in RA patients. However, primed neutrophils from CD177 negative individuals also express mPR3 and are susceptible to anti-PR3-mediated oxidative burst suggesting that recruitment of $CD177^-$ independent mPR3 is involved in anti-PR3-induced neutrophil activation.

Introduction

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

In resting neutrophils, PR3 is contained intracellularly in azurophilic granules. However, in many individuals a membrane bound form of PR3 (mPR3) can also be detected in a subset of neutrophils making it accessible for ANCA binding. In the general population, the percentage of mPR3 expressing neutrophils ranges from 0 to 100%.[9] Within a given individual, the percentage of $mPR3^{high}$ neutrophils is constant in time and is not affected by neutrophil activation, disease activity or therapy, suggesting involvement of genetic factors in the regulation of mPR3 expression.[9-11]

The mechanism of membrane PR3 expression is still under investigation. Colocalization of mPR3 with other membrane proteins, such as β2-integrin and FcγRIIIb, has been reported previously.[12,13] In recent studies, CD177 (NB1) has been proposed as the mPR3 receptor on the neutrophil surface.[14,15] CD177 is a neutrophil specific, GPI-anchored glycoprotein, compartmentalized in secondary (specific) granules.[16] Concurrent with mPR3, CD177 also shows differential expression on the neutrophil surface, with percentages of $CD177^{+}$ neutrophils ranging from 0 to 100%.[17] It has also been observed that mPR3 colocalizes with CD177 on the neutrophil membrane and the subpopulation of neutrophils expressing CD177 is identical to that expressing mPR3.[15] Interestingly, the intracellular content of PR3 is similar in all neutrophil subsets regardless of mPR3 expression status, while CD177 molecules are only present in lysates of neutrophils expressing CD177 on their membrane.[10,15] Collectively, these observations suggest that CD177 may be a determinant of membrane expression of PR3 and, as such, influences the potential of neutrophils to be activated by PR3-ANCA. Indeed, increased percentages of mPR3 expressing neutrophils have been observed in AAV patients, and a high percentage of mPR3-expressing cells is a risk factor for relapse in WG.[11,18,19] However, expression levels of CD177 have not

been consistently studied in patient populations, and its influence on ANCA-induced neutrophil activation has not been described.
Therefore, we assessed the differential expression of CD177 and mPR3 on neutrophils from AAV patients in comparison with disease and healthy controls, and investigated the role of CD177 expression in neutrophil activation by PR3-ANCA.

Patients and Methods

Study population

For the assessment of neutrophil CD177 expression, consecutive patients with ANCA-associated systemic vasculitis (n=53), rheumatoid arthritis (n=26) and systemic lupus erythematosus (n=30) recruited from our out-patient clinic, as well as healthy controls (n=31) recruited from laboratory personnel, were included. Individuals with pregnancy, G-CSF treatment or bacterial infection were excluded.
ANCA-associated systemic vasculitis (AAV): A diagnosis of Wegener's granulomatosis (WG), Churg Strauss syndrome (CSS) or microscopic polyangiitis (MPA) was based on the Chapel Hill definitions.[20] ANCA specificity for PR3 or MPO was determined by capture ELISA. ANCA titers were determined by indirect immunofluorescence (IIF) assay on ethanol-fixed neutrophils.[21,22]
Rheumatoid arthritis (RA): A diagnosis of RA was established on the American College of Rheumatology (ACR) criteria for definite RA.[23]
Systemic lupus erythematosus (SLE): A diagnosis of SLE was based on the revised ACR-criteria for this disease.[24]
Demographic characteristics of patients and controls are given in Table 1.
All patients and controls gave informed consent and the study was approved by the Medical Ethical Committee of the hospital.

Antibodies

PR3 was detected using three murine monoclonal antibodies; PR3G-3,[25] 4A3 (Wieslab, Lund, Sweden), and CLB12.8 (CLB, Amsterdam, The Netherlands). MEM166 (BD Pharmingen, San Diego, CA) was used for CD177 detection. Irrelevant isotype IgG1 (MCG1; IQProducts, Groningen, The Netherlands) and IgG2a (MCG2a; IQProducts, Groningen, The Netherlands) antibodies were used as control. FITC- and PE-conjugated goat anti-mouse secondary antibodies (Southern Biotechnology Associates Inc., Birmingham, USA) were used for FACS analysis. Human IgG fractions were isolated from PR3-ANCA-positive sera of WG patients, anti-glomerular basement membrane (GBM)-positive sera of patients

with Goodpasture syndrome, and sera of age-matched healthy donors using a protein G column (MabTrap G II; Pharmacia Biotech, Uppsala, Sweden) following manufacturer's instructions.

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

	HC	PR3-ANCA	MPO-ANCA	SLE	RA
Number	31	32	21	30	26
Gender (n)					
Male	17	19	12	5	4
Female	14	13	9	25	22
Age (mean±SD)	39±11	57±13	61±14	45±15	56±12

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

Isolation and priming of neutrophils

Neutrophils were isolated from peripheral blood and primed following routine procedures published previously.[19] Briefly, neutrophils were isolated from heparinized venous blood by centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Contaminating erythrocytes were lysed with ice-cold ammonium chloride buffer. Cells were washed and resuspended in cold Hanks' balanced salt solution (HBSS) without Ca^{2+}/Mg^{2+} ($HBSS^{-/-}$; GIBCO/Life Technologies, Breda, The Netherlands). Next, cells were resuspended in RPMI 1640 (Cambrex BioScience, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS; BioWhittaker Europe, Verviers, Belgium) and 50 µg/ml of gentamicin (Gibco, Paisley, UK), to obtain 106 cells/ml.

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

Measurement of membrane-bound PR3 and CD177 expression

Membrane expression of PR3 and CD177 was measured by flowcytometry. The complete procedure was performed on ice to avoid cell activation. After priming, each sample, containing 1×106 isolated neutrophils, was washed with ice-cold HBSS-/- / 1% FCS and centrifuged at 1,200×g, 4˚C for 3 min. Cell pellets were

incubated with 0.5 mg/ml heat-aggregated goat immunoglobulin (HAGG; Sigma, Zwijndrecht, The Netherlands) for 15 min to block Fc receptors on the cell surface. Next, cells were loaded with saturating dose of monoclonal antibodies against human PR3 (PR3G-3) or CD177 (MEM166). An irrelevant isotype IgG1 mouse monoclonal antibody (MCG1) was used as control. Where indicated, 4A3 and CLB12.8 were also used for PR3 detection. After 30 min, unbound antibodies were washed off and cells were resuspended in PE-conjugated secondary antibody diluted with 0.5 mg/ml HAGG, and incubated in the dark for another 30 min. Next, cells were washed and resuspended in washing buffer. Fluorescence intensity was measured immediately by FACS Calibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and calibrated using CellQuestTM software (Becton Dickinson). The results were analyzed using Win-List software package (Verity Software House, Topsham, ME), and figures were edited with FlowJo™ analysis software (Treestar, Ashland, OR). Expression levels are presented as mean fluorescence intensity of 10,000 counted cells corrected for nonspecific binding of isotype control antibodies. Bimodal expression was defined as the presence of 10~90% $mPR3^{high}/CD177^{+}$ cells, respectively, while percentages out of this range were defined as monomodal. Definitions for monomodal-$mPR3^{high}$ and monomodal-$mPR3^{low}$ were based on data from a previous study.[19]

Detection of intracellular level of PR3 and CD177

Isolated neutrophils were suspended in $HBSS^{-/-}$ to obtain a concentration of 1×106 cells/ml. The cell suspensions were incubated with 10 µg/ml cytochalasin B at 37˚C for 5 min, and primed with 2 ng/ml rhTNF-α at 37˚C for 15 min. Next, all neutrophils were fixed and permeabilized with Fix&Perm kit (Caltag Laboratories, Burlingame, CA) following the manufacturer's instructions. Next, intracellular levels of PR3 and CD177 were detected by incubation with anti-PR3 (PR3G-3) or anti-CD177 antibody (MEM166) at saturating concentrations. MCG1 was used as negative control. After 30 min incubation at room temperature, unbound antibodies were washed off and cells were incubated with PE-conjugated secondary antibody diluted with 0.5 mg/ml HAGG in the dark for another 30 min. After a next washing step, cells were resuspended in washing buffer. Fluorescence intensity was measured immediately by FACS Calibur and calibrated with CellQuestTM software. Levels of PR3 and CD177 expression were given as MFI and corrected for non-specific binding as detected from isotype control antibody incubation.

Dihydrorhodamine 123 oxidation assay

Intracellular hydrogen peroxide produced during neutrophil activation was measured by the dihydrorhodamine 123 (DHR 123) oxidation assay as described previously.[26] Isolated neutrophils from healthy donors were suspended in HBSS with Ca^{2+}/Mg^{2+} to a concentration of 2.5×106 cells/ml and incubated with cytochalasin B (5 µg/ml, Serva Electrophoresis, Heidelberg, Germany) at 37˚C, to enhance oxygen radical production. Next, cells were loaded with 1 µg/ml DHR 123 (Molecular Probes Europe) and kept at 37˚C for 15 min. Sodium azide (NaN_3, 2 mM) was added in order to prevent intracellular breakdown of H_2O_2 by catalase. Part of the DHR 123 loaded cells were incubated for 15 min in the presence of a priming concentration of rhTNF-α (2 ng/ml). Next, neutrophils were stimulated for 1 hour with anti-PR3 antibody (PR3G-3, 5 µg/ml) or an irrelevant mouse IgG1 monoclonal antibody (MCG1) at the same concentration. Where indicated, neutrophils were also stimulated with 200 µg/ml of PR3-$ANCA^+$ or irrelevant human IgG fractions (anti-GBM or normal IgG). Stimulation with 0.2 µM phorbol myristate acetate (PMA; Sigma, Zwijndrecht, The Netherlands) for 30 min was performed as positive control. Reaction was stopped with ice-cold HBSS-/-. Finally, cells were resuspended in washing buffer and fluorescence intensity, resulting from the intracellular oxidation of DHR 123 into the fluorescent rhodamine 123 (R 123), was measured using a FACS Calibur and calibrated using CellQuestTM software.

Statistical analysis

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

Results

Expression of CD177 correlates with mPR3 expression on primed neutrophils

Combining data from healthy controls and patient groups (n=140), 120 donors were identified that displayed a bimodal expression of mPR3. In agreement with other studies,[14,15] CD177 and mPR3 expression on primed neutrophils showed a strong correlation (data not shown). However, neutrophil priming was an essential procedure for assessing the correlation between mPR3 and CD177 expression, as priming with TNF-α is necessary for standardized assessment of mPR3 expression as shown before.[19] In 19 of the 31 healthy donors (61%), isolated

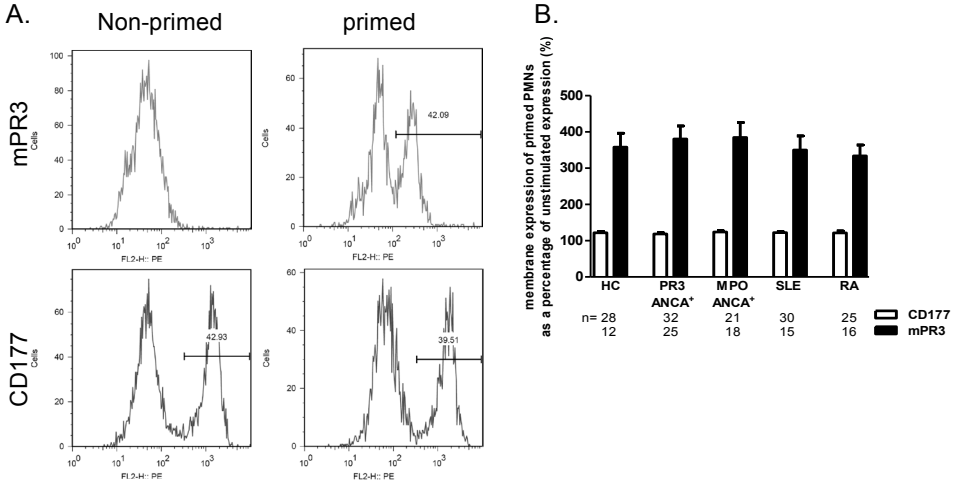

Figure 1. Effect of TNF-α-stimulation on CD177+/mPR3high expressing neutrophils. CD177/mPR3 expression on TNF-α-primed and non-primed neutrophils was measured by flowcytometry. (A) shows a representative example of TNF-α-induced bimodal pattern of mPR3 expression in comparison to CD177 expression. (B) Extent of TNF-α-induced upregulation of mPR3 and CD177 on CD177+/mPR3high neutrophils from donors with bimodal expression of CD177 or mPR3. Results show membrane expression of primed neutrophils expressed as percentage of mean fluorescence intensity (MFI) measured on non-primed neutrophils. HC: Healthy Control; PR3-ANCA+: PR3-ANCA-associated vasculitis; MPO-ANCA+: MPO-ANCA-associated vasculitis; SLE: Systemic Lupus Erythematosus; RA: Rheumatoid Arthritis.

neutrophils showed monomodal-negative expression of mPR3 without priming, while, after incubation with TNF-α for 15 min, 12 of these donors turned into bimodal patterns with a low- and a high-expression subset (Figure 1A). Percentages of $mPR3^{high}$ neutrophils ranged from 38 to 85%, and remained constant in repeated tests within a given individual (data not shown). The amount of mPR3 on the neutrophil surface, given as mean fluorescence intensity (MFI), was also remarkably upregulated by TNF-α stimulation (Figure 1B). Effect of neutrophil priming on mPR3 recruitment was comparable between patient groups (Table 2 and Figure 1B). Therefore, priming with TNF-α revealed the potential of neutrophils to increase expression of mPR3. In contrast to mPR3 expression, percentages of $CD177^{+}$ neutrophils were not modified by neutrophil priming (Table 2). The amount of membrane expressed CD177 was only slightly upregulated after priming, with a mean increase of MFI of 21±19% of the expression level on non-primed neutrophils (Figure 1B).

Table 2. Bimodal expression of mPR3 and CD177 induced by TNF-α priming

	HC	PR3-ANCA	MPO-ANCA	SLE	RA
Total number	31	32	21	30	26
mPR3					
before/after priming (n)	12/24	25/31	18/21	15/27	16/22
CD177					
before/after priming (n)	28/28	32/32	21/21	30/30	25/25

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

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

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

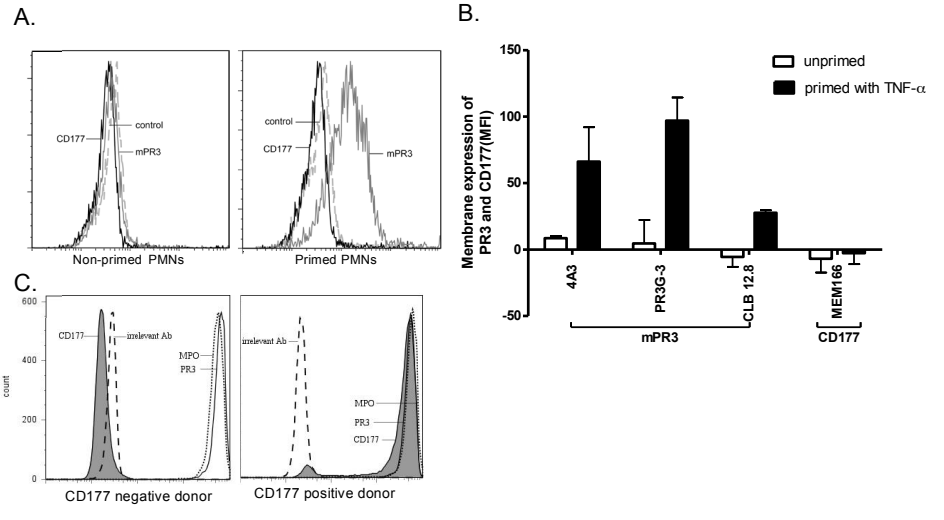

Figure 2. TNF-α-induced mPR3 expression is detected on CD177 negative neutrophils. Membrane expression of mPR3 and CD177 on neutrophils was measured by flowcytometry in parallel. **(A)** CD177 and mPR3 expression on non-primed and primed neutrophils from a CD177 negative donor. Figure shows a representative of duplicated experiments with 3 $CD177^-$ donors. **(B)** mPR3 on neutrophils from $CD177^-$ donors was detected with 4A3, PR3G-3 and CLB12.8 anti-PR3 antibodies, and $PR3\text{-}ANCA^+$ IgG fractions, in comparison with CD177 expression detected with MEM166 (anti-CD177). Results show mean+1SD of mPR3 and CD177 expression level (MFI) of 3 $CD177^-$ donors after correcting for nonspecific binding. **(C)** Histograms showing intracellular content of CD177 (grey-colored) and PR3 (solid line) in CD177 negative and positive donors measured by flowcytometry. An isotype control IgG_1 (dashed line) and anti-MPO antibody (dotted line) were used as negative and positive control, respectively. The figure shows a representative example of 3 independent experiments with different donors.

which was detected using three different monoclonal antibodies (Figure 2A and 2B). Absence of CD177 expression was confirmed by intracellular FACS staining. In contrast, PR3 and MPO were uniformly detected in these neutrophils (Figure 2C). These results demonstrate that mPR3 on the neutrophil surface can be independent of CD177 expression.

Percentages of mPR3^{high} and CD177 expressing neutrophils in inflammatory diseases

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

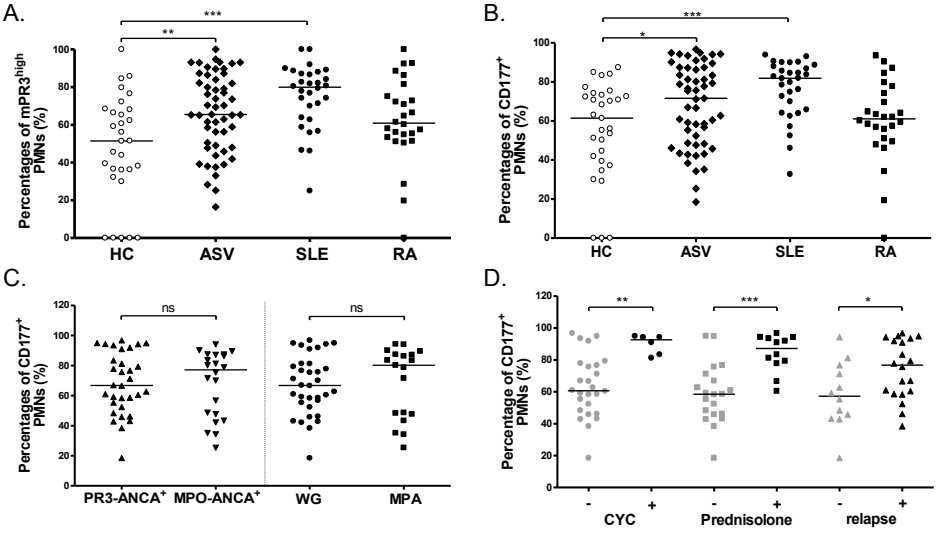

Figure 3. Percentages of CD177 expressing neutrophils are increased in ASV and SLE patients. Membrane expression of CD177 and mPR3 were measured on isolated neutrophils by flowcytometry. Results are presented as percentages of CD177^{+}/mPR3^{high} neutrophils in the total neutrophil population. Percentages of mPR3^{high} (A) and CD177^{+} (B) neutrophils were compared among healthy controls (n=31), and patients with ASV (n=53), SLE (n=30) and RA (n=26). (C) Percentages of CD177^{+} neutrophils were compared between MPO-ANCA^{+} (n=22) and PR3-ANCA^{+} (n=32) ASV patients, and between WG (n=33) and MPA (n=19) ASV phenotypes. (D) Comparison of CD177^{+} neutrophils between PR3-ANCA positive patients with or without treatment with cyclophosphamide (CYC) or prednisolone and patients with or without relapsing disease. ns: not significant, *: p<0.05, **: p<0.01, ***: p<0.001. Horizontal line denotes the median.

higher in AAV (71%, range 18~97%) and SLE (82%, range 33~94%) patients than in healthy controls (62%, range 0~87%) and RA patients (61%, range 0~93%) (Figure 3B). Within the AAV patient group, the expression profile was also analyzed according to ANCA specificity and AAV phenotypes. There were no statistical differences in the percentages of $CD177^+$ neutrophils between patients with MPO-ANCA (77%, range 25~94%) and PR3-ANCA (67%, range 18~97%) associated vasculitis, or between patients with WG (67%, range 18~97%) and MPA (80%, range 25~94%) (Figure 3C).

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

Neutrophil activation by PR3-ANCA

Neutrophil activation induced by a mouse anti-human PR3 monoclonal antibody (PR3G-3) or by PR3-$ANCA^+$ IgG fractions was evaluated by measuring the oxidative burst using the dihydrorhodamine assay. In agreement with previous studies, oxidative burst was detected only when neutrophils were primed with TNF-α (data not shown).[26,27] Neutrophils from CD177 negative donors (n=3) could be activated by anti-PR3 antibody and showed comparable levels of neutrophil activation as neutrophils from donors with moderate expression of CD177 (40~70% positive; n=3) and high expression of CD177 (>70% positive; n=3) (Figure 4A). Moreover, no correlation was observed between the levels of neutrophil oxidative burst induced by anti-PR3 antibody and percentages of $CD177^+$ neutrophils (data not shown). Next, CD177 positive and negative neutrophils from donors with bimodal expression of CD177 were sorted by flow cytometry. The purity of each sorted sample was more than 95% (Figure 4B). These subsets were used for induction of the oxidative burst by anti-PR3 antibody. The results were comparable for the CD177 negative and positive subpopulations (Figure 4C).

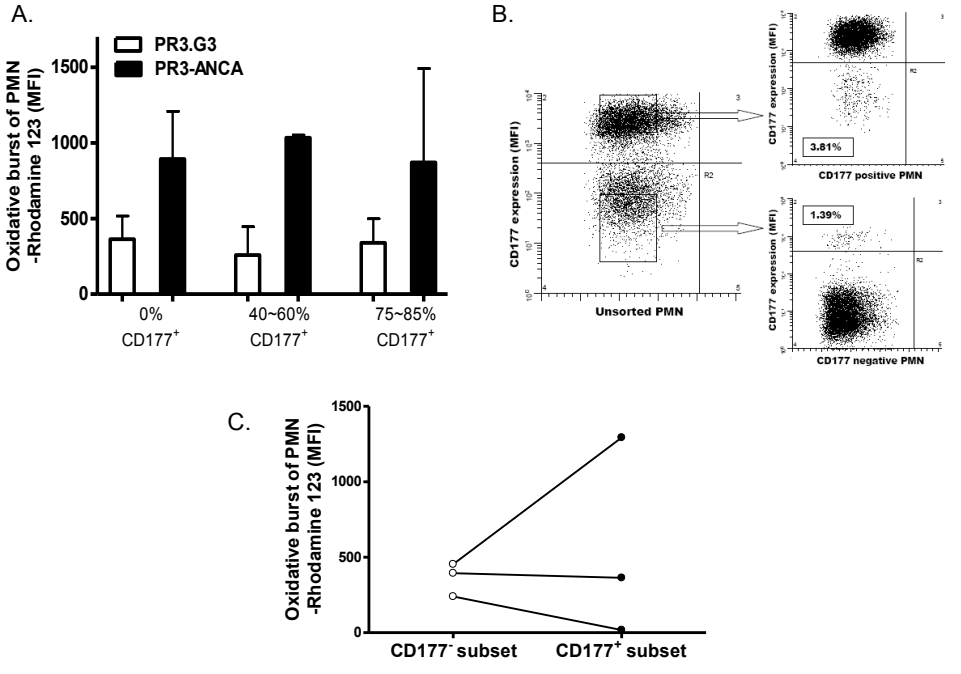

Figure 4. Anti-PR3-antibody-mediated neutrophil activation is independent of CD177 expression. Oxidative burst was assessed by DHR 123 oxidation assay and activation level is presented as MFI of Rhodamine 123. (A) Levels of neutrophil activation were compared among individuals with CD177 negative neutrophils (0% $CD177^+$, n=3), neutrophils with moderate expression of CD177 (40~70% $CD177^+$, n=3) and high expression of CD177 (>70% $CD177^+$, n=3). (B) CD177 negative and positive neutrophils from the same donor were sorted by moflo flow cytometry, purity of each sorted sample was more than 95%. Figure shows a representative example of 3 independent experiments. (C) Levels of neutrophil activation induced by anti-PR3 antibody were compared between $CD177^-$ and $CD177^+$ neutrophils from 3 individual donors.

Discussion

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

neutrophil activation, as analyzed by DHR 123 oxidation, could also be induced in CD177 negative neutrophils, and, among healthy individuals, there was no correlation between the level of neutrophil activation and the percentage of CD177 expressing neutrophils.

Although the pathogenesis of AAV is still unclear, it has been shown that ANCA are pathogenic in animal models and that the antibodies in vitro activate cytokine-primed neutrophils leading to oxidative burst and neutrophil degranulation.[3,4] Membrane-bound PR3, which is accessible for ANCA binding, shows heterogeneous expression on the neutrophil surface.[9] The percentage of $mPR3^{high}$ neutrophils is increased in AAV patients and is a risk factor for relapse of WG.[11,18,29] Recent studies have suggested that CD177 acts as a receptor of mPR3 by showing that mPR3 expression is highly correlated with CD177 expression and mPR3 colocalizes with CD177 on the neutrophil membrane.[14,15] It is tempting to speculate that increased proportions of mPR3 expressing neutrophils in AAV patients reflect upregulated expression of CD177. Furthermore, it is relevant in view of the pathogenesis of AAV whether $CD177^{+}/mPR3^{high}$ neutrophils act differently from $CD177^{-}/mPR3^{low}$ neutrophils when encountering PR3-ANCA, for instance, in PR3-ANCA mediated neutrophil activation.

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

It has been shown that the percentage of mPR3-expressing neutrophils cannot be modified by disease activity or treatment.[11,18] In agreement, we did not find any effect of treatment on the proportion of $CD177^{+}$ neutrophils in patients with SLE and MPO-ANCA associated vasculitis. However, in patients with PR3-ANCA associated vasculitis, patients treated with cyclophosphamide (CYC) or prednisolone showed significantly higher percentages of CD177 expressing neutrophils than patients without these treatments (Figure 3D). In our disease control groups, treatment with prednisolone did not increase the percentage of $CD177^{+}$ neutrophils in patients with SLE, and neither CYC nor prednisolone

induced upregulation of CD177 in patients with MPO-ANCA$^+$ vasculitis, suggesting that increased CD177 expression is not a consequence of treatment, but probably associated with other factors. In patients with PR3-ANCA$^+$ vasculitis, we did not find any correlation between the percentages of CD177$^+$ neutrophils and disease activity or disease duration. However, we found that patients who had suffered from relapsing disease showed significantly higher percentages of CD177$^+$ neutrophils than patients without relapses. Concurrently, four out of six patients treated with CYC and 10 out of 12 patients treated with prednisolone had relapsing disease, probably explaining differences in CD177 expression between patients on CYC or prednisolone and those without these treatments. Therefore, treatment with immunosuppresives and corsticosteroids has, probably, in itself no effect on CD177/mPR3 expression. Moreover, the current study confirmed our previous observation that a high proportion of mPR3 (and CD177) expressing neutrophils is associated with relapse in WG.[18]

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

CD177 is a heterophilic binding partner of CD31 which is expressed on endothelial cells suggesting a role of CD177 in neutrophil migration.[41] Apart from this, the biological function of CD177 is largely unknown. Furthermore, CD177 negative individuals, about 3% of Caucasians, are healthy, although detailed studies on neutrophil function in these individuals are lacking. In contrast, PR3 executes multiple biological functions such as regulation of granulopoiesis, microbicidal activity, degradation of extracellular matrix, and modulation of inflammatory factors.[42,43] It has been shown, in vivo, that PR3 is essential for immune complex-mediated neutrophil activation by cleaving anti-inflammatory progranulin into its inactive form, and both PR3 and elastase deficiency diminish neutrophil infiltration into the inflammatory site.[44] Apparently, enzymatic activity of PR3 is

required in most of its important actions, and it is probably protected from endogenous inhibitors by anchoring at the neutrophil membrane instead of being secreted in a soluble form.[45,46] Therefore, one may expect that mPR3 expression also occurs in healthy persons with CD177 deficiency. In our study, PR3 was detected by 3 different MoAbs on the surface of primed neutrophils from CD177 negative donors. Membrane-bound PR3 expression on $mPR3^{low}$ neutrophils has also been observed in other studies,[9,19,47] and we have shown, previously, that the extent of TNF-α-induced mPR3 upregulation on $mPR3^{low}$ neutrophils is comparable with that of neutrophil elastase.[19] These results suggest that CD177 is not an exclusive receptor of mPR3 and the binding site(s) for CD177-independent mPR3 expression are still open for discussion. It has been shown that mPR3 colocalizes with activated β2-integrin and FcγR in the neutrophil membrane.[12,13,48] PR3 can insert into the membrane lipid bilayer via the hydrophobic region.[49] Elastase, a proteinase homologous to PR3, binds to chondroitin sulphate (CS)- and heparin sulphate (HS)- containing proteoglycans in the neutrophil membrane, and PR3 is competitive for these binding sites.[50] In a recent study, Witko-Sarsat et al. assumed mPR3 to be a peripheral membrane protein and predicted an interfacial binding site in the hydrophobic region, through molecular dynamics simulation on a membrane model.[46] All these data suggest that mPR3 binds to the neutrophil membrane via its cationic nature and functionally colocalizes with some receptor molecules, such as integrins, FcγR and CD177, for signal transduction.

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

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

vasculitis and suggest different mechanisms of membrane-binding of PR3 other than to CD177.

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