

# Function and Regulation of SPLUNC1 Protein in *Mycoplasma* Infection and Allergic Inflammation<sup>1</sup>

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Respiratory infections, including *Mycoplasma pneumoniae* (Mp), contribute to asthma pathobiology. To date, the mechanisms underlying the increased susceptibility of asthmatics to airway Mp infection remain unclear. Short palate, lung, and nasal epithelium clone 1 (SPLUNC1) protein is a recently described large airway epithelial cell-derived molecule that was predicted to exert host defense activities. However, SPLUNC1 function and regulation in an infectious or allergic milieu are still unknown. We determined host defense and anti-inflammatory functions of SPLUNC1 protein in Mp infection and the regulation of SPLUNC1 by Mp and allergic inflammation (e.g., IL-13). SPLUNC1 function was examined in Mp or human airway epithelial cell cultures by using SPLUNC1 recombinant protein, overexpression and RNA interference. Human and mouse bronchial epithelial SPLUNC1 was examined using immunostaining, Western blotting, ELISA, laser capture microdissection, and real-time PCR. Mouse models of Mp infection and allergic inflammation and air-liquid interface cultures of normal human primary bronchial epithelial cells were used to study SPLUNC1 regulation by Mp and IL-13. We found that: 1) SPLUNC1 protein decreased Mp levels and inhibited epithelial IL-8 production induced by Mp-derived lipoproteins; 2) normal human and mouse large airway epithelial cells expressed high levels of SPLUNC1; and 3) although Mp infection increased SPLUNC1, IL-13 significantly decreased SPLUNC1 expression and Mp clearance. Our results suggest that SPLUNC1 serves as a novel host defense protein against Mp and that an allergic setting markedly reduces SPLUNC1 expression, which may in part contribute to the persistent nature of bacterial infections in allergic airways. *The Journal of Immunology*, 2007, 179: 3995–4002.

**A** typical bacterial infections in the airways, such as those caused by *Mycoplasma pneumoniae* (Mp),<sup>3</sup> can be involved in the pathogenesis of chronic lung diseases including asthma (1–4). Our clinical studies have demonstrated that nearly 40% of chronic stable asthmatics are positive for Mp (1). Only those asthmatics positive for Mp who were treated with the macrolide antibiotic clarithromycin showed improved lung function, further supporting the involvement of this pathogen in asthma (5).

The mechanisms by which asthmatics are susceptible to infectious pathogens including Mp remain unclear. Airway epithelial cells represent the first line of host defense against pathogens by producing a large array of mediators. Palate, lung, and nasal epi-

thelium clone (PLUNC) proteins are a recently described family of proteins that have been predicted to exert host defense functions (6). Among the 10 PLUNC proteins described so far, short PLUNC1 (SPLUNC1) has been localized to the large airway epithelium in humans and mice (6). In cultured human primary airway epithelial cells, SPLUNC1 represents up to 10% of total proteins in the epithelial lining fluid (7). Although these studies suggest a pivotal role for SPLUNC1 protein in airway epithelial biology, the functions of SPLUNC1 protein in host defense against bacterial infections have not been determined. Given the interactions of allergic inflammation with infection and our observation that asthmatics are susceptible to Mp infections, a better understanding of the role of SPLUNC1 in host defense is imperative. In the present study, we hypothesize that SPLUNC1 protein exerts host defense and anti-inflammatory properties against Mp and that allergic inflammation including Th2 cytokines such as IL-13 would decrease SPLUNC1 production, leading to a persistent Mp infection. Our hypothesis was tested in a murine model of allergic asthma and in an air-liquid interface culture system of human primary bronchial epithelial cells. Our results suggest that: 1) SPLUNC1 protein exerts host defense (e.g., inhibition of Mp growth) and anti-inflammatory (e.g., IL-8 production) properties; 2) normal human and mouse large airway epithelial cells express high levels of SPLUNC1; and 3) although Mp infection increases SPLUNC1 expression, allergic inflammation including IL-13 decreases SPLUNC1 expression and Mp clearance.

## Materials and Methods

### Generation of recombinant mouse SPLUNC1 (mSPLUNC1) protein

The cDNA fragment encoding mature mSPLUNC1 (258 aa) was amplified by PCR and ligated to pGEX-4T-2 (Amersham Biosciences). The final

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<sup>3</sup> Abbreviations used in this paper: Mp, *Mycoplasma pneumoniae*; ALI, air-liquid interface; BAL, bronchoalveolar lavage; BPI, bactericidal/permeability increasing (protein); ffu, focus-forming unit; hSPLUNC1, human SPLUNC1; LCM, laser capture microdissection; mSPLUNC1, mouse SPLUNC1; Pam3CSK4, (S)-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys<sub>4</sub>-OH, 3HCl; PLUNC, palate, lung, and nasal epithelium clone; PLO, pleuropneumonia-like organism; shRNA, short hairpin RNA; SPLUNC1, short PLUNC1; VSV-G, vesicular stomatitis virus envelope glycoprotein.

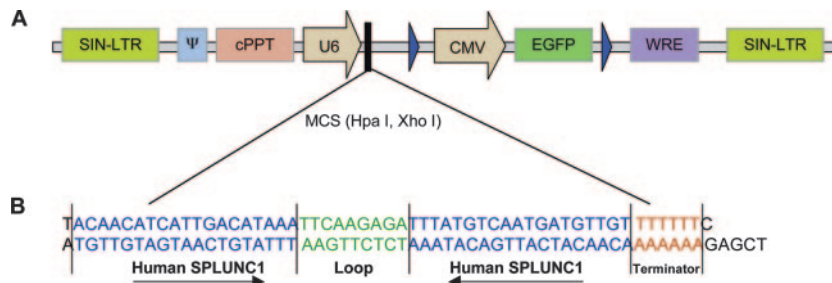


**FIGURE 1.** Schematic representation of pLL3.7 and an insertion of an oligonucleotide encoding hSPLUNC1 shRNA. **A**, Structure of pLL3.7. SIN-LTR, self-inactivating long terminal repeat;  $\psi$ , HIV packaging signal; cPPT, central polypurine track; U6, U6 (RNA polymerase III) promoter; MCS, multiple cloning site such as *HpaI* and *XhoI*; CMV, cytomegalovirus (RNA polymerase II) promoter; EGFP, enhanced GFP; WRE, woodchuck hepatitis virus response element. **B**, Oligonucleotide that encodes an shRNA that targets the expression of hSPLUNC1.

clones were verified by restriction digestion and DNA sequencing. The recombinant plasmid containing the SPLUNC1 gene was transformed to Rosetta (DE3) cells (Novagen). The expression of GST-SPLUNC1 was induced by adding isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) to 8 liters of growing culture (37°C) at an OD<sub>600</sub> of 0.8. After 4 h of additional growth, cells were harvested and resuspended in buffer A (20 mM Tris-HCl (pH 7.2), 500 mM NaCl, and 1 mM DTT) and protease inhibitors (Invitrogen Life Technologies). After cell lysis through a continuous flow French press and a low-speed spin, the soluble fraction was loaded onto GST affinity beads. After an intensive wash with buffer A, thrombin was added for 24 h of incubation. Most of SPLUNC1 was precipitated after thrombin cutting. The remaining soluble SPLUNC1 protein was further purified by a combination of GST beads and a Superdex-200 column (8, 9). The SPLUNC1 protein sequence was verified by N-terminal sequencing.

#### Mp preparation

Mp (strain FH; American Type Culture Collection 15531) was grown in SP-4 broth for 5 days at 37°C. The adherent Mp was harvested, spun, resuspended in PBS with 20% FBS, and frozen at -80°C in aliquots that were subsequently used to infect epithelial cells and different groups of mice in a consistent manner (10, 11). On the infection day, frozen Mp aliquots were thawed, spun, resuspended in SP-4 broth, and incubated for 2 h at 37°C. Mp was then spun at 6,000 rpm for 5 min and resuspended in sterile saline to yield the indicated concentrations for infecting cells or mice.

#### Host defense activity of recombinant mSPLUNC1 protein

We tested the host defense activity of SPLUNC1 protein by incubating Mp ( $4 \times 10^4$  CFU/ml) with recombinant mSPLUNC1 protein (1–50  $\mu$ g/ml; within the range of SPLUNC1 protein levels in mouse bronchoalveolar lavage (BAL) fluid) in 96-well plates (100  $\mu$ l of SP-4 broth per well) for 2 h, a typical time for bactericidal assay in Gram-negative bacteria (12). Mp in the supernatants was then plated on pleuropneumonia-like organism (PPLO) agar plates and incubated at 37°C with 5% CO<sub>2</sub> for a week to quantify Mp.

#### Effects of recombinant mSPLUNC1 protein on airway epithelial IL-8 production induced by Mp-derived lipoproteins

Our previous studies have shown that Mp-derived lipoproteins increased epithelial production of mucins (13). Further, in a pilot study we found that Mp-derived proteins (0.1, 0.5, and 2.0  $\mu$ g/ml) increased IL-8 production in NCI-H292 cells (a human airway epithelial cell line) in a dose-dependent manner at 48 h posttreatment. Moreover, we similarly performed a SPLUNC1 dose (0.1, 0.5, and 2  $\mu$ g/ml) response to determine an optimal SPLUNC1 protein dose in inhibiting IL-8 production that was induced by lipoproteins (2  $\mu$ g/ml). It was found that SPLUNC1 at 2  $\mu$ g/ml showed a maximal inhibition of IL-8 production in response to the lipoproteins (J. G. Rino, unpublished data). To test whether SPLUNC1 protein exerts any anti-inflammatory effects on Mp-derived lipoproteins, NCI-H292 in 24-well tissue culture plates ( $2 \times 10^4$  cells/well in 300  $\mu$ l of serum-free RPMI 1640) were cultured overnight. The next day, recombinant mSPLUNC1 protein (2  $\mu$ g/ml) or BSA (2  $\mu$ g/ml; irrelevant protein control) was added into NCI-H292 cells simultaneously treated with PBS (control) or Mp-derived lipoproteins (2  $\mu$ g/ml) or (S)-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys<sub>4</sub>-OH, 3HCl (Pam3CSK4) (0.2  $\mu$ g/ml; a synthetic TLR2 ligand from InvivoGen) as a TLR2 agonist con-

trol. After 48 h of culture, supernatants were collected for IL-8 protein measurement by using an IL-8 ELISA kit (R&D Systems).

#### Overexpression of human SPLUNC1 (hSPLUNC1) protein in airway epithelial cells

To confirm our findings using the recombinant mSPLUNC1 protein, we overexpressed hSPLUNC1 protein in NCI-H292 cells under the immersed culture condition. Using Lipofectamine 2000, cells in 24-well culture plates were transfected with a full-length hSPLUNC1 cDNA construct (generated by PCR and confirmed by DNA sequencing) in a pcDNA3.1 vector (0.3  $\mu$ g/well) or an empty pcDNA3.1 vector (0.3  $\mu$ g/well) (Invitrogen Life Technologies). One day after the transfection, cells were incubated in the absence or presence of Mp (10 CFU/cell) for two additional days. Mp in the supernatants was seeded on PPLO plates for quantitative culture. Secreted SPLUNC1 protein in the supernatants was measured using a direct ELISA. Cell lysates were analyzed for intracellular SPLUNC1 protein using Western blotting.

#### The effects of hSPLUNC1 RNA interference on Mp growth and IL-8 production in airway epithelial cells

A vesicular stomatitis virus envelope glycoprotein (VSV-G) pseudotyping approach was used to transduce SPLUNC1 short hairpin RNA (shRNA) encoded in a lentiviral vector (pLL3.7;  $10^7$  focus-forming units (ffu)/ml) to NCI-H292 cells. Antiparallel pairs of human SPLUNC1 oligonucleotides were ordered from IDT Laboratories and SPLUNC1 shRNA encoded in pLL3.7 was generated as previously reported (14–16) (Fig. 1). Briefly, NCI-H292 cells were cultured in 6-well culture plates ( $2 \times 10^5$  cells/well) under the immersed condition until ~60% when they were transduced with either pLL3.7-shSPLUNC1 (50 ffu/cell) or pLL3.7-firefly luciferase (an irrelevant control; 50 ffu/cell) once daily for three consecutive days. Forty-eight hours after the last transduction, cells from each condition were collected for SPLUNC1 Western blotting to verify SPLUNC1 protein knock-down. The remaining cells were treated with or without Mp at 10 CFU/cell for 48 h, and supernatants were collected to quantify Mp and IL-8 protein secretion.

#### Mouse model of Mp infection

All experimental animals used in this study were covered by a protocol approved by our Institutional Animal Care and Use Committee. On day 0, female BALB/c mice (8 wk of age) were treated with either Mp or saline (control). Before Mp or saline, all mice were i.p. anesthetized with Avertin (ethanol) at 0.25 g/kg. Mice in the infected group were inoculated intranasally with 50  $\mu$ l of Mp at  $\sim 1 \times 10^8$  CFU. A 50- $\mu$ l inoculation of saline was similarly given to the mice in the control groups. Mice were sacrificed 4 and 72 h after Mp or saline treatment. Lungs were frozen for laser capture microdissection (LCM) procedure.

To examine the in vivo effects of SPLUNC1 on lung Mp clearance and inflammation, we performed a SPLUNC1 blocking experiment in female BALB/c mice using an anti-mouse SPLUNC1 Ab (University of Texas Southwestern Medical Center, Dallas, TX). In a pilot study, SPLUNC1 Ab at various doses (1, 5, and 25  $\mu$ g/mouse) was tested for its effects on lung Mp clearance at day 3 postinfection. It was found that SPLUNC1 Ab at 5 and 25  $\mu$ g/mouse similarly delayed Mp clearance compared with the control mice (R. Breed, unpublished data). The specificity and neutralizing activity of the anti-mouse SPLUNC1 Ab were verified by demonstrating its specific binding to native SPLUNC1 protein in naive BALB/c mouse BAL

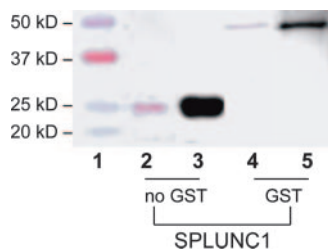


**FIGURE 2.** Collated images showing purity and specificity of recombinant mouse SPLUNC1 protein by using Ponceau S staining (lanes 2 and 4) and Western blot (lanes 3 and 5). Lane 1, Protein standard, Ponceau S staining; lanes 2 and 3, SPLUNC1 protein without GST; lanes 4 and 5, GST-SPLUNC1 fusion protein.

fluid using Western blotting and immunoprecipitation and by revealing the reduced activity (79% inhibition) of recombinant mouse SPLUNC1 protein pretreated with the anti-mouse SPLUNC1 Ab in inhibiting Mp growth. Three groups of mice were studied: group 1, saline (control); group 2, SPLUNC1 Ab in the form of mouse whole serum (5  $\mu$ g/mouse) Mp ( $10^8$  CFU/mouse); and group 3: nonimmunized normal mouse serum (control, 5  $\mu$ g/mouse) plus Mp ( $10^8$  CFU/mouse). SPLUNC1 Ab or control serum was given intranasally to mice 2 h before Mp infection and was repeated once daily on days 1 and 2 after Mp treatment. On day 3 after Mp or saline treatment, BAL fluid was collected for Mp culture and neutrophil counts.

#### Mouse model of allergic airway inflammation

We used a well-established mouse model of allergic airway inflammation (17, 18) to study the effects of an *in vivo* allergic milieu on SPLUNC1 expression and lung tissue Mp clearance. Female BALB/c mice (8 wk of age) were *i.p.* sensitized twice in a span of 14 days (day 1 and 14) by injection of 20  $\mu$ g of OVA emulsified in 2.25 mg of aluminum hydroxide (AlmImject; Pierce). Fourteen days after the last OVA sensitization, mice were placed in a Plexiglas chamber and challenged once daily for three consecutive days with 1% aerosolized OVA for 30 min using an ultrasonic nebulizer (DeVilbiss). Two days after the last OVA challenge, mice were either sacrificed for SPLUNC1 detection using LCM or intranasally inoculated with 50  $\mu$ l of saline (control) or Mp at  $10^8$  CFU/mouse. Additional controls included OVA naive mice with saline or Mp inoculation. Seven days after saline or Mp treatment, mice were sacrificed and lungs were homogenized for Mp culture and CFU counting.

To determine whether the Th2 cytokines IL-13 and IL-4 are contributing factors in SPLUNC1 modulation under the allergic milieu, we performed an IL-13 and IL-4 neutralization experiment in OVA-challenged mice. IL-4 and IL-13 neutralizing Abs (each at 10  $\mu$ g/mouse; R&D Systems) or isotype control (goat IgG; 20  $\mu$ g/mouse) were intranasally inoculated to BALB/c mice 2 h after each OVA challenge and once more a day before Mp ( $10^8$  CFU/mouse) infection. On day 7 after Mp treatment, lungs were processed for SPLUNC1 mRNA detection and Mp culture.

#### Laser capture microdissection

LCM was performed to capture mouse airway epithelial cells for detection of SPLUNC1 mRNA expression. Mouse lungs were frozen at optimal cutting temperature and stored at  $-80^{\circ}\text{C}$  for the preparation of frozen sections. Frozen sections at a thickness of 6  $\mu$ m were stained with HistoGene (Arcturus), and LCM was performed using the Arcturus Veritas microdissection system. Large airway (diameter  $\geq 300$   $\mu$ m) epithelial cells were captured onto Macro LCM caps that were then placed into a tube and incubated with extraction buffer for subsequent total RNA extraction using the PicoPure RNA isolation kit (Arcturus) (19). The extracted RNA was used for quantitative real-time RT-PCR of SPLUNC1 mRNA.

#### Air-liquid interface (ALI) culture of normal human primary bronchial epithelial cells

Normal human primary bronchial epithelial cells at passage one were purchased from Cambrex to perform ALI culture to determine the effects of the Th2 cytokine IL-13 on SPLUNC1 expression and Mp clearance. It is noteworthy that ALI culture is the best cell culture system that mimics *in vivo* airway epithelial cell biology. ALI culture was performed by plating the expanded second passage of epithelial cells onto collagen-coated 12-well Transwell plates at  $4 \times 10^4$  cells/cm<sup>2</sup> as previously reported (20, 21). After a week in the immersed culture condition, epithelial cells reached

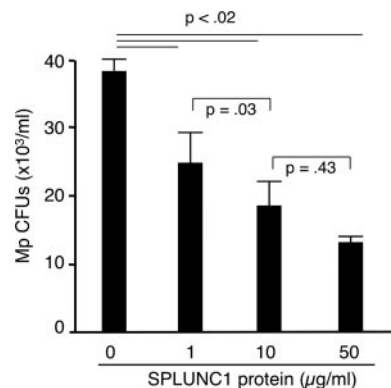


**FIGURE 3.** A 2-h incubation of recombinant mouse SPLUNC1 protein with Mp markedly reduced levels of bacterial growth in 96-well culture plates. Data are expressed as means  $\pm$  SEM from five independent replicates.

100% confluence and were shifted to an ALI condition by reducing the apical medium volume to 50  $\mu$ l. From day 0 of ALI, cells were stimulated in triplicate every 48 h with the addition of IL-13 (10 ng/ml) or PBS (as a negative control) into the apical surface (50  $\mu$ l) and the lower chamber (1.2 ml) for 10 days. ALI conditions were maintained for 10 days because previous studies have demonstrated that culture for 10 days is required for the mucociliary differentiation of human airway epithelial cells. The rationale for us to stimulate cells with IL-13 at 10 ng/ml is that our previous dose-response study has shown that this dose is optimal to reduce prostaglandin E synthase, an enzyme involved in anti-inflammatory cascades (21).

On day 10 of ALI culture, cells in some Transwell plates were collected for the determination of SPLUNC1 mRNA by using real-time RT-PCR. The apical surface of bronchial epithelial cells was rinsed with 200  $\mu$ l of sterile PBS that was then used for the measurement of secreted SPLUNC1 protein by using the ELISA. Cells in the remainder of the Transwell plates were then treated with Mp (10 CFU/cell) at the apical surface in the continuous presence or absence of IL-13 in antibiotic-free culture medium for 7 days. On day 7 after Mp treatment, the apical surface of bronchial epithelial cells was rinsed with 200  $\mu$ l of sterile PBS that was then serially diluted with Mp culture medium (SP4 broth) and plated on PPLO plates for Mp culture and CFU counting.

#### Quantitative real-time RT-PCR

Levels of human and mouse SPLUNC1 mRNA in epithelial cells and lung tissues were determined by reverse transcription followed by real-time quantitative PCR. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). Reverse transcription was performed using 1  $\mu$ g of total RNA and random hexamers in a 50- $\mu$ l reaction (Applied Biosystems). Primers and probes for mouse (GenBank accession no. NM\_011126) and human (GenBank accession no. NM\_016583) SPLUNC1 genes were designed using Primer Express software (Applied Biosystems). The primers were: mSPLUNC1, 5'-GGTCTTGTGACAGATCCTGATG-3' (forward), 5'-CTACGGGCATATTTACGTTGAGTGT-3' (reverse), and 5'-CGTCTCTATGTCCACCATCCCTCTGGGC-3' (probe); and hSPLUNC1: 5'-GGGCCTGTTGGGCATTCT-3' (forward), 5'-CCTCCTCCAGGCTTCAGAT-3' (reverse), and 5'-AAACCTTCGCTCCTGGA-3' (probe).

PCR was performed on the ABI Prism 7700 sequence detection system. The 25- $\mu$ l PCR contained 30 ng of cDNA, 100 nM fluorogenic probe, and 200 nM primers and other components from the TaqMan RT-PCR kit. The housekeeping gene *GAPDH* (human) or 18S rRNA (mouse) was also evaluated. The comparative threshold cycle ( $C_T$ ) method was used to determine the relative gene expression levels (22).

#### Western blot analysis

Mouse lung tissues or airway epithelial cells were homogenized in Western lysis buffer with protease inhibitors. Twenty micrograms of protein lysate was electrophoresed on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, blocked with 5% nonfat milk in Tris buffer (pH 7.6) with 0.1% Tween 20, and then incubated with a primary Ab (SPLUNC1 or  $\beta$ -actin) overnight at  $4^{\circ}\text{C}$ . After washes, the membranes were incubated with anti-IgG Ab conjugated with HRP and detected by

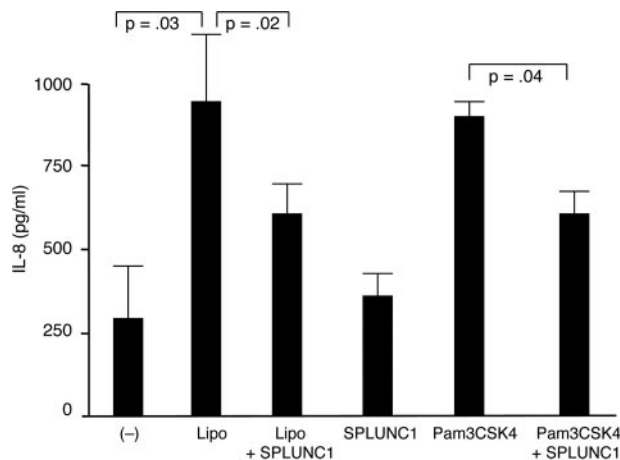


**FIGURE 4.** Recombinant mouse SPLUNC1 protein inhibited human bronchial epithelial cell (NCI-H292 cell line) IL-8 production that was induced by Mp-derived lipoproteins (Lipo) or a synthetic TLR2 ligand Pam3CSK4. Data are expressed as mean  $\pm$  SEM from five independent replicates.

using the chemiluminescence system (23). Densitometry was then performed to quantify SPLUNC1 protein in relation to  $\beta$ -actin.

#### Direct ELISA of SPLUNC1 protein

To measure the secretion levels of SPLUNC1 protein, we developed a direct SPLUNC1 ELISA. Because a high homology ( $\sim 80\%$ ) of SPLUNC1 protein has been identified between mice and humans (24) and we also found cross-reactivity to mouse recombinant SPLUNC1 protein by both human and mouse SPLUNC1 mAbs, we used recombinant mSPLUNC1 protein as a standard of the ELISA. Recombinant mSPLUNC1 protein, supernatants of cultured epithelial cells, or mouse BAL fluid were coated onto a 96-well Immulon 2HB plate followed by incubations with anti-SPLUNC1 Ab (1  $\mu$ g/ml; R&D Systems), biotinylated anti-mouse Ab, and an avidin-biotin peroxidase complex. The plate was developed using a peroxidase substrate (tetramethylbenzidine) and read on a plate reader. The  $R^2$  of standard curves was between 0.94 and 0.99.

#### Immunohistochemistry

Immunohistochemistry was performed as previously described (25) to localize airway epithelial SPLUNC1 protein in lung tissues. Briefly, 4% paraformaldehyde-fixed and paraffin-embedded lungs were dewaxed, hydrated, and then incubated with mAb (1/50 dilution) against human SPLUNC1 protein. After an overnight incubation at 4°C with SPLUNC1 Ab, lung tissue sections were washed and then incubated with biotinylated anti-mouse Ab followed by an avidin-biotin-peroxidase complex. Thereafter, 0.03% aminoethylcarbazole in 0.03%  $H_2O_2$  was used to develop a peroxide-dependent red color reaction.

#### Statistical analysis

If the data were normally distributed, they were presented as means  $\pm$  SEM and compared between the groups using ANOVA. When the data were not normally distributed, the data were expressed as medians with interquartile (25–75%) ranges and the comparisons between the groups were performed using the Wilcoxon rank-sum test. Paired  $t$  tests were performed to analyze cell culture results under different conditions such as medium alone vs IL-13 stimulation. A two-tailed  $p$  value of  $<0.05$  was considered statistically significant.

## Results

### Host defense and anti-inflammatory properties of SPLUNC1 protein

At the cDNA and amino acid levels, mouse and human SPLUNC1 shares  $\sim 80\%$  of identity (25). Thus, it is predicted that SPLUNC1 proteins from the two species may have similar functions. The purity and specificity of recombinant mSPLUNC1 protein used in the current study were verified by Western blot analysis using the anti-mouse as well as anti-human SPLUNC1 Abs (Fig. 2).

Experiments aimed at revealing the functions of mouse and human SPLUNC1 proteins yielded the following results.

**Recombinant mSPLUNC1 protein inhibits Mp growth.** A 2-h incubation of recombinant mSPLUNC1 protein at various concentrations significantly reduced Mp growth in a dose-dependent manner (Fig. 3). Our data thus suggest the host defense activity of SPLUNC1 protein.

**Recombinant mSPLUNC1 protein reduces IL-8 production by human airway epithelial cells.** Having shown the host defense activity of SPLUNC1 protein, we next examined whether SPLUNC1 protein could directly inhibit airway epithelial cell IL-8 production induced by Mp-derived lipoproteins. IL-8 has been shown to be an important mediator in host innate immunity, and previous studies have demonstrated the induction of IL-8 by Mp in cultured lung epithelial cells (26). Fig. 4 shows that Mp-derived lipoproteins increased the epithelial production of IL-8, which was significantly reduced by mSPLUNC1 protein. Additionally, SPLUNC1 inhibited IL-8 production that was induced by Pam3CSK4, a synthetic TLR2 ligand. SPLUNC1 protein alone had no effects on IL-8 production. Therefore, our results suggest that SPLUNC1 protein uses a non-host defense mechanism to suppress an inflammatory response to Mp-derived lipoproteins or a TLR2 ligand.

**Overexpression of human SPLUNC1 protein decreases Mp growth on human airway epithelial cells.** Although NCI-H292 cells express SPLUNC1, their SPLUNC1 levels are less ( $\sim 32$ -fold

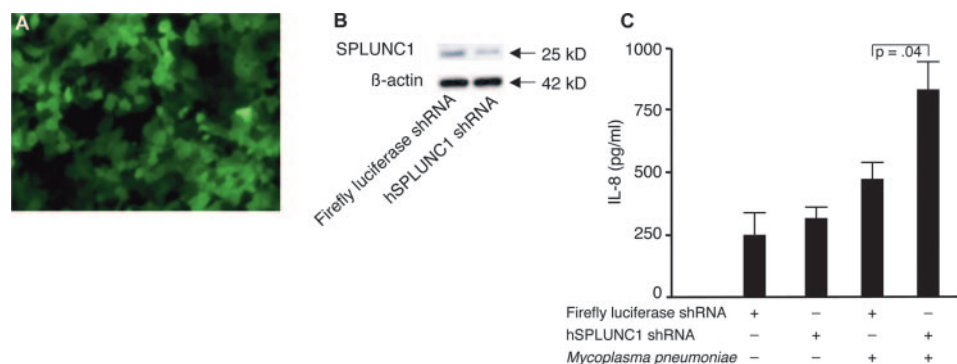


**FIGURE 5.** RNA interference of SPLUNC1 in NCI-H292 cells using the VSV-G pseudotyping approach to transduce hSPLUNC1 shRNA encoded in a lentiviral vector (pLL3.7). *A*, hSPLUNC1 shRNA in the pLL3.7-GFP vector were successfully transduced into NCI-H292 cells. The green cells indicate the GFP-positive cells. *B*, SPLUNC1 protein was reduced in hSPLUNC1 shRNA-transduced NCI-H292 cells as compared with firefly luciferase shRNA-transduced cells. *C*, Transduction of hSPLUNC1 shRNA enhanced Mp-induced IL-8 production by NCI-H292 cells. Data are expressed as mean  $\pm$  SEM from five independent replicates.

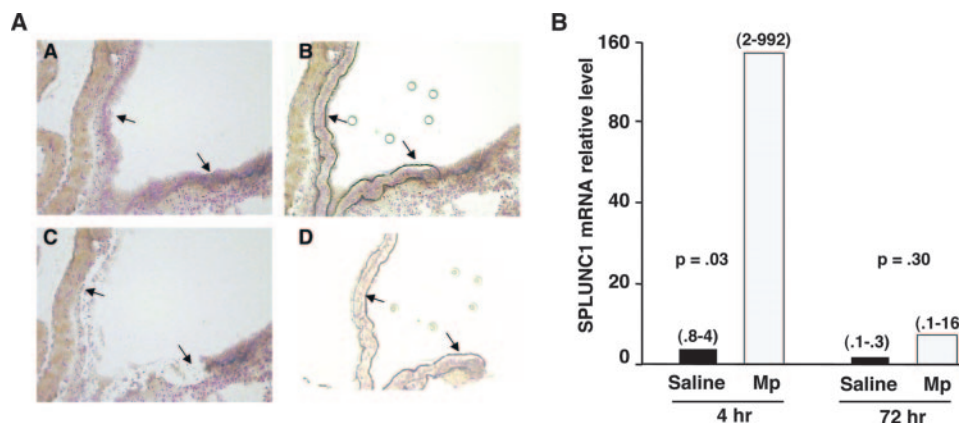


**FIGURE 6.** Mp infection in mice increases SPLUNC1 expression by airway epithelial cells. *A*, An example of LCM procedure in a mouse lung tissue frozen section. *Section B*, Airway epithelium was selected with laser fired on the Macro cap. *Section C*, Airway epithelial cells removed from the tissue after LCM. *Section D*, Epithelial cells captured on the cap ready for total RNA extraction. *B*, Mouse large airway epithelial cells were microdissected using the LCM and processed for the RNA extraction. Mp significantly increased SPLUNC1 mRNA expression at 4 h, but not 72 h, after the infection;  $n = 6$  mice/group. Data are expressed as medians (25–75% range).

lower at the mRNA level) than those by normal human primary bronchial epithelial cells under the ALI culture. Thus, we overexpressed SPLUNC1 protein in NCI-H292 cells by transfecting the cells with a plasmid vector (pcDNA3.1) containing full-length hSPLUNC1 cDNA to examine the effects of human SPLUNC1 protein on Mp growth. First, hSPLUNC1 cDNA transfection at 24 h led to an increase in secreted SPLUNC1 protein levels in hSPLUNC1 cDNA-transfected cells as compared with control cells ( $1325$  ( $568$ – $2038$ ) ng/ml vs  $130$  ( $0$ – $402$ ) ng/ml,  $p = 0.03$ ;  $n = 4$ ). Second, after 48 h of Mp infection, Mp levels in the supernatants of hSPLUNC1 cDNA-transfected cells were lower ( $45.6 \pm 2.7\%$  reduction,  $p = 0.01$ ) than those in control cells.

**SPLUNC1 RNA interference enhances Mp growth and IL-8 production in human airway epithelial cells.** To further reveal a role of SPLUNC1 protein in Mp growth and inflammatory cytokine production, we attempted to knock down SPLUNC1 expression by using a VSV-G pseudotyping approach to transduce hSPLUNC1 shRNA encoded in a lentiviral vector (pLL3.7) to NCI-H292 epithelial cells. At 48 h after transduction, based on GFP expression by NCI-H292 cells under the confocal microscope,  $73 \pm 8\%$  cells were transduced with the SPLUNC1 shRNA (Fig. 5A). SPLUNC1 protein in pLL3.7-shSPLUNC1-transduced cells (Fig. 5B), as evaluated by Western blot analysis and densitometry, was significantly suppressed ( $55 \pm 4\%$  reduction vs control cells,  $p = 0.04$ ;  $n = 5$ ). After 48 h of Mp infection, levels of Mp in the supernatants of pLL3.7-shSPLUNC1-transduced cells were higher ( $3.1 \pm 0.9$ -fold,  $p = 0.02$ ) than those in control cells. Moreover, with Mp infection, supernatant IL-8 protein levels were higher in pLL3.7-shSPLUNC1-transduced cells than in control cells (Fig. 5C). These results suggest that a reduction of epithelial SPLUNC1 expression could lead to an impaired Mp clearance coupled with an enhanced production of the proinflammatory cytokine IL-8.

#### SPLUNC1 expression in normal large airway epithelial cells

Similarly as in previous studies (7), SPLUNC1 protein was localized on human large airway surface epithelial cells and submucosal glandular cells. SPLUNC1 protein was also observed on mouse large airway epithelial cells. Because mice have fewer submucosal glands in the airways than humans, large airway epithelial cells are the major source of SPLUNC1 protein. SPLUNC1 protein was also detectable in BAL fluid of naive adult (8-wk old) BALB/c mice at a high level ( $4.3 \pm 0.7$   $\mu$ g/ml using the ELISA and  $3.1 \pm 0.6$   $\mu$ g/ml using the Western blot analysis;  $n = 5$ ).

SPLUNC1 mRNA was easily detectable in cultured (ALI culture) normal human primary bronchial epithelial cells. It was noted that levels of SPLUNC1 expression was dependent on the degree of cell differentiation. SPLUNC1 mRNA levels from cells harvested at day 17 of ALI culture were  $\sim 2$ -fold higher than those obtained at day 10 ( $p = 0.04$ ;  $n = 5$ ), which was also reflected at the protein levels in the apical supernatants (SPLUNC1 protein levels: day 17 at  $710.0 \pm 195.0$   $\mu$ g/ml, vs day 10 at  $264.5 \pm 5.5$   $\mu$ g/ml,  $p = 0.008$ ).

#### Mp infection increases SPLUNC1 expression in mice

Mp infection at 4 h, but not 72 h, significantly increased mouse large airway epithelial SPLUNC1 mRNA expression as analyzed by LCM and RT-PCR (Fig. 6, A and B).

To define the role of an up-regulated SPLUNC1 expression by Mp infection, we performed a SPLUNC1 neutralization experiment in Mp-infected mice. As illustrated in Fig. 7, SPLUNC1 neutralizing Ab treatment in Mp-infected mice led to an increased Mp burden and neutrophil count in BAL fluid. Our data indicate that in vivo blockade of SPLUNC1 protein in Mp-infected mice significantly impairs lung innate defense against bacterial infection accompanied by more inflammatory cell recruitment into the lung.

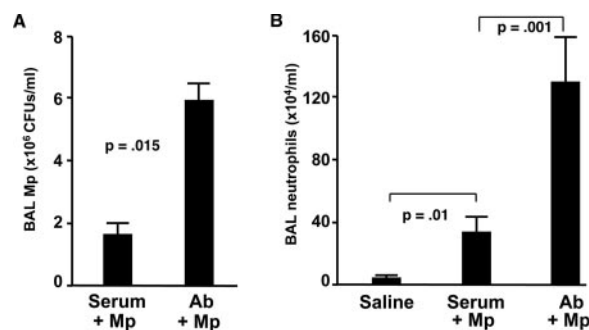


**FIGURE 7.** In Mp-infected mice intranasal mouse monoclonal SPLUNC1 Ab treatment increased Mp (*A*) and neutrophils (*B*) in BAL fluid at day 3 postinfection as compared with the control (normal mouse serum treatment);  $n = 5$ – $7$  mice/group. Data are expressed as means  $\pm$  SEM.

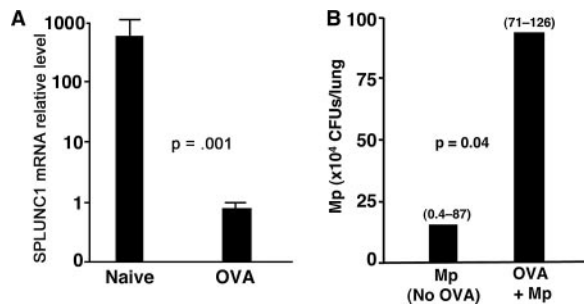


**FIGURE 8.** In BALB/c mice, allergic inflammation reduced SPLUNC1 expression but increased lung tissue Mp load. *A*, Large airway epithelial cells from OVA-naïve or OVA-challenged mice were microdissected using LCM and processed for RNA extraction. SPLUNC1 mRNA was significantly reduced in OVA-challenged mice (day 2 after the last OVA challenge) compared with naïve ones;  $n = 4$  mice/group. Data are expressed as means  $\pm$  SEM. *B*, Lung tissue Mp load in OVA-challenged and Mp-infected mice was markedly higher than that in Mp-infected mice alone (no OVA). In OVA-challenged mice, Mp was intranasally inoculated at day 2 after the last OVA challenge and then sacrificed at day 7 after Mp treatment (day 9 after OVA challenge). OVA-naïve mice received an intranasal Mp inoculation at day 0 and then sacrificed at day 7 after Mp treatment;  $n = 6$  mice/group. Data are expressed as medians (25–75% range).

#### *An allergic milieu decreases SPLUNC1 expression and Mp clearance in mouse lungs*

In mice with allergic airway inflammation, SPLUNC1 mRNA expression by isolated large airway epithelial cells using the LCM approach was markedly reduced ( $\sim 800$ -fold) at day 2 after the last OVA challenge (Fig. 8A). Interestingly, lung tissue Mp load in OVA-challenged and Mp-infected mice was significantly higher on day 7 postinfection than that in OVA-naïve mice infected with Mp (Fig. 8B).

Administration of IL-13 and IL-4 neutralizing Abs in allergic mice increased lung tissue SPLUNC1 mRNA expression (2.7-fold increase,  $p = 0.04$ ) but decreased lung Mp levels (6.3-fold reduction,  $p = 0.002$ ). These data suggest that Th2 cytokines contributed to impaired SPLUNC1 expression and Mp clearance in an allergic setting.

#### *Th2 cytokine IL-13 decreases SPLUNC1 expression and Mp clearance in cultured human airway epithelial cells*

To examine the direct effects of an allergic milieu (e.g., IL-13) on SPLUNC1 expression and Mp clearance from airway epithelium, Mp was inoculated onto the apical surface of normal human primary bronchial epithelial cells under the ALI conditions with or

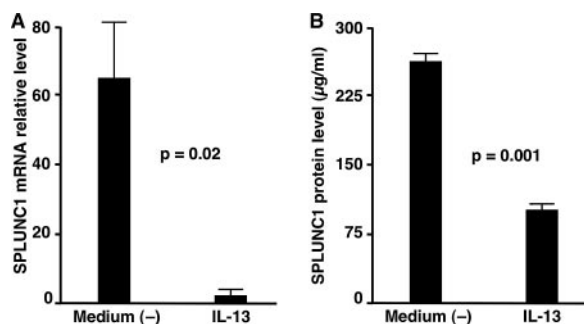


**FIGURE 9.** A 10-day IL-13 (10 ng/ml) treatment in normal human bronchial epithelial cells significantly reduced the levels of SPLUNC1 mRNA (*A*) and protein secretion (*B*) at the apical surface of cells under the ALI culture conditions. Data are expressed as mean  $\pm$  SEM from five independent replicates.

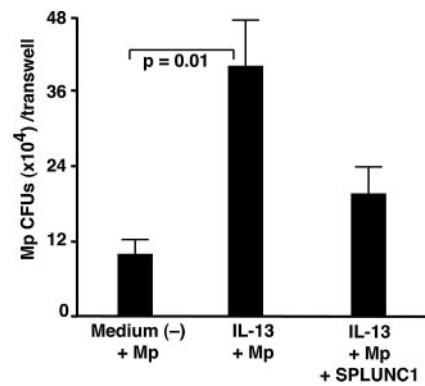


**FIGURE 10.** In Mp-infected normal human bronchial epithelial cells under the ALI culture conditions, IL-13 treatment significantly increased the levels of Mp at the apical surface. Recombinant mouse SPLUNC1 protein in part reduced the Mp levels. Data are expressed as mean  $\pm$  SEM from five independent replicates.

without IL-13 stimulation. As shown in Fig. 9, *A* and *B*, 10 days of IL-13 treatment significantly reduced SPLUNC1 mRNA expression as well as protein secretion into the apical surface. At day 7 postinfection, Mp load in the apical supernatants of IL-13-treated cells was significantly higher than that of Mp-infected cells without IL-13 (Fig. 10). We also tested whether exogenous mSPLUNC1 protein would restore an impaired Mp clearance in IL-13-treated cells. mSPLUNC1 protein was added to Mp-infected and IL-13-treated cells every other day after Mp infection at 150  $\mu$ g/ml (or 7.5  $\mu$ g per transwell in 50  $\mu$ l), a dose approximately equivalent to the loss of SPLUNC1 protein in IL-13-treated cells at day 10 of ALI culture. It was found that exogenous SPLUNC1 protein reduced Mp levels in IL-13-treated cells by  $\sim 50\%$  (Fig. 10).

## Discussion

Our current study has, for the first time, offered direct evidence showing host defense (i.e., inhibition of Mp growth) and anti-inflammatory (i.e., lipoproteins-induced IL-8) functions of SPLUNC1 protein. Further, we have demonstrated that SPLUNC1 expression could be differently regulated under various conditions. Although Mp up-regulates SPLUNC1 expression, an allergic setting such as IL-13 significantly inhibits its expression and delays the bacterial clearance.

One of the key areas in the study of SPLUNC1 protein is to determine its functions. PLUNC proteins are a recently described family of host defense molecules. The PLUNC-encoding genes have been mapped to chromosome 20 in humans and to chromosome 2 in mice. Based on protein size, they are categorized into short (25 kDa) and long (50 kDa) PLUNCs (6, 24). Due to its structural homology to bactericidal/permeability-increasing (BPI) protein, SPLUNC1 has been predicted to have the host defense activity (27). Previous studies have identified BPI protein as an important mediator in killing Gram-negative bacteria and inhibiting LPS-induced proinflammatory cytokine production (28). However, the exact function of SPLUNC1 protein remains unknown.

In the present study, we determined whether SPLUNC1 protein has any host defense activity against Mp or inhibits epithelial inflammatory responses to Mp-derived components such as lipoproteins. Our experiments using recombinant SPLUNC1 protein (mouse), SPLUNC1 overexpression (human), and suppression via RNA interference (human) have demonstrated that SPLUNC1 protein inhibited Mp growth. Moreover, SPLUNC1 protein was shown to reduce epithelial IL-8 (a chemokine involved in host

defense against bacterial infection via recruiting neutrophils) production in response to Mp-derived lipoproteins. Together, our current study has provided direct evidence demonstrating both host defense and anti-inflammatory effects of SPLUNC1 protein. Thus, SPLUNC1 protein should be a new member of listed host defense substances (e.g., cathelicidins and  $\beta$ -defensins) that have been shown to be important host innate factors against respiratory infections (29, 30). It is noteworthy that the mechanisms underlying the host defense/anti-inflammatory effects of SPLUNC1 have not been studied in detail in our current study. A preliminary study from our laboratory suggests that Mp incubation with SPLUNC1 protein for 2 h reduced the mRNA expression of Mp P1 adhesin, a major molecule used by mycoplasma to adhere to airway epithelial cells (H. W. Chu, unpublished observation). Other mechanisms, including the effects of SPLUNC1 protein on P1 protein expression and Mp membrane integrity, need to be determined. Furthermore, we recently performed a binding assay of SPLUNC1 protein to lipoproteins (0.1, 0.5, and 2  $\mu$ g/ml) on a 96-well plate. We observed that SPLUNC1 bound to Mp-derived lipoproteins (0.1, 0.5, and 2  $\mu$ g/ml). We thus propose that the direct binding of SPLUNC1 protein to lipoproteins may subsequently prevent lipoproteins from binding to their receptors (e.g., TLR2) on the cell surface, leading to dampened cell activation (e.g., IL-8 production).

SPLUNC1 distribution and secretion data from our study have not only confirmed but also extended previous findings (7, 24). Our results further suggest that SPLUNC1 protein is constitutively expressed by human and mouse large airway epithelial cells. In human subjects, both airway surface and glandular epithelial cells were shown to express SPLUNC1 protein. Interestingly, we found that SPLUNC1 expression in cultured human bronchial epithelial cells is increasing with the degree of mucociliary cell differentiation, which is consistent with the work by Campos and coworkers (7). In mice that have fewer submucosal glands in the airways than humans under the physiologic and pathologic conditions, SPLUNC1 was detected only on airway surface epithelial cells. Further, levels of secreted SPLUNC1 protein were very high at the apical surface of cultured human primary epithelial cells and mouse BAL fluid. Collectively, our data and those from others strongly suggest that SPLUNC1 may be an important mediator in maintaining airway mucosal homeostasis. Future studies are warranted to measure SPLUNC1 protein in BAL fluid of human subjects with or without various lung diseases (e.g., infection and asthma).

Unlike other host defense substances (e.g., defensins), regulation of SPLUNC1 expression under an infection has not been examined. In the present study, we demonstrated that Mp infection increased SPLUNC1 expression. A neutralizing SPLUNC1 Ab treatment before Mp infection in mice led to an impaired Mp clearance from the lung, which was accompanied by an increased neutrophil recruitment into the lung. Our data suggest that a timely up-regulation of SPLUNC1 expression after an infection could be critical to an efficient clearance of the invading pathogen.

Respiratory viral and bacterial infections contribute to the pathogenesis of chronic lung diseases such as asthma. One of the fundamental research questions in deciphering the interplay of infection and asthma is: which innate pathways are deficient in asthmatic airways so an infection could prevail? Although the effects of allergic inflammation or Th2 cytokines on airway hyperreactivity and remodeling (e.g., fibrosis and mucus production) have been relatively well investigated (31–33), little is known about the role of a Th2 cytokine milieu in regulating the airway epithelial innate immune response to infections. Early observations suggested that SPLUNC1 protein decreased in the nasal lavage fluid of human

subjects exposed to allergens and irritants such as cigarette smoke (34–38). However, a direct role of an allergic milieu in regulating SPLUNC1 in the context of an infection was not investigated. We hypothesized that allergic inflammation or a Th2 cytokine milieu such as IL-13 directly reduces host innate molecules (i.e., SPLUNC1), thus preventing an efficient clearance of the invading pathogens from the lung. Studies from our mouse model of airway allergic inflammation (or “asthma”) revealed a significant reduction of airway SPLUNC1 expression. Further, IL-13 was shown to directly inhibit SPLUNC1 expression and secretion by normal human primary airway epithelial cells. Interestingly, reduction of epithelial SPLUNC1 expression in allergic mice or IL-13-treated cells was accompanied by impaired clearance of Mp. The addition of exogenous recombinant SPLUNC1 protein was shown to restore the clearance of Mp from the epithelial surface. Together, our data clearly suggest that an allergic milieu may be detrimental to the host innate immune response to respiratory bacterial infections in part through the inhibition of SPLUNC1 production. To definitely dissect a role of SPLUNC1 in bacterial infections in allergic mice, future studies are needed to administer recombinant SPLUNC1 protein into airways of mice with allergen challenge, mice treated with Th2 cytokines such as IL-13, and in transgenic mice with overexpression of a Th2 cytokine (e.g., IL-13).

We realize that although we studied the regulation of SPLUNC1 under infection (i.e., Mp) and allergic settings, the involved molecular mechanisms (e.g., signaling pathways) have not been fully examined in our current study. Nevertheless, we have obtained preliminary data suggesting a role of TLR2 in regulating SPLUNC1 expression (Q. Wu, unpublished data). First, we found that TLR2-deficient mice demonstrated less SPLUNC1 induction in response to Mp infection as compared with the wild-type mice. Second, in cultured NCI-H292 cells, Mp and Pam3CSK4 (a synthetic TLR2 ligand) increased SPLUNC1 mRNA expression in a dose-dependent manner. With regard to SPLUNC1 regulation by IL-13, the impact of IL-13 on epithelial cell composition should be considered. In our previous studies (20), IL-13 was shown to increase the number of mucus-producing goblet cells. In the current studies, we measured the mRNA levels of MUC5AC, a major subtype of mucins, in cells treated with and without IL-13. Similar to our previous findings, IL-13 increased MUC5AC mRNA on day 17 of ALI culture as compared with nonstimulated cells (13.8-fold,  $p < 0.05$ ). The opposite regulation of MUC5AC and SPLUNC1 by IL-13 is interesting and deserves future study. We may speculate that IL-13-induced change of cell composition could contribute to the reduction of SPLUNC1 expression in that goblet cells produce less SPLUNC1. However, this needs to be verified in future studies. In the absence of IL-13, as we presented in *Results*, SPLUNC1 expression levels increase (2-fold) as cells differentiate into mucociliary phenotype, which is accompanied by increased MUC5AC mRNA (3.5-fold). Thus, increased mucus goblet cells may not necessarily result in less SPLUNC1.

We are also aware of an additional limitation of our current study in that only one strain of bacteria (i.e., Mp) was used to study the host defense function of SPLUNC1 protein. We may propose that SPLUNC1 could also exert a similar host defense activity against other strains of bacteria. Indeed, we incubated *Escherichia coli* ( $10^3$ – $10^6$  CFU/ml) with SPLUNC1 protein at the same concentrations (1, 10, and 50  $\mu$ g/ml) as used in the Mp experiments for 2 h and found that SPLUNC1 protein had a minimal (<15%) inhibition on *E. coli* growth. These results suggest that unlike what was initially predicted, SPLUNC1 protein has selective inhibition of microorganisms. The efficiency of SPLUNC1 protein in affecting the growth of other species of bacteria (e.g., *Haemophilus influenzae*, *Pseudomonas aeruginosa*) remains to be tested.

In summary, our current study has revealed the host defense and anti-inflammatory activity of SPLUNC1 against the atypical bacterium *Mycoplasma pneumoniae*. Further, we demonstrated that an infection increased SPLUNC1 expression. In contrast, an allergic milieu decreased SPLUNC1 expression and subsequent bacterial clearance. Results from this study should shed light on future research aimed at eradicating persistent airway infections in chronic lung diseases such as asthma and chronic obstructive pulmonary disease (COPD).

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## Disclosures

The authors have no financial conflict of interest.

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