Antineutrophil Cytoplasmic Antibodies Induce Monocyte IL-8 Release
Role of Surface Proteinase-3, α1-antitrypsin, and Fcγ Receptors

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Abstract
Cytoplasmic antineutrophil cytoplasmic antibodies (cANCA) that accompany the neutrophilic vasculitis seen in Wegener’s granulomatosis (WG), are directed against proteinase-3 (PR-3), a serine proteinase which is located in azurophilic granules of neutrophils and monocytes. PR-3, when expressed on the surface of TNFα–primed neutrophils, can directly activate neutrophils by complexing cANCA and promoting concomitant Fcγ receptor (FcγR) cross-linking. Although the neutrophil’s pathogenic role in WG has been studied, the role of the monocyte has not been explored. The monocyte, with its ability to release cytokines and regulate neutrophil influx, also expresses PR-3. Therefore, the monocyte may play a significant role in WG via the interaction of surface PR-3 with cANCA, inducing cytokine release by the monocyte. To test this hypothesis, monocytes were studied for PR-3 expression and for IL-8 release in response to cANCA IgG. PBMC obtained from healthy donors displayed dramatic surface PR-3 expression as detected by immunohistochemistry and flow cytometry in response to 0.5-h pulse with TNFα (2 ng/ml). Purified monoclonal anti–PR-3 IgG added to TNFα–primed PBMC induced 45-fold more IL-8 release than an isotype control antibody. Furthermore, alpha 1–antitrypsin (α1-AT), the primary PR-3 antiproteinase, inhibited the anti–PR-3 induced IL-8 release by 80%. Importantly, Fab and F(ab)2 fragments of anti–PR-3 IgG, which do not result in Fcγ receptor cross-linking, do not induce IL-8 release. As a correlate, IgG isolated from cANCA positive patients with WG induced six times as much PBMC IL-8 release as compared to IgG isolated from normal healthy volunteers. Consistent with PR-3 associated IL-8 induction, α1-AT significantly inhibited this effect. These observations suggest that cANCA may recruit and target neutrophils through promoting monocyte IL-8 release. This induction is mediated via Fcγ receptor cross-linking and is regulated in part by α1-AT. (J. Clin. Invest. 1997. 100:1416–1424.)

Key words: Wegener’s granulomatosis • serine proteinase • chemokine • receptor cross-linking

Introduction
Wegener’s granulomatosis (WG) is a disease distinguished pathologically by a disseminated necrotizing granulomatous vasculitis, typically involving the upper and lower respiratory tracts and the kidney. WG is characterized by the production of IgG auto-antibodies against cytoplasmic components of neutrophils. These specific cytoplasmic antineutrophil cytoplasmic antibodies (cANCA) are directed against proteinase-3 (PR-3), a protease located in azurophilic granules of neutrophils and monocytes, and expressed on the surface of TNFα–stimulated neutrophils (1, 2). The interaction of cANCA with the surface-expressed PR-3 on neutrophils results in neutrophil activation, as evidenced by the release of oxygen radicals, via concurrent binding of the surface of PR-3 and FcγRIIa receptors (1, 3). Importantly, alpha 1–antitrypsin (α1-AT) is the primary serum protein that complexes PR-3, and recent studies have also associated cANCA with α1-AT deficiency (4, 5).

Although the pathogenic role of the neutrophil has been explored in WG, the role of the monocyte has yet to be evaluated. The monocyte is a potent regulator and producer of cytokines, especially when stimulated via endotoxin or Fcγ receptor cross-linking (6). In this context, we have demonstrated that FcγR cross-linking produces IL-8 in monocytes (7). Therefore, cANCA binding to PR-3 expressed on the surface of the monocyte, may result in FcγR cross-linking and subsequent IL-8 release. IL-8, a powerful neutrophil chemoattractant and hallmark of many inflammatory diseases (8, 9), may be critical to the pathogenesis of the vasculitis that defines WG. Thus, we hypothesize that cANCA plays a pivotal role in inducing monocyte IL-8 release by binding to monocyte PR-3 and subsequently cross-linking Fcγ receptors.

Methods

Cell purification
Peripheral blood mononuclear cells (PBMC). PBMC were purified from normal, healthy volunteers. Heparinized (heparin sodium, 15 U/ml; Elkins-Sinn, Inc., Cherry Hill, NJ) blood was obtained (60 ml), and PBMC were purified using polysucrose/sodium diatrizoate (Histoaque; Sigma Diagnostics, St. Louis, MO) density gradient centrifugation. The PBMC were counted, washed, and resuspended at a concentration of 5 × 10^6/ml in RPMI 1640 (BioWhittaker, Walkersville, MD)/5% FBS (HyClone, Logan, UT) with 10 μg/ml of polymyxin B (Roerig Pfizer, New York) to bind any contaminating endotoxin. By flow cytometry analysis, the composition of the PBMC consisted of 20% monocytes and 80% lymphocytes.

Cell culture conditions
Anti–PR-3 antibody induces IL-8 release by primed monocytes. PBMC were either cultured alone (unprimed cells) or with 2 ng/ml

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Abbreviations used in this paper: α1-AT, alpha 1–antitrypsin; cANCA, cytoplasmic antineutrophil cytoplasmic antibodies; PR-3, proteinase-3; TBS, Tris-buffered saline; WG, Wegener’s granulomatosis.

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TNFα (gift from Knoll Pharmaceutical, Whippany, NJ) for 0.5 h (primed cells). Both the primed cells and the unprimed cells, were washed with RPMI 1640 after 0.5 h to rid the cells of TNFα, and new media was added. An IgG, mouse monoclonal antibody to proteinase-3 (aPR-3; Research Diagnostics Inc., Flanders, NJ) was treated to remove contaminating endotoxin using End-X B-15 beads (Associates of Cape Cod Inc., Woods Hole, MA), and after treatment was confirmed to have < 10 µg/ml endotoxin by chromogenic limulus amebocyte lysate assay (Associates of Cape Cod Inc.). The monoclonal anti–PR-3 antibody was added to the primed and unprimed PBMC at a concentration of 1 µg/ml, while MOPC 21 (Sigma Diagnostics), a mouse monoclonal IgG, isotype control (1 µg/ml), was also added to primed and unprimed PBMC as a control. The PBMC incubated with either anti–PR-3 IgG or the isotype control IgG were suspended in RPMI 1640/5% heat-inactivated FCS/polyvinyl B (10 µg/ml) and incubated overnight at 37°C with 5% CO₂. The supernatants were removed and IL-8 release was measured by standard ELISA.

Released IL-8 induced by anti–PR-3 antibody is newly synthesized and time dependent. To determine if the IL-8 released by the primed mononuclear cells was newly synthesized, and to further evaluate the kinetics of this release, PBMC were isolated and primed with TNFα for 0.5 h as described above. After washing, either isotype control IgG (1 µg/ml), anti–PR-3 antibody (1 µg/ml), or anti–PR-3 antibody (1 µg/ml) and cycloheximide (1 µg/ml) (Sigma Chemical Co., St. Louis, MO) was added to the primed PBMC. Supernatants and cells were harvested at time points 0, 0.5, 1, 2, 4, 8, 16, and 24 h during the incubation. The cells were lysed in Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0]) containing 1-µg/ml aprotinin, 1-µg/ml leupeptin, 1-µM phenylmethylsulfonyl fluoride, and 1-µg/ml Pepstatin A. IL-8 of the supernatants and lysates was then measured by ELISA.

Specificity of anti–PR-3 antibody induction of monocyte IL-8 production. In order to evaluate if monocytes are vulnerable to activation by other antibodies since they are primed, studies were performed to determine the specificity of the response to the anti–PR-3 antibody. PBMC were isolated and cultured and then washed with RPMI 1640, either anti-CD 43 antibody (1 µg/ml) (Pierce Chemical Co., Rockford, IL) or anti–PR-3 antibody was added to the cells and the cells cultured for 16 h. The supernatants were harvested and IL-8 release was measured by standard ELISA.

TNFα dose response to anti–PR-3 antibody by primed monocytes. PBMC were isolated as described above. PBMC were primed with varying doses of TNFα (0.156 µg/ml, 0.312 µg/ml, 0.625 µg/ml, 1.25 µg/ml, 2.5 µg/ml, or 5 µg/ml) for 0.5 h. The cells were then washed with RPMI 1640, and anti–PR-3 antibody was added to them. The cells were then cultured for 16 h, supernatants removed, and IL-8 release was measured by ELISA.

Inhibition of anti–PR-3 antibody induces monocyte IL-8 production by α1-AT. To determine if α1-AT prevented the interaction of surface expressed PR-3 with anti–PR-3 antibody, PBMC were primed with TNFα at 2 ng/ml for 0.5 h. Then, the PBMC were incubated for 1 h at 37°C, 5% CO₂ with or without human pooled α1-AT (Miles Laboratories, Inc., Elkhart, IN) at the physiological concentration of 2 mg/ml. Next, mononclonal anti–PR-3 (1 µg/ml) was added to the cells that were cultured for 16 additional hours at 37°C, 5% CO₂. After the incubation, the cell-free supernatants were harvested and evaluated for IL-8 by ELISA.

IL-8 ELISA
IL-8 release was measured by a sandwich ELISA developed in our laboratory as described previously (7). Briefly, the ELISA uses a mouse monoclonal anti–human IL-8 antibody (R & D Systems, Minneapolis, MN) as the capture antibody and a rabbit anti–human IL-8 antibody (Endogen, Inc., Woburn, MA) to complex the antigen. The complex was detected colorimetrically by an enzymatic reaction between a goat anti–rabbit IgG conjugated to horseradish peroxidase (Sigma Diagnostics) and o-phenylenediamine (OPD) (Sigma Diagnostics). The ELISAs were read by comparison to human recombinant IL-8 (R&D Systems), using best fit software (Immunosoft; Dynatech Laboratories, Inc., Chantilly, VA) on an Apple Ile personal computer. Neither anti–PR-3 IgG or α1-AT interfered with ELISA readings when tested alone.

Immunohistochemistry
PBMC (5 × 10⁶/ml) that had been primed for 0.5 h with TNFα (2 ng/ml), or unprimed, were deposited on a microscope slide by cytoprep centrifugation. The slides were fixed for 10 min in acetone at 4°C and then hydrated for 5 min with Tris-buffered saline (TBS) (0.05 M, pH 7.6). Horse serum (20%) was placed on the cells for 10 min at 37°C in a humidity chamber to block nonspecific binding of the antibody. Iso- type control IgG (10 µg/ml) or anti–PR-3 IgG (10 µg/ml) was placed on the cells and incubated in the humidity chamber at 37°C for 30 min. The slides were washed with TBS, incubated with a biotinylated anti–mouse IgG (Vector Laboratories, Inc., Burlingame, CA) (dilution of 1:200) at 37°C for 10 min in a humidity chamber, and then washed again with TBS. Horseradish peroxidase avidin D conjugate was added to the cells and incubated at 37°C for 10 min in a humidity chamber. After being washed with TBS, the slides were then placed in an acetate buffer solution (0.02 M, pH 5.2) at 20°C for 5 min. The slides were then developed in 3-amin-9-ethylcarbazole, which had been activated with 1% hydrogen peroxide, for 1 min. The development was inhibited by the acetate buffer. The cells were then counterstained with hematoxylin for 1 min, and the slides fixed with glycerin jelly.

Flow cytometry
PBMC (5 × 10⁶/ml), either primed with TNFα for 0.5 h or unprimed, were prepared for flow cytometry analysis to ensure surface expression of PR-3. After the priming, the cells were washed with centrifugation (3,500 g) for 1 min. The cells were then washed gently three times with 1% FBS. Human IgG (Sigma Diagnostics) (30 mg/ml) was incubated with the cells for 15 min at 4°C. Subsequently, anti–PR-3 IgG (40 µg/ml), or isotype control IgG (40 µg/ml), or Fab or F(ab’); fragments of anti–PR-3 IgG or isotype control (5 µg/ml) was added to the PBMC and incubated for 1 h at 4°C. Separately, primed and unprimed PBMC were cultured with α1-AT (2 mg/ml) 1 h before the addition of the anti–PR-3 IgG. Next, the cells were again washed with 1% FBS, and goat anti–mouse fluorescein isothiocyanate conjugated antibody (Sigma Diagnostics) (1:25) was added to the cells for 0.5 h. After the appropriate time, the cells were washed with 1% FBS and subsequently fixed with 2% paraformaldehyde.

Flow cytometry analysis was performed on the Coulter Elite Flow Cytometer (Coulter Corp., Miami, FL) using 488-nm 15-mw air-cooled Argon laser. A total of 20,000 gated monocytes were discriminated from lymphocytes using forward versus side (90°) light scatter characteristics. Fluorescence light emission was collected with a 525-nm bandpass filter. Data files were stored as list mode FCS 2.0 format and extended analysis was performed using the Coulter Elite Immuno-4 software (Coulter Corp).

Production of anti–PR-3 Fab fragments
To examine if the IL-8 production by mononuclear cells is mediated by FcyR cross-linking, Fab fragments, which lack Fc domains and do not cross-link Fcy receptors, were produced by papain cleavage of either anti–PR-3 IgG or the IgG isotype control. The IgG isotype control or the anti–PR-3 antibody (0.1 mg/ml) was mixed with 50-mM cysteine and 1-mM EDTA. Papain (Sigma Chemical Co.) (10 mg/ml) was added and incubated for 10 h at 37°C. After the prescribed time, iodoacetamide (Sigma Chemical Co.) (75 mM) was added to inactivate the papain.

Iodoacetamide was removed, the inactivated papain, the IgG isotype control with or without inactivated papain, the IgG isotype control Fab fragments, the anti–PR-3 IgG with or without inactivated papain, the IgG isotype control Fab fragments, which lack Fc domains and do not cross-link Fcy receptors, were produced by papain cleavage of either anti–PR-3 IgG or the IgG isotype control. The IgG isotype control or the anti–PR-3 antibody (0.1 mg/ml) was mixed with 50-mM cysteine and 1-mM EDTA. Papain (Sigma Chemical Co.) (10 mg/ml) was added and incubated for 10 h at 37°C. After the prescribed time, iodoacetamide (Sigma Chemical Co.) (75 mM) was added to inactivate the papain. Once Fab fragments were obtained, the inactivated papain, the IgG isotype control with or without inactivated papain, the IgG isotype control Fab fragments, the anti–PR-3 IgG with or without inactivated papain, and the anti–PR-3 Fab fragments were cultured with primed PBMC (5 × 10⁶/ml) for 16 h at 37°C, 5% CO₂. The supernatants were then harvested and a standard IL-8 ELISA performed.

Cytoplasmic Antineutrophil Cytoplasmic Antibodies Induce Monocyte IL-8 Release
The generation of Fab fragments was confirmed by 15% SDS-PAGE (10) and immunoblotting (Bio-Rad Laboratories, Richmond, CA) as described previously (11). The size of the heavy chain was evaluated with a rabbit anti-mouse Fc antibody conjugated with horseradish peroxidase (Pierce Chemical Co.). Bound antibody was detected by a chemiluminescence kit (Amersham International, Little Chalfont, UK) using the manufacturer’s protocol, with the primary antibody at a concentration of 1:100,000. The exposure time was 3 min. The ability of Fab fragments to recognize surface expressed PR-3 on monocytes was confirmed by flow cytometry analysis.

Production of anti–PR-3 F(ab’)_2 fragments

In order to evaluate if the IL-8 production by the monocytes can be mediated by the anti–PR-3 F(ab’)_2 portion of the antibody, anti–PR-3 F(ab’)_2 fragments were produced by ficin cleavage of either anti–PR-3 IgG or the IgG isotype control. Using the IgG F(ab’)_2 Preparation Kit (Pierce Chemical Co.), either IgG isotype control or anti–PR-3 antibody (0.1 mg/ml) was added to the IgG, mild elution buffer. A 2-ml column of immobilized ficin was equilibrated with 1-mM cysteine. Either the IgG isotype control or anti–PR-3 antibody solution was then applied to the ficin column and allowed to digest at 37°C for 20 h. The digest was then eluted, and applied to a protein A column to separate the F(ab’)_2 fragments from undigested IgG and Fc fragments.

Once F(ab’)_2 fragments were obtained, the IgG isotype control, the IgG isotype control F(ab’)_2 fragments, the anti–PR-3 IgG, and the anti–PR-3 F(ab’)_2 fragments were cultured with primed PBMC (5 × 10^6/ml) for 16 h at 37°C, 5% CO_2. The supernatants were then harvested and a standard IL-8 ELISA performed.

The generation of F(ab’)_2 fragments was confirmed by 7.5% SDS-PAGE (10) and immunoblotting (Bio-Rad Laboratories) as described previously (11). The size of the F(ab’)_2 was evaluated with a rabbit anti-mouse F(ab’)_2 antibody conjugated with horseradish peroxidase.

Figure 1. Dose-dependent mononuclear cell release of IL-8 by TNFα. PBMC were primed with TNFα (2 ng/ml) for 0.5 h, washed, and then cultured for 16 h. Supernatants were assayed for IL-8 release by ELISA. The results represent four subjects and are reported as mean±SEM.

Figure 2. (a) Immunohistochemistry demonstrates surface expression of PR-3 on monocytes. Mononuclear cells, either unprimed or primed with TNFα (2 ng/ml) for 0.5 h, were fixed with acetone. The cells were stained with murine IgG1 isotype control or anti–PR-3 IgG followed by biotinylated anti-mouse IgG and horseradish peroxidase avidin D. The slides were then developed with 3-amino-9-ethylcarbazole solution and counterstained with hematoxylin: (A) unprimed monocyte (M) with murine IgG1 isotype control; (B) primed monocyte (M) with murine IgG1 isotype control; (C) unprimed monocyte (M) with anti–PR-3 IgG; note the stained PR-3 located in perinuclear cleft location; and (D) primed monocyte (M) with anti–PR-3 IgG. Note the surface and cytoplasmic staining of the PR-3. The results shown are representative of seven experiments. (b) Flow cytometry demonstrates surface expression of PR-3 on monocytes. PBMC, either unprimed or primed with TNFα (2 ng/ml) for 0.5 h, were analyzed by flow cytometry using (A) IgG Isotype control (40 μg/ml), or (B) anti–PR-3 IgG (40 μg/ml). Separately, α1-AT (2 mg/ml) was incubated with the PBMC 1 h before the addition of anti–PR-3 IgG (C). The results are representative of five experiments.
Monocytes, primed to express surface PR-3, release IL-8 in response to anti-PR-3 IgG

Induction of monocyte surface PR-3. In order to independently study the significance of cANCA as a monocyte activator, it was important to determine whether monocytes could be induced to express PR-3 on their surface without concomitant production of IL-8. After pulse stimulation for 0.5 h with increasing doses of TNFα, PBMC cultured for 16 h demonstrated IL-8 release in a dose-dependent fashion (Fig. 1). From this dose–response curve, it was determined that TNFα at 2 ng/ml for 0.5 h was the optimal dose to induce the monocytes to express surface PR-3 with minimal IL-8 release. Surface PR-3 expression by the TNFα primed monocytes was confirmed by immunohistochemistry (Fig. 2a) and by flow cytometry (Fig. 2b). Flow cytometry demonstrated that 80% of the monocytes were positive for surface PR-3 after 0.5-h stimulation with TNFα (2 ng/ml).

Anti–PR-3 IgG induces PBMC to release IL-8. Next, purified mouse anti–PR-3 IgG or isotype control antibody (1 μg/ml) was added to TNF-pulsed mononuclear cells (5 × 10⁶ cells per milliliter). The monoclonal anti–PR-3 IgG induced 45 times as much IL-8 release as the isotype control and 23 times more IL-8 than unprimed cells (anti–PR-3 IgG with primed cells: 49.5 ± 6.2 ng/ml, isotype control IgG with primed cells: 1.1 ± 0.2 ng/ml, anti–PR-3 IgG with unprimed cells: 2.2 ± 0.7 ng/ml, P < 0.0001) (Fig. 3). This effect was not due to contaminating endotoxin since the concentration of polymyxin B used in these experiments prevented PBMC IL-8 release induced by as much as 0.1 μg/ml of LPS (data not shown). Chromogenic limulus amebocyte lysate assay showed endotoxin to be < 4.5 pg/ml in the anti–PR-3 IgG preparations after End-X treatment. Additionally, the presence of lymphocytes did not appear to affect the anti–PR-3 IgG induced IL-8 release by monocytes, as studies performed on purified monocyte preparations did not differ from results with PBMC. (results not shown).

To document that the released IL-8 is functional, neutrophil chemotaxis was performed. Only anti–PR-3 supernatants attracted neutrophils and this effect was inhibited by the use of anti-IL-8 antibodies. Furthermore, the antigenic IL-8 levels correlated with the amount of chemotaxis (r = 0.94).

Released IL-8 induced by anti–PR-3 antibody is newly synthesized. To confirm that the IL-8 released by the primed monocytes was newly synthesized, anti–PR-3 antibody (1 μg/ml), isotype control antibody (1 μg/ml), or anti-PR-3 antibody (1 μg/ml) and cycloheximide was added to unprimed and primed PBMC. After a 16-h culture, the supernatants were harvested, and cells were lysed. The lysates did not contain IL-8 as compared to the supernatants (0 ng/ml versus 143.2 ng/ml) (Fig. 4A). In addition, the presence of cycloheximide, a known inhibitor of new protein synthesis, completely inhibited the anti–PR-3 induced IL-8 release by the monocytes.

To further evaluate the kinetics of the IL-8 release, PBMC, primed with TNFα, were cultured with either anti–PR-3 IgG (1 μg/ml) or isotype control IgG (1 μg/ml), and supernatants were harvested at multiple time points. The anti–PR-3 IgG–induced IL-8 release is first detected at 2 h and continues to steadily increase over the 24 h of the study. (Fig. 4b).

Anti–PR-3 antibody induction of monocyte IL-8 release is antibody specific. In order to evaluate if priming predisposes monocytes to IL-8 release in response to antibodies directed against other surface proteins, unprimed or primed PBMC were cultured for 16 h with either anti-CD 43 IgG (1 μg/ml) or anti–PR-3 IgG (1 μg/ml). The primed PBMC did not release IL-8 in response to the anti-CD 43 antibody (Fig. 4c). This implies that the primed monocyte does not react to all antibodies directed against surface proteins with the release of IL-8.

TNFα dose response to anti–PR-3 IgG by primed mono-
cytes. Since TNFα priming is necessary for the response, experiments were performed to determine the critical concentration of TNFα that was needed to get effective priming. PBMC were pulsed with varying doses of TNFα (0.002 ng/ml, 0.02 ng/ml, 0.2 ng/ml, or 2 ng/ml) for 0.5 h and cultured with either anti–PR-3 IgG (1 μg/ml) or isotype control antibody (1 μg/ml) for 16 h. A dose of 0.2 ng/ml was the lowest amount of TNFα that primed the monocyte, as evidenced by anti–PR-3 induced IL-8 release (Fig. 4d).

Exogenous α1-AT inhibits release of IL-8 by PBMC in response to anti–PR-3 IgG
Since α1-AT prevented anti–PR-3 binding to surface PR-3 (Fig. 2b), we tested to see if the addition of a physiological

Figure 4. (a) Released IL-8 induced by anti–PR-3 antibody is newly synthesized. PBMC were either unprimed (open bars) or primed with TNFα (2 ng/ml) (solid bars) for 0.5 h. After washing with RPMI 1640 to rid the cells of TNFα, either isotype control IgG (1 μg/ml), anti–PR-3 IgG (1 μg/ml), or anti–PR-3 IgG (1 μg/ml) and cycloheximide (1 μg/ml) was added to the cells for 16 h. The supernatants were then harvested and the cells lysed. The lysates and the supernatants were then assayed for IL-8 by standard ELISA. The results shown are representative of two experiments. (b) Kinetic study of released IL-8 induced by anti–PR-3. PBMC, primed with TNFα, were cultured with either anti–PR-3 IgG (1 μg/ml) or isotype control IgG (1 μg/ml) for 16 h. Cell-free supernatants were harvested at multiple time points and assayed for antigenic IL-8 release by ELISA. (c) Anti-CD43 IgG does not induce monocyte IL-8 release after priming. PBMC unprimed (open bars) or primed with TNFα for 0.5 h (solid bars) were cultured for 16 h with either anti-CD43 antibody (1 μg/ml) or anti–PR-3 IgG (1 μg/ml). Cell-free supernatants were harvested and antigenic IL-8 release was assayed by ELISA. The results shown represent mean±SEM for two subjects. (d) Sensitivity of monocytes to TNFα priming for anti–PR-3 effect. PBMC were primed with varying doses of TNFα for 0.5 h, and then cultured with either anti–PR-3 IgG (1 μg/ml) or isotype control IgG (1 μg/ml) for 16 h. Cell-free supernatants were assayed for antigenic IL-8 release by ELISA.
Table I. Inhibition of PBMC IL-8 Release by α1-AT

<table>
<thead>
<tr>
<th>Condition</th>
<th>Unprimed mononuclear cells</th>
<th>Primed mononuclear cells</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.9±0.5</td>
<td>2.8±0.9</td>
</tr>
<tr>
<td>IgG, isotype control</td>
<td>0.6±0.2</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>Anti-PR-3 IgG</td>
<td>2.2±0.7</td>
<td>49.5±6.2</td>
</tr>
<tr>
<td>Anti-PR-3 + α1-AT4</td>
<td>6.1±2.2</td>
<td>9.8±2.3</td>
</tr>
</tbody>
</table>

*PBMC were either maintained in cell culture (unprimed cells) or induced to express surface PR-3 by TNFα for 0.5 h (primed cells). Anti-PR-3 IgG or isotype control (1 μg/ml) was added to the cells and the cells cultured for 16 h in an α1-AT–deficient state. Supernatants were assayed for antigenic IL-8 release by ELISA. The primed PBMC in the presence of anti–PR-3 IgG released a significant amount of IL-8 as compared to the unprimed PBMC (P < 0.0001). α1-AT (2 mg/ml) was added to the cells for 1 h before the addition of the anti–PR-3 IgG (1 μg/ml), and after culture for 16 h, the supernatants assayed for IL-8 release. The addition of α1-AT to the primed cells resulted in a significant reduction in IL-8 release (P < 0.0001).

To investigate the mechanism by which anti–PR-3 IgG induces IL-8 release in PBMC, Fab fragments of anti–PR-3 IgG or the isotype control were generated using papain cleavage. Immunoblots confirmed that Fab fragments were successfully produced by demonstrating conversion of the 50-kD Fc portion of the IgG to 25 kD (Fig. 5a). Importantly, the papain cleavage did not result in a change in the binding ability of the Fab fragment to the surface expressed PR-3, as demonstrated by flow cytometry (Fig. 5b). The Fab fragments of anti–PR-3 IgG or IgG isotype control were added to primed monocytes and cultured for 16 h. In comparison to the whole monoclonal anti–PR-3 IgG, its Fab fragment did not induce PBMC to release IL-8 (0 ng/ml versus 34.7±7.7 ng/ml, P < 0.0001) (Fig. 5c).

Since Fab fragments of anti–PR-3 IgG are monovalent, it is possible that the observed lack of IL-8 induction (Fig. 5b) is due to the inability of Fab anti–PR-3 antibody to cross-link surface PR-3. To address this important concern, F(ab′)2 fragments of anti–PR-3 IgG or the isotype control were generated using ficin cleavage. F(ab′)2 fragments were successfully produced by demonstrating a 105-kD F(ab′)2 fragment on immunoblots (Fig. 6a). The ficin cleavage did not result in a change in the binding ability of the F(ab′)2 fragment to the surface expressed PR-3, as demonstrated by flow cytometry (Fig. 6b). These F(ab′)2 fragments of anti–PR-3 IgG or IgG isotype control were added to primed monocytes and cultured for 16 h. In comparison to the whole monoclonal anti–PR-3 IgG, its F(ab′)2 fragment did not induce PBMC to release IL-8 (8.3 ng/ml versus 104.5±17.7 ng/ml, P < 0.0001) (Fig. 6c). Increasing the concentration of F(ab′)2 fragments (up to 5 mg/ml) did not induce IL-8 release (results not shown).

This observed requirement for the intact Fc portion of IgG suggests that anti–PR-3 induces PBMC to release IL-8 by cross-linking surface Fc receptors. The lack of effect by F(ab′)2 antibody demonstrates that the cross-linking of surface PR-3 alone is not sufficient to induce IL-8.

Table II. PBMC Release of IL-8 in Response to Isolated IgG from WG Patients and Inhibition of Release by α1-AT

<table>
<thead>
<tr>
<th>Condition</th>
<th>Unprimed mononuclear cells</th>
<th>Primed mononuclear cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3±0.3</td>
<td>8.3±2.0</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>12.4±7.2</td>
<td>24.1±2.4</td>
</tr>
<tr>
<td>WG IgG</td>
<td>9.1±3.7</td>
<td>155.7±13.1</td>
</tr>
<tr>
<td>Normal IgG + α1-AT4</td>
<td>8.9±0.5</td>
<td>36.7±2.6</td>
</tr>
<tr>
<td>WG IgG + α1-AT4</td>
<td>15.8±12.2</td>
<td>47.5±12.9</td>
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</table>

*IgG purified from patients with WG and normal healthy donors was added to PBMC as described in Table I. The cells were cultured in an α1-AT deficient condition for 16 h and the supernatants analyzed for IL-8 release by ELISA (n = 3). The IgG isolated from WG patients induced a significant release of IL-8 as compared to IgG from healthy donors (P < 0.0001). α1-AT (2 mg/ml) was added to the mononuclear cells before the addition of the IgG (n = 3). With the addition of α1-AT, this release was significantly inhibited (P < 0.0001).

Discussion

WG is a disease that is characterized pathologically by disseminated neutrophilic vasculitis. In addition, patients with WG often produce autoantibodies (cANCA) against PR-3, a serine proteinase located in the primary granules of neutrophils and...
monocytes. The role of cANCA in the pathogenesis of WG has been debated, but recent studies have demonstrated that cANCA can directly stimulate TNFα-primed neutrophils, suggesting a direct role for these antibodies (1, 2, 12).

This study confirms for the first time that cANCA can induce monocytes to release IL-8, and thus may play an important regulatory role in this disease process. We demonstrate that monocytes express surface PR-3 after pulse priming with TNFα, and that monoclonal anti–PR-3 IgG then induces a 45-fold increase in functional IL-8 release compared to control IgG. Importantly, only the primed monocytes, which expressed surface PR-3, release IL-8 in response to the anti–PR-3 IgG, implying that the surface interaction of the PR-3 with the anti–PR-3 IgG is critical. Furthermore, the addition of 1-AT specifically and significantly decreases anti–PR-3–induced IL-8 release. We speculate that the 1-AT may inhibit IL-8 release by binding to the surface expressed PR-3, thus preventing the interaction of the antigen and antibody. This hypothesis is supported by the flow cytometry data that demonstrates that exogenous 1-AT blocks the surface detection of PR-3 as measured by flow cytometry.

By cleaving the Fc component of monoclonal anti–PR-3 IgG by either papain or ficin digestion, inducing Fab and F(ab')2 fragments, respectively, we were able to eliminate the ability of the anti–PR-3 IgG to induce IL-8 release by monocytes. These data suggest that both the recognition of PR-3 by anti–PR-3 IgG and Fc:Fc receptor engagement are necessary for anti–PR-3 IgG induction of monocyte IL-8. These results are consistent with our recent observation that monocyte Fc receptor cross-linking induces IL-8 (7).

That the effect of the cANCA occurs via Fc receptor cross-linking is controversial. Previous studies show that F(ab')2 fragments of cANCA can induce a respiratory burst in neutrophils and increased neutrophil mediated injury to endothelial cells that imply that antibody interaction with PR-3 directly activates neutrophils (13, 14). However, Porges et al. demonstrated that FcyRIIa cross-linking is critical to cANCA mediated neutrophil activation (3). Our experiments with monocytes support the requirement for Fc receptor cross-linking. The induction of IL-8 was completely inhibited by removal of the Fc component. To document that Fc removal did not prevent PR-3 interaction, we felt it was critical to document that our Fab and F(ab')2 antibodies were still capable of binding monocyte surface PR-3 (Figs. 5b and 6b). Therefore, Fc receptor cross-linking is necessary for cANCA mediated IL-8 induction for the monocyte. Unexpectedly, however, TNFα–primed monocytes did not release IL-8 in response to whole antibody CD43, a constitutive monocyte surface marker.
There are a number of potential explanations why anti-CD43 did not induce IL-8. It is possible that the binding of cANCA to the surface bound PR-3 provides a part of the activation signal (as it does for neutrophil respiratory burst) but that IL-8 release also requires Fcγ receptor cross-linking for complete induction. This explanation appears unlikely since we have shown previously that Fcγ receptor cross-linking alone is sufficient to potently induce IL-8 release (7). It may also be that CD43 binding prevents Fcγ receptor activation. For example, it is conceivable that CD43 binding induces rapid removal of surface IgG by either pinocytosis or shedding that prevents Fcγ receptor engagement. Another possibility is that anti-CD43 antibody binding induces an inhibitory signal that prevents IL-8 release. These questions deserve future investigation. It is clear, however, that Fcγ receptor engagement provides a critical if not essential role in the cANCA-mediated monocyte IL-8 induction.

Finally, to demonstrate that human anti–PR-3 antibody may have a function in disease, IgG isolated from WG patients with high titers of cANCA induced a sixfold increase in IL-8 release in primed PBMC as compared to control IgG. Reinforcing the critical role for PR-3, exogenous pooled human α1-AT inhibits this effect. These data imply that in vivo circulating cANCA IgG can stimulate IL-8 release by monocytes which have been induced to express PR-3. Thus, cANCA may amplify local inflammatory reactions via monocyte activation, serving to target and recruit neutrophils in WG.

The requirement for monocyte priming is potentially relevant to the natural history of cANCA related disorders. Frequently, patients with WG report a viral prodrome before the onset of vasculitic syndromes (15). There is also an increased incidence of these diseases in the winter when respiratory tract infections are common (15). Additionally, treatment with trimethoprim-sulphamethoxazole reduces the incidence of exacerbations in patients with WG (16–18). These clinical observations support the concept that priming of the monocyte or neutrophil by TNFα may be required to induce surface expression of PR-3, the critical antigen. After this priming, surface PR-3 can be antigenic and antibodies to PR-3 generated. The mechanisms for this putative antigenicity are not known.

It is intriguing to hypothesize that defective PR-3-α1-AT interactions may promote cANCA production. Supporting this possibility that altered binding of α1-AT to PR-3 is critical in cANCA-mediated disease, there is an epidemiological association of PR-3 with the Pi Z allele of α1-AT (4, 5). In this respect, Pi Z α1-AT has impaired antiprotease function (19). Thus, ineffective α1-AT or heightened binding of cANCA to PR-3 favors interaction of cANCA with PR-3, and results in Fcγ receptor cross-linking, release of IL-8 by the monocyte, and subsequent neutrophil recruitment and tissue injury. In
keeping with this hypothesis, a recent study demonstrated that cANCA-positive vasculitis patients with the PiZ gene (α1-AT deficiency heterozygote) had more disseminated disease and a significantly higher mortality when compared to patients with a normal α1-AT genotype (20).

In our experiments, the addition of α1-AT at physiological doses consistently decreased cANCA induced IL-8 release by PR-3 expressing monocytes. In this manner, α1-AT may indirectly regulate inflammation by suppressing the inflammatory cascade induced by cANCA. An anti-inflammatory role for antiproteases has been reported by a recent evaluation of secretory leukoprotease inhibitor (SLPI) in inhibiting IL-8 measured in the airways of cystic fibrosis patients. It is conceivable that a part of the SLPI (22).

In summary, this study demonstrates that monocytes, after being induced to express surface PR-3, release significant amounts of biologically active IL-8 when exposed to either monoclonal anti–PR-3 IgG or IgG from WG patients with high titers of cANCA. The mechanism of this interaction is Fcγ receptor mediated, and is prevented by the addition of α1-AT or cleavage of the Fc domain from IgG. These studies suggest that monocyte surface PR-3 expression may be a critical event in the pathogenesis of cANCA associated diseases.

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