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Antimicrobial Activity of PLUNC 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: 000–000.

The ability of the host to avoid infection depends largely on mechanisms of innate immunity. This rapid-response system acts efficiently without prior exposure to a pathogen (1) and is initiated when bacterial products are detected. LPS, a cell-wall component of Gram-negative bacteria, is an agonist for innate immune response through activation of the TLR4 signaling cascade. Exposure to LPS results in a production of proinflammatory and anti-inflammatory mediators by myeloid lineage and other cell types including epithelial cells. In humans, bactericidal/permeability-increasing protein (BPI) and LPS-binding protein (LBP) can bind LPS and modulate the host response to Gram-negative bacterial infections. Although BPI and LBP belong to the same protein family (i.e., BPI protein family), their functions are antagonistic: LBP exhibits proinflammatory activity, whereas BPI displays anti-inflammatory activity and direct bactericidal action (2, 3). Two additional lipid transfer LPS-binding plasma proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), are included in the BPI protein family based on predicted structural similarity (4, 5).

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 pathology. Zhou et al. (12) found that PLUNC protein binds LPS and inhibits the growth of Pseudomonas aeruginosa in vitro. García-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 Scgb1a1 promoter and these mice were challenged with *P. aeruginosa*.

**Materials and Methods**

**Generation of Scgb1a1-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 Scgb1a1 (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 hPLUNC cDNA (0.78 kb). The forward primer included the protein translational start codon and 12 nucleotides of flanking sequence (5′-ATA AGA AGG CGG CCT AAT AGC AAA GAT TTC AGG GCA ACG TGT GCC-3′), whereas the reverse primer covered the translational stop site (5′-ATA AGA AGG CGG CCT TCC GGT GAC GAC ACC CCT GGC ACC TCA-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 Scgb1a1 genomic fragment in pUC19 (kindly supplied by Dr. Magnus Nord, Karolinska Institutet, Stockholm, Sweden) was modified by site-directed mutagenesis to include an NcoI site upstream of the Scgb1a1 translational start site and resulted in generation of pNotI-9.Scgb1a1. In contrast with the short (2.1-kb) Scgb1a1 promoter, the 9.6-kb construct included 3′ and 2.5 kb of 5′ and 3′ gene-flanking sequence, respectively. The hPLUNC cDNA was cloned into the NotI site of pNotI-9.6Scgb1a1 and the construct verified by restriction endonuclease digestion and sequencing. The 10.38-kb Scgb1a1-hPLUNC cDNA fragment was isolated by isolation with SplI and transgenic generated by microinjection into FVB/N mouse oocytes. 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 Scgb1a1 gene and the Scgb1a1-hPLUNC TG were distinguished as 450- and 545-bp amplicons using a mouse Scgb1a1 promoter-specific forward primer (5′-GTT GGC AAG TCT ACA GTT GC-3′), a PLUNC coding region forward primer (5′-GAC GTC AGT GAT TCC TGG CC-3′), and in combination with a Scgb1a1 intron l-specific reverse primer (5′-GAA GAG GAC CCT GGG CAC TCA-3′).

**Animal householdry**

The Scgb1a1-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 microisolation cages housed in an American Association for Accreditation of Laboratory Animal Care-accredited facility. Protocols and studies involving animals were conducted in accordance with National Institutes of Health guidelines and approved by Institutional Animal Care and Use Committee at the University of Pittsburgh.

**Real-time PCR analysis**

Total RNA was isolated from various tissues of WT and transgenic mice by a single-step acid guanidinium thiocyanate extraction method (15). Quantitative RT-PCR (qRT-PCR) (ABI7700; Applied Biosystems, Foster City, CA) was performed using human-specific PLUNC primers (forward: 5′-TTC AGG GCA ACG TGT GCC-3′; reverse: 5′-TAG TCC GTG GAT CAG CAT GTT AAC A-3′; probe: 5′-56FAM-CTG GTC AAT GAG CAA AGG CAG-3′; or mouse-specific plunc primers (forward: 5′-TGC TAT CCT TGG CTT GGA CAG-3′; reverse: 5′-GAC GTC AGT GAT TCC TGG CC-3′). In combination with a Scgb1a1 intron l-specific reverse primer (5′-GAA GAG GAC CCT GG GAC TCA-3′), 9.6-kb amplified as follows: 95˚C for 12 min, 40 cycles; 95˚C for 15 s; 60˚C for 1 min). Three replicates were used to calculate the average cycle threshold for the transcript of interest and for a transcript for normalization (glyceraldehyde-3-phosphate dehydrogenase (GUSB); Assays on Demand; Applied Biosystems). Relative mRNA abundance was calculated by the ∆∆ cycle threshold (Ct) method.

**Gram-negative bacteria *P. aeruginosa***

The *P. aeruginosa* strain (PA01, ATCC BAA-47) was used for all experiments. *P. aeruginosa* obtained from a single colony was stored in aliquots at −80˚C in 20% glycerol/Luria-Bertani (LB) broth. For each experiment, an aliquot of bacteria was thawed, inoculated into 10 ml LB, and incubated (6; 37˚C) with shaking. An aliquot was then diluted 1:100 into 100 ml LB broth and incubated (16; 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 resuspended in PBS, and the OD (OD600) was adjusted to ~0.74, ~2 × 10^9 bacteria/ml. Five serial 10-fold dilutions in PBS were prepared immediately before use. Duplicate bacterial samples were mixed with 50 µl PBS, or bronchoalveolar lavage fluid (BALF) from either transgenic or WT control mice were prepared and placed on ice. An equal volume of the 2 × 10^9 bacteria/ml solution was added to the wells. Samples were mixed and incubated (2; 37˚C) with shaking. Three 50-µl 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 µl) were preincubated (1 h; 23˚C) with 7.5 µl normal rabbit serum or with anti-PLUNC serum with gentle agitation, and CFU were calculated.

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

Sex-matched 6- 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 de-position of ~2 × 10^6 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 × 10^9 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-µl 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 on 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).

**Bacterial clearance**

Bacterial deposition was determined by harvesting the right lungs immediately postinfection (n = 4 mice/group). Bacterial clearance was assayed 4 and 24 h postinfection (n = 5–6 mice/group). The right lungs or spleens were placed into 1 ml sterile PBS and kept on ice before homogenization. Six serial 10-fold dilutions were prepared and 50-µl aliquots placed on LB agar plates. Each dilution was plated in triplicate. Plates were inverted and incubated overnight at 37˚C. The number of viable bacteria in the lung and spleen was determined and expressed as CFU per lung.

**Immunohistochemistry**

Lungs were inflation fixed in situ with 4% paraformaldehyde (10 cm H2O; 24–h incubation) 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-µl 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 on 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).
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 $\pm$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 (PA01, 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). Pre-immune 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. PA01 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 $\mu$g 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 PA01-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-

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**FIGURE 1.** Generation and assessment of hPLUNC gene expression in Scgb1a1-PLUNC transgenic mice. A, TG construct. B, Real-time TaqMan RT-PCR analysis of hPLUNC mRNA abundance in tissues from WT and transgenic mice. Relative expression was determined by the ΔΔCt method using mouse GUS-B RNA as a control (mean ± SD; n = 10). hPLUNC was highly expressed in intrapulmonary airways of transgenic mice.

**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).

**FIGURE 3.** Expression and secretion of hPLUNC is significantly increased in Scgb1a1-PLUNC transgenic mice. A, Western blot analysis was used to assess protein expression of PLUNC in BAL and total lung homogenate from 6-wk-old WT and Scgb1a1-PLUNC transgenic (TG) mice. Proteins were separated by SDS-PAGE and blotted onto PVDF membranes. Blots were incubated with 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).

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**TABLE 1.** Expression and secretion of hPLUNC is significantly increased in Scgb1a1-PLUNC transgenic mice. A, Western blot analysis was used to assess protein expression of PLUNC in BAL and total lung homogenate from 6-wk-old WT and Scgb1a1-PLUNC transgenic (TG) mice. Proteins were separated by SDS-PAGE and blotted onto PVDF membranes. Blots were incubated with 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 clearance was ∼15-fold greater (*p < 0.05) in lung homogenates from Scgb1a1-hPLUNC transgenic mice (2.6 ± 0.29 × 10^7 CFU/lung/ml) as compared with that from WT control mice (3.9 ± 0.34 × 10^7 CFU/lung/ml) (Fig. 7A). Thus, control mice had 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.

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 (∼1.4-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

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.

FIGURE 5. Exposure to P. aeruginosa increases the mRNA expression of mPlunc. Murine Plunc expression was analyzed 6 and 24 h after aerosolized exposure of P. aeruginosa PAO1 strain to FVB/N WT mice, and all data are expressed as fold induction over saline-exposed mice for time point at 6 h postexposure. Levels of mPlunc were quantified in lung homogenates by real-time RT-PCR and determined by the ΔΔCt method using mouse GUS-B RNA as a control. Results are mean ± SEM from two experiments; n = 6–8 mice for each group. *p < 0.001 and **p < 0.05 when the PAO1-exposed mouse group is compared with the saline-exposed mouse group at 6 and 24 h postexposure.

FIGURE 6. Pretreatment with anti-PLUNC Ab increases susceptibility of WT mice to bacterial infection. WT FVB/N mice were intranasally instilled (10 and 25 µg/mouse) with control rabbit IgG or neutralizing mouse anti-PLUNC Ab (nAb) 2 h before exposure to ∼2 × 10^6 CFU/lung aerosolized P. aeruginosa (PAO1). The number of bacteria in lung homogenates was determined 24 h after bacterial exposure. Anti-PLUNC Ab treatment resulted in a significant increase in CFU counts compared with IgG control. Results are mean ± SEM from two experiments; n = 5 mice for each group. *p < 0.05 and **p < 0.05 when two different neutralization Ab concentrations were compared with their respective IgG control group.
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 littermates (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...
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, 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 \textit{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 \textit{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 significance 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 < 0.01 for WT to TG comparisons at each time point.

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 \textit{P. aeruginosa} when bacteria were coincubated with BALF from Scgb1a1-hPLUNC transgenic mice. Furthermore, both in vitro and

FIGURE 10. Overexpressed hPLUNC in Scgb1a1-PLUNC mice enhances the survival from PAO1-induced acute pneumonia. WT and TG mice were infected with \textit{P. aeruginosa} as described at a concentration of \(1 \times 10^9\) 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 levels similar to that of lysozyme in traheobronchial 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 overexpressed 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 proinflammatory 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.

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