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The Presence of a Matrix-Derived Neutrophil Chemoattractant in Bronchiolitis Obliterans Syndrome after Lung Transplantation[1,2]

Matthew T. Hardison,[3]* F. Shawn Galin,[3]* Christopher E. Calderon,* Uros V. Djekic,* Suzanne B. Parker,† Keith M. Wille,‡ Patricia L. Jackson,* Robert A. Oster,‡ K. Randall Young,‡ J. Edwin Blalock,[4]*§¶ and Amit Gaggar[4]*‡§

Lung transplantation is a therapeutic modality frequently used in end-stage lung disease. Unfortunately, lung transplant recipients have poor clinical outcomes, often due to the development of bronchiolitis obliterans syndrome (BOS). This process is often characterized by the pathologic findings of obliterative bronchiolitis: neutrophil influx and extracellular matrix remodeling leading to luminal obstruction and airway inflammation. The molecular mechanisms underlying BOS are poorly understood and disease-specific biomarkers are lacking. We report that in addition to increased levels of IL-8, the level of the neutrophil chemoattractant proline-glycine-proline (PGP) is elevated in BOS patient bronchoalveolar lavage (BAL) fluid. The enzymes responsible for generating PGP, matrix metalloproteases 8 and -9 and prolyl endopeptidase, are also elevated in these samples. Together, IL-8 and PGP account for most of the neutrophil chemoattractant capacity seen in BOS BAL fluid. Using specific neutralizing Abs to both IL-8 and PGP, we demonstrate that PGP is a prominent neutrophil chemoattractant found in BAL fluid from individuals at the time of diagnosis of BOS. These findings highlight the influence of a matrix-derived neutrophil chemoattractant in post-transplantation BOS and provide opportunities for the development of unique diagnostics and therapeutics to potentially improve disease outcomes. ***The Journal of Immunology,*** **2009, 182: 4423–4431.**

With the increasing prevalence of end-stage chronic pulmonary conditions and lack of disease-modifying medications, lung transplantation is often the only therapeutic modality available for affected individuals (1). Unfortunately, complications are frequent and result in reduced long-term preservation of graft function and patient survival (1, 2). Chronic allograft rejection accounts for poor rates of patient survival. More than 50% of all lung transplant recipients will eventually develop this condition (2). The clinical correlate of this condition is known as bronchiolitis obliterans syndrome (BOS).[5] It is a clinical diagnosis of exclusion made with decline in lung function (3–5). It is manifested histologically as obliterative bronchiolitis (OB), a fibroproliferative process which targets airways. Five-year survival after chronic rejection is 30% (6).

The specific pathogenic mechanism of OB is poorly understood but there is damage to both epithelial cells and subepithelial structures (7). Neutrophils are a prominent cell type found in the bronchoalveolar lavage (BAL) of BOS patients (8), which may have the capacity of inducing ongoing airway remodeling and inflammation. Chemokines are thought to be important effectors in cellular recruitment in the development of chronic rejection. Specifically, glutamate-leucine-arginine (ELR) positive CXC chemokines, important in neutrophil recruitment (9), may play an important role in the pathogenesis of this condition. Patients with BOS demonstrate increased IL-8 levels in BAL fluid (10, 11). CXCR2 ligands have been shown to be important in early neutrophil recruitment and ongoing vascular remodeling in OB (12).

Due to the degree of remodeling seen in OB, interest has turned to the role of proteases in the development of this condition. Several chronic inflammatory conditions are characterized by an imbalance of proteases with their naturally occurring antiproteases (13). We have recently reported a proteolytic pathway for the generation of a collagen-derived neutrophil chemoattractant (proline-glycine-proline (PGP)) (14), which involves the efforts of matrix metalloproteases (MMP-8 and MMP-9) and a serine protease,

*Department of Physiology and Biophysics, †Department of Pathology, ‡Department of Medicine, and §Gregory Fleming James Cystic Fibrosis Center, University of Alabama at Birmingham, AL 35294; and ¶Division of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

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[3] M.T.H. and F.S.G. have contributed equally to this work.

[4] Address correspondence and reprint requests to Dr. J Edwin Blalock and Dr. Amit Gaggar Gregory Fleming James Cystic Fibrosis Center, University of Birmingham, 1918 University Boulevard, MCLM 898, Birmingham, AL 35294-0005. E-mail addresses: blalock@uab.edu and agaggar@uab.edu

[5] Abbreviations used in this paper: BOS, bronchiolitis obliterans syndrome; BAL, bronchoalveolar lavage; FEV1, forced expiratory volume 1 s; FVC, forced vital capacity; MMP, matrix metalloprotease; N-α-PGP, N-terminal acetylated PGP; PE, prolyl endopeptidase; PGP, proline-glycine-proline; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; CF, cystic fibrosis; ECM, extracellular matrix; COPD, chronic obstructive pulmonary disease; IPF, interstitial pulmonary fibrosis.

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Table I. *Patient characteristics*[a]

Parameter	Nonrejection Transplant Controls	Acute Rejection	BOS
No.	7	8	7
Age	58.4 years (1.9)	48.3 years (3.7)	48.6 years (7.3)
Gender	58% male 42% female	38% male 62% female	58% male 42% female
Race	86% Caucasian, 14% AA[b]	75% Caucasian, 25% AA	86% Caucasian, 14% AA
Type of transplant (single vs bilateral)	86% single, 14% bilateral	87% single, 13% bilateral	72% single, 28% bilateral
Indication for lung transplant	28% IPF 58% COPD 14% CF	37% IPF 50% COPD 13% Pulm HTN	58% IPF 14% COPD 28% CF
Average time of lavage from date of transplant	1431 days (464)	1203 days (299)	3 mo before diagnosis: 878 days (205) At time of diagnosis: 961 days (208)
Lung function	FEV_1 = 1.47L (0.1) FEV_1% = 56.2 (5.1) FVC = 2.30L (0.08) FVC% = 64.1 (5.2)	FEV_1 = 1.57L (0.25) FEV_1% = 55.9 (5.9) FVC = 2.34L (0.29) FVC% = 58.4 (5.9)	3 mo before diagnosis: FEV_1 = 2.17L (0.24) FEV_1% = 73.1 (8.4) FVC = 2.94L (0.37) FVC% = 72.0 (8.0) At time of diagnosis: FEV_1 = 1.71L (0.21) FEV_1% = 58 (6.0) FVC = 2.53L (0.28) FVC% = 63.8 (7.2)
Immune suppression regimen	72% Prograf, prednisone, and Cellcept 28% Prograf, prednisone, and azathioprine	63% Prograf, prednisone, and Cellcept 37% Gengraf, prednisone, and Cellcept	86% Prograf, prednisone, and Cellcept 14% Gengraf, prednisone, and Cellcept

[a] Patient populations are presented in three columns with SEMs noted in parentheses. Data are presented as either percentages or means ± SEM.
[b] AA, African American; Pulm HTN, pulmonary hypertension.

prolyl endopeptidase (PE) (15, 16). PGP acts on CXCR1 and 2 receptors on neutrophils, similar to IL-8, causing neutrophil influx into areas of injury (14, 15).

Despite these intriguing findings, the impact of this matrix-derived system of inflammation in clinical disease is not currently known. The aim of the present study was to probe for the presence of these peptides and the proteases required for their generation in lung transplantation recipients to determine their potential role in the neutrophilic inflammation observed in BOS. In this report, we demonstrate that the appropriate enzymes required for PGP generation are present at both increased levels and activity in BOS samples compared with other transplant populations. We note a significant correlation between both MMP-9 activity with PGP levels and PE activity with PGP levels in BOS, supporting a direct relationship between these enzymes and PGP generation in this condition. These samples demonstrate increased capacity for neutrophil chemotaxis and have elevated levels of both IL-8 and PGP. Using an Ab against PGP, we are able for the first time to demonstrate the presence of PGP-mediated chemotaxis in BOS. Finally, we highlight the combined effects of IL-8 and PGP in BOS BAL fluid and suggest the possibility of a change in chemokine predominance seen during the development of BOS. These findings suggest a novel mechanism of neutrophilic inflammation in BOS and may identify disease biomarkers (PE, PGP) to characterize this condition.

Materials and Methods

Patient populations

The University of Alabama at Birmingham (UAB) Institutional Review Board approval (IRB X051014005) was obtained before all studies involving human participants and samples. All patients had BAL samples and basic clinical information collected with a unique patient identifier to maintain patient confidentiality. The diagnosis of BOS was made on a decline in forced expiratory volumes 1 s (FEV_1) to <80% of baseline over a 3-wk period without other identified etiologies. Diagnosis of acute rejection was ≥A2 grade rejection based off of a transbronchial biopsy. A1 grade rejection was not included into any population studied. All study patients had both a negative BAL culture for bacteria and virus. Clinical data are summarized in Table I.

Bronchoscopy and BAL processing

Bronchoscopy and BAL (4 × 25 ml of 0.9% NaCl for a total of 100 ml instilled, ~30–40 ml were recovered) were performed in all patients at 3-mo intervals and remnant lavage samples were stored in the Core Facility for Collection, Processing, and Storage of Alveolar Fluid (IRB X041026004). These samples were centrifuged at 200 × *g* for 10 min and the cell-free BAL was stored in −80°C until analysis. These samples underwent routine microbiologic testing, examining for bacteria (Gram stain and culture) and the presence of viruses (respiratory and CMV) in samples.

Materials

Coomassie Brilliant Blue R-250 was obtained from Bio-Rad. HyClone PBS (1×). 0067M (PO_4) without Ca^{2+} and without Mg^{2+} was from HyClone. Goat anti-rabbit Ig-HRP human adsorbed Ab was from Southern Biotechnology Associates. Albumin from bovine serum, Cohn V fraction, was obtained from Sigma-Aldrich. Z-Gly-Pro-pNA was from Chem Impex Internation. DMEM was from Mediatech.

Gelatin zymography

Porcine skin gelatin (Sigma-Aldrich) at 1 mg/ml was added to a 7.5% SDS-polyacrylamide solution before casting. Biologic samples were aliquoted and diluted in nonreducing sample buffer, and 25 μl of sample was added to each lane. All samples were electrophoresed at 12 mÅ for 1 h. Following electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h at 4°C, then incubated in 50 mM Tris-Cl for 16 h at 37°C. Gels were stained in Coomassie blue for 30 min and subsequently destained for 2 h.

MMP-8 and MMP-9 activity assay

Briefly, MMP-8- and -9-specific ELISA-based activity assays were used to quantify specific MMP activity (R&D Systems). Samples were diluted to fit the manufacturer's sensitivity for individual kits. Both samples and recombinant enzyme standards were prepared and incubated for 2 h at room temperature in 96-well plates coated with mAb for MMP of interest. After incubation, samples and standards were activated with 1 mM 4-aminophenylmercuric acetate, a chemical activator of MMPs, and further incubated

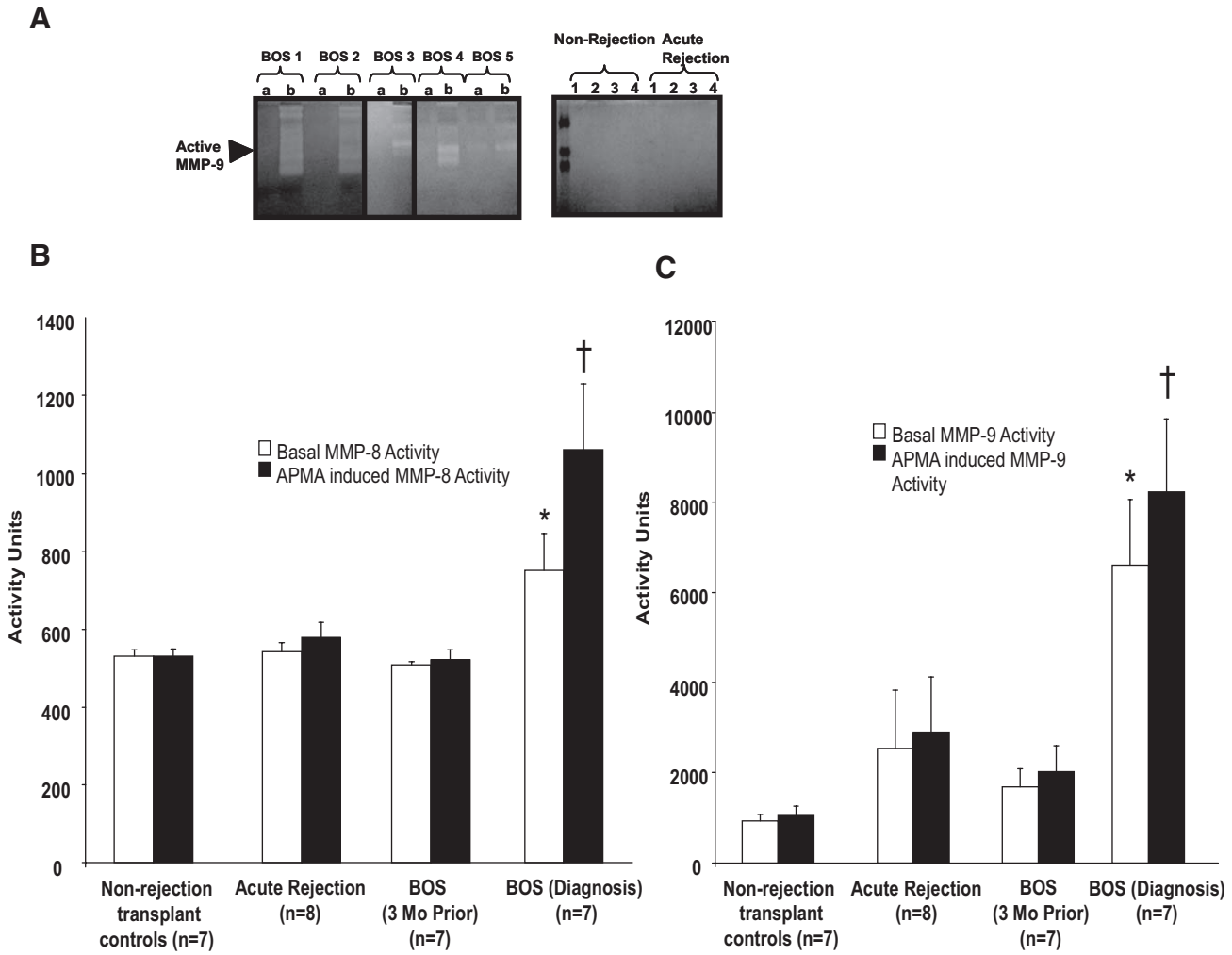

FIGURE 1. *A,* Zymogram of representative patient BAL. *Left,* BOS samples are paired at 3 mo before (*a*) and at time of diagnosis (*b*) per individual. Five of seven individuals displayed increased gelatinolytic activity compared with their prior matched samples at the time of BOS. *Right,* Representative zymogram of four nonrejection individuals and four acute rejection individuals. Overall, none of the nonrejection (zero of seven) or acute rejection (zero of eight) samples displayed any detectable gelatinolytic activity. *B*, MMP-8 activity in patient populations. BOS samples collected at the time of diagnosis had elevated levels of both basal (∗, $p < 0.05$) and total (†, $p < 0.05$) MMP-8 activity compared with BOS (3 mo before) and to other transplant populations. *C*, MMP-9 activity in patient populations. The activity of both the basal (∗, $p < 0.05$) and total (†, $p < 0.05$) forms of MMP-9 are elevated in BOS BAL at the time of diagnosis compared with BOS (3 mo before) and other transplant populations.

for 2 h at 37°C. After incubation, a fluorogenic substrate (Fluor-Pro-Leu-Gly-Leu-Ala-Arg-NH2) was placed in each well and the plate was incubated at 37°C for 18 h. The plate was then read on a spectrophotometer (excitation and emission wavelength of 320 and 405, respectively, SpectraMax Gemini; Molecular Devices) and data were quantified using standard curves provided with the kits.

Myeloperoxidase (MPO) assay

Briefly, commercially available assays kits were used to quantify MPO activity in clinical samples (Calbiochem). Samples were diluted to fit the manufacturer's sensitivity for the kit. Both samples and MPO standards were prepared and placed on 96-well plates coated with polyclonal Ab directed to human MPO. After a 2-h incubation, detection reagent was placed in wells for 1 h. The samples' absorbance was then measured at 450 nm wavelength.

Western blot

All samples were electrophoresed through SDS-polyacrylamide gels (both reducing and nonreducing conditions) and electroblotted onto nitrocellulose membranes. Membranes were blocked in PBS (pH 7.4) containing 5% BSA for 1 h. Once washed, they were incubated with primary Ab (rabbit anti-PE) for 1 h at room temperature. The polyclonal rabbit anti-PE Ab was made for us by EZ Biolab against a synthetic peptide representing residues 190–219 of mouse PE. This Ab detects recombinant human (whose sequence differs from mouse by one residue between aa 190 and 219), but not bacterial PE (whose sequence completely differs from human or mouse PE at residues 190–219). After incubation, samples were washed and incubated with goat anti-rabbit-HRP secondary Ab for 1 h. Immunoblots were then developed using ECL chemiluminescent kits (Pierce).

PE activity assay

Twenty microliters of BAL was incubated with a specific substrate (2 mM benzylcarboxy-glycine-proline-*p*-nitroaniline) at 37°C and 5% CO_2 and cleavage of *p*-nitroaniline from the substrate by PE was detected using a spectrophotometer at 410 nm and compared with a generated standard curve for PE activity.

IL-8 levels

Briefly, IL-8 ELISA kits were used to quantify the IL-8 levels in clinical samples (R&D Systems). Samples were diluted to fit the manufacturer's sensitivity for the kit. Fifty microliters of the samples or standards was added to 96-well plates coated with mAb against IL-8 for 2 h at room temperature. Thereafter, 100 μl of IL-8 polyclonal Ab conjugated with HRP was added to the wells and incubated for 1 h. Finally, hydrogen peroxide/chromogen was added to each well and, after 30 min, the absorbance was read at a wavelength of 450 nm.

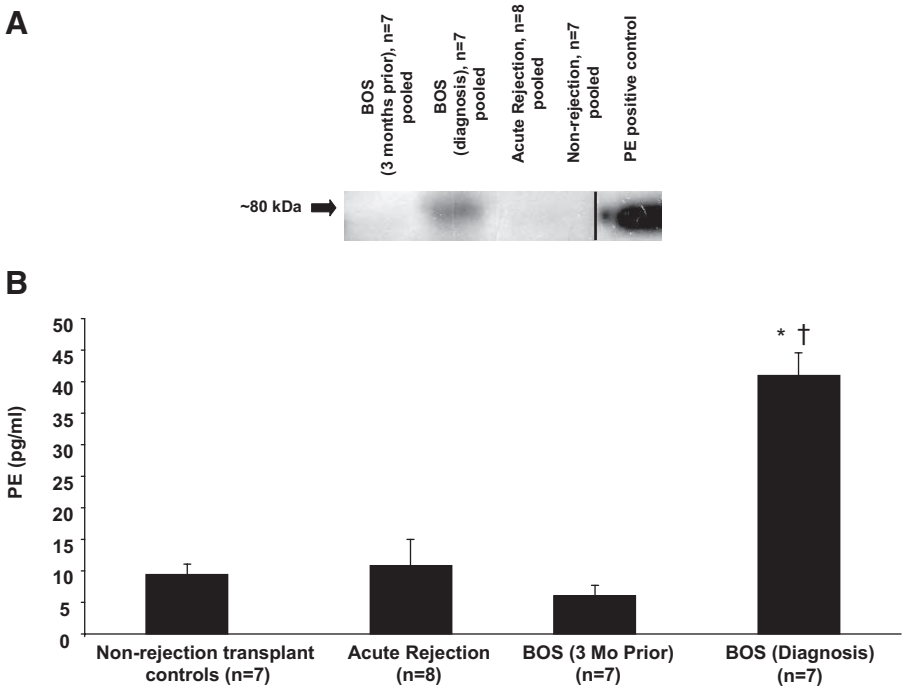

FIGURE 2. *A*, PE detection in pooled BAL fluid from study populations. BAL fluid was probed using a specific polyclonal Ab for PE. Only the BOS samples collected at the time of diagnosis detected PE (~80 kDa). *B*, PE activity in patient BAL fluid samples relative to transplant controls. BOS samples at the time of diagnosis demonstrate significantly elevated PE activity compared with matched samples collected 3 mo before diagnosis (†, $p < 0.01$) and other transplant populations (*, $p < 0.01$).

Electrospray ionization liquid chromatography/mass spectrometry/mass spectrometry for PGP detection

PGP and N-terminal acetylated PGP (N-α-PGP) were measured in BAL samples using a MDS Sciex (Applied Biosystems) API-4000 spectrometer equipped with a Shimadzu HPLC. HPLC was done using a 2.1 × 150-mm Develosi C30 column (with buffer A: 0.1% formic acid and buffer B: acetonitrile plus 0.1% formic acid: 0–0.6 min 20% buffer B/80% buffer A, then increased over 0.6–5 min to 100% buffer B/0% buffer A). Background was removed by flushing with 100% isopropanol plus 0.1% formic acid. Positive electrospray mass transitions were at 270-70 and 270-116 for PGP and 312-140 and 312-112 of N-α-PGP.

Neutrophil chemotaxis assay

Lavage sample was placed in the bottom wells of a 3-μm, 96-well polycarbonate filter plate (Millipore) in 150 μl of DMEM. Two × 10^5 neutrophils were added in 100 μl of DMEM to the top portion. These were incubated for 1 h at 37°C in 5% CO_2. The upper portion of the plate was removed and micrographs of the migrated cells were made with an Olympus IX70 microscope. Migration was standardized from cell counts such that chemotactic index = cells per high-powered field (experimental)/cells per high-powered field (medium control), as previously described (14). Chemokinesis experiments were preformed using BOS BAL fluid in the upper chamber with BOS BAL fluid in the lower chamber ("checkerboard").

For neutralization assays, anti-IL-8 neutralization Abs were purchased from R&D Systems. A polyclonal anti-PGP-neutralizing Ab was made for us by EZ-Biolabs by coupling N-α-PGP via its C terminus to keyhole limpet hemocyanin. This polyclonal Ab reacts with both N-α-PGP and PGP.

Statistical analysis

Descriptive statistics such as means and SEMs for quantitative measures and frequencies and proportions for qualitative measures were derived. Nonparametric testing was used to compare populations in this study. Comparisons between two groups were performed using the exact Wilcoxon rank sum test for unpaired data and the exact Wilcoxon signed-rank test were used for paired data. In addition, comparisons of proportions between groups were performed using the Fisher exact test. Spearman correlation analysis was used to compare relationships between 1) PE activity and PGP, 2) MMP-9 activity and PGP, 3) change in FEV_1 and change in PGP, and 4) change in forced vital capacity (FVC) and change in PGP. Calculations were made using SAS (version 9.1.3; SAS Institute) and SPSS (version 14; SPSS). Values of $p \leq 0.05$ were considered statistically significant.

Results

Gelatinolytic activity of proteases was increased in BOS lung transplant patients

To investigate the presence of PGP in clinical lung transplant samples, we began by examining whether or not enzymes necessary for PGP production are present in BAL fluid samples from transplant recipients. Using gelatin zymography, we demonstrated that BAL fluid from BOS patients at the time of diagnosis had a marked increase in gelatinolytic activity as compared with other transplant populations, with a major molecular weight band consistent with the active MMP-9 isoform. This activity was also noticeably increased when compared with matched samples 3 mo before the diagnosis of BOS (Fig. 1*A*). We have previously demonstrated that MMP-8 or MMP-9 activity seem to be required for PGP generation and that these enzymes can act in concert for more efficient generation of PGP (15, 16). We next examined both the activity and concentrations of these enzymes in our transplant populations. MMP-8 concentrations were elevated in BOS BAL fluid samples compared with other populations ($p < 0.05$). The majority of increased enzyme was detected as a zymogen form that was inducible by 4-aminophenylmercuric acetate (Fig. 1*B*; $p < 0.05$). In contrast to MMP-8, MMP-9 is constitutively active in BAL fluid of all populations. Interestingly, the average MMP-9 concentrations (146 ng/ml) in all populations were ~10-fold higher than the concentration of MMP-8 (16 ng/ml). However, like MMP-8, MMP-9 activity was severalfold lower in all other patient samples including BOS BAL fluid from 3 mo before diagnosis (Fig. 1*C*; $p < 0.05$). These data suggest that MMP-9 probably plays a prominent role in the generation of PGP after lung transplantation.

PE detection and activity were increased in BOS BAL fluid samples when compared with other patient groups

PE, a serine protease, has been previously described to have a central role in PGP generation (15). We examined whether this enzyme, not previously described in transplantation pathology,

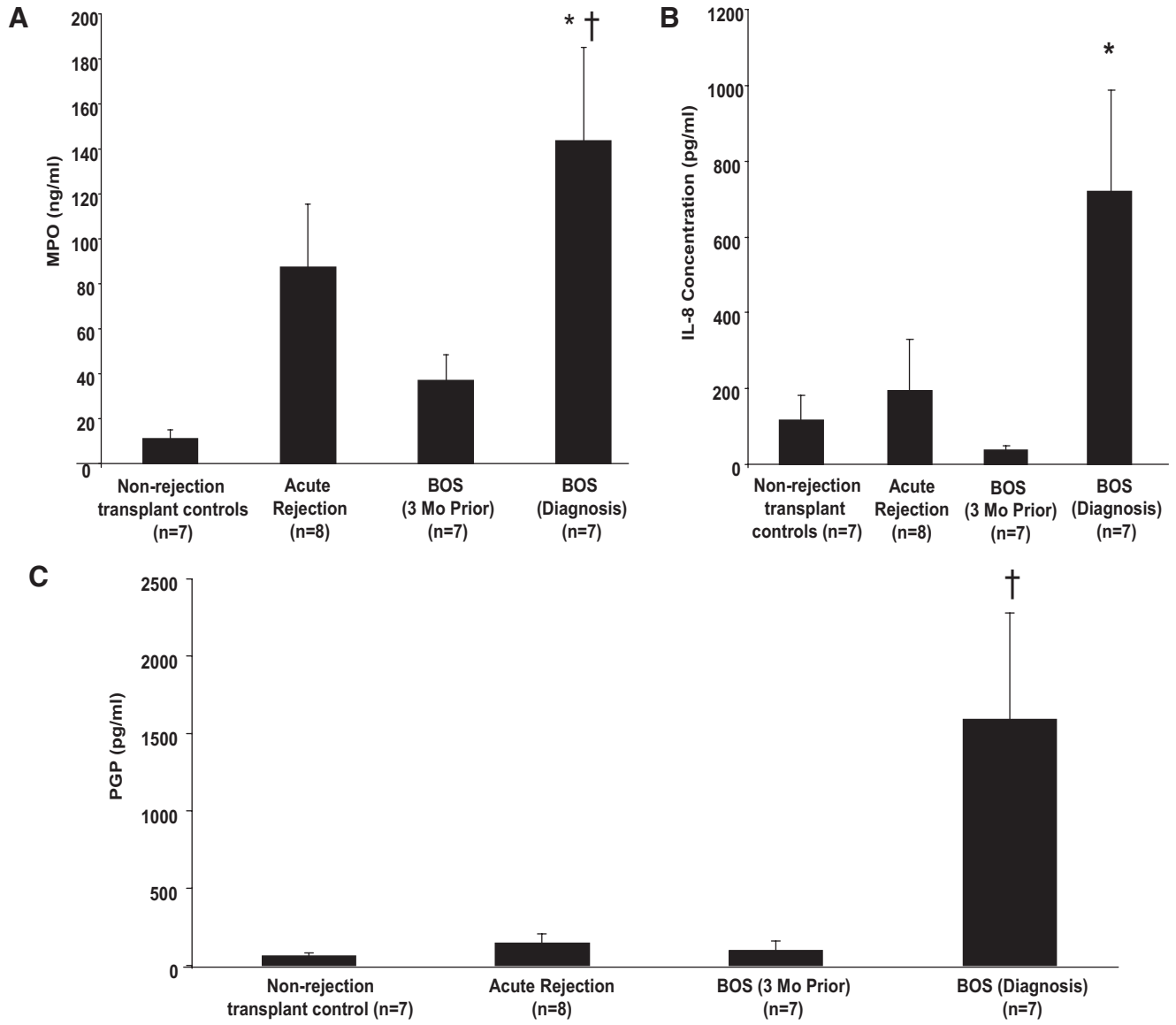

FIGURE 3. *A*, MPO in BAL fluid samples relative to transplant controls. BOS samples from the time of diagnosis are significantly elevated compared with both nonrejection transplant controls (∗, $p < 0.05$) and matched samples collected 3 mo before diagnosis (†, $p < 0.05$). *B*, IL-8 levels in BAL fluid from lung transplant populations. BOS BAL fluid collected at the time of diagnosis demonstrated a 6-fold increase in IL-8 levels compared with samples 3 mo before diagnosis(∗, $p < 0.05$). *C*, PGP levels in BAL fluid from lung transplant populations. BOS samples demonstrated a >16-fold increase in PGP compared with the individual's prior matched samples and other transplant populations (†, $p < 0.05$).

was present in the transplant patient samples. Western blot analysis identified an 80-kDa band (consistent with the molecular mass of PE) only present in the BOS samples taken at the time of diagnosis (Fig. 2*A*). The increased detection of this enzyme was complemented by 4-fold increased activity in BOS BAL fluid at the time of diagnosis compared with normal transplant patients or acute rejection ($p < 0.01$) and when compared with matched samples from 3 mo before BOS diagnosis ($p < 0.01$; Fig. 2*B*). These findings confirm that all of the necessary enzymes for PGP generation were found in BOS BAL fluid.

Classic and novel chemokine levels were elevated in BOS BAL fluid samples

As previously mentioned, BAL from BOS individuals have demonstrated increased polymorphonuclear leukocyte (PMN) counts (8). Indeed, when MPO, a surrogate of neutrophil influx (17), is measured in the transplant patient populations, there was an increased amount seen in BOS vs other groups ($p < 0.05$; Fig. 3*A*). To determine the neutrophil chemoattractants responsible for the increased neutrophil levels observed in these samples, we first examined the presence of IL-8. Levels of IL-8 were ~20-fold higher in BOS samples compared with the matched samples obtained 3 mo before diagnosis ($p < 0.05$; Fig. 3*B*) and higher than in samples from control populations. We also examined the presence of the neutrophil chemoattractant PGP in clinical disease samples using a mass spectrometry technique: electrospray ionization liquid chromatography-tandem mass spectrometry (14–16). PGP levels were 16-fold higher in BOS samples compared with their matched samples 3 mo before diagnosis ($p < 0.05$) and significantly higher compared with other transplant populations ($p < 0.05$; Fig. 3*C*). Only the non-acetylated form of PGP was found in BAL from lung transplant patients (in contrast with our previous reports of N-α-PGP detection in samples from cystic fibrosis (CF) patients).

Change in PGP correlated with change in lung function in BOS BAL fluid samples

We next examined whether changes in PGP concentrations correlated with changes in lung function in BOS individuals. We observed a correlation coefficient (r) of -0.83 ($p < 0.05$) for change in FVC and a r of -0.67 for change in FEV_1 ($p = 0.14$). These coefficients were somewhat better than those observed with IL-8 change vs change in FEV_1 ($r = -0.56$; $p = 0.25$) or change in FVC ($r = -0.73$; $p = 0.10$) in these samples.

PGP levels correlated with both MMP and PE activity in BOS BAL fluid samples

As we have previously described in a murine model and in CF sputum samples ex vivo (15, 16), PGP generation involves the coordinated activity of MMPs and PE. When we examined the correlation between MMP-9 activity and PGP levels in BOS BAL fluid, the samples demonstrated a strong correlation ($r = 0.86$, $p < 0.05$; Fig. 4*A*). Similarly, PE activity demonstrated a strong correlation with PGP concentrations in these samples ($r = 0.96$, $p < 0.01$; Fig. 4*B*). This exponential correlation is in keeping with the relationship of an enzyme and its product. Of note, similar r values were seen between MMP-9 activity/PGP and PE activity/PGP in the matched samples 3 mo before BOS, although these correlation coefficients were not as robust as that seen at the time of BOS. These findings suggest that these enzymes operate in vivo to generate PGP over the course of the development of BOS following lung transplantation.

BOS BAL samples were highly chemotactic for peripheral blood neutrophils ex vivo

Since we have demonstrated the presence of two neutrophil chemoattractants (IL-8 and PGP) in clinical samples, we examined the neutrophil chemotactic potential of BAL fluid from our study populations. BAL fluid from each of the transplant patient groups was used to perform a chemotaxis assay ex vivo on peripheral blood neutrophils (isolated from normal, nontransplant controls). The BOS samples taken at the time of diagnosis demonstrated a 16-fold higher chemotactic index relative to medium control ($p < 0.01$) and was also higher than transplant controls ($p < 0.05$) and pre-BOS patient population ($p < 0.05$; Fig. 5). These results are consistent with chemotactic activity of both IL-8 and PGP in these populations' BAL fluid. To verify that these results were due to chemotaxis and not chemokinesis, placement of BOS BAL fluid in the upper well reduced BOS fluid-mediated chemotaxis to the lower well by 82% ($p < 0.05$; data not shown).

Blockade of IL-8 and PGP ablated the chemoattractive ability of BOS BAL fluid samples

To determine the degree by which each of these chemokines alone or in concert influenced the chemotactic properties of BOS BAL fluid, we examined pooled samples at the time of BOS with the matched control samples from 3 mo before diagnosis. We investigated the samples' ability to induce neutrophil chemotaxis in the presence of a specific neutralizing IL-8 Ab and a specific PGP-neutralizing Ab developed in our laboratory. Chemotaxis neutralizing curves for each Ab were determined. For the anti-PGP Ab incubated with 100 μg/ml PGP, 1/5000 dilution gave 11% inhibition, 1/1000 dilution gave 92% inhibition, and 1/100 dilution gave 100% inhibition. For the anti-IL-8 Ab incubated with 10 ng/ml IL-8, 1/5000 dilution gave 26% inhibition, 1/1000 dilution gave 90% inhibition, and 1/100 gave 100% inhibition. These values led to the usage of a 1/1000 dilution of each Ab for neutralization experiments. Of note, these Abs demonstrated no cross-reactivity with the alternate chemokine (Fig. 6, *A* and *B*). When both Abs were used in combination in either the 3 mo before or at the time of diagnosis BOS BAL, we observed almost complete inhibition of chemotaxis activity ($p < 0.05$). This suggests that PGP, along with IL-8, are the major PMN chemoattractants in BOS BAL fluid.

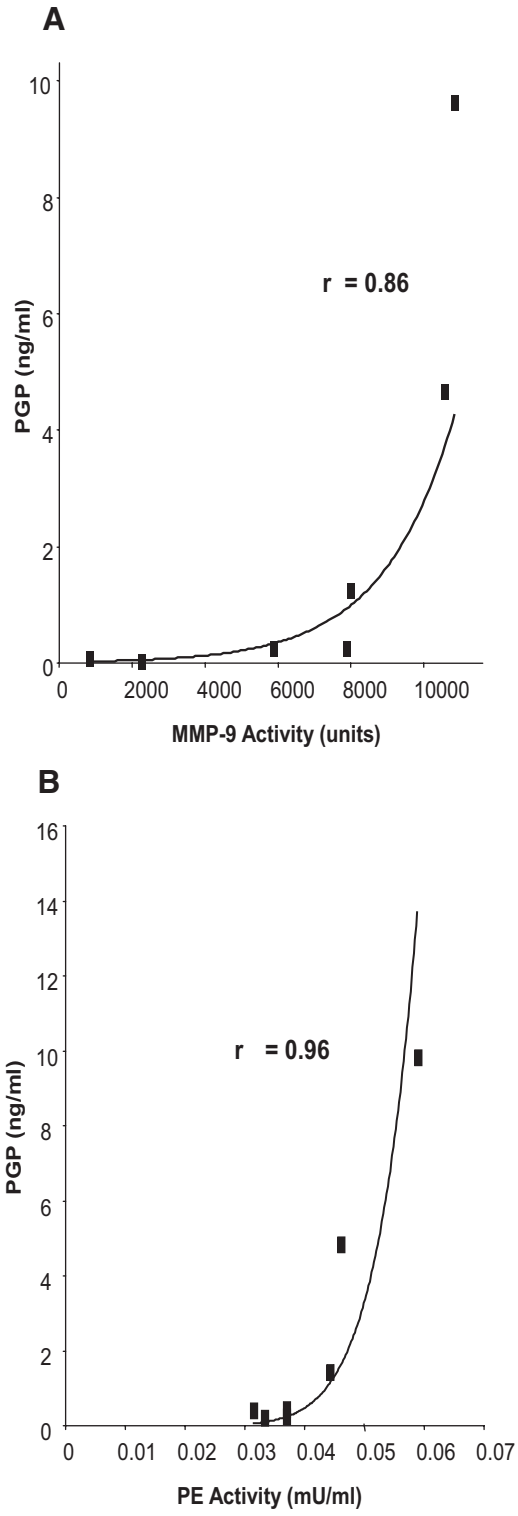

FIGURE 4. *A*, Correlation of MMP-9 activity and PGP levels. The PGP levels and MMP-9 activity in BOS samples taken at the time of diagnosis displayed a strong correlation r value of 0.86 ($p < 0.05$). *B*, Correlation of PE activity and PGP levels. The PGP levels and PE activity in BOS samples taken at the time of diagnosis displayed a strong correlation r value of 0.96 ($p < 0.01$).

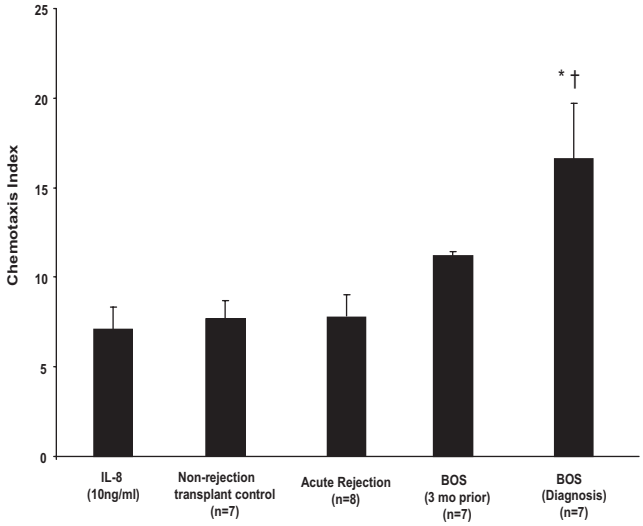

FIGURE 5. Neutrophil chemotaxis of BAL fluid from transplant populations. Samples collected at the time of BOS diagnosis demonstrated 16-fold increase in neutrophil chemotaxis relative to medium control and was significantly elevated compared with BOS 3 mo before diagnosis (†, $p < 0.05$) and to other transplant populations (*, $p < 0.05$).

By using the specific neutralizing Abs to both PGP and IL-8, we hoped to be able to determine the chemoattractant profile for both time points of the disease. In the samples collected 3 mo before diagnosis, the neutralizing Abs for IL-8 and PGP caused approximately an 81and 30% reduction in chemotaxis, respectively (Fig. 6*C*). When matched samples taken at the time of diagnosis were examined, IL-8-neutralizing Ab reduced the chemotactic ability of the samples by ~28%, while the PGP-neutralizing Ab caused a 53% inhibition of neutrophil migration. Only the anti-PGP Ab used in BOS samples at diagnosis demonstrated statistical significance with regard to chemotactic inhibition ($p < 0.05$). In contrast, only the IL-8 Ab used in BOS samples 3 mo before diagnosis demonstrated statistical significance with regard to chemotactic inhibition ($p < 0.05$). These data, therefore, provide some evidence for the importance of PGP as a neutrophil chemoattractant seen in BOS and suggest a shift from an IL-8-biased neutrophil chemotaxis to a PGP-biased neutrophil chemotaxis during the development of BOS.

Discussion

Chronic rejection/BOS in lung transplantation remains the major source of morbidity and mortality in lung allograft recipients (2). Although the role of neutrophils in BOS pathogenesis is not well understood, there is emerging evidence for PMN-derived oxidant injury and protease imbalance in this disease population, potentially leading to airway remodeling (18). The determination of unique pathways involved in this inflammatory and remodeling response may serve to identify unique biomarkers and to elucidate specific therapeutic targets.

Our work confirms that lavage samples from individuals with BOS have increased proteolytic activity. Although different families of proteases have been implicated in the end-organ damage described in postlung transplantation pathology, recent attention has turned to MMPs as mediators of lung damage (19, 20). Although MMP dysregulation appears to contribute to extracellular matrix reorganization in many conditions (21, 22), few studies have investigated MMPs in postlung transplant organ pathology. Studies have noted increased MMP-9 expression in ischemia reperfusion injury, in lung transplant patients with clinical airway obstruction, and in patients with BOS (23–25). There is also evidence that both MMP-8 and MMP-9 are increasingly elevated before the development of BOS in posttransplant individuals (26).

We demonstrate increased MMP-8 and MMP-9 levels and activity in lavage samples from patients at the time of BOS compared with matched samples from 3 mo before diagnosis. Using a novel polyclonal anti-PE Ab and an enzymatic assay to determine PE activity, we describe, for the first time, that PE is both detected and up-regulated in BOS samples. Our current and previous work suggest that increased activity of these enzymes in clinical samples is important for PGP liberation from collagen in vitro and in vivo (15). The statistically significant correlation coefficients seen with either MMP-9 or PE with PGP levels strongly suggest that these enzymes are operative in PGP production in BOS.

There is also increasing evidence that components of the extracellular matrix may serve as modulators of airway inflammation in lung transplantation rejection. Wilkes and colleagues (27, 28) have described the role of cryptic epitopes of type V collagen as a mediator of immune responses seen during chronic lung transplantation rejection. Attention has also turned to alternate pathways of inflammation involving fragmentation of matrix scaffolding proteins. Nonspecific collagen-derived fragments have been reported to induce neutrophil chemotaxis in murine models (29, 30). In addition, elastin fragments ending with proline-glycine demonstrate the capacity to cause fibroblast and monocyte chemotaxis and, to a lesser degree, neutrophil chemotaxis in models of emphysema (31).

BAL fluid samples from patients at the time of BOS also demonstrate an increased capability to attract neutrophils compared with all other transplant populations. These samples also exhibited high levels of IL-8, a neutrophil chemoattractant well characterized in BOS. Traditional CXCR ligands are felt to play an important role in both neutrophil influx and propagation of injury via activation of other cellular inflammatory mechanisms (10–12). In this study, we show that a extracellular matrix-derived neutrophil chemoattractant, PGP, is elevated in clinical samples from individuals with BOS. To our knowledge, this is the first characterized extracellular matrix (ECM) fragment described in transplantation rejection with the capability to drive neutrophilic inflammation.

We have also developed a novel detection and inhibitory Ab to PGP which distinguishes this peptide from IL-8. Using this reagent and an IL-8-specific Ab, we demonstrate that during the course of the development of BOS, there is an emerging role of PGP as a prominent neutrophil chemoattractant. Together IL-8 and PGP account for virtually all of the chemotactic activity seen in BOS BAL fluid (Fig. 6*C*) and may represent logical therapeutic targets. These findings expand our understanding of the role of the ECM as modulators of immune response in lung transplantation rejection, demonstrating that peptide fragments from ECM structural proteins may serve as important mediators of neutrophil recruitment. Our findings also support the concept that collagen-derived chemoattractants might have an increased role in neutrophil influx at the time of airflow reduction in BOS due to the airway remodeling seen in the disease.

One obvious question is whether pretransplant diagnosis may affect posttransplant PGP levels. Although we observe differences in PGP values between populations pretransplant (CF>chronic obstructive pulmonary disease (COPD)>interstitial pulmonary fibrosis (IPF)), all patients with BOS had higher PGP levels compared with other patients with similar pretransplant diagnoses in either the acute rejection or nonrejection populations. These data suggest that the posttransplantation pathology plays a more prominent role in PGP generation than the pretransplant diagnosis.

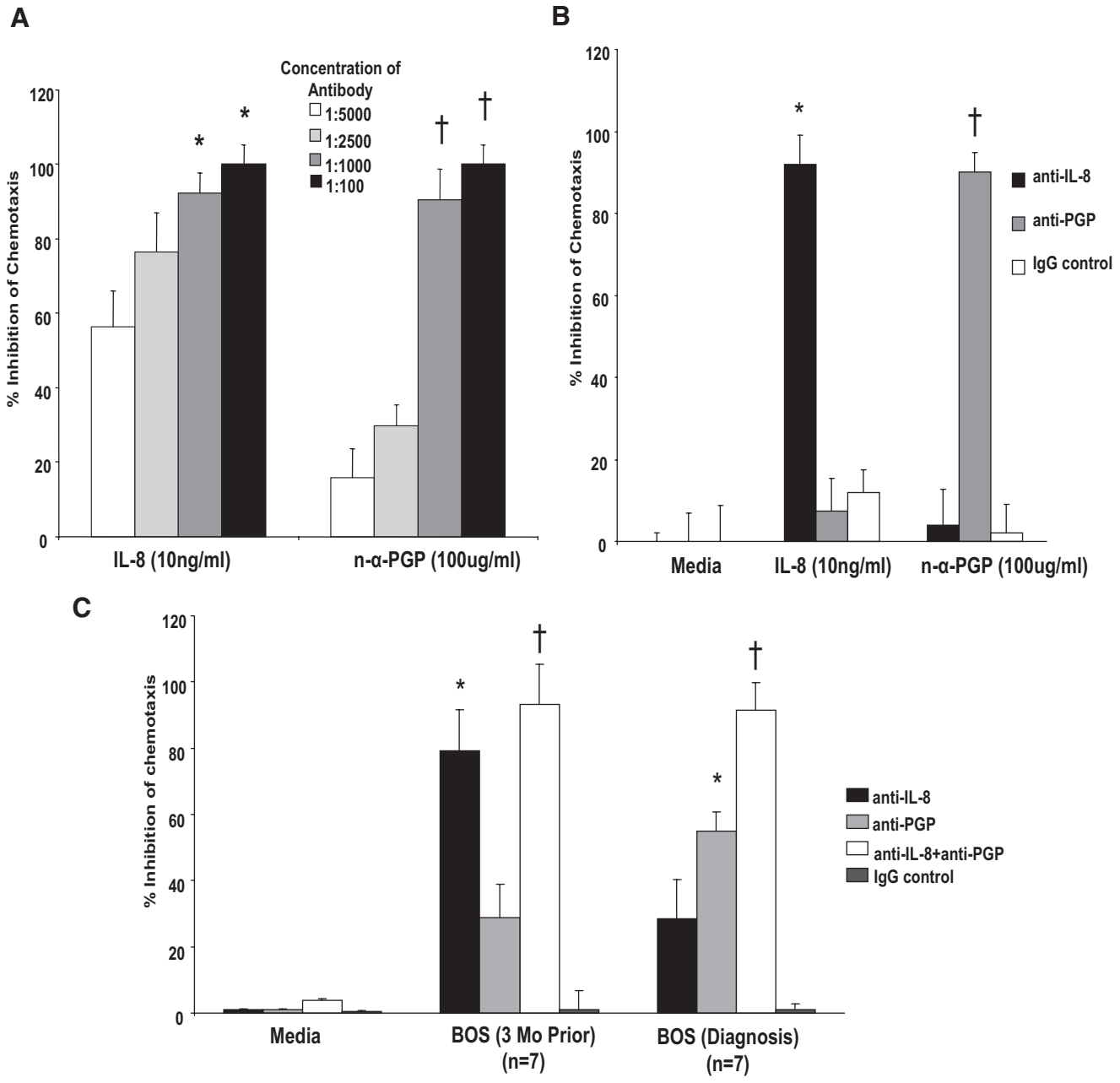

FIGURE 6. *A*, PGP- and IL-8- neutralizing Abs do not cross-react with the alternate chemokine. The IL-8- and PGP-specific neutralizing Abs (1/1000 each) completely inhibited IL-8- and PGP-mediated chemotaxis, respectively (∗†, $p < 0.01$). Neither Ab cross-reacts with the alternate chemokine. *B*, Relative IL-8 and PGP chemotaxis in BOS BAL fluid. IL-8-neutralizing Ab (1/1000) created an 80.6 and 29.6% inhibition of BOS (3 mo before) and BOS (at diagnosis) induced PMN chemotaxis, respectively. In contrast, the inhibition by the PGP-neutralizing Ab (1/1000) was 28.2% (in the 3-mo prior samples) and 53.4% (in the samples at time of diagnosis). Together, the Abs were able to almost completely ablate the chemotactic capacity of the BAL fluid collected 3 mo before (93.2%) and at the time of diagnosis (91.4%) of BOS (†, $p < 0.05$). Of the individual Abs, anti-IL-8-neutralizing Ab demonstrated a statistically significant reduction in chemotaxis for BOS (3 mo before diagnosis), whereas anti-PGP-neutralizing Ab demonstrated a statistically significant reduction in chemotaxis for BOS at diagnosis (∗, $p < 0.05$).

There are certain limitations to our study. Although most of the presented data is statistically significant between populations studied, the small numbers per group make it difficult to interpret these results for larger cohorts. Also, although correlations were seen between changes in lung function and changes in chemoattractants in BOS individuals, these findings should be taken with caution as they may represent epiphenomena. Examination of larger populations from multiple centers may allow for determination of the specificity and sensitivity of the levels/activity of PGP, PE, and MMPs in the development of BOS. In addition, future prospective studies with larger cohorts may examine if, over time, PGP levels may predict loss of lung function in posttransplantation individuals. As such, these longitudinal studies of PGP production in chronic rejection/BOS may also be useful to increase our understanding of disease onset and progression and possibly prognosis.

In summary, we demonstrate that PGP is increased in the progressive development of BOS in lung transplant recipients. The elevated levels in BAL fluid in BOS patients, along with the high correlation with generating enzymes and diagnosis of disease, make PGP a potential biomarker and therapeutic target for this condition. We have also shown that, although traditional chemokines regulate neutrophils before BOS, matrix degradation

products, such as PGP, have an emerging role in the PMN influx seen in clinical disease. The identification of this novel neutrophil chemoattractant pathway in chronic rejection following lung transplantation sets the stage for future studies to examine the role of this peptide in disease pathogenesis.

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Disclosures

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References

1. Arcasoy, S. M., and R. M. Kotloff. 1999. Lung transplantation. *N. Engl. J. Med.* 340: 1081–1089.
2. Trulock, E. P., L. B. Edwards, D.O. Taylor, M. M. Boucek, B. M. Keck, and M. I. Hertz. 2006. Registry of the international society for heart and lung transplantation: Twenty-Third Official Adult Lung and Heart-Lung Transplantation Report 2006. *J. Heart Lung Transplant.* 25: 880–892.
3. Estenne, M., J. R. Maurer, A. Boehler, J. J. Egan, A. Frost, M. I. Hertz, G. B. Mallory, G. I. Snell, and S. Yousem. 2002. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. *J. Heart Lung Transplant.* 21: 297–310.
4. Lama, V. N., S. Murray, R. J. Lonigro, G.B. Toews, A. Chang, C. Lau, A. Flint, K. M. Chan, and F. J. Martinez. 2007. Course of FEV1 after onset of BOS in lung transplant recipients. *Am. J. Respir. Crit. Care Med.* 175: 1192–1198.
5. Estenne, M., and M. I. Hertz. 2002. Bronchiolitis obliterans after human lung transplantation. *Am. J. Respir. Crit. Care Med.* 166: 440–444.
6. Lau, C. L., and G. A. Patterson. 2003. Current status of lung transplantation. *Eur. Respir. J.* 22(Suppl. 47): 57s–64s.
7. Boehler, A., and M. Estenne. 2003. Post-transplant bronchiolitis obliterans. *Eur. Respir. J.* 22: 1007–1018.
8. Elssner, A., and C. Vogelmeier. 2001. The role of neutrophils in the pathogenesis of obliterative bronchiolitis after lung transplantation. *Transplant. Infect. Dis.* 3: 168–176.
9. Weathington, N., and J. E. Blalock. 2005. The biology of CXC chemokines and their receptors. *Curr. Top. Membr.* 55: 49–71.
10. DiGiovine, B., J. P. Lynch III, F. J. Martinez, A. Flint, R. I. Whyte, M. D. Iannettoni, D. A. Arenberg, M. D. Burdick, M. C. Glass, C. A. Wilke, et al. 1996. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. *J. Immunol.* 157: 4194–4202.
11. Elssner, A., F. Jaumann, S. Dobmann, J. Behr, M. Schwaiblmair, H. Reichenspurner, H. Fürst, J. Briegel, and C. Vogelmeier. 2000. Elevated levels of interleukin-8 and transforming growth factor-β in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells: Munich Lung Transplant Group. *Transplantation* 70: 362–367.
12. Belperio, J. A., M. P. Keane, M. D. Burdick, B. Gomperts, Y. Y. Xue, K. Hong, J. Mestas, A. Ardehali, B. Mehrad, R. Saggar, et al. 2005. Role of CXCR2/CXCR2 ligands in vascular remodeling during bronchiolitis obliterans syndrome. *J. Clin. Invest.* 115: 1150–1162.
13. Gadek, J. D., and E. R. Pacht. 1990. The protease-antiprotease balance within the human lung. *Lung* 168(Suppl.): 552–564.
14. Weathington, N. M., A. H. van Houwelingen, B. D. Noerager, P. L. Jackson, A. D. Kraneveld, F. S. Galin, G. Folkerts, F. P. Nijkamp, and J. E. Blalock. 2006. A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat. Med.* 12: 317–323.
15. Gaggar, A., P. L. Jackson, B. N. Noerager, P. J. O'Reilly, D. B. McQuaid, S. M. Rowe, J. P. Clancy, and J. E. Blalock. 2008. A novel proteolytic cascade generates an extracellular matrix-derived chemoattractant in chronic neutrophilic inflammation. *J. Immunol.* 180: 5662–5669.
16. Malik, M., C. S. Bakshi, K. McCabe, S. V. Catlett, A. Shah, R. Singh, P. L. Jackson, A. Gaggar, D. W. Metzger, J. A. Melendez, et al. 2007. Matrix metalloproteinase-9 activity enhances host susceptibility to pulmonary infection with type A and B strains of *Francisella tularemia*. *J. Immunol.* 178: 1013–1020.
17. Tauber, E., Y. Herouy, M. Goetz, R. Urbanek, E. Hagel, and D. Y. Koller. 1999. Assessment of serum myeloperoxidase in children with bronchial asthma. *Allergy* 54: 177–182.
18. Hirsch, J., A. Elssner, G. Mazyr, K. L. Mairer, I. Bittmann, J. Behr, M. Sxhwaiblmair, H. Reichenspurner, H. Furst, J. Briegel, and C. Vogelmeier. 1999. Bronchiolitis obliterans syndrome after (heart-) lung transplantation. *Am. J. Respir. Crit. Care Med.* 160: 1640–1646.
19. Sternlicht, M. D., and Z. Werb. 2001. How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell Dev. Biol.* 17: 463–516.
20. Stamenkovic, I. 2003. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J. Pathol.* 200: 448–464.
21. Gaggar, A., Y. Li, N. Weathington, M. Winkler, P. L. Jackson, J. E. Blalock, and J. P. Clancy. 2007. Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients. *Am, J. Physiol.* 293: L96–L104.
22. Finlay, G. A., K. J. Russell, K. J. McMahon, E. M. D'Arcy, J. B. Masterson, M. X. Fitzgerald, and C. M. O'Conner. 1997. Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid from emphysematous patients. *Thorax* 52: 502–506.
23. Yano, M., Y. Omoto, Y. Yamakawa, Y. Nakashima, M. Kiriyama, Y. Saito, and Y. Fujii. 2001. Increased MMP-9 activity and mRNA expression in lung ischemia-reperfusion injury. *J. Heart Lung Transplant.* 20: 679–686.
24. Beeh, K. M., J. Beier, O. Kornmann, P. Micke, and R. Buhl. 2001. Sputum levels of MMP-9 and TIMP-1 and their ratio correlate with airway obstruction in lung transplant recipients: relation to TNF-α and IL-10. *J. Heart Lung Transplant.* 20: 1144–1151.
25. Hübner, R. H., S. Meffert, U. Mundt, H. Böttcher, S. Freitag, N. E. El Mokhtari, T. Pufe, S. Hirt, U. R. Fölsch, and B. Bewig. 2005. MMP-9 in bronchiolitis obliterans syndrome after lung transplantation. *Eur. Respir. J.* 25: 494–501.
26. Smith, G. N. Jr., E. A. Mickler, K. K. Payne, J. Lee, M. Duncan, J. Reynolds, B. Foresman, and D. S. Wilkes. 2007. Lung transplant metalloproteinase levels are elevated prior to bronchiolitis obliterans syndrome. *Am. J. Transplant.* 7: 1856–1861.
27. Haque, M. A., T. Mizobuchi, K. Yasufuku, T. Fujisawa, R. R. Brutkiewicz, Y. Zheng, K. Woods, G. N. Smith, Jr., O. W. Cummings, K. M. Heidler, et al. 2002. Evidence for immune responses to a self-antigen in lung transplantation: role of type V collagen-specific T cells in the pathogenesis of lung allograft rejection. *J. Immunol.* 169: 1542–1549.
28. Burlingham, W. J., R. B. Love, E. Jankowska-Gan, L. D. Haynes, Q. Xu, J. L. Bobadilla, K. C. Meyer, M. S. Hayney, R. K. Braun, D. S. Greenspan, et al. 2007. IL-17-dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants. *J. Clin. Invest.* 117: 3498–3506.
29. Postlethwaite, A. E., J. M. Seyer, and A. H. Kang. 1978. Chemotactic attraction of human fibroblasts to type I, II, and III collagen and collagen-derived peptides. *Proc. Natl. Acad. Sci. USA* 75: 871–875.
30. Riley, D. J., R. A. Berg, R. A. Soltys, J. S. Kerr, H. N. Gueds, S. F. Curren, and D. L. Laskin. 1988. Neutrophil response following intratracheal instillation of collagen peptides into rat lungs. *Exp. Lung Res.* 14: 549–563.
31. Senior, R. M., G. L. Griffin, R. P. Mecham, D. S. Wrenn, K. U. Prasad, and D. W. Urry. 1984. Val-Gly-Val-Ala-Pro-Gly, a repeating peptide in elastin, is chemotactic for fibroblasts and monocytes. *J. Cell Biol.* 99: 870–874.