Absence of SP-A modulates innate and adaptive defense responses to pulmonary influenza infection

ANN MARIE LeVINE,1,2 KEVAN HARTSHORN,3 JAMES ELLIOTT,1 JEFFREY WHITSETT,1 AND THOMAS KORFHAGEN1
1Divisions of Pulmonary Biology and 2Critical Care Medicine, Cincinnati, Ohio 45229-3039; and 3Department of Medicine and Pathology, Boston University School of Medicine, Boston, Massachusetts 02118

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LeVine, Ann Marie, Kevan Hartshorn, James Elliott, Jeffrey Whitsett, and Thomas Korfhagen. Absence of SP-A modulates innate and adaptive defense responses to pulmonary influenza infection. Am J Physiol Lung Cell Mol Physiol 282:L563–L572, 2002;10.1152/ajplung.00280.2001.—Mice lacking surfactant protein-A [SP-A(+/−)] and wild type SP-A(+/+) mice were infected with influenza A virus (IAV) by intranasal instillation. Decreased clearance of IAV was observed in SP-A(−/−) mice and was associated with increased pulmonary inflammation. Treatment of SP-A(−/−) mice with exogenous SP-A enhanced viral clearance and decreased lung inflammation. Uptake of IAV by alveolar macrophages was similar in SP-A(−/−) and SP-A(+/+) mice. Myeloperoxidase activity was reduced in isolated bronchoalveolar lavage neutrophils from SP-A(−/−) mice. B lymphocytes and activated T lymphocytes were increased in the lung and spleen, whereas T helper (Th) 1 responses were increased [interferon-γ, interleukin (IL)-2, and IgG2a] and Th2 responses were decreased (IL-4, and IL-10, and IgG1) in the lungs of SP-A(−/−) mice 7 days after IAV infection. In the absence of SP-A, impaired viral clearance was associated with increased lung inflammation, decreased neutrophil myeloperoxidase activity, and increased Th1 responses. Because the airway is the usual portal of entry for IAV and other respiratory pathogens, SP-A is likely to play a role in innate defense and adaptive immune responses to IAV.

In the human lung, SP-A is expressed in alveolar type II cells, serous cells in tracheobronchial glands, and in nonciliated bronchiolar cells (17). In vitro, SP-A stimulates macrophage chemotaxis (35) and enhances the binding of bacteria and viruses to alveolar macrophages (34). SP-A enhances macrophage phagocytosis of herpes simplex virus type 1 (HSV-1; see Ref. 31) and binds to HSV-1-infected cells (32). Mannose-binding lectin, conglutinin, SP-A, and SP-D neutralize influenza virus (2, 5, 11, 13, 14), although neutralization by SP-A occurs through binding of the viral hemagglutinin (HA) to sialic acid on the SP-A molecule rather than through carbohydrate-binding activity of SP-A (5).

Influenza A virus (IAV) infection is acquired primarily by inhalation, generally causing infection of the upper respiratory tract. During infection, virus spreads to the lower respiratory tract and may result in pneumonia. Influenza infections are most frequent in children and young adults. Deaths from IAV infection occur most frequently in the very young (<1 yr), the elderly, and persons of all ages with underlying heart or lung disease (26). Prematurity has been associated with decreased SP-A levels in bronchoalveolar lavage fluid (BALF; see Ref. 8). Bronchopulmonary dysplasia and cystic fibrosis have been associated with decreased SP-A concentrations in the lung (3, 25), conditions that may increase susceptibility to infection by respiratory viruses such as IAV. In addition, viral pneumonia has been associated with decreased SP-A in BALF (23), which may further exacerbate the viral infection and increase susceptibility to bacterial superinfection.

Specific and nonspecific immune mechanisms take part in the immune response to influenza virus. IAV is a lytic infection and causes the breakdown of the blood-tissue barrier early in infection, resulting in the influx of macrophages, neutrophils, and natural killer (NK) cells into the lung. Specific immune responses to IAV are initiated by the influx of virus-specific T lymphocytes and antibody production. Cytotoxic T lympho-
cytes (CTL) are thought to be involved in viral clearance by direct cytolysis of virus-infected cells (37). Defects in neutrophil and monocyte chemotactic, oxidative, and bacterial killing functions have been documented in IAV infection (10, 18). In vitro, SP-A enhanced uptake of IAV by neutrophils; however, SP-A did not protect neutrophils from the inhibitory effects of IAV on the respiratory burst (15).

In spite of considerable in vitro evidence that SP-A is involved in innate host defense, its role in vivo has only recently been demonstrated. SP-A-deficient [SP(−/−)] mice produced by targeted gene inactivation are susceptible to bacterial and respiratory syncytial virus pneumonia (20, 21). In the present study, SP-A(−/−) mice were infected intranasally with IAV. Clearance of IAV was delayed, and lung inflammation increased in SP-A(−/−) mice in vivo.

METHODS

Animal husbandry. The murine SP-A gene locus was targeted by homologous recombination as previously described; the lungs of SP-A(−/−) mice were lacking detectable SP-A (20). SP-A(−/−) and SP-A-sufficient [SP-A(+/+)] mice were maintained in strain 129. Animals were housed and studied under Institutional Animal Care and Use Committee-approved protocols in the animal facility of the Children’s Hospital Research Foundation (Cincinnati, OH). Male and female mice of ~20–25 g (42–56 days old) were used.

Preparation of IAV. IAV strain H3N2 A/Philadelphia/82 (Phil/82) (H3N2) was a gracious gift from E. M. Anders (University of Melbourne, Melbourne, Australia) and was 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 (11). Virus stocks were dialyzed against PBS, separated into aliquots, and stored at −70°C until used. HA titers were determined by titration of virus samples in PBS followed by the addition of thoroughly washed human erythrocytes. The potency of each viral stock was measured by HA inhibition assay (Sigma, St. Louis, MO) according to the manufacturer’s directions. Quantitative IAV cultures of lung homogenates were performed 3 days after intranasal inoculation of mice with IAV following immediately by intratracheal inoculation with PBS or SP-A (100 μg). Because previous studies demonstrated that 100 μg SP-A restored resistance to bacterial pneumonia in SP-A(−/−) mice (22), this dose was used for the present study.

Bronchoalveolar lavage. Lung cells were recovered by bronchoalveolar lavage (BAL). Animals were killed as described for viral clearance, and lungs were lavaged three times with 1 ml of sterile PBS at 2,000 rpm for 10 min and resuspended in 600 μl of PBS; total cells were stained with trypan blue and counted under light microscopy. Differential cell counts were performed on cytoospin preparations stained with Diff-Quick (Scientific Products, McGaw Park, IN).

Cytokine production. Lung homogenates were centrifuged at 2,000 rpm, and the supernatants were stored at −20°C. Tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, macrophage inflammatory protein (MIP)-2, and interferon (IFN)-γ were quantitated 3 and 5 days after IAV infection. IL-2, IL-4, IL-10, and IL-12 were measured 7 days after IAV infection using murine sandwich enzyme-linked immunosorbent assay (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 with 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 by flow cytometry. After infection (2 h), macrophages from BALF were incubated in buffer (PBS, 0.2% BSA fraction V, and 0.02% sodium azide) with phycoerythrin-conjugated murine CD16/CD32 antibodies (PharMingen, San Diego, CA) for 1 h on ice and washed two times in fresh
buffer. Trypan blue (0.2 mg/ml) was added to quench fluorescence of extracellular cell. Cell-associated fluorescence was measured on a FACSScan flow cytometer using CELLQuest software (Becton-Dickinson, San Jose, CA). For each sample of macrophages, 20,000 cells were counted in duplicate, and the results were expressed as the percentage of macrophages with label.

**Lymphocytes in BALF.** Lymphocytes in BALF were measured after intranasal IAV infection, staining of cells with fluorescent antibodies, and evaluation of cell-associated fluorescence by flow cytometry. Cells from BALF were incubated in fluorescence-activated cell sorter (FACS) buffer (PBS, 0.2% BSA fraction V, and 0.02% sodium azide) with rat anti-mouse CD16/CD32 antibodies (Fc Block), and separate aliquots were stained with FITC-conjugated mouse CD4 (T-helper lymphocytes), CD8 (CTL), CD19 (B lymphocytes), and CD56 (NK cells) antibodies (PharMingen) for 1 h on ice. Specific markers on T lymphocytes were evaluated by double staining with phycoerythrin-conjugated mouse CD3 (T cell receptor) and FITC conjugate mouse CD6 (T cell activation marker) or CD6 (Fc receptor) antibodies (PharMingen), and a lymphocyte gate was used. Cell-associated fluorescence was measured on a FACSscan flow cytometer using CELLQuest software (Becton-Dickinson). For each sample, 10,000 events were analyzed, and the results were expressed as the percentage of CD4+, CD8+, CD19+, and CD56+ lymphocytes in BALF or the percentage of CD3+ cells expressing CD6 or CD16.

**Isolation of spleen cells.** Seven days after IAV infection, mouse spleens were removed, passed through a 70-μm nylon cell strainer (Fisher Scientific, Pittsburgh, PA) in 10 ml of fresh Hank’s balanced salt solution with 1% FCS and 10 mM HEPES, aspirated through a 21-gauge needle, and centrifuged at 800 g for 5 min at 4°C. Erythrocytes in the pellet were lysed with erythrocyte lysis buffer (Life Technologies, Rockville, MD), and spleen cells were resuspended in FACS buffer. Spleen lymphocytes were stained as described for BALF lymphocytes, and fluorescent staining was measured by flow cytometry.

**Cell-mediated cytotoxicity assay.** Cytotoxicity of splenic lymphocytes was measured using the CytoTox96 cytotoxicity assay (Promega, Madison, WI), a colorimetric assay that measures lactate dehydrogenase (LDH) release from lysed cells. EL4 cells (H-2b; ATCC, Manassas, VA) were grown in RPMI with 10% FCS and 10 mM HEPES, aspirated through a 21-gauge needle, and centrifuged at 3,500 rpm for 5 min, and serum was collected and stored at −20°C. Concentrations of total immunoglobulin, IgM, and the IgG subclasses IgG1 and IgG2a in mouse serum were measured by an ELISA using an isotype-specific kit (Southern Biotechnology, Birmingham, AL) with sensitivities of 2 μg/ml. Ninety-six-well plates were coated with 10 μg of anti-mouse immunoglobulin overnight and then blocked for 1 h at room temperature with 1% BSA. Serum samples were equalized for total protein, diluted (1:1,000 or 1:2,000) in PBS, and added to the plate. Alkaline phosphatase-labeled isotype antibodies were used for detection, and standard curves (31 ng to 4 μg total protein) were generated for each isotype. All samples were run in duplicate, and the concentrations of the samples were calculated by graphing absorbance vs. concentrations of the standard.

**Neutrophil myeloperoxidase activity.** Myeloperoxidase (MPO) activity was measured in BAL neutrophils 3 days after intranasal infection with IAV at a concentration of 10⁵ 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-A(−/−) mouse was used. Blood obtained from uninjected SP-A(−/−) and SP-A(+/+ ) mice was separated on a gradient of neutrophil isolation medium (NIM-1; Cardinal Associates, Santa Fe, NM) to isolate blood neutrophils and was assayed after stimulation with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) or was left unstimulated. Neutrophils were added to homogenate buffer [100 mM sodium acetate (pH 6.0), 20 mM EDTA (pH 7.0), and 1% hexadecyltrimethylammonium bromide (HETAB)] 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% HETAB, 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 for a period of 4 min. Readings were the average of at least three individual wells, and MPO activity was reported as maximum MPO activity per min per 3 × 10⁶ neutrophils.

**SP-D concentrations.** Concentrations of SP-D in BALF were determined with an ELISA. After IAV infection (5 days), lungs from infected and uninfected SP-A(+/+) and SP-A(−/−) mice were lavaged with 2 ml of sterile saline. SP-D concentrations were measured in a double-antibody 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, immunoglobulins, and MPO activity were compared using ANOVA and Student’s t-test. Findings were considered statistically significant at probability levels <0.05.

**RESULTS**

**Pulmonary pathology after IAV administration.** Intranasal administration of IAV (10⁵ ff) was well tolerated, and all animals survived the study period. Mice infected with IAV had weight loss over 4 days postinfection, with 1.5 ± 1.0 and 3.6 ± 1.5% weight loss in the SP-A(+/+) and SP-A(−/−) mice, respectively (means ± SE). SP-A(−/−) mice had increased total cell counts in BALF 3 and 5 days after IAV infection (Fig. 1). Baseline total cell counts in BALF from controls inoculated with PBS were 9.6 ± 1.1 × 10⁶ and 8.8 ± 0.4 × 10⁵ for the SP-A(+/+) and SP-A(−/−) mice, respectively (means ± SE). A significantly greater percentage of polymorphonuclear neutrophils was detected in BALF...
SP-A(-/-) MICE AND INFLUENZA

from SP-A(-/-) compared with SP-A(+/+) mice 3 and 5 days postinfection (Fig. 1). Pulmonary inflammation was not observed in wild-type mice inoculated with sterile PBS (n = 5; data not shown).

Decreased viral clearance in SP-A(-/-) mice. Quantitative IAV cultures of lung homogenates were performed 3 and 5 days after inoculation of the animals with IAV. SP-A(-/-) mice had significantly increased viral titers of IAV in the lung 3 and 5 days after infection compared with SP-A(+/+) mice (Fig. 1). Systemic dissemination of IAV was assessed by quantitative culture of the spleen, and no IAV was isolated from the spleens of either group 5 days after IAV infection.

SP-A inhibits hemagglutination activity and virus infectivity in vitro. The hemagglutination inhibition assay was used to test the ability of the human SP-A used for treatment in vivo to inhibit HA activity of the Phil/82 strain of IAV in vitro. SP-A at a concentration of 812 ± 187 ng/ml completely inhibited HA activity of 40 HA U/ml of the Phil/82 strain of IAV. The foci assay was used to determine the effect of SP-A on the ability of IAV to infect monolayers of MDCK. SP-A at a concentration of 7.2 µg/ml reduced the number of foci to 36 ± 14% of control. These results are comparable to those previously reported using SP-A in vitro (5, 15). Data represent mean ± SE for n = 3 preparations.

Cytokine concentrations in lung homogenates. After IAV infection (3 and 5 days), the proinflammatory cytokines TNF-α, IL-1β, and IL-6 were significantly increased in lung homogenates from SP-A(-/-) compared with SP-A(+/+) mice (Fig. 2). IFN-γ was also increased in the lungs of SP-A(-/-) mice after viral infection. Concentrations of IFN-γ 5 days after IAV infection were 91 ± 20 and 696 ± 82 pg/ml for SP-A(+/+) and SP-A(-/-) mice, respectively (P < 0.05). MIP-2, a neutrophil chemoattractant, was significantly increased in lung homogenates from SP-A(-/-) mice after viral infection (Fig. 2).

Exogenous SP-A increased viral clearance and decreased lung inflammation in SP-A(-/-) mice. After infection (3 days), the clearance of IAV in the SP-A(-/-) mice was significantly enhanced when IAV was coadministered with SP-A (100 µg). Cytokine levels in lung homogenates (TNF-α, IL-6, and IFN-γ) were significantly reduced in lungs of SP-A(-/-) mice treated with SP-A (Fig. 3).

Macrophage phagocytosis of IAV. Phagocytosis of FITC-labeled IAV by alveolar macrophages was similar in SP-A(+/+) and SP-A(-/-) mice. The percentage of macrophages with fluorescent IAV was 11.1 ± 1.9 and 9.6 ± 2.8% in SP-A(+/+) and SP-A(-/-) mice, respectively, 2 h after IAV infection, suggesting that macrophage phagocytosis of IAV is not a major factor in the decreased clearance of IAV observed in the SP-A(-/-) mice.

CD4+ and CD8+ cells. After IAV infection, CD4+ (helper T lymphocytes, Th) and CD8+ (CTL) cells were measured in BALF and from the spleen. CD4+ cells in BALF were similar in SP-A(-/-) and SP-A(+/+) mice 3 and 5 days after IAV infection; however, 7 days after infection, significantly less CD4+ cells were present in BALF from SP-A(-/-) mice (Fig. 4). The greatest in-

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Fig. 1. Increased total cell counts, neutrophils, and viral titers in lung of surfactant protein-A-deficient [SP-A(-/-)] mice. Lung cells were recovered by bronchoalveolar lavage (BAL), stained with trypan blue, and counted under light microscopy. Cytospin preparations of bronchoalveolar lavage fluid were stained with Diff-Quik to identify macrophages, lymphocytes, and polymorphonuclear (PMN) leukocytes. A: SP-A(-/-) (filled bars) mice had increased total cell counts in BAL fluid (BALF) 3 and 5 days after influenza A virus (IAV) infection compared with wild-type [SP-A(+/+)] mice (hatched bars). B: percentage of neutrophils in BALF was significantly greater 3 and 5 days after administration of 10⁶ fluorescent foci (ff) IAV to SP-A(-/-) compared with SP-A(+/+) mice. C: IAV titers were determined by quantitative plaque assays of lung homogenates. Viral titers of IAV were significantly greater 3 and 5 days after administration of 10⁶ ff IAV for SP-A(-/-) compared with SP-A(+/+) mice. Data are means ± SE with n = 10 mice/group. *P < 0.05 compared with SP-A(+/+) mice on the same day.

Fig. 2. Increased proinflammatory cytokines in lung homogenates from SP-A(-/-) mice after IAV infection. Concentrations of tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6, and macrophage inflammatory protein (MIP)-2 were assessed in lung homogenates from SP-A(-/-) (filled bars) and SP-A(+/+) (hatched bars) mice. Increased concentrations of the proinflammatory cytokines TNF-α, IL-6, IL-1β, and MIP-2 were found in lung homogenates from the SP-A(-/-) mice 3 and 5 days after IAV infection. Data are expressed as pg/ml and represent means ± SE with n = 10 mice/group. *P < 0.05 compared with SP-A(+/+) mice on the same day.
crease in BALF CD8+ cells was observed 7 days after IAV infection for both groups. After infection (3 days), CD8+ cells were increased in BALF from SP-A(−/−) compared with SP-A(+/+). BALF CD8+ and CD4+ T lymphocytes were measured in BALF by flow cytometry with fluorescein isothiocyanate (FITC)-conjugated mouse CD4 and phycoerythrin (PE)-conjugated mouse CD8 antibodies. A: CD4+ cells were decreased in BALF from SP-A(−/−) (filled bars) compared with SP-A(+/+)(hatched bars) mice 7 days after IAV infection. B: 3 days after IAV infection, increased CD8+ cells were observed in BALF from SP-A(−/−) mice. CD4+ and CD8+ T lymphocytes in BALF were similar for uninfected SP-A(+/+) and SP-A(−/−) mice. Data represent means ± SE with n = 8 mice/group. *P < 0.05 compared with untreated SP-A(−/−) mice on the same day.

**Lymphocytes in BALF and spleen.** Surface markers on lymphocytes from BALF and spleen were measured by flow cytometry. CD6, a receptor for T cell activation, and CD16, a receptor important for signaling for antibody production, were assessed on CD3+ T lymphocytes. CD6 expression on T lymphocytes from BALF and spleen was significantly greater in SP-A(−/−) mice. CD16 expression was similar for BALF T lymphocytes and increased on T lymphocytes from the spleen of SP-A(−/−) mice. The percentage of CD19+ (B lymphocytes) and CD56+ (NK cells) was measured in BALF and spleen. B lymphocytes were increased in BALF and spleen of SP-A(−/−) compared with SP-A(+/+). The percentage of NK cells in BALF and spleen was similar in both groups (Table 1).

**Cytotoxic activity of spleen lymphocytes.** Cytolytic potential of spleen lymphocytes was assessed in an LDH release assay with IAV-infected EL4 cells as targets. Lymphocytes from the spleen of IAV-infected SP-A(−/−) and SP-A(+/+) mice exhibited a comparable degree of specific cytolytic activity on virus-infected

**Table 1. Lymphocytes in BALF and spleen**

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<th>CD6 Lymphocytes, %</th>
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<td></td>
<td>CD16</td>
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<td>BALF</td>
<td>SP-A(−/−)</td>
<td>4.1 ± 0.2*</td>
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<td>SP-A(+/)</td>
<td>2.0 ± 0.3</td>
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<td>Spleen</td>
<td>SP-A(−/−)</td>
<td>12.2 ± 1.5*</td>
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Data are means ± SE with n = 8 mice/group. After influenza A virus infection, (7 days) bronchoalveolar lavage fluid (BALF) and spleen cells were recovered as described in METHODS and were stained with specific fluorescent antibodies. Cell-associated fluorescence assessed by flow cytometry. CD6, a receptor for T lymphocyte activation, and CD16, a receptor important for signaling for antibody production, were assessed on CD3+ T lymphocytes. CD6 expression on T lymphocytes from BALF and spleen was significantly greater in surfactant protein A-deficient [SP-A(−/−)] mice. CD16 expression was similar for BALF T lymphocytes; however, it was increased on T lymphocytes from the spleen of SP-A(−/−) compared with SP-A(+/+) mice. B lymphocytes (CD19+) were increased in BALF and spleen of SP-A(−/−) compared with SP-A(+/+) mice. The finding of increased B lymphocytes in the spleen of SP-A(−/−) mice is consistent with increased Ig levels in the serum. The percentage of natural killer cells (CD56+) in BALF and spleen were similar between the two groups. *P < 0.05 compared with SP-A(+/+) mice.

**Fig. 3.** SP-A enhanced clearance and decreased proinflammatory cytokines from the lung of SP-A(−/−) mice. IAV titers were determined by quantitative plaque assay of lung homogenates 3 days after infection with 10⁶ fI IAV. A: viral titers in the lung were significantly reduced in SP-A(−/−) mice treated with SP-A (100 µg; crosshatched bars) compared with untreated SP-A(−/−) mice (filled bars). B: concentrations of TNF-α, IL-6, and interferon (IFN)γ were assessed in lung homogenates after treatment with SP-A, TNF-α, IL-6, and IFN-γ concentrations were significantly reduced in SP-A(−/−) mice treated with SP-A (100 µg; crosshatched bars) compared with untreated SP-A(−/−) mice (filled bars). Data are means ± SE with n = 8 mice/group. ∗P < 0.05 compared with untreated SP-A(−/−) mice on the same day.

**Fig. 4.** CD4+ and CD8+ T lymphocytes in BALF after IAV infection. After IAV infection, CD4+ and CD8+ T lymphocytes were measured in BALF by flow cytometry with fluorescein isothiocyanate (FITC)-conjugated mouse CD4 and phycoerythrin (PE)-conjugated mouse CD8 antibodies. A: CD4+ cells were decreased in BALF from SP-A(−/−) (filled bars) compared with SP-A(+/+)(hatched bars) mice 7 days after IAV infection. B: 3 days after IAV infection, increased CD8+ cells were observed in BALF from SP-A(−/−) mice. CD4+ and CD8+ T lymphocytes in BALF were similar for uninfected SP-A(+/+) and SP-A(−/−) mice. Data represent means ± SE with n = 8 mice/group, UI, uninfected.
target cells in vitro (Fig. 5). These results suggest that SP-A is not a critical determinant of the specific cytotoxic T lymphocyte response in the spleen.

**Increased immunoglobulins in serum of SP-A(−−) mice.** Total immunoglobulins, IgM, IgG, and its subclasses were measured in serum. After IAV infection (7 days), total immunoglobulins, IgM, and the IgG subclass IgG2a were significantly increased in serum from SP-A(−−) compared with SP-A(+/+) mice. IgG subclass IgG1 was significantly less in the serum of SP-A(−−) mice; therefore, in the absence of SP-A, Th1 responses to IAV predominated in the lung.

**Increased neutrophil MPO activity in SP-A(−−) mice.** After IAV infection, MPO activity from isolated BAL neutrophils was significantly decreased in SP-A(−−) compared with SP-A(+/+) mice (Fig. 7). Control neutrophils isolated from the blood of uninfected SP-A(+/+) mice had similar MPO activity, whereas BAL neutrophils from IAV-infected SP-A(−−) mice had MPO activity from PMA-stimulated blood neutrophils was similar for SP-A(−−) and SP-A(+/+) mice (data not shown).

**IAV infection enhances SP-D accumulation in the lung.** Concentrations of SP-D in BALF increased 12-fold in SP-A(+/+) mice and 7-fold in SP-A(−−) mice 5 days after IAV infection (Fig. 8). The results are consistent with previous studies demonstrating increased SP-D levels in the lung of wild-type mice early after IAV infection (24).
The target text is a scientific manuscript discussing the role of SP-A in the lung following influenza A virus (IAV) infection. The text explains the immunological response, including increased inflammation and cytokine production, and the enhancement of viral clearance by SP-A. The authors also describe the use of SP-A as a therapeutic agent in vivo and in vitro to modulate immune responses. The manuscript includes experimental data visualized in a figure showing the decrease in neutrophil myeloperoxidase (MPO) activity in SP-A(-/-) mice compared to wild-type mice after IAV infection. The data suggest that SP-A plays a critical role in the initial pulmonary host defense against IAV, enhancing viral clearance and modulating innate and adaptive immune responses.

**DISCUSSION**

Pulmonary clearance of intranasally administered IAV was reduced in SP-A(-/-) mice compared to wild-type mice. Pulmonary inflammation was increased in SP-A(-/-) mice compared to wild-type controls, as indicated by increased total cell counts and proinflammatory cytokines in the lung after IAV infection. Treatment with exogenous SP-A enhanced viral clearance and decreased lung inflammation. In the absence of SP-A, association of IAV with alveolar macrophages was similar to wild-type levels. Activated T lymphocytes and B lymphocytes were increased in the lung and spleen of SP-A(-/-) mice and were associated with increased serum immunoglobulins. Th1 cytokines were increased and Th2 cytokines decreased in the lung in the absence of SP-A. Neutrophil MPO activity was decreased in SP-A(-/-) mice, suggesting that neutrophil clearance of IAV may be impaired. These findings support the concept that SP-A plays an important role in the initial pulmonary host defense against IAV and modulates innate and adaptive immune responses to the virus.

Clearance of IAV from the lungs of SP-A(-/-) mice was impaired, supporting the importance of SP-A in viral host defense of the lung. SP-A is a member of the C-type lectin family of polypeptides that includes mannose-binding lectin, conglutinin, and SP-D. C-type lectins share structural features, including collagenous NH2-terminal and “globular” COOH-terminal domains, the latter serving as a carbohydrate recognition domain that functions in opsonization. Influenza virus has two membrane glycoproteins, the HA and neuraminidase. Collectins bind to oligosaccharides on influenza virus glycoproteins, neutralizing the virus, with heavily glycosylated strains of viruses being the most sensitive to SP-A (15). Binding of SP-A to IAV likely enhances viral clearance by binding to the virus, perhaps blocking access of cell surface receptors used by the virus, thus interfering with internalization. In addition, SP-A agglutinated IAV (15), which may also enhance viral removal from the lung through mucociliary and phagocytic clearance. Phagocytosis of IAV by alveolar macrophages was similar in SP-A(-/-) and SP-A(+/+) mice in vivo, a finding that contrasts with in vitro studies demonstrating that SP-A enhanced the association of IAV with alveolar macrophages (4). The reasons for this discrepancy are unclear; however, we chose early time points to assess macrophage phagocytosis. Because large quantities of ingested FITC-labeled virus were necessary to detect macrophage fluorescence, we were unable to assess uptake at a lower inoculum or later time point. Nevertheless, the finding that phagocytosis of IAV was similar in SP-A(-/-) and wild-type mice suggests that SP-A is not a critical determinant for macrophage clearance of IAV in vivo under our experimental conditions.

After IAV infection, inflammatory cells and proinflammatory cytokine concentrations were increased in the lungs of SP-A(-/-) mice. SP-A(-/-) mice were able to mount an immune response to IAV infection; however, the inflammatory response was increased compared with wild-type controls. Increased cytokine production may lead to increased numbers of cells in BALF after viral infection. Increased cytokines, TNF-α, IL-1β, IL-6, and IFN-γ have been demonstrated in a mouse model of IAV infection in association with lymphocytic and mononuclear infiltrates in the lung (16). In the absence of SP-A, cytokine responses were similar to that observed in previous experimental conditions.
mouse models of IAV infection; however, cytokine production and inflammation were increased in SP-A(−/−) compared with wild-type controls. The increased numbers of neutrophils found in lungs from SP-A(−/−) mice after infection may also contribute to the increase in inflammatory cytokines.

During lung injury, concentrations of SPs may be influenced by changes in SP-A synthesis or degradation. SP-A levels were reduced in BALF from children with viral pneumonia (23). In the present study, IAV clearance was enhanced and proinflammatory cytokines decreased in SP-A(−/−) mice receiving 100 μg SP-A. In a murine model of idiopathic pneumonia syndrome after bone marrow transplant, exogenous SP-A (100 μg) administered to wild-type mouse lungs suppressed lung inflammation and decreased pulmonary edema (36). Thus enhancement of alveolar levels of SP-A during IAV infection may augment viral clearance and limit the tissue-damaging production of inflammatory mediators.

SP-D, another collectin family member, bound, agglutinated, and enhanced the association of neutrophils with IAV (15). In previous studies, SP-D concentrations in the lungs of wild-type mice increased after IAV infection (24). Similarly, in the current study, SP-D levels increased in the lungs of wild-type and SP-A(−/−) mice after IAV infection, but the increase in SP-D was less in the lungs of SP-A(−/−) mice. Clearance of IAV from the lungs of SP-A(−/−) mice was impaired, and enhanced production of SP-D in the lung was not sufficient to compensate for the absence of SP-A. These findings suggest that SP-D may play a role that differs from SP-A in IAV clearance, and both proteins may be necessary for optimal viral clearance from the lung.

Activated T lymphocytes were increased in the lung and spleen in SP-A(−/−) mice. Recent in vitro studies support a role of SP-A in modulating the adaptive immune responses (6). In vitro, SP-A inhibited proliferation of human peripheral blood and tonsillar mononuclear cells after stimulation with either phytohemagglutinin or anti-CD3 (6) and inhibited allergen-stimulated lymphocyte proliferation (33). In a murine model of idiopathic pneumonia syndrome, SP-A suppressed T cell immune responses and T cell-dependent macrophage activation (36). In the current study, decreased Th cells were observed in BALF from SP-A(−/−) mice. In contrast, the percentage of CTL and NK cells was similar to the wild type after IAV infection, suggesting that SP-A regulation of CTL and NK cells is not a critical determinant for pulmonary clearance of IAV. The production of the potent T cell mitogen IL-2 was inhibited by SP-A in vitro (6). The present observation that IL-2 levels are increased in the lungs of SP-A(−/−) mice supports these in vitro findings. IL-12, which stimulates IL-2 production, was also increased in the lungs of SP-A(−/−) mice after IAV infection. Mononuclear phagocytes and dendritic cells produce IL-12, which is a key inducer of cell-mediated immune responses that play a critical role in lung defense against viral infection but can also cause tissue damage (28). SP-A inhibited activation of macrophages in vivo (36), which may decrease IL-12 production and serve an anti-inflammatory role to control the inflammatory response and limit tissue damage during viral infection.

In the absence of SP-A, Th1 responses were increased (IFN-γ, IL-2, and IgG2a) and Th2 responses were decreased (IL-4, IL-10, and IgG1; see Refs. 7 and 29). After IAV infection, IL-12, which promotes Th1 responses, was increased in SP-A(−/−) mice. Because SP-A has an important role in the initial innate host defense response, impaired early viral clearance may stimulate an exaggerated adaptive Th1 immune response. Alternatively, SP-A may suppress lymphocyte proliferation and macrophage activation, promoting Th2 responses to reduce inflammation and tissue damage in the lung during viral infection.

B lymphocytes were increased in the lung and spleen of SP-A(−/−) mice in association with increased levels of serum immunoglobulins. Expression of CD16, a receptor for antibody signaling, was also increased on splenic T lymphocytes. Adaptive immunity against viral infections is mediated by antibodies that block virus binding and entry into host cells and by CTL that eliminate the infection by killing infected cells. Although in vitro studies suggest that SP-A may stimulate the production of IgA, IgG, and IgM from splenocytes (19), total immunoglobulin levels were increased in the serum of SP-A(−/−) mice. Distinct subclasses of immunoglobulins were altered in SP-A(−/−) mice after IAV infection. IgM, an antibody produced in the early primary immune response to viral antigens, was increased in the absence of SP-A. Interestingly, IgG1, induced by the Th2 cytokine IL-4, was decreased, and IgG2a, induced by the Th1 cytokine IFN-γ, was increased in the absence of SP-A. These findings suggest that SP-A may potentiate the capacity of specific cytokines to promote production of particular immunoglobulin isotypes or SP-A may act directly on the B lymphocyte or Th cell to stimulate production of a particular immunoglobulin isotype. Alternatively, decreased viral clearance in the absence of SP-A may favor specific cytokines that promote production of a particular immunoglobulin isotype.

Neutrophil accumulation was greater in the lungs of the SP-A(−/−) than in SP-A(+/+) mice after infection. However, MPO activity of these neutrophils was decreased in the absence of SP-A. Defects in neutrophil chemotactic, oxidative, and bacterial killing functions have been documented after pulmonary IAV infection (12), which may underlie a predisposition to bacterial superinfections (1). SP-D but not SP-A inhibits the effects of IAV on the neutrophil respiratory burst responses in vitro (15). In the current study, it is unclear whether neutrophil MPO activity was decreased because of the absence of SP-A or because of impaired clearance and increased IAV titers in the lung.

In summary, in the absence of SP-A, IAV clearance from the lung was impaired. Lung inflammation was
more severe in SP-A(-/-) mice, suggesting that SP-A plays a role in modulating cytokine production and inflammatory responses during viral infection. Th1 responses were increased, whereas Th2 responses were decreased in SP-A(-/-) mice. Exogenous SP-A restored viral clearance in the SP-A(-/-) mice. Because the airway is the usual portal of entry for influenza virus and other respiratory pathogens, the local production of SP-A is likely to play a role in innate defense responses to inhaled viruses.

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