Persistence of LPS-Induced Lung Inflammation in Surfactant Protein-C–Deficient Mice

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Pulmonary surfactant protein-C (SP-C) gene-targeted mice (*Sftpc*^{−/−}) develop progressive lung inflammation and remodeling. We hypothesized that SP-C deficiency reduces the ability to suppress repetitive inflammatory injury. *Sftpc*^{+/+} and *Sftpc*^{−/−} mice given three doses of bacterial LPS developed airway and airspace inflammation, which was more intense in the *Sftpc*^{−/−} mice at 3 and 5 days after the final dose. Compared with *Sftpc*^{+/+} mice, inflammatory injury persisted in the lungs of *Sftpc*^{−/−} mice 30 days after the final LPS challenge. *Sftpc*^{−/−} mice showed LPS-induced airway goblet cell hyperplasia with increased detection of Sam pointed Ets domain and FoxA3 transcription factors. *Sftpc*^{−/−} type II alveolar epithelial cells had increased cytokine expression after LPS exposure relative to *Sftpc*^{+/+} cells, indicating that type II cell dysfunction contributes to inflammatory sensitivity. Microarray analyses of isolated type II cells identified a pattern of enhanced expression of inflammatory genes consistent with an intrinsic low-level inflammation resulting from SP-C deficiency. SP-C-containing clinical surfactant extract (Survanta) or SP-C/phospholipid vesicles blocked LPS signaling through the LPS receptor (Toll-like receptor [TLR] 4/CD14/MD2) in human embryonic kidney 293T cells, indicating that SP-C blocks LPS-induced cytokine production by a TLR4-dependent mechanism. Phospholipid vesicles alone did not modify the TLR4 response. *In vivo* deficiency of SP-C leads to inflammation, increased cytokine production by type II cells, and persistent inflammation after repetitive LPS stimulation.

Keywords: surfactant protein-C; LPS; lung inflammation; type II cells; Toll-like receptor 4

Surfactant protein-C (SP-C) is an abundant 3.5-kD surfactant-associated protein expressed and secreted by alveolar type II epithelial cells. The mature airspace form of SP-C is highly

CLINICAL RELEVANCE

This work demonstrates that surfactant protein-C (SP-C)–deficient mice have unresolved inflammation after protracted LPS exposure that models chronic inflammation. The loss of SP-C increases the inflammatory response of alveolar type II cells to LPS that is controlled, in part, by SP-C inhibiting Toll-like receptor 4 signaling.

hydrophobic and palmitoylated at adjacent amino terminal cysteine residues (1). Mutations in the gene encoding human SP-C (*SFTPC*) have been shown to cause familial interstitial lung disease (ILD). Affected individuals express an altered proSP-C, leading to reduced levels of mature SP-C in the airspace (2–4). SP-C deficiencies without detectable mutations in the protein-coding region of *SFTPC* or due to promoter mutations have also been reported, and represent a true null condition (5–7). These individuals also develop ILD, including idiopathic pulmonary fibrosis (IPF). SP-C deficiency–related disease may arise as an acute childhood or as a late-onset disease in adulthood (3). Because SP-C is expressed exclusively in alveolar type II cells, the associated ILD/idiopathic pulmonary fibrosis presumably originates from a primary defect in the epithelium. SP-C deficiency increases susceptibility to viral- or bacterial-induced exacerbations in affected children (5, 6, 8–10). SP-C–deficient mice (*Sftpc*^{−/−}) developed a strain-specific pulmonary phenotype with age that was similar to the human disease (11). *Sftpc*^{−/−} mice were more susceptible to challenge with respiratory syncytial virus (RSV) and *Pseudomonas aeruginosa*, and showed a more robust inflammatory reaction compared with strain-matched wild-type *Sftpc*^{+/+} mice (12, 13). The patient and mouse model data show that SP-C is an innate immune molecule providing both antimicrobial and anti-inflammatory function in the lung.

SP-C has been shown to interact with bacterial LPS and reduce macrophage cytokine activity *in vitro* (14). The precise signaling pathways and role of SP-C in response to LPS *in vivo* have not been determined. In the present study, we identify a role of SP-C in reducing LPS-mediated signaling through the TLR4/CD14/MD2 receptor complex *in vitro* and in reducing lung inflammation *in vivo*. The data suggest that inflammatory exacerbations in individuals with SP-C deficiency may respond to treatment with SP-C.

MATERIALS AND METHODS

Mice

The *Sftpc*^{−/−} and *Sftpc*^{+/+} mice were maintained on a 129S6 background (11). Animals were housed in a pathogen-free barrier facility to minimize risk of inflammation. Routine serotyping of sentinel animals was negative for the presence of bacterial and viral pathogens. However, these mice were not germ free, and were still exposed to endogenous

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bacterial flora. Experiments were performed under animal use protocols approved by the Cincinnati Children's Hospital Institutional Animal Care and Use Committee (Cincinnati, OH). All mice were studied at 6 to 8 weeks of age.

In Vivo Model of Recurrent LPS Exposure

Escherichia coli LPS type 0111:B4 (List Biologicals, Campbell, CA) was used for animal exposures. LPS was delivered by noninvasive oral aspiration, as described previously (13). Briefly, mice were lightly anesthetized and suspended by upper incisors on a 45°-angle incline board. The tongue was extended with forceps and 100 μ l of LPS or PBS was placed into the oral cavity. Mice were monitored until fluid aspiration followed by several additional chest retractions was observed; 10 to 12 mice of each genotype were exposed for each end point. Mice were administered either a single 8- μ g dose of LPS to assess threshold sensitivity due to SP-C deficiency or were serially administered 8 μ g LPS interspaced by 2 days of recovery before the second and third doses. Mice were observed for 3, 5, or 30 days after the final LPS exposure before assessing recovery from injury. In a separate experiment, an SP-C-enriched surfactant extract was transiently replaced before LPS challenge. Mice were given a single 25- μ g dose of Survanta (Abbvie Inc., North Chicago, IL) as an exogenous source of SP-C before LPS exposure, and the cell counts and myeloperoxidase activity levels determined in the bronchoalveolar lavage fluid (BALF) 24 hours later as an indicator of neutrophil activity and cellular inflammation.

Lung Histology and Immunohistochemistry

The lungs of mice were perfused with PBS via the pulmonary artery. Perfused lungs were inflation fixed with 10% buffered formalin via a tracheal cannula at a set pressure of 20 cm H₂O. Paraffin sections (5 μ m) of lungs from mock-exposed (PBS) or LPS-exposed mice (five per group) were processed and stained with hematoxylin-eosin to visualize morphology and to assess the extent of LPS-induced injury. Immunostaining with polyclonal antibodies to mouse FoxA3 and Sam pointed Ets domain (SPDEF) was used to evaluate goblet cell-like transformation of airway epithelial cells.

Determination of Host Defense Protein Levels after Repetitive LPS Exposure

BALF from the lungs of mice exposed to repetitive PBS or LPS exposure was normalized to total protein. For Western blot analysis of BALF, 50 μ g of protein was separated by electrophoresis on 10–20% Tris-Glycine gels and the blots probed with antibodies to known abundant molecules with antimicrobial activity.

Preparation of SP-C-Phospholipid Complexes

Native SP-C was purified by C8 liquid chromatography of bovine lung lavage, as previously described in the supplemental MATERIALS AND METHODS (15).

Determination of SP-C and *E. coli* LPS Interactions

The synthetic phospholipid liposomes with or without the incorporated 5% wt/wt SP-C (prepared as described in supplemental MATERIALS AND METHODS) were incubated with commercially available *E. coli* 0111:B4 LPS-conjugated with FITC (Sigma F-3665; Sigma-Aldrich, St. Louis, MO) and the fluorescence monitored to detect LPS binding.

Isolation of Alveolar Type II Cells for Microarray Analysis and In Vitro LPS Response

Cell isolation procedures for culture and RNA extraction and microarray analysis are provided in the online supplement.

Cell Transfection and SP-C Effect on NF- κ B Signaling

Human embryonic kidney (HEK) 293T cells were transiently transfected with plasmids to reconstruct the TLR4-mediated signaling. LPS-stimulated TLR4 signaling was detected by monitoring luciferase

activity from an NF- κ B-responsive reporter plasmid. Detailed methodology for this experiment is available in the online supplement.

RESULTS

Repetitive LPS Injury Induces Increased Inflammation in Lungs of SP-C Null Mice

Dose-finding experiments were used to titrate the amount of LPS that resulted in inflammatory differences between wild-type and SP-C-deficient mice. Three sequential 8- μ g LPS challenges produced a progressive, chronic inflammation for both genotypes. The resolution of inflammation was evaluated on Days 3 and 5 after the final administration. The lungs of saline vehicle-treated *Sftpc*^{+/+} or *Sftpc*^{-/-} mice after the third and final PBS challenge appeared normal (Figures 1A and 1B). Diffuse cellular infiltrates were observed in the lungs of LPS-challenged *Sftpc*^{+/+} mice on Day 3 after challenge (Figure 1C, *left panels*), but appeared more intense in the lungs of the treated *Sftpc*^{-/-} mice (Figure 1D). Lung sections of the *Sftpc*^{-/-} mice had an increase in vessels with perivascular edema and cellular infiltration compared with the *Sftpc*^{+/+} mice (Figure 1C). Fragmentation of alveolar septae was observed in areas of macrophage accumulation (Figure 1D, *arrows*). By Day 5 after challenge, the inflammation was diminished in the lungs of *Sftpc*^{+/+} mice (Figure 1E). However, pronounced perivascular/bronchiolar and both diffuse and consolidated alveolar cell infiltrates remained in the lungs of the *Sftpc*^{-/-} mice (Figure 1F). Airway goblet cell morphology as an indicator of inflammation was detected in the lungs of *Sftpc*^{-/-} mice at Day 3 and remained at Day 5 (Figures 1F and 2). Low-power images are provided to demonstrate the extent of inflammation in the lungs of *Sftpc*^{+/+} and *Sftpc*^{-/-} mice on recovery Day 3 after repetitive LPS (*see* Figure E1 in the online supplement). Diffuse alveolar cellular inflammation and more clustered perivascular, peribronchiolar cellular inflammation was present in the lungs of recovering *Sftpc*^{-/-} mice (Figure E1B). A semi-quantitative scoring of all mice in each control PBS or repetitive LPS exposure groups was performed to indicate the overall observable histopathology, and is reported in Table E1. These findings indicate that, upon repetitive LPS challenge, the *Sftpc*^{-/-} mice did not resolve LPS-induced inflammation as rapidly as *Sftpc*^{+/+} mice.

SP-C Null Mice Express Transcription Factors Associated with Goblet Cell Transformation after LPS Injury

Immunostaining for the transcription factor, SPDEF, was detected in the airway epithelia of *Sftpc*^{-/-} mice at Day 3 after LPS exposures, whereas no expression was detected in *Sftpc*^{+/+} mice (compare Figures 2C and 2D). Faint immunostaining for the transcription factor, Foxa3, was detected in a few cells lining the airways of saline-treated *Sftpc*^{-/-} mice. These data are consistent with previous studies showing that the airways of *Sftpc*^{-/-} mice are predisposed to inflammatory changes. The intensity of staining and number of Foxa3-positive cells was increased in the airways of the LPS-exposed *Sftpc*^{-/-} mice in comparison to the exposed *Sftpc*^{+/+} mice at Day 3 (Figure 2E versus Figure 2F, *black nuclei*). Cytoplasmic alcian blue staining that denotes acidic mucin glycoprotein production was similarly increased in intensity and colocalized with the Foxa3-positive and adjacent airway epithelia of LPS-exposed *Sftpc*^{-/-} mice (Figures 2E and 2F).

Effect of SP-C Deficiency on Long-Term Recovery after LPS Exposure

The lungs of LPS-exposed mice were examined 30 days after the sequential LPS exposures to determine if long-term recovery is

Figure 1. Lung histopathology of surfactant protein-C *Sftpc*^{+/+} and *Sftpc*^{-/-} mice during recovery from repeated LPS exposure. Images are of hematoxylin and eosin (H&E) staining of lung sections from *Sftpc*^{+/+} (A, C, and E, left) or *Sftpc*^{-/-} (B, D, and F, right) after the last of three doses of PBS (A and B, top) and either 3 days (C and D, middle) or 5 days after final LPS dose (E and F, bottom). Day 3—arrowheads indicate morphology of airway epithelium. Arrows identify alveolar accumulation of inflammatory cells and region of alveolar septal fragmentation indicative of air-space injury. Day 5—diffuse alveolar infiltrates were present in LPS-exposed *Sftpc*^{-/-} mice. Partial airway obstruction by inflammatory cells was present in the lungs of *Sftpc*^{-/-} mice (arrowheads [F]). Images, 20× original magnification, five mice each group.

impaired. The histopathology of lungs from *Sftpc*^{+/+} mice uniformly appeared free of inflammation (Figures 3A and 3C). In contrast, residual tissue and cellular inflammation was detected in the lungs of all *Sftpc*^{-/-} mice. Focal sites of perivascular/bronchiolar cell accumulation, diffuse alveolar mixed cell infiltrates, and a limited number of airways with hyperplastic goblet cells were observed (Figures 3B and 3D). These findings were consistent with a delayed resolution after a “protracted” low-level inflammation. Experiments were performed to determine if replacement of SP-C could reduce the persistence of inflammation in the lungs of SP-C–deficient mice. A single instillation of the surfactant extract, Survanta, as a source of exogenous SP-C (containing minimal SP-B) decreased the inflammatory response to a low-dose LPS challenge (16). BALF total cell counts were decreased in Survanta-treated *Sftpc*^{-/-} mice relative to PBS control *Sftpc*^{-/-} mice and relative to *Sftpc*^{+/+} Survanta-treated mice (Figure E2A). The reduction in Survanta-treated LPS-exposed *Sftpc*^{+/+} mice did not reach statistical significance. Myeloperoxidase (MPO) activity was quantified as an index of neutrophil activity. The MPO activity was increased in the BALF of LPS exposed *Sftpc*^{-/-} mice relative to levels detected in BALF from *Sftpc*^{+/+} mice, consistent with the observed vigorous neutrophil influx. Survanta treatment decreased MPO activity of LPS *Sftpc*^{-/-} mice, but did not reduce MPO activity in BALF from treated LPS-exposed *Sftpc*^{+/+} mice (Figure E2C).

LPS Does Not Alter Levels of Other Innate Immune Molecules in SP-C Null Mice

Using Western blot analyses, the relative amount of surfactant SP-A and SP-D, the antimicrobial proteins, lysozyme

and lactoferrin, and the Clara cell secretory protein were unchanged in the BALF of LPS-challenged *Sftpc*^{+/+} and *Sftpc*^{-/-} mice on Day 3 after final challenge (Figure 4A). The LPS-induced increase of lactoferrin and modest decrease in Clara cell secretory protein expression was similar between the two genotypes of mice (Figure 4B). These results indicate that there was no altered production of known abundant defensive–anti-inflammatory molecules in BALF due to the absence of SP-C.

SP-C Null Mice Have Intrinsic Pulmonary Inflammation and Their Type II Cells Are Hyperresponsive to LPS

Functional and genetic analysis demonstrated that the lack of SP-C in mice promotes inflammation with age and upon infection. These findings implicate a deficit in type II cells in responding to proinflammatory ligands as an underlying cause of the observed injury. To test the hypothesis that SP-C deficiency alters type II cell homeostasis and alveolar defense, type II cells isolated from *Sftpc*^{+/+} and *Sftpc*^{-/-} mice were maintained in culture and exposed to a low dose (5 ng) and high dose (100 ng) of LPS. The culture media was assayed for proinflammatory mediators. Type II cells from *Sftpc*^{-/-} mice had increased basal expression of the cytokines IL-6, TNF- α , and keratinocyte chemoattractant (KC) before LPS stimulus (Figure 5). This finding is consistent with the intrinsic low-level pulmonary neutrophilia reported in unchallenged adult *Sftpc*^{-/-} mice (12). Increased cytokine expression by *Sftpc*^{-/-} type II cells was detected after 4-hour LPS exposure (data not shown), with larger increases after 24 hours of LPS exposure (Figure 5). Expression of IL-1 β , IL-6, TNF- α , and KC was increased in type II cells from the

Figure 2. Airway goblet cell morphology in *Sftpc*^{-/-} mice after repeated LPS exposure. Micrographs are of airways from *Sftpc*^{+/+} mice (A, C, and E [left]) or *Sftpc*^{-/-} mice (B, D, and F [right]) after immunostaining for Sam pointed Ets domain (SPDEF; A–D) or FoxA3 (E and F) 5 days after final PBS treatment (A and B, top) or LPS treatment (C–F). Airway epithelial cells of PBS-treated *Sftpc*^{+/+} or *Sftpc*^{-/-} mice (top) were negative for SPDEF expression (A and B) and FoxA3 expression (data not shown). SPDEF expression was only detected in the epithelium of LPS-exposed *Sftpc*^{-/-} mice (C versus D). Limited FoxA3 (dark nuclei) and alcian blue (blue cytoplasm) staining was detected in the epithelium of LPS-exposed *Sftpc*^{+/+} mice, and was more extensive in the airway epithelium of LPS-exposed *Sftpc*^{-/-} mice (E versus F). Images, 40× original magnification, five mice each group.

Sftpc^{-/-} mice relative to the response of *Sftpc*^{+/+} type II cells exposed to either dose of LPS. Increased cytokine expression by *Sftpc*^{-/-} cells was statistically significant at the 5-ng LPS and 100-ng LPS dose. These findings suggest that the observed inflammatory phenotype originates from the SP-C-deficient alveolar epithelial cells.

Microarray analysis of gene expression by type II cells isolated from *Sftpc*^{+/+} and *Sftpc*^{-/-} mice showed an expression pattern

consistent with increased inflammation in *Sftpc*^{-/-} mice compared with *Sftpc*^{+/+} mice. Differentially expressed genes from strain-matched *Sftpc*^{+/+} and *Sftpc*^{-/-} mice (false discovery rate < 0.01; fold change > 1.5) were then further filtered by expression enrichment in normal type II cells. The relative enrichment was determined by genome-wide expression distribution analysis using archived microarrays of isolated, wild-type mouse type II epithelial cells (*n* = 11). The analysis identified a set of genes

Figure 3. Long-term resolution of LPS-induced injury is impaired. Images are of H&E-stained sections from the lungs of two representative *Sftpc*^{+/+} (A and C) and *Sftpc*^{-/-} mice (B and D) 30 days after the final repetitive LPS challenge. Pulmonary histopathology of *Sftpc*^{+/+} mice was limited to occasional inflammatory cells in the parenchyma. Persistent peribronchiolar cellular infiltrates, diffuse alveolar infiltrates, including enlarged macrophages and alveolar interstitial thickening, were present (B), and goblet cell morphology was still observed in the airways of the *Sftpc*^{-/-} mice (D). Images, 40× original magnification.

Figure 4. Expression of pulmonary innate defense molecules in the airspace is unchanged between *Sftpc*^{+/+} and *Sftpc*^{-/-} mice after repetitive LPS challenge. Western blot analysis was used to determine the levels of known airspace and airway protective molecules in bronchoalveolar lavage fluid (BALF) from *Sftpc*^{+/+} and *Sftpc*^{-/-} mice on Day 3 after final repetitive LPS challenge (A). The luminescence values for each protein indicate that the relative abundance was similar between genotypes of control or LPS-exposed mice (B). Samples were normalized to total protein in the BALF. **P* < 0.001; ***P* < 0.02.

altered in *Sftpc*^{-/-} type II cells relative to the strain-matched *Sftpc*^{+/+} cells that were also different in a large cohort of previous type II cell preparations. This comparison reduced the detection of changes due to variable contamination by other cell types.

From this analysis, the expression of 220 genes was increased in *Sftpc*^{-/-} cells, whereas 192 were decreased in *Sftpc*^{-/-} cells relative to *Sftpc*^{+/+} cells (Figure 6A). Among these, 122 genes were functionally related to immune response and inflammation based on gene ontology classification (66 were increased and 56 were decreased in the *Sftpc*^{-/-} versus *Sftpc*^{+/+} mice). A subset of these genes is composed of key components in the LPS/TLR signaling pathway (Figure 6B). As shown in Figure 6B, the LPS-binding protein, lipocalin 2, immunity-related GTPases (Ilgp1, Ifitm2, and RAB27a), TLR4, and TLR4 chaperone, HSP90B1, were up-regulated. Lipocalin 2 is induced by LPS/TLR4, and regulates innate microbial defense (17). The increased GTPases are members of a diverse family that control lysosomal-related secretory function and pathogen inactivation (18, 19). Several additional Toll-related downstream genes were increased at a lower threshold of significance (*P* < 0.05), and are listed outside of the cluster in Figure 6B. Within the cluster were several genes more broadly related to epithelial inflammation. Decreased expression of genes related to control of effective immunity could account in part for the overt inflammation response to modest challenge seen with SP-C deficiency. The

epsilon isoform of NF-κB inhibitor blocks nuclear translocation of NF-κB (20). Laptm5 mediates lysosome-related sorting, and has been reported to regulate TLR4-dependent cytokine secretion (21). A reduction of these factors may account for the inappropriate inflammatory response by the *Sftpc*^{-/-} type II cell. Tnfrsf13c (BAFF-R) is a B cell-activating factor that is decreased and may reduce the transition from innate to adaptive response (22). Taken together, these results support the observation that mice lacking SP-C and housed in pathogen-free microisolators have a baseline lung inflammation in the absence of a known stimulus, and that inflammation originates from an extensive alteration in the expression of protective genes by type II cells. Other functionally related pathways were altered in the type II cells from *Sftpc*^{-/-} mice, indicating that SP-C deficiency changes cellular homeostasis beyond the innate protective functions related to the observed decrease in resolving inflammation (Tables E2–E4).

SP-C Inhibition of LPS Signaling Requires Expression of the LPS Receptor

The LPS receptor was reconstituted in HEK293T cells by transfection with TLR4, CD14, and MD2 expression plasmids and cotransfection with NF-κB-responsive reporter plasmid, endothelial leukocyte adhesion molecule-luciferase (ELAM-Luc). Transfected cells were stimulated with LPS with or without SP-C vesicles. Preincubation with synthetic phospholipid vesicles containing 5% purified SP-C inhibited the LPS-stimulated luciferase activity (Figure 7A, lanes 2 versus 3). Similarly, the SP-C containing clinical surfactant extract inhibited the LPS-induced luciferase activity (Figure 7A, lane 4) (16, 23). However, preincubation with phospholipid vesicles alone did not affect LPS-stimulated luciferase activity (Figure 7B, lane 5). Incubation with an antibody to CD14 blocked the LPS-stimulated luciferase activity, confirming the specificity of TLR4-CD14-driven NF-κB inflammatory gene activation (Figure 7A, lane 5). Thus, both sources of SP-C, the purified-reconstituted and commercial, enriched extract reduced the LPS-stimulated signaling. The synthetic phospholipid vesicles alone did not influence either basal ELAM-Luc activity or LPS-stimulated ELAM-Luc activity (Figure 7B). In separate cell transfection experiments, the lipid A component alone induced a much lower activation of the TLR4-mediated ELAM-Luc readout than with complete LPS. We were unable to show blocking of this modest lipid A-stimulated ELAM-Luc with our SP-C preparations (data not shown).

To distinguish between the SP-C regulating TLR4/LPS interactions or inhibition of intracellular events downstream of TLR4 activation, HEK293 cells were transfected with a plasmid expressing the accessory factor, MyD88. In this system, constitutive MyD88 expression activates signaling events to increase NF-κB-luciferase activity independent of extracellular LPS binding to the membrane TLR4 complex. SP-C:phospholipid vesicles did not reduce the intracellular cytosolic MyD88-driven ELAM-Luc activity (Figure 7C), indicating that SP-C does not act via the soluble mediator/components of LPS-initiated signaling.

SP-C may also limit alveolar concentrations of proinflammatory mediators by binding to bacterial LPS. *E. coli* is a source of environmental LPS and a pulmonary pathogen to compromised individuals. Coincubation of liposomes that contain 5% SP-C with FITC-*E. coli* LPS yielded increased fluorescence relative to control liposome-binding reactions without SP-C. This result indicates that SP-C complexes with *E. coli* LPS, potentially sequestering inspired irritants to prevent or reduce alveolar inflammation (Figure 7D).

Figure 5. Inflammatory cytokine response of isolated *Sftpc*^{+/+} (shaded bars) and *Sftpc*^{-/-} (solid bars) type II cells exposed to LPS: Type II cells were maintained in culture for 3 days on matrigel matrix and then exposed to PBS or two concentrations of LPS (5 or 100 ng). Cytokine levels in the culture media (pg/ml) were determined by ELISA 24 hours after LPS stimulation.

DISCUSSION

Familial SP-C-mediated ILD and other forms of ILD present as a progressive disease hypothesized to result from recurrent injury. The LPS of gram-negative bacteria is a potent inducer of human inflammatory lung diseases, and our previous studies showed robust inflammation in the lungs of *Sftpc*^{-/-} mice after gram-negative bacterial infection. To determine the role of SP-C with LPS inflammation, we used a model of repetitive LPS exposure. The present data show that SP-C confers protective activity by inhibiting signaling through the TLR4-LPS receptor and in part regulates acute and persistent inflammatory responses in the intact lung.

As summarized in the introduction, SP-C deficiency diseases result from a variety of mutations causing aberrant pro forms, reducing levels of mature SP-C in the airspace (2–4), or decreasing *SFTPC* gene expression (5–7). *SFTPC*-related ILD is highly heterogeneous in both emergence and severity of disease. Family members with a defined *SFTPC* mutation were reported where ILD was detected during infancy, childhood, or adulthood that progressed to pulmonary fibrosis (3, 4). This highly variable phenotype suggests that other modifier genes influence the timing and morbidity from a specific *SFTPC* lesion. The majority of reported *SFTPC*-linked ILD has identified mutations that disrupt proSP-C mRNA or protein processing to induce cellular misfolded protein responses. A smaller group of individuals have reduced SP-C levels and decreased mRNA expression without the generation of aberrant forms of the proprotein. Specifically, a single study identified three infants with respiratory distress syndrome associated with mutations in the *SFTPC* promoter that decreased expression without introducing any distortion of SP-C biosynthesis (7). Together, these reports suggest a compound disease pathogenesis from both the absence of mature SP-C and/or from cellular stress induced by malformed proSP-C. The *Sftpc*^{-/-} mice are a true null model, and are informative about injury or compromised function due to reduced proSP-C and/or mature SP-C. The persistent/protracted LPS-induced inflammation observed with the *Sftpc*^{-/-} model may not fully represent the impact of reoccurring inflammation in affected patients where the alveolus is compromised by both the loss of SP-C activity as well as type II cell stress from aberrant SP-C biosynthesis.

Cellular and tissue inflammation induced by repetitive LPS exposure rapidly resolved in *Sftpc*^{+/+} mice, but persisted in the lungs of *Sftpc*^{-/-} mice at 5 days and 30 days after challenge. In a separate study, daily aerosolized LPS exposure was used to model chronic inflammation in mice. The chronic LPS exposure resulted in parenchymal injury and persistent airway remodeling disease similar to our results (24). The pulmonary pathology of *Sftpc*^{-/-} mice after sequential LPS challenges was similar to the morphological changes that occurred with *Pseudomonas aeruginosa* infection (12).

The repetitive LPS-induced injury provoked an unanticipated airway goblet cell metaplasia in challenged *Sftpc*^{-/-} animals. Spdef and Foxa3 promote goblet cell transdifferentiation and mucin production. Expression of these two critical transcription factors colocalized precisely to the regions of altered morphology. A pattern of pronounced airway epithelial inflammation and expression of goblet cell markers was reported after both *Pseudomonas* and RSV infection of *Sftpc*^{-/-} mice (12, 13). SP-C is present in tracheal aspirates, consistent with migration of surfactant up the airway from the primary site of secretion. SP-C specifically enhances immune recognition and neutralization of influenza A virus by the nasal epithelium, further supporting an immune-protective role for SP-C at the proximal respiratory epithelia (25). Collectively, these findings indicate that SP-C influences homeostasis along the alveolar and conducting respiratory epithelium. The inflammatory gene signature from arrays of enriched type II cells, in conjunction with the increased parenchymal and airway injury, support the interpretation that SP-C regulates innate protection along the entire respiratory surface.

Both SP-A and SP-D null mice had increased expression of proinflammatory cytokines, which was reduced by instillation of isolated SP-A and SP-D, respectively. SP-A and SP-D regulate LPS-induced cytokine responses through binding to LPS and TLR4 (26–29). In the present study, the SP-A and SP-D levels, and those of other antimicrobial molecules (lysozyme and lactoferrin) (30), were not different in BALF recovered from *Sftpc*^{+/+} and *Sftpc*^{-/-} mice. Expression of *Sftpa* and *Sftpd* mRNA in type II cells was not changed in the microarray analysis.

Prior studies have shown that exposure of isolated human type II cells in culture to LPS precipitated a vigorous cytokine

Figure 6. SP-C–dependent gene expression: (A) Changes in gene expression related to inflammation in type II cells from *Sftpc*^{−/−} mice. The pattern of gene expression is depicted as a heat map on the left, with green indicating increased expression and red indicating decreased expression. The fold change and statistical value of genes that were increased in the *Sftpc*^{−/−} type II cell preparations are listed on the right. (B) Biological association networks of up-regulated genes in *Sftpc*^{−/−} versus *Sftpc*^{+/+} type II cells. The functional relationships of genes changed by SP-C deletion were analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). Solid line indicates a direct relationship; dashed line indicates an indirect relationship. Nodes shaded in gray indicate significantly up-regulated genes (*Sftpc*^{−/−} versus *Sftpc*^{+/+}). In the absence of SP-C, several genes from the Toll-like receptor (TLR) 4 signaling pathway were significantly up-regulated. Genes with increased expression linked to inflammation or LPS/TLR4 signaling are indicated by the oval. A small subset of additional related Toll signaling genes that approached the *P* = 0.01 value are listed to the right.

release, demonstrating that this cell type is central to regulating the proinflammatory stasis of the alveolus (31). Using similar type II cell culture conditions, LPS stimulated a greater accumulation of cytokines IL-1 β , TNF- α , and KC in the media of *Sftpc*^{−/−} compared with *Sftpc*^{+/+} type II cells. Comparative microarray analysis of isolated type II cells identified *Sftpc*-dependent alteration of genes linked to inflammatory activity. The comparison of type II cells isolated from *Sftpc*^{−/−} to *Sftpc*^{+/+} littermates were compared and filtered against expression levels from an additional 11 distinct type II cell isolations from wild-type mice was used to reveal changes specifically due to loss of SP-C and minimize changes that might result from cell contamination during isolation. The *Sftpc*^{−/−}-dependent changes include genes that both sense LPS and initiate TLR signaling, as well as immune protective genes that participate in pathogen clearance. The signature of inflammatory-related genes in *Sftpc*^{−/−} type II cells included a group of genes with decreased relative expression known to repress steps in NF- κ B-related inflammatory/pathogen responses. Such a decrease may contribute to the escalating and sustained inflammation seen in SP-C-deficient mice. The finding of a widespread change in

inflammation and immunoprotective-related gene expression implicates SP-C as a central regulator of type II cell homeostasis and reaction to inflammatory ligands. The additional changes in functional groupings of gene expression detected in *Sftpc*^{−/−} type II cells are included as supplemental data (Tables E2–E4).

The present data show that an intact LPS receptor (TLR4/CD14/MD2) was required for SP-C inhibition of NF- κ B-mediated expression. The TLR4-activated signaling was reduced by both purified SP-C phospholipid vesicles and by the commercial surfactant extract, Survanta. TLR4 is a type I receptor that interacts with intracellular adaptors, including MyD88, to initiate signaling. SP-C did not influence intracellular signaling initiated directly from MyD88 in the absence of the LPS receptor. Thus, SP-C inhibitory activity requires membrane (lipid vesicle) structures, and not free cytosolic components, consistent with the extreme hydrophobic nature of mature SP-C.

Using a sensitive fluorescence assay, the purified native SP-C bound to LPS of the opportunist pulmonary pathogen *E. coli*. This finding is consistent with previous studies showing SP-C binding with *Salmonella* LPS (14, 32). These data suggest that SP-C likely associates with a range of gram-

Figure 7. SP-C inhibits LPS-induced TLR4-mediated gene expression. Potential inhibition of TLR4 activity was assessed in transfected human embryonic kidney (HEK) 293 cells using synthetic phospholipid vesicles with and without SP-C or Survanta, a surfactant extract containing SP-C. (A) SP-C is required to reduce inflammatory signaling: LPS stimulated TLR4-mediated luciferase activity. Pretreatment with either of the SP-C-containing vesicles or Survanta reduced the LPS-induced activity (SP-C, *P* = 0.03; Survanta, *P* = 0.02). Inclusion of an antibody to the CD14 component of the TLR4 signaling complex ablated the LPS-induced NF- κ B-mediated luciferase activity (*P* = 0.003; *n* = 5). (B) The phospholipid vesicle mixture without SP-C does not inhibit the LPS-induced luciferase activity. Survanta, CD14 blocking antibody, or phospholipid vesicles alone

did not alter baseline ELAM-Luc reporter activity (*n* = 5). (C) SP-C does not block intracellular-cytosolic MyD88-mediated NF- κ B-induced activity. SP-C inhibition experiments were repeated with HEK293 cells transfected with the cytosolic accessory factor, MyD88, with variable amounts of SP-C-phospholipid vesicles or the phospholipid vesicles alone (*n* = 3). (D) SP-C increases binding of FITC-labeled *E. coli* LPS. The recovered fluorescence of 0111:B4 LPS incubated with liposomes was increased by incorporation of SP-C. Values are from four replicates $\pm$ SEM.

negative endotoxins. Thus, the influence of SP-C on alveolar inflammation is complex and involves binding to trace amounts of LPS and blocking of TLR4-mediated inflammatory signaling of LPS receptors.

Recently, minor species of surfactant phospholipids, palmitoyl-oleoyl-phosphatidylglycerol and phosphatidylinositol, were found to inhibit LPS-induced signaling in macrophages and reduced lung inflammation *in vivo* (33). In the current study, phospholipid vesicles comprised of the surfactant phospholipids dipalmitoyl-phosphatidylcholine, dipalmitoyl oleoyl-phosphatidylcholine did not reduce the LPS activation of TLR4 signaling, indicating that these two abundant surfactant phospholipid species do not independently exert anti-inflammatory activity. In a separate study, Survanta was shown to inhibit LPS signaling *in vitro* by blocking translocation of TLR4 to lipid rafts in A549 cells (34). Future studies will determine if SP-C reconstituted with defined lipids redirects TLR4 localization in response to LPS. Thus, although SP-A and SP-D and minor surfactant phospholipid species influence LPS-induced inflammation, the current findings are consistent with an essential role for SP-C in protection from repetitive LPS injury.

Sftpc^{-/-} mice were found to be more susceptible to infection with the respiratory pathogen, RSV (13). RSV induces inflammation by double-stranded RNA activation through the TLR family member, TLR3. TLR3 expression was increased in the lung of *Sftpc*^{-/-} mice, and SP-C was shown to specifically block TLR3-mediated signaling in HEK293 cells, comparable to the inhibition of reconstituted TLR4 signaling in this study. The RSV-infected *Sftpc*^{-/-} mice had long-term residual inflammation that is similar to the persistent inflammation detected after repetitive LPS challenge. These data indicate that SP-C is required to both reduce lung inflammation and enhance resolution of inflammation induced by distinct microbes. In a recent study, sustained TLR3 activation and long-term lung inflammation was achieved using three sequential exposures to double-stranded RNA similar to the current LPS protocol (35). Together, these findings indicate that the infections linked to exacerbations of lung disease in affected individuals may in part be due to recurrent or unremitting signaling by multiple TLR isoforms that include TLR3 and -4.

In summary, the SP-C-deficient mice exhibit an underlying inflammation that increases with repetitive LPS exposure as well as bacterial or viral infection. *Ex vivo* analysis of isolated type II cells identified a pattern of gene expression and cytokine elaboration consistent with an intrinsic activated inflammatory status.

Mechanisms of LPS inhibition include binding to LPS and reduced signaling through the LPS receptor. LPS is a contaminant of environmental particulates, including house dust mite and cockroach byproducts known to induce asthma and allergic disease. LPS represents a common, potentially continuous inciting challenge to susceptible individuals. Diminished levels of SP-C in affected individuals could similarly impair resolution of inflammation from a variety of microbial sources mediated by Toll receptors and stimulate progressive interstitial disease. Considered together, the data suggest that treatment with SP-C preparations may be a useful adjuvant in reducing lung inflammation in patients with forms of SP-C-deficient diseases.

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