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Cathepsin-G Interferes with Clearance of *Pseudomonas aeruginosa* from Mouse Lungs

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ABSTRACT: The cystic fibrosis airway is susceptible to *Pseudomonas aeruginosa* infection, which stimulates an intense inflammatory response leading to airway obstruction and bronchiectasis. Neutrophils migrate into the airway, and once there, release high concentrations of neutral serine proteases during phagocytosis and in death. In particular, neutrophil elastase is central to progression of bronchiectasis by interfering with bacterial clearance and directly perpetuating the inflammatory response in the airway. Using a murine model of endobronchial inflammation, we found that a different neutrophil-derived serine protease, cathepsin G, inhibited the host's ability to clear *Pseudomonas* from the lung, based on a 1-log reduction in bacteria recovered from cathepsin G-deficient mice. Higher antibody concentrations were found in respiratory epithelial lining fluid from mice lacking cathepsin G, but there was no difference in other opsonins, such as surfactant proteins A and D. Chemokine levels measured in the lung correlated with bacterial burden and not the animal's genotype, indicating that airway inflammation was not affected by the presence (or absence) of specific serine proteases. These findings suggest that cathepsin G interferes with airway defenses, showing that proteases other than neutrophil elastase have roles in the pathogenesis of suppurative airway diseases.
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Persistent lung infection, typically with *Pseudomonas aeruginosa*, and an unremitting inflammatory response are the major causes of morbidity and mortality in cystic fibrosis (CF). The exaggerated inflammatory response is characterized by a massive influx of neutrophils across the respiratory epithelium (1). This neutrophil excess brings with it an abundance of serine proteases, such as neutrophil elastase (NE), cathepsin G (CG), and proteinase 3, which are lytic enzymes with a wide range of substrates. Intracellular NE has been shown to be necessary for bacterial killing and host defense (2,3). However, during phagocytosis or in death, neutrophils release their contents into the airways, and active enzymes overwhelm native antiproteases contributing to the pathogenesis of CF lung disease. In particular, NE has been shown to incite inflammatory responses (4,5), interfere with bacterial clearance (6,7), and disrupt innate immunity (8,9).

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The roles of other neutrophil-derived serine proteases are less clear, but they are likely to be clinically relevant and could have effects distinct from NE. An abundant protein in human and murine neutrophils (10) CG activates airway epithelial cells and induces secretion from airway submucosal glands (11). It has a number of potential substrates in the CF lung and has been shown to cleave components of the extracellular matrix *in vitro*, although less efficiently than NE (12). Moreover, it can regulate innate immunity and inflammation through cleavage or proteolytic inactivation of collectins, cytokines, complement, and cell surface receptors (13,14). Cathepsin G has been shown to be important for neutrophils to respond to chemotactic signals (15), and has some antimicrobial activity against pathogens (16,17).

In these experiments, we examined the effects of CG in the infected lung, testing the hypothesis that CG interferes with bacterial clearance and escalates the pulmonary inflammatory response. Unlike previous studies which used antiproteases to indiscriminately neutralize the effects of serine proteases (6,18), we examined bacterial clearance and airway inflammation *in vivo* in the absence of selected proteases using a well-established model of *Pseudomonas* endobronchitis in mice genetically altered to lack NE or CG.

MATERIALS AND METHODS

Animals. Protease-deficient ($ne^{-/-}cg^{+/+}$, $ne^{+/+}cg^{-/-}$, and $ne^{-/-}cg^{-/-}$) mice previously created by homologous recombination were used in these experiments (2,19,20). To control for mouse genetic background, protease-sufficient mice ($ne^{+/+}cg^{+/+}$) of the same strain were used as controls, *i.e.* wild-type SvJ strain was used as controls in experiments with $ne^{-/-}cg^{+/+}$ and $ne^{-/-}cg^{-/-}$ mice, and wild-type 129/SvJ strain served as control for the $ne^{+/+}cg^{-/-}$ mice. The genotype of individual animals was established by Southern blot hybridization of genomic DNA isolated from the animal's tail using established protocols. Finally, all research conformed to National Institutes of Health guidelines, and the protocol was reviewed and approved by the Washington University School of Medicine Animal Studies Committee.

Murine model of endobronchial inflammation. An adaptation of the agarose bead method was used to create a neutrophilic endobronchitis in mice (21). Mice underwent intratracheal inoculation with sterile agarose beads or beads embedded with nonmucoid *P. aeruginosa* (PAO1). A silastic catheter was inserted transtracheally and 50 μL of a 1:10 dilution of bead slurry (5.0×10^4 *P. aeruginosa* CFU/lung) was instilled into the right mainstem bronchus.

Bronchoalveolar lavage. Based on previous data examining maximum bacterial burden and inflammation in this infection model, bronchoalveolar

Abbreviations: BAL, Bronchoalveolar lavage; **CG,** cathepsin G; **CF,** cystic fibrosis; **NE,** neutrophil elastase; **SP,** surfactant protein

lavage (BAL) was performed 3 d after infection using established techniques (21).

ELISA for secreted cytokines. Pro-inflammatory cytokine, TNF-α, and CXC chemokines (MIP-2 and KC) were measured in BAL fluid and lung homogenates *in vivo* using commercially available ELISA (R & D Systems, Minneapolis, MN) as described previously (21). Protein concentrations were corrected for the volume of respiratory epithelial lining fluid (ELF) recovered by measuring urea dilution (22).

Phagocyte isolation. Mice lacking neutrophil serine proteases and their wild-type littermates underwent intraperitoneal injection with 15% glycogen, and 3 h later, peritoneal exudates were collected and washed with Hanks buffered saline solution. Polymorphonuclear cells were separated from other inflammatory cells by Ficoll density gradient centrifugation, and contaminating erythrocytes were removed by hypotonic lysis (2). To isolate murine macrophages, the animals underwent intraperitoneal injection with thioglycollate and underwent peritoneal washings 3 d later using established techniques (23).

Measurement of cathepsin G and neutrophil elastase activity. Based on established techniques, functional activity of serine proteases in cell lysates and BAL samples from experimental animals were measured using specific peptide chromogenic substrates of NE and CG, respectively (Elastin Products, Owensville, MO) (23).

Immunoblot analyses for murine immunoglobulins. Using nonreducing conditions, proteins from BAL fluid were separated by SDS-PAGE and transferred onto nylon membranes using standard techniques. The blots were blocked with PBS, pH 7.4, 0.03% polyoxyethylenesorbitan monolaurate, and 10% (wt/vol) dry skim milk for 1h at room temperature, then incubated at room temperature with horseradish peroxidase conjugated, goat-derived, anti-mIgG or anti-mIgA antibodies (Sigma Chemical Co., St. Louis, MO). The membranes were washed, then Western blot enhanced chemiluminescence detection solution (Amersham, Arlington Heights, IL) was applied for 1 min. Luminescence emitted from the filter was detected by exposure to photographic film.

ELISA for murine antibodies in BAL fluid. Lavage fluid was analyzed for mouse antibodies using established ELISA. To assess for specific anti-*Pseudomonas* antibodies, *P. aeruginosa* was grown to an OD of 0.5 at 650 nm, 100 μL of the bacterial culture was applied to each well of a 96-well flat-bottomed ELISA plate, and incubated overnight at 4°C. The cells were washed with PBS containing 0.5% BSA and 0.005% polyoxyethylenesorbitan monolaurate, then blocked with 0.5% BSA in PBS. The wells were washed, and 50 μL of BAL fluid was applied to each well for 1 h at room temperature. After additional washings, bound mIgG or mIgA was detected by incubation with 1:1000 dilution of horseradish peroxidase-conjugated anti-mouse IgG or IgA secondary antibody (Sigma Chemical Co. Immunochemicals, St. Louis, MO). Irrelevant, conjugated anti-mouse antibody was used as controls. Treated with TMB Microwell Peroxidase Substrate system, absorbance was measured at 450 nm.

Surfactant protein A and Dd analyses. Quantitative analyses for SP-A and SP-D in BAL fluid were performed using a double-capture ELISA as described previously (24,25).

Quantitative bacteriology. To examine the effect of neutrophil proteases on bacterial clearance, right lung lobes of mice inoculated with infected agarose beads were removed aseptically, homogenized in 1 mL PBS, and cultured quantitatively using serial dilution on tryptic soy agar plates (21).

Flow cytometry. Mice were infected with green fluorescent protein (GFP)-expressing PAO1 using the agarose bead model, and 3 d after bacterial inoculation the lungs were perfused to clear intravascular leukocytes and erythrocytes from the pulmonary circulation. The right lung was removed, homogenized briefly in 1 mL PBS, and passed through a preseparation filter (Miltenyi Biotec, Auburn, CA) to obtain a single cell suspension. Isolated cells were incubated with R-phycoerythrin (RPE)-conjugated rat-derived, anti-mouse neutrophil antibody (Serotec, Raleigh, NC) (1:10 dilution) for 1h at 4°C in the dark. After incubation, cells were washed with 1 mL cold PBS containing 1% fetal bovine serum and 0.1% sodium azide, then centrifuged at 2000 rpm for 5 min. The process was repeated twice, and cells were re-suspended in 500 μL 1% paraformaldehyde. The degree of cell fluorescence was identified using EPICS-Elite ESP Coulter Fluorescence Activated Cell Sorter.

Statistical analysis. Data are expressed as means $\pm$ SE (SEM). Statistical comparisons between control and the various protease-deficient groups were made using unpaired Student *t*-tests or single-factor ANOVA. For samples that were not normally distributed, Kruskal-Wallis or Mann-Whitney tests were applied. The relationship between pulmonary inflammation and bacterial burden was examined by determining the Pearson product moment correlation coefficient and least squares linear regression analysis.

RESULTS

Serine protease content of murine neutrophils. In the initial experiments, the serine protease phenotype of neutrophils from the different mouse models was confirmed. As expected, wild-type neutrophils contained high amounts of both NE (38 μmol/10^6 pmn) and CG (70 μmol/10^6 pmn). Cells isolated from NE-deficient mice expressed CG (40 μmol/10^6 pmn) but no NE, while CG-deficient mice contained NE (61 μmol/10^6 pmn) but undetectable CG activity.

Bacterial clearance and endobronchial inflammation in protease-deficient mice. To examine the roles of NE and CG in bacterial clearance from the lungs of protease-deficient mice, animals with the same genetic background and deficient in NE ($cg^{+/+}ne^{-/-}$) (2) or both neutrophil proteases ($cg^{-/-}ne^{-/-}$) (20) were challenged with *Pseudomonas*-laden agarose beads. Wild-type mice ($cg^{+/+}ne^{+/+}$) of the same strain background, replete with neutrophil proteases, served as controls.

Before infection, pulmonary histology was normal and similar between mouse genotypes (data not shown). After inoculation with *P. aeruginosa*-laden beads, there was no difference between mouse genotypes in clinical outcomes, including survival (no mortality in any group after infection), histopathologic appearance of the lungs (**data not shown**), and weight loss ($cg^{+/+}ne^{+/+}$: 17.9 $\pm$ 4.0%, $n = 16$; $cg^{+/+}ne^{-/-}$: 21.7 $\pm$ 4.4%, $n = 14$; and $cg^{-/-}ne^{-/-}$: 20.2 $\pm$ 3.1, $n = 16$). Three days after intratracheal inoculation with *P. aeruginosa*, lungs were removed and quantitative bacterial examinations of lung homogenates were performed to determine whether the bacterial burden differed among the genotypes. No difference was detected in the bacterial loads in lungs from $cg^{+/+}ne^{+/+}$ and $cg^{+/+}ne^{-/-}$ mice, but there was a log reduction in viable bacteria recovered from $cg^{-/-}ne^{-/-}$ mice (Fig. 1*A*). This phenotype was also found in the infected lungs of CG-deficient mice compared with isogenic, wild-type, control mice (Fig. 1*B*).

Before infection, there was no difference in neutrophil concentrations in BAL fluid of protease-deficient and wild-type mice (Fig. 2). The influx of neutrophils into the lungs of protease-deficient and wild-type mice after infection was similar, which confirmed that neutrophil transmigration was unaffected by the absence of serine proteases (Fig. 2).

Lung homogenates and BAL fluid supernatants from mice of each genotype were assayed for inflammatory markers in lungs. The CXC chemokine KC (but not MIP-2) concentration measured in whole lung homogenates was significantly higher in wild-type animals compared with protease-deficient mice (Fig. 3). No difference in TNF-α levels was measured in lungs from protease-deficient and -replete mice (**data not shown**). Bronchoalveolar lavage levels of all cytokines and chemokines did not differ between protease genotypes (**data not shown**). MIP-2 and KC tissue concentrations correlated with bacterial burden in the lungs (Fig. 4), suggesting that airway inflammation was only indirectly related to the presence (or absence) of a particular serine protease.

Effect of serine proteases on opsonins in the murine lung. Antibody levels measured in BAL fluid using ELISA and immunoblot analyses were significantly greater in CG-deficient compared with wild-type littermates inoculated with

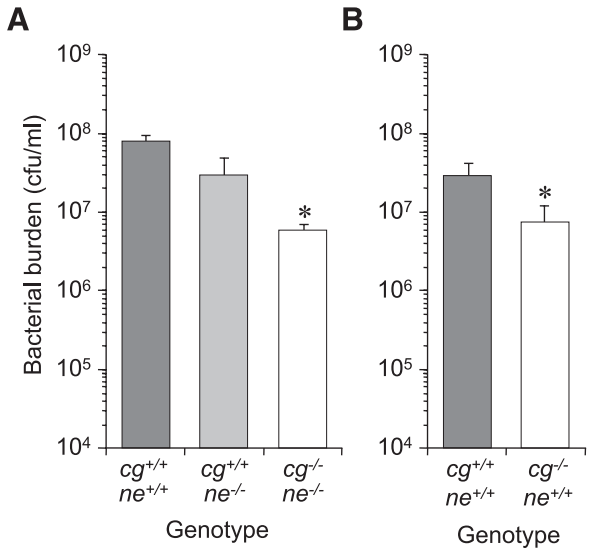

Figure 1. (*A*) Bacterial load in right lung homogenates from $cg^{+/+}ne^{+/+}$ ($n = 16$), $cg^{+/+}ne^{-/-}$ ($n = 14$), and $cg^{-/-}ne^{-/-}$ ($n = 16$) mice 3 d after intratracheal inoculation with *Pseudomonas*-laden beads. The mice lacking both serine proteases had significantly lower bacterial burden compared with wild-type controls (Kruskal Wallis test, $p = 0.01$). (*B*) Bacterial burden in the lungs from isogenic $\boldsymbol{cg^{+/+}ne^{+/+}}$ ($n = 10$) and $\boldsymbol{cg^{-/-}ne^{+/+}}$ ($n = 10$), mice following intratracheal instillation of *Pseudomonas*. Right lung homogenates were obtained 3 d after infection. Mice lacking CG ($cg^{-/-}$) had significantly lower bacterial counts than that detected in CG-replete mice ($cg^{+/+}$) (Mann Whitney test, $p = 0.01$).

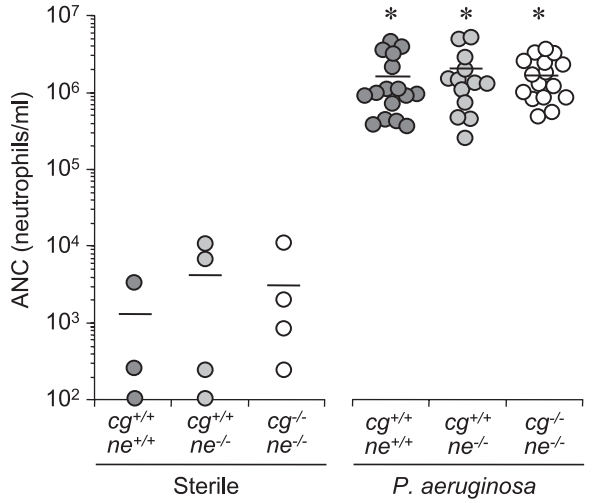

Figure 2. Absolute neutrophil concentrations in the lungs from $cg^{+/+}ne^{+/+}$, $cg^{+/+}ne^{-/-}$, and $cg^{-/-}ne^{-/-}$ mice inoculated with sterile or *Pseudomonas*-laden beads. Data represented as means. A marked increase in lavage ANC was found in all mouse genotypes after infection (ANOVA, $p < 0.01$), but there was no difference in neutrophil influx between mice replete or deficient in serine proteases.

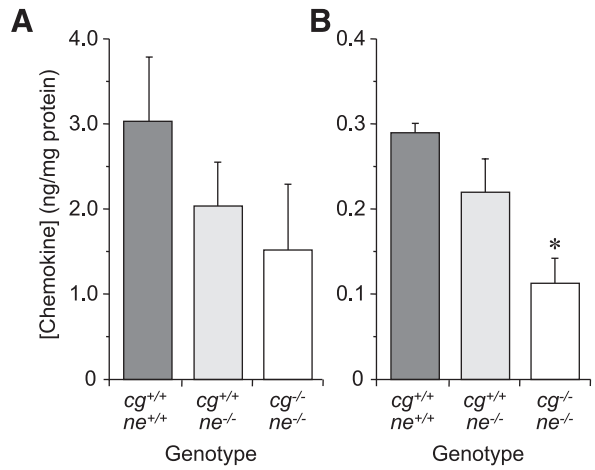

Figure 3. Chemokine (*A*) **MIP-2** and (*B*) **KC** concentrations in lung homogenates from $cg^{+/+}ne^{+/+}$, $cg^{+/+}ne^{-/-}$, and $cg^{-/-}ne^{-/-}$ mice 3 d after intratracheal instillation of *Pseudomonas*-laden beads. Data represented as mean ± SEM. The mice lacking both serine proteases had lower tissue KC concentrations compared with wild-type controls (Kruskal Wallis test, $p = 0.03$), while differences in MIP2 levels did not achieve statistical significance ($p = 0.15$).

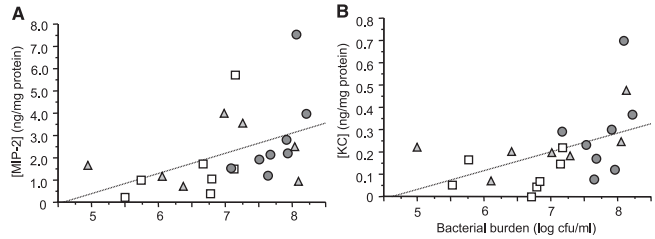

Figure 4. The relationship between bacterial load and CXC chemokine (*A*) MIP-2 and (*B*) **KC** concentrations in lung homogenates from isogenic $cg^{+/+}ne^{+/+}$ (**circles**), $cg^{+/+}ne^{-/-}$ (**triangles**) and $cg^{-/-}ne^{-/-}$ (**squares**) mice 3 d after *Pseudomonas* infection. Bacterial burden correlated with MIP-2 concentrations, irrespective of the animals genotype ($r = 0.5$, $p = 0.03$). Similar results were found with KC ($r = 0.5$, $p = 0.01$).

Pseudomonas-laden agar beads (Fig. 5 *A*). A similar relationship was noted between isogenic, CG-deficient and CG-replete mice (Fig. 5 *B*). Our analyses did not reveal a difference in airway immunoglobulin concentrations between NE-replete and NE-deficient strains. In addition, there was no difference in antibody concentrations in the lungs of uninfected, protease-deficient and -replete mice (**data not shown**).

Even though maintained in a full-barrier facility, the mice had been previously exposed to *P. aeruginosa*, a ubiquitous, water-borne organism, in their environment based on the presence of circulating anti-*P. aeruginosa* antibodies (Fig. 6 *A*). Specific, anti-*Pseudomonas* IgG and IgA was found in BAL fluid, especially after infection, which was markedly reduced in wild-type, protease-replete mice (Fig. 6 *B*).

CG may degrade other opsonins in the lung, resulting in defective bacterial killing and clearance at the airway surface. Collectins, specifically surfactant proteins-A (SP-A) and -D (SP-D), were considered to be potentially susceptible to proteolytic cleavage, resulting in gaps in airway immunity (25,26). SP-A and SP-D levels were measured in lavage fluid from $cg^{+/+}ne^{+/+}$, $cg^{+/+}ne^{-/-}$, and $cg^{-/-}ne^{-/-}$ mice, which did not reveal a difference between protease-replete and protease-deficient animals (Fig. 7).

Bacterial uptake by phagocytes from cathepsin G-deficient mice. We examined the *in vivo* uptake of GFP-expressing *P. aeruginosa* PAO1 by neutrophils recruited into infected lungs of $cg^{+/+}ne^{+/+}$ and $cg^{-/-}ne^{+/+}$ mice. Using flow cytometry, pulmonary neutrophils isolated from BAL fluid of CG-deficient mice generally had greater percentage of neutrophils with ingested fluorescent bacteria than CG-replete animals ($n = 5$ mice per group, $cg^{+/+}ne^{+/+}$, 6.7 ± 1.9%;

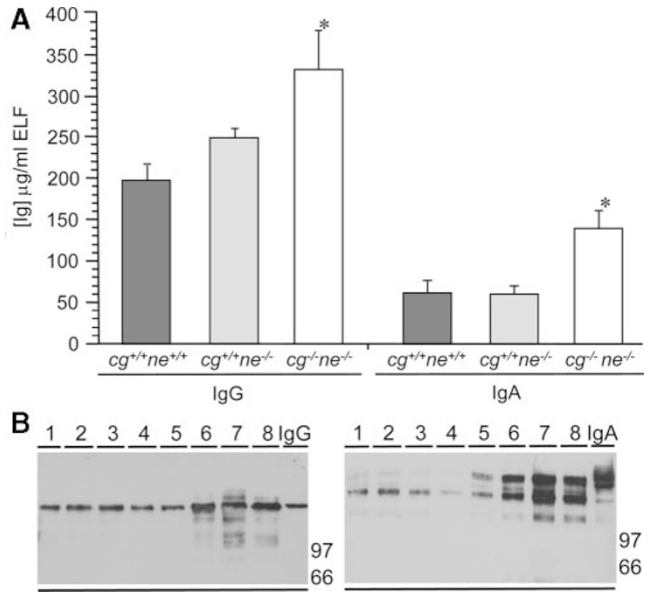

Figure 5. (*A*) Antibody levels in the lungs of $cg^{+/+}ne^{+/+}$ ($n = 16$), $cg^{+/+}ne^{-/-}$ ($n = 14$), and $cg^{-/-}ne^{-/-}$ ($n = 16$) mice 3 d after *P. aeruginosa* infection. The concentrations of **IgG** and **IgA** in respiratory ELF from $cg^{-/-}ne^{-/-}$ mice were significantly higher compared with $cg^{+/+}ne^{+/+}$ or $cg^{+/+}ne^{-/-}$ mice (Kruskal Wallis test, $p = 0.05$ and $p < 0.001$, respectively). Similar differences between ELF levels in $cg^{-/-}ne^{-/-}$ and $cg^{+/+}ne^{+/+}$ or $cg^{+/+}ne^{-/-}$ were found for IgM concentrations in BAL fluid. (*B*) Immunoblot analysis of murine antibodies secreted into the lung. Bronchoalveolar lavage fluid from randomly selected $cg^{+/+}ne^{+/+}$ (lanes **1 – 4**) and $cg^{-/-}ne^{+/+}$ (5–8) mice 3 d after infection. Purified mouse antibodies were used as positive controls (**IgG** and **IgA**). Molecular weight standards (kDa) are shown.

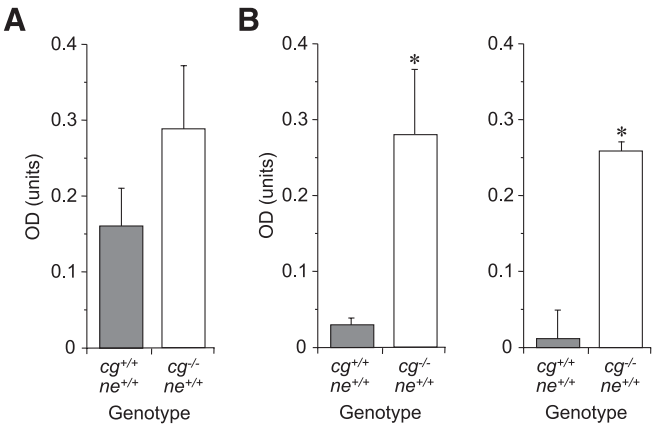

Figure 6. (*A*) Circulating anti-*Pseudomonas* IgG in isogenic $cg^{+/+}ne^{+/+}$ and $cg^{-/-}ne^{+/+}$ mice. There was no difference in blood levels of *Pseudomonas*-specific IgG between $cg^{-/-}ne^{+/+}$ and $cg^{+/+}ne^{+/+}$ (wild-type) mice. (*B*) Presence of anti-*Pseudomonas* IgA and IgG in BAL fluid from isogenic $cg^{+/+}ne^{+/+}$ and $cg^{-/-}ne^{+/+}$ mice 3 d after bacterial challenge. Mice lacking CG ($cg^{-/-}ne^{+/+}$) have higher *Pseudomonas*-specific IgG and IgA levels in ELF compared with wild-type mice (Student *t*-test, $p < 0.05$).

and $cg^{-/-}ne^{+/+}$, 13.2 ± 5.1%), but the difference was not significant.

Finally, cell surface proteins are vulnerable to proteolytic cleavage (27), which could potentially interfere with phagocytic function, but there were no differences in expression of scavenger receptor, integrin-α_m chain, and Fcγ III/II receptor on the surface of cells isolated from $cg^{+/+}ne^{+/+}$ and $cg^{-/-}ne^{+/+}$ mice (**data not shown**).

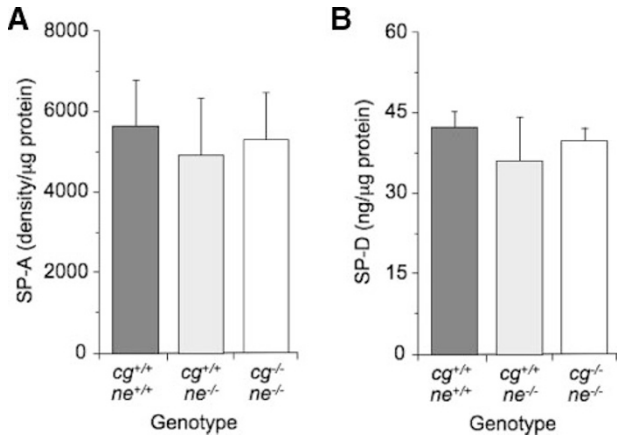

Figure 7. Collectin **SP-A** (*A*) and **SP-D** (*B*) levels measured in BAL fluid from protease-deficient and wild-type mice after *P. aeruginosa* infection. Three days after intratracheal instillation of *Pseudomonas*-laden beads, $cg^{+/+}ne^{+/+}$ ($n = 16$), $cg^{+/+}ne^{-/-}$ ($n = 14$), and $cg^{-/-}ne^{-/-}$ ($n = 16$) mice underwent lavage. Similar SP-A and SP-D concentrations were measured in BAL fluid, regardless of the mouse genotype.

DISCUSSION

Neutrophil elastase has been shown to contribute to pathogenesis of suppurative airway diseases by interfering with bacterial clearance and inciting greater inflammation. However, we found that CG independently hindered the host's ability to clear *P. aeruginosa* from the airway in a mouse model, perhaps related to protease-created defects in extracellular bacterial killing at the epithelial surface. Also, our results indicate that serine protease-mediated effects on airway inflammation are primarily a response to increased *P. aeruginosa* burden in the lungs, and not due to direct interaction between specific proteases and epithelial cells. Finally, there were no differences in neutrophil concentrations within BAL fluid collected from wild-type and protease-deficient mice before and after *P. aeruginosa* infection, showing that the absence of CG or NE did not affect neutrophil migration into the airway.

Given the substantial overlap in substrates and neutralizing antiproteases, it has been difficult to separate the effect of a single protease *in vivo*. We had the opportunity to reexamine previous observations using animal models completely devoid of individual proteases, in that these animals were genetically altered to be totally deficient of NE, CG, or both. Despite the absence of NE or CG, these animals were phenotypically identical to wild-type mice until challenged with bacteria. Previous studies using these mice have shown that serine proteases (2,19) are important for Gram-negative bacterial killing, but pulmonary infection was not specifically assessed in these models. Given the confinement of *P. aeruginosa* to lungs, earlier studies did not effectively model infection typical of CF.

The *Pseudomonas* endobronchitis model described in this report allowed us to control the host's genetic background as well as the genotype and virulence factors expressed by infecting organisms, factors difficult to manipulate or evaluate in humans. This approach provides another advantage for these experiments since the agarose beads physically impede the mucociliary escalator. The lung had to rely on alternative mechanisms to clear bacteria from the lungs, including innate and adaptive immunity

of the airways. Nevertheless, it is unclear whether the difference in bacterial load of CG-deficient mice is related to better *P. aeruginosa* killing or inhibition of bacterial growth, which will be examined in future studies.

Although the protease-deficient mice are housed under pathogen-free conditions, these animals had clearly been exposed to *P. aeruginosa*, a common environmental bacterium. While mice lacking CG had consistently lower bacterial loads, indicating more efficient bacterial clearance or killing, and higher concentrations of specific antibodies in the ELF, it is unclear whether antibody-mediated defenses are important to control *P. aeruginosa* infection. Many anti-*Pseudomonas* antibodies collected from infected CF patients are nonopsonic (28).

The phagocytic system affords protection against bacterial invasion, yet it also contributes to epithelial cell injury and damage. The extracellular release of catalytically active enzymes can have deleterious effects on the respiratory epithelium, and the protease burden in the bronchiectatic airway overwhelms the existing antiprotease defense. Proteases at the epithelial surface can inhibit or destroy components needed for airway defenses (29), resulting in defective bacterial killing and clearance. Indeed, though relatively resistant to destruction by serine protease, β-defensin can be inactivated by cysteine proteases (30). The antimicrobial enzyme, lactoferrin, is cleaved by cysteine proteases in CF sputa, resulting in a loss of antimicrobial activity against *P. aeruginosa* (31).

Two recent reports described reduced SP-A and SP-D levels in CF BAL fluid (25,26). Collectins are susceptible to proteolysis by NE and CG, leading investigators to postulate that unopposed neutrophil-derived serine proteases digest SP-A and create a breach in CF airway defenses. However, we did not find any differences in SP-A and SP-D concentrations in BAL fluid collected from protease-deficient and replete mice after infection with bacteria. The discrepancy of our findings with those described in patients was surprising, and are likely related to differences in the chronicity of *Pseudomonas* infection and intensity of the inflammatory response in the CF lung compared with that seen in the murine agar bead model.

Neutrophil elastase has been shown to stimulate epithelial inflammation, inducing the CXC-chemokine, IL-8, through a NF-κB-dependent pathway (4,5), and we expected to find less inflammation in the lungs of protease-deficient mice following infection. Conversely, neutrophil-derived serine proteases cleave *P. aeruginosa* flagellin, a potent inflammatory stimulus (32), and degradation of this virulence factor blunted expression of host defense genes in airway cell lines (20). In this model, the proteases only indirectly affected inflammatory response of the lungs following *P. aeruginosa* infection. Our results showed that serine protease-mediated effects on airway inflammation in the *Pseudomonas*-agar bead model was a response to increased bacterial burden in the lungs and not due to direct interaction between specific proteases and epithelial cells. Because the bacteria are encased in agar beads, it is possible that for some *P. aeruginosa*, their microenvironment was altered (*i.e.* hypoxia) such that flagellin was not expressed (33).

In conclusion, although NE has a principal role in the pathogenesis of suppurative airway diseases, such as CF, we found that CG also contributes to the infectious complications. We have shown that neutral serine proteases have differential effects in the infected lung. Specifically, CG hindered clearance of *P. aeruginosa* from the murine lung, which we suspect are related to gap(s) in airway defenses created by high concentrations of unopposed protease. The resultant increase in bacterial load then stimulated a greater inflammatory response from the epithelium. We also expect that such mouse models will permit identification of candidate proteins central to bacterial clearance in the airway and could potentially provide new therapeutic targets.

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