Neutrophils from vasculitis patients exhibit an increased propensity for activation by anti-neutrophil cytoplasmic antibodies

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Neutrophils from vasculitis patients exhibit an increased propensity for activation by anti-neutrophil cytoplasmic antibodies

Short title: ANCA stimulation of neutrophils

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Key words: Autoimmunity, ANCA associated vasculitis, reactive oxygen species, degranulation.

Abbreviations: ANCA associated vasculitis (AAV), anti-neutrophil cytoplasmic antibody (ANCA), Birmingham vasculitis activity score (BVAS), eosinophilic granulomatosis with polyangiitis (EGPA), granulomatosis with polyangiitis (GPA), healthy control (HC), microscopic polyangiitis (MPA), myeloperoxidase (MPO), neutrophil extracellular trap (NET), phorbol 12-myristate 13-acetate (PMA), polymorphonuclear leukocyte (PMN), proteinase 3 (PR3), reactive oxygen species (ROS), superoxide dismutase (SOD), tumour necrosis factor alpha (TNFα).

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Summary

Anti-neutrophil cytoplasmic antibodies (ANCA) are thought to be pathogenic in ANCA associated vasculitis (AAV) by stimulating polymorphonuclear leukocytes (PMNs) to degranulate and produce reactive oxygen species (ROS). The aim of this study was to investigate if PMNs from AAV patients are more readily stimulated by ANCA compared with PMNs from healthy controls (HCs). Differences in ANCA characteristics that can account for different stimulation potential were also studied.

PMNs from 5 AAV patients and 5 HCs were stimulated with 10 different IgGs, purified from PR3-ANCA positive patients, and ROS production, degranulation and neutrophil extracellular trap (NET) formation was measured. ANCA levels, affinity, and clinical data of the AAV donors were recorded. The results show that PMNs from AAV patients produce more intracellular ROS (p=0.019), but degranulate to a similar extent as PMNs from HCs. ROS production correlated with NET formation. Factors that may influence the ability of ANCA to activate PMNs include affinity and specificity for N-terminal epitopes. In conclusion, our results indicate that PMNs from AAV patients in remission behave quite similar to HC PMNs, with the exception of a greater intracellular ROS production. This could contribute to more extensive NET formation and thus an increased exposure of the ANCA autoantigens to the immune system.
**Introduction**

ANCA Associated Vasculitides (AAV) is a group of autoimmune diseases comprising granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA). They are characterized by autoantibodies against the neutrophil granule proteins proteinase 3 (PR3) or myeloperoxidase (MPO). These anti-neutrophil cytoplasmic antibodies, or ANCAs, can activate primed neutrophils to degranulate and produce reactive oxygen species (ROS) [1], induce formation of Neutrophil Extracellular Traps (NETs) [2] and mediate the release of microparticles from polymorphonuclear leukocytes (PMNs) [3]. The exact mechanisms are not completely understood, but activation by ANCA seems to involve both the Fc portion of the antibody, as well as the Fab portion binding to its antigen on the neutrophil surface [4,5]. However, there are also reports indicating that ANCA are internalized into the PMNs after binding, but the importance of this remains unknown [6]. Activation of neutrophils by ANCA is thought to play a major role in the pathogenesis and progress of these diseases. The pathogenicity of ANCA was first proved in vivo in a MPO-ANCA mouse model [7]. More recently, a PR3-ANCA mouse model has been described [8]. However, ANCA levels alone cannot predict relapses conclusively [9], and there is an unmet need for better methods to assess the degree of disease and risk of relapse. Several attempts have been made to map both MPO [10] and PR3 for particularly harmful epitopes, and several conformational epitopes have been found [11,12]. This information will be of importance for understanding the underlying mechanisms of these diseases and could also lead to better methods for assessing the disease course. Furthermore, IgG subclass specificity also matters for ANCA-mediated activation of the neutrophil [13].
To our knowledge there are no systematic studies of PMNs from AAV patients and their response to ANCA stimulation. In this article we aimed to investigate if IgG from PR3-ANCA positive patients more or less easily activates neutrophils from AAV patients compared with neutrophils from healthy donors. Furthermore we characterize IgG purified from different patients to determine why some ANCA are more potent activators than others.
Material and Methods

Blood samples and patients

PMNs were isolated from PR3-ANCA positive AAV patients in stable clinical remission, as assessed by a Birmingham Vasculitis Activity Score (BVAS) of 0 [14]. Serum samples for IgG purification were obtained from AAV patients with PR3-ANCA specificity and varying activity. The PR3-ANCA levels in a standard direct ELISA (Wieslab) ranged between 6-991 IU/mL (Table 1). Healthy control (HC) IgG was used as negative controls in some assays. Written informed consent was retrieved from all donors and these studies were conducted with permission from the Ethical Review Board, Lund, Sweden.

Table 1. Clinical and biochemical characteristics of IgG preparations from AAV patients.

<table>
<thead>
<tr>
<th>IgG</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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<tr>
<td>BVAS</td>
<td>18</td>
<td>12</td>
<td>6</td>
<td>19</td>
<td>17</td>
<td>16</td>
<td>63</td>
<td>63</td>
<td>15</td>
<td>16</td>
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<tr>
<td>Age (years)</td>
<td>45.6</td>
<td>56.9</td>
<td>38.6</td>
<td>67.0</td>
<td>71.4</td>
<td>58.9</td>
<td>65.8</td>
<td>55.8</td>
<td>75.1</td>
<td>59.1</td>
</tr>
<tr>
<td>Direct ELISA PR3-ANCA (IU/ml)</td>
<td>991</td>
<td>63</td>
<td>6</td>
<td>12</td>
<td>63</td>
<td>19</td>
<td>35</td>
<td>64</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>Capture ELISA PR3-ANCA (IU/ml)</td>
<td>745</td>
<td>320</td>
<td>179</td>
<td>0</td>
<td>590</td>
<td>203</td>
<td>188</td>
<td>320</td>
<td>142</td>
<td>96</td>
</tr>
<tr>
<td>Affinity (ml/µg)</td>
<td>15.4</td>
<td>11.1</td>
<td>2.5</td>
<td>2.8</td>
<td>2.1</td>
<td>2.1</td>
<td>2.7</td>
<td>3.2</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Activation score</td>
<td>99.3</td>
<td>60.5</td>
<td>30.1</td>
<td>34.2</td>
<td>24.1</td>
<td>11.7</td>
<td>25.4</td>
<td>49.3</td>
<td>32.0</td>
<td>47.8</td>
</tr>
</tbody>
</table>

IU/ml: International units/ml; ANCA: anti-neutrophil cytoplasmic antibody; BVAS: Birmingham Vasculitis Activity Score. Activation score represents a summarized value from ROS, degranulation and NET formation assays. See text for details.
IgG purification

Serum or plasma samples were spun down for 5 min at 10,000 g and clear supernatants were added to NAb protein G spin columns (Thermo Scientific, Rockford, IL, USA) that were prepared according to the manufacturer’s instructions. The samples were incubated in room temperature with rotation for 20 min. Columns were washed with binding buffer (20 mM sodium phosphate buffer, pH 7.0) 3 times and eluted with 0.1 M glycine-HCl, pH 2.7. Eluates were neutralized with 0.1 volumes 1 M Na2HPO4, pH 8-9. Buffer was exchanged with PD Miditrap G-25 columns (GE Healthcare, Uppsala, Sweden) to phosphate buffered saline (PBS) without Ca2+ and Mg2+ (Hyclone, South Logan, Utah, USA). IgG were concentrated to 2 mg/mL using Vivaspin 4 mL columns, 10,000 MWCO (Sartorium stedim biotech, Goettingen, Germany). The concentration was measured using a Nanodrop®. IgG aliquots were frozen at -20°C and they were spun down at 12,000 g for 10 min before use to avoid IgG aggregates in the assays.

PMN purification

Heparinized blood was collected and sedimented with 2 % Dextran T500 (Pharmacosmos, Holbæk, Denmark) in 0.9 % NaCl followed by density gradient centrifugation using Lymphoprep™ (Axis-Shield, Oslo, Norway) at 4°C. All subsequent steps were performed at this temperature. The remaining erythrocytes were lysed using water as hypotonic solution for a maximum of 30 s to leave PMNs intact. Ionic concentration was restored by adding 1 volume 1.8 % NaCl.

Membrane PR3

Purified PMNs were stained with monoclonal antibodies against CD177-FITC (Serotech, Stockholm, Sweden) and PR3-Alexa647 (Eurodiagnostica, Malmö, Sweden)
and analysed using a FACSCanto II (BD Biosciences, Stockholm, Sweden) as described previously [15]. Both percentage of PR3 positive cells and MFI of the positive cells were recorded.

**ROS assay**

Purified PMNs were adjusted to 2x10^6 cells/mL in HBSS++ (Hyclone) and primed with tumour necrosis factor alpha (TNFα) (R&D Systems, Abingdon, UK, 2 ng/ML) for 15 min at 37°C followed by Cytochalsin B (Sigma-Aldrich, Steinheim, Germany, 5 µg/mL) for 5 min at 37°C. PMNs were then stimulated with the 10 different purified AAV IgG (200 µg/mL), a monoclonal PR3 antibody (a kind gift from Dr Zhao, Beijing, 5 µg/mL), phorbol 12-myristate 13-acetate (PMA) (Glycotope Biotechnology, Heidelberg, Germany 100 ng/mL) or PBS at 37°C. This was done in duplicate samples in white 96 well polystyrene plates (Thermo Scientific) in the presence of luminol (Sigma-Aldrich, Fluka, 1 µg/mL) and the scavengers superoxide dismutase (SOD) (Sigma-Aldrich, 5 U/mL) and catalase (Sigma-Aldrich, 2400 U/mL) to detect intracellular ROS, or isoluminol (Sigma-Aldrich, 1 µg/mL) alone to detect extracellular ROS. PMNs were stimulated for totally 70 min and luminescence, as a measure of ROS production, was monitored in a microplate reader (Tristar LB941, Berthold Technologies, Bad Wildbad, Germany) every 10 min. Each well was measured for 0.1 s with no filter. The software MicroWin 2000 was used for analysis. We chose to report the value after 20 min stimulation, since this time-point corresponds to the maximum stimulation in most cases (Fig. 1b).

**Degranulation assay**
Purified PMNs were adjusted to 6x10^6 cells/mL in KRG buffer (Krebs-Ringer buffer with glucose: 130 mM NaCl, 5 mM KCl, 1.27 mM MgSO₄, 0.95 mM CaCl₂, 10 mM NaH₂PO₄/Na₂HPO₄, pH 7.4, 5 mM glucose). PMNs were primed with Cytochalasin B (5 µg/mL) for 5 min followed by TNFα (2 ng/mL) for 15 min. The cells were then stimulated with IgG (200 µg/mL) for 15 min. All stimulations were performed at 37°C, gently shaking. As controls, cells stimulated with PMA (4 µg/mL) or only buffer were used. Stimulations were stopped by adding 1 volume ice-cold KRG buffer with 0.5 mM PMSF (Sigma-Aldrich) and spun down at 250g, 6 min at 4°C. Supernatants were collected. Pellets were resuspended in 1 volume ice-cold KRG buffer with 0.5 mM PMSF. 1 volume of Elisa Dilution Buffer (EDB: 0.5 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄/KH₂PO₄, 1% (w/v) BSA, 1% (v/v) Triton-X100, 0.05% NaN₃, pH 7.2) with 0.5 mM PMSF were added to both supernatant and pellet before freezing at -20°C. Release values are calculated as amount of protein in supernatant compared to total amount of protein (in supernatant + pellet) and reported as percentages.

**Degranulation ELISA**

5 different ELISAs, with supernatants and pellets from the degranulation assay, were performed to detect markers of different granula as has been described before [16]. Albumin was used to detect secretory vesicles, gelatinase for gelatinase granula, lactoferrin for specific granula, MPO for azurophilic granula and PR3, mainly present in azurophilic granula but also others.

**Albumin:** Clear 96 well polystyrene plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with rabbit anti albumin IgG (Dako, Glostrup, Denmark, 1:2500) in coating buffer (0.0106 M Na₂CO₃, 0.0393 M NaHCO₃, 0.02 % NaN₃) over night at 4°C. ELISA
plates were blocked in incubation buffer (0.137 M NaCl, 8 mM Na2HPO4 x 2H2O, 2.7 mM KCl, 1.5 mM KH2PO4, 0.02 % NaN3, 0.05 % Tween 20) for 1 h gently shaking at room temperature, all subsequent incubations were done under these conditions. Plates were washed 3 times with ELISA wash (0.9 % NaCl, 0.05 % Tween 20) between each incubation step. Supernatants and pellets were diluted in incubation buffer to 1:10 and 1:20 respectively. A standard curve with two-fold serial dilutions of albumin (Kabivitrum, 250-3.9 ng/mL) was included in each plate. After sample incubation a detection antibody was added: rabbit anti albumin IgG (Dako, 1:2000), biotinylated with NHS-D-Biotin (Sigma-Aldrich) followed by secondary reagent ImmunoPure® Avidin-AP (Thermo Scientific, 1:1000). A coloured product was developed using substrate phosphatase tablets (Sigma-Aldrich) diluted in substrate solution (1 M dietanolamine pH 9.8, 0.5 mM MgCl x 6H2O, 0.02 % NaN3). Absorbance was measured at 405 nm in a plate reader (Epoch, Biotek Instruments, Bad Friedrichshall, Germany). All other ELISAs were performed the same way except that blocking and dilutions were done in EDB unless otherwise stated. Supernatants and pellets were diluted 1:500 and 1:2000 respectively for all following ELISAs. Other specific differences are indicated here:

**Gelatinase:** Antibody for coating were mouse anti MMP-9 IgG1, MAB936, R&D systems, clone 36020, 2 µg/mL. For the standard curve, gelatinase (ENZO, Plymouth Meeting, PA, USA, 5-0.08 ng/mL) was used. The detection antibody was polyclonal goat anti MMP9, biotinylated (R&D systems, 1:2000) and secondary reagent ExtrAvidin® Peroxidase (Sigma-Aldrich. 1:20000, diluted in PBS). Substrate was 3,3’,5,5’-Tetramethyl-benzidine Liquid Substrate (TMB) (Sigma-Aldrich) and absorbance were measured at 370 nm.
**Lactoferrin:** Antibody for coating was polyclonal sheep anti lactoferrin (Biogenesis, Poole, UK, 2µg/mL). Lactoferrin (Wieslab, Lund, Sweden, 200-3.1 ng/mL) was used for the standard curve. Detection antibody: rabbit anti lactoferrin (Sigma-Aldrich, 1:4000), secondary reagent: Goat anti rabbit IgG-AP (Sigma-Aldrich, 1:15000).

**MPO:** Antibody for coating was 2B11 (MPO specific mAB purified from a hybridoma, 1µg/ml). MPO (a kind gift from Dr I. Olsson, haematology dept., Lund University, Sweden, 100-1.56 ng/mL) was used for the standard curve. Detection antibody: polyclonal rabbit anti MPO (Dako, 1:2000), secondary reagent: Swine anti rabbit IgG-AP (Dako, 1:1000).

**PR3:** Antibodies for coating were 4A5 and 4A3 (PR3 specific mABs purified from hybridomas), 1.5µg/mL each. PR3 (Wieslab, 200-3.1 ng/mL) was used for the standard curve. Detection antibody: rabbit anti PR3, affinity purified from serum (Wieslab, 1:1200), secondary reagent: Swine anti rabbit IgG-AP (Dako, 1:1000).

**NET formation assay**

PMNs were isolated from a HC using Percoll density gradient separation (GE Healthcare, Uppsala, Sweden). Cell concentration was adjusted to 0.5x10^6 cells/mL in RPMI 1640 medium (Gibco, Paisley, UK) containing 2 % fetal bovine serum (FBS, Pasching, Austria) and seeded onto a 0.001 % poly-l-lysine (Sigma-Aldrich) pre-treated 96-well plate (Sarstedt, Nümbrecht, Germany). Cells were primed with TNFα (Sigma-Aldrich, 2 ng/mL) for 15 min at 37°C. They were then stimulated with IgG, 200 µg/ml for up to 7 h and extracellular DNA were measured with Sytox Green (Invitrogen, Paisley, UK, 2.5 µg/mL) using a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT, USA). As a negative control, healthy control IgG
(Beriglobin, CSL Behring, Marburg, Germany, 200 µg/mL) was used. PMA (Sigma-Aldrich, 20 nM) was used as a positive control. Each IgG was measured in 1 or 2 experiments with the same PMN donor.

**Activation score**

An activation score was calculated for each IgG using the values for intracellular and extracellular ROS, release of gelatinase and MPO and NET formation. They were normalized, setting the highest value in each assay to 100 % and the lowest to 0 %, and mean values (5 HC and 5 AAV PMNs) for all 5 assays were calculated and regarded as the activation score for each IgG.

**Epitope mapping ELISA**

Clear 96 well polystyrene plates (Maxisorp, Nunc) were coated over night with 0.5 µg/mL of the different recombinant mouse/human chimeric PR3 as previously described [17,18]. The 8 different chimeric proteins consisted of mouse (m) and human (H) sequence in parts of 1/3, 1/2 or 2/3 and named thereafter. For example the chimera HHm consists of 2/3 human parts in the N-terminal region and 1/3 mouse PR3 in the C-terminal region. After washing plates in ELISA wash twice, 50 µg/mL IgG, diluted in incubation buffer with BSA (0.05 % Tween 20, 0.02 % NaN₃, 0.2 % BSA in PBS, pH 7.3-7.4), were added to the plates. All incubations were performed for 1 h at room temperature and thereafter washed twice in ELISA wash. A goat anti human Fc specific AP conjugated antibody (Sigma-Aldrich, 1:7000) was used to detect bound IgG. Substrate phosphatase tablets (Sigma-Aldrich) diluted in substrate solution were used to detect colour change at 405 nm. To be able to compare different chimeras, a
background for each chimera, consisting of the median value of each particular chimera, was subtracted from the ELISA values.

**PR3 affinity ELISA**

All 10 AAV IgG were diluted to give the same absorbance in PR3 ELISA after 1 h development time. The IgG were mixed and preincubated over night at 4°C with increasing concentrations of PR3 (Wieslab, 0 to 2 µg/mL). Clear 96 well polystyrene plates (Maxisorp, Nunc) were coated with 1.5 µg/mL each of the 4A5 and 4A3 mABs in coating buffer over night at 4°C. After blocking the ELISA plates with incubation buffer with BSA, PR3 (Wieslab, 0.5µg/mL) was added to the plates for 1 h at room temperature and then washed 3 times in ELISA wash (the same conditions were employed for all subsequent incubations). The IgG samples with PR3 were then added, followed by a goat anti human Fc specific AP conjugated antibody (Sigma-Aldrich, 1:15000). Substrate phosphatase tablets (Sigma-Aldrich) diluted in substrate solution were used to detect colour change at 405 nm. Absorbance values for each IgG were normalized to the same scale, setting the first value (incubated with 0 µg/mL PR3) to 100 % and last value (incubated with 2 µg/mL PR3) to 0 %. The PR3 concentration of the 50 % absorbance value for each IgG was read and the inverse value of this was interpreted as the affinity for PR3.

**Statistical analysis**

All statistical calculations were done using GraphPad Prism, version 6.0a. To compare groups, Mann-Whitney test was performed. For correlations, Spearman correlation coefficients were calculated.
Results

ANCA stimulation of PMNs

To determine if PMNs from AAV patients are more readily activated by ANCA, ROS assays, measuring intracellular and extracellular ROS, and a degranulation assay were performed in parallel. PMN from each donor (5 AAV and 5 HC) were stimulated with 10 IgGs purified from PR3-ANCA positive AAV patients.

Intracellular ROS production was measured by stimulating primed PMNs with IgG in the presence of luminol together with the scavengers SOD and catalase. The results showed significantly higher intracellular ROS production in AAV patients compared with HC (p=0.019, Mann-Whitney, Fig. 1a). Extracellular ROS was measured using isoluminol instead, since it is not able to cross membranes. The extracellular ROS production did not differ between the two groups. A representative experiment is shown in figure 1b. There was a substantial variation in the amount of intracellular and extracellular ROS obtained with different stimulating IgGs, however there was no significant difference when comparing to what degree the individual IgG stimulated HC and AAV PMNs (Fig. 1c-d).

Degranulation was measured as percentage protein released from activated PMN in relation to total amount of protein detected. No significant differences were seen for any of the five measured granular proteins when comparing PMNs from AAV patients to HCs (Fig. 2a). AAV IgG stimulated HC PMNs to a greater extent than IgG purified from HC sera (Fig. 2b). The variation in the individual 10 AAV IgG preparations is
shown in Fig. 3a-e. There were no significant differences between AAV and HC PMNs when studying individual AAV IgG stimulations.

The ability of the purified AAV IgG to induce NET formation in HC PMNs was determined by measuring the release of extracellular DNA using a fluorometric assay. The ability to induce DNA release differed considerably between individual IgG preparations (Fig. 4a). NET formation correlated with ROS production (intracellular ROS: \( r_s = 0.733, p = 0.031 \) and extracellular ROS: \( r_s = 0.750, p = 0.026 \)) (Fig. 4b-c).

To be able to compare the activation potential of the different AAV IgG preparations, an activation score was constructed based on the levels of intracellular and extracellular ROS, gelatinase and MPO release and NET formation (see materials and methods and table 1 for details). The activation score did not correlate with disease activity, BVAS.

The membrane expression of PR3 (mPR3) did not differ significantly between HCs and AAV PMNs (63% and 67% respectively). No significant associations between the percentages of mPR3 positive PMNs with either ROS production or degranulation (data not shown) were found. Moreover, there was no correlation with MFI of mPR3 positive cells and activation of the PMNs.

**Importance of ANCA levels, epitope specificity and affinity**

A possible source of the variations in PMN activation capacity between different IgG preparations could be differences in the proportion of IgG being ANCA. However, when comparing results from the different functional assays performed above with
ANCA levels, no significant correlations were found. This was true regardless if ANCA was measured with a direct ELISA (data not shown) or with a capture ELISA (Fig. 5a). Commercially available PR3 ELISAs are designed to measure all epitope specificities and previous studies indicate that certain epitope specificities of PR3-ANCA are more pathogenic than others [11,12]. To investigate this, we used 8 different mouse-human chimeric PR3 proteins previously developed by our group [17,18], and tested all IgGs for reactivity towards the different parts of the PR3 molecule. Special emphasis was put on two of the chimeras: HHm (2/3 human sequence starting in the N-terminal part and 1/3 mouse sequence) and Hm (1/2 human sequence, 1/2 mouse sequence) as they include an epitope defined by Silva et al. (designated ‘epitope 1’ by them). This same epitope corresponds to an epitope proposed by Kuhl et al. to be pathogenic, since it seems to interfere with α1-antitrypsin binding and perhaps PR3’s enzymatic activity [11,12]. It includes the amino acids at position 35-39 and 72-78 in the PR3 molecule. When combining the epitope ELISA results from HHm and Hm, we found significant correlations between reactivity to these chimeras and gelatinase release (r_s=0.649, p=0.049) as well as lactoferrin release (r_s=0.661, p=0.044, Fig. 5b-c). No other significant correlations, including the activation score, were found. The epitope assay did not correlate with ROS and NET assays. No correlations were found when any of the eight individual constructs were correlated with any of the activation assays.

Another independent factor possibly influencing the activation of PMNs is affinity, which was measured using a competition ELISA. The IgGs were incubated with increasing concentrations of PR3 over night before tested in a PR3 ELISA. The results show that PR3 affinity correlated with the activation score (r_s=0.758, p=0.015, Fig. 5d)
as well as with total ROS production (luminol assay, $r_s=0.721$, $p=0.023$, data not shown). Extracellular ROS alone, gelatinase and lactoferrin release had a tendency to correlate with affinity, but did not reach statistical significance. There was no correlation between affinity and ANCA levels (as measured by standard commercial PR3 ELISAs), emphasizing the importance of affinity for ANCA-mediated activation of neutrophils.
Discussion

In this study we aimed to decipher whether PMNs from AAV patients behave differently in response to ANCA stimulation compared with PMNs from HCs. We could see an increased intracellular ROS production in comparison with HC PMNs, whereas extracellular ROS production and degranulation were similar. The reason for increased ROS production is unknown, but we speculate that AAV patients’ PMNs, even though in clinical remission, are exposed to a low-grade activation or priming. It has previously been shown that the cytokine profile of patients in active phase as well as remission phase is different from healthy donors [19]. There might be other explanations for this including intrinsic defects in the PMNs in AAV patients. AAV patients have an increased membrane expression of PR3 on their neutrophils and this has been connected to their higher degree of stimulation [20,21]. However, PMNs from AAV patients in this study did not have significantly more PR3 on the surface than the HC PMNs. All patients received low dose glucocorticoids, but that this has any stimulatory effect on ROS production is unlikely, since glucocorticoids have been shown to reduce ROS production in PMNs [22].

Why only intracellular and not extracellular ROS production differed remains elusive and it must be emphasized that our data is based on a small number of observations. The major ROS producing complex in phagocytes is the NADPH oxidase that resides in the plasma membrane (5 %) and in granule membranes (95 %) in neutrophils. Activation of NADPH oxidase in the plasma membrane leads to production of ROS that is released to the extracellular space. NADPH oxidase activation in the phagosome, by fusion of NADPH oxidase containing granules to the phagosome, is also a well-established
defence mechanism, however there also exists intracellular non-phagosomal ROS production that seems to play an important roll for cell signalling and immune regulation [23]. It is also known that the assembly is somewhat different for extracellular and intracellular ROS production. For example the cytosolic component p40phox is needed for intracellular, but not extracellular NADPH oxidase assembly [24]. There is also evidence for involvement of different isoforms of PI3K and PLC in intracellular and extracellular ROS production, suggesting that different stimuli distinguish between these pathways [24,25]. Moreover, ROS produced in one compartment can stimulate ROS production in another; so-called ROS induced ROS release. If the balance between the antioxidant and oxidant system is disturbed, excessive ROS could possibly initiate a self-perpetuating cycle leading to more damage [26].

NET formation has previously been described to be dependent on ROS production [27]. A role for NET formation in AAV has also been proposed since purified AAV IgG was found to induce more NET formation compared with HC IgG. In the same study it was also shown that AAV patients had more circulating NET-remnants, in terms of MPO-DNA complexes [2]. Our data support this finding since NET formation correlates with ROS release. Increased NET formation may be of importance for the development of ANCA, since NETs give rise to an increased antigen exposure for the immune system. This could, in a pro-inflammatory milieu, supposedly break tolerance towards PR3 or MPO.
Our 10 different AAV IgGs exhibited different activation potential on PMNs. It is highly unlikely that this is due to differences in cytokine profiles [19] or different treatment regimens since the IgG was purified and soluble factors would be eliminated. Moreover, the activity of the patients, as measured by BVAS, from whom the IgG was purified, did not influence the results in any of our assays. More surprisingly, ANCA levels assessed by standard direct PR3-ANCA or capture PR3-ANCA ELISAs did not correlate with any of our data. On the other hand, the antibody affinity for PR3 correlated with the activation score and this indicates that ANCA affinity is more important than ANCA levels for PMN activation. A previously identified epitope region in the PR3 molecule has been suggested to be of importance for AAV [11,12]. In this study, we could confirm this, since degranulation correlated with reactivity to our chimeras that included this epitope. These results suggest that ANCA with certain epitope specificities are more potent PMN activators than others and that these epitopes are situated in the N-terminal region of PR3.

In conclusion, PMNs from AAV patients show an increased intracellular ROS production, but otherwise behave in a similar way as PMNs from HCs, opening the door for further studies within the field of cell signalling and involvement of ROS pathophysiology in these diseases.

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**Conflict of interest**

The authors declare no conflict of interest.
References


Fig. 1. ROS activation of PMNs. (a) PMNs from AAV patients (n=5) and healthy controls (HCs) (n=5) stimulated with 10 AAV IgG at 200 µg/mL. The bars represent mean values of 10 stimulations read after 20 min incubation. PBS is the negative control. AAV PMNs gave rise to more intracellular ROS production (measured with luminol + scavengers) than PMNs from HCs (p=0.019). Extracellular ROS was measured with isoluminol. (b) Representative graph showing ROS production over time in PMNs (from an AAV patient, filled circles, from a HC open circles) stimulated with one representative AAV IgG. Filled lines represent extracellular ROS and dotted lines intracellular ROS. (c-d) The characteristic of each IgG is shown with a mean value of 5 experiments in each donor group (HC and AAV PMNs). PMA is excluded from extracellular ROS graphs for clarity since these values were much higher than for IgG stimulations. There were no significant difference between HC and AAV PMNs in any individual IgG. RLU is relative luminescence units. Statistics were calculated using the Mann-Whitney test. Error bars represent standard deviations.
Fig. 2. Degranulation. (a) Degranulation of 5 different granula markers after stimulation of PMNs from AAV patients (n=5) and healthy controls (HCs (n=5)) with 10 AAV IgG. No significant differences were detected. 37 ctrl represents PMNs from both HC and AAV PMN donors that were stimulated with buffer only at 37°C. Release is the amount of protein in supernatant compared with total amount of protein. (b). AAV IgG compared to HC IgG in ability to stimulate HC PMNs to degranulate. The same 10 AAV IgG as in (a) were tested on 5 different HC PMNs (median of 5 is reported) compared to 7 HC IgG (for albumin) or 5 HC IgG (for gelatinase, lactoferrin, PR3 and MPO). Median of several experiments with the same IgG is reported in cases where the same IgG had been tested on several HC PMNs. All different granula markers were released to a higher degree when stimulated with AAV IgG compared to HC IgG: albumin (p=0.0097), gelatinase (p=0.0027), lactoferrin (p=0.0007), PR3 (p=0.0047), MPO (p=0.0007). Statistics were calculated using the Mann-Whitney test. Error bars represent standard deviations.
Fig. 3. (a-e) Degranulation characteristics of the 10 different AAV IgG preparations. Each bar represents a mean value of 5 experiments in each donor group (HC and AAV PMNs). No significant difference was seen between HC and AAV PMNs for any IgG. PMA was used as a positive control. 37 ctrl represents PMNs that were stimulated with buffer only at 37°C. Statistics were calculated using the Mann-Whitney test. Error bars represent standard deviations.
Fig. 4. NET formation. (a) NET formation measured as the degree of extracellular DNA after stimulation with AAV IgG in a fluorometric assay. 1 or 2 experiments per IgG were performed and in cases of 2, a mean value of these is shown. IgG number 4 is missing from NET formation experiments. (b-c) Intracellular ROS (luminol +scavengers) and extracellular ROS (isoluminol) correlates with NET formation; $r_s=0.733$, $p=0.031$ and $r_s=0.750$, $p=0.026$, respectively. Spearman correlation coefficient, $r_s$ was calculated.
Fig. 5. (a) Activation score do not correlate with capture PR3-ANCA levels. (b-c) Gelatinase and lactoferrin degranulation correlates with the human-mouse chimeric PR3 proteins HHm and Hm added together; $r_s=0.649$, $p=0.049$ and $r_s=0.661$, $p=0.044$, respectively. (d) Activation score correlates with PR3 affinity: $r_s=0.758$, $p=0.015$. Spearman correlation coefficient, $r_s$ was calculated.