Surfactant Dysfunction in SP-A^{-/-} and iNOS^{-/-} Mice with Mycoplasma Infection

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Surfactant dysfunction was studied in C57BL/6 (B6), B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice with pulmonary mycoplasma infection (10⁷ colony-forming units). Cell-free bronchoalveolar lavage (BAL) from uninfected B6.SP-A^{-/-} versus B6 mice had a reduced content of very large aggregates (VLA) and an increase in intermediate large aggregates (ILA), with no difference in total large aggregates (LA = VLA + ILA). However, LA from uninfected B6.SP-A^{-/-} versus B6 mice contained less protein and were more sensitive to inhibition by serum albumin and lysophosphatidylcholine in pulsating bubble studies *in vitro*. Infection with *Mycoplasma pulmonis* caused significant lung injury and surfactant abnormalities in B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice at 24, 48, 72 h after infection compared with uninfected mice of the same strain. Analyses of time-pooled data indicated that mycoplasma-infected B6.SP-A^{-/-} and B6.iNOS^{-/-} mice had significantly lower levels of LA and higher protein/phospholipid ratios in BAL compared with infected B6 mice. Infected B6.iNOS^{-/-} versus B6 mice also had increased minimum surface tensions on the pulsating bubble and decreased levels of surfactant protein (SP)-B in BAL. These results indicate that pulmonary mycoplasma infection *in vivo* causes lung injury and surfactant abnormalities that are dependent in part on iNOS and SP-A. In addition, SP-A deficiency modifies surfactant aggregate content and lowers the inhibition resistance of LA surfactant *in vitro* compared with congenic normal mice.

Keywords: lung injury; minimum surface tension; SP-B; lipid aggregate fractions; *M. pulmonis*

Mycoplasma pneumoniae (mycoplasmas) accounts for a significant fraction (10–20%) of community-acquired pneumonias in the general population, as well as being a frequent cause of tracheobronchitis and other upper respiratory symptoms (1). Mycoplasma-induced pneumonia was once thought to be relatively uncommon in children under five and in older adults, but is now recognized as affecting a broad spectrum of patient age groups (1). In addition to causing a primary pneumonia, mycoplasmas are known to exacerbate the pathophysiology of asthma

CLINICAL RELEVANCE

Mycoplasmas account for a large fraction of pneumonias, and may cause severe systemic problems, such as arthritis. In this article, we show that mycoplasmas damage the pulmonary surfactant, an essential component of normal lung function.

and chronic obstructive pulmonary disease (COPD) (2). Less common, but severe associated conditions precipitated by mycoplasma infection include acute lung injury (ALI), acute respiratory distress syndrome (ARDS), pericarditis, myocarditis, hemolytic anemia, and encephalitis (3).

Infection of C57BL/6 (B6) mice with *Mycoplasma pulmonis* reproduces the essential features of human respiratory mycoplasmosis. Alveolar macrophage (AM) activation has been demonstrated to be essential for the killing of *M. pulmonis* *in vitro* (4). Both surfactant protein (SP)-A and inducible nitric oxide synthase (iNOS) are thought to be important in pulmonary defenses against respiratory pathogens such as mycoplasmas. The present paper investigates lung surfactant abnormalities in mice with respiratory mycoplasma infection, and examines the specific importance of SP-A and iNOS in mycoplasma-induced surfactant dysfunction through the study of B6.SP-A^{-/-} and B6.iNOS^{-/-} mice. In addition, experiments also assess the specific aggregate composition, surface activity, and inhibition resistance of surfactant from uninfected B6.SP-A^{-/-} mice compared with uninfected B6 mice.

Lung SP-A plays multiple roles in pulmonary biology, and related protein forms are present in all vertebrate classes, including most primitive amphibious fish (5). SP-A has calcium-dependent activity in increasing the aggregation and molecular order of phospholipids, including promoting tubular myelin formation in lung surfactant in conjunction with SP-B (6, 7). SP-A also enhances the ability of lung surfactant extracts to resist biophysical inhibition by plasma proteins *in vitro* (7–10). As a member of the collectin family of host defense proteins, SP-A also contributes to innate pulmonary immunity (11–13). We have previously studied *M. pulmonis* infection in B6 mice and showed that SP-A is necessary for maximal mycoplasma killing (4, 14, 15) by AM by upregulating the production of NO and reactive oxygen-nitrogen intermediates in these cells (14, 15). Both SP-A^{-/-} and iNOS^{-/-} mice have a decreased ability to clear intratracheally instilled mycoplasmas. However, SP-A and iNOS-dependent abnormalities in pulmonary surfactant have not been investigated in detail in mycoplasma infection as is done here using congenic B6.SP-A^{-/-} and B6.iNOS^{-/-} as well as B6 mice. Studies in mycoplasma-infected mice test the hypothesis that a deficiency in SP-A or iNOS leads to a significant increase in the severity of mycoplasma-induced surfactant dysfunction in the respective knockout models compared with B6 mice. An additional hypothesis

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tested was that surfactant from uninfected B6.SP-A^{-/-} mice will have an altered distribution of VLA and ILA compared with uninfected B6 mice, and will be more sensitive to biophysical inhibition by lysophosphatidylcholine (LPC) and albumin *in vitro*.

MATERIALS AND METHODS

Animals

C57BL/6Ncr (B6) mice were obtained from the National Cancer Institute (Frederick, MD). C57BL/6 SP-A^{-/-} (B6.SP-A^{-/-}) N10 mice were generated from 129 × BS SP-A^{-/-} mice provided by Drs. Whitsett and Korfhagen (University of Cincinnati, Ohio) (16) by backcrossing for at least 10 generations onto the B6 background (15). Mice were bred at the University of Alabama at Birmingham (UAB), and genotype was characterized from tail DNA as described previously (15). C57BL (C57BL/6J-*Nos2^{tm1Lau}*) transgenic mice lacking the gene for iNOS (B6.iNOS^{-/-}) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) (14) and were bred at UAB in autoclaved microisolator cages (Lab Products, Maywood, NJ). Mice of both sexes were used, and animals were maintained in a sterile environment and provided with autoclaved food (Agway, Syracuse, NY) and water *ad libitum* until being studied at 8–12 wk of age (20–25 g body weight). All mice were monitored and found to be negative for murine pathogens by the Health Surveillance Facility at UAB (17).

Mycoplasma Infection Model

Animal experiments were performed according to protocols and guidelines approved by the Institutional Animal Care and Use Committee guidelines at the University of Alabama at Birmingham (UAB). For mycoplasma infection, mice were inoculated intranasally with the UAB CT strain of *M. pulmonis* (18), while control mice received an equal 50- μ l intranasal volume of sterile broth. An inoculate dose of 10⁷ colony-forming units (cfu) was confirmed by serial dilution and plating on agar for 7 d (4). The dose of 10⁷ cfu was chosen because exposure to lower amounts of mycoplasma did not result in a reproducible level of injury to the pulmonary surfactant system in B6 mice. All mouse strains were studied at 24, 48, and 72 h after inoculation with *M. pulmonis*.

Bronchoalveolar Lavage

After being killed at a designated time point, mice were lavaged with a total of 3 ml of 0.15 M NaCl, given in three equally divided 1-ml aliquots. Recovered aliquots of lavage fluid were pooled on ice, centrifuged immediately at 150 × *g* for 10 min to remove cells, and the cell-free supernatant was then frozen at -20°C for biochemical and biophysical analyses.

Aggregate Subfractions

In the majority of studies, cell-free bronchoalveolar lavage (BAL) was centrifuged at 12,500 × *g* for 30 min to sediment the total large aggregate (LA) fraction. In the subset of experiments investigating aggregate subfractions, cell-free BAL was initially centrifuged at 1,500 × *g* for 10 min to pellet very large aggregates (VLA) and the supernatant was then re-centrifuged at 12,500 × *g* for 30 min to pellet intermediate large aggregates (ILA). The fraction of smaller aggregates remaining in the supernatant after centrifugation at 12,500 × *g* for 30 min was also examined in terms of its content relative to LA, VLA, and ILA.

Biochemical Measurements in BAL

Total phospholipid in cell-free BAL and aggregate fractions was measured by the assay of Ames (19), and total protein was determined using the colorimetric assay of Lowry and coworkers (20) modified by the addition of 15% SDS to allow accurate quantitation of protein in the presence of lipid. Total hydrophobic surfactant protein was measured by the modified Lowry assay following the extraction of cell-free BAL or centrifuged aggregates into chloroform:methanol by the method of Bligh and Dyer (21). The composition of phospholipid classes in cell-free BAL and LA was determined on the basis of phosphate measurements after extraction and one-dimensional thin-layer chromatography (TLC) on 250- μ m thick silica gel G (Analtech, Newark, DE) using a

solvent system of chloroform:methanol:2-propanol:triethylamine:water (30:9:25:25:7 by volume) (22).

Surface Activity Measurements

The overall surface tension lowering ability of surfactant dispersions was measured during cycling at a physiologic rate of 20 cycles/min at 37 ± 0.5°C on a pulsating bubble surfactometer (General Transco, Largo, FL) (7, 23). Cell-free BAL or centrifuged aggregates were evaporated under nitrogen and then resuspended with hand vortexing in 0.15 M NaCl plus 2 mM CaCl₂ at the desired phospholipid concentration (1.0 or 2.0 mg/ml). When present, inhibitors were added to resuspended cell-free BAL or surfactant aggregates and incubated for 30 min at 37°C before surface activity measurements. Substances studied for inhibitory effects on the activity of surfactant from uninfected B6.SP-A^{-/-} versus B6 mice were bovine serum albumin (BSA) (Fraction V, 3 mg/ml; Sigma Chemical Company, St. Louis, MO) or C18:1 lysophosphatidylcholine (LPC) (0.125 mg/ml; Sigma Chemical). Aliquots of surfactant or surfactant/inhibitor mixtures were introduced into a 40- μ l plastic sample chamber mounted on the pulsator unit of the surfactometer, and an air bubble (communicating with ambient air) was formed and repetitively oscillated between maximum and minimum radii of 0.55 and 0.4 mm at a rate of 20 cycles of compression–expansion per min. The pressure drop across the air–water interface of the bubble was measured by a precision pressure transducer, and surface tension at minimum bubble radius (minimum surface tension) was calculated as a function of time of pulsation using the Laplace equation for a spherical interface (7, 24).

In experiments testing the direct effects of *M. pulmonis* on surface activity, BAL from uninfected mice of a given strain was exposed to 2 × 10⁷ cfu/ml of mycoplasma organisms *in vitro* for ≥ 48 h at room temperature. BAL was then centrifuged at 12,500 × *g* for 30 min to pellet LA, and minimum surface tension was measured on the pulsating bubble apparatus (37°C, 20 cycles/min, 50% area compression).

SP-B Levels in BAL

SP-B levels were specifically examined in BAL for each mouse strain at 48 h after inoculation with mycoplasmas (10⁷ cfu/mouse). SP-B in cell-free BAL was detected as described by Griese and colleagues (25). For SP-B detection, the protein content each sample was determined with the BioRad Protein Assay Kit (BioRad, Richmond, CA) based on the method of Bradford (26). Five micrograms of total protein were separated under nonreducing conditions on 12% Bis-Tris gels using a Criterion Cell and Blotter System (BioRad, Carlsbad, CA). Two gels were prepared in parallel for each sample, and after electrophoresis one was stained with SYPRO Ruby protein gel stain (Invitrogen, Carlsbad, CA) and the other subjected to Western blotting. For immunodetection, the proteins in the gels were transferred onto a PVDF membrane with a Criterion Blot module in a wet buffer system (all from BioRad). Overnight blotting was with the primary rabbit polyclonal anti-SP-B antibody (1:1,000 dilution; Chemicon International, Inc., Temecula, CA) and horseradish peroxidase–conjugated goat anti-rabbit polyclonal anti-IgG (1:10,000 dilution; Amersham Biosciences, Piscataway, NJ). We used an ECL Detection system to detect SP-B, which appeared as a band at 18–20 kD.

Statistical Analyses

All data are expressed as mean ± 1 SEM. The pooled variance *t* test was used to assess statistical significance for statistical analysis of discrete data on biochemical composition and aggregate content. Quantitative measures (surface tension, phospholipids levels, etc.) data were analyzed between mouse strains by one-way ANOVA with Tukey's HSD (Honest Significant Difference) *post hoc* procedure to identify points of significant difference. In some cases, data were analyzed by ANOVA followed by Tukey's multi-group comparison of the means after log conversion or by Kruskal Wallis ANOVA and Pearson's correlation of the means for nonparametric data (Analytical Software, St. Paul, MN). In all statistical analyses, differences were considered significant if the *P* value was < 0.05.

RESULTS

Aggregate Content of BAL from Uninfected B6.SP-A^{-/-} versus B6 Mice

In an initial set of studies, cell-free BAL obtained from groups of 10–15 uninfected B6.SP-A^{-/-} mice was pooled and assessed for aggregate composition compared to pooled lavage from uninfected B6 mice. Uninfected B6.SP-A^{-/-} had a reduced percentage of BAL phospholipid in the VLA fraction sedimenting at $1,500 \times g$ for 10 min as compared with B6 mice ($31 \pm 6\%$ versus $52 \pm 6\%$ of total BAL phospholipid, $P < 0.05$) (Figure 1). However, this decrease in VLA in uninfected B6.SP-A^{-/-} mice was offset by an increase in ILA obtained by centrifuging the supernatant of the $1,500 \times g$ spin at $12,500$ (i.e., aggregates sedimenting between $1,500 \times g$ and $12,500 \times g$). B6.SP-A^{-/-} mice had $40 \pm 6\%$ of total BAL phospholipid in the ILA pellet compared to only $22 \pm 3\%$ for B6 mice (Figure 1; $P < 0.01$). Due to the offsetting changes in VLA and ILA, there was no difference between uninfected B6.SP-A^{-/-} and B6 mice in the content of total large surfactant aggregates (LA = VLA + ILA) sedimenting from BAL at $\leq 12,500 \times g$. Consistent with this, uninfected B6.SP-A^{-/-} and B6 mice also had no difference in smaller aggregates remaining in the supernatant after centrifugation at $12,500 \times g$ (aggregates sedimenting at $>12,500 \times g$, Figure 1).

Phospholipid and Protein Composition of BAL and Aggregates from Uninfected B6.SP-A^{-/-} versus B6 Mice

Total phospholipid in cell-free BAL was equivalent for uninfected B6.SP-A^{-/-} and B6 mice (0.18 ± 0.01 mg/ml and 0.17 ± 0.02 mg/ml, respectively). In addition, the phospholipid class distributions and total protein levels in whole cell-free BAL did not differ between these mice (Tables 1 and 2). However, centrifuged LA from uninfected B6.SP-A^{-/-} mice had a significantly lower total protein content compared with uninfected B6 mice (4.9 ± 1.7 versus $9.3 \pm 1.4\%$ by weight relative to phospholipid, $P < 0.05$ Table 2). The lower protein content of LA surfactant from uninfected B6.SP-A^{-/-} versus B6 mice in Table 2 is consistent with the lack of SP-A in the knockout animals. LA from uninfected B6.SP-A^{-/-} and B6 mice had equal levels of chloroform-extracted hydrophobic protein indica-

Figure 1. Content of different aggregate subfractions in surfactant from uninfected B6.SP-A^{-/-} and B6 mice. Surfactant aggregate fractions are: VLA sedimented by centrifugation at $1,500 \times g$ for 10 min; ILA obtained when the supernatant after the $1,500 \times g$ centrifugation was spun at $12,500 \times g$ for 30 min; and smaller aggregates that did not sediment at $12,500 \times g$. * $P < 0.05$ and # $P < 0.01$ compared with B6 mice. Data are mean $\pm$ SEM for $n = 3$.

TABLE 1. PHOSPHOLIPID COMPOSITION OF WHOLE BRONCHOALVEOLAR LAVAGE SURFACTANT AND CENTRIFUGED LARGE AGGREGATES FROM UNINFECTED B6.SP-A^{-/-} AND B6 MICE

Phospholipid class	B6 mice		B6.SP-A ^{-/-} mice	
	Cell-free BAL	LA	Cell-free BAL	LA
Lyso-PC	0.30 $\pm$ 0.3	0.90 $\pm$ 0.6	0.90 $\pm$ 0.4	0.60 $\pm$ 0.4
SPH	4.90 $\pm$ 1.0	3.70 $\pm$ 1.1	4.30 $\pm$ 1.3	4.40 $\pm$ 1.7
PC	83.8 $\pm$ 1.5	82.5 $\pm$ 2.0	83.1 $\pm$ 2.3	83.4 $\pm$ 2.7
PI	3.20 $\pm$ 0.8	3.10 $\pm$ 1.4	2.40 $\pm$ 0.9	2.60 $\pm$ 1.3
PE	1.50 $\pm$ 0.7	1.50 $\pm$ 0.4	1.90 $\pm$ 1.1	1.80 $\pm$ 0.7
PG	5.60 $\pm$ 1.3	7.40 $\pm$ 0.5	6.20 $\pm$ 1.8	6.30 $\pm$ 1.9
R	0.60 $\pm$ 0.7	0.90 $\pm$ 0.3	1.20 $\pm$ 0.5	0.90 $\pm$ 0.9

Definition of abbreviations: BAL, bronchoalveolar lavage; LA, large aggregate; Lyso-PC, lyso-phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; R, residual; SPH, sphingomyelin.

BAL samples from uninfected B6 and B6.SP-A^{-/-} mice were immediately centrifuged at $150 \times g$ for 10 min to remove cells. For each mouse strain, BAL from three to six individual animals was pooled and total LA were obtained by centrifugation of at $12,500 \times g$ for 30 min. This LA fraction included both VLA (sedimenting at $1,500 \times g$) and ILA (sedimenting between $1,500 \times g$ and $12,500 \times g$) in Figure 1. Phospholipid classes were defined by thin layer chromatography with a solvent system of chloroform:methanol:2-propanol:triethylamine:water (30:9:25:25:7 by volume) (see MATERIALS AND METHODS). Data are mean $\pm$ SEM for $n = 3$ pooled samples. No significant differences in phospholipid composition were present between uninfected B6 and B6.SP-A^{-/-} mice.

tive of SP-B/C (Table 2). Additional analyses not included in Table 2 showed that VLA and ILA from uninfected B6.SP-A^{-/-} also had a reduced total protein content as compared with B6 mice (6.4 ± 1.0 versus $8.2 \pm 0.6\%$ by weight for VLA and 4.7 ± 1.1 versus $7.5 \pm 0.6\%$ by weight for ILA, respectively; $P < 0.05$). These findings of reduced protein in VLA and ILA from uninfected B6.SP-A^{-/-} versus B6 mice are again consistent with the lack of SP-A in the knockout animals.

Surface Activity (Uninhibited) of BAL and Aggregate Fractions from Uninfected B6.SP-A^{-/-} versus B6 Mice

The surface activity of each form of surfactant studied (VLA, ILA, LA, or whole BAL) for uninfected B6.SP-A^{-/-} and B6 mice increased as surfactant phospholipid concentration increased from 1 mg/ml to 2 mg/ml (Figure 2). However, none of the surface tension lowering curves for comparable forms of

TABLE 2. PERCENTAGES OF TOTAL PROTEIN AND HYDROPHOBIC PROTEIN IN CELL-FREE BRONCHOALVEOLAR LAVAGE AND CENTRIFUGED LARGE AGGREGATES FROM UNINFECTED B6.SP-A^{-/-} AND B6 MICE

	Total Protein (wt %)	Hydrophobic Protein (wt %)
Cell-free BAL		
B6	54.1 $\pm$ 1.0	1.85 $\pm$ 0.15
B6.SP-A ^{-/-}	50.5 $\pm$ 6.0	1.77 $\pm$ 0.14
Pelleted LA		
B6	9.29 $\pm$ 1.35	1.77 $\pm$ 0.11
B6.SP-A ^{-/-}	4.87 $\pm$ 1.72*	1.72 $\pm$ 0.18

Definition of abbreviations: BAL, bronchoalveolar lavage; LA, large aggregate.

Protein is expressed in wt % relative to total phospholipid. Cell-free BAL from three to six uninfected B6.SP-A^{-/-} or B6 mice was pooled, and large aggregates (LA = VLA + ILA) were obtained by centrifugation at $12,500 \times g$ for 30 min as in the legend to Table 1. Free plasma- or cell-derived proteins in BAL remain in the supernatant of this centrifugation. Hydrophobic protein was measured following extraction into chloroform (21). Data are mean $\pm$ SEM for $n = 3$ pooled samples.

* $P < 0.05$ compared with uninfected B6 mice.

Figure 2. Dynamic surface tension lowering of surfactant aggregates from uninfected B6.SP-A^{-/-} and B6 mice. Surface activity was measured on a pulsating bubble surfactometer (37°C, 20 cycles/min, 50% area compression) at a uniform surfactant phospholipid concentration (1.0 or 2.0 mg/ml in 0.15 M NaCl + 2 mM CaCl₂). (A) Resuspended BAL. (B) Resuspended VLA pelleted from BAL by centrifugation at 1,500 × *g* for 10 min. (C) Resuspended ILA obtained from BAL by centrifugation of the VLA supernatant at 12,500 × *g* for 30 min. (D) Resuspended total large aggregates (LA = VLA + ILA). Data are mean ± SEM for *n* = 4–9.

surfactant (VLA, LA, ILA, or whole BAL) differed significantly between uninfected B6.SP-A^{-/-} and B6 mice by ANOVA at either phospholipid concentration (Figures 2A–2D). At a fixed phospholipid concentration, the relative surface activity of the different forms of surfactant studied from both uninfected B6.SP-A^{-/-} and B6 mice was ordered as: VLA > LA > ILA > whole BAL (Figure 2). This behavior is consistent with the known concentration-dependent activity of lung surfactants and the fact that larger aggregate forms of surfactant exhibit the greatest surface activity (*see Refs. 7, 27, and 28 for review*).

Inhibition Resistance of Whole BAL and LA from Uninfected B6.SP-A^{-/-} versus B6 Mice

Resuspended BAL and LA from uninfected B6.SP-A^{-/-} versus B6 mice had less ability to resist inhibition when incubated with either BSA (3 mg/ml; Figure 3) or C18:1 LPC (0.125 mg/ml; Figure 4). For both types of mice, the greatest inhibitor-induced reductions in surface activity were found at a low surfactant concentration of 1 mg phospholipid/ml (Figures 3A and 4A), and inhibition was decreased at a higher concentration of 2 mg phospholipid per milliliter (Figures 3B and 4B). However, at either phospholipid concentration, whole BAL and LA from uninfected B6.SP-A^{-/-} versus B6 mice had elevated minimum surface tensions in the presence of BSA or LPC (Figures 3 and 4).

Effects of Mycoplasma Infection on Lung Injury and Surfactant Content in B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} Mice

Following studies of surfactant aggregates and their activity in pooled surfactant from uninfected B6.SP-A^{-/-} versus B6

mice, remaining experiments focused on the effects of mycoplasma infection on lung injury and surfactant abnormalities in B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice. All strains of mice infected with intranasal mycoplasma (10⁷ cfu) had evidence of lung injury and surfactant dysfunction (Table 3). Mycoplasma-infected B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice had higher levels of BAL total protein, higher protein/phospholipid ratios in BAL, and lower levels of LA in BAL at all time points of injury studied (24, 48, 72 h) compared with uninfected mice of the same strain (Table 3). However, no significant intrastrain differences were observed for any BAL parameter across the three time points of injury studied (Table 3). Thus, data for BAL parameters at all three injury times were combined for each mouse strain and analyzed statistically to examine differences between mycoplasma-infected and uninfected mice. Analyses of time-pooled data for each mouse strain were consistent with the results already noted at individual times in Table 3—that is, mycoplasma-infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice all had significant decreases in LA in BAL (Figure 5), increased total protein levels in BAL (Figure 6), and increased protein/phospholipid ratios in BAL (Figure 7), compared to uninfected mice of the same strain. Minimum surface tension values on the bubble surfactometer were also elevated after 0.25 min and 20 min of pulsation for resuspended LA's from infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice compared with uninfected mice of the same strain (Figures 8A and 8B).

In addition to documenting lung injury and surfactant dysfunction in mycoplasma-infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and

Figure 3. Inhibition resistance to albumin for surfactant from uninfected B6.SP-A^{-/-} and B6 mice. (A) Surfactant concentration of 1 mg/ml phospholipid. (B) Surfactant concentration of 2 mg/ml phospholipid. Bovine serum albumin (BSA, 3.0 mg/ml) was added to resuspended BAL or LA and incubated for 30 min at 37°C before being studied on the bubble apparatus. Surface activity in the absence of BSA is shown earlier in Figures 2A and 2D. **P* < 0.05 and #*P* < 0.01 compared with B6 mice at the same time of pulsation. Data are mean ± SEM for *n* = 4–9.

B6 mice compared to uninfected same-strain controls, analyses of time-pooled injury data also identified significant differences between mouse strains in responses to mycoplasma infection. One-way ANOVA analysis followed by *post hoc* Tukey's HSD testing indicated that mycoplasma-infected B6.SP-A^{-/-} and B6.iNOS^{-/-} mice had significantly decreased levels of LA in BAL (Figure 5), and increased ratios of protein to phospholipid in BAL (Figure 7), compared with mycoplasma-infected B6 mice. Infected B6.SP-A^{-/-} mice also had increased levels of total protein in BAL compared with infected B6 mice (Figure 6). In addition, resuspended LA from infected B6.iNOS^{-/-} mice had increased minimum surface tensions after 0.25 min or 20 min of bubble pulsation compared with infected B6 mice (Figure 8). Differences in LA surface activity for infected B6.SP-A^{-/-} mice versus B6 mice approached, but did not reach, statistical significance (*P* = 0.06; Figure 8).

To identify whether direct exposure to mycoplasmas contributed significantly to lung surfactant activity deficits in the absence of an inflammatory response, BAL samples from uninfected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice were exposed to *M. pulmonis* *in vitro* (2 × 10⁷ cfu/ml) for 48 h. Mycoplasma-exposed BAL were then centrifuged to pellet large surfactant aggregates, and surface tension-lowering ability was assessed in comparison to control (unexposed) aggregates. As shown in Table 4, direct exposure of surfactant from B6 mice to myco-

Figure 4. Inhibition resistance to LPC for surfactant from uninfected B6.SP-A^{-/-} and B6 mice. (A) Surfactant concentration of 1 mg/ml phospholipid. (B) Surfactant concentration of 2 mg/ml phospholipid. C18:1 LPC (0.125 mg/ml) was added to resuspended cell-free BAL or LA and incubated for 30 min at 37°C before bubble measurements. Surface activity data in the absence of LPC are shown earlier in Figures 2A and 2D. **P* < 0.05 and #*P* < 0.01 compared with B6 mice at the same time of pulsation. Data are mean ± SEM for *n* = 4–9.

plasma organisms did not alter surface activity. In addition, only minor decreases in surface activity were apparent for mycoplasma-exposed surfactant from B6.SP-A^{-/-} and B6.iNOS^{-/-} mice. All surfactant samples exposed to mycoplasma *in vitro* reached minimum surface tension values of < 1 mN/m after 20 min of bubble pulsation, with the exception of B6.iNOS^{-/-} mice (which still reached a very low mean minimum surface tension of 1.7 mN/m; Table 4).

Levels of SP-B in BAL at 48 h after Mycoplasma Infection

Western blotting studies of BAL using specific anti-SP-B antibodies revealed a single band just below 20 kD, corresponding to the dimer form of SP-B. Respiratory mycoplasma infection caused a significant increase in the level of SP-B in BAL from B6 mice compared to uninfected same-strain controls at 48 h after inoculation (Figure 9). SP-B levels in BAL were unchanged for mycoplasma-infected B6.SP-A^{-/-} mice at the 48-h time point compared with uninfected B6.SP-A^{-/-} mice. In contrast, there was a significant decrease of about 40% in the levels of SP-B in BAL from mycoplasma-infected B6.iNOS^{-/-} mice compared with uninfected controls at 48 h after inoculation with mycoplasma (Figure 9). It should be stressed that equal amounts of proteins (5 µg) were loaded in each lane. Because of the very low levels of SP-B in mouse BAL, we were unable to detect it when we stained the 12% Bis-Tris gels with SYPRO Ruby protein gel

TABLE 3. PHOSPHOLIPID, PROTEIN, AND LARGE AGGREGATE CONTENT OF CELL-FREE BRONCHOALVEOLAR LAVAGE FROM MICE AT 24, 48, AND 72 h AFTER INFECTION WITH *M. pulmonis*

Groups	Phospholipid (mg/ml)	Protein (μg/ml)	Protein/PL (%)	LA (%)
Uninfected				
B6	0.19 ± 0.01	134 ± 10	70 ± 3	58 ± 1
B6.SP-A ^{-/-}	0.18 ± 0.03	151 ± 42	77 ± 6	59 ± 2
B6.iNOS ^{-/-}	0.15 ± 0.01	126 ± 8	84 ± 5	58 ± 2
24 h after infection				
B6	0.21 ± 0.02	225 ± 19	113 ± 10	43 ± 2
B6.SP-A ^{-/-}	0.19 ± 0.01	221 ± 14	115 ± 4	41 ± 2
B6.iNOS ^{-/-}	0.15 ± 0.01	240 ± 20	169 ± 23	40 ± 2
48 h after infection				
B6	0.22 ± 0.02	207 ± 30	98 ± 13	50 ± 1
B6.SP-A ^{-/-}	0.21 ± 0.02	275 ± 38	133 ± 12	43 ± 2
B6.iNOS ^{-/-}	0.15 ± 0.01	218 ± 21	145 ± 13	42 ± 2
72 h after infection				
B6	0.17 ± 0.02	191 ± 19	115 ± 15	45 ± 3
B6.SP-A ^{-/-}	0.23 ± 0.03	343 ± 63	147 ± 13	39 ± 2
B6.iNOS ^{-/-}	0.19 ± 0.01	287 ± 20	154 ± 8	39 ± 3

Definition of abbreviations: LA, large aggregate; PL, phospholipid.

Data are means ± SEM for *n* = 8–10. Infection was with 10⁷ cfu of mycoplasma given intranasally at time 0, and total LA were obtained from cell-free BAL by centrifugation at 12,500 × *g* for 30 min (see MATERIALS AND METHODS). Tabulated values for protein, protein/PL ratio, and LA content were all statistically significant (*P* < 0.05 or less) for mycoplasma-infected B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice at 24, 48, and 72 h compared with uninfected animals of the same strain. Values for phospholipid, protein, protein/PL ratio, and LA were not significantly different between the three types of infected mice by ANOVA at a fixed time of injury. However, when data at all three time points of mycoplasma injury were pooled for each strain, statistical differences between B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice were found as discussed in the text (cf. Figures 5–8).

stain. We thus normalized the digitized signal of the SP-B band seen in the Western blot to a protein of about 85 kD seen when gels were stained with SYPRO Ruby. The results were identical to those shown in Figure 9. Thus, we feel confident that the

Figure 5. Large aggregate content in BAL (as % of total phospholipids) for B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice with and without mycoplasma infection. Mice were either infected with mycoplasmas (10⁷ cfu; shaded bars) or inoculated with sterile broth (solid bars) for 24–72 h. Data are mean ± SEM, with the numbers of mice analyzed in a given group shown within the bars. *P* values for infected versus uninfected for each mouse strain are shown below the x-axis (*t* tests), while *P* values among the various strains of infected mice are indicated in the graph (one-way ANOVA followed by *post hoc* Tukey's HSD test).

Figure 6. Protein values in BAL (μg/ml) for B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice with and without mycoplasma infection. Mice were either infected with mycoplasmas (10⁷ cfu; shaded bars) or inoculated with sterile broth (solid bars) for 24–72 h. Data are mean ± SEM, with the numbers of mice analyzed in a given group shown within the bars. *P* values for infected versus noninfected for each mouse strain are shown below the x-axis (*t* tests), while *P* values among the various strains of infected mice are indicated in the graph (one-way ANOVA followed by *post hoc* Tukey's HSD test).

decrease of SP-B levels seen in the BAL of iNOS^{-/-} mice was not due to unequal loading of the gels.

DISCUSSION

The results of this study demonstrate that pulmonary mycoplasma infection in congenic B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice leads to surfactant dysfunction, with decreases in both the content and surface activity of large surfactant aggregates in BAL. Mycoplasma-infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6

Figure 7. Protein to phospholipid ratios in BAL (μg/ml) for B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice with and without mycoplasma infection. Mice were either infected with mycoplasmas (10⁷ cfu; shaded bars) or inoculated with sterile broth (solid bars) for 24–72 h. Data are mean ± SEM, with the numbers of mice analyzed in a given group shown within the bars. *P* values for infected versus noninfected for each mouse strain are shown below the x-axis (*t* tests), while *P* values among the various strains of infected mice are indicated in the graph (one-way ANOVA followed by *post hoc* Tukey's HSD test).

Figure 8. Minimum surface tension (mN/m) values for large aggregates (LA) from B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice with and without mycoplasma infection. Mice were either infected with mycoplasmas (10^7 cfu; shaded bars) or inoculated with sterile broth (solid bars) for 24–72 h and surface tension data at all three times were combined for statistical analysis. Minimum surface tension was measured for LA resuspended in 0.15 M NaCl + 2 mM CaCl₂ after 0.25 min (A) or 20 min (B) of pulsation on a bubble surfactometer (37°C, 20 cycles/min, 50% area compression, 1 mg/ml phospholipid). Values are mean $\pm$ 1 SEM, with the total number of mice shown inside each bar for the indicated group. *P* values for infected versus uninfected mice for a given strain are shown below the x-axis (*t* tests), while *P* values among various means of strains of infected mice are indicated in the graph (one-way ANOVA followed by *post hoc* Tukey's HSD test).

mice all had decreased levels of LA, increased levels of total protein, and increased protein/phospholipid ratios in BAL compared with uninfected mice of the same strain (Table 3, Figures 5–7). Resuspended LA from mycoplasma-infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice also had decreased surface activity (increased minimum surface tension values) during pulsation on a bubble surfactometer compared with uninfected same-strain controls (Figure 8). Additional analyses of time-pooled data combining results at 24, 48, and 72 h after mycoplasma infection for each mouse strain documented significant interstrain differences in the severity of mycoplasma-induced surfactant dysfunction and lung injury. Infected B6.SP-A^{-/-} and B6.iNOS^{-/-} mice had significantly lower levels of LA and higher protein/phospholipid ratios

in BAL compared with infected B6 mice (Figures 5 and 7). Mycoplasma-infected B6.SP-A^{-/-} mice also had increased levels of protein in BAL compared with infected B6 mice (Figure 6), and LA from infected B6.iNOS^{-/-} mice had increased minimum surface tensions compared with infected B6 mice after 0.25 or 20 min of bubble pulsation (Figure 8). Infected B6.iNOS^{-/-} mice had significantly decreased levels of SP-B in BAL compared with uninfected same-strain controls, while levels of SP-B were unchanged for infected versus uninfected B6.SP-A^{-/-} mice and significantly increased for infected versus uninfected B6 mice (Figure 9). These results are consistent with the overall interpretation that both SP-A and iNOS contribute to limiting lung surfactant abnormalities in the presence of mycoplasma infection.

Our studies investigating the importance of SP-A and iNOS in uninfected and mycoplasma-infected mice used congenic animals all having the B6 background. Previous investigations of surfactant function in SP-A-deficient mice have been performed using animals on the outbred Black Swiss background (16, 29). While the use of outbred mice is often justified as being representative of the human (outbred) population, there are inherent differences within outbred mouse populations that complicate interstrain comparisons such as those involving responses to mycoplasma-induced lung injury (15). Mouse strains differ dramatically in resistance to infection with *M. pulmonis*, with B6 mice being comparatively resistant to disease (30). In-depth knowledge about the specific impact of murine genetic background on surfactant function is lacking, and SP-A and iNOS knockout mice backcrossed onto the B6 background (N10) were thus compared directly with B6 (SP-A^{+/+}) mice to control for unknown genetic factors that might impact the surfactant system.

The surface activity deficits measured here for LA surfactant from mycoplasma-infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice (Figure 8) most likely underestimate the severity of functional deficits actually present in the alveoli of infected animals *in vivo*. Activity studies in mycoplasma-infected animals used LA obtained by centrifugation of cell-free BAL at $12,500 \times g$, a centrifugation force that does not sediment free proteins (plasma- or cell-derived proteins) in whole BAL. Due to material constraints in processing mouse lavages, centrifuged LA were resuspended for activity studies in 0.15 M NaCl + 2 mM CaCl₂ as opposed to the original BAL supernatants that became depleted during assays and handling. For mycoplasma-infected animals, original BAL samples contained substantial amounts of injury-induced free protein from the alveolar spaces (Figure 6), and this free protein was thus not present when LA surface activity was measured (Figure 8). Since plasma- and cell-derived proteins are known to be inhibitory to lung surfactant activity (see Refs. 7 and 31 for review), it is highly probable that surface activity deficits in all injured mice would have been more severe than shown in Figure 8 if all the original BAL protein had been present during bubble measurements. This effect would be expected to be most pronounced for BAL from mycoplasma-infected B6.SP-A^{-/-} mice, which had the largest amounts of total protein originally present in whole BAL (Figure 6). Future physiological studies of pulmonary mechanics in different strains of mycoplasma-infected mice would be helpful in assessing the functional importance of the surface activity differences reported here.

Direct exposure to mycoplasmas did not cause significant reductions in the surface activity of lavaged lung surfactant *in vitro* (Table 4), indicating that the surfactant abnormalities found during mycoplasma infection were largely associated with the underlying lung injury. The surface activity detriments measured in resuspended LA from mycoplasma-infected mice (Figure 8) could result from several mechanisms. Although free proteins in BAL remain in the supernatant during centrifugation

TABLE 4. MINIMUM SURFACE TENSION OF RESUSPENDED LARGE SURFACTANT AGGREGATES EXPOSED TO MYCOPLASMA ORGANISMS *IN VITRO*

Samples	Minimum Surface Tension (mN/m) at Time (min)							
	0.25	0.5	1	2	5	10	15	20
B.6	18.5 ± 0.5	17.6 ± 0.5	16.0 ± 0.6	15.0 ± 0.7	12.3 ± 1.3	8.2 ± 1.5	2.4 ± 0.7	< 1
B.6 + Myco	18.3 ± 0.4	17.4 ± 0.4	16.2 ± 0.4	15.1 ± 0.4	12.6 ± 0.5	6.1 ± 0.7	2.3 ± 0.6	< 1
SP-A ^{-/-}	19.1 ± 0.5	18.3 ± 0.5	17.0 ± 0.6	16.0 ± 0.6	12.3 ± 1.0	8.7 ± 1.3	4.5 ± 1.9	< 1
SP-A ^{-/-} + Myco	22.0 ± 0.7	21.2 ± 0.8	19.7 ± 0.9	18.3 ± 1.0	14.9 ± 0.9	11.5 ± 1.1	5.9 ± 1.3	< 1
iNOS ^{-/-}	19.8 ± 0.7	18.9 ± 0.7	18.0 ± 0.8	17.0 ± 0.9	14.6 ± 1.4	12.0 ± 1.9	4.5 ± 1.3	< 1
iNOS ^{-/-} + Myco	24.1 ± 0.6	23.3 ± 0.6	22.0 ± 0.5	20.1 ± 0.6	16.7 ± 0.7	12.3 ± 0.9	7.1 ± 1.3	1.7 ± 0.6

Cell-free lavage from B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice was exposed *in vitro* to mycoplasma (2×10^7 organisms per ml) for 48 h. Exposed and control (unexposed) BAL was centrifuged at $12,500 \times g$ for 30 min, and the large aggregate surfactant pellet was resuspended in 0.9% NaCl + 2.0 mM CaCl₂ by vortexing at a concentration of 1.0 mg phospholipid/ml for pulsating bubble measurements (37°C, 20 cycles/min, 50% area compression). Data are mean ± SEM for $n = 6-9$ for each group.

at $12,500 \times g$ as noted above, recent work in rodents with aspiration lung injury (32) has demonstrated that some plasma- or cell-derived proteins in BAL become associated with or incorporated into surfactant aggregates so as to impair surface activity. In addition, surfactant aggregates from animals with lung injury can also exhibit changes in lipids and proteins as a result of

interactions with inhibitors such as phospholipases, proteases, or reactive oxygen/nitrogen species. For example, degradation of surfactant phospholipids in injured lungs generate increased levels of inhibitory lysophosphatidylcholine in surfactant aggregates that can impair surface activity, as has been reported in rats with aspiration (32). The specific protein and lipid composition of

Figure 9. Western blotting detection of SP-B in BAL from B6, B6.SP-A^{-/-}, and B6.iNOS^{-/-} mice with and without mycoplasma infection. Mice were either infected with mycoplasmas (10^7 cfu) or inoculated with sterile broth for 48 h. Five micrograms of total protein were separated under nonreducing conditions on 12% Bis-Tris gels. For immunodetection, the proteins in the gels were transferred onto a PVDF membrane and immunostained with the primary rabbit polyclonal anti-SP-B antibody (1:1,000 dilution) and horseradish peroxidase-conjugated goat anti-rabbit polyclonal anti-IgG. SP-B was then detected with the ECL Detection system. *Upper panels:* Each lane represents a Western blot from the indicated strain. Contr., inoculated with sterile broth; Myc., infected with mycoplasmas. *Arrows and numbers on the left column* indicate molecular weights (kD) based on molecular weight standards. *Lower panels:* Optical densities (absolute units) of digitized blots shown in the upper panels. Values are means ± 1 SEM; number of mice for each group: wild type = 5; iNOS^{-/-} = 9; SP-A^{-/-} = 5. * $P < 0.05$ compared with the corresponding control value.

LA from mycoplasma-infected animals was not assessed in the current study in order to avoid pooling of BAL samples that would have hampered other assessments of strain-dependent lung injury (aggregate composition was assessed only in pooled lavage from uninfected B6.SP-A^{-/-} and B6 mice in a subset of studies in Tables 1 and 2 and in Figure 1).

Although comparisons between mycoplasma-infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice (Figures 5–8) implicate SP-A and iNOS as being important in mitigating some aspects of surfactant dysfunction and lung injury in this condition, additional mediators and factors are almost certainly also involved. Our prior studies have investigated mycoplasma lung injury in iNOS-deficient mice (15, 33, 34), with an emphasis on the roles of reactive nitrogen species in inflammatory tissue injury as well as alterations in cellular sodium transport. Infection of mice with *M. pulmonis* induces an infiltration of polymorphonuclear cells (PMNs) in the airways and alveoli, together with an increase in alveolar macrophages (AMs) (e.g., 14, 15, 33, 34). Prior work on innate host defenses against respiratory mycoplasmas has identified the alveolar macrophage as a primary effector cell in early mycoplasma killing *in vivo* (30) and *in vitro* (4). *In vitro* studies with activated alveolar macrophages from mycoplasma-resistant B6 mice indicate that SP-A and nitric oxide (NO) are important in mycoplasma killing (4). NO and other reactive nitrogen species including peroxynitrite are still present in B6.SP-A^{-/-} and B6.iNOS^{-/-} mice. Our experiments did not specifically assess physiological detriments in B6.SP-A^{-/-} mice associated with the loss of the immunoprotective effects of SP-A as a member of the collectin family of host defense proteins (11–13, 35). Decreased clearance of mycoplasmas in B6.SP-A^{-/-} and/or B6.iNOS^{-/-} mice may have contributed to lung injury and surfactant abnormalities, although neither mouse strain exhibited a substantial increase in lung injury severity over time after infection (e.g., at 24, 48, and 72 h in Table 3).

We have previously published measurements of several histologic lung lesion indices for different strains of mice infected with mycoplasmas under conditions identical to those used here (15, 33). Lungs from mycoplasma-infected mice were coded randomly and scored subjectively for lesion severity on the basis of: (1) neutrophilic exudates in the airway lumina; (2) hyperplasia-dysplasia of the mucosal epithelium; (3) peribronchial and perivascular lymphoid accumulations; and (4) inflammatory infiltrations within the alveoli. At 72 h after infection, B6.iNOS^{-/-} mice had significantly higher indices for all lesion parameters compared with B6 mice (33). These results are consistent with the concept that pulmonary epithelial injury induced by the immune/inflammatory response contributes to surfactant abnormalities in B6.iNOS^{-/-} mice. In contrast, our prior work did not identify significant differences between mycoplasma-infected B6 and B6.SP-A^{-/-} mice in histologic indices of lung injury despite the presence of higher numbers of mycoplasmas within the lungs of the knockout mice (15). However, functional changes in injured lungs can precede the appearance of overt histologic changes (e.g., rabbits exposed to hyperoxia develop significant increases in alveolar permeability before the appearance of interstitial and alveolar edema or structural changes to the blood gas barrier based on histology [56, 57]). The importance of the immune/inflammatory response in mycoplasma-induced lung injury is indicated by our prior work showing that infection of wild-type but not MPO^{-/-} mice with mycoplasmas leads to decreased sodium-dependent fluid clearance across their alveolar epithelium *in vivo* (58). Detailed future investigations of inflammatory mediator responses during pulmonary mycoplasma infection would be helpful in clarifying the contributions of the immune/inflammatory response to surfactant dysfunction in this condition.

To help understand specific actions of SP-A that might contribute to surfactant abnormalities, a subset of studies focused on specific aggregation and inhibition resistance characteristics in lavaged surfactant from uninfected B6.SP-A^{-/-} versus B6 mice (Figures 1–4, Tables 1 and 2). Pooled lavage from uninfected B6.SP-A^{-/-} mice showed significant changes in the size-distribution of surfactant aggregates relative to uninfected congenic B6 mice. BAL from uninfected B6.SP-A^{-/-} mice had a significantly reduced content of VLA sedimenting at 1,500 × g, but an increased content of ILA sedimenting between 1,500 × g and 12,500 × g (Fig. 1). The content of total large aggregates (LA = VLA + ILA) did not differ between uninfected B6.SP-A^{-/-} and B6 mice (Figure 1), and LA content in these mice was also the same as in uninfected B6.iNOS^{-/-} mice (Table 3). LA from B6.SP-A^{-/-} mice had reduced levels of total protein compared to B6 mice (Table 2), but there were no differences in terms of phospholipid class distribution (Table 1) or content of hydrophobic protein (Table 2).

The finding of compensatory increases in ILA in BAL from uninfected B6.SP-A^{-/-} versus B6 mice (Figure 1) extends earlier studies on the composition, activity, and metabolism of pulmonary surfactant in outbred SP-A^{-/-} mice that reported a decreased content of BAL aggregates obtained by centrifugation at 40,000 × g with and without 0.8 M sucrose (16, 29, 36). The aggregates studied here (VLA, ILA, and LA) reflect microstructural populations discriminated primarily by size as opposed to density, and represent the most active fractions of normal native surfactant (e.g., 7, 27, 28). These aggregate forms are the basis for several active clinical exogenous surfactants such as Infasurf (CLSE), which is currently used in treating surfactant deficiency and dysfunction in humans (7). Changes in VLA and ILA in B6.SP-A^{-/-} versus B6 mice could involve modifications in aggregate size/structure within the alveolar lumen, or aggregate changes associated with altered surfactant synthesis, reuptake, or recycling in type II pneumocytes that are normally regulated by SP-A (6, 7, 11, 37). It is also possible that some aggregate changes in SP-A^{-/-} mice could involve contributions induced by SP-D, which is found in normal amounts in lung homogenates from these mice (16). SP-D does not participate in the biophysics of normal surfactant containing SP-A (7), and it is not present in normal type II cell lamellar bodies (38, 39). However, SP-D has structural similarities to SP-A, including an N-terminal collagenous domain and a C-terminal carbohydrate recognition domain (40, 41). SP-D has been reported to bind and agglutinate carbohydrates but not to affect the surface activity of phospholipids (42).

Our finding that BAL and LA obtained from uninfected B6.SP-A^{-/-} versus B6 mice were more sensitive to inhibition by LPC and BSA *in vitro* (Figures 3 and 4) is consistent with previous studies showing that SP-A improves inhibition resistance in hydrophobic lung surfactant extracts (8–10). SP-A has also been shown to mitigate surfactant dysfunction from peroxynitrite (43) and Cu-Zn superoxide dismutase (44), and to enhance phospholipid adsorption and film respreading *in vitro* (45–49). The effects of SP-A in enhancing inhibition resistance likely involve cooperative interactions with SP-B in forming tubular myelin (50, 51), plus the facilitation of less specific phospholipid aggregation in the aqueous phase (e.g., 51–55). It has previously been reported that minimum surface tension was increased in films of lung surfactant from SP-A^{-/-} mice compared with Black Swiss mice in the presence of albumin on the Wilhelmy balance during cycling at slow rate (3 min/cycle) (29). Functional assessments in the present study utilized a pulsating bubble surfactometer, which measures a more physiologically relevant combination of adsorption and dynamic surface activity at a cycling rate (20 cycles/min) and area compression (50%) reflective of the lungs *in vivo* (7, 23, 24). The decreased inhibition resistance found for

surfactant from B6.SP-A^{-/-} mice in the presence of albumin and LPC in pulsating bubble studies (Figures 3 and 4) is consistent with this being a contributor to surfactant activity deficits in mycoplasma-infected B6.SP-A^{-/-} mice (Figure 8).

Although surfactant from uninfected B6.SP-A^{-/-} mice had less resistance to inhibition by albumin and LPC *in vitro*, LA from these animals maintained a normal phospholipid composition and a normal total hydrophobic protein content (Tables 1 and 2). Due to this normal content of active hydrophobic components, as well as a compensatory increase in ILA (Figure 1), surfactant from B6.SP-A^{-/-} mice maintained near-normal surface activity in the absence of inhibitors (Figure 2). This finding is consistent with previous studies showing that lung compliance is effectively normal in uninfected outbred SP-A^{-/-} mice (16). However, in the presence of mycoplasma infection, significant differences in the level of an important specific surfactant protein (SP-B) were found in BAL from B6.SP-A^{-/-} and B6.iNOS^{-/-} mice compared to infected B6 mice (Figure 9). At 48 h after infection with mycoplasma, infected B6 mice had a significant increase in SP-B in BAL compared with uninfected controls, while levels of SP-B were unchanged in infected B6.SP-A^{-/-} mice and significantly decreased in infected B6.iNOS^{-/-} mice compared to uninfected same-strain controls (Figure 9). Multiple studies have documented that SP-B is the most active of the hydrophobic surfactant proteins in facilitating adsorption and dynamic surface activity in endogenous surfactant (*see Refs. 7 and 31 for review*). Because of its combination of nonpolar and polar residues, amphipathic SP-B can interact with both the fatty chains and headgroups of phospholipid molecules. It is very likely that mycoplasma-induced reductions in SP-B in infected iNOS^{-/-} mice, in particular, were an important contributor to the decreased surface activity found in LA from these animals (Figure 8).

In summary, the present study has demonstrated that mycoplasma-infected B6.SP-A^{-/-}, B6.iNOS^{-/-}, and B6 mice all had lung injury with increased protein and protein/phospholipid ratios in BAL, depleted LA surfactant, and impaired surface activity compared with uninfected mice of the same strain. Moreover, mycoplasma-infected B6.SP-A^{-/-} and B6.iNOS^{-/-} mice had more severe lung injury and surfactant dysfunction compared with B6 mice based on analyses of time-pooled data from 24, 48, and 72 h after infection, indicating a role for SP-A and iNOS in this disease. Abnormalities in lung surfactant in infected B6.iNOS^{-/-} mice included a significantly decreased level of SP-B in BAL, which correlated directly with decreased surface activity in surfactant aggregates in BAL from these mice. An additional likely contributor to surface activity deficits in mycoplasma-infected B6.SP-A^{-/-} mice is that lavaged surfactant from SP-A-deficient mice is shown here to have a decreased ability to resist biophysical inactivation by albumin and lysophosphatidylcholine when examined *in vitro*.

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

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References

- Cassell GH, Gray GC, Waites KB. Mycoplasma infections. In Fauci AS, Braunwald E, Isselbacher KJ, Martin JB, Kasper DL, Hauser SL, Longo DL, editors. Harrison's principles of internal medicine, 14th ed. New York: McGraw Hill Companies, Inc.; 1998. pp. 1052–1055.
- Waites KB, Talkington DF. Mycoplasma pneumoniae and its role as a human pathogen. *Clin Microbiol Rev* 2004;17:697–728.
- Cartner SC, Lindsey JR, Gibbs-Erwin J, Cassell GH, Simecka JW. Roles of innate and adaptive immunity in respiratory mycoplasmosis. *Infect Immun* 1998;66:3485–3491.
- Hickman-Davis JM, Lindsey JR, Zhu S, Matalon S. Surfactant protein A mediates mycoplasmicidal activity of alveolar macrophages. *Am J Physiol* 1998;274:L270–L277.
- Daniels CB, Orgeig S. Pulmonary surfactant: the key to the evolution of air breathing. *News Physiol Sci* 2003;18:151–157.
- Hawgood S, Poulain FR. The pulmonary collectins and surfactant metabolism. *Annu Rev Physiol* 2001;63:495–519.
- Notter RH. Lung surfactants: basic science and clinical applications. New York: Marcel Dekker, Inc.; 2000.
- Cockshutt AM, Weitz J, Possmayer F. Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins *in vitro*. *Biochemistry* 1990;19:8424–8429.
- Venkitaraman A, Hall S, Whitsett J, Notter R. Enhancement of biophysical activity of lung surfactant extracts and phospholipid-apoprotein admixtures by surfactant protein A. *Chem Phys Lipids* 1990;56:185–194.
- Yukitake K, Brown CL, Schlueter MA, Clements JA, Hawgood S. Surfactant apoprotein A modifies the inhibitory effect of plasma proteins on surfactant activity *in vivo*. *Pediatr Res* 1995;37:21–25.
- Mason RJ, Greene K, Voelker DR. Surfactant protein A and surfactant protein D in health and disease. *Am J Physiol* 1998;275:L1–L13.
- Wright JR. Immunomodulatory functions of surfactant. *Physiol Rev* 1997;77:931–962.
- Crouch E, Wright JR. Surfactant proteins A and D and pulmonary host defense. *Annu Rev Physiol* 2001;63:521–554.
- Hickman-Davis J, Gibbs-Erwin J, Lindsey JR, Matalon S. Surfactant protein A mediates mycoplasmicidal activity of alveolar macrophages by production of peroxynitrite. *Proc Natl Acad Sci USA* 1999;96:4953–4958.
- Hickman-Davis JM, Gibbs-Erwin J, Lindsey JR, Matalon M. Role of surfactant protein A in nitric oxide production and mycoplasma killing in congenic C57BL/6 mice. *Am J Respir Cell Mol Biol* 2004;30:319–325.
- Korfhagen TR, Bruno MD, Ross GF, Huelsman KM, Ikegami M, Jobe AH, Wert SE, Stripp BR, Morris RE, Glasser SW, *et al.* Altered surfactant function and structure in SP-A gene targeted mice. *Proc Natl Acad Sci USA* 1996;93:9594–9599.
- Faulkner CB, Simecka JW, Davidson MK, Davis JK, Schoeb TR, Lindsey JR, Everson MP. Gene expression and production of tumor necrosis factor alpha, interleukin 1, interleukin 6, and gamma interferon in C3H/HeN and C57BL/6N mice in acute Mycoplasma pulmonis disease. *Infect Immun* 1995;63:4084–4090.
- Davidson MK, Lindsey JR, Parker RF, Tully JG, Cassell GH. Differences in virulence for mice among strains of Mycoplasma pulmonis. *Infect Immun* 1988;56:2156–2162.
- Ames BN. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* 1966;8:115–118.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275.
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911–917.
- Touchstone JC, Chen JC, Beaver KM. Improved separation of phospholipids in thin-layer chromatography. *Lipids* 1980;15:61–62.
- Enhörning G. Pulsating bubble technique for evaluation of pulmonary surfactant. *J Appl Physiol* 1977;43:198–203.
- Hall SB, Bermel MS, Ko YT, Palmer HJ, Enhörning GA, Notter RH. Approximations in the measurement of surface tension with the oscillating bubble surfactometer. *J Appl Physiol* 1993;75:468–477.
- Griese M, Schumacher S, Tredano M, Steinecker M, Braun A, Guttentag S, Beers MF, Bahuau M. Expression profiles of hydrophobic surfactant proteins in children with diffuse chronic lung disease. *Respir Res* 2005;6:80.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
- Putman E, Creuwels LAJM, Van Golde LMG, Haagsman HP. Surface properties, morphology and protein composition of pulmonary surfactant subtypes. *Biochem J* 1996;320:599–605.
- Putz G, Goerke J, Clements JA. Surface activity of rabbit pulmonary surfactant subfractions at different concentrations in a captive bubble. *J Appl Physiol* 1994;77:597–605.
- Ikegami M, Korfhagen TR, Whitsett JA, Bruno MD, Wert SE, Wada K, Jobe AH. Characteristics of surfactant from SP-A-deficient mice. *Am J Physiol* 1998;275:L247–L254.

30. Hickman-Davis JM, Michalek SM, Gibbs-Erwin J, Lindsey JR. Depletion of alveolar macrophages exacerbates respiratory mycoplasmosis in mycoplasma-resistant C57BL mice but not mycoplasma-susceptible C3H mice. *Infect Immun* 1997;65:2278–2282.
31. Wang Z, Holm BA, Matalon S, Notter RH. Surfactant activity and dysfunction in lung injury. In Notter RH, Finkelstein JN, Holm BA, editors. Lung injury: mechanisms, pathophysiology, and therapy. Boca Raton: Taylor Francis Group, Inc.; 2005. pp. 297–352.
32. Davidson BA, Knight PR, Wang Z, Chess PR, Holm BA, Russo TA, Hutson A, Notter RH. Surfactant alterations in acute inflammatory lung injury from aspiration of acid and gastric particulates. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L699–L708.
33. Hickman-Davis JM, Lindsey JR, Matalon S. Cyclophosphamide decreases nitrotyrosine formation and abrogates nitric oxide production by alveolar macrophages in mycoplasmosis. *Infect Immun* 2001;69:6401–6410.
34. Hickman-Davis JM, McNicholas-Bevensee C, Davis IC, Davis GC, Bosworth CB, Matalon S. Reactive species mediate inhibition of alveolar type II sodium transport during mycoplasma infection. *Am J Respir Crit Care Med* 2006;173:719–726.
35. Lawson PR, Reid KBM. The roles of surfactant proteins A and D in innate immunity. *Immunol Rev* 2000;173:66–78.
36. Ikegami M, Korfhagen TR, Bruno MD, Whitsett JA, Jobe AH. Surfactant metabolism in surfactant protein A-deficient mice. *Am J Physiol* 1997;272:L479–L485.
37. Haagsman HP. Surfactant protein A and D. *Biochem Soc Trans* 1994;22:100–106.
38. Crouch E, Parghi D, Kuan S-F, Persson A. Surfactant protein D: subcellular localization in non-ciliated bronchiolar epithelial cells. *Am J Physiol* 1992;263:L60–L66.
39. Voorhout WF, Veenendaal T, Kuroki Y, Ogasawara Y, van Golde LMG, Geuze HJ. Immunocytochemical localization of surfactant protein D (SP-D) in type II cells, Clara cells, and alveolar macrophages of rat lung. *J Histochem Cytochem* 1992;40:1589–1597.
40. Crouch E, Persson A, Chang D, Heuser J. Molecular structure of pulmonary surfactant protein D (SP-D). *J Biol Chem* 1994;269:17311–17319.
41. Kuroki Y, Voelker DR. Pulmonary surfactant proteins. *J Biol Chem* 1994;269:25943–25946.
42. Persson A, Chang D, Crouch E. Surfactant protein D is a divalent cation-dependent carbohydrate-binding protein. *J Biol Chem* 1990;265:5755–5760.
43. Haddad IY, Ischiropoulos H, Holm BA, Beckman JS, Baker JR, Matalon S. Mechanisms of peroxynitrite-induced injury to pulmonary surfactants. *Am J Physiol* 1993;265:L555–L564.
44. Haddad IY, Nieves-Cruz B, Matalon S. Inhibition of surfactant function by copper-zinc superoxide dismutase (CuZn-SOD). *J Appl Physiol* 1997;83:1545–1550.
45. Ross GF, Notter RH, Meuth J, Whitsett JA. Phospholipid binding and biophysical activity of pulmonary surfactant-associated protein SAP-35 and its non-collagenous C-terminal domains. *J Biol Chem* 1985;261:14283–14291.
46. Yu SH, Possmayer F. Adsorption, compression, and stability of surface films of natural, lipid extract, and reconstituted pulmonary surfactants. *Biochim Biophys Acta* 1993;1167:264–271.
47. Schurch S, Possmayer F, Cheng S, Cockshutt AM. Pulmonary SP-A enhances adsorption and appears to induce surface sorting of lipid extract surfactant. *Am J Physiol* 1992;263:L210–L218.
48. Taneva S, McEachren T, Stewart J, Keough KM. Pulmonary surfactant protein SP-A with phospholipids in spread monolayers at the air-water interface. *Biochemistry* 1995;34:10279–10289.
49. Hawgood S, Benson BJ, Schilling J, Damm D, Clements JA, White RT. Nucleotide and amino acid sequences of pulmonary surfactant protein SP 18 and evidence for cooperation between SP 18 and SP 28–36 in surfactant lipid adsorption. *Proc Natl Acad Sci USA* 1987;84:66–70.
50. Suzuki Y, Fujita Y, Kogishi K. Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig lung surfactant. *Am Rev Respir Dis* 1989;140:75–81.
51. Williams MC, Hawgood S, Hamilton RL. Changes in lipid structure produced by surfactant proteins SP-A, SP-B, and SP-C. *Am J Respir Cell Mol Biol* 1991;5:41–50.
52. Efrati H, Hawgood S, Williams MC, Hong K, Benson BJ. Divalent cation and hydrogen ion effects on the structure and surface activity of pulmonary surfactant. *Biochemistry* 1987;26:7986–7993.
53. Hawgood S, Benson BJ, Hamilton RJ. Effects of a surfactant-associated protein and calcium ions on the structure and surface activity of lung surfactant lipids. *Biochemistry* 1985;24:184–190.
54. Veldhuizen R, Yao L, Hearn S, Possmayer F, Lewis J. Surfactant-associated protein A is important for maintaining surfactant large-aggregate forms during surface-area cycling. *Biochem J* 1996;313:835–840.
55. Voorhout WF, Veenendaal T, Haagsman HP, Verkleij AJ, van Golde LMG, Geuze HJ. Surfactant protein A is localized at the corners of the pulmonary tubular myelin lattice. *J Histochem Cytochem* 1991;39:1331–1336.
56. Matalon S, Egan EA. Effects of 100% O₂ breathing on permeability of alveolar epithelium to solute. *J Appl Physiol* 1981;50:859–863.
57. Nickerson PA, Matalon S, Farhi LE. An ultrastructural study of alveolar permeability to cytochrome c in the rabbit lung: Effect of exposure to 100% O₂ at one atmosphere. *Am J Pathol* 1981;102:1–9.
58. Hickman-Davis JM, McNicholas-Bevensee C, Davis IC, Davis GC, Bosworth CB, Matalon S. Reactive species mediate inhibition of alveolar type II sodium transport during mycoplasma infection. *Am J Respir Crit Care Med* 2006;173:334–344.