

Antineutrophil Cytoplasmic Antibodies Induce Monocyte IL-8 Release

Role of Surface Proteinase-3, α 1-antitrypsin, and Fc γ Receptors

David R. Ralston, Clay B. Marsh, Melissa P. Lowe, and Mark D. Wewers

Division of Pulmonary and Critical Care Medicine, The Ohio State University, Columbus, Ohio 43210

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 TNF α -primed neutrophils, can directly activate neutrophils by complexing cANCA and promoting concomitant Fc γ receptor (Fc γ R) 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 TNF α (2 ng/ml). Purified monoclonal anti-PR-3 IgG added to TNF α -primed PBMC induced 45-fold more IL-8 release than an isotype control antibody. Furthermore, alpha 1-antitrypsin (α 1-AT), the primary PR-3 antiprotease, 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 Fc γ 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, α 1-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 Fc γ receptor cross-linking and is regulated in part by α 1-AT. (*J. Clin. Invest.* 1997. 100:1416–1424.) **Key words:** Wegener's granulomatosis • serine protease • chemokine • receptor cross-linking

Introduction

Wegener's granulomatosis (WG)¹ 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 TNF α -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 Fc γ RIIa receptors (1, 3). Importantly, alpha 1-antitrypsin (α 1-AT) is the primary serum protein that complexes PR-3, and recent studies have also associated cANCA with α 1-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 Fc γ receptor cross-linking (6). In this context, we have demonstrated that Fc γ R cross-linking produces IL-8 in monocytes (7). Therefore, cANCA binding to PR-3 expressed on the surface of the monocyte, may result in Fc γ R 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 Fc γ 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 (Histopaque; 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 μ g/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

Address correspondence to Mark D. Wewers, M.D., Division of Pulmonary and Critical Care Medicine, 1654 Upham Drive, The Ohio State University Medical Center, Columbus, Ohio 43210. Phone: 614-293-4925; FAX: 614-293-4799; E-mail: wewers.2@osu.edu

Received for publication 4 September 1996 and accepted in revised form 11 July 1997.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9738/97/09/1416/09 \$2.00

Volume 100, Number 6, September 1997, 1416–1424
<http://www.jci.org>

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

TNF α (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 TNF α , and new media was added. An IgG₁ mouse monoclonal antibody to proteinase-3 (α PR-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 pg/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 μ g/ml, while MOPC 21 (Sigma Diagnostics), a mouse monoclonal IgG₁ isotype control (1 μ g/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/polymyxin B (10 μ g/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 TNF α for 0.5 h as described above. After washing, either isotype control IgG (1 μ g/ml), anti-PR-3 antibody (1 μ g/ml), or anti-PR-3 antibody (1 μ g/ml) and cycloheximide (1 μ g/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- μ g/ml aprotinin, 1- μ g/ml leupeptin, 1- μ M phenylmethanesulfonyl fluoride, and 1- μ g/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 once they are primed, studies were performed to determine the specificity of the response to the anti-PR-3 antibody. PBMC were isolated as described above. PBMC were either cultured alone or primed with 2 ng/ml TNF α for 0.5 h. After washing with RPMI 1640, either anti-CD 43 antibody (1 μ g/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.

TNF α dose response to anti-PR-3 antibody by primed monocytes. PBMC were isolated as described above. PBMC were primed with varying doses of TNF α (0.156 μ g/ml, 0.312 μ g/ml, 0.625 μ g/ml, 1.25 μ g/ml, 2.5 μ g/ml, or 5 μ g/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 α 1-AT. To determine if α 1-AT prevented the interaction of surface expressed PR-3 with anti-PR-3 antibody, PBMC were primed with TNF α 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 α 1-AT (Miles Laboratories, Inc., Elkhart, IN) at the physiological concentration of 2 mg/ml. Next, monoclonal anti-PR-3 (1 μ g/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 Diag-

nostics). 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 IIe personal computer. Neither anti-PR-3 IgG or α 1-AT interfered with ELISA readings when tested alone.

Immunohistochemistry

PBMC (5×10^6 /ml) that had been primed for 0.5 h with TNF α (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. Isotype control IgG (10 μ g/ml) or anti-PR-3 IgG (10 μ g/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-amino-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 (5×10^6 /ml), either primed with TNF α 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 harvested by 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 μ g/ml), or isotype control IgG (40 μ g/ml), or Fab or F(ab')₂ fragments of anti-PR-3 IgG or isotype control (5 μ g/ml) was added to the PBMC and incubated for 1 h at 4°C. Separately, primed and unprimed PBMC were cultured with α 1-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 Fc γ R cross-linking, Fab fragments, which lack Fc domains and do not cross-link Fc γ 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 (5×10^6 /ml) for 16 h at 37°C, 5% CO₂. The supernatants were then harvested and a standard IL-8 ELISA performed.

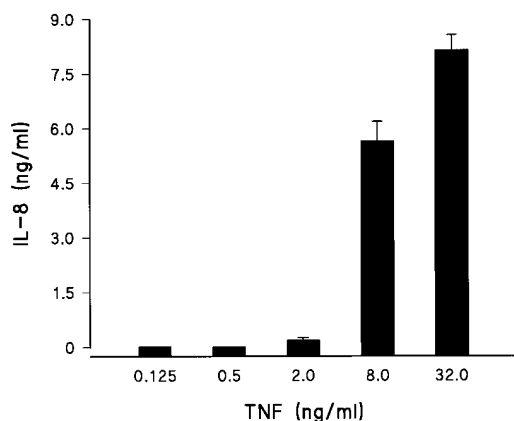


Figure 1. Dose-dependent mononuclear cell release of IL-8 by TNF α . PBMC were primed with TNF α (2 ng/ml) for 0.5 h, washed, and then cultured for 16 h. Supernatants were assayed for IL-8 release by ELISA. The results represent four subjects and are reported as mean \pm SEM.

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 IgG₁ 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 IgG₁ 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% CO₂. 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

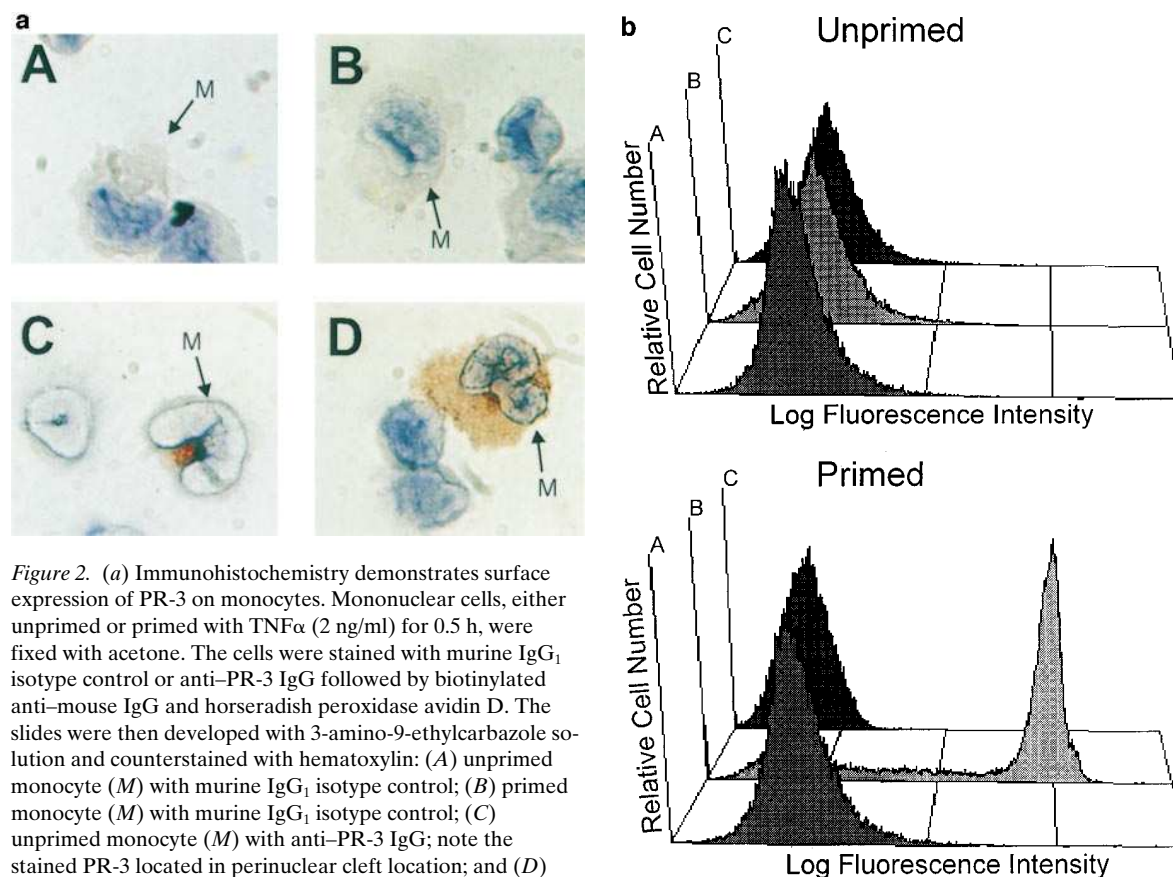


Figure 2. (a) Immunohistochemistry demonstrates surface expression of PR-3 on monocytes. Mononuclear cells, either unprimed or primed with TNF α (2 ng/ml) for 0.5 h, were fixed with acetone. The cells were stained with murine IgG₁ isotype control or anti-PR-3 IgG followed by biotinylated anti-mouse IgG 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 TNF α (2 ng/ml) for 0.5 h, were analyzed by flow cytometry using (A) IgG Isotype control (40 μ g/ml), or (B) anti-PR-3 IgG (40 μ g/ml). Separately, α 1-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.

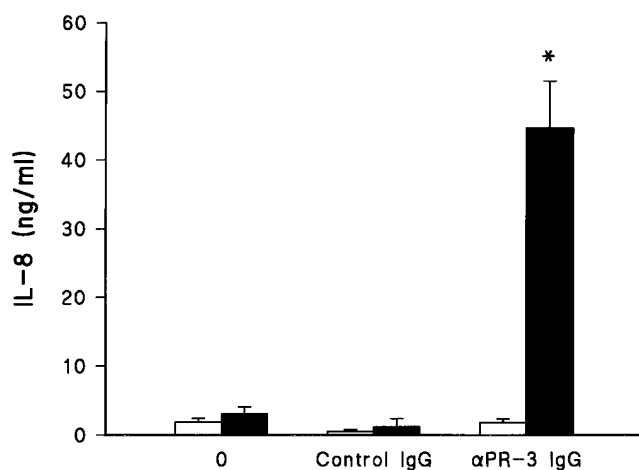


Figure 3. Anti-PR-3 IgG induces IL-8 release in primed mononuclear cells. Mononuclear cells were either primed to express surface PR-3 by TNF α (2 ng/ml) for 0.5 h (primed cells, *solid bars*), or maintained in cell culture (unprimed cells, *open bars*). After washing of both sets of cells with RPMI 1640 (to rid the primed cells of TNF α), anti-PR-3 IgG (1 μ g/ml) was added to the cells for 16 h. Murine IgG₁ served as an isotype control. Cell-free supernatants were assayed for antigenic IL-8 release. The results shown represent mean \pm SEM for eight subjects. * $P < 0.0001$.

(Pierce Chemical Co.). Bound antibody was detected by a chemiluminescence kit (Amersham International) using the manufacturer's protocol, with the primary antibody at a concentration of 1:100,000. The ability of F(ab')₂ fragments to recognize surface expressed PR-3 on monocytes was confirmed by flow cytometry analysis.

Isolation of human IgG from WG patients and normal volunteers

Three serum samples with cANCA levels greater than 1:320 were obtained (courtesy of William Becker, D.O., Department of Pathology, The Ohio State University, Columbus, OH). 1 ml of the cANCA sera and normal volunteer sera was passed over a protein A-agarose column (Bio-Rad Laboratories). The column was washed with 10 column volumes of 100-mM Tris (pH 8.0) and 10-mM Tris (pH 8.0). The IgG was then eluted with 100-mM glycine (pH 3.0), collected, and quantified by O. D. at 280 nm on a DU-50 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The purified cANCA IgG from the three patients and two normal patients was incubated with unprimed or primed PBMC (5×10^6 /ml) isolated from an O negative blood-type healthy volunteer in the presence or absence of α 1-AT (2 mg/ml). An O negative blood type was required to prevent IL-8 release because of ABO incompatibility between the WG patients serum and PBMC. The cells were cultured for 16 h at 37°C, 5% CO₂, after which the cell-free supernatants were harvested, and IL-8 was quantitated by ELISA.

Statistical analysis

All data were expressed as mean \pm SEM. ANCOVA with Tukey's post-hoc testing was used to compare conditions (Systat, Evanston, IL). Statistical significance was defined as a P value < 0.05 .

Results

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 in-

duced to express PR-3 on their surface without concomitant production of IL-8. After pulse stimulation for 0.5 h with increasing doses of TNF α , 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 TNF α 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 TNF α 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 TNF α (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 μ g/ml) was added to TNF-pulsed mononuclear cells (5×10^6 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 μ g/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 effect 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 μ g/ml), isotype control antibody (1 μ g/ml), or anti-PR-3 antibody (1 μ g/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 TNF α , were cultured with either anti-PR-3 IgG (1 μ g/ml) or isotype control IgG (1 μ g/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 IgG₁ (1 μ g/ml) or anti-PR-3 IgG₁ (1 μ g/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.

TNF α dose response to anti-PR-3 IgG by primed mono-

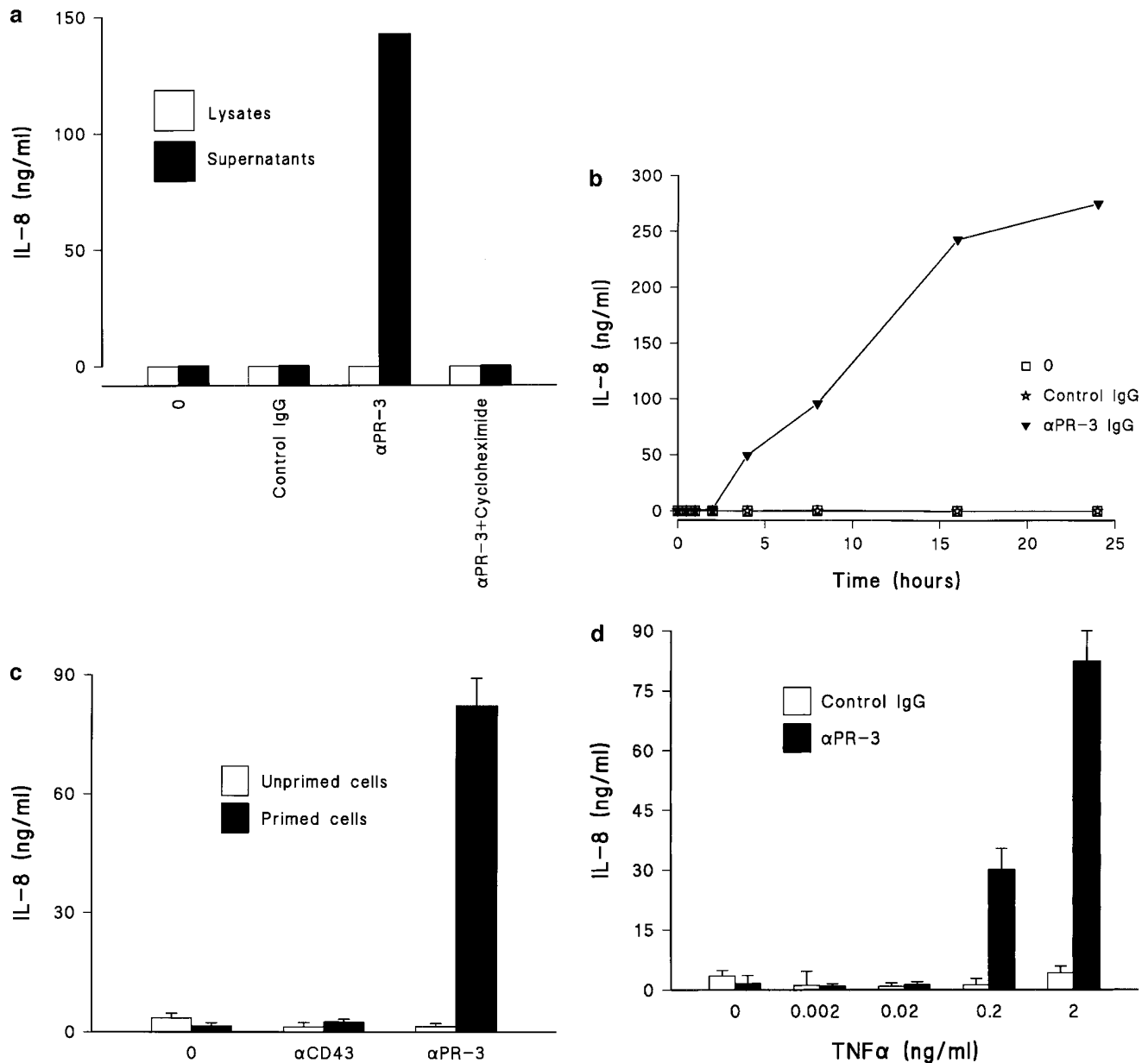


Figure 4. (a) Released IL-8 induced by anti-PR-3 antibody is newly synthesized. PBMC were either unprimed (*open bars*) or primed with TNF α (2 ng/ml) (*solid bars*) for 0.5 h. After washing with RPMI 1640 to rid the cells of TNF α , either isotype control IgG (1 μ g/ml), anti-PR-3 IgG (1 μ g/ml), or anti-PR-3 IgG (1 μ g/ml) and cycloheximide (1 μ g/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 TNF α , were cultured with either anti-PR-3 IgG (1 μ g/ml) or isotype control IgG (1 μ g/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 TNF α for 0.5 h (*solid bars*) were cultured for 16 h with either anti-CD43 antibody (1 μ g/ml) or anti-PR-3 IgG (1 μ g/ml). Cell-free supernatants were harvested and antigenic IL-8 release was assayed by ELISA. The results shown represent mean \pm SEM for two subjects. (d) Sensitivity of monocytes to TNF α priming for anti-PR-3 effect. PBMC were primed with varying doses of TNF α for 0.5 h, and then cultured with either anti-PR-3 IgG (1 μ g/ml) or isotype control IgG (1 μ g/ml) for 16 h. Cell-free supernatants were assayed for antigenic IL-8 release by ELISA.

cytes. Since TNF α priming is necessary for the response, experiments were performed to determine the critical concentration of TNF α that was needed to get effective priming. PBMC were pulsed with varying doses of TNF α (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 μ g/ml) or isotype control antibody (1 μ g/ml) for 16 h. A dose of 0.2 ng/ml was the lowest amount of TNF α

that primed the monocyte, as evidenced by anti-PR-3 induced IL-8 release (Fig. 4 d).

Exogenous α 1-AT inhibits release of IL-8 by PBMC in response to anti-PR-3 IgG

Since α 1-AT prevented anti-PR-3 binding to surface PR-3 (Fig. 2 b), we tested to see if the addition of a physiological

Table I. Inhibition of PBMC IL-8 Release by α 1-AT

Condition*	IL-8 (ng/ml)	
	Unprimed mononuclear cells	Primed mononuclear cells
Control	1.9 \pm 0.5	2.8 \pm 0.9
IgG ₁ isotype control	0.6 \pm 0.2	1.1 \pm 0.2
Anti-PR-3 IgG	2.2 \pm 0.7	49.5 \pm 6.2
Anti-PR-3 + α 1-AT [‡]	6.1 \pm 2.2	9.8 \pm 2.3

*PBMC were either maintained in cell culture (unprimed cells) or induced to express surface PR-3 by TNF α for 0.5 h (primed cells). Anti-PR-3 IgG or isotype control (1 μ g/ml) was added to the cells and the cells cultured for 16 h in an α 1-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 ($P < 0.0001$). [‡] α 1-AT (2 mg/ml) was added to the cells for 1 h before the addition of the anti-PR-3 IgG (1 μ g/ml), and after culture for 16 h, the supernatants assayed for IL-8 release. The addition of α 1-AT to the primed cells resulted in a significant reduction in IL-8 release ($P < 0.0001$).

dose of α 1-AT would inhibit the interaction of PR-3 with anti-PR-3 IgG on monocytes and thereby decrease PBMC IL-8 release. To assess α 1-AT's role, unprimed and primed PBMC were cultured in 5% FBS, mimicking α 1-AT-deficient conditions. Exogenous α 1-AT (2 mg/ml) was then incubated with primed PBMC for 1 h before the addition of anti-PR-3 IgG. The addition of α 1-AT decreased IL-8 release by 80% from the primed mononuclear cells cultured with cANCA (49.5 \pm 6.2 ng/ml versus 9.8 \pm 2.3 ng/ml, $P < 0.0001$) (Table I). This reduction was specific for the anti-PR-3 IgG interaction with PR-3 since the α 1-AT did not inhibit PBMC IL-8 release induced by 16 h TNF α incubation (2 ng/ml) in the absence of anti-PR-3 IgG (TNF α alone, 11.9 ng/ml, TNF α with α 1-AT 19.5 ng/ml).

Table II. PBMC Release of IL-8 in Response to Isolated IgG from WG Patients and Inhibition of Release by α 1-AT

Condition*	IL-8 (ng/ml)	
	Unprimed mononuclear cells	Primed mononuclear cells
Control	0.3 \pm 0.3	8.3 \pm 2.0
Normal IgG	12.4 \pm 7.2	24.1 \pm 2.4
WG IgG	9.1 \pm 3.7	155.7 \pm 13.1
Normal IgG + α 1-AT [‡]	8.9 \pm 0.5	36.7 \pm 2.6
WG IgG + α 1-AT [‡]	15.8 \pm 12.2	47.5 \pm 12.9

*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 α 1-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 ($P < 0.0001$). [‡] α 1-AT (2 mg/ml) was added to the mononuclear cells before the addition of the IgG ($n = 3$). With the addition of α 1-AT, this release was significantly inhibited ($P < 0.0001$).

Anti-PR-3 IgG induces primed PBMC to release IL-8 through Fc γ receptor cross-linking

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. 5 a). 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. 5 b). The Fab fragments of anti-PR-3 IgG or IgG₁ 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 \pm 7.7 ng/ml, $P < 0.0001$) (Fig. 5 c).

Since Fab fragments of anti-PR-3 IgG are monovalent, it is possible that the observed lack of IL-8 induction (Fig. 5 b) 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. 6 a). 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. 6 b). 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 \pm 17.7 ng/ml, $P < 0.0001$) (Fig. 6 c). 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 γ 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.

IgG from cANCA-positive patients with WG induces primed PBMC to release IL-8

To determine if cANCA from patients with WG could also induce primed PBMC to release IL-8, sera from patients with WG and known cANCA values $> 1:320$ were obtained along with control sera from normal healthy donors. The isolated IgG from the WG patients and normal donors was incubated for 16 h with either resting or primed mononuclear cells in the presence or absence of exogenous α 1-AT (Fig. 7). The cANCA containing IgG induced the primed PBMC to release six times more IL-8 than the IgG control (155.7 \pm 13.1 ng/ml versus 24.1 \pm 2.4 ng/ml, $P < 0.0001$). Importantly, the addition of α 1-AT at a physiological dose (2 mg/ml) reduced the IL-8 release by 70% (155.7 \pm 13.1 ng/ml versus 47.5 \pm 12.9 ng/ml, $P < 0.0001$). These data demonstrate that cANCA IgG from patients with WG can induce and regulate the release of IL-8 by PR-3 expressing PBMC.

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

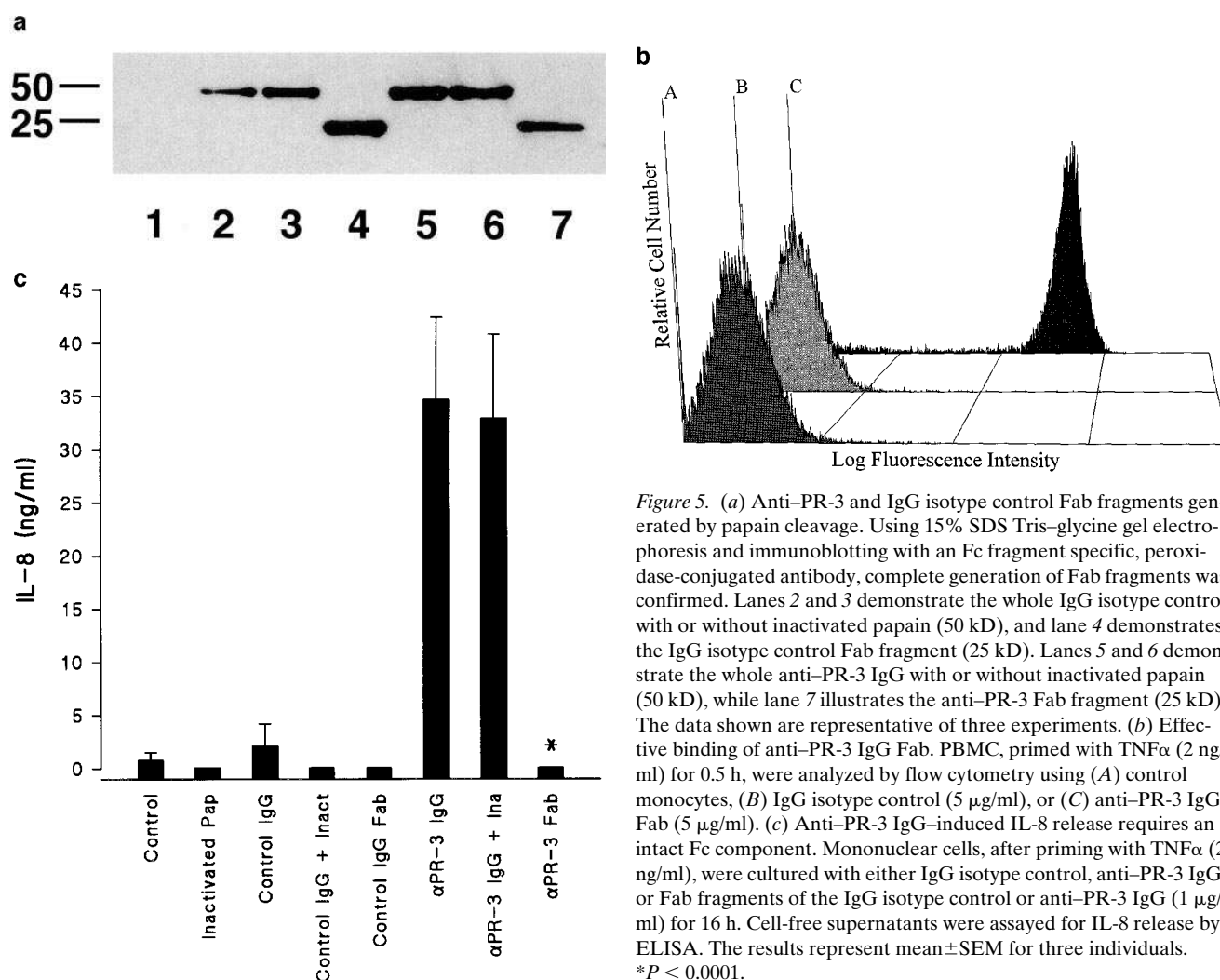


Figure 5. (a) Anti-PR-3 and IgG isotype control Fab fragments generated by papain cleavage. Using 15% SDS Tris-glycine gel electrophoresis and immunoblotting with an Fc fragment specific, peroxidase-conjugated antibody, complete generation of Fab fragments was confirmed. Lanes 2 and 3 demonstrate the whole IgG isotype control with or without inactivated papain (50 kD), and lane 4 demonstrates the IgG isotype control Fab fragment (25 kD). Lanes 5 and 6 demonstrate the whole anti-PR-3 IgG with or without inactivated papain (50 kD), while lane 7 illustrates the anti-PR-3 Fab fragment (25 kD). The data shown are representative of three experiments. (b) Effective binding of anti-PR-3 IgG Fab. PBMC, primed with TNF α (2 ng/ml) for 0.5 h, were analyzed by flow cytometry using (A) control monocytes, (B) IgG isotype control (5 μ g/ml), or (C) anti-PR-3 IgG Fab (5 μ g/ml). (c) Anti-PR-3 IgG-induced IL-8 release requires an intact Fc component. Mononuclear cells, after priming with TNF α (2 ng/ml), were cultured with either IgG isotype control, anti-PR-3 IgG, or Fab fragments of the IgG isotype control or anti-PR-3 IgG (1 μ g/ml) for 16 h. Cell-free supernatants were assayed for IL-8 release by ELISA. The results represent mean \pm SEM for three individuals. * $P < 0.0001$.

monocytes. The role of cANCA in the pathogenesis of WG has been debated, but recent studies have demonstrated that cANCA can directly stimulate TNF α -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 TNF α , 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 α 1-AT specifically and significantly decreases anti-PR-3-induced IL-8 release. We speculate that the α 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 α 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 γ 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 γ receptor cross-linking induces IL-8 release (7).

That the effect of the cANCA occurs via Fc γ 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 Fc γ RIIa cross-linking is critical to cANCA mediated neutrophil activation (3). Our experiments with monocytes support the requirement for Fc γ 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 γ receptor cross-linking is necessary for cANCA mediated IL-8 induction for the monocyte. Unexpectedly, however, TNF α -primed monocytes did not release IL-8 in response to whole antibody CD43, a constitutive monocyte surface marker.

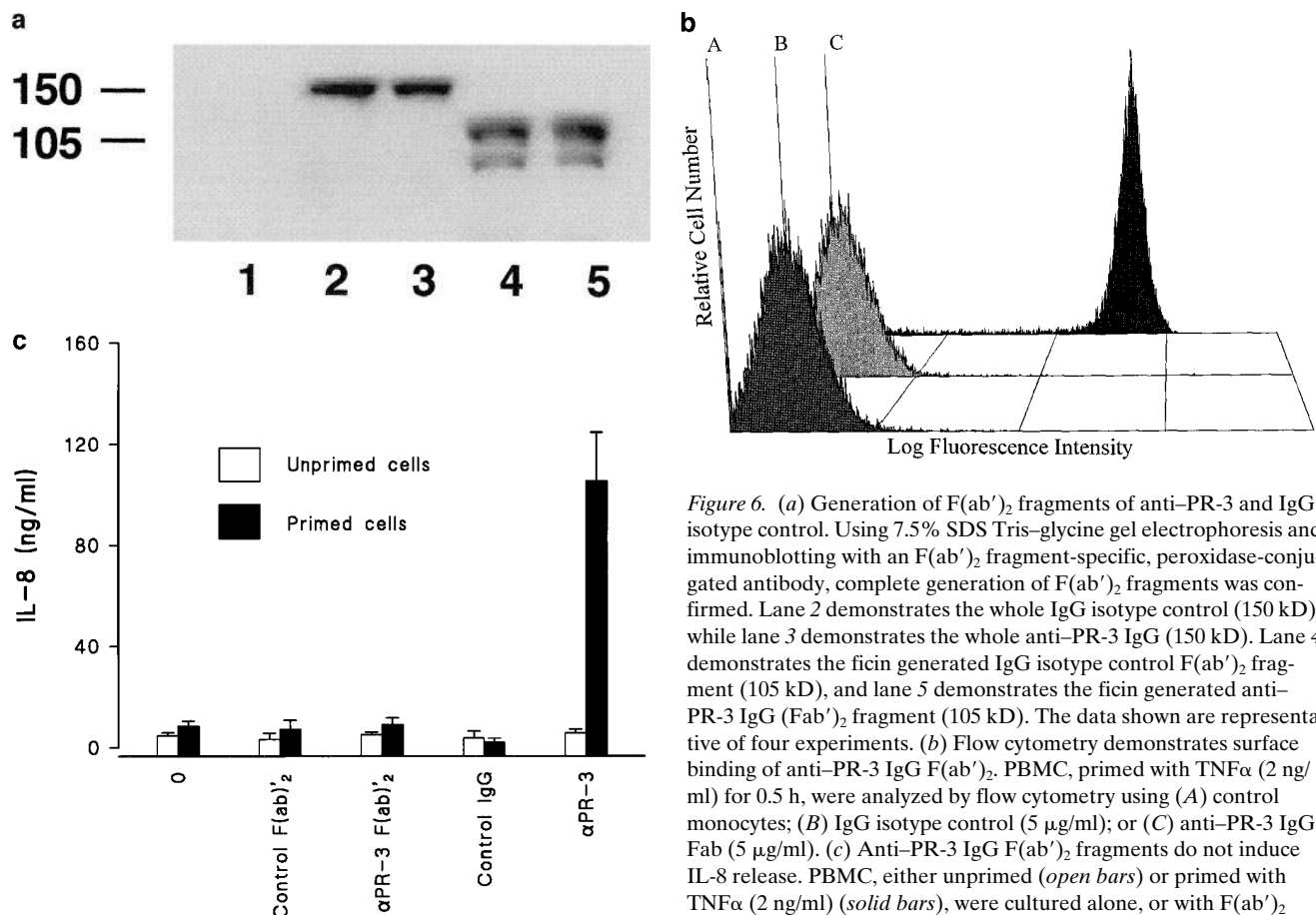


Figure 6. (a) Generation of $F(ab')_2$ fragments of anti-PR-3 and IgG isotype control. Using 7.5% SDS Tris-glycine gel electrophoresis and immunoblotting with an $F(ab')_2$ fragment-specific, peroxidase-conjugated antibody, complete generation of $F(ab')_2$ fragments was confirmed. Lane 2 demonstrates the whole IgG isotype control (150 kD), while lane 3 demonstrates the whole anti-PR-3 IgG (150 kD). Lane 4 demonstrates the ficin generated IgG isotype control $F(ab')_2$ fragment (105 kD), and lane 5 demonstrates the ficin generated anti-PR-3 IgG ($F(ab')_2$) fragment (105 kD). The data shown are representative of four experiments. (b) Flow cytometry demonstrates surface binding of anti-PR-3 IgG $F(ab')_2$. PBMC, primed with $TNF\alpha$ (2 ng/ml) for 0.5 h, were analyzed by flow cytometry using (A) control monocytes; (B) IgG isotype control (5 μ g/ml); or (C) anti-PR-3 IgG Fab (5 μ g/ml). (c) Anti-PR-3 IgG $F(ab')_2$ fragments do not induce IL-8 release. PBMC, either unprimed (open bars) or primed with $TNF\alpha$ (2 ng/ml) (solid bars), were cultured alone, or with $F(ab')_2$ fragments of the IgG isotype control (1 μ g/ml), or $F(ab')_2$ fragments

of anti-PR-3 IgG (1 μ g/ml), or intact IgG isotype control (1 μ g/ml), or intact anti-PR-3 IgG (1 μ g/ml), for 16 h. Cell-free supernatants were assayed for IL-8 release by ELISA. The results represent mean \pm SEM for three individuals.

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 Fc γ receptor cross-linking for complete induction. This explanation appears unlikely since we have shown previously that Fc γ receptor cross-linking alone is sufficient to potently induce IL-8 release (7). It may also be that CD43 binding prevents Fc γ receptor activation. For example, it is conceivable that CD43 binding induces rapid removal of surface IgG by either pinocytosis or shedding that prevents Fc γ 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 Fc γ 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 α 1-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-sulfamethoxazole reduces the incidence of relapses in patients with WG (16–18). These clinical observations support the concept that priming of the monocyte or neutrophil by $TNF\alpha$ 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- α 1-AT interactions may promote cANCA production. Supporting this possibility that altered binding of α 1-AT to PR-3 is critical in cANCA-mediated disease, there is an epidemiological association of PR-3 with the Pi Z allele of α 1-AT (4, 5). In this respect, Pi Z α 1-AT has impaired antiprotease function (19). Thus, ineffective α 1-AT or heightened binding of cANCA to PR-3 favors interaction of cANCA with PR-3, and results in Fc γ receptor cross-linking, release of IL-8 by the monocyte, and subsequent neutrophil recruitment and tissue injury. In

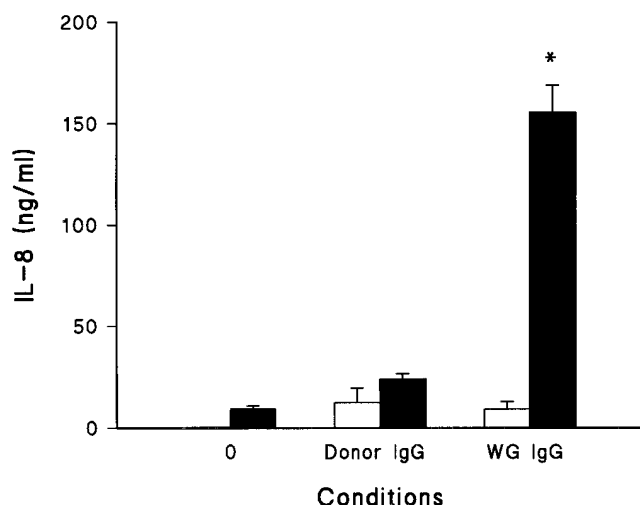


Figure 7. IgG obtained from WG patients induces release of IL-8 by primed mononuclear cells. IgG purified from patients with WG (WG IgG) and normal healthy donors (Donor IgG) was added to mononuclear cells with (solid bars) or without (open bars) TNF α priming (2 ng/ml). The cells were cultured either alone or with additives for 16 h, and the supernatants analyzed for IL-8 release by ELISA. The results represent the mean \pm SEM of serum used from three patients. * $P < 0.0001$.

keeping with this hypothesis, a recent study demonstrated that cANCA-positive vasculitis patients with the PiZ gene (α 1-AT deficiency heterozygote) had more disseminated disease and a significantly higher mortality when compared to patients with a normal α 1-AT genotype (20).

In our experiments, the addition of α 1-AT at physiological doses consistently decreased cANCA induced IL-8 release by PR-3 expressing monocytes. In this manner, α 1-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 in cystic fibrosis (21). In this study, the aerosolized antiprotease, secretory leukoprotease inhibitor (SLPI), reduced IL-8 measured in the airways of cystic fibrosis patients. It is conceivable that a part of the SLPI anti-inflammatory action occurred by blocking PR-3 mediated Fc γ receptor activation. In an additional study, aerosolized α 1-AT, when given to cystic fibrosis patients, suppressed neutrophil elastase in the respiratory epithelial lining fluid and IL-8 release, further supporting the regulatory role for antiproteases (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 Fc γ receptor mediated, and is prevented by the addition of α 1-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.

Acknowledgments

The authors are grateful to William Becker, D.O. of The Ohio State University Medical Center, Division of Pathology for providing the

human cANCA serum samples. In addition, we would like to acknowledge O.J. Trask and The Ohio State University Comprehensive Cancer Center Analytical Cytometry Laboratory, which is supported in part by PHS NIH P30 CA-16058, for their assistance.

This work was supported by grant HL 40871 from the National Institutes of Health, a Postdoctoral Fellowship Award from the American Heart Association, Ohio Affiliate, and by the Francis Families Foundation. C.B. Marsh is a Parker B. Francis Fellow in Pulmonary Research.

References

- Falk, R.J., R.S. Terrell, L.A. Charles, and J.C. Jennette. 1990. Antineutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc. Natl. Acad. Sci. USA* 87:4115-4119.
- Charles, L.A., M.L.R. Caldas, R.J. Falk, R.S. Terrell, and J.C. Jennette. 1991. Antibodies against granule proteins activate neutrophils *in vitro*. *J. Leukocyte Biol.* 50:539-546.
- Porges, A.J., P.B. Redecha, W.T. Kimberly, E. Csernok, W.L. Gross, and R.P. Kimberly. 1994. Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc γ IIa. *J. Immunol.* 153:1271-1280.
- Esnault, V.L.M., A. Testa, M. Audrain, C. Roge, M. Hamidou, J.H. Barrier, R. Sesboue, J.-P. Martin, and P. Lesavre. 1993. Alpha 1-antitrypsin genetic polymorphism in ANCA-positive systemic vasculitis. *Kidney Int.* 43:1329-1332.
- Elzouki, A.-N.Y., M. Segelmark, J. Wieslander, and S. Eriksson. 1994. Strong link between the α 1-antitrypsin PiZ allele and Wegener's granulomatosis. *J. Intern. Med.* 236:543-548.
- Wewers, M.D. 1997. Cytokines and macrophages. In *Cytokines in Health and Disease: Physiology and Pathophysiology*. D.G. Remick and J.S. Friedland, editors. Marcel Dekker, Inc., New York. 339-355.
- Marsh, C.B., J.E. Gadek, G.C. Kindt, S.A. Moore, and M.D. Wewers. 1995. Monocyte Fc gamma receptor cross-linking induces IL-8 production. *J. Immunol.* 155:3161-3167.
- Strieter, R.M., and S.L. Kunkel. 1994. Acute lung injury: the role of cytokines in the elicitation of neutrophils. *J. Invest. Med.* 42:640-651.
- Strieter, R.M., A.E. Koch, V.B. Antony, R.B. Fick, Jr., T.J. Standiford, and S.L. Kunkel. 1994. The immunopathology of chemotactic cytokines: the role of interleukin-8 and monocyte chemoattractant protein-1. *J. Lab. Clin. Med.* 123:183-197.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Herzyk, D.J., A.E. Berger, J.N. Allen, and M.D. Wewers. 1992. Sandwich ELISA formats designed to detect 17 kDa IL-1 beta significantly underestimate 35 kDa IL-1 beta. *J. Immunol. Methods.* 148:243-254.
- Ewert, H.E., J.C. Jennette, and R.J. Falk. 1991. The pathogenic role of antineutrophil cytoplasmic antibodies. *Am. J. Kidney Dis.* 28:188-195.
- Keogan, M.T., V.M. Esnault, A.J. Green, C.M. Lockwood, and D.L. Brown. 1992. Activation of normal neutrophils by anti-neutrophil cytoplasmic antibodies. *Clin. Exp. Immunol.* 90:228-234.
- Savage, C.O., B.E. Pottinger, G. Gaskin, C.D. Pusey, and J.D. Pearson. 1992. Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. *Am. J. Pathol.* 141:335-342.
- Falk, R.J., S. Hogan, T.S. Carey, and J.C. Jennette. 1990. Clinical course of anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and systemic vasculitis. The Glomerular Disease Collaborative Network. *Ann. Intern. Med.* 113:656-663.
- DeRemee, R.A., T.J. McDonald, and L.H. Weiland. 1985. Wegener's granulomatosis: observations on treatment with antimicrobial agents. *Mayo. Clin. Proc.* 60:27-32.
- Stegeman, C.A., J.W.C. Tervaert, P.E. DeJong, and C.G.M. Kallenberg. 1996. Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. *N. Engl. J. Med.* 335:16-20.
- DeRemee, R.A. 1996. Editorial: empiricism and Wegener's granulomatosis. *N. Engl. J. Med.* 335:54-55.
- Ogushi, F., G.A. Fells, R.C. Hubbard, S.D. Straus, and R.G. Crystal. 1987. Z-type alpha 1-antitrypsin is less competent than M1-type alpha 1-antitrypsin as an inhibitor of neutrophil elastase. *J. Clin. Invest.* 80:1366-1374.
- Segelmark, M., A.-N. Elzouki, J. Wieslander, and S. Eriksson. 1995. The PiZ gene of α 1-antitrypsin as a determinant of outcome in PR3-ANCA-positive vasculitis. *Kidney Int.* 48:844-850.
- McElvaney, N.G., H. Nakamura, P. Birrer, C.A. Hebert, W.L. Wong, M. Alphonso, J.B. Baker, M.A. Catalano, and R.G. Crystal. 1992. Modulation of airway inflammation in cystic fibrosis. *In vivo* suppression of interleukin-8 levels on the respiratory epithelial surface by aerosolization of recombinant secretory leukoprotease inhibitor. *J. Clin. Invest.* 90:1296-1301.
- McElvaney, N.G., R.C. Hubbard, P. Birrer, M.S. Chernick, D.B. Caplan, M.M. Frank, and R.G. Crystal. 1991. Aerosol α 1-antitrypsin treatment for cystic fibrosis. *Lancet (N. Am. Ed.)* 337:392-394.