Immunopathology and Infectious Diseases

Pseudomonas aeruginosa Exotoxin Pyocyanin Causes Cystic Fibrosis Airway Pathogenesis

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The cystic fibrosis (CF) airway bacterial pathogen *Pseudomonas aeruginosa* secretes multiple virulence factors. Among these, the redox active exotoxin pyocyanin (PCN) is produced in concentrations up to 100 $\mu\text{mol/L}$ during infection of CF and other bronchiectatic airways. However, the contributions of PCN during infection of bronchiectatic airways are not appreciated. In this study, we demonstrate that PCN is critical for chronic infection in mouse airways and orchestrates adaptive immune responses that mediate lung damage. Wild-type FVBN mice chronically exposed to PCN developed goblet cell hyperplasia and metaplasia, airway fibrosis, and alveolar airspace destruction. Furthermore, after 12 weeks of exposure to PCN, mouse lungs down-regulated the expression of T helper (Th) type 1 cytokines and polarized toward a Th2 response. Cellular analyses indicated that chronic exposure to PCN profoundly increased the lung population of recruited macrophages, CD4^+ T cells, and neutrophils responsible for the secretion of these cytokines. PCN-mediated goblet cell hyperplasia and metaplasia required Th2 cytokine signaling through the Stat6 pathway. In summary, this study establishes that PCN is an important *P. aeruginosa* virulence factor capable of directly inducing pulmonary pathophysiology in mice, consistent with changes observed in CF and other bron-

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Cystic fibrosis (CF) is one of the most common fatal genetic disorders among the Caucasian population, affecting ~30,000 individuals in the United States alone. CF is caused by mutations in the gene encoding the CF transmembrane regulator, which mediates anion (predominantly chloride, Cl^-) conductance. Because of temperature-dependent misfolding and misprocessing in the cytoplasm, the most common CF transmembrane regulator mutation, ΔF508 , exhibits reduced levels of CF transmembrane regulator localization to the apical membrane of lung epithelial cells, causing reduced levels of Cl^- secretion.^{1,2} The main pathological feature of CF airways is the accumulation of thick, inspissated mucus, which has been attributed to mechanisms including excessive airway water and sodium absorption by airway epithelia leading to airway surface liquid volume depletion, increased mucus concentration, mucus adhesion to airway surfaces, and delayed mucus transport.^{1,2} Defective mucociliary clearance has severe consequences in the lung as patients develop mucus obstruction of large and small airways, goblet cell hyperplasia, neutrophilic infiltration, and poor bacterial clearance, ultimately leading to scarring and airway fibrosis.^{1,2} The major clinical problem for CF patients is a progressive loss of lung function caused by chronic lung infection with mucoid *Pseudomonas aeruginosa*, resulting in the death of >80%

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Figure 1. Biosynthesis and profiling of PCN in clinical isolates of *P. aeruginosa* from CF patients. **A:** PCN is synthesized from phenazine carboxylic acid via enzymatic modification by PhzM and PhzS. **B:** The majority of *P. aeruginosa* CF clinical isolates overproduced PCN.

of patients.^{1,2} The repeated cycles of pro- and anti-inflammatory responses triggered by *P. aeruginosa*-associated surface antigens, as well as secreted exoproducts, progressively compile the damage on CF lungs.^{1–4}

Lung damage in *P. aeruginosa*-infected CF airways has been proposed to be partially because of imbalances between oxidants and antioxidants and between protease and antiprotease activities.^{1–4} In normal airways, the antioxidant capacity exceeds the level of oxidant formation because of the presence of a variety of antioxidants including enzymes, vitamins, metal chelators and thiols. Collectively, these antioxidants protect cellular components from oxidative damage. In *P. aeruginosa*-infected CF airways, there is an abundant neutrophilic inflammatory response stimulated by both host and bacterial factors. Dysregulated inflammatory responses lead to high levels of cytotoxic phagocyte-derived reactive oxygen species (ROS). ROS are also produced by the redox-cycling activity of pyocyanin (PCN), a blue-colored tricyclic phenazine (Figure 1A) that is produced in concentrations up to 100 $\mu\text{mol/L}$ by *P. aeruginosa* in CF airways.⁵ Notably, PCN-mediated ROS inhibit catalase activity, deplete cellular antioxidant reduced glutathione, and increase the oxidized reduced glutathione in the bronchiolar epithelial cells.^{3,4} Excessive and continuous production of ROS and inhibition of antioxidant mechanisms overwhelm the antioxidant capacity, leading to tissue damage.

As an immunomodulator, PCN inhibits ciliary beating of airway epithelial cells,⁵ nitric oxide production by macro-

phages and endothelial cells,⁶ prostacyclin production by endothelial cells,⁷ oxidation of leukotriene B₄ by neutrophils,⁸ and eicosanoid metabolism by platelets.⁹ PCN also enhances superoxide production,¹⁰ increases apoptosis in neutrophils,^{11,12} and inactivates α 1-protease inhibitor.¹³ In addition, PCN increases calcium signaling in human airway epithelial cells, stimulates interleukin (IL)-8 release, and inhibits regulated on activation normal T cell expressed and secreted and monocyte chemoattractant protein-1 release in human epithelial cells.^{14–17} PCN furthermore inhibits the expression of IL-2 and its receptor.¹⁸ In animal models, PCN stimulates IL-8 release, neutrophil influx, and bronchoconstriction in sheep and decreases tracheal mucus velocity in sheep, guinea pigs, and baboons.^{19–22}

Recently, we provided direct evidence that PCN participates in *P. aeruginosa* virulence using PCN-deficient mutants that were found to be attenuated in their ability to infect mouse lungs in an acute pneumonia model of infection when compared with isogenic wild-type bacteria.²³ These mutants also were less competitive than isogenic parental wild-type bacteria during competitive mixed infection using the agar bead model of chronic lung infections.²³ Thus, the production of PCN appears to confer a growth and/or survival advantage in mixed culture settings. These studies provide the most direct evidence for the importance of PCN in the *P. aeruginosa*-infected airway.

PCN biosynthesis is regulated by the intercellular process of bacterial communication known as quorum sensing,^{3,4} a process that is also critical for biofilm formation and full virulence.²⁴ PCN biosynthesis is increased during *P. aeruginosa* growth in biofilms *in vitro*.^{25,26} As biofilms are the predominant mode of *P. aeruginosa* growth within CF airways,^{1,2,4,27} not surprisingly, PCN concentrations of up to 100 $\mu\text{mol/L}$ have been detected in pulmonary secretions of CF patients.⁵ Because the major CF pathogen is *P. aeruginosa*, lung epithelia and macrophages are constantly being exposed to PCN, which contribute to oxidative stress and immunomodulation. In this study, we examined whether continuous exposure of mouse lungs to PCN could result in some pathological features commonly found in CF airways of humans.

Materials and Methods

P. aeruginosa Strains, Growth Conditions, and PCN Purification

Wild-type *P. aeruginosa* strain PA01 is the most widely used laboratory isolate.²⁸ The genome of PA01 has been sequenced and annotated (www.pseudomonas.com/gbrowse_index.jsp). The PCN-deficient mutants ΔphzM and ΔphzS were derived from PA01.²³ All *P. aeruginosa* strains were grown in Luria broth at 37°C with or without 30 $\mu\text{g/ml}$ gentamicin. Sputum samples were collected from patients following approval by the Institutional Review Board at the University of Cincinnati. *P. aeruginosa* isolates were cultured by serially diluting and plating sputa on *Pseudomonas* Isolation Agar (Difco, Sparks,

MD). PCN was purified from late stationary phase culture of PA01 with successive rounds of $\text{CHCl}_3/0.2\text{ N}$ hydrochloric acid extraction as described previously.²⁹ The final PCN preparations were dissolved in sterile water. PCN preparations were determined to have no detectable levels of LPS as determined by the E-TOXATE (Sigma-Aldrich, St. Louis, MO) and QCL-1000 lysosomal acid lipase (BioWhittaker, Walkersville, MD) assays, or homoserine lactone autoinducers *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone as measured by high-performance liquid chromatography, or peptidoglycan by enzyme-linked immunosorbent assay (ELISA) using an anti-peptidoglycan monoclonal antibody (MAB983; Millipore, Bedford, MA) or CpG by Toll-like receptor (TLR)9 activation assays as described previously.³⁰ The presence of *P. aeruginosa* flagellin (TLR5 agonist) and slime-glycolipoprotein (TLR2 agonist) have been ruled out by TLR2 and TLR5 activation assays in HeLa cells as described previously.³¹ Final PCN preparations were diluted to $1\text{ }\mu\text{g}/\mu\text{l}$ concentration in sterile H_2O .

PCN Purification from CF P. aeruginosa Clinical Isolates

To examine PCN production, frozen stocks of CF *P. aeruginosa* isolates were cultured in King's medium A broth by shaking for 12 hours at 37°C .³² Bacterial cells were removed by centrifugation, and supernatants were collected. PCN in the supernatants was quantified using a Beckman DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA) with the following formula: PCN concentration $[\text{mg}/\text{ml}] = \text{OD}_{690\text{ nm}} \div 16$.

Model of Chronic Mouse Lung Infection

Chronic mouse infections were performed as described previously.²³ Briefly, stationary phase wild-type PA01 and isogenic PCN-deficient mutants *phzM* and *phzS* were entrapped in agar beads by mixing with heavy mineral oil (Sigma-Aldrich) at 52°C and stirred vigorously for 6 minutes, followed by cooling the mixture on ice for 10 minutes. The bacteria-containing beads were recovered by centrifugation ($9000 \times g$ for 20 minutes at 4°C), followed by extensive washing in PBS. The beads were passively filtered through sterile $200\text{-}\mu\text{m}$ -diameter hole nylon mesh and then verified for size (70- to $150\text{-}\mu\text{m}$ diameter) and uniformity by microscope examination. An aliquot of beads was homogenized and plated onto Luria broth agar plates to determine the number of colony-forming units. A $100\text{-}\mu\text{l}$ inoculum containing 1×10^5 colony-forming units of viable *P. aeruginosa* entrapped in agar beads was introduced into the lungs of adult FVBN mice (9 weeks old, groups of eight) via the trachea nonsurgically by a 21-gauge blunt-end needle to the back of the tongue above the tracheal opening. Successful delivery of the beads to the lungs was manifested by choking the mouse immediately after instillation followed by rapid breathing.

Model of Chronic PCN Exposure in FVBN Mice

Nine-week-old wild-type FVBN and C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, IN) and $\text{Stat6}^{-/-}$ (C57BL/6 background) (The Jackson Laboratory, Bar Harbor, ME) were housed in positively ventilated microisolator cages with automatic recirculating water, located in a room with laminar, high efficiency particle accumulation-filtered air. The animals received autoclaved food, water, and bedding. Mice used in experimental procedures were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. PCN (10 or $25\text{ }\mu\text{g}$ in $25\text{ }\mu\text{l}$) was intranasally inoculated into the lungs of groups of 12 FVBN mice anesthetized with isoflurane three times a week for the intervals of 3, 6, and 12 weeks. C57BL/6 mice and $\text{Stat6}^{-/-}$ mice were inoculated with PCN for 3 weeks. Control mice were exposed to $25\text{ }\mu\text{l}$ of sterile water. These time points were chosen based on our pilot studies demonstrating PCN-induced goblet cell hyperplasia, pulmonary fibrosis, and airspace destruction in FVBN mice, where clear differences in the lung histopathology, cytokine and immune cell profiles can be detected between the PCN-treated and control mice.

Bronchoalveolar Lavage and Assessment of Infiltrating Pulmonary Leukocytes

Bronchoalveolar lavage (BAL) was performed to obtain cells for fluorescence-activated cell sorting analysis of leukocyte subsets. The trachea was exposed and intubated with a 1.7-mm outer diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mmol/L EDTA in 1-ml aliquots. Two milliliters of PBS was instilled per mouse. Total cell counts were determined using a cell counter. Differential cell analysis was performed by flow cytometry to determine the absolute number and type of lymphocytes, macrophages, and neutrophils. Specifically, analysis of CD4, CD62L, CD8, and T cell receptor (TCR) β expression were used to distinguish naive CD4 T cells (CD4^+ , $\text{CD62L}^{\text{high}}$, and $\text{TCR } \beta^+$), nonnaive (antigen experienced) CD4 T cells (CD4^+ , $\text{CD62L}^{\text{low}}$, and $\text{TCR } \beta^+$), naive CD8 T cells (CD8^+ , $\text{CD62L}^{\text{high}}$, and $\text{TCR } \beta^+$), and nonnaive CD8 T cells (CD8^+ , $\text{CD62L}^{\text{low}}$, and $\text{TCR } \beta^+$). Fc receptor binding was blocked with anti-murine CD16/CD32 (FcR III/II ; BD Pharmingen, San Diego, CA). In a separate panel, analysis of Gr-1, F4/80, and CD11b expression were used to distinguish neutrophils (CD11b^+ and Gr-1 $^{\text{bright}}$), resident macrophages (F4/80^+ and CD11b^-), and infiltrating macrophages (F4/80^+ and CD11b^+). In a final panel, analysis of TCR δ , DX5, CD4, and TCR β were used to identify $\gamma\delta$ T cells ($\text{TCR } \delta^+$, CD4^+ , and $\text{TCR } \beta^-$), natural killer (NK) cells (DX5^+ , CD4^- and $\text{TCR } \beta^-$), and NK T cells (DX5^+ , CD4^+ and $\text{TCR } \beta^+$). The experiments were conducted on three separate occasions using at least six mice, and statistical analyses were performed as indicated.

ELISA

Cytokine protein levels in BAL or lung homogenates were determined by ELISA according to the manufacturer's protocols (Invitrogen, Carlsbad, CA).

Histopathology Evaluation of Mouse Lung Tissues

After BAL, lung tissues were collected for histopathological analyses. Briefly, a cannula was inserted in the trachea, and the lung was instilled with 10% phosphate-buffered formalin at a constant pressure (25 cm H₂O). The trachea was ligated, and the inflated lung was immersed in fixative for 24 hours for histological analysis and then embedded in paraffin wax. Five-micrometer paraffin wax sections were adhered to slides and stained with H&E, Masson's Trichrome, PAS reagent, and Alcian blue. The pathological inflammation, fibrosis, alveolar space destruction, and goblet cell hyperplasia in a mid-sagittal section from the lung were evaluated under light microscope. A blinded pathologist examined tissue sections independently at the Department of Pathobiology, University of Illinois at Urbana-Champaign.

Hydroxyproline Assay

Collagen deposition was estimated by measuring the hydroxyproline content of the whole lung in PCN-treated or control mice (groups of six) as described previously.³³ Briefly, a 500- μ l sample of lung homogenate was added to 1 ml of 6 N HCl for 8 hours at 120°C. To a 5- μ l sample of the digested lung, 5 μ l of citrate/acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid (pH 6.0)) and 100 μ l of chloramine-T solution (282 mg of chloramine-T, 2 ml of *n*-propanol, 2 ml of distilled water, and 16 ml of citrate/acetate buffer) were added. The resulting samples were incubated at room temperature for 20 minutes before 100 μ l of Ehrlich's solution (Aldrich, Milwaukee, WI), 9.3 ml of *n*-propanol, and 3.9 ml of 70% perchloric acid were added. These samples were incubated for 15 minutes at 65°C, and cooled samples were read at 550 nm in a DU 640 spectrophotometer (Beckman Coulter). Hydroxyproline concentrations were calculated from a hydroxyproline standard curve (0 to 100 μ g of hydroxyproline/ml).

Assessment of Goblet Cell Hyperplasia and Mucus Overproduction

Mucus cell development along the airway epithelium was quantified in paraffin-embedded tissue sections (4 to 8 μ m) stained with PAS. Parasagittal sections (*n* = 5 mice/group) were analyzed by bright-field microscopy using an image analysis software program (ImagePro Plus; Media Cybernetics, Silver Spring, MD) to derive an airway mucus index (MI) reflective of both the amount of mucus per airway and the number of airways affected. The mucus content of all of the airways per section (20 to 30,

proximal to distal) was measured from groups of four to five animals as published previously.³⁴ An imaging program (Image ProPlus; Media Cybernetics) was used to quantify the area and intensity of PAS staining per airway. The airway MI was calculated by summing the ratio of the PAS-positive epithelial area to the total epithelial area per section and dividing by the number of airways per section.

Mean Linear Intercept Determination

Mean linear intercept (MLI) was calculated on H&E-stained sections of mouse lung as described previously.^{35,36} Ten fields per section from five sections per mouse (five to eight mice per group) were evaluated, both vertically and horizontally, independently, and each by two investigators. Measurements of the two investigators were not significantly different. Large airways and blood vessels were excluded from the measurements.

Immunohistochemistry of IL-4 and IL-13

Paraffin-embedded mouse lung sections (4 to 8 μ m) were stained with rat anti-mouse IL-4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-mouse IL-13 polyclonal antibody (Santa Cruz Biotechnology) and visualized using the avidin-biotin complex kit (Vector Laboratories, Burlingame, CA) according to protocols supplied by the manufacturers.

Quantitative RT-PCR of Mucin Biosynthesis Gene MUC5AC

A human pulmonary mucoepidermoid carcinoma cell line, NCI-H292, was purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a humidified, 5% CO₂-supplemented air-containing incubator at 37°C. On reaching visual confluence, the cells were treated with control medium (RPMI 1640), 25 μ g/ml PCN, or with medium supplemented with human recombinant IL-4 (10 ng/ml; Endogen, Rockford, IL) or human recombinant IL-13 (25 ng/ml; Thermo Fisher Scientific, Rockford, IL) for 24 hours. Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated from 5 μ g of total RNA using the ThermoScript RT-PCR system (Invitrogen). Quantitative RT-PCR analyses of MUC5AC and GAPDH mRNA were performed using the appropriate oligo primers and ABI PRISM 7700 sequence detection system. The MUC5AC primer sequences were as follows: forward, 5'-TGTTCTATGAGGGCTGCGTCT-3', and reverse, 5'-ATGTCGTGGGACGCACAGA-3'. The glyceraldehyde-3-phosphate dehydrogenase primer sequences were as follows: forward, 5'-AGTGGATATTGTTGCCATCA-3', and reverse, 5'-GAAGATGGTGATGGGATTTC-3'. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for normalizing MUC5AC mRNA levels in control and exper-

imental samples. Quantitative RT-PCR was performed using the Power SYBR Green PCR Master Mix Applied Biosystems, Foster City, CA).

Statistics

Parametric data were analyzed for statistical significance by Student's *t*-tests, with differences between means considered significant when $P < 0.05$.

Results

Clinical Isolates of *P. aeruginosa* from CF Airways Produce More PCN Than Laboratory Strain PA01

A study performed in 1988 involving 13 CF and other bronchiectasis patients demonstrated that *P. aeruginosa* was capable of producing PCN at concentrations as high as 27 $\mu\text{g/ml}$ in their sputum.⁵ These data suggested a role for PCN in the pathogenesis of CF and other bronchiectasis airways. However, extensive use of antibiotics to treat chronic CF infection by *P. aeruginosa* may affect PCN production. We determined the PCN production ability of freshly isolated CF strains from anonymous *P. aeruginosa*-colonized CF patients at the University of Cincinnati Adult CF Clinic. *P. aeruginosa* isolates were cultured overnight in King's Medium A, and PCN was extracted and quantified. As shown in Figure 1B, 9 of the 12 CF isolates produced more PCN than laboratory strain PA01, and one strain produced equivalent amounts of PCN as PA01. Two strains produced less PCN than PA01. These results suggest that many CF clinical isolates of *P. aeruginosa* may overproduce PCN.

PCN Is Important for Chronic Lung Infection

One of the main features of CF pathogenesis is poor bacterial clearance from the airways.^{1,2,4} High amounts of PCN in CF sputum⁵ and increased ability of PCN secretion by CF isolates of *P. aeruginosa* (Figure 1B) suggest that PCN may play important roles during chronic infection in CF and other bronchiectasis lungs. We have previously shown that PCN plays an important role during acute lung infection.²³ Because *P. aeruginosa* infection of CF and other bronchiectasis airways is chronic, demonstration of the requirement of PCN during chronic infection would suggest that PCN plays a role in the pathogenesis of these diseased airways. We have also previously shown that PCN-deficient strains *phzM* and *phzS* were less competitive than their isogenic parental wild-type PA01 in an agar bead model of chronic lung infection.²³ We determined the requirement of PCN during *in vivo* single infection with wild-type PA01 and its isogenic PCN-deficient mutants *phzM* and *phzS* (Figure 2A). When infected singly with 1×10^5 bacteria embedded in agarose beads, FVBN mice were unable to clear wild-type PA01. By 7 days postinfection, viable counts of PA01 increased by ~ 0.8 logs (Figure 2A). In contrast, the

Figure 2. PCN-deficient mutants of *P. aeruginosa* are attenuated in causing chronic respiratory tract infections. **A:** Male FVBN mice (9 weeks old, groups of eight) were intratracheally infected with agarose-embedded wild-type *P. aeruginosa* strain PA01 or isogenic PCN-deficient mutants *phzM* or *phzS* or genetically complemented *pucP26phzM* or *pucP26phzS*. One group of mice infected with *phzM* or *phzS* was given exogenous PCN (50 μg) intranasally every day for 7 days. Attenuation is defined as the $\log_{10}$ difference in colony-forming units between PA01 and mutant bacteria recovered from lung tissue 7 days after inoculation. The mean $\pm$ SE was shown. *P* values: *phzM* (0.007) and *phzS* (0.009) against PA01. *P* values: *phzM* (0.687) and *phzS* (0.616) against PA01 when *phzM* and *phzS* mutants infected lungs were exogenously supplied with PCN. *P* values: *pucP26phzM* (0.481) and *pucP26phzS* (0.538) against PA01. **B:** H&E-stained lung sections of FVBN mice infected with wild-type PA01 and PCN-deficient mutant *phzM*. **C:** The profile of proinflammatory cytokine KC in PA01-infected versus *phzM*- or *phzS*-infected mice. * $P < 0.001$ when comparing PA01 against *phzM* or *phzS*.

bacterial load of PCN-deficient *phzM* and *phzS* was attenuated by 2.1 and 1.9 logs, respectively (without PCN; Figure 2A). We next examined if exogenously added PCN could complement PCN deficiency in the mutant strains. Indeed, exogenous PCN restored virulence to mutant strains *phzM* and *phzS*, and numbers of viable bacteria recovered were comparable with the wild-type PA01

Figure 3. Chronic exposure of mouse lungs to PCN results in goblet cell hyperplasia. **A:** FVBN mice (groups of 12) were exposed to PCN (10 or 25 μ g in 25 μ l, three times per week, for 3, 6, and 12 weeks). Control mice were exposed to 25 μ l of sterile H₂O for the same duration. After BAL, the lungs were perfused with buffered formalin, embedded in paraffin, sectioned, and double-stained with PAS. No mucin staining was detected in the conducting airways of control mice. **B:** After 12 weeks of exposure to 10 μ g/ml PCN, small amounts of goblet cells were present as indicated by PAS-stained mucins (red arrows). **C:** After 6 weeks of exposure to 25 μ g/ml PCN, goblet cells were present in conducting airways (red arrows). **D:** After 12 weeks of 25 μ g/ml PCN instillation, mouse lungs developed goblet cell hyperplasia with numerous goblet cells stained positive for mucins (red arrows). **E:** Goblet cell metaplasia in a small airway. **F:** Airway MI of control and PCN-exposed mice. Lung sections from control and PCN-exposed mice were PAS stained. Airway MI was determined as described in *Materials and Methods*. Six weeks PCN (**P* < 0.05) and 12 weeks PCN (**P* < 0.05), respectively, when compared with control mice.

(with PCN; Figure 2A). Most importantly, the ability of *phzM* and *phzS* mutants to chronically infect the lungs was restored by providing copies of the wild-type *phzM* (*pucP26-phzM*) or *phzS* (*pucP26-phzS*) gene *in trans* (genetic complementation; Figure 2A). Histological analysis suggests that PA01- or PAOC-fliC-infected mouse lungs showed severe congestion with increased infiltration of leukocytes (data not shown; Figure 2B). In contrast, *phzM*- or *phzS*-infected mice developed alveolitis with lesser amount of immune cell infiltration (data not shown; Figure 2B). Analysis of the neutrophilic chemokine KC showed that PA01 infection induced significantly higher levels of KC at 6- and 12-hour postinfection than the *phzM*- and *phzS*-infected mice (Figure 2C). Collectively, these results indicate that PCN plays an important role during chronic lung infection by *P. aeruginosa*.

Mouse Lungs Chronically Exposed to Clinically Relevant Concentrations of PCN Develop Goblet Cell Hyperplasia

PCN at levels reaching 27 μ g/ml has been detected in sputa of CF and bronchiectasis patients.⁵ Due to the capa-

bility of PCN to cause oxidative damage and immunomodulate the cytokine profiles of lung epithelia^{3,4,14–17,29}, we investigated whether chronic exposure to PCN could induce pathological features consistent within CF airways, including goblet cell hyperplasia, airway fibrosis, alveolar airspace destruction, and leukocyte influx in mouse lungs. One of the most prominent pathological features of airways in CF patients is the accumulation of thick mucus caused by excessive airway water and sodium absorption by airway epithelia and by excessive mucin secretion caused by goblet cell hyperplasia.^{1,2,4} Thus, we examined if chronic PCN exposure resulted in increased number of goblet cells. FVBN mice were intranasally inoculated three times per week with two different clinically relevant concentrations of PCN (10 or 25 μ g resuspended in 25 μ l of sterile H₂O) for 3, 6, and 12 weeks. Previous studies have shown that ~50% of intranasally inoculated volume entered the airways.^{37–39} Control mice were treated with 25 μ l of sterile H₂O. The airway epithelia of FVBN wild-type mice exposed to sterile H₂O appeared normal, with no detectable goblet cells (Figure 3A). Similarly, no goblet cells were detected in mouse lungs after exposure to 10 μ g/ml PCN for 3 or 6 weeks

(data not shown). After 12 weeks of exposure to 10 $\mu\text{g/ml}$ PCN, however, mouse lungs developed mild goblet cell hyperplasia (Figure 3B, red arrows). Mouse lungs exposed to a higher clinically relevant concentration of PCN (25 $\mu\text{g/ml}$) developed more robust airway remodeling, with observable goblet cells as early as 6 weeks following chronic PCN exposure (Figure 3C, red arrows). By 12 weeks post-PCN exposure, the number of mucin-producing goblet cells had increased significantly (Figure 3D, red arrows). In addition, PAS staining revealed the presence of goblet cells within small airways indicative of goblet cell metaplasia (Figure 3E), which was not observed in lung sections of control mice (data not shown).

We compared the airway MI in both control and PCN-exposed mice. After 3 weeks of PCN exposure, the levels of airway mucus were not significantly altered in FVBN mice. In contrast, after 6 and 12 weeks of exposure to 25 $\mu\text{g/ml}$ PCN, the amounts of airway mucus were significantly increased by 5- and 22.5-fold, respectively (Figure 3F). Again, these results confirm that chronic exposure of murine airways to PCN reproduces and mimics the goblet cell hyperplasia and metaplasia of CF and other bronchiectasis airways.

Mouse Lungs Chronically Exposed to Clinically Relevant Concentrations of PCN Develop Airway Fibrosis

Advanced stage CF patients, particularly those with severe forms of CF transmembrane regulator mutations (eg, ΔF508), invariably develop varying degrees of pulmonary fibrosis, especially bronchiolar fibrosis. Therefore, we determined whether chronic exposure to PCN could cause airway fibrosis. When lung sections were examined by Masson's trichrome staining, the control mice had normal airway and alveolar architecture (Figure 4A). Similarly, following 6 or 12 weeks of exposure to 10 $\mu\text{g/ml}$ PCN (Figure 4, B and C) or a 3-week exposure to 25 $\mu\text{g/ml}$ PCN (Figure 4D), the mice did not develop airway fibrosis. In contrast, after 6 to 12 weeks of exposure to 25 $\mu\text{g/ml}$ PCN, mice developed diffuse and patchy airspace consolidations. Increased amounts of blue-staining collagens were visible immediately adjacent to peribronchial and perivascular regions (Figure 4, E and F). By 12 weeks, there were numerous large areas of consolidated air space that extended from bronchi and bronchioles (Figure 4F). Within the consolidated areas, fibroblasts were evident. Large numbers of mononuclear cells were also observed within the perivascular and peribronchial regions (see cell analysis below). In some rare but severe cases, the fibrosis extended to the entire lobe of the lung (Figure 4G), and large amounts of collagen deposition were visible (Figure 4H).

We compared the amount of extracellular matrix within the lungs of the control and PCN-treated mice by hydroxyproline assays.³² After 3 weeks of exposure to 25 $\mu\text{g/ml}$ PCN, the amounts of collagen within exposed lungs were no different from control lungs (Figure 4I). By 6 and 12 weeks, the PCN-exposed lungs had accumulated 27 and 62% more collagen, respectively, than the lungs

from control mice (Figure 4I). These biochemical results confirm the histopathological observations that chronic exposure to PCN causes airway fibrosis in FVBN mice.

Chronic Exposure to PCN Causes Alveolar Airspace Destruction

Previously, PCN was shown to cause apoptosis in neutrophils and cultured lung epithelial cells.¹⁰ Because apoptosis is one of the major mechanisms that contributes to alveolar airspace destruction,^{40,41} we examined whether chronic PCN exposure causes destruction of alveolar spaces. As shown in Figure 5A, the alveolar spaces of control mice were intact. After 6 and 12 weeks of exposure to 10 $\mu\text{g/ml}$ PCN or after 3 weeks of exposure to 25 $\mu\text{g/ml}$ PCN, the amount of airspace destruction was not significantly different from that of control mice (Figure 5, A–C). In contrast, significant airspace destruction was clearly visible after 6 and 12 weeks of exposure to 25 $\mu\text{g/ml}$ PCN (Figure 5, D and E). We quantified alveolar airspace enlargement by the MLI method.^{35,36} The MLI of control mice was $27.0 \pm 1.2 \mu\text{m}$. After 6 weeks of PCN treatment, the MLI was increased 34% to $36.2 \pm 2.6 \mu\text{m}$, whereas the MLI of the 12-week PCN-exposed mice was increased by 56% to $42.1 \pm 1.2 \mu\text{m}$ (Figure 5F). These results confirm that chronic exposure to PCN causes alveolar airspace destruction.

Chronic Exposure to PCN Polarized the Pulmonary Cytokine Profiles toward a T Helper Type 2 Response

There is increasing evidence that the development of goblet cell hyperplasia^{42,43} and pulmonary fibrosis^{44–46} within airways is mainly driven by an imbalance between T helper (Th) type 1 and Th2 cytokines. An overabundance of Th2 cytokines polarizes the lung toward goblet cell hyperplasia and fibrosis, rather than injury resolution. On the basis of the observation that chronic exposure to PCN induces goblet cell hyperplasia and airway fibrosis, we predicted that chronic PCN exposure could polarize the cytokine profiles toward a Th2 response. We examined the expression of the chemokine macrophage-inflammatory protein (MIP)-1 α and Th1 cytokines interferon- γ (IFN- γ) and IL-12p70 (Figure 6), and Th2 and immunoregulatory cytokines IL-13, transforming growth factor- β (TGF- β), IL-4, and IL-10 (Figure 7) in the BAL from control mice and mice chronically exposed to 25 $\mu\text{g/ml}$ PCN. As shown in Figure 6, control mice expressed low or nondetectable levels of IFN- γ and basal levels of IL-12 p70. After 3 weeks of PCN exposure, there was no discernable difference in levels of IFN- γ (Figure 6A), but there was a 56% increase in IL-12 p70 (Figure 6B). After 6 weeks of chronic PCN exposure, levels of IFN- γ increased 100-fold (Figure 6A), whereas the amounts of IL-12 p70 returned to basal levels (Figure 6B). After 12 weeks of PCN exposure, IFN- γ levels within BAL were almost nondetectable (Figure 6A), whereas levels of IL-12 p70 steadily de-

Figure 4. Chronic exposure to PCN causes fibrosis in conducting airways. **A:** Lung sections (as described in Figure 3) were stained for collagen with Masson's trichrome. The presence of collagen is indicated by blue color in the lung sections. Lung sections from control mice indicate very low, basal levels of collagen. **B and C:** Lung sections from mice exposed to 10 µg/ml PCN for 6 and 12 weeks, showing basal levels of collagen. **D:** After 3 weeks of exposure to 25 µg/ml PCN, mouse lungs showed basal levels of collagen. **E:** After 6 weeks of exposure to 25 µg/ml PCN, increasing levels of collagens, particularly at the peribronchi and perivascular areas, are clearly visible in the lungs. **F:** After 12 weeks of exposure to 25 µg/ml PCN, fibrosis has spread into parenchyma of the lung immediately adjacent to bronchi and bronchioles. **G:** Masson's trichrome staining of lung sections from a 12-week PCN-instilled (25 µg/ml) mouse that developed severe pulmonary fibrosis where consolidation has expanded to entire lobe of the lung. **H:** A fibrotic area from **G** magnified to $\times 40$, indicating the blue-stained collagen (see **white arrow**). **I:** Chronic exposure to PCN results in overproduction of extracellular matrix within mouse lungs. The amounts of lung collagen were determined by hydroxyproline assays using whole lung homogenates from six FVB/N mice chronically exposed to PCN. Data are the mean $\pm$ SE, $n = 6$. P values: 3 weeks PCN (0.95); 6 weeks PCN (0.0015); 12 weeks PCN (9.1×10^{-6}). C = control mice.

creased by a factor of 23-fold when compared with control mice (Figure 6B).

Because macrophages are known to produce both pro- and anti-inflammatory cytokines, we next compared levels of the macrophage and monocyte chemokine MIP-1 α within the BAL of control and PCN-exposed mice. After 6 and 12 weeks of PCN exposure, levels of MIP-1 α were significantly increased by 24- and 4.5-fold higher, respectively, in the BAL when compared with control mice (Figure 6C). The increased levels of MIP-1 α would be expected to play a role in attracting the infiltration of F4/80 $^{+}$ /CD11b $^{+}$ macrophages into the lungs.

We further examined profiles of Th2 (IL-4 and IL-13) and immunoregulatory (IL-10 and TGF- β) cytokines. Levels of IL-13 and TGF- β decreased slightly at 3 and 6 weeks post-PCN exposure (Figure 7, A and B). However, by 12 weeks, levels of both IL-13 and TGF- β were twofold higher as compared with those in control mice. In addition,

the levels of IL-4 and IL-10 increased steadily throughout the PCN exposure and were determined to be 3.1- and 4-fold higher than that observed in control mice at 12 weeks, respectively (Figure 7, C and D). Taken together, these results are consistent with the histopathological observations (Figures 3 and 4) and suggest that chronic exposure to PCN polarized Th1 cytokines toward Th2 and immunoregulatory cytokines, favoring the development of goblet cell hyperplasia and pulmonary fibrosis.

Profiling of Immune Cells during Chronic PCN Exposure

We also examined the immune cell populations in BAL obtained from the PCN-exposed mouse lungs that might be responsible for the production of Th1 and Th2 cytokines. Conventional CD4 $^{+}$ and CD8 $^{+}$ T cells as well as

Figure 5. Chronic exposure of mouse lungs to PCN causes alveolar airspace destruction. **A:** Lung sections from control or PCN-exposed mice (as described in Figure 3) were stained with H&E. Control mice with normal alveolar space. Magnification: $\times 10$. **B and C:** After 6 or 12 weeks exposure to 10 $\mu\text{g/ml}$ PCN, alveolar space destruction and enlargement was not statistically different from the control mice. Magnification: $\times 10$. **D:** Alveolar space destruction and enlargement in mouse lungs following 6 weeks of exposure to 25 $\mu\text{g/ml}$ PCN. Magnification: $\times 10$. **E:** After 12 weeks of exposure to 25 $\mu\text{g/ml}$ PCN, mouse lungs developed severe alveolar space destruction and enlargement. Magnification: $\times 10$. **F:** MLI measurement of alveolar space destruction and enlargement. MLI was determined in mouse lungs exposed to 25 $\mu\text{g/ml}$ PCN. MLI was calculated as described in *Materials and Methods*. Six weeks PCN ($*P < 0.05$) and 12 weeks PCN ($*P < 0.001$), respectively, when compared with control mice.

NK cells are important sources of immunoregulatory cytokines.^{44–47} We found that the absolute numbers of nonnaïve CD4⁺ and CD8⁺ T cells began to increase 6 weeks post-PCN exposure. The increase in both T cell populations was most dramatic at 12 weeks post-PCN exposure. We further observed nearly twice as many CD4⁺ T cells than CD8⁺ T cells at both points in time (compare Figure 8, A and B), suggesting that CD4⁺ T

cells are the predominant type of T cells within PCN-exposed lung. However, the number of NK cells was not significantly altered within the time intervals of chronic PCN exposure (data not shown).

Because macrophages are capable of secreting both pro- and anti-inflammatory cytokines, continuous exposure to PCN may also change their cytokine profiles to a primarily Th2 type. We found that within the BAL of PCN-

Figure 6. Chronic exposure to PCN alters the profiles of MIP-1 α and Th1 cytokines IFN- γ and IL-12 p70 in the lungs. **A:** Cytokine concentrations were quantified by ELISA using BAL samples from control and PCN-exposed mice (25 $\mu\text{g/ml}$, Figure 3). The P values were derived by comparing cytokine levels in the BAL of PCN-exposed versus control mice. PCN increased the IFN- γ levels within BAL after 6 weeks exposure ($*P < 0.05$), but the cytokine levels returned to near normal after 12 weeks PCN exposure ($*P < 0.05$). **B:** PCN caused an early increase (3 weeks PCN, $*P < 0.05$) and then declining levels of IL-12 p70 (12 weeks PCN, $**P < 0.001$). **C:** PCN caused a transient increase in the levels of MIP-1 α (6 weeks PCN, $P < 0.01$; 12 weeks PCN, $P < 0.05$).

Figure 7. Cytokine profiles demonstrate a Th2 polarization in mouse lung chronically exposed to PCN. After BAL, cell-free supernatants were collected from control and PCN-exposed mice (25 μ g/ml; Figure 3). The amounts of cytokines were quantified by ELISA. PCN exposure decreased the levels of TGF- β (**A**) and IL-13 (**B**) in mouse lungs at initial stages of disease, but significantly increased the levels of TGF- β (**A**) and IL-13 (**B**) at more severe stages of PCN-induced lung pathogenesis. *P* values: TGF- β (3 weeks, *P* = 0.28; 6 weeks ***P* < 0.01; 12 weeks PCN; **P* < 0.01). IL-13 (3 weeks, *P* = 0.11; 6 weeks, ***P* < 0.001; 12 weeks PCN; **P* < 0.001). The *P* values were derived by comparing cytokine levels in the BAL of PCN-exposed versus control mice. **C:** PCN induced a continuous increase of IL-10 in mouse lungs (3 weeks PCN, **P* < 0.01; 6 and 12 weeks PCN; ***P* < 0.001). **D:** PCN induced a significant increase of IL-4 in mouse lungs (3 weeks PCN, *P* = 0.73; 6 and 12 weeks PCN, **P* < 0.001).

exposed lungs, the population of resident macrophages (F4/80⁺/CD11b⁺) decreased as early as 3 weeks and continued to drop fivefold lower at 6 weeks post PCN exposure when compared with control mice (Figure 8C). The numbers of resident macrophages recovered slightly at 12 weeks but were still significantly lower than control mice (Figure 8C). The population of recruited (infiltrating

F4/80⁺/CD11b⁺) macrophages stayed at basal levels at 3 and 6 weeks after PCN exposure. By 12 weeks, there was a dramatic increase in the population of recruited macrophages (Figure 8D).

Neutrophil infiltration is another hallmark of chronic CF disease.^{1,2} Therefore, we examined whether mouse lungs that were chronically exposed to PCN had in-

Figure 8. Enumeration of leukocyte subsets in mouse lungs chronically exposed to PCN. **A:** Cells were collected from BAL samples of control and PCN-treated mice (25 μ g/ml; Figure 3), labeled with antibodies specific for the indicated cell types, and analyzed using flow cytometry. Chronic exposure to PCN increases the numbers of nonnaïve CD4⁺ cells in mouse lungs (**P* < 0.05 for 12 week PCN). **B:** Chronic exposure to PCN increases nonnaïve CD8⁺ cells (*P* < 0.01 for 6 week PCN; **P* < 0.001 for 12 week PCN). **C:** Chronic exposure to PCN decreases the number of resident macrophages (**P* < 0.05 for 6 week PCN; ***P* < 0.01 for 12 week PCN). **D:** Chronic exposure to PCN increases the number of infiltrating macrophages (*P* < 0.05 for 12 week PCN). **E:** Chronic exposure to PCN increases the influx of neutrophils (**P* < 0.05 for 6 and 12 week PCN). The *P* values were derived by comparing cells in the BAL of control and PCN-exposed mice.

Figure 9. Chronic exposure to PCN failed to induce goblet cell hyperplasia in the lungs of Stat6^{-/-} mice. Wild-type C57BL/6 and Stat6^{-/-} mice (groups of eight) were inoculated with 25 μ g/ml PCN or sterile H₂O (controls). **A:** No mucin is detected in the airways of control wild-type mice exposed to sterile H₂O. **B–D:** After 3 weeks of exposure to 25 μ g/ml PCN, wild-type mice developed goblet cell hyperplasia in large airways (**B** and **C**) and metaplasia in small airways (**D**), as indicated by PAS-stained mucins (**arrows**). **E:** No mucin staining was detected in the airways of Stat6^{-/-} mice. **F:** Wild-type mice exposed to PCN have a significantly higher MI than control and Stat6^{-/-} mice (**P* < 0.001 when compared with Stat6^{-/-} and control mice).

creased neutrophil numbers. The numbers of neutrophils were significant increased at 6 and 12 weeks post-PCN exposure (Figure 8E). Taken together, these results suggest that chronic exposure to PCN may be chemotactic toward CD4⁺ and CD8⁺ T cells, macrophages, and neutrophils.

PCN Mediates Goblet Cell Hyperplasia through Th2 Cytokine-Mediated Stat6 Signaling

Th2 cytokines IL-4 and IL-13 are known to regulate the development of goblet cell hyperplasia through the Stat6-mediated signaling pathway.^{48–52} As PCN induces Th2 cytokine secretion, which favors the development of goblet cell hyperplasia, we examined whether PCN mediates this effect through the Stat6 signaling pathway. Wild-type C57BL/6 and isogenic Stat6^{-/-} mice were exposed to 25 μ g/ml PCN for 3 weeks. Wild-type control mice instilled with same volume of sterile H₂O did not develop goblet cell hyperplasia (Figure 9A). In contrast, wild-type mice instilled with PCN developed goblet cell hyperplasia in larger conducting airways (Figure 9, B and C) and metaplasia in small airways (Figure 9D). Significantly, PCN did not cause goblet cell hyperplasia and metaplasia in the lungs of Stat6^{-/-} mice (Figure 9E). The airway MI in wild-type mice exposed to PCN is significantly higher than Stat6^{-/-} mice exposed to PCN or control wild-type mice exposed to sterile H₂O (Figure 9F). Three weeks of PCN exposure did not induce pulmonary fibrosis and emphysema in wild-type and Stat6^{-/-} mice (data not shown). These results suggest PCN causes goblet cell

hyperplasia and metaplasia by activating the Stat6 signaling pathway.

Next, we compared the profiles of Th2 cytokines in BAL fluids of wild-type and Stat6^{-/-} mice. PCN failed to induce the secretion of IL-4 and IL-13 in Stat6^{-/-} mice (Figure 10, A and B). Similarly, exposure to sterile H₂O did not induce IL-4 and IL-13. In contrast, PCN exposure induced significant levels of IL-4 and IL-13 in the wild-type mice. The secretion of IL-13 and IL-4 was confirmed by immunohistochemistry analysis (Figure 10, D–I). Wild-type mice exposed to PCN secreted significantly higher levels of IL-13 (Figure 10E, arrows) and IL-4 (Figure 10H, arrows) when compared with wild-type controls (Figure 10, D and G) or Stat6^{-/-} mice (Figure 10, F and I). These results strongly suggest PCN causes goblet cell hyperplasia and metaplasia by inducing the secretion of IL-4 and IL-13, which activate the Stat6 signaling pathway.

We next examined whether PCN, IL-4 or IL-13 could individually induce the transcription of the mucin biosynthesis gene MUC5AC in the human pulmonary mucociliary carcinoma cell line, NCI-H292. Quantitative RT-PCR results suggest that PCN and IL-4 could induce the transcription of the mucin biosynthesis *MUC5AC* gene by 4-fold, whereas IL-13 could induce the transcription of MUC5AC by 2.2-fold (Figure 10C).

Finally, we compared the immune cell populations in BAL obtained from the PCN-exposed wild-type and Stat6^{-/-} mouse lungs that might be responsible for the production of Th2 cytokines. After 3 weeks of exposure to PCN, we found that the absolute numbers of nonnaive CD4⁺ T cells and infiltrating macrophages were signifi-

Figure 10. Induction of Th2 cytokines by PCN is attenuated in Stat6^{-/-} mice. The amounts of IL-13 and IL-4 in the BAL of control and PCN-exposed wild-type and Stat6^{-/-} mice were quantified by ELISA. **A** and **B**: Wild-type mice exposed to sterile H₂O (control) or Stat6^{-/-} mice exposed to PCN did not induce IL-13. In contrast, 3 weeks of PCN (25 μ g/ml) exposure induced significant expression of IL-13 (**A**) and IL-4 (**B**) in wild-type mouse lungs. * P < 0.001. The P values were derived by comparing cytokine levels in the BAL of PCN-exposed wild-type versus wild-type control or Stat6^{-/-} mice. **C**: Exposure to PCN, IL-4, or IL-13 induced the expression of mucin biosynthesis gene *MUC5AC* in the human pulmonary mucopidermoid carcinoma cell line, NCI-H292. **D–F**: PCN induced the secretion of IL-4 in wild-type C57BL/6 mice (**E**, arrows) but not in Stat6^{-/-} mice (**F**). **G–I**: PCN induced the secretion of IL-13 in wild-type C57BL/6 mice (**H**, arrows) but not in Stat6^{-/-} mice (**I**). Wild-type mice exposed to sterile H₂O (control) did not elaborate IL-4 or IL-13 (**D** and **G**).

cantly higher in wild-type mice than in Stat6^{-/-} mice (Figure 11, A and B). In contrast, the levels of CD8⁺ T cells and neutrophils were not significantly different. These results suggest that CD4⁺ T cells and infiltrating macrophages are most probably responsible for the secretion of Th2 cytokines activating the Stat6 signaling pathway leading to goblet cell hyperplasia.

Discussion

PCN is an important redox active exotoxin that is secreted in such high concentrations (up to 27 μ g/ml) in the sputum in bronchiectasis airways with *P. aeruginosa* infection that it often determines the color of expectorated sputa.⁵ However, the importance of PCN during chronic

Figure 11. Leukocyte subset numbers are mediated by STAT6 in mouse lungs chronically exposed to PCN. **A**: Three weeks of PCN exposure increases the numbers of nonnaive CD4⁺ cells significantly in the lungs of wild-type mice but not Stat6^{-/-} mice (** P < 0.01). No significant difference in the numbers of nonnaive CD8⁺ cells was observed. **B**: Three weeks of PCN exposure increases the numbers of infiltrating macrophages significantly in the lungs of wild-type mice but not Stat6^{-/-} mice (* P < 0.05). No significant difference in the number of neutrophils was observed. The P values were derived by comparing cells in the BAL of wild-type and Stat6^{-/-} mice exposed to 25 μ g/ml PCN.

P. aeruginosa infection *in vivo* is unknown. In this study, we demonstrate that under *in vitro* conditions, the majority of the clinical CF *P. aeruginosa* isolates produce more PCN than the laboratory strain PA01. In addition, PCN-deficient *P. aeruginosa* mutants *phzM* and *phzS* were attenuated in their ability to infect mouse lungs compared with the isogenic parental wild-type strain PA01 and less able to induce the proinflammatory neutrophilic cytokine KC. Furthermore, we demonstrate for the first time that chronic exposure to PCN results in multiple pulmonary pathologies that are consistently encountered within the lungs of CF patients, including goblet cell hyperplasia and metaplasia, airway fibrosis, and alveolar space destruction, as well as the influx of macrophages, neutrophils, and lymphocytes. The amount of immune cell influx as a result of PCN instillation is ~10% of the of immune cells in mice infected with *P. aeruginosa* cells,¹² and the mechanisms of PCN-mediated pathogenesis may be independent of neutrophils. Because the development of goblet cell hyperplasia and pulmonary fibrosis are largely caused by excess of Th2 and immunoregulatory cytokines in the lung, increased infiltration of macrophages and CD4⁺ T cells that secrete Th2 cytokines are predicted to contribute to the development of goblet cell hyperplasia and airway fibrosis. These observations are confirmed by our study using the Stat6^{-/-}-knockout mice, which failed to induce Th2 cytokines and to develop goblet cell hyperplasia and metaplasia after exposure to PCN.

The pathogenesis of goblet cell hyperplasia is multifactorial. It is closely related to the hypersecretion of Th2 cytokines triggered by microbial infection and allergens^{42,43,51–57} through the Stat6 signaling pathway. Paradoxically, Th2 cell-mediated mucus production acts positively in the protection of mucosal surfaces from pathogens, but it may also be detrimental by contributing to airway obstruction during chronic inflammatory processes, especially in CF airways. Two Th2 cytokines, IL-4 and IL-13, play crucial roles during the pathogenesis of goblet cell hyperplasia, through induction of various mitogen-activated protein kinase signaling pathways that result in the activation of transcription factors STAT6, nuclear factor- κ B, Sp1, and Ap1, which positively regulate mucin biosynthesis.^{42,43,48–52,55,58–62} Transgenic mice deficient in STAT6, IL-4, or IL-13 fail to develop allergen-induced goblet cell hyperplasia.^{48–52,55,57} Additional cytokines, including IL-1 β , IL-9 and IL-17, all positively activate the transcription of mucin biosynthetic genes.^{53–57} Furthermore, IL-5 induces a CD4⁺ T cell-dependent airway mucus production in the lung.³⁴ Another molecule involved in the pathogenesis of goblet cell hyperplasia is the winged helix transcription factor FOXA2.⁶³ Selective deletion of *Foxa2* in respiratory epithelial cells causes airspace enlargement, goblet cell hyperplasia, increased mucin, and neutrophilic infiltration, pathological features that strikingly resemble mouse lungs chronically exposed to PCN. Furthermore, the loss of surfactant protein C also causes mice to develop goblet cell hyperplasia.⁶⁴ Finally, oxidative stress has been shown to induce the epidermal growth factor-dependent mucin genes expression.⁶⁵ Collectively, our data and

published results suggest that PCN and PCN-induced ROS may induce goblet cell hyperplasia by enhancing the secretion of Th2 cytokines such as IL-4 and IL-13, which in turn activates Stat6 signaling and downstream transcriptional activators of mucin biosynthesis. It remains to be seen whether exposure to PCN suppresses the expression of FoxA2, a transcriptional repressor of mucin biosynthesis.

Most of our current understanding of pulmonary fibrosis, especially the immune mechanisms and mediators involved, is derived from the bleomycin and fluorescein isothiocyanate exposure experimental models in mice, as well as from patients with idiopathic pulmonary fibrosis.^{44–47,66} The pathology of pulmonary fibrosis demonstrates features of dysregulated and abnormal repair with exaggerated vascular remodeling, fibroproliferation, and increased deposition of extracellular matrix, all leading to progressive fibrosis and loss of lung function. In contrast to interstitial fibrosis mediated by bleomycin- and fluorescein isothiocyanate-exposed mice,^{44–47,66} PCN-mediated pulmonary fibrosis in FVB mice occurred primarily in conducting airways (bronchial and bronchiolar), with gradual and moderate spread to surrounding areas adjacent to the conducting airways. Only in the most severe cases, the fibrotic growth spread to the entire lobe, involving the pleural areas of the lung. These observations suggest that clinically relevant doses of PCN induce a differing pattern of injury than the bleomycin model. In addition, bleomycin generally induces an acute and massive interstitial pulmonary fibrosis in experimental mouse models of idiopathic pulmonary fibrosis. In contrast, chronic PCN exposure induces lung pathologies that more closely mimic the chronic injury experienced by CF patients. Thus, PCN-mediated chronic colonization of *P. aeruginosa* in immunologically dysfunctional CF lungs may be of advantage to the bacteria whose likely goal is to create a stable niche for colonization and long-term survival. Interestingly, although C57BL/6 mice develop goblet cell hyperplasia and metaplasia after 3 weeks of exposure to PCN, these lungs do not develop pulmonary fibrosis or emphysema. These results may be due to inherent differences in the genetic makeup of FVB and C57BL/6, which render the latter more prone to PCN-mediated goblet cell hyperplasia and metaplasia.

Similar to the pathogenesis of goblet cell hyperplasia, the balance between Th1 and Th2 cytokines and chemokines seems to play a critical role in pulmonary fibrosis. For example, the Th1 cytokines IFN- γ and IL-12 p70 strongly suppress the production of extracellular matrix proteins collagen and fibronectin.⁶⁷ In contrast, the Th2 cytokines IL-4 and IL-13 induce the expression of fibroblast-derived type I and III procollagens by selectively stimulating the production and activation of TGF- β .⁶⁸ Chemokines and their receptors also have been shown to be essential components of pro- (Th1) and anti-inflammatory (Th2) mediated responses.⁶⁹ For example, the Th2 chemokine CCL17 secreted by pulmonary epithelial cells has an important role in the development of pulmonary fibrosis. Cells that express CCR4 are more characteristic of the Th2 response, and the neutralization of CCL17 (CCR4 ligand) led to reduced numbers of in-

trapulmonary macrophages and Th2 T cells (eg, CD4⁺) and attenuated pulmonary fibrosis,⁷⁰ most likely due to decreased concentration of Th2 cytokines. In contrast, CXCR3, which is expressed by pulmonary NK and CD8⁺ T cells, is both necessary and sufficient to produce IFN- γ , which is ultimately critical in polarizing the immune response to injury toward resolution rather than progressive fibrosis.⁴⁷ These published results suggest that macrophages and Th2 T cells are the primary cell types responsible for secretion of Th2 cytokines that drive pulmonary fibrosis. In our study, we demonstrate that continuous exposure to PCN, which can cause oxidative damage^{3,4,29,71,72} and cytokine imbalance^{7–9,14–16} within lung epithelia, induced dramatic influx of activated macrophages and CD4⁺ T cells, which are predicted to contribute to the pathogenesis of lung fibrosis.

We have shown that chronic exposure to PCN induces destruction of the alveolar airspace in mice. Airspace destruction is partially mediated by proteolytic events mediated by neutrophil elastases as well as by epithelial apoptosis.⁷³ Although *P. aeruginosa* also secretes exoproteases that can degrade innate immunity proteins (eg, surfactant protein SP-A) *in vitro*,⁷⁴ its relevance in airspace destruction is debatable because the secretion of *P. aeruginosa* proteases are down-regulated during the chronic stage of infection.^{2,4} More likely, PCN-mediated apoptosis may directly kill type II cells or indirectly by release of elastases through the lysis of apoptotic neutrophils.^{10,11} In addition, inhibition of α -1 protease inhibitor by PCN is likely to cause an imbalance between protease-antiprotease activities in the CF airways and contribute to the airspace destruction.¹³ Alternatively, PCN-induced infiltration of cytotoxic T cells can induce apoptosis in target cells by cross-linking of the Fas expressed on the target cell membrane with the Fas ligand expressed on the surface of effector cells and by the release of two cytotoxic granules, perforin and granzyme B, from the effector cells.⁷³ Perforin is the cytotoxic pore forming protein that mediates target cell lysis by cytotoxic T cells, whereas granzyme B is a serine protease released by cytotoxic cells, which triggers a series of biochemical events that lead to apoptosis. Although NK cells also induce apoptosis through perforin and granzyme B, they are unlikely to play a role in the PCN-mediated airspace destruction because PCN does not appear to induce the influx of NK cells into the lungs.

In summary, this study demonstrates that PCN is a significant contributor to lung destruction during chronic *P. aeruginosa* infection of bronchiectasis airways. Mouse lungs chronically exposed to PCN develop some of the important pathological features that resemble some of the common features of the CF airways chronically infected with *P. aeruginosa*. These features include goblet cell hyperplasia, peribronchial and peribronchiolar fibrosis, and distal airspace destruction. PCN-mediated airway pathogenesis appears to involve Th2 cytokine signaling through the Stat6 pathway. Our results support the hypothesis that PCN, which is secreted in prodigious amounts in the CF airways, contributes to the accelerated decline in lung function of CF and other bronchiectasis patients once they are infected with *P. aeruginosa*.

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