Antimicrobial Activity of PLUNC Protects against Pseudomonas aeruginosa Infection

Lina Lukinskienė, Yang Liu, Susan D. Reynolds, Chad Steele, Barry R. Stripp, George D. Leikauf, Jay K. Kolls and Y. Peter Di

*J Immunol* 2011; 187:382-390; Prepublished online 1 June 2011;  
doi: 10.4049/jimmunol.1001769  
http://www.jimmunol.org/content/187/1/382
Antimicrobial Activity of PLUNC Proteins Protects against Pseudomonas aeruginosa Infection

Lina Lukinskiene,* Yang Liu,* Susan D. Reynolds,† Chad Steele,‡ Barry R. Stripp,§ George D. Leikauf,* Jay K. Kolls,¶ and Y. Peter Di*†

Epithelial antimicrobial activity may protect the lung against inhaled pathogens. The bactericidal/permeability-increasing protein family has demonstrated antimicrobial activity in vitro. PLUNC (palate, lung, and nasal epithelium associated) is a 25-kDa secreted protein that shares homology with bactericidal/permeability-increasing proteins and is expressed in nasopharyngeal and respiratory epithelium. The objective of this study was to determine whether PLUNC can limit Pseudomonas aeruginosa infection in mice. Transgenic mice (Scgb1a1-hPLUNC) were generated in which human PLUNC (hPLUNC) was directed to the airway epithelium with the Scgb1a1 promoter. The hPLUNC protein (hPLUNC) was detected in the epithelium throughout the trachea and bronchial airways and in bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid from transgenic mice exhibited higher antibacterial activity than that from wild type littermates in vitro. After in vivo P. aeruginosa challenge, Scgb1a1-hPLUNC transgenic mice displayed enhanced bacterial clearance. This was accompanied by a decrease in neutrophil infiltration and cytokine levels. More importantly, the overexpressed hPLUNC in Scgb1a1-hPLUNC transgenic mouse airway significantly enhanced mouse survival against P. aeruginosa-induced respiratory infection. These data indicate that PLUNC is a novel antibacterial protein that likely plays a critical role in airway epithelium-mediated innate immune response. The Journal of Immunology, 2011, 187: 382–390.

Based on predicted structural homology, palate, lung, and nasal epithelium associated (PLUNC) has been added to the BPI protein family. PLUNC protein is expressed specifically in the nasopharyngeal and respiratory epithelium (6–8). The PLUNC genes are clustered on human chromosome 20 and encode eight different proteins that share some predicted structural similarity (9). PLUNC or short PLUNC protein 1 (SPLUNC1) is the first identified PLUNC protein that has multiple alternative names including secretory protein in upper respiratory tracts, lung-specific protein X, and nasopharyngeal carcinoma-related protein.

All BPI protein family members are defined by a similarly predicted three-dimensional structure with a single predicted disulphide bond and a conserved exon structure (7). However, the amino acid homology among members of the BPI protein family is typically ≈15–30% (10). The biological function of PLUNC is not known, but structural similarities to BPI family proteins suggest that PLUNC may be involved in defense responses in the airways. Chu et al. (11) reported that human and mouse bronchial epithelial cells expressed PLUNC (hSPLUNC1 and mSPlunc1), and that exogenously applied PLUNC decreased Mycoplasma pneumoniae levels and lessened IL-8 production in vitro. Conversely, PLUNC small interfering RNA enhanced M. pneumoniae growth and IL-8 production. IL-13 significantly decreased PLUNC and M. pneumoniae clearance in epithelial cell cultures, suggesting that decreased PLUNC expression during allergic responses may contribute to asthma pathobiology. Zhou et al. (12) found that PLUNC protein binds LPS and inhibits the growth of Pseudomonas aeruginosa in vitro. Garcia-Caballero et al. (13) reported that PLUNC may serve as an airway surface liquid volume sensor by regulating epithelial sodium ion-channel activity. Gakhar et al. (14) demonstrated that PLUNC has surfactant activity, and that this activity may account for the low surface tension of airway secretions. What was lacking in these studies is a demonstration that PLUNC has antimicrobial activity in vivo, and that additional PLUNC may be protective during infection. To determine whether PLUNC can limit P. aeruginosa infection in the lung, transgenic mice (Scgb1a1-hPLUNC) were generated in...
which human PLUNC (hPLUNC) was directed to the airway epithelium with the Scgb1al promoter and these mice were challenged with *P. aeruginosa*.

**Materials and Methods**

**Generation of Scgb1al-hPLUNC transgenic mice**

Constitutive expression of hPLUNC protein in the mouse respiratory epithelium was achieved through generation of mice harboring a transgene (TG) composed of the hPLUNC cDNA under the transcriptional control of the mouse Scgb1al (also known as Clara cell secretory protein, or CCSP) promoter (Fig. 1A). Total human airway epithelial cell RNA was used as a template for RT-PCR amplification of PLUNC cDNA (0.78 kb). The forward primer included the protein translational start codon and 12 nucleotides of flanking sequence (5'-ATA ATG CCG CCG CCT AAG AGC AAA GAT GTT TC-3'), whereas the reverse primer covered the translational stop site (5'-ATA ATG CCG CCG CAC TCT GTG GAC AAA CTG-3'). This cDNA coded for the active and mature PLUNC protein. The PCR product was gel purified, cloned using the pCR2.1-TOPO cloning system (Invitrogen, Carlsbad, CA), and sequenced. A 9.6-kb Scgb1al genomic fragment in pUC19 (kindly supplied by Dr. Magnus Nord, Karolinska Institute, Stockholm, Sweden) was modified by site-directed mutagenesis to include an NotI site upstream of the hPLUNC promoter and these mice were obtained from a single colony was stored in liquid nitrogen. One of 19 offspring was positive for the TG, as assessed by PCR genotyping and confirmed by Southern blot analyses of tail DNA. Subsequently, genotype was determined by PCR. The endogenous Scgb1al gene and the Scgb1al-hPLUNC transgene were distinguished as 450- and 545-bp amplicons using a mouse Scgb1al promoter-specific forward primer (5'-GGT GCC AAC AGC ACG TCT AGC CCT-3')} for the generation of pNotI-9. The PCR product was gel purified, cloned using the pCR2.1-TOPO cloning system (Invitrogen, Carlsbad, CA), and sequenced. A 9.6-kb Scgb1al genomic fragment in pUC19 (kindly supplied by Dr. Magnus Nord, Karolinska Institute, Stockholm, Sweden) was modified by site-directed mutagenesis to include an NotI site upstream of the hPLUNC promoter and these mice were obtained from a single colony was stored in liquid nitrogen. One of 19 offspring was positive for the TG, as assessed by PCR genotyping and confirmed by Southern blot analyses of tail DNA. Subsequently, genotype was determined by PCR. The endogenous Scgb1al gene and the Scgb1al-hPLUNC transgene were distinguished as 450- and 545-bp amplicons using a mouse Scgb1al promoter-specific forward primer (5'-GGT GCC AAC AGC ACG TCT AGC CCT-3') and the construct verified by restriction enzyme digestion and sequencing. The 10.38-kb Scgb1al+/-hPLUNC cDNA fragment was isolated by digestion with SpH1 and transgenic generated by microinjection into FVB/N mouse blastocysts. The plasmid was linearized with NotI, transfected into FVB/N blastocysts, and the transgenic founders were identified by Southern blot analyses of tail DNA. Approximately 12 months were required for the generation of a stable transgenic line.

**Animal husbandry**

The Scgb1al-hPLUNC TG was maintained in a hemizygous state by breeding with wild type (WT) FVB/N mice. Mendelian transmission frequencies were observed, and no overt consequences of the TG on growth, breeding, or survival were noted. Mice were maintained in a specific pathogen-free status in 12-h light/dark cycle conditions. All procedures were conducted using mice 8–12 wk of age maintained in ventilated microisolator caging (Harlan Industries, Indianapolis, IN) as per the Guidelines for the Care and Use of Laboratory Animals (NIH). Mice were genotyped by PCR using primers specific for the hPLUNC transgene. Mice were identified by tail DNA and ear punch. Mice were housed in isolators, fed ad libitum, and drinking water was supplied ad libitum. At least 2 weeks of acclimation were required before experiments were performed.

**Bacterial clearance**

Bacterial deposition was determined by harvesting the right lungs immediately postinfection (4–6 h postinfection) and the recovered fluid was pooled (pool 2). Cells from the two pools were recovered through centrifugation at 300 × *g* for 30 min and resuspended in 0.5 ml PBS. A 50-ml aliquot was stained with an equal volume of 0.4% trypan blue (Invitrogen, San Diego, CA) and cells counted with a hemocytometer. An additional aliquot was placed onto glass microscope slides (Shandon Cytospin; Thermo Fisher, Pittsburgh, PA), stained with Diff-Quick, and cell differential determined microscopically. Protein concentration in pool 1 was determined by DC Protein assay using BSA standards (Bio-Rad, Hercules, CA).

**Immunohistochemistry**

Lungs were inflation fixed in situ with 4% paraformaldehyde (10 cm H2O; 10 min) with chest cavity open. The lower right lobe was embedded in 100 ml LB broth and incubated (6 h; 37°C). Bacteria were washed twice and resuspended in 10 ml PBS containing 10 mM magnesium chloride.

**CFU assay**

The number of CFU was determined by serial dilution and quantitative culture on LB agar plates. *P. aeruginosa* was reisolated in PBS, and the OD (OD260) was adjusted to ~0.74, ~2 × 10^11^ bacteria/ml. Five serial 10-fold dilutions in PBS were plated immediately before use. Duplicate bacterial samples were mixed with 50 ml PBS, or bronchoalveolar lavage fluid (BALF) from either transgenic or WT control mice were prepared and plated on ice. An equal volume of the 2 × 10^9^ bacteria/ml solution was added to the wells. Samples were mixed and incubated (2 h; 37°C) with shaking. Three 50-ml aliquots with different concentrations (no dilution, 5-fold dilution, and 10-fold dilution) from each well were plated on LB agar plates. Plates were inverted and incubated overnight at 37°C. Colonies were counted and the number of colonies per plate determined. For Ab neutralization studies, mouse BALF samples (50 ml) were preincubated (1 h; 23°C) with 7.5 ml normal rabbit serum or with anti-PLUNC serum with gentle agitation, and CFU were calculated.

**In vivo exposure of mice to *P. aeruginosa***

Six-week- to 8-wk-old WT and transgenic mice were coexposed to *P. aeruginosa* aerosol using an inhalation exposure system (model A42X; Glas-Col, Terre Haute, IN) as previously described (16). Mice were placed in a compartmentalized mesh basket (5 chambers, 20 mice/chamber capacity) and exposed (45 min), followed by cloud decay (15 min) and decontamination (5 min; UV irradiation). Preliminary studies demonstrated that exposure to 10^9^ bacteria/ml for 45 min resulted in a bacterial deposition of ~2 × 10^7^ CFU/lung immediately after exposure (5 independent experiments, n = 5–6/experimental group, 1.6–2.5 × 10^6^, median and 25th–75th percentile). For survival test, female 6- to 8-wk-old WT and transgenic mice were infected with an intratracheal instillation of *P. aeruginosa* at a concentration of 1 × 10^6^ CFU/mouse.

**BAL and cell differential counts**

At 4 or 24 h after aerosol exposure, mice (5–6 mice/group) were anesthetized with 2.5% tribromoethanol (Avertin). The trachea was cannulated, the lungs were lavaged (1 ml PBS twice), and the BALF samples pooled (pool 1). The lungs were lavaged an additional five times with 1 ml PBS and the recovered fluid was pooled (pool 2). Cells from the two pools were recovered through centrifugation at 300 × *g* and resuspended in 0.5 ml PBS. A 50-ml aliquot was stained with an equal volume of 0.4% trypan blue (Invitrogen, San Diego, CA) and cells counted with a hemocytometer. An additional aliquot was placed onto glass microscope slides (Shanon Cytospin; Thermo Fisher, Pittsburgh, PA), stained with Diff-Quick, and cell differential determined microscopically. Protein concentration in pool 1 was determined by DC Protein assay using BSA standards (Bio-Rad, Hercules, CA).

**Immunohistochemistry**

Lungs were inflation fixed in situ with 4% paraformaldehyde (10 cm H2O; 10 min) with chest cavity open. The lower right lobe was embedded in paraffin and 5-μm sections prepared. Sections were cleared with xylene, hydrated to water, and equenched in 3% H2O2 (10 min). Ag retrieval was performed in 10 mM citrate buffer (20 min; 100°C; pH 6.0). Sections were blocked in 0.5% casein in 0.05% PBS/Tween 20 solution (1 h) and incubated (18 h; 4°C) with hPLUNC Ab (R&D Systems, Minneapolis, MN). Negative control serum was used as a control. After extensive washing with PBS, sections were incubated (2 h; 23°C) with biotinylated donkey anti-goat Ab (Pierce Biotechnology, Rockford, IL) diluted in blocking solution. Sections were washed (30 min; 23°C) and incubated with streptavidin-HRP.
Antimicrobial Activity of Plunc Against P. aeruginosa

Expression of hPLUNC protein in mouse airways was assessed by immunohistochemical staining of paraffin-embedded trachea and lung sections from WT control and transgenic (Scgb1a1-hPLUNC, TG) littermates using an Ab that detects both hPLUNC and mouse Plunc (mPlunc). Expression of endogenous mPlunc was detected in the tracheal and bronchial epithelium (data not shown) but was absent in bronchiolar cells and alveolar epithelial cells of WT control mice (Fig. 2A, 2C). In transgenic Scgb1a1-hPLUNC mice, PLUNC was detected in tracheal (data not shown), bronchial, and bronchiolar epithelial cells (Fig. 2B, 2D). The distribution of Scgb1A1 protein was not altered (Fig. 2E, 2F).

It has been demonstrated that PLUNC is secreted into lumen of human airway and can be detected in sputum (6). To determine whether hPLUNC is similarly secreted in transgenic mice, we used Western blot analysis to examine BALF and lung homogenate from WT and TG littermates. SCGB1A1 protein, also known as Clara cell secretory protein (CCSP), was used as a loading control for secreted BALF proteins and GAPDH was used to normalize tissue proteins in lung homogenates. mPlunc is not normally expressed in intrapulmonary airway epithelium, and very little protein could be detected in lung homogenate from control littermates. Total PLUNC protein (mouse and human) in BALF was ≥10-fold greater in transgenic mice as compared with control littermates (Fig. 3). These results indicate that hPLUNC was highly expressed in intrapulmonary airways, and that it was effectively secreted into the luminal space.

Enhanced antibacterial activity in BALF from Scgb1a1-hPLUNC transgenic mice

To determine whether BALF from transgenic TG mice had increased antibacterial activity, we assessed P. aeruginosa (PAO1, ATCC BAA-47) colony formation in BALF obtained from WT control and Scgb1a1-hPLUNC transgenic littermates. CFU decreased 2.9-fold in cultures treated with BALF from transgenic mice compared with that from control littermates (Fig. 4). Primary normal rabbit serum IgG or rabbit anti-PLUNC IgG were preincubated with BALF and antibacterial assay was performed to determine the contribution of hPLUNC and mouse Plunc to this bactericidal activity. Ab neutralization by neutralizing anti-PLUNC IgG decreased total bactericidal activity of WT control BALF, indicating that endogenous mouse Plunc was responsible for a portion of the activity (Fig. 4). In addition, the bactericidal activity of Ab-treated Scgb1a1-hPLUNC BALF was not different from Ab-treated BALF from WT control littermates. These results demonstrated that mPlunc is active in the airway, and that the additional hPLUNC is secreted to and is active in the airway lumens in Scgb1a1-hPLUNC transgenic mice.

mPlunc enhanced host defense in mouse lung against inhaled P. aeruginosa

To evaluate the effect of inhaled P. aeruginosa on mPlunc expression, we assessed lung mRNA levels by qRT-PCR analysis. PAO1 bacterial exposure significantly increased Plunc expression at both 6 (5.1-fold; p = 0.0002) and 24 h (2.5-fold; p = 0.021) postexposure (Fig. 5), but the induction of Plunc at 6 h postexposure was higher than 24 h postexposure. To determine the functional significance of increased plunc in mouse lung after bacterial infection, we pretreated the mice with specific anti-PLUNC Ab to neutralize the antimicrobial activity of PLUNC in mouse airways. The Ab concentration at either 10 or 25 µg did not show any noticeable difference in affecting bacterial susceptibility. However, a pretreatment on mice with either concentration of neutralizing Ab significantly increased the mouse susceptibility to PAO1-induced bacterial infection when compared with the
control mice that were pretreated with nonspecific IgG (Fig. 6). There was an at least 3-fold increase in bacterial burden of mouse lung when mice were pretreated with the neutralizing Ab. These data further supported the important role of PLUNC in host defense against *P. aeruginosa*-induced lung infection.

**Improved bacterial clearance in Scgb1a1-hPLUNC transgenic mice against inhaled *P. aeruginosa***

To determine whether hPLUNC expression could alter bacterial infection in vivo, WT control and *Scgb1a1*-hPLUNC transgenic littermates were coexposed to saline or *P. aeruginosa* aerosols, and deposition and clearance were monitored. The aerosolized exposure protocol resulted in a deposition of $2.26 \pm 0.39 \times 10^6$ CFU/lung *P. aeruginosa* and was consistent among exposures. Immediately after exposure, bacterial deposition was equivalent between transgenic mice and WT control littermates (Fig. 7). This exposure concentration enabled us to examine PLUNC-mediated antimicrobial activity after a reversible respiratory infection. Because a whole-body exposure method was used, a systemic bac-

**FIGURE 2.** hPLUNC protein is expressed in bronchiolar airway epithelial cells in *Scgb1a1*-PLUNC transgenic mice. Cellular localization of PLUNC protein in mice was assessed by immunohistochemistry (A, B; diaminobenzidine staining) and immunofluorescence microscopy (C, D: Alexa 488; E, F: Alexa 594) on lung sections of wild-type (A, C, E) and transgenic (B, D, F) mice using anti-PLUNC Ab (A–D) and anti-SCGB1A1 Ab (E, F). Signal was not detected when parallel sections from transgenic mice were incubated with preimmune goat serum (data not shown). PLUNC was detected in airway epithelial cells (arrowheads) of transgenic but not WT mice. Original magnification ×400 (A, B) and ×100 (C–F).
teremia was also assessed by determining the CFU in spleen homogenates 24 h postinfection. Less than 500 CFU were detected in spleens of mice, and no statistical difference was detected between the transgenic mice as compared with their control littermates.

Four hours postexposure, WT control mice became slightly lethargic and demonstrated signs of infection (e.g., ruffled fur and hunched back), whereas Scgb1a1-hPLUNC transgenic littermates displayed more active behavior and little observable signs of infection. The bacterial clearance was ∼3-fold greater (p < 0.05) in lung homogenates from Scgb1a1-hPLUNC transgenic mice (2.31 ± 0.41 × 10^5 CFU/lung/ml) as compared with that from WT control mice (6.83 ± 0.57 × 10^5 CFU/lung/ml) (Fig. 7A). At 24 h after exposure, WT control and Scgb1a1-hPLUNC transgenic mice exhibited similar activity levels. However, bacteria cleared 80%, whereas transgenic mice had cleared 98% of the initial bacterial burden. These data suggest that PLUNC acts early in infection and promotes efficient clearance of the initial bacterial inoculum.

**FIGURE 4.** BALF from transgenic mice exhibits enhanced antimicrobial activity. BALF from unchallenged WT and TG littermates (first and second bars, respectively) was mixed with P. aeruginosa (PAO1) as described in Materials and Methods and CFU were assessed. Neutralizing Ab against PLUNC was preincubated with WT or TG BALF (third and fourth bars, respectively) for 1 h before mixing with bacteria and CFU counts were assessed. Results are the mean and SD from three independent experiments (n = 8 for each experiment). *p < 0.05, **p < 0.05, and ***p < 0.05 when the transgenic mouse group is compared with the control WT mouse group.

**Decreased inflammatory cell infiltration in Scgb1a1-hPLUNC transgenic mouse lung after inhaled P. aeruginosa**

BALF total cell count (2–4 × 10^5 cells/lung) or differentials (≥99% macrophages) from unexposed Scgb1a1-hPLUNC transgenic mice were not different from those from WT control mice. Four hours after bacterial exposure, the total inflammatory cell counts in BALF increased in both TG and WT control mice, but the increase was higher in WT mice than in TG mice. The increased macrophage number between WT (3.1 ± 1.4 × 10^6; n = 18) and TG (3.6 ± 1.6 × 10^6; n = 18) mice was not statistically different, but the total neutrophils in WT control mice (26.1 ± 2.8 × 10^6; n = 18) were slightly greater (∼1.44-fold; p < 0.05) than from Scgb1a1-hPLUNC transgenic mice (18.1 ± 2.3 × 10^6; n = 18) (Fig. 7B). However, 24 h postexposure, the total neutrophils were >2-fold (p < 0.01) greater in BALF from WT control mice (16.2 ± 2.0 × 10^6; n = 18) as compared with BALF from Scgb1a1-hPLUNC transgenic mice (6.90 ± 0.6 × 10^6; n = 18) (Fig. 7B). There were no significant differences in the total macrophage number between TG and WT mice at 24 h after PAO1-induced bacterial infection. These observations were supported by...
histological analysis of lung tissue. Lungs from uninfected WT control mice (Fig. 8A) were not different from those from Scgb1a1-hPLUNC transgenic mice (Fig. 8C). However, 24 h after bacterial exposure, lung tissues from WT control mice (Fig. 8B) contained more areas of focal peribronchial and alveolar neutrophil infiltrates than lungs from Scgb1a1-hPLUNC transgenic littersmates (Fig. 8D).

Decreased proinflammatory cytokines in Scgb1a1-hPLUNC transgenic mice after P. aeruginosa exposure

BALF samples were collected and cytokine concentrations were determined by Bio-Plex assay to determine whether production of inflammatory cytokines varied 4 or 24 h postexposure. Without bacterial exposure, BALF cytokine levels from WT control mice were not different from those from Scgb1a1-hPLUNC transgenic mice, and most cytokine levels were not detected (data not shown). Postexposure, the spectrum of BALF cytokines detected did not vary by genotype, but the magnitude of the response was consistently lower in BALF from Scgb1a1-hPLUNC transgenic mice as compared with that from WT control mice (Fig. 9). At 4 h postexposure, IL-1β levels were lower in BALF from Scgb1a1-hPLUNC transgenic mice as compared with that from WT control mice (Fig. 9A). At 24 h postexposure, the difference was greater and additional cytokines including IL-6 (Fig. 9B) and chemotactic cytokines, chemokine (C-X-C motif) ligand 1 (also known as KC; Fig. 9C) and chemokine (C-X-C motif) ligand 2 (also known as MIP-2; Fig. 9D), were significantly lower in BALF from Scgb1a1-hPLUNC transgenic mice as compared with that from WT control mice. We did not observe significant differences in the production of IL-2, IL-3, IL-10, and IL-12(p40) in steady-state or P. aeruginosa-challenged WT and Scgb1a1-hPLUNC transgenic mice (data not shown).

Enhanced survival in Scgb1a1-hPLUNC transgenic mice after P. aeruginosa exposure

To determine whether PLUNC affects susceptibility to P. aeruginosa-induced lung infection, groups of female 6- to 8 wk-old WT and Scgb1a1-hPLUNC transgenic mice were challenged through intratracheal instillation with various doses (10^7, 10^8, and 10^9 CFU/mouse) of P. aeruginosa PAO1. We did not observe any mortality when concentrations of PAO1 at 10^7 and 10^8 CFU/lung were administered (data not shown). When mice were challenged with 10^9 CFU/animal of PAO1, Scgb1a1-hPLUNC transgenic mice displayed enhanced survival and decreased lethality (log-rank test, p = 0.001; Fig. 10). All WT mice succumbed to PAO1-induced lung infection within 48 h (mean survival time, 41.6 ± 1.3 h), whereas 5 of 10 Scgb1a1-hPLUNC transgenic mice survived the challenge and were still alive 1 wk postinfection. These results indicate that the overexpressed PLUNC is essential to an enhanced survival and resistance to P. aeruginosa infection.

Discussion

Effective host defense against microbial invasion requires an innate immune system whose response is both rapid and independent of prior exposure (18). Several secreted proteins participate in this innate immune response (19), and data presented in this article indicate that PLUNC is a member of this group. In this study, we successfully expressed hPLUNC protein in the airway epithelium of transgenic mice using the mouse Scgb1a1 promoter. Scgb1a1-hPLUNC transgenic mice expressed hPLUNC mRNA and hPLUNC protein in the airway epithelium and secreted hPLUNC was detected in BALF. Expression of hPLUNC did not alter lung structure or function, nor did it change the expression and secretion of the major mouse airway secretory protein SCGB1A1. However, hPLUNC was associated with enhanced bacterial killing and decreased inflammation after exposure with a major human airway pathogen, the Gram-negative bacteria P. aeruginosa (PAO1). Our results provide evidence of a role for PLUNC in mitigation of bacterial infection both in vitro and in vivo. Although other pathogens were not tested in the study, the transgenic...

FIGURE 7. Scgb1a1-PLUNC transgenic mice are resistant to P. aeruginosa-induced lung infection. Age-matched WT and TG littersmates were infected with ~2 × 10^6 CFU P. aeruginosa (PAO1) per lung by inhalation. A, CFU in lung homogenates were determined at the indicated recovery time points. *p < 0.01 for WT to TG comparisons. B, Total neutrophils in BALF from WT compared with TG mice 4 and 24 h after bacterial exposure (*p < 0.05). Total neutrophils were significantly lower in TG mice 24 h postinfection (**p < 0.01). Results are mean ± SD (n = 5–6) from three independent experiments.

FIGURE 8. Decreased neutrophilic infiltration Scgb1a1-PLUNC mice after bacterial exposure. WT and Scgb1a1-PLUNC mice were exposed to saline (A, C) or aerosolized P. aeruginosa (B, D). Lung tissues were harvested at 24 h after exposure, fixed and H&E-stained for histological evaluation. No histological differences were observed between WT and TG mice before bacterial exposure. After bacterial exposure, TG mice showed less neutrophil infiltration. Original magnification ×40. Sections are representative of at least five mice studied at each time point from each infection.
mice generated in this study represent a useful animal model for future investigations to identify PLUNC-mediated antimicrobial activity in the airway.

Several lines of evidence suggest that PLUNC-mediated antibacterial activity is an important aspect of the innate immune response in the airway. First, PLUNC is structurally related to BPI and LBP, proteins that have been demonstrated to be important to host defense against Gram-negative bacteria (20–22). Second, PLUNC belongs to the short-peptide subfamily of PLUNC family proteins and has homology to the N-terminal domains of BPI (23–25). Peptides derived from this region of BPI have previously been shown to possess direct bactericidal activity (23, 26). Using a bioactivity assay to test antimicrobial functions of secretory proteins in BALF, we demonstrated a significant decrease in CFU counts of *P. aeruginosa* when bacteria were coincubated with BALF from *Scgb1a1-hPLUNC* transgenic mice. Furthermore, both in vitro and

**FIGURE 9.** Decreased proinflammatory cytokine production in *Scgb1a1-PLUNC* mice after PAO1 exposure. WT and TG mice were infected with *P. aeruginosa* as described. Cytokines concentrations in BALF were measured using a Bio-Plex assay and are reported in pg/ml. Values were determined for 4 and 24 h after bacterial exposure. Results from the measurements of IL-1β (A) and MIP2 (D) showed significant difference between WT and TG mice both 4 h and 24 h after bacterial challenge. Measurements of IL-6 (B) and KC (C) only exhibited statistical significance between WT and TG mice 24 h after bacterial challenge. Striped bars represent WT mice; solid bars represent *Scgb1a1-PLUNC* TG mice. Results are mean ± SD from three experiments (n = 5–6 mice). *p < 0.05, **p < 0.05 for WT to TG comparisons at each time point.

**FIGURE 10.** Overexpressed hPLUNC in *Scgb1a1-PLUNC* mice enhances the survival from PAO1-induced acute pneumonia. WT and TG mice were infected with *P. aeruginosa* as described at a concentration of 1 × 10^9 CFU/mouse of PAO1 strain. Survival is represented by Kaplan–Meier survival curves, and there was a statistically significant difference between survival curves of WT and TG groups (p = 0.001, log rank test).
in vivo Ab neutralization studies indicated that the increased antibacterial activity was due to PLUNC. Therefore, the decreased sensitivity in Scgb1a1-hPLUNC transgenic mice may have partially attributed to the direct killing activity. These data suggested that expression of hPLUNC in mouse airways increased mouse resistance to pathogens.

Because BPI is normally expressed in inflammatory cells but not airway epithelial cells, these data may also suggest that critical aspects of the innate response can be mediated by similar proteins that are secreted by different cell types. Moreover, the augmentation of protection by the additional hPLUNC suggests that PLUNC or BPI congeners could provide a novel anti-infection therapy. PLUNC-mediated antibacterial activity highlights its functional significance and supports the notion that PLUNC plays an important role in innate immunity against respiratory infection.

PLUNC is one of the most abundant secretory proteins in nasal drainage, BALF, and in primary epithelial cell cultures (6, 27, 28). The significant amount of PLUNC secreted into the airway lumen and its antibacterial activity indicated that PLUNC plays a critical role in airway epithelial cell-based innate immunity. The enhanced antibacterial activity in Scgb1a1-PLUNC transgenic mice correlated with expression of hPLUNC in mouse airways. The magnitude of enhanced antibacterial activity (~3-fold) was similar to previously reported antibacterial activity for lysozyme, a known antimicrobial protein in airway secretions (29). Because PLUNC is an abundant component of the antimicrobial fraction of nasal secretions (30), is present at high levels in airway secretions (6, 27, 28, 31, 32), is expressed at levels similar to that of lysozyme in tracheobronchial submucosal glands, and has antimicrobial activity comparable with that of lysozyme, it is reasonable to propose that PLUNC is an important component of the innate response to airway pathogens that originates from airway epithelial cells. It has been suggested that PLUNC is a novel airway secretory protein with a surfactant protein function that modulates surface tension and inhibits biofilm formation (14). Therefore, one of the potential PLUNC-mediated antimicrobial mechanisms may be overexpression of hPLUNC in Scgb1a1-PLUNC transgenic mice that enhanced the mucociliary clearance and disrupted the biofilm formation of P. aeruginosa after PAO1 challenged respiratory infection. The enhanced survival of Scgb1a1-PLUNC transgenic mice compared with their WT littermates after a high dose of P. aeruginosa challenge further indicates an important antimicrobial activity of PLUNC to P. aeruginosa-induced lung infection and supports the notion that PLUNC plays a critical role in airway epithelium-mediated innate immune response.

PLUNC may possess anti-inflammatory activities that are important in modulating airway innate immune response. Our data demonstrate a lower degree of neutrophil recruitment and less lung inflammation in PAO1-exposed Scgb1a1-PLUNC transgenic mice than their WT littermates and suggest that constitutive overexpression of hPLUNC in mouse airways resulted in more efficient bacterial killing. The decreased inflammation noted in Scgb1a1-PLUNC transgenic mice could have been a direct consequence of more efficient bacterial clearance. The potential binding of PLUNC to LPS may modulate LPS-mediated inflammatory response. It has been suggested that PLUNC binds directly to LPS from Escherichia coli (28). A structurally related protein murine parotid secretory protein (PSP), also called SPLUNC2, has been shown to bind to bacterial membranes (33).

Cytokines play an important role in regulation and modulation of immunological and inflammatory processes. Unlike an in vitro cell culture system that represents a single cell type, the use of a transgenic mouse model provided an opportunity to identify coordinated changes in cytokine production between inflammatory and epithelial cells after exposure to aerosolized P. aeruginosa. Normally, after recognition of microbial products, TLR-mediated signaling results in the production of TNF-α and IL-1β, two early-responsive cytokines that regulate subsequent recruitment of neutrophils (34–36). These substances usually play beneficial roles in the body’s defense systems. However, excess cytokine production may contribute to an exacerbated inflammatory response and could even contribute to severe inflammation and dysfunction in other organs if these inflammatory mediators are released excessively into the bloodstream. Therefore, a well-regulated and balanced production of inflammatory mediators is critical to an effective local and systemic host defense. In our studies, proinflammatory cytokines such as IL-1β, IL-6, and TNF-α were nearly undetectable before bacterial exposure but were significantly increased after challenge. Enhanced clearance of inhaled bacteria in Scgb1a1-PLUNC mice also correlated with decreased production of pro-inflammatory cytokines (including IL-1β, TNF-α, and IL-6) after bacterial challenge. Another proinflammatory chemokine, KC, has been shown to substantially increase the accumulation of neutrophils in the lungs after the intratracheal administration of LPS (37), and we observed significantly lower levels of KC secretion in BALF from Scgb1a1-PLUNC transgenic mice when compared with WT control mice after P. aeruginosa exposure. The lower amount of KC in BALF of Scgb1a1-PLUNC transgenic mice might also partially account for decreased neutrophil infiltration and later resulted in lesser damage to the lungs in transgenic mice as compared with their WT littermates. Although the decrease in proinflammatory cytokine production noted in P. aeruginosa-exposed Scgb1a1-PLUNC transgenic mice is likely to be a consequence of antibacterial activities of hPLUNC, it is also possible that the lower cytokine levels in Scgb1a1-PLUNC transgenic mice could be because of unanticipated roles for hPLUNC in regulation of cytokine production in epithelial or inflammatory cells, or both. Further studies to identify potential bioactivity of PLUNC in association with inflammatory response are worthy of future investigation.

Our data suggest that expression of hPLUNC provides an airway epithelial cell-specific protection against opportunistic pathogens such as P. aeruginosa. Although the major difference between transgenic mice and their WT littermates is most likely due to expression of hPLUNC, we could not rule out the possibility that other antimicrobial proteins/peptides may also act synergistically with ectopically expressed hPLUNC to enhance the bacterial killing effect. The enhanced bacterial clearance observed in Scgb1a1-PLUNC transgenic mice may be because of interaction of overexpressed PLUNC with other antimicrobial peptides such as defensins and/or antimicrobial proteins such as lysozyme to potentiate its antibacterial activities.

In conclusion, this study demonstrated antibacterial activity of PLUNC against airway bacterial pathogens using both in vitro and in vivo approaches. We also demonstrated that Scgb1a1-PLUNC transgenic mice exhibited enhanced survival and improved resistance to P. aeruginosa infection, one of the most common airway infections associated with chronic colonization in cystic fibrosis patients. Given the emergence of highly resistant bacteria pathogens and the increasing population of immunocompromised patients, the treatment of bacterial infection has and will continue to be challenging. A better understanding of airway epithelial cell-initiated host defense may provide an alternative approach to efficiently combat airway bacterial infection.

Acknowledgments
We thank Dr. Jonathan Chao for making constructs for the generation of Scgb1a1-PLUNC transgenic mice and Dr. Thomas Zhao for technical assistance in dissecting mice.
Disclosures
The authors have no financial conflicts of interest.

References