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Surfactant Protein D Enhances Clearance of Influenza A Virus from the Lung In Vivo¹

Ann Marie LeVine,^{2*†} Jeffrey A. Whitsett,^{*} Kevan L. Hartshorn,[‡] Erika C. Crouch,[§] and Thomas R. Korfhagen^{*}

Mice lacking surfactant protein D (SP-D^{-/-}) and wild-type mice (SP-D^{+/+}) were infected with influenza A virus (IAV) by intranasal instillation. IAV infection increased the endogenous SP-D concentration in wild-type mice. SP-D-deficient mice showed decreased viral clearance of the Phil/82 strain of IAV and increased production of inflammatory cytokines in response to viral challenge. However, the less glycosylated strain of IAV, Mem/71, which is relatively resistant to SP-D in vitro, was cleared efficiently from the lungs of SP-D^{-/-} mice. Viral clearance of the Phil/82 strain of IAV and the cytokine response were both normalized by the coadministration of recombinant SP-D. Since the airway is the usual portal of entry for influenza A virus and other respiratory pathogens, SP-D is likely to play an important role in innate defense responses to IAV. *The Journal of Immunology*, 2001, 167: 5868–5873.

Surfactant protein D (SP-D)³ is a member of the collectin family of the mammalian C-type lectins that also includes mannose-binding protein, conglutinin, and surfactant protein A (SP-A) (1, 2). There is increasing evidence that the collectins are involved in innate host defense against various bacterial, fungal, and viral pathogens. The collectins form multimeric structures resembling C1q (the first component of the complement cascade), and consist of collagenous N-terminal domains and globular C-terminal, carbohydrate binding domains (2). Collectins bind carbohydrate surfaces of many micro-organisms mediating phagocytosis and killing by phagocytic cells (3).

In the lung, SP-D is produced primarily by alveolar type II cells, tracheobronchial glands, and nonciliated bronchiolar cells (4). In vitro, SP-D interacts with bacteria, fungi, and viruses. SP-D binds to alveolar macrophages (5) and binds and increases macrophage association with *Escherichia coli* (6), *Pseudomonas aeruginosa* (7), *Mycobacterium tuberculosis* (8), and *Pneumocystis carinii* (9). In vitro, mannose binding protein, conglutinin, SP-A, and SP-D neutralize influenza A virus (IAV) and enhance the association of neutrophils with IAV (10–14).

IAV infection is airborne and is primarily an infection of the upper respiratory tract. However, during infection virus spreads to the lower respiratory tract and may result in viral pneumonia or

predispose to secondary bacterial infections. Influenza infections are most frequent in children and young adults, yet deaths are most frequent in the very young (<1 yr), the elderly, and persons of all ages with underlying heart or lung disease (15). Bronchopulmonary dysplasia has been associated with decreased secretion of SP-D (16), and cystic fibrosis has been associated with decreased SP-D concentrations in pulmonary washes (17), conditions that may increase susceptibility to infection by respiratory viruses such as IAV.

Specific as well as nonspecific immune mechanisms take part in the host response to influenza virus. IAV infection is a lytic infection and causes breakdown of the blood-tissue barrier early in infection, resulting in the influx of macrophages, neutrophils, and NK cells into the lung. Specific immune responses to IAV are initiated by the influx of virus-specific T lymphocytes and Ab production, and CTL are thought to be involved in viral clearance by direct cytolysis of virus-infected cells (18). Neutrophils also play an important role in viral clearance from the lung. Mice irradiated to reduce the number of peripheral polymorphonuclear leukocytes have increased viral titers after influenza infection of the lung (19). Defects in neutrophil and monocyte chemotactic, oxidative, and bacterial killing functions have been documented in IAV infection (20, 21). In vitro, neutrophil dysfunction resulting from IAV exposure is diminished when the virus is preincubated with SP-D (14). On the other hand, SP-D has been reported to have no effect on IAV uptake by alveolar macrophages (22).

Although there is compelling evidence that SP-D enhances host defense against viruses in vitro, its role in the clearance of viral pathogens in vivo has not been demonstrated. In the present study SP-D-deficient mice were infected intranasally with SP-D-sensitive and -resistant strains of IAV. Rescue experiments were performed using highly purified recombinant SP-D. IAV clearance, lung inflammation, cytokine production, and uptake of virus by macrophages and neutrophil activity were compared in SP-D^{-/-} and SP-D^{+/+} mice in vivo.

Materials and Methods

Animal husbandry

SP-D^{-/-} mice were produced by targeted gene inactivation (23). Lungs of SP-D^{-/-} mice do not contain detectable SP-D. National Institutes of Health Swiss Black SP-D^{+/+} and SP-D^{-/-} mice were studied. Mice were

Divisions of ^{*}Pulmonary Biology and [†]Critical Care Medicine, Children's Hospital Medical Center, Cincinnati, OH 45229; [‡]Department of Medicine and Pathology, Boston University School of Medicine, Boston, MA 02118; and [§]Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110

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² Address correspondence and reprint requests to Dr. Ann Marie LeVine, Division of Pulmonary Biology, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail address: levia0@chmcc.org

³ Abbreviations used in this paper: SP-D, surfactant protein D; SP-A, surfactant protein A; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; ff, fluorescent foci; IAV, influenza A virus; MIP-2, macrophage-inflammatory protein 2; MPO, myeloperoxidase.

housed in barrier containment and remained virus free as assessed by serology. Studies were reviewed and approved by the institutional animal care and use committee of the Children's Hospital Research Foundation (Cincinnati, OH). Male and female mice of approximately 20–25 g (35–42 days old) were used.

Preparation of IAV

IAV strain H₃N₂ A/Philippines/82 (Phil/82) and H3N1 Mem71_H-Bel_N (Mem71) were gifts from E. M. Anders to K. Hartshorn (University of Melbourne, Melbourne, Australia) and were grown in the chorioallantoic fluid of 10-day-old embryonated hen's eggs. Allantoic fluid was harvested after 48 h of incubation and was clarified by centrifugation at 1,000 × *g* for 40 min, followed by centrifugation at 135,000 × *g* to precipitate viruses. The virus-containing pellets were resuspended and purified on a discontinuous sucrose density gradient as previously described (24). Virus stocks were dialyzed against PBS, separated into aliquots, and stored at –70°C until used.

The potency of each viral stock was measured by the fluorescent foci assay (24) after samples were thawed from frozen storage at –70°C. Several stocks were used which varied from 5 × 10⁸ to 5 × 10⁹ fluorescent foci/ml.

FITC labeling of IAV

FITC stock was prepared at 1 mg/ml in 1 mol/L sodium carbonate, pH 9.6. FITC-labeled virus (Phil/82) was prepared by incubating concentrated virus stocks with FITC (10/1 mixture (v/v) of virus in PBS with FITC stock) for 1 h, followed by dialysis of the mixture for 18 h against PBS.

Viral clearance of influenza

Mice were lightly anesthetized with isoflurane and inoculated intranasally with 10⁵ fluorescent foci (ff) of IAV in 50 μl PBS. Quantitative IAV cultures of lung homogenates were performed 3, 5, 7, and 10 days after inoculation of the animals with IAV. The entire lung was removed, homogenized in 2 ml sterile PBS, quick-frozen, weighed, and stored at –80°C. Madin-Darby canine kidney cell monolayers were prepared in 96-well plates for the viral focus assay as previously described (24). The layers were incubated with lung homogenates diluted in PBS containing 2 mM calcium for 45 min at 37°C, and the monolayers washed three times in virus-free DMEM containing 1% penicillin and streptomycin. The monolayers were incubated for 7 h at 37°C in DMEM and repeatedly washed, and the cells were fixed with 80% (v/v) acetone for 10 min at –20°C. The monolayers were then incubated with mAb directed against IAV nucleoprotein (mAb A-3) and then with rhodamine-labeled goat anti-mouse IgG. Fluorescent foci were counted directly under fluorescent microscopy. The resulting titer was divided by the lung weight and reported as ff per gram of lung.

Treatment with human SP-D

Human SP-D was isolated as previously described (25). Briefly, CHO-K1 cells (ATCC CCL-61; American Type Culture Collection, Manassas, VA) were transfected with a full-length human cDNA in the pEE14 mammalian expression vector. Secreted SP-D was isolated by maltosyl-agarose affinity chromatography, and SP-D dodecamers were resolved from larger multimers and trimers by gel filtration chromatography under nondenaturing conditions. Proteins were concentrated by rechromatography on maltosyl-agarose. Bound proteins were eluted in HEPES-buffered saline containing 10 mM EDTA and stored at –80°C. The protein concentration was estimated using a dye binding assay with BSA as standard. The level of endotoxin contamination was quantified using an end-point chromogenic microplate assay (Chromogenix, Molndal, Sweden) with *E. coli* O111:B4 endotoxin as a standard. The endotoxin content of the purified recombinant proteins used for these experiments was <2 ng/ml for stock solutions. Quantitative IAV cultures of lung homogenates were performed 3 days after intranasal inoculation of mice with IAV, followed by intratracheal inoculation with PBS or SP-D (5 μg).

Bronchoalveolar lavage (BAL)

Lung cells were recovered by BAL. Animals were sacrificed as described for viral clearance, and lungs were lavaged three times with 1 ml sterile PBS. The fluid was centrifuged at 2000 rpm for 10 min and resuspended in 600 μl PBS, and total cells were stained with trypan blue and counted under light microscopy. Differential cell counts were performed on cyto-spin preparations stained with Diff-Quick (Dade Behring, Newark, DE).

Cytokine production

Lung homogenates were centrifuged at 2000 rpm, and the supernatants were stored at –20°C. TNF-α, IL-1β, IL-6, and macrophage inflammatory protein 2 (MIP-2) were quantitated using murine sandwich ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. All plates were read on a microplate reader (Molecular Devices, Menlo Park, CA) and analyzed with the use of a computer-assisted analysis program (Softmax, Molecular Devices). Only assays having standard curves with a calculated regression line value >0.95 were accepted for analysis.

Phagocytosis of IAV

Phagocytosis of IAV by macrophages *in vivo* was measured by intranasally infecting mice with FITC-labeled IAV, followed by evaluation of cell-associated fluorescence with a flow cytometer. Two hours after infection, macrophages from BAL fluid (BALF) were incubated in buffer (PBS, 0.2% BSA fraction V, and 0.02% sodium azide) with PE-conjugated murine CD16/CD32 Abs (BD Pharmingen, San Diego, CA) for 1 h on ice and washed twice in fresh buffer. Trypan blue (0.2 mg/ml) was added to quench fluorescence of extracellular FITC. Cell-associated fluorescence was measured on a FACScan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA). For each sample of macrophages, 20,000 cells were counted in duplicate, and the results are expressed as the percentage of macrophage phagocytosis.

CD4 and CD8 T lymphocytes in BALF

CD4 and CD8 T lymphocytes were measured after intranasal IAV infection and staining of cells in BALF with fluorescent Abs, followed by evaluation of cell-associated fluorescence by flow cytometry. Three days after infection, cells from BALF were incubated in buffer (PBS, 0.2% BSA fraction V, 0.02% sodium azide) with rat anti-mouse CD16/CD32 Abs (Fc Block), FITC-conjugated mouse CD4, and PE-conjugated mouse CD8 Abs (BD Pharmingen) for 1 h on ice and washed twice in fresh buffer. Cell-associated fluorescence was measured on a FACScan flow cytometer using CellQuest software (BD Biosciences). For each sample 20,000 cells were counted, and the results are expressed as the percentage of CD4 and CD8 T lymphocytes in BALF.

Neutrophil myeloperoxidase (MPO) activity

MPO activity was measured in BAL neutrophils and whole lung 3 days after intranasal infection with IAV at a concentration of 10⁶ ff. A higher concentration of virus was used to provide adequate neutrophils to study. BALF from three wild-type mice was pooled to provide sufficient neutrophils, whereas a single SP-D^{–/–} mouse was used. Blood obtained from uninfected SP-D^{+/+} mice was separated on a gradient of neutrophil isolation medium (NIM-1, Cardinal Associates, Santa Fe, NM) to isolate blood neutrophils. Neutrophils were added to homogenate buffer (100 mM sodium acetate (pH 6.0), 20 mM EDTA (pH 7.0), 1% hexadecyl trimethylammonium bromide) in a 96-well microtiter plate in a final volume of 50 μl. The neutrophil mixtures were incubated at 37°C for 1 h to lyse the neutrophils and allow release of MPO from the granules. Assay buffer (100 μl) containing 1 mM H₂O₂, 1% homogenate buffer (see above), and 3.2 mM 3,3',5,5'-tetramethylbenzidine was added to each well, and readings were taken at 650 nm using a Thermomax microplate reader (Molecular Devices) for a period of 4 min. Lungs were harvested, weighed, and homogenized in 3 ml homogenate buffer, sonicated for 15 s, then centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatants were diluted 1/15 in the homogenate buffer, mixed with an equal volume of assay buffer, and read at 650 nm over 4 min. Readings were the average of at least three individual wells, and MPO activity was reported as maximum MPO activity per 4 min per 3 × 10³ neutrophils or MPO activity per gram of lung for isolated neutrophils or whole lung, respectively.

SP-D concentrations

Concentrations of SP-D in lung homogenates were determined by ELISA. Three and 5 days after infection with IAV, lungs from infected and uninfected wild-type mice were removed and homogenized in 2 ml PBS. SP-D concentrations were measured in a double-Ab ELISA using rabbit and guinea pig anti-SP-D sera. Each assay plate included a standard curve generated with purified mouse SP-D. All samples were run in duplicate, and the concentrations of the samples were calculated by graphing absorbance vs concentrations of the standard.

Statistical methods

Lung viral titers, total cell counts, cytokines, MPO activity, and SP-D levels were compared using ANOVA and Student's *t* test. Findings were considered statistically significant at $p < 0.05$.

Results

Pulmonary pathology after IAV administration

Intranasal administration of IAV (10^5 ff) was well tolerated. Mice infected with IAV lost weight during the 4 days after infection. The percentage of weight loss was greater for the SP-D^{-/-} mice with $14.4 \pm 1.4\%$ compared with $1.5 \pm 1.0\%$ for the SP-D^{+/+} mice (mean \pm SEM; $n = 6$ mice; $p < 0.05$ compared with SP-D^{+/+} mice). Increased total cell counts in BALF were observed in SP-D^{-/-} mice 3 and 5 days after IAV infection (Fig. 1). Baseline total cell counts in BAL fluid from controls inoculated with PBS (days 3 and 5) or uninfected controls were not significantly different for the SP-D^{+/+} and SP-D^{-/-} mice (Fig. 1). A significantly greater percentage of polymorphonuclear leukocytes was detected in BAL fluid from SP-D^{-/-} compared with SP-D^{+/+} mice 3 and 5 days postinfection (Fig. 1).

Viral clearance in SP-D^{-/-} mice

Quantitative IAV cultures of lung homogenates were performed 3, 5, 7, and 10 days after inoculation of the animals with IAV. In-

creased viral titers of IAV (Phillipines/82) were observed in the lungs of SP-D^{-/-} mice 3, 5, 7, and 10 days after infection compared with SP-D^{+/+} mice (Fig. 2). Intratracheal coadministration of recombinant SP-D ($5 \mu\text{g}$) enhanced clearance of A/Phillipines/82 (H₃N₂) (Phil/82) virus from the lung of SP-D^{-/-} compared with untreated SP-D^{-/-} mice (Fig. 2).

Strains of influenza virus differ in the extent of glycosylation of surface glycoproteins. To examine whether strain-dependent changes in glycosylation influence SP-D-dependent clearance, infection by Mem71_{H-Bel_N} (H3N1; Mem71) was compared with that by Phil/82. Previous studies have shown that Mem71, which has a less glycosylated hemagglutinin than Phil/82, is relatively resistant to the effects of SP-D in vitro (25). Mem71 shows less SP-D-dependent viral agglutination and hemagglutination inhibition, decreased enhancement of neutrophil uptake and activation, and decreased inhibition of infectivity compared with SP-D-sensitive viral strains. Significantly, titers in the lung of Mem71 were similar for SP-D^{-/-} ($6.8 \times 10^3 \pm 1.9 \times 10^3$ ff/g lung) and SP-D^{+/+} ($5.9 \times 10^3 \pm 1.4 \times 10^3$) mice 3 days after viral infection (mean \pm SEM; eight mice per group).

Cytokine levels in lung homogenates

Three and 5 days after IAV infection, proinflammatory cytokines TNF- α , IL-1 β , and IL-6 were significantly increased in lung homogenates from SP-D^{-/-} compared with SP-D^{+/+} mice (Fig. 3). IFN- γ was increased in the lungs of SP-D^{-/-} mice compared with SP-D^{+/+} mice after IAV infection. Lungs from the SP-D^{-/-} mice had the greatest concentration of IFN- γ 5 days after IAV infection

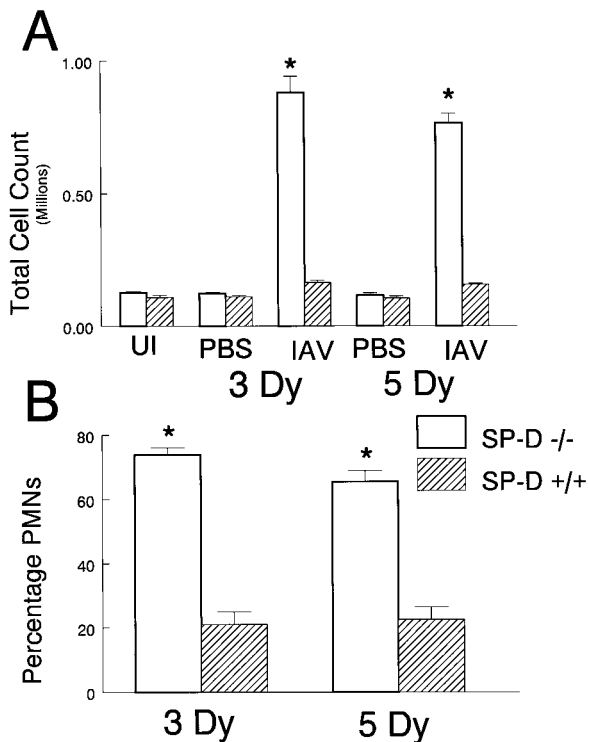


FIGURE 1. Increased total cell counts and neutrophils in BAL fluid from SP-D^{-/-} mice. Lung cells were recovered by BAL, stained with trypan blue, and counted under light microscopy. Cytospin preparations of BALF were stained with Diff-Quik to identify macrophages, lymphocytes, and polymorphonuclear leukocytes (PMNs). Baseline total cell counts from uninfected (UI) or PBS-inoculated (3 and 5 days (Dy)) controls were not significantly different in SP-D^{-/-} (□) and SP-D^{+/+} (▨) mice (A). SP-D^{-/-} mice had increased total cell counts in BAL fluid 3 and 5 days after IAV infection compared with SP-D^{+/+} mice (A). The percentage of neutrophils in BAL fluid was significantly greater 3 and 5 days after administration of 10^5 ff IAV to SP-D^{-/-} (□) compared with SP-D^{+/+} (▨) mice (B). Data are means \pm SEM ($n = 8$ mice/group). *, $p < 0.05$ compared with SP-D^{+/+} mice.

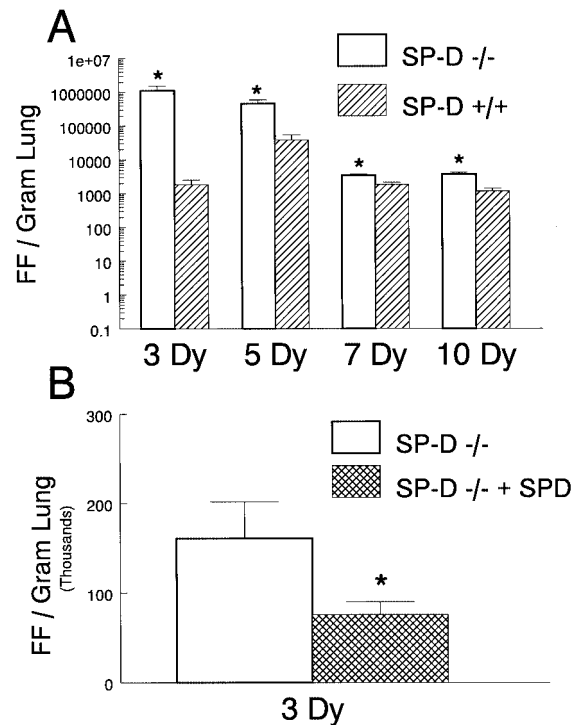


FIGURE 2. Increased viral titers in lung homogenates from SP-D^{-/-} mice. IAV titers were determined by quantitative plaque assays of lung homogenates. Viral titers of IAV were significantly greater 3, 5, 7, and 10 days (Dy) after administration of 10^5 ff IAV for SP-D^{-/-} (□) compared with SP-D^{+/+} (▨) mice (A). Data are means \pm SEM ($n = 10$ mice/group). *, $p < 0.05$ compared with SP-D^{+/+} mice. Intratracheal administration of recombinant SP-D ($5 \mu\text{g}$) enhanced clearance of IAV virus from the lung of SP-D^{-/-} (▨) compared with untreated SP-D^{-/-} (□) mice (B). Data are means \pm SEM of 10 mice/group. *, $p < 0.05$ compared with untreated SP-D^{-/-} mice.

with 91 ± 20 and 2398 ± 176 pg/ml for SP-D^{+/+} and SP-D^{-/-} mice, respectively (mean \pm SEM; $p < 0.05$). MIP-2, a neutrophil chemoattractant, was significantly greater in lung homogenates from SP-D^{-/-} mice after viral infection (Fig. 3). Intratracheal treatment with SP-D significantly reduced TNF- α and IL-6 levels in the lung (Fig. 3). Basal cytokine levels in the lungs of control mice inoculated with sterile PBS were low or absent and were not different in SP-D^{-/-} and SP-D^{+/+} mice (data not shown).

Macrophages phagocytosis of IAV

Phagocytosis of FITC-labeled IAV by alveolar macrophages was assessed by flow cytometry. Uptake of virus was similar in SP-D^{+/+} and SP-D^{-/-} mice (11.1 ± 1.9 and $9.4 \pm 1.9\%$ phagocytosis, respectively) 2 h after IAV infection (mean \pm SEM). These results suggest that macrophage phagocytosis of IAV is not a major contributor to the decreased clearance of IAV seen in the absence of SP-D in vivo.

CD4 and CD8 T lymphocytes in BALF

Three days after IAV infection, CD4 (Th lymphocytes) and CD8 (CTL) cells were measured in BALF. There was no difference in the percentages of CD4 and CD8 T lymphocytes in BALF between SP-D^{-/-} and SP-D^{+/+} mice (Fig. 4). The fractions of CD4 and CD8 T lymphocytes in BALF were similar in uninfected SP-D^{+/+} and SP-D^{-/-} mice (Fig. 4).

Decreased neutrophil MPO activity in SP-D^{-/-} mice

MPO is stored in specific granules of neutrophils. Neutrophil accumulation in the lung was quantitated by measuring MPO activity in lung homogenates, and neutrophil function was assessed by measuring levels of MPO associated with neutrophils recovered in lung lavage. Although MPO activity was greater in the lungs of SP-D^{-/-} mice after IAV infection, MPO activity from isolated

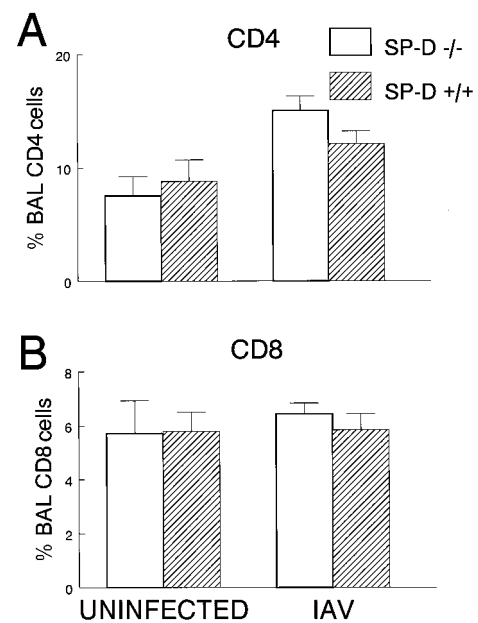


FIGURE 4. CD4 and CD8 T lymphocytes in BALF after IAV infection. Three days after IAV infection, CD4 and CD8 T lymphocyte subsets were measured in BALF by flow cytometry with FITC-conjugated mouse CD4 and PE-conjugated mouse CD8 Abs. There was no difference in the percentages of CD4 (graph A) and CD8 (graph B) T lymphocytes in BALF between SP-D^{-/-} (□) and SP-D^{+/+} (▨) mice. CD4 and CD8 T lymphocytes in BALF were similar in uninfected SP-D^{+/+} and SP-D^{-/-} mice. Data are the mean \pm SEM ($n = 8$ mice/group). *, $p < 0.05$ compared with SP-D^{+/+} mice.

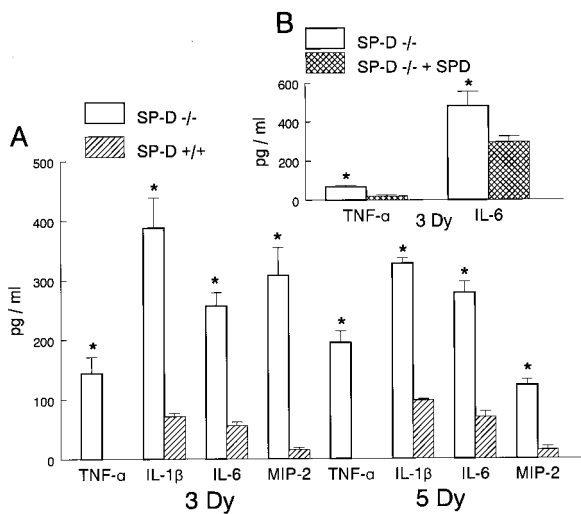


FIGURE 3. Increased proinflammatory cytokines in lung homogenates from SP-D^{-/-} mice after IAV infection. Concentrations of TNF- α , IL-1 β , IL-6, and MIP-2 were assessed in lung homogenates from SP-D^{-/-} (□) and SP-D^{+/+} (▨) mice. Increased concentrations of the proinflammatory cytokines TNF- α , IL-6, IL-1 β , and MIP-2 were found in lung homogenates from SP-D^{-/-} mice 3 and 5 days (Dy) after IAV infection (A). Data are expressed as picograms per milliliter and are means \pm SEM of 10 mice/group. *, $p < 0.05$ compared with SP-D^{+/+} mice. Intratracheal treatment with SP-D significantly reduced TNF- α and IL-6 levels in the lungs of treated SP-D^{-/-} (▨) compared with untreated SP-D^{-/-} (□) mice (B). Data are expressed as picograms per milliliter and are means \pm SEM of 10 mice/group. *, $p < 0.05$ compared with untreated SP-D^{-/-} mice.

BAL neutrophils was significantly decreased in SP-D^{-/-} compared with SP-D^{+/+} mice (Fig. 5). Control neutrophils isolated from the blood of uninfected SP-D^{+/+} mice had significantly greater MPO activity compared with BAL neutrophils from IAV-infected SP-D^{-/-} mice and significantly less MPO activity compared with BAL neutrophils from IAV-infected SP-D^{+/+} mice (Fig. 5).

IAV infection enhances SP-D accumulation in the lung

Concentrations of SP-D in lung homogenates were increased approximately 2-fold 3 days following IAV infection in SP-D^{+/+} mice (Fig. 6). Five days after IAV infection, SP-D concentrations in the lung of infected SP-D^{+/+} mice decreased to concentrations similar to those in uninfected SP-D^{+/+} mice.

Discussion

Pulmonary clearance of intranasally administered Phil/82 strain of IAV was reduced in SP-D^{-/-} mice compared with SP-D^{+/+} mice. However, the less glycosylated strain Mem71, which is relatively resistant to SP-D in vitro, was cleared efficiently from the lungs of SP-D^{-/-} mice. In addition, the coadministration of recombinant SP-D normalized viral clearance. Thus, the impaired clearance of IAV can be directly attributed to the deficiency of SP-D rather than to other aspects of the SP-D null phenotype or more global host defense deficits. In this regard we have previously demonstrated that SP-D null mice show no impairment of clearance of group B streptococcus and *Hemophilus influenzae* (26). Pulmonary inflammation was increased in SP-D-deficient mice compared with wild-type controls, as indicated by increased total cell counts and proinflammatory cytokines in the lung after IAV infection. Neutrophil MPO activity was decreased in SP-D^{-/-} mice, suggesting that neutrophil clearance of IAV may be impaired. Pulmonary IAV

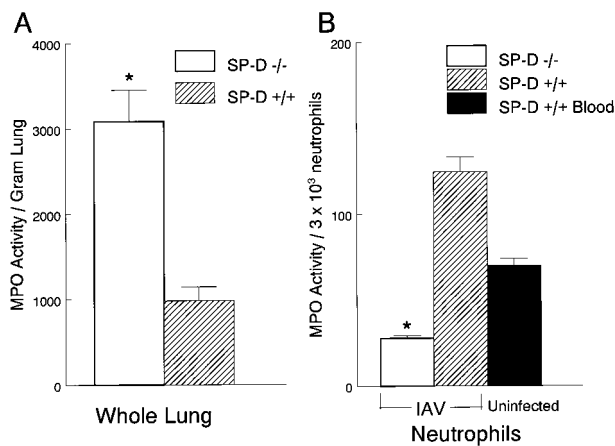


FIGURE 5. Neutrophil MPO activity was decreased in SP-D^{-/-} mice. MPO activity was measured in whole lung or BAL neutrophils 3 days after intranasal infection with IAV at a concentration of 10⁶ ff. Lung MPO activity was measured to estimate total neutrophil influx in the lung. MPO activity was greater in lung homogenates from SP-D^{-/-} (□) compared with SP-D^{+/+} (▨) mice (A). Isolated blood neutrophils from uninfected wild-type mice were used as controls. Neutrophils were lysed to allow release MPO from the granules, and MPO activity was measured as described in *Materials and Methods*. MPO activity in BAL neutrophils was significantly decreased in SP-D^{-/-} compared with SP-D^{+/+} mice 3 days after IAV infection. Blood neutrophils from uninfected SP-D^{+/+} (■) mice had significantly greater MPO activity compared with SP-D^{-/-} BAL neutrophils and less MPO activity compared with SP-D^{+/+} BAL neutrophils after infection (B). Data are means ± SEM (*n* = 8 mice/group). *, *p* < 0.05 compared with SP-D^{+/+} mice.

infection increased SP-D concentrations in wild-type mice. These findings demonstrate that SP-D plays an important role in the initial pulmonary host defense against certain strains of IAV *in vivo*.

Impaired clearance of IAV from the lungs of SP-D^{-/-} mice supports the importance of SP-D in host defense. SP-D is a member of the C-type lectin family of polypeptides that includes mannose binding protein, conglutinin, and SP-A. C-type lectins share structural features, including collagenous N-terminal and globular

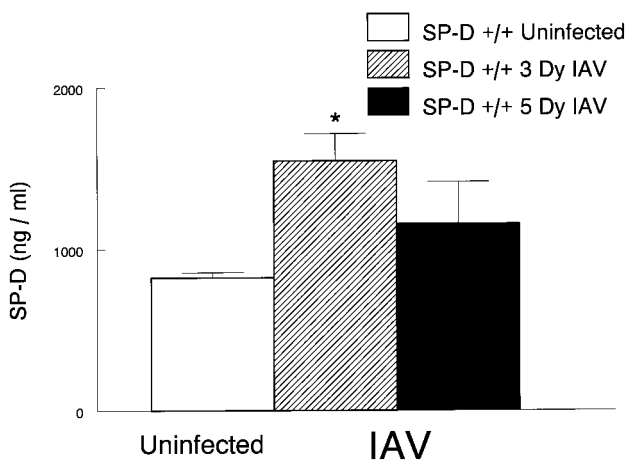


FIGURE 6. Increased SP-D concentrations in the lung following IAV infection. Concentrations of SP-D in lung homogenates were determined with an ELISA. Three days (Dy) after IAV infection, SP-D concentrations in the lung of SP-D^{+/+} (▨) mice were significantly greater than those in uninfected SP-D^{+/+} (□) mice. Five days after IAV infection, SP-D concentrations in the lung of SP-D^{+/+} mice (■) decreased to levels similar to those in uninfected SP-D^{+/+} mice. Data are means ± SEM (*n* = 10 mice/group). *, *p* < 0.05 compared with uninfected SP-D^{+/+} mice.

C-terminal domains, the latter serving as a carbohydrate recognition domain that functions in opsonization. In the presence of calcium, SP-D binds to a variety of glycoconjugates, including di- and monosaccharides such as maltose, glucose, and mannose (2). Influenza virus has two membrane glycoproteins, hemagglutinin and neuraminidase. Collectins bind to oligosaccharides on influenza virus glycoproteins and neutralize virus infectivity *in vitro*; more heavily glycosylated strains of viruses are the most sensitive (27). SP-D may enhance viral clearance by binding to the carbohydrate side chain of IAV, blocking access of cell surface receptors to the receptor binding site and thus interfering with virus internalization by host cells, or it may inhibit viral replication at a later stage within the cell. In addition, SP-D binds and agglutinates IAV, which may in part enhance viral removal from the lung through mucociliary and phagocytic clearance. However, the finding that uptake of the virus by alveolar macrophages is not reduced suggests that SP-D binding, aggregation, and uptake by the alveolar macrophages are not critical determinants of the decreased viral killing noted in SP-D^{-/-} mice.

Phagocytosis of IAV by alveolar macrophages was similar for SP-D^{-/-} and wild-type mice *in vivo*. Since macrophage phagocytosis is part of the early, nonspecific immune response, an early time point was chosen to assess macrophage phagocytosis; however, the optimal time point for assessing viral phagocytosis by macrophages is unknown. In addition, large quantities of ingested FITC-labeled virus are necessary to detect macrophage fluorescence. As indicated in the introduction, previous studies have suggested that SP-D does not enhance the uptake of some strains of IAV by alveolar macrophages *in vitro* (22). In the absence of SP-D, macrophage phagocytosis of IAV was similar to that in wild-type mice, suggesting that SP-D is not a critical determinant for macrophage clearance of IAV *in vivo*.

After IAV infection, markers of inflammation, including inflammatory cells and cytokines, were increased in the lungs of SP-D^{-/-} mice, and exogenous recombinant SP-D reduced IAV-induced cytokine production. SP-D^{-/-} mice are able to mount an immune response to IAV infection; however, the response is greatly increased compared with that of wild-type controls. Increased cytokine production may reflect increased cells in BALF after viral infection. Uninfected SP-D^{-/-} mice have modestly increased numbers of alveolar macrophages in the lung; however, proinflammatory cytokine concentrations are not substantially increased (28). Increased cytokines, TNF- α , IL-1 β , IL-6, and IFN- γ , have been demonstrated in a mouse model of IAV infection associated with lymphocytic and mononuclear infiltrates in the lung (29). The cytokine response to IAV was similar in SP-D^{-/-} mice with elevated TNF- α , IL-1 β , IL-6, and IFN- γ levels; however, pulmonary cytokine responses to IAV were significantly greater in SP-D^{-/-} mice than in wild-type mice. Augmented inflammatory responses have also been observed following bacterial challenge (26). These findings are consistent with our general hypothesis that SP-D plays important anti-inflammatory roles *in vivo*. It is possible that these effects serve to minimize collateral damage to lung tissue while enhancing uptake or clearance.

Cytotoxic T cells play an important role in IAV clearance from the lung by direct cytolysis of virus-infected cells (18). *In vitro*, SP-D inhibits IL-2-dependent, mitogen-stimulated, T lymphocyte proliferation (30). In the absence of SP-D, the percentages of CD4 and CD8 T lymphocytes in BALF were similar to those in wild-type mice after IAV infection. The current study examined the number of T lymphocytes present in BAL fluid following IAV infection; however, the function and activation state of the T lymphocytes were not examined. In addition, an early time point was chosen to examine T lymphocytes in BAL fluid, which may have

failed to recognize the difference in the specific T lymphocyte response that occurs later after IAV infection.

After IAV infection, pulmonary neutrophil accumulation was greater in SP-D^{-/-} mice than in wild-type mice. However, neutrophil MPO activity normalized per cell was decreased after IAV infection in SP-D-deficient mice. Because the levels were normal in blood neutrophils, it is likely that the recruited cells have undergone a greater degree of degranulation in response to the viral challenge in the absence of SP-D. Defects in neutrophil chemotactic, oxidative, and bacterial killing functions have been documented in IAV infection (31). In addition, it has been shown in animal models that there is a correlation between impairment of the function of these cells and predisposition to bacterial superinfection (32). SP-D enhances the uptake and specific oxidative response to internalized virus in vitro. In addition, SP-D decreases the inhibitory effects of IAV on neutrophil respiratory burst responses (14). Although the effects of SP-D and IAV on neutrophil degranulation and MPO activity have not yet been characterized in vitro, the findings emphasize the potential importance of neutrophils for the initial host response to IAV and suggest that SP-D may alter the neutrophil response to internalized virus.

In summary, in the absence of SP-D, IAV viral clearance from the lung was impaired. Lung inflammation was more severe in SP-D^{-/-} mice, suggesting that SP-D plays a role in modulating cytokine production and inflammatory responses during viral infection. In addition, SP-D binds and agglutinates IAV that may also play a role by enhancing IAV removal from the lung through mucociliary clearance and enhanced recruitment and activation of polymorphonuclear leukocytes. Since the airway is the usual portal of entry for influenza virus and other respiratory pathogens, the local production of SP-D is likely to play a role in innate defense responses to inhaled viruses.

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