Surfactant Proteins A and D Enhance Pulmonary Clearance of *Pseudomonas aeruginosa*

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Surfactant protein (SP)-A and SP-D, members of the collectin family, are involved in innate host defenses against various bacterial and viral pathogens. In this study, we asked whether SP-A and SP-D enhance clearance of a nonmucoid strain of *Pseudomonas aeruginosa* from the lungs. We infected mice deficient in SP-A (SP-A^{-/-}), SP-D (SP-D^{-/-}) and both pulmonary collectins (SP-AD^{-/-}) by intratracheal administration of *P. aeruginosa*. Six hours after infection, bacterial counts were significantly higher in SP-A^{-/-}, SP-D^{-/-}, and SP-AD^{-/-} compared with wild-type (WT) mice. Forty-eight hours after infection, bacterial counts were significantly higher in SP-A^{-/-} mice compared with WT mice and in SP-AD^{-/-} mice compared with WT, SP-A^{-/-}, and SP-D^{-/-} mice. Phagocytosis of the bacteria by alveolar macrophages was decreased in SP-A^{-/-} and SP-D^{-/-} mice. Levels of macrophage inflammatory peptide-2 and IL-6 were more elevated in the lungs of SP-D and SP-AD^{-/-} mice compared with WT mice. There was more infiltration by neutrophils in the lungs of SP-D^{-/-} compared with WT and SP-A^{-/-} mice 48 h after infection. This study shows that SP-A and SP-D enhance pulmonary clearance of *P. aeruginosa* by stimulating phagocytosis by alveolar macrophages and by modulating the inflammatory response in the lungs. These findings also show that the functions of SP-A and SP-D are not completely redundant *in vivo*.

Keywords: pneumonia; *Pseudomonas aeruginosa*; surfactant proteins

Surfactant protein (SP)-A and SP-D are members of the collectin family of innate immune proteins (1). Collectins contain an N-terminal collagen-like domain and a C-terminal calcium-dependent lectin domain, also known as carbohydrate recognition domain. SP-A and SP-D, the pulmonary collectins, are produced by alveolar type II cells, nonciliated bronchiolar cells, and tracheobronchial glands. They are involved in the innate host defense against various bacterial and viral pathogens. Despite these similarities, it is unknown whether SP-A and SP-D have redundant, additive, or synergic functions *in vivo*.

A number of studies have shown that SP-A and SP-D contribute to clearance of bacteria by several mechanisms *in vitro* and *in vivo*. *In vitro*, SP-A and SP-D bind carbohydrate structures on the surface of microorganisms. SP-D aggregates some gram-positive and gram-negative bacteria, including strains of *Pseudomonas aeruginosa* (2). SP-A and SP-D opsonize bacteria and increase their phagocytosis by alveolar macrophages (AM) (3–5). SP-A also stimulates macrophages directly without binding to the bacteria and increases phagocytosis of various bacteria, including strains of *P. aeruginosa* (6, 7). SP-A and SP-D increase phagocytosis of gram-positive and gram-negative bacteria by

neutrophils (8). In addition, recent data suggest that both pulmonary collectins have a direct antimicrobial effect against gram-negative bacteria (9, 10).

In vivo, the role of pulmonary collectins has been investigated after intratracheal infection of gene-targeted mice (11–13). These studies suggest that SP-A but not SP-D increases clearance of bacteria from the lungs (14). SP-A-deficient (SP-A^{-/-}) mice had decreased pulmonary clearance of group B streptococcus, *Hemophilus influenzae*, *Klebsiella pneumoniae*, and a mucoid strain of *P. aeruginosa*. SP-A^{-/-} mice also displayed more severe pulmonary inflammation measured by higher levels of cytokines and had increased pulmonary infiltration by neutrophils compared with wild type (WT) mice. SP-D-deficient (SP-D^{-/-}) mice had increased pulmonary inflammation after infection with group B streptococcus or *H. influenzae* but cleared these bacteria from their lungs as efficiently as WT mice.

Despite considerable evidence that SP-A and SP-D have a role in host defense against bacteria, there is limited information about their role in clearance of *P. aeruginosa* from the lungs in animal models (12) and in human disease. In humans, *P. aeruginosa* is a major cause of pneumonia in patients with impaired host defenses (15). It is one of the most common causes of pneumonia in intensive care units and has a high mortality rate. *P. aeruginosa* is also the most prevalent pathogen in cystic fibrosis (CF), where it chronically colonizes the lung, eventually causing respiratory failure and death. SP-A and SP-D levels are decreased in bronchioalveolar lavage fluid from patients with CF (16).

In this study, we asked whether SP-A and SP-D enhance clearance of *P. aeruginosa* from the lungs. We infected SP-A^{-/-} mice, SP-D^{-/-} mice, and mice deficient in both pulmonary collectins (SP-AD^{-/-}) by intratracheal administration of a nonmucoid strain of *P. aeruginosa*. We chose a nonmucoid strain because nonmucoid strains of *P. aeruginosa* are involved in nosocomial pneumonia and in the early stages of CF. Bacterial clearance from the lungs, phagocytosis by AM, modulation of cytokine production, and neutrophil influx in the lungs were determined.

MATERIALS AND METHODS

Mice

SP-A^{-/-}, SP-D^{-/-}, and SP-AD^{-/-} mice were generated from embryonic stem cells targeted with replacement-type vectors as previously described (17–19). All three strains were backcrossed 10 generations onto a C57/BL6 background. WT C57/BL6 mice were generated from heterozygous matings. Mice were housed in barrier containment and were weaned on Day 21. The protocols were approved by the Committee for Animal Research of the University of California San Francisco.

Preparation of Bacteria

The nonmucoid laboratory strain of *P. aeruginosa* PAK was provided by Dr. Jeanine Wiener-Kronish from University of California San Francisco. *Pseudomonas* strain PAK transfected with a plasmid expressing green fluorescent protein was provided by Dr. Suzanne Fleiszig from University of California Berkeley. The bacteria were grown from

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frozen stocks on trypticase soy agar plates. Before each experiment, bacteria were inoculated in a dialysate of tryptic soy broth supplemented with 10 mM nitrilotriacetic acid (Sigma Chemical, St. Louis, MO), 1% glycerol, and 100 mM monosodium glutamate and grown at 37°C for 13 h in a shaking incubator. Cultures were centrifuged, and the bacterial pellet was washed twice in Ringer's lactate solution. The bacterial concentration was adjusted by spectrophotometry and confirmed by plating out serial dilutions on sheep blood agar plates.

Intratracheal Inoculation

Mice were anesthetized at 21 d of age with inhaled methoxyflurane. Fifty microliters of bacterial solution containing 5×10^6 colony forming units (cfu) were instilled slowly in the lungs through a gavage needle (Modified animal feeding needle, 24 G; Popper and Sons, Inc., New Hyde Park, NY) inserted into the trachea via the oropharynx. The proper insertion of the needle in the trachea was confirmed by palpation of the tip of the blunt needle through the skin as previously described (20).

Bacterial Clearance

Quantitative cultures of lung and spleen homogenates were performed 6 and 48 h after infection. Mice were exsanguinated after a lethal intraperitoneal injection of sodium pentobarbital. Blood was removed from the pulmonary circulation by injection of 2 ml of Ringer's lactate into the pulmonary artery. Lungs and spleens were collected and homogenized in 1 ml of Hepes (50 mM; pH 8). Serial dilutions of lungs and spleen homogenates were plated on sheep blood agar plates to quantify the bacteria.

In Vivo Phagocytosis by AM

P. aeruginosa strain PAK expressing green fluorescent protein (PAK-GFP) was grown as described previously, and 5×10^6 cfu were instilled in the lungs. One hour after infection, the lungs were lavaged six times with 0.4 ml of Tris-buffered saline containing 0.25 mM EDTA and EGTA. Bronchoalveolar lavage (BAL) fluid was centrifuged 15 min at $300 \times g$ at 4°C. Cytospins were stained with a nuclear dye (TO-PRO-3; Invitrogen, Carlsbad, CA) and examined by confocal microscopy. Serial sections through > 100 randomly chosen AM were examined to determine the percentage of AM with intracellular bacteria.

In Vitro Phagocytosis by AM

Phagocytosis by AM was evaluated *in vitro* using described methods with some modifications (21). AM were harvested from adult WT and SP-D^{-/-} mice by BAL with Tris-buffered saline containing 0.25 mM EDTA and EGTA. AM were washed three times with RPMI 1640 and counted using trypan blue. WT and SP-D^{-/-} AM (2×10^5 cells) were mixed with PAK-GFP (2×10^6 cfu) in RPMI with or without 2 µg/ml of mouse recombinant SP-D. The incubation was performed for 30 min at 37°C in the presence of 2 mM CaCl₂ and 5% FCS. Phagocytosis was stopped by adding ice cold RPMI, and the cells were washed three times. Cytospins were prepared and observed under confocal microscopy. Serial sections through > 100 randomly chosen AM were examined to determine the percentage of AM with intracellular bacteria.

Cytokines

Levels of IL-6, macrophage inflammatory peptide (MIP)-2, and TNF-α were measured in lung homogenates 6 h after infection. After addition of protease inhibitors, lung homogenates were centrifuged for 15 min at 14,000 rpm. Levels of cytokines were measured in the supernatant by sandwich ELISA (Quantikine ELISA kit; R&D Systems, Minneapolis, MN).

Myeloperoxidase Assay

Neutrophil influx in the lungs was determined by measuring myeloperoxidase (MPO) activity in lung homogenates as previously described (22). Lung homogenates were centrifuged for 30 min at 14,000 rpm. The pellet was resuspended in 0.5% cetyltrimethylammonium chloride and centrifuged as described previously. A 10-fold dilution of the supernatant in 10 mM citrate buffer pH 5.0 was used for the reaction. Aliquots of 60 µl of samples were mixed with equal volumes of the substrate (3,3',5,5'-tetramethylbenzidine dihydrochloride, 3 mM; resorcinol, 120 µM; and H₂O₂, 2.2 mM) for 2 min. The reaction was stopped by adding 150 µl of ice-cold 2 M H₂SO₄. Optical density (OD) was measured at 450 nm. Background OD was measured at 450 nm by adding the stop solution first, then the samples, then the substrate. The enzymatic activity in the lungs was calculated by subtracting the background OD and was expressed as a change of OD/min.

Histology

Forty-eight hours after infection, lungs were fixed at an inflation pressure of 20 cm H₂O by intratracheal injection of a solution containing

Figure 1. Bacterial counts in the lungs 6 and 48 h after infection. Quantitative cultures were performed by plating serial dilutions of lung homogenates. Six hours after infection with 5×10^6 cfu of *P. aeruginosa*, bacterial counts were significantly higher in SP-D^{-/-} ($n = 11$), SP-A^{-/-} ($n = 11$), and SP-AD^{-/-} mice ($n = 10$) compared with WT mice ($n = 14$). Forty-eight hours after infection, bacterial counts were significantly higher in SP-A^{-/-} mice ($n = 8$) compared with WT mice ($n = 13$) and in SP-AD^{-/-} mice ($n = 7$) compared with WT, SP-D^{-/-} ($n = 12$), and SP-A^{-/-} mice. Data are expressed as log cfu per lungs and represent means $\pm$ SEM. * $P < 0.05$ versus WT mice. *** $P < 0.05$ versus WT, SP-A^{-/-}, and SP-D^{-/-} mice.

2% glutaraldehyde and 1% paraformaldehyde in a 0.1 M phosphate buffer. The lungs were then prepared for sectioning using standard techniques. Sections were stained with toluidine blue.

SP-A and SP-D Levels

SP-A and SP-D levels were measured by Western blot in supernatant from lung homogenates of WT mice 6 and 48 h after infection. For SP-A and SP-D detection, 40- and 10- μ l of samples were loaded on SDS polyacrylamide gels, respectively. SP-A and SP-D levels were measured using specific polyclonal antibodies against recombinant mouse SP-A and SP-D. Proteins were visualized by enhanced chemiluminescence detection (Amersham, Arlington Heights, IL) after incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Chemicon International, Temecula, CA).

Statistical Methods

The variables cfu/lungs and cfu/spleen were not normally distributed. Therefore, $\log_{10}$ transformation was used for statistical analysis. ANOVA followed by the Student-Newman-Keuls test was used for comparisons between the four genotypes. Chi square test was used for survival analysis. Findings were considered statistically significant at $P < 0.05$.

RESULTS

Bacterial Clearance and Survival

Six hours after infection, WT mice had started clearing bacteria from their lungs (Figure 1). In contrast, bacterial counts were higher than the inoculum in SP-A $^{-/-}$, SP-D $^{-/-}$, and SP-AD $^{-/-}$ mice. The difference in lung bacterial counts was significant between WT mice and the three other genotypes at 6 h after infection. Another set of mice was infected with the same inocu-

lum and studied until 48 h after infection. Survival at 48 h was 100% in WT ($n = 13$) and SP-D $^{-/-}$ ($n = 12$) mice. Survival was 80% in SP-A $^{-/-}$ mice ($n = 10$) and 70% in SP-AD $^{-/-}$ mice ($n = 10$). The difference in survival rate between the four genotypes did not reach statistical significance (chi square P value of 0.057). Bacterial clearance from the lungs was measured in the mice that had survived until 48 h. SP-A $^{-/-}$ mice had significantly higher lung bacterial counts compared with WT mice, and SP-AD $^{-/-}$ mice had significantly higher lung bacterial counts compared with the three other genotypes. The systemic spread of *P. aeruginosa* to the spleen was not different between the genotypes at 6 or 48 h. These findings indicate that SP-A $^{-/-}$ and SP-D $^{-/-}$ mice have decreased clearance of *P. aeruginosa* from the lungs compared with WT mice and that double knockout mice for SP-A and SP-D have an additional decrease in bacterial clearance compared with single knockout mice.

In Vivo Phagocytosis by AM

To evaluate phagocytosis by AM, we collected BAL fluid 1 h after infection with PAK-GFP. Cytospins were observed under confocal microscopy, and serial sections through AM were performed to quantify the percentage of AM that had intracellular bacteria. The percentage of AM that had intracellular bacteria was highest in WT mice (Figure 2). It was significantly lower in SP-A $^{-/-}$, SP-D $^{-/-}$, and SP-AD $^{-/-}$ mice. There was also a significant difference in phagocytosis between SP-A $^{-/-}$ and SP-AD $^{-/-}$ mice. Our results indicate that SP-A and SP-D stimulate phagocytosis of *P. aeruginosa* by AM *in vivo*.

Figure 2. Phagocytosis by AM *in vivo*. Phagocytosis of GFP expressing *P. aeruginosa* was measured *in vivo* by collecting BALF 1 h after infection. Cytospin preparations were observed under confocal microscopy to quantify the percentage of AM that had intracellular bacteria. The percentage of AM that had intracellular bacteria was the highest in WT mice. It was significantly lower in SP-A $^{-/-}$, SP-D $^{-/-}$, and SP-AD $^{-/-}$ mice. There was also a significant difference in phagocytosis between SP-A $^{-/-}$ and SP-AD $^{-/-}$ mice. Data are expressed as means $\pm$ SEM with $n = 5-7$ mice per genotype. * $P < 0.05$ versus WT mice. ** $P < 0.05$ versus WT and SP-A $^{-/-}$ mice.

Figure 3. Phagocytosis by AM *in vitro*. AM from WT and SP-D $^{-/-}$ mice were incubated for 30 min at 37°C with PAK-GFP in the absence or in the presence of rSP-D. Cytospin preparations were observed under confocal microscopy to quantify the percentage of AM that had intracellular bacteria. A lower percentage of SP-D $^{-/-}$ AM had intracellular bacteria compared with WT AM. With the addition of rSP-D, a higher percentage of SP-D $^{-/-}$ and WT AM had intracellular bacteria. However, even with the addition of rSP-D, there was a lower percentage of SP-D $^{-/-}$ AM versus WT AM with intracellular bacteria. Data are expressed as means $\pm$ SEM of three separate experiments performed in duplicate. * $P < 0.05$ versus SP-D $^{-/-}$ AM. ** $P < 0.05$ versus SP-D $^{-/-}$ AM, WT AM, and SP-D $^{-/-}$ AM with rSP-D.

Figure 4. Cytokine levels in lung homogenates. Levels of IL-6, MIP-2, and TNF- α were measured in lung homogenates by ELISA at 6 h after infection. Levels of MIP-2 and IL-6 were significantly higher in SP-D^{-/-} and in SP-AD^{-/-} mice compared with WT mice. Additionally, levels of MIP-2 were significantly higher in SP-AD^{-/-} compared with SPA^{-/-} mice. There was no significant difference in levels of TNF- α between the four genotypes. Data are expressed in pg/ml and represent means $\pm$ SEM with $n = 10$ mice per genotype. * $P < 0.05$ versus WT mice. ** $P < 0.05$ versus WT and SP-A^{-/-} mice.

In Vitro Phagocytosis by AM

Because AM from SP-D^{-/-} mice have been shown to have an abnormal phenotype (18), we further characterized the defect

Figure 5. Myeloperoxidase activity in lung homogenates. Neutrophil influx in the lungs was determined by measuring MPO activity. There was higher MPO activity in SP-D^{-/-} mice compared with WT and SP-A^{-/-} mice. MPO activity was also higher in SP-AD^{-/-} compared with WT mice. Data are expressed as a delta OD 450 per minute and represent means $\pm$ SEM with $n = 7$ –12 mice per genotype. * $P < 0.05$ versus WT mice. ** $P < 0.05$ versus WT and SP-A^{-/-} mice.

in phagocytosis observed in SP-D^{-/-} mice by an *in vitro* assay. AM from WT and SP-D^{-/-} mice were incubated with PAK-GFP in the absence or in the presence of recombinant SP-D (rSP-D). After 30 min of incubation, a lower percentage of SP-D^{-/-} AM had intracellular bacteria compared with WT AM (Figure 3). With the addition of rSP-D, the percentage of AM with intracellular bacteria increased for SP-D^{-/-} and WT AM. However, even with the addition of rSP-D, the percentage of AM with intracellular bacteria was lower for SP-D^{-/-} versus WT AM. These results suggest that the defect in phagocytosis observed in SP-D^{-/-} mice is due to the absence of SP-D and to functional changes in AM.

Cytokines

To assess the pulmonary inflammatory response in collectin-deficient mice, cytokine levels were determined in lung homogenates 6 h after infection. Our laboratory has previously shown that cytokine levels in lung homogenates of uninfected mice are low and do not differ between genotypes. Six hours after infection, cytokine levels were elevated in all four genotypes (Figure 4). Levels of MIP-2 and IL-6 were significantly higher in SP-D^{-/-} and in SP-AD^{-/-} mice compared with WT mice. Additionally, levels of MIP-2 were significantly higher in SP-AD^{-/-} compared with SPA^{-/-} mice. Levels of TNF- α tended to be higher in SP-D^{-/-} and SP-AD^{-/-} mice compared with WT and SP-A^{-/-} mice, but the difference was not statistically significant. In summary, our results show that SP-D^{-/-} and SP-AD^{-/-} mice have higher levels of pulmonary cytokines after infection with *P. aeruginosa*. This suggests that SP-D has a stronger effect than SP-A in modulating cytokine production.

Myeloperoxidase Activity

To further describe the pulmonary inflammatory response in collectin-deficient mice, we measured the influx of neutrophils in the lungs by determination of MPO activity. MPO activity was very low in the lungs of uninfected animals and did not differ between the genotypes (data not shown). Forty-eight hours

after infection, MPO activity was significantly higher in SP-D^{-/-} and SP-AD^{-/-} mice compared with WT mice and in SP-D^{-/-} mice compared with SP-A^{-/-} mice (Figure 5). Therefore, SP-D^{-/-} mice have an increased pulmonary infiltration by neutrophils after infection with *P. aeruginosa*.

Lung Histology

When mice were 21 d of age, we did not detect significant abnormality in lung architecture or any infiltration by inflammatory cells in uninfected mice of each genotype. Forty-eight hours after infection, lungs were infiltrated with macrophages and polymorphonuclear neutrophils. Qualitatively, the infiltration was more prominent in the lungs of SP-A^{-/-}, SP-D^{-/-}, and SP-AD^{-/-} mice compared with WT mice (Figure 6).

SP-A and SP-D Levels

SP-A and SP-D levels were assessed 6 and 48 h after infection in the supernatant of lung homogenates from WT mice. Compared with uninfected controls, there was a dramatic decrease in SP-A levels 6 and 48 h after infection. To detect SP-A in the lungs of infected mice and to look for degradation products, 40 μ l of lung homogenates were used in the assay. This gave a signal beyond the linear range for the controls. Therefore, we were not able to quantify the decrease in SP-A levels. No degradation products of SP-A were seen with the polyclonal antibody used. SP-D levels were decreased by 20% and 25% at 6 and 48 h after infection ($P < 0.05$ versus uninfected controls). No degradation products of SP-D were visualized.

DISCUSSION

Our results show that SP-A and SP-D, the pulmonary collectins, contribute to clearance of a nonmucoid strain of *P. aeruginosa* from the lungs in mice. Nonmucoid strains of *P. aeruginosa* are a major cause of nosocomial pneumonia in intensive care units and are involved in the early stages of CF (15). In the noncompromised host, *P. aeruginosa* is cleared from the lungs by innate immune mechanisms that involve the mucociliary apparatus, phagocytic cells, and antimicrobial molecules. This study indi-

cates that the pulmonary collectins modulate innate immunity against *P. aeruginosa* *in vivo*.

Previous studies have shown that SP-A^{-/-} mice had decreased clearance of bacteria from their lungs after intratracheal infection (11–13). In contrast, in prior studies, SP-D^{-/-} mice cleared gram-positive and gram-negative bacteria from their lungs as efficiently as WT mice (13). The present study confirms the clearance defect observed in SP-A^{-/-} mice but shows that SP-D^{-/-} mice have decreased pulmonary clearance of *P. aeruginosa* at an early time point (Figure 1). Furthermore, SP-AD^{-/-} mice have decreased clearance of *P. aeruginosa* compared with SP-A^{-/-} or SP-D^{-/-} mice. The differences between our study and previous reports may be related to differences in age and strain of mice and differences in species and strain of bacteria. Previous studies performed a bacterial challenge in collectin-deficient mice at an age of 35–42 d (11–13). At this age, ongoing pulmonary inflammation and macrophage activation in SP-D^{-/-} mice could have modified their response to infection. In the present study, mice were infected at an age of 21 d to minimize the potential effect of their lung phenotype on their response to infection.

One of the most well characterized functions of SP-A and SP-D is their ability to act as opsonins and enhance the uptake of micro-organisms by phagocytic cells (3–5). *In vitro*, previous experiments have shown that SP-A and SP-D bind some but not all strains of *P. aeruginosa* and increase their phagocytosis by AM (2, 4, 5). SP-A can also increase phagocytosis by stimulating macrophages directly without binding to the bacteria (6, 7). In the present study, SP-A^{-/-} and SP-D^{-/-} mice had decreased phagocytosis of PAK-GFP by AM compared with WT mice (Figure 2). SP-AD^{-/-} mice had decreased phagocytosis of PAK-GFP by AM compared with WT and SP-A^{-/-} mice. Therefore, our results show that SP-A and SP-D stimulate phagocytosis of *P. aeruginosa* by AM *in vivo*.

SP-A and SP-D can modulate the inflammatory response to pathogens by regulating the production of cytokines and the influx of inflammatory cells in the lungs (1). This was confirmed in our study, but SP-A^{-/-} and SP-D^{-/-} mice differed in their

Figure 6. Lung histology. Sections of the lungs were prepared 48 h after infection with 5×10^6 cfu of *P. aeruginosa*. There was more infiltration by inflammatory cells in the lungs of SP-A^{-/-} (B), SP-D^{-/-} (C), and SP-AD^{-/-} (D) mice compared with WT mice (A). These sections are representative of three different animals per genotype.

Figure 7. SP-A and SP-D levels. SP-A and SP-D levels were assessed in lung homogenates of WT mice by Western blotting. At 6 and 48 h after infection, there was a dramatic decrease in SP-A levels and a smaller but significant decrease in SP-D levels compared with uninfected mice ($P < 0.05$, $n = 6$ mice per group).

inflammatory response. We found that after infection with *P. aeruginosa*, SP-D $^{-/-}$ mice had increased pulmonary inflammation compared with WT mice. On qualitative histology, SP-D $^{-/-}$ mice had increased pulmonary infiltration by inflammatory cells (Figure 6). SP-D $^{-/-}$ mice also had higher levels of MPO activity (Figure 5) and higher levels of MIP-2 and IL-6 (Figure 4) in their lungs compared with WT mice. This increased production of MIP-2 could have contributed to the increased influx of neutrophils observed in SP-D $^{-/-}$ mice. In SP-A $^{-/-}$ mice, on histology, there was also an increased pulmonary infiltration after infection with *P. aeruginosa*. However, SP-A $^{-/-}$ mice did not have a statistically significant increase in infiltration of the lungs by neutrophils, as measured by MPO activity at 48 h. Pulmonary levels of MIP-2 and IL-6 tended to be higher in SP-A $^{-/-}$ mice compared with WT mice, but the difference was not statistically significant. Previous studies have shown that SP-A and SP-D can upregulate or downregulate cytokine production by macrophages *in vitro* (23–26). The nature of these effects depends on various factors, including the type of pathogen or stimulus, the type and the state of activation of the cell, and the cellular receptor that is engaged. Our results show that SP-D $^{-/-}$ but not SP-A $^{-/-}$ mice had a significantly increased pulmonary inflammatory response after infection with a nonmucoid strain of *P. aeruginosa*, despite a bacterial load that tended to be higher in SP-A $^{-/-}$ than in SP-D $^{-/-}$ mice. These data suggest that, in addition to promoting bacterial clearance, SP-D downregulates the pulmonary inflammatory response.

The phenotypes of SP-A $^{-/-}$ and SP-D $^{-/-}$ mice have been reported previously. Although uninfected SP-A $^{-/-}$ mice are largely indistinguishable from WT mice except for the absence of a surfactant fraction called tubular myelin (27), SP-D $^{-/-}$ mice develop patchy pulmonary inflammation, airspace remodeling, and surfactant accumulation (18). These apparently noninfectious changes start early in life but are pronounced by 6 wk of age (28). The mice used for this study were 3 wk of age. At this age, pulmonary cytokine levels, MPO activity, and lung histology were similar in WT mice compared with SP-D $^{-/-}$ mice. In SP-D $^{-/-}$ mice, the number of AM increases progressively after birth. These macrophages progressively enlarge and accumulate intracellular lipids. Our results show that AM from SP-D $^{-/-}$ mice had decreased phagocytosis of *P. aeruginosa* compared with AM from WT mice *in vitro* (Figure 3). This defect in phagocytosis observed in SP-D $^{-/-}$ mice was partially rescued by the administration of rSP-D. Taken together, these data suggest that the decrease in clearance of *P. aeruginosa* observed in SP-A $^{-/-}$ and SP-D $^{-/-}$ mice is due to the absence of the collectins. However, functional changes in AM of SP-D $^{-/-}$ mice may have contributed to decreased bacterial clearance.

SP-A and SP-D levels were measured 6 and 48 h after infection in the lungs of WT mice (Figure 7). Compared with uninfected mice, there was a dramatic decrease in SP-A levels as early as 6 h after infection. There was a small but significant decrease in SP-D levels at 6 and 48 h. At least four different mechanisms could explain this decrease in SP-A and SP-D levels. First, several reports indicate that *P. aeruginosa* can secrete

proteases that degrade SP-A and SP-D *in vitro* (29). In our experiments, degradation of SP-A and SP-D by bacterial enzymes could have occurred *in vivo*. However, we did not detect any immunoreactive degradation products for SP-A or SP-D with the polyclonal antibodies used. Second, SP-A has a relatively short half-life (30). Acute lung injury and damage to alveolar type II cells and Clara cells by the bacteria could have resulted in decreased production of SP-A and SP-D. Third, other investigators have found that phagocytes and epithelial cells can degrade lung collectins (31, 32) and that clearance of SP-D by neutrophils was increased after inflammation induced by lipopolysaccharide (33). Finally, as in previous studies, we measured SP-A and SP-D in the supernatant of lung homogenates after centrifugation (34). The fraction of SP-A and SP-D that was bound to the surface of the bacteria and to various cell types removed during centrifugation was not measured. This could have contributed to an underestimation of the collectin levels in the lungs of infected mice. In summary, our data show that despite having decreased collectin levels after acute pulmonary infection with *P. aeruginosa*, WT mice cleared bacteria more efficiently than SP-A $^{-/-}$ and SP-D $^{-/-}$ mice.

The concentrations of pulmonary collectins required to clear bacteria *in vivo* are unknown. Low levels of pulmonary collectins have been associated with several human diseases, including CF (16), pneumonia (35, 36), and chronic lung disease of prematurity (37, 38). Infection and inflammation contribute to the pathogenesis of CF and chronic lung disease of prematurity. Therefore, SP-A and SP-D may play an important role in the defense against bacterial infections in humans.

In conclusion, this study shows that SP-A and SP-D enhance pulmonary clearance of *P. aeruginosa* by stimulating phagocytosis by AM and by modulating the inflammatory response in the lungs. This is the first report of a bacterial challenge in mice deficient in both SP-A and SP-D. More bacteria remained in the lungs in SP-AD $^{-/-}$ mice compared with SP-A $^{-/-}$ and SP-D $^{-/-}$ mice (Figure 1). In addition, markers of inflammation, including the histologic appearance of the lungs, tended to be more pronounced in SP-AD $^{-/-}$ compared with SP-A $^{-/-}$ or SP-D $^{-/-}$ mice (Figures 2, 4, and 6). Therefore, our results are consistent with a model where SP-A and SP-D have overlapping but not identical functions *in vivo*. These findings may be clinically important in human diseases associated with decreased levels of SP-A and SP-D.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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