Surfactant protein C-deficient mice are susceptible to respiratory syncytial virus infection

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Surfactant protein C-deficient mice are susceptible to respiratory syncytial virus infection. Am J Physiol Lung Cell Mol Physiol 297: L64–L72, 2009. Patients with mutations in the pulmonary surfactant protein C (SP-C) gene develop interstitial lung disease and pulmonary exacerbations associated with viral infections including respiratory syncytial virus (RSV). Pulmonary infection with RSV caused more severe interstitial thickening, air space consolidation, and goblet cell hyperplasia in SP-C-deficient (Sftpc−/−) mice compared with SP-C replete mice. The RSV-induced pathology resolved more slowly in Sftpc−/− mice with lung inflammation persistent up to 30 days postinfection. Polymorphonuclear leukocyte and macrophage counts were increased in the bronchoalveolar lavage (BAL) fluid of Sftpc−/− mice. Viral titer and viral F and G protein mRNA were significantly increased in both Sftpc−/− and heterozygous Sftpc+/− mice compared with controls. Expression of Toll-like receptor 3 (TLR3) mRNA was increased in the lungs of Sftpc−/− mice relative to Sftpc+/− mice before and after RSV infection. Consistent with the increased TLR3 expression, BAL inflammatory cells were increased in the Sftpc−/− mice after exposure to a TLR3-specific ligand, poly(I:C). Preparations of purified SP-C and synthetic phospholipids blocked poly(I:C)-induced TLR3 signaling in vitro. SP-C deficiency increases the severity of RSV-induced pulmonary inflammation through regulation of TLR3 signaling.

Interstitial lung disease; Toll-like receptor 3

PULMONARY SURFACTANT is a complex of phospholipids and proteins that is synthesized by pulmonary epithelial cells and secreted onto the alveolar surface. In addition to reducing surface tension, pulmonary surfactant also contributes to host defense of the lung against inhaled pathogens. Surfactant protein C (SP-C) is a hydrophobic lipopeptide that enhances surface activity in vitro and contributes to host defense. The SP-C encoding gene (SFTPC) is expressed at high levels only in pulmonary type II cells producing a 21-kDa proprotein that is processed to a mature 35-amino acid peptide. Structurally, the SP-C peptide is composed of a hydrophobic valine-rich domain from amino acid residues 9–23 that adopts an α-helical structure in a lipid environment. The short NH2-terminal domain of SP-C is palmitoylated at adjacent cysteine residues 5 and 6 further enhancing the hydrophobicity of the mature form of SP-C (24, 35). SP-C is enriched in commercial surfactant extracts used to treat human neonatal respiratory distress syndrome.

The penetrance of lung diseases due to SP-C mutations is highly variable including respiratory distress in term newborn infants, interstitial lung disease (ILD) in older children, and ILD or pulmonary fibrosis in adults. SP-C-related ILD was reported in association with family histories of lung disease suggesting a genetic mechanism (1, 8, 25, 32). The molecular basis of the phenotype is complex. The underlying cause of SP-C-related ILD has been attributed to deficiency of mature SP-C or aberrant forms of proSP-C. Familial examples of SP-C-deficient ILD include individuals with no detectable mutations in the gene (1, 33). The majority of affected individuals carry mutations in the SP-C gene that alter proSP-C processing and reduce levels of mature SP-C in the air space (3, 24). The severity and age of onset of disease vary among family members carrying a single defined mutation. Some individuals had acute neonatal respiratory distress, whereas others developed a chronic disease (8, 32). Such intrafamily variability implies that other factors or modifier genes influence disease progression. Consequently, the spectrum of SP-C-related lung diseases reflects a variety of mutations that reduce SP-C expression, produce mutant forms of proSP-C, and potentially impair type II cell function.

Pulmonary infection in SP-C-deficient individuals has been temporally linked to acute exacerbations, pulmonary insufficiency, and hospitalization. Patient histories are remarkable for recurrent neonatal and childhood pulmonary infections that include influenza virus and respiratory syncytial virus (RSV) (1, 7, 9, 32). RSV is a common respiratory pathogen that infects infants in the first year of life as well as immunocompromised patients and the elderly. RSV infection in at-risk individuals can be severe and result in mortality (26). The lack of a protective vaccine and subsequent reinfection by RSV in some individuals suggest that adaptive immunity is incomplete. The report of severe and recurrent infections in SP-C-deficient patients suggests that SP-C has a protective role in lung defense against viral pathogens.

Considering a possible role of RSV in pulmonary exacerbations in patients with SP-C deficiency, we tested the hypothesis that SP-C influences innate immune responses to RSV infection. In the present study, we demonstrated that RSV infection increased pulmonary inflammation in the absence of SP-C, at least in part by an SP-C-mediated inhibition of Toll-like receptor 3 (TLR3) signaling.

MATERIALS AND METHODS

Mice. The Sftpc gene was inactivated by gene targeting in embryonic stem cells used to generate Sftpc−/− mice (12). The Sftpc−/− allele was backcrossed onto the 129S6/SvEvTac (Taconic Farms,
129S6) and FVB/NJ (Jackson Labs, FVB/N) strains of mice to generate congenic Sftpc<sup>−/−</sup> lines (13, 17). Strain- and age-matched wild-type mice (Sftpc<sup>+/+</sup>) were used for controls. Mice heterozygous for the disrupted allele (Sftpc<sup>+/−</sup>) were used in limited studies. Animals were housed in a barrier facility to minimize inflammation, and sentinel animals were negative for bacterial and viral pathogens. Animals were studied under protocols approved by the Children’s Hospital Research Foundation Institutional Animal Care and Use Committee.

Preparation of virus. An isolate of RSV used in previous infection studies of SP-A (Sftpa)- and SP-D (Sftpd)-deficient mice was propagated in HEp-2 cells maintained in Eagle’s minimal essential media supplemented with glutamine, amphotericin, streptomycin, penicillin G, and 10% low immunoglobulin FBS (10% EMEM) (20). The virus was allowed to adsorb to subconfluent monolayers of HEp-2 cells at 37°C for 1 h and then maintained for 3 days at 37°C until extensive cytopathic effects were visible throughout the monolayer. The contents of the flask were scrapped, sonicated on ice, and clarified by centrifugation at 400 g for 10 min. The supernatants were distributed into 1-ml aliquots, quick-frozen, and stored at −80°C. Titer of stock virus for infection and RSV titer of lung homogenates were determined by quantitative plaque assay. HEp-2 cells were seeded onto 24-well dishes. At ~40% confluency, the wells were infected in triplicate with 10-fold serial dilution of stock RSV or tissue homogenates diluted in 10% EMEM and incubated at 37°C and 5% CO<sub>2</sub>. After 48 h, media was removed, and cells were washed twice in PBS, fixed with 4% paraformaldehyde, and stained with an antibody specific for the RSV F protein at a 1:2,000 dilution (Fitzgerald Industries International), followed by 1:1,000 secondary antibody, anti-mouse IgG<sub>2b</sub> (BD Pharmingen). Subsequently, plaques were counted, and the titers were determined.

Viral infection. Mice were infected by noninvasive oral aspiration as described previously (36). Briefly, mice were lightly anesthetized and suspended by upper incisors on a 45-degree angle incline board. The tongue was extended with forceps, 100 μl of virus preparation or PBS. Lavage solution in 7:1:1 methanol/chloroform/water (vol/vol/vol) was added to visualize morphology. To assess viral-induced injury, paraffin sections of lungs from mock-infected and RSV-infected mice on day 3 postinfection was resolved on SDS-PAGE under reducing conditions. Following transfer to PVDF membrane, proteins were probed with an anti-rat SP-A or anti-mouse SP-D rabbit polyclonal antibody overnight at 4°C. Subsequently, plaques were counted, and the titers were determined.

Lung morphology-immunohistochemistry. To assess viral-induced injury, paraffin sections of lungs from mock-infected and RSV-infected mice were processed and stained with hematoxylin and eosin to visualize morphology. Immunostaining with a polyclonal antibody to mouse Clec3 (Abcam) was used to evaluate goblet cell-like cell formation to airway epithelial cells following RSV infection. The primary antibody was used at 1:25,000 dilution with a goat anti-mouse biotinylated secondary antibody 1:1,000 dilution (Vector Laboratories) on tissue sections following citrate antigen retrieval.

Quantification of bronchoalveolar lavage fluid-associated inflammatory cells. Bronchoalveolar lavage fluid (BALF) was isolated by sequential lavage of five mice per genotype at 4 h and at days 1, 3, 5, 7, and 9 post-RSV infection with three 1-ml aliquots of PBS. Lavage was collected from media-instilled control mice at 4 h and on days 1 and 7. Cells were pelleted at 750 g for 5 min, and the pellets resuspended in 150 μl of ice-cold PBS. Cells were enumerated using a hemocytometer to determine total BALF cell concentrations, and aliquots of cells were collected by cytopsin followed by (Giemsa-Gru¨nall, Diff-quik, Dade Behring) staining to determine differential cell counts. Five fields per BALF sample at two dilutions were counted for each mouse lavage. The data from each group of five mice were averaged, and the significance of the difference between groups was determined by Student’s t-test. Supernatant of BALF was mixed with protease inhibitors, frozen, and subsequently used for assessing the concentrations of inflammatory mediators.

Analysis of gene expression. Lung tissue was harvested from five mice per genotype at each time point and snap-frozen for RNA isolation and subsequent synthesis of first-strand cDNA and RT-PCR analysis of gene expression. PCR reactions were normalized to total RNA pooled from all five mice. Expression of RSV F and G protein and TLR3 genes was normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) levels. PCR reactions were run under similar conditions with a 58°C annealing temperature for the F, G, and GAPDH reactions and 55°C for TLR3 reactions. F and GAPDH reactions were continued for 30 cycles and the G and TLR3 reactions for 35 cycles. The gene-specific primers used were as follows: RSV F protein, 5′ CCA GCA AAG TGT TAG ACC TCA AAA, 3′ AAT CGC ACC CGT TAG AAA ATG; RSV G protein, 5′ CTC GGC AAA CCA AAG ATG CA, 3′ GCA GAT AGC CCA GGA GTT; TLR3, 5′ CTT CAG ACT CAC CAA TCC CGT; TLR3, 3′ TCC CCT CGC TCT TTG TAT GMEAC; GAPDH, 5′ CAA CTT TGG CAT TGT GGA AG, 3′ TCC ACC ACC CTT TTG CTG TA.

Detection of SP-A and SP-D by Western blot. Fifty micrograms of BALF protein collected from media-instilled mice or RSV-infected mice on day 3 postinfection was resolved on SDS-PAGE under reducing conditions. Following transfer to PVDF membrane, proteins were probed with an anti-rat SP-A or anti-mouse SP-D rabbit polyclonal antibody overnight at 1:1,000 or 1:7,500 dilution, respectively. Membranes were reacted with the appropriate horseradish peroxidase-conjugated secondary antibody (Calbiochem, La Jolla, CA) for 1 h. Protein-antibody complexes were detected with an enhanced chemiluminescent system (Pierce, Rockford, IL) and quantified using Gel-Pro Analyzer software (MediaCybernetic, Bethesda, MD).

Delivery of double-stranded (ds) RNA in vitro and in vivo. Poly(I:C) (Sigma-Aldrich) was resuspended at 2.5 mg/ml in sterile water and frozen in aliquots. Five to seven mice of each genotype were challenged with either 40 μg/g body wt, by oral aspiration of poly(I:C), or equal volume of vehicle alone. Lungs were collected for morphological analysis on day 3 postadministration. The same stock poly(I:C) was used for stimulation of TLR3 activity in the HEK-293 cell transient transfection experiments as described below.

Preparation of SP-C-phospholipid complexes. Native SP-C was purified by C8 liquid chromatography of bovine lung lavage, as previously described (11). Silver stain of SDS-PAGE gels indicated a single band at ~4.200 Da. Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry of the purified protein identified a single peak of m/z 4,200 Da. Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry of the purified protein identified a single peak of m/z 4,200 Da. Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry of the purified protein identified a single peak of m/z 4,200 Da. Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry of the purified protein identified a single peak of m/z 4,200 Da. Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry of the purified protein identified a single peak of m/z 4,200 Da. Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry of the purified protein identified a single peak of m/z 4,200 Da. Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry of the purified protein identified a single peak of m/z 4,200 Da.
RESULTS

Model selection. Sftpc−/− mice on the FVB/N background have normal lungs and do not develop ILD, and were used to determine whether SP-C influenced RSV-induced lung pathology (17). In contrast, 129S6 Sftpc−/− mice developed spontaneous lung inflammation and remodeling that increases with age. In the current study, Sftpc−/− (129S6) mice were used at 6–8 wk of age, before severe lung pathology is evident in this strain (13). No apparent changes in overall activity or mortality of the mice were detected during the study.

RSV-induced histopathology. Lung pathology was more severe in both strains of RSV-infected Sftpc−/− mice relative to Sftpc+/+ mice. Lung morphology of either FVB/N Sftpc−/− or Sftpc+/+ mice appeared normal the first day following infection. Mild perivascular and alveolar infiltration was detected in the lungs of Sftpc−/+ mice on day 3 that was decreased on day 5 and virtually absent on day 7 postinfection. RSV-induced changes to lung histology were more severe in Sftpc−/− mice and included both tissue and cellular inflammation. Changes in macrophage morphology and areas of interstitial thickening were detected in the lungs of FVB/N Sftpc−/− mice on day 3 postinfection and were most pronounced on day 5 (Fig. 1). Lung morphology was unchanged in mock-infected Sftpc+/+ and Sftpc−/− mice (Fig. 1).

Airway inflammation was increased in the lungs of RSV-infected Sftpc−/− mice compared with Sftpc+/+ mice. Goblet cell hyperplasia was more pronounced in the airways of FVB/N and 129S6 Sftpc−/− mice than in the airways of infected strain-matched Sftpc+/+ mice. Goblet cell differentiation was confirmed by immunostaining with an antibody specific to the mucin granule protein Clea3 (Fig. 2). Clea3-positive cells were virtually absent from the airways of the mock-infected animals. There was a small amount of Clea3 staining in airway epithelium of RSV-infected FVB/N Sftpc−/− mice (shown in Fig. 2B). Clea3 staining was markedly increased in the FVB/N Sftpc−/− lungs wherein Clea3 was detected in the majority of epithelial cells in large airways (Fig. 2D). Staining was more pronounced in the lungs of infected 129S6 Sftpc−/− compared with FVB/N Sftpc−/− mice. Clea3-positive cells extended to bronchoalveolar junctions of the 129S6 Sftpc−/− mice, whereas Clea3 staining in the lungs of infected 129S6 Sftpc+/+ mice was confined to small numbers of cells in the larger airways (compare Fig. 2, E and F with inset in F).

Pulmonary inflammation. Total cell counts of BALF collected from mice were similar 4 h after RSV infection but increased in BALF from FVB/N Sftpc−/− relative to Sftpc+/+ mice on days 1–9 postinfection (Fig. 3). Cells were primarily mononuclear, with increased numbers of polymorphonuclear leukocytes and lymphocytes in the Sftpc−/− BALF. Mononuclear cells in BAL from Sftpc−/− mice were enlarged and foamy, a finding that contrasted with smaller macrophages seen in Sftpc+/+ mice (Fig. 1).

RSV clearance and viral gene expression in the lungs of infected mice. Viral titers were increased in the lung homogenates of FVB/N Sftpc−/− lungs 3 and 5 days after infection, indicating that virus was cleared from the lungs less efficiently than from Sftpc+/+ mice (Fig. 4). Expression of both the RSV F and G genes was increased in the lungs of Sftpc−/− mice over the levels detected in Sftpc+/+ mice (Fig. 5). RSV F and G gene expression levels in the lungs of infected heterozygous (Sftpc−/+ ) mice were intermediate to the levels detected in Sftpc+/+ and Sftpc−/− mice. This finding is consistent with an SP-C haploinsufficiency affecting viral gene expression.

RSV infection does not alter pulmonary collectin levels in BALF of Sftpc−/− mice. The relative concentration of SP-A and SP-D was determined by Western blot analysis on day 3 post-RSV infection. SP-A levels were similar in the BALF of control Sftpc+/+ and Sftpc−/− mice instilled with media. There was a modest proportional increase in SP-A in the BALF of RSV-infected mice of both genotypes (Fig. 6, top). The increase was not statistically significant (media Sftpc+/+ to media Sftpc−/+ P = 0.67, RSV Sftpc+/+ to RSV Sftpc−/+ P = 0.62, media Sftpc−/+ to RSV Sftpc−/+ P = 0.13, unpaired t-test). SP-D levels were even more uniform between Sftpc+/+ and Sftpc−/+ mice in the BALF of media-instilled (control) mice and RSV-infected mice (Fig. 6, bottom). This finding is consistent with the pronounced inflammation and histopathological changes in the lungs of infected Sftpc−/− mice resulting from an SP-C deficiency and not due to changes of the innate protective collectins. Baseline SP-A and SP-D levels were previously shown to be similar in the BALF of Sftpc−/− and Sftpc+/+ mice (12, 13).

Long-term effects from RSV infection in Sftpc−/− mice. To assess the sequelae of chronic RSV infection, FVB/N Sftpc+/+ and Sftpc−/− mice were infected with RSV and maintained in a pathogen-free environment for 30 days. The RSV-induced injury was resolved in the Sftpc+/+ mice. In the lungs of Sftpc−/− mice, the interstitial changes induced by RSV infection were resolved, but discrete focal areas of perivascular and diffuse alveolar infiltrates remained (Fig. 7). The residual inflammation indicates a delayed resolution of inflammation following in SP-C-deficient lungs.

TLR3 expression and the injury response to TLR3-specific ligand are increased in 129S6 Sftpc−/− mice. Because viral dsRNA mediates TLR3 signaling, TLR3 expression and the response to the TLR3-specific ligand poly(I:C) were assessed in the 129S6 Sftpc−/− mice that develop inflammatory injury with age. Baseline TLR3 RNA was increased in the lungs of the control, uninfected 129S6 Sftpc−/− mice relative to the Sftpc+/+ mice and further increased by RSV infection (Fig. 8). Exposure of Sftpc+/+ and Sftpc−/− mice to the TLR3 ligand poly(I:C) by aspiration caused mild inflammation in scattered areas of the lungs of Sftpc+/+ mice, but, overall, histological changes were unremarkable.
In contrast, cell infiltration was more extensive throughout the lungs of Sftpc<sup>−/−</sup> mice with areas of consolidation (Fig. 9). Increased response to TLR3 ligand stimulation in vivo is consistent with increased TLR3 levels in the lungs of Sftpc<sup>−/−</sup> mice.

**SP-C inhibits ligand-stimulated TLR3 activity in vitro.** SP-C interference with dsRNA-induced TLR3 activity was determined in the TLR3-negative HEK-293 cell line using either a purified SP-C synthetic phospholipid preparation or commercial SP-C containing surfactant extract (Survanta). Transient transfection with a constitutive TLR3 expression plasmid and an NF-κB promoter-dependent luciferase reporter plasmid (ELAM-luc) was used to assess TLR3-dependent NF-κB signaling (Fig. 10). Addition of poly(I:C) stimulated a 10-fold increase in luciferase activity over the luciferase activity of transfected but nonstimulated cells. Preexposure to Survanta reduced luciferase activity by 30%. Preexposure to the SP-C:DPPC:POPC vesicles reduced the TLR3-initiated luciferase activity by 80% (P < 0.02). Pretreatment with the DPPC:POPC lipid vesicles alone had no affect on

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**Fig. 1.** Respiratory syncytial virus (RSV)-induced pulmonary pathology. Lungs from FVB/N Sftpc<sup>+/+</sup> and Sftpc<sup>−/−</sup> mice infected with 4 × 10<sup>7</sup> pfu RSV and from media-instilled control mice were prepared for histology and stained with hematoxylin and eosin. *a:* photomicrographs from day 5 post-RSV infection reveal mild perivascular and alveolar infiltration in Sftpc<sup>+/+</sup> mice (A and B) with increased infiltration in the lungs of Sftpc<sup>−/−</sup> mice (C and D). Representative low-magnification images are shown in A and C (×20 magnification), and high-magnification images in B and D (×40 magnification). High-magnification images demonstrate the mixed cell infiltrates and pronounced interstitial changes in the lungs of Sftpc<sup>−/−</sup> mice (D). *Insets* in B and D are high-magnification images that indicate the enlarged appearance of macrophages (arrows) and increased interstitial changes including thickened alveolar septum (arrowheads) in the lungs of infected Sftpc<sup>−/−</sup> mice relative to Sftpc<sup>+/+</sup> mice. *b:* the morphology of lungs from untreated and mock-infected adult FVB/N Sftpc<sup>+/+</sup> (A and C) and Sftpc<sup>−/−</sup> (B and D) mice appeared normal with no overt inflammation. Representative images of untreated mice are shown at the top; mock-infected mice are shown at the bottom (×20 magnification).
poly(I:C) stimulation indicating that inhibition of poly(I:C)-stimulated luciferase activity required SP-C.

**DISCUSSION**

By several parameters, RSV infection caused greater inflammation in Sftpc−/− mice including increased inflammatory cell influx, increased viral gene transcription, reduced clearance of virus, and delayed resolution of inflammation. Although the pulmonary response to RSV infection is complex, the present study demonstrates a role for SP-C in the innate immunity of the lung to RSV. Increased TLR3 gene expression and increased inflammation by a TLR3 ligand in Sftpc−/− mice are consistent with increased viral-induced disease due in part to enhanced TLR3 signaling in the absence of SP-C. Additionally, SP-C blocked TLR3 signaling in vitro. These findings support the concept that SP-C contributes to alveolar defense in RSV infection by regulating TLR3 activity.

SFTPC-related disease has been associated with mutations that occur de novo or that are present in inheritable forms of ILD. Two cases of SP-C deficiency without a defined mutation have also been reported (1, 33). Expression of hereditary SFTPC-related ILD is complex, usually occurring in an autosomal dominant pattern, but some examples of asymptomatic carriers with undetectable disease are known (32). SFTPC-associated lung diseases include point mutations that produce single amino acid substitutions, frame shifts, or deletions of entire exons by alternative splicing (3, 25, 32). Regardless of the nature of the genetic lesion, the consequences of the SFTPC mutations are twofold. The mutations produce corresponding structurally altered proSP-C proteins that potentially elicit a misfolded protein response and render affected cells more susceptible to challenge. RSV infection of human embryonic kidney cells transfected with a mutant proSP-C construct increased cell death consistent with viral sensitivity due to the aberrant proprotein (6). It is currently unclear if the expression of misfolded proSP-C in vivo equals the level of expression achieved in the transfected cell study to produce an increased viral response. Importantly, all mutations impair proSP-C processing, which results in deficiency of the mature SP-C in the air space. The Sftpc−/− mice are null animals that do not produce either proSP-C or mature SP-C and model the alveolar SP-C deficiency that occurs in patients.

The inciting factors associated with pulmonary exacerbations in ILD in affected SFTPC-deficient individuals include...
RSV infection (1, 7, 9, 32). RSV is one of the most common human respiratory pathogens, with nearly all children infected in early childhood. RSV infection causes bronchiolitis and interstitial pneumonia in infants (10, 22, 26). Reinfection can occur, suggesting that the adaptive immunity to RSV is incomplete. Studies from other laboratories further support a role of SP-C in protection from RSV. Polymorphisms of the SFTPC gene have been linked to neonatal respiratory distress, and specific haplotypes of SFTPC were identified in children hospitalized with severe RSV infection (18, 27). Bovine RSV vaccine inoculation resulted in 30% mortality in a strain of cattle with naturally occurring SP-C deficiency (30).

The histological findings indicate that the inflammatory response of both airway and alveolar compartments of Sftpc/H11002 mice is increased upon RSV challenge. Injury in RSV-infected Sftpc/H11002 mice consisted of a robust mixed cell infiltration of the pulmonary parenchyma, areas of extensive

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**Fig. 3.** Increased total cell counts in bronchoalveolar lavage fluid (BALF) from FVB/N Sftpc/H11002 mice. Cells were recovered by low-speed centrifugation of BALF, resuspended in 150 μl of PBS and an aliquot stained with trypan blue, and counted under light microscopy. RSV-infected Sftpc/H11002 mice had greater cell counts on days 1–9. Data are presented as means ± SE. *P < 0.05 compared with Sftpc/H11001 mice, 5 mice/group.

**Fig. 4.** Increased RSV in the lungs of FVB/N Sftpc/H11002 mice. Viral titers were determined by quantitative plaque assays of lung homogenates from Sftpc/H11001 and Sftpc/H11002 mice. Viral titers were increased in the lung homogenates from Sftpc/H11002 mice compared with Sftpc/H11001 mice on days 3 and 5 postinfection and undetectable by day 7. Day 3 values were significant (*P < 0.05). Data are presented as means ± SE.

**Fig. 5.** Viral gene expression is increased in the lungs of FVB/N Sftpc/H11002 mice. Expression of RSV genes encoding F and G proteins was measured by RT-PCR of RNA isolated from the lungs of Sftpc/H11002 mice 3 days after mock infection (media) or RSV infection (5 mice/genotype). RSV F gene expression was increased in the lungs of heterozygote +/- mice and -/- mice. RSV G gene expression was present in +/- and -/- lungs. Viral gene expression was not detected in the lungs of control (media) mice.

**Fig. 6.** Pulmonary collectin levels in BALF after RSV infection. The concentrations of SP-A and SP-D in the BALF of Sftpc/H11001 and Sftpc/H11002 mice were determined by Western blot analysis and densitometry. SP-A levels were similar between both genotypes of mice. There was a proportional increase in SP-A levels after RSV infection that was not statistically different between the Sftpc/H11001 and Sftpc/H11002 mice. SP-D levels were identical in the BALF of media-instilled and RSV-infected Sftpc/H11001 and Sftpc/H11002 mice. Data are presented as means ± SE (n = 3 mice/group).
interstitial and airway inflammation, and goblet cell hyperplasia. In contrast, the \textit{Sftpce}^{-/-} mice had a limited cellular inflammatory response to RSV, minimal airway inflammation, and no interstitial changes. Goblet cell hyperplasia and expression of the mucin granule protein Clec3 were increased in airway epithelia of both strains of the RSV-infected \textit{Sftpce}^{-/-} mice relative to the infected \textit{Sftpce}^{+/+} mice. Inflammatory response to RSV infection is exaggerated and resolution of lung pathology is delayed in response to RSV infection is unresolved in the SP-C-deficient lung relative to recovery in the normal lung. Photomicrographs are representative of 5 mice/time point.

Viral clearance from the lungs of FVB/N and 129S6 \textit{Sftpce}^{-/-} mice was delayed, and viral gene expression persisted longer in the lungs of infected FVB/N \textit{Sftpce}^{-/-} mice than \textit{Sftpce}^{+/+} mice. RSV-induced inflammation was unresolved at 30 days postinfection. Thus, the acute response to infection was impaired in the \textit{Sftpce}^{-/-} mice, and long-term resolution of disease was delayed. Repeat RSV infection in rats resulted in inflammation and sustained RSV gene expression in the lungs without virus replication and has been termed abortive replication (5). Detailed studies of RSV gene expression will be required to determine if the observed long-term inflammation in infected \textit{Sftpce}^{-/-} mice results from persistent RSV gene expression.

SP-C is not unique in a protective role against RSV infection. Other surfactant proteins affect the response to RSV infection. SP-A (\textit{Sftpa}) and SP-D (\textit{Sftpd}) gene-targeted mice had increased susceptibility to RSV infection (19, 20). The increased cellular inflammatory response and the time course of the acute response seen in \textit{Sftpce}^{-/-} mice were similar to the results of RSV infection in \textit{Sftpda}^{-/-} and \textit{Sftpd}^{-/-} mice, wherein similar mixed cell infiltrates were reported. The pronounced interstitial changes and airway inflammation observed in the infected \textit{Sftpce}^{-/-} mice were not detected in RSV-infected \textit{Sftpda}^{-/-} and \textit{Sftpd}^{-/-} mice, suggesting a more severe injury to the lungs of the \textit{Sftpce}^{-/-} mice. Both \textit{Sftpda}^{-/-} and \textit{Sftpd}^{-/-} mice had decreased clearance of RSV in doses similar to those used in this study (19, 20). SP-A levels were increased in the BALF of wild-type outbred Black Swiss mice infected with RSV (19). In the present study, RSV infection was assessed in genetically uniform inbred FVB/N mice. A modest increase of SP-A (\textit{Sftpa}) and SP-D (\textit{Sftpd}) after RSV infection in this study is likely due to genetic differences between the inbred FVB/N strain and the Black Swiss mice as previously reported. The increased injury observed in the lungs of infected FVB/N \textit{Sftpce}^{-/-} mice thus appears related to SP-C deficiency and not
to significant changes in the levels of SP-A and SP-D. SP-C is structurally distinct from the pulmonary collectins. SP-A and SP-D can oligomerize, bind, and crosslink a variety of pathogens via their lectin domains. SP-C does not contain such a motif and likely contributes to RSV clearance by a distinct mechanism. Together, these results indicate that the intact alveolar innate response to RSV is complex and has multiple components (SP-A, -C, -D) that mediate viral clearance, impede viral gene expression, and limit acute and abrogate persistent inflammation.

In families affected by a single specific SFTP mutation, the disease profile can vary from onset and death in infancy to respiratory insufficiency throughout childhood or adult onset of ILD with pulmonary fibrosis (7, 8, 25, 32). Infection of the two Sftp−/− strains were used in an attempt to distinguish if RSV sensitivity was increased by SP-C deficiency in lungs without apparent intrinsic injury (FVB/N) or was increased in SP-C-deficient lungs predisposed to inflammatory disease (129S6). We found that the response to RSV infection was increased in Sftp−/− mice on both backgrounds. In contrast, the response of young Sftp−/− mice to Pseudomonas infection was strain specific (14). 129S6 Sftp−/− mice had impaired Pseudomonas clearance, whereas there was no difference between FVB/N Sftp−/− or Sftp+/+ mice. These distinct responses to bacterial and viral infection suggest that genetic modifiers of Sftp−/− sensitivity may be pathogen specific.

SP-C was reported to reduce LPS-induced cytokine production. Our previous study demonstrated that SP-C was a component of innate defense against pulmonary infection with Pseudomonas (14). LPS, Pseudomonas, and RSV induce inflammation through TLR signaling (4, 15, 21, 34). Infected cells detect viral nucleic acids by two distinct mechanisms that employ both membrane-associated sensing (TLR3) and soluble cytoplasmic RNA helicase (RIG-I) sensors (20). We hypothesized that SP-C modulates TLR3 activity to control alveolar inflammation during RSV infection. TLR3 RNA expression and RSV-stimulated TLR3 RNA expression were increased in the lungs of 129S6 Sftp−/− mice over Sftp+/+ mice. Expression of RIG-I and RIG-I-related genes were unchanged in the lungs of Sftp−/− mice. Conceptually, this finding is consistent with the highly hydrophobic SP-C selectively interacting with vesicle-associated sensors such as TLR3 but not components of the freely soluble alternative RIG-1 pathway. The TLR3-specific dsRNA ligand poly(I:C) increased lung inflammation in Sftp−/− mice. Collectively, these findings indicate that a functional increase of TLR3 activity in vivo could mediate the increased RSV-induced injury and protracted inflammation in the lungs of Sftp−/− mice. SP-C-containing phospholipid vesicles inhibited TLR3-dependent NF-κB signaling in cell culture experiments, indicating that SP-C directly blocks the defined TLR3 pathway by which RSV products stimulate expression of inflammatory response genes (4, 21).

RSV genes and TLR3 gene expression in the lungs of Sftp++ mice were intermediate to expression levels in the lungs of either the Sftp+/- or Sftp−/−-infected animals (Figs. 5 and 8). The inflammatory injury to Sftp+/- mice was also intermediate in severity to the Sftp+/- and Sftp−/− mice on day 5 postinfection (images not shown). SP-C levels in the lungs of Sftp+/- heterozygous mice were previously determined to be one-half that of wild-type mice (12). The current results are consistent with increased susceptibility to RSV in SP-C haploinsufficient mice. Hyperoxic injury in Sftp+/- mice was enhanced by SP-C deficiency, suggesting that combined partial SP-B, SP-C deficiencies may have subtle effects
that are only detected upon stress (17). SP-C is decreased during pulmonary infection and inflammation, supporting the concept that a transient SP-C decrease from infection superimposed on a preexisting intrinsic SP-C deficiency in heterozygous individuals may exacerbate an otherwise mild RSV lung disease (2, 16).

In summary, pulmonary clearance of RSV was diminished in Sftpc−/− mice associated with increased and prolonged inflammation. Increased TLR3 RNA levels in Sftpc−/− lungs and increased dsRNA-induced injury in vivo support a role for SP-C in regulating TLR3 levels. Additionally, phospholipid mixtures containing purified SP-C blocked TLR3-stimulated NF-κB activity in vitro showing that SP-C inhibits TLR3 activity. An inability to limit viral-induced inflammation may contribute to the severe forms of SP-C-associated lung disease seen in patients with recurrent exacerbations and infection.

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