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The effect of serum on mPr3 and CD177 expression on neutrophils

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Abstract

: Proteinase 3 (Pr3) is a serine protease that is stored primarily in azurophilic granules and secretory vesicles in neutrophils. The high affinity Pr3 receptor, CD177, is expressed on a subset of neutrophils (ranging from 0-100%), but usually only half of the circulating neutrophils express CD177 in most normal individuals. As a serine proteinase, Pr3 is controlled by a variety of inhibitors, including alpha-1-antitripsin (AAT). We investigated if the surface expression of Pr3 and CD177 was affected by stimulation of neutrophils in the presence or absence of serum. **Methods**: Neutrophil cells were isolated from healthy donors. The cells counted and stimulated with fMLP only or cytochalasin B followed by addition of fMLP, and compared to unstimulated controls, in the presence or absence of 100 % autologous serum. The expression of Pr3 and CD177 was analysed by flow cytometery. **Results**: The expression of membrane bound Pr3 (mPr3) by neutrophils was still detectable in the presence of serum however, the expression was reduced on both $mPr3^{low}$ and $mPr3^{high}$ cells in comparison to cells incubated in the absence of serum. No significant increase was observed in mPr3 expression following stimulation with fMLP in either the presence or absence of serum. In comparison, stimulation with cytochalasin B combined with fMLP resulted in a 9-fold increase (P<0.0001) in the $Pr3^{high}$ cells compared with unstimulated cells in the absence of serum. This increase was only 3-fold (P< 0.002) in the presence of serum.

Conclusion: Despite the ability of purified AAT to inhibit Pr3 binding to CD177, significant surface Pr3 was still found on the surface of CD177-positive neutrophils when stimulated in the presence of 100% autologous serum, but only following maximal stimulation of neutrophils.

Key words: Proteinase 3, CD177, neutrophils, ANCA-associated vasculitis

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Introduction

Pr3 and other serine proteases are proteolytic enzymes involved in destruction of pathogens within the phagolysosome and in producing tissue damage when secreted extracellularly. It has been identified as the major anti-neutrophil cytoplasmic auto-antigen (ANCA) which is found in patients with ANCA-associated diseases and it is an important factor in myeloid differentiation (1,2,3). Pr3 is stored in neutrophil azurophilic granules and also in the secretory granules thus, can be easily mobilised to the neutrophil surface following moderate and maximal stimulation (4). It is a peripheral membrane protein, does not comprise a transmembrane domain and needs to cooperate with other membrane proteins or lipids to maintain a stable membrane anchorage. Moreover, Pr3 seems to be a protein with specific hydrophobic properties which interact with lipid bilayers by hydrophobic insertion (5). Several membrane partners of Pr3 have been identified, such as CD16/FcγRIIIb, but among Pr3 partners NB1, a glycosylphosphatidylinositol (GPI)-anchored membrane protein has been shown to be the best candidate as the surface receptor. NB1 (also known as CD177), has an identical biomodal expression pattern to surface Pr3 with a variable expression on 0-100% of neutrophils in the population, therefore mPr3/CD177 complex may have implications in systemic vasculitis autoimmune disorders (e.g. Wegener's granulomatosis; WG).

Circulating neutrophils in patients with active WG express Pr3 on their cell surface, and the binding of Pr3-ANCA to membrane-bound Pr3 is followed by neutrophil activation that results in the release of reactive oxygen species and proteolytic enzymes. Like the other serine proteases, Pr3 is inhibited by serpins, predominantly α-1-antitrypsin (AAT). AAT, the major physiological proteinase inhibitor in serum, inhibits a broad variety of serine proteases (especially human neutrophil elastase; HNE and Pr3) and is present in serum at 1.5-3.5 g/L (6). In the presence of AAT, Pr3 remains on the neutrophil surface and can bind ANCA, resulting in neutrophil activation and release of neutrophil granule contents, with higher levels of membrane Pr3 associated with greater responsiveness to Pr3-ANCA stimulation (7).

A previous study demonstrated that AAT can impair the binding and activation of neutrophils by anti-Pr3 antibodies from both healthy control and individuals with WG which suggests that mPr3 activity and the protease-antiprotease balance are implicated in neutrophil activation

during WG (8). Korkmaz *et al.* (9) has shown that addition of purified AAT to Pr3-bound NB1 (CD177) transfected CHO cells completely removed Pr3 from the surface of CD177 receptor-expressing CHO cells. Furthermore he demonstrated that the binding of CD177 possibly occurs via the single hydrophobic cluster on the surface of Pr3. The membrane-bound human neutrophil elastase (mHNE) is rapidly cleared from the surface of activated neutrophils by AAT and by EPI-hNE4, a low molecular weight recombinant inhibitor, with which it forms soluble, inactive complexes. The behaviour of mPr3 clearly differs from that of mHNE, which explains why it may be a preferential target for autoantibodies and so contributes to the pathogenicity of Wegener disease (10, 11, 12).

Material and methods

Materials

The bacterial peptide f-met-leu-phe (fMLP), cytochalasin B, Dextran and Percoll were obtained from Sigma-Aldrich. PE-conjugated goat anti-mouse immunoglobulin (rPE-GAM) was from DAKO. Monoclonal anti-Pr3 antibody (G2) was from Hycult biotech. Unconjugated isotype IgG control was from eBioscience. APC CD16 and monoclonal anti-CD177 (MEM-166) were from Biolegend.

Neutrophils isolation and stimulation

Neutrophils of healthy human volunteers were isolated from citrated anti-coagulated blood (1/10 volume 3.8 sodium citrate) by dextran (6%) sedimentation and Percoll gradient centrifugation. The $1x10^6$ cell/ml was washed once in Hank's Balanced Salt Solution (HBSS; Lonza) without Ca^{+2} and Mg^{+2} and twice in HBSS with Ca^{+2} and Mg^{+2}. Cell surface CD177 and Pr3 expression were compared for unstimulated and stimulated cells resuspended in HBSS or in 100% autologous serum. Neutrophils were stimulated with fMLP only, or cytochalasin B followed by addition of fMLP, and compared to unstimulated controls.

Flow cytometry

Cell surface expression of CD177 and Pr3, were detected by mouse monoclonal antibodies, relative to isotype matched controls, which were in turn detected by PE-conjugated goat anti-mouse immunoglobulins on unfixed neutrophils. Neutrophils were identified in mixed

leukocyte populations by a third staining step using APC-conjugated mouse monoclonal CD16, which is highly expressed on neutrophils. All samples were run on a FACScalibur and data for 10,000 cells collected and analysed by sub-population gating using CellQuest software the following day.

Statistical analysis

Data in this study was analysed using GraphPad Prism 5.01 software (GraphPad software Inc). Results were viewed as statistically significant when the P value < 0.05.

Results

The effect of serum on mPr3 and CD177 expression on neutrophils

Neutrophils were taken from healthy donors and stimulated with fMLP only or cytochalasin B (cytoB) followed by addition of fMLP (maxima stimulation), and compared to unstimulated control cells in the presence or absence of 100% autologous serum. These natural agonists are potent inducers of neutrophil degranulation and activation.

Consistent with the results from other studies, we observed that unstimulated neutrophils, isolated from healthy individuals, expressed varying levels of Pr3 on their cell surface (17, 18). As mentioned before the percentage of Pr3 and CD177 expression on the neutrophil surface is variable among individuals ranging from 0-100%. However, our results showed that in some individuals (n=2) the expressions of Pr3 was bimodal (the presence of both $mPR3^{low}$ and $mPR3^{high}$ populations within one individual) where as in others expression was monomodal (one uniform population of neutrophils), which does not reflect the CD177 expression (figure 1). The CD177 low subset represents low or possibly negative CD177 expression, whereas the high subset expresses a substantial amount of CD177.

Following, stimulation especially with fMLP combined with cytoB, neutrophils that expressed a monomodal mPr3 on unstimulated cells, were induced to express an increased sub-population of neutrophils with low mPR3 expression (figure 1 donors 2&4).

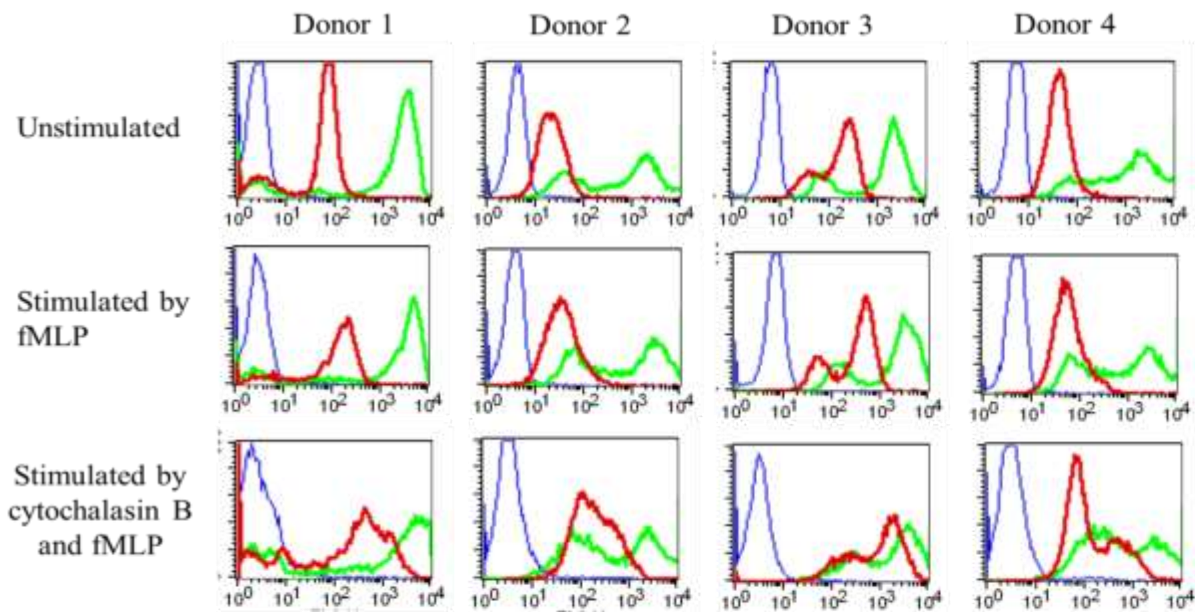

Figure 1. Patterns of mPr3 CD177 expression on surface neutrophils from different donors detected by flow cytometry. The blue line represents nonspecific binding of isotype-matched control. The red line shows binding of monoclonal anti-Pr3 antibody (G2) and green line shows binding of monoclonal anti-CD177 (MEM-166) both antibodies were detected by PE-conjugated goat anti-mouse antibody (rPE-GAM) in different tubes prepared in parallel, but histograms are overlaid to compare the expression distribution.

The results demonstrated that Pr3 and CD177 expression was still detectable in the presence of serum (figure 2A). The levels of CD177 showed no change in expression in the presence of serum, compared with HBSS (no serum) controls. While the mPr3 expression on unstimulated neutrophils in the presence of serum appeared to be less than that on cells in the absence of serum, this difference was not statistically significant. Both mPr3 populations showed reduction in mean cellular fluorescence (MCF) in the presence of serum and it reduced the level of Pr3low population to around that of the background isotype control staining (Pr3low= 5.02 and control= 4.21; figure 2B). No significant increase was observed in mPr3high expression following stimulation with fMLP in either the presence or absence of serum.

In contrast, stimulation with cytochalasin B combined with fMLP resulted in a 9-fold increase ($P<0.0001$) in the Pr3high cells compared with unstimulated cells in the absence of serum. This increase was only 3-fold ($P< 0.002$) when stimulation occurred in the presence of serum (Figure 3). When the data is transformed to represent the percent increase in mPr3high expression (to remove variation in staining procedure on separate days), the effect of serum on decreasing surface Pr3 expression post-stimulation is very clear and reproducible (Figure 4).

No significant increase was observed in mPr3low expression following stimulation with fMLP in either the presence or absence of serum. However, stimulation of the cells with cytochalasin B combined with fMLP resulted in a 3.8-fold increase (P=0.03) in the Pr3low cells compared with unstimulated cells in the absence of serum. The statistical significance of this change is lost in the presence of serum, despite the average increase being 2-fold following maximal stimulation (Figure 5). In fact the only condition that showed a significant increase in mPr3low subpopulation expression was following stimulation of the cells by cytochalasin B & fMLP in the absence of serum (P=0.03).

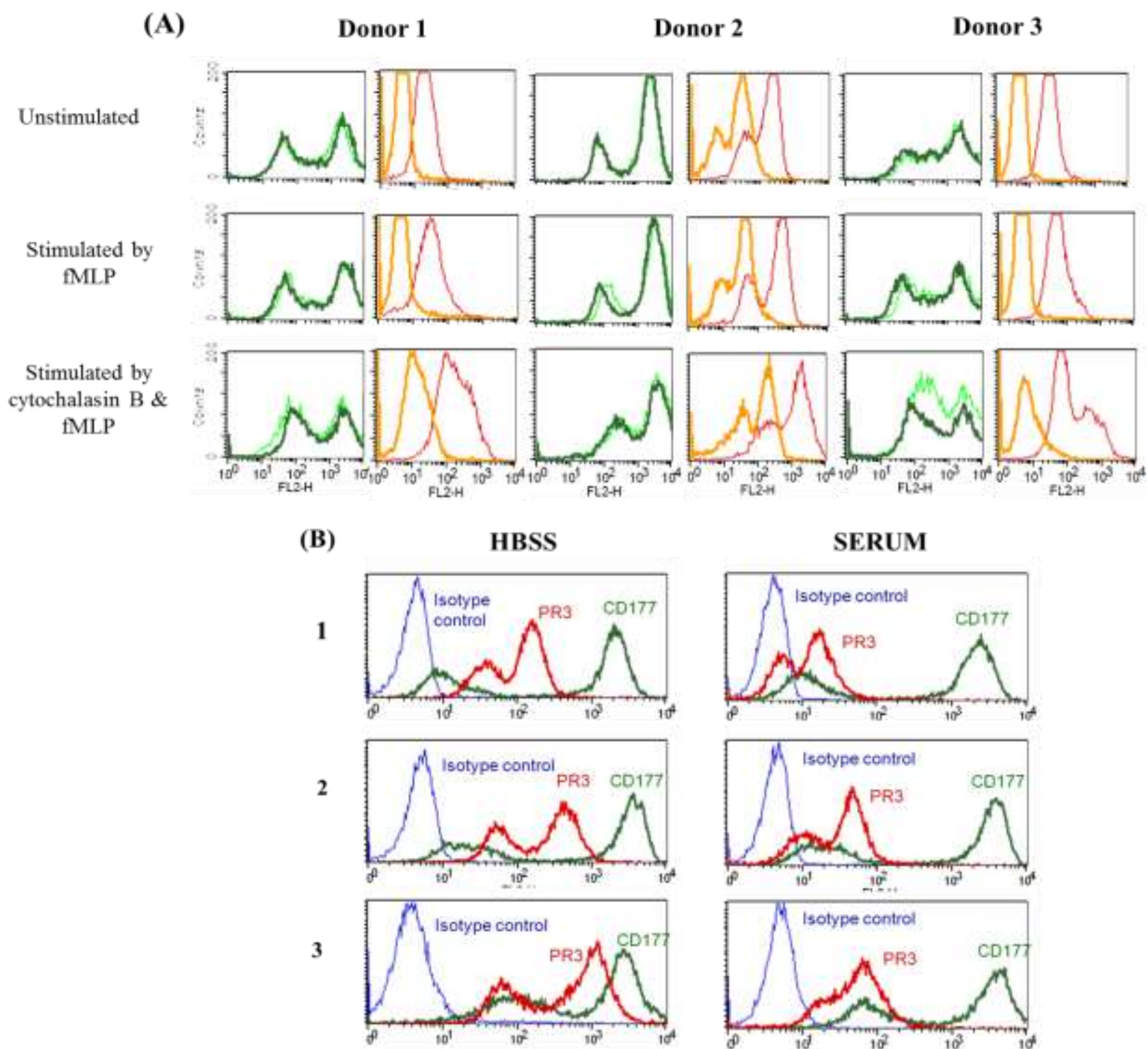

Figure 2. A) Surface expression of CD177 and Pr3 on neutrophils in presence or absence of serum from three different donors.

The histograms show the fluorescence intensity of neutrophil surface expression of Pr3 (red line in presence of serum and orange line in presence of HBSS), CD177 (dark green line in presence of serum and light green line in presence of HBSS). The levels of CD177 showed no change in expression in the presence of serum, compared with HBSS whereas the cells showed a decrease in the levels of Pr3 expression. B) These histograms show the expression of mPr3 and CD177 from a single donor comparing CD177 (green) and Pr3 (red) expression relative to the isotype background control (blue). Neutrophils were unstimulated (1) or stimulated with fMLP (2) or stimulated with Cyto B in combination with fMLP(3).(n=7).

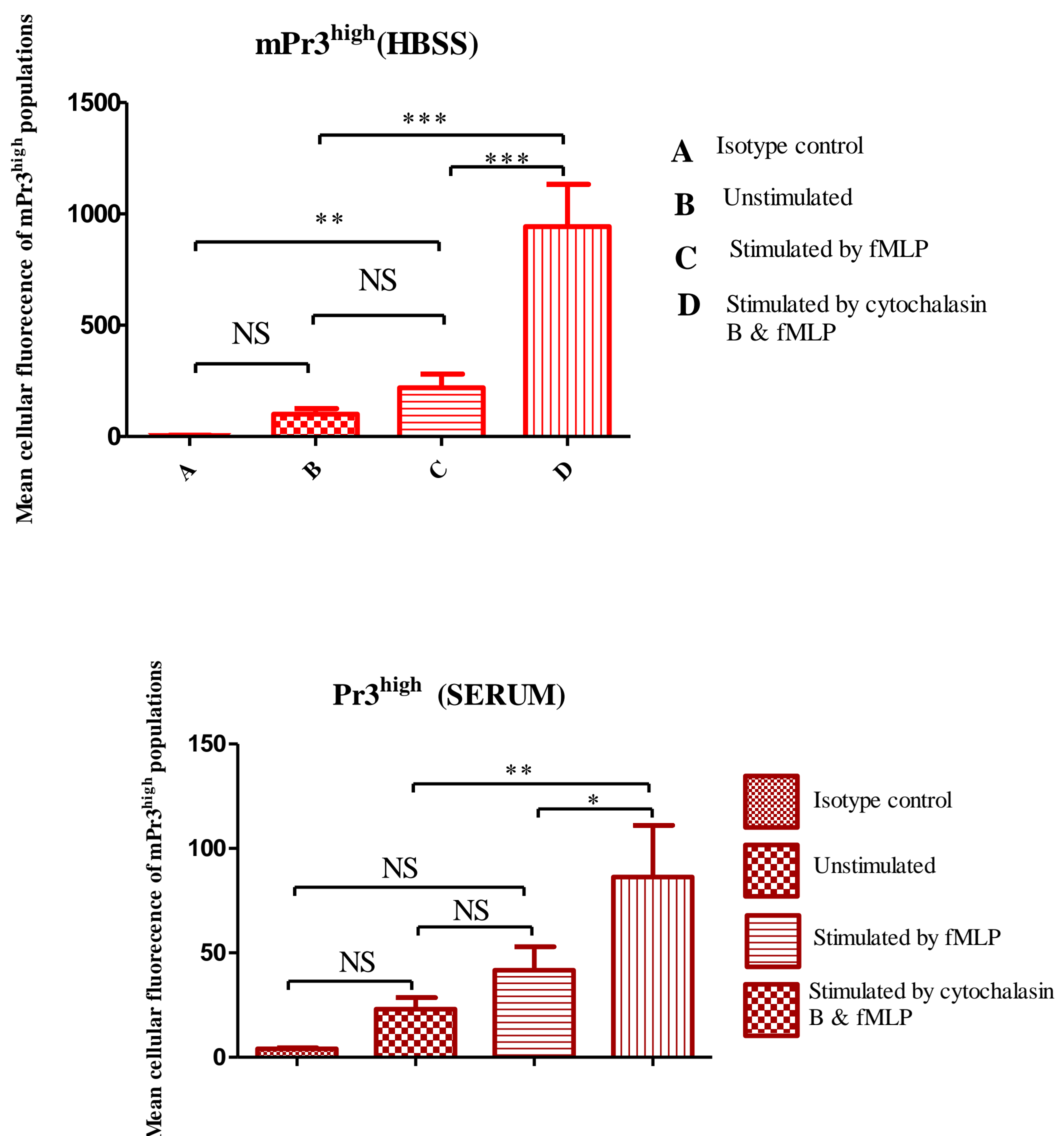

Figure 3. Bar graphs show the mean cellular fluorescence of mPr3^{high} populations in presence of HBSS or serum. Neutrophils were unstimulated and stimulated with fMLP only or cytoB in combination with fMLP (n=7).

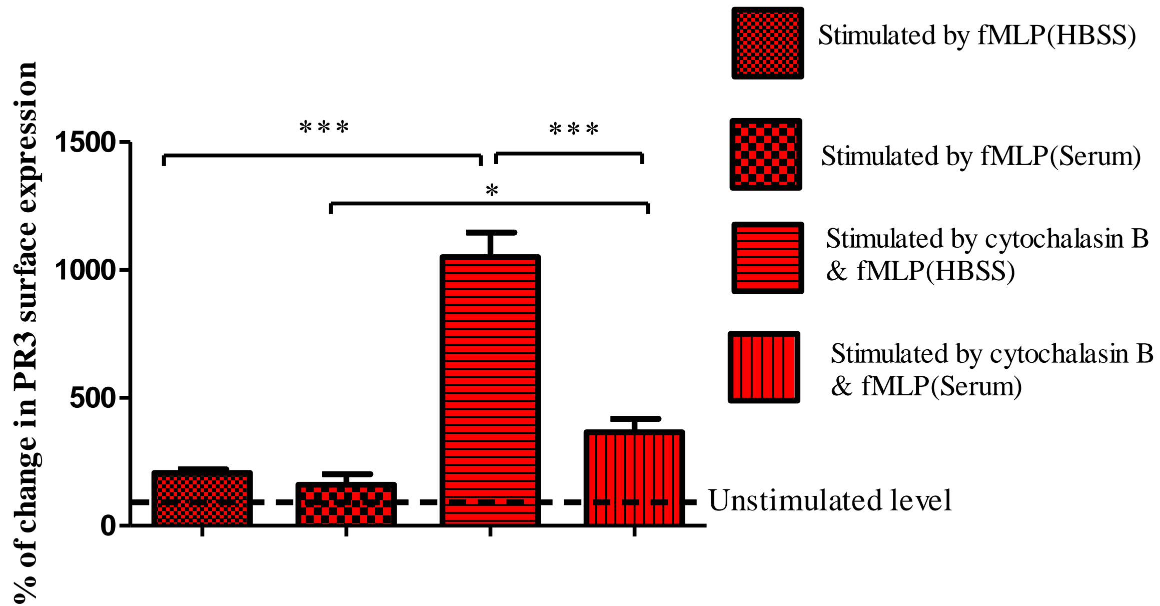

Figure 4. The percentage of change in Pr3 surface expression in presence of HBSS or serum. The percentage of change is shown by dividing the level of detected mPr3 on surface of stimulated cells (with fmlp only or in combination with cytoB) by the amount of mPr3 detected on unstimulated cells. Data expressed as mean, error bars demonstrate SEM, *= p< 0.05; ***=p<0.001 (n=7).

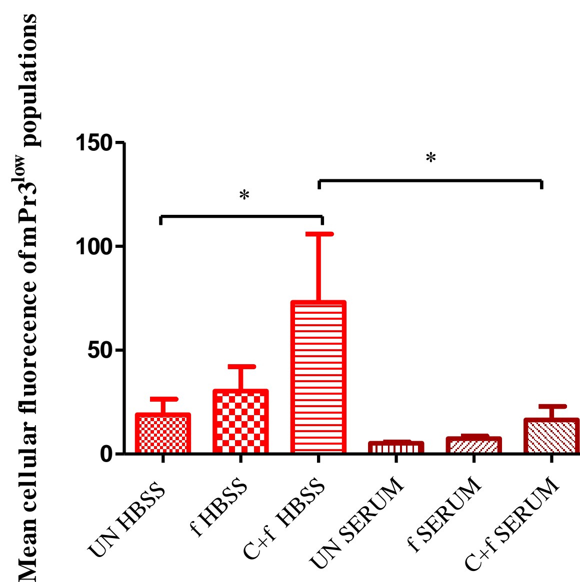

Figure 5. Percentage of Pr3 expressing neutrophils ($Pr3^{low}$ neutrophils) before (UN) stimulation with fMLP (f) or with cytoB & fMLP (C+f) in presence and absence of serum. The data are presented as mean cellular fluorescence ± SEM for five healthy donors (P=0.03).

These results indicate that serum (likely due to the AAT present) at physiological concentrations can reduce or remove Pr3 from the surface of neutrophils; however, for cells that express high levels of Pr3, this reduction does not result in complete loss of surface Pr3, whereas the decrease in Pr3 on the surface on $mPr3^{low}$ sub-populations result in a decrease below measurable residual amounts. Whether these surface levels of Pr3 that remain in the presence of serum retain enzymatic activity is unknown.

As mentioned previously, the levels of CD177 showed no change in expression in the presence of serum, compared with the absence of serum as seen in figure 3.6. Moreover, the statistical analysis shows no significant difference in expression of $CD177^{low}$ and $CD177^{high}$ between the two conditions, despite the significant increase in CD177 expression observed after stimulation with cytochalasin B & fMLP in presence or absence of serum (figure 6). These data indicate that serum (most likely AAT contained within) acts by reducing Pr3 surface expression by dissociating the Pr3/CD177complex rather than removing the Pr3/CD177 complex.

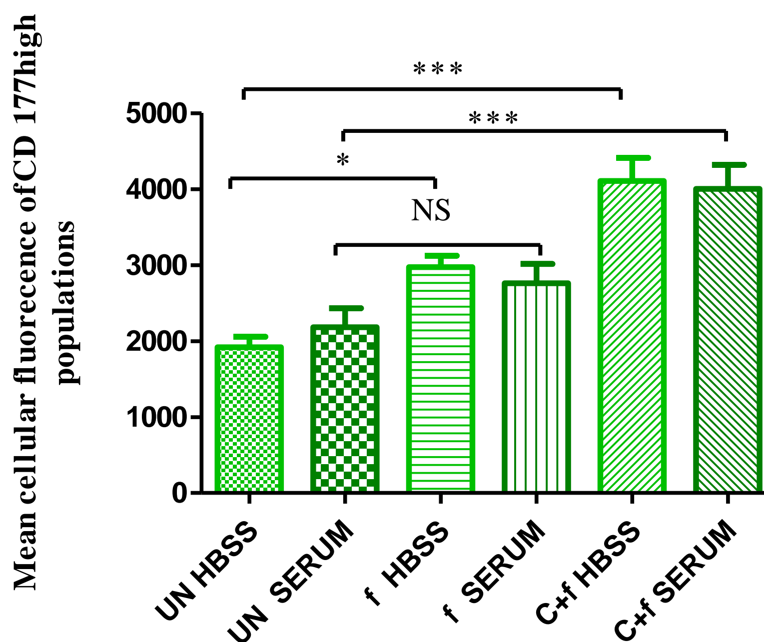

Figure 6. Comparison of CD177 expression populations in presence of HBSS or serum. Mean cellular fluorescence of $CD177^{low}$ (A) and $CD177^{high}$ (B) expression as measured by flow cytometry. Data expressed as mean, error bars demonstrate SEM, *= $p< 0.05$; **=$p<0.01$; ***=$p<0.001$ (n=7). UN= unstimulated; f= fMLP stimulated; C+f= cytochalasin B and fMLP stimulated.

Discussion

In this study, we analysed the surface expression of CD177 and mPr3 in the presence of physiological inhibitors present in serum before and after neutrophil stimulation. It was confirmed that circulating human neutrophils have a bimodal distribution of Pr3 and CD177 on their plasma membrane which varies between individuals (13,14,15).

We did find that not all individuals have bimodal distribution of Pr3, but that these cells sometimes separated into a low and high mPr3 population after maximal stimulation. Hu and co-workers has demonstrated that isolated adult neutrophil samples change from monomodal to bimodal mPr3 expression following 15 minutes with TNF-α (16). In contrast all CD177 expression was bimodal irrespective of stimulation. A study by Halbwachs-Mercarelli *et al.,* 1995 (17) demonstrated that the proportion of freshly isolated neutrophils that expresses Pr3 varies considerably between donors (0–95%), but is extremely stable for each individual over prolonged periods of time.

Our data enhance the earlier funding that Pr3 is present on the surface of quiescent neutrophils (17,18). These results go against the findings of Yang *et al.,* (19) who state that circulating neutrophils do not demonstrate mPr3. Many studies have found that the expression of Pr3 on CD177-positive neutrophil cell surfaces is upregulated by multiple proinflammatory mediators including TNF-α, PMA, LPS, fMLP and GMCSF (20,17,4,21, 22). Pr3 is a Neutrophil Serine proteases (NSP) that is stored in the granules of circulating neutrophils. Moreover, it is located within secretory vesicles that readily fuse with the plasma membrane.

CD177 is also found in the same intracellular compartments as Pr3 (secondary granule and secretory vesicles) and this supports the hypothesis that Pr3 and CD177 may share the same mode of trafficking (23,13). However, CD177 is also found in high abundance on the cell surface of some neutrophils, raising the possibility that intracellular CD177 and Pr3 translocate individually from different sources and associate at the membrane surface. The low levels of mPr3 and CD177 may translocate to the membrane as an already preformed complex from an intracellular pool; however, most of the surface CD177 is not associated with Pr3. So whether these complexes dissociate at the cell surface and following stimulation, Pr3 binds again to CD177 is unknown (24,25). The hydrophobic patch predicted to be on the surface of the Pr3 molecule was shown to be important for its CD177 binding (9) .The higher percentage of mPr3/CD177-double positive neutrophil is associated with an increased risk for

and worse outcome of ANCA vasculitis. Therefore, dissociation of Pr3 and CD177, or disrupting Pr3/CD177 complexes, would have obvious therapeutic implication for Pr3/ANCA-mediated neutrophil activation.

Pr3 activity is inhibited by variety of natural inhibitors, such as AAT (a major physiological proteinase inhibitor in serum) and elafin. AAT binds covalently to all serine proteases and therefore exhibits effects that go beyond the Pr3-CD177 interaction. A previous study by Korkmaz *et al.* (8) has demonstrated that addition of purified AAT to Pr3-bound CD177 transfected CHO cells completely removed Pr3 from the surface of CD177 receptor-expressing CHO cells. Our results illustrate that serum has no effect on the levels of CD177 expression before or after stimulation for either $CD177^{low}$ or $CD177^{high}$ sub-populations. Levels of mPr3 on unstimulated neutrophils in HBSS and serum were similar (very low), while a significant reduction in mPr3 was noted following stimulation in the presence of serum when compared to cells stimulated in the absence of serum.

This suggests that Pr3 upregulated to the cell membrane following stimulation may be more easily removed than Pr3 that is already bound to the membrane before stimulation. Nonetheless, one should note that there is still a significant increase in the $mPr3^{high}$ population following stimulation in the presence of serum (which reflects the physiological conditions of neutrophil stimulation) indicating that regardless of the presence of physiological inhibitors, increased surface Pr3 would still occur with stimulation and may play an important role in the physiological processes of neutrophils. However, we have not ruled out the possibility that other molecules in serum may have protected the mPr3, or inhibited AAT function, or that given the bait-loop mechanism of AAT that the surface mPr3 may lack enzymatic activity. Our result also indicated that the inhibitors in serum disassociated Pr3 only from the surface of neutrophils without removing the expression of the high affinity receptor (CD177). Korkmaz *et al.* (12) reported that constitutive mPr3 (mPr3 on resting neutrophils) is inactive and is not able to interacting with AAT so it can remain at the surface of quiescent circulating neutrophils even in the presence of huge amounts of inhibitor. In addition they suggested induced mPr3 (mPr3 after priming or stimulation of neutrophils) is inhibited and removed by AAT as was shown using cells that stably expressed the CD177 receptor. However, as I mention above stimulating neutrophil in presence of serum showed significant increase in Pr3 expression which suggested that AAT is not able to remove all induced mPr3.

Summary

In this study we have presented that mPr3 is still detectable on the surface of neutrophils in the presence of serum but it showed a marked decrease relative to levels bound in the absence of serum. Moreover the levels of mPr3 on stimulated cells (with fMLP or cytochalasin B + fMLP) in serum still demonstrated binding above background staining levels. This indicates that regardless of the presence of physiological inhibitors, Pr3 bound to the cell surface of neutrophils retains its presence, and because of that it may play an important role in the physiologic processes of neutrophils. In addition, the levels of CD177 showed no change in expression in the presence of serum, compared with the absence of serum, demonstrating decreased Pr3 surface staining is not an artefact of failure to detect surface neutrophil markers and that serum enhances the dissociation from the high affinity surface receptor. The results published by different research groups may be differentially influenced the method of neutrophil preparation which may alter mPr3 surface expression and CD16 shedding.

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