The Role of Surfactant Protein A in Bleomycin-induced Acute Lung Injury

Hisatsugu Goto¹, Julie G. Ledford¹, Sambuddho Mukherjee¹, Paul W. Noble², Kristi L. Williams^{1,3}, and Jo Rae Wright¹

¹Department of Cell Biology, and ³Department of Immunology, Duke University Medical Center, Durham, North Carolina; and ²Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Duke University School of Medicine, Durham, North Carolina

Rationale: Surfactant protein A (SP-A) is a collectin family member that has multiple immunomodulatory roles in lung host defense. SP-A levels are altered in the bronchoalveolar lavage (BAL) fluid and serum of patients with acute lung injury and acute respiratory distress syndrome, suggesting the importance of SP-A in the pathogenesis of acute lung injury.

Objectives: Investigate the role of SP-A in the murine model of noninfectious lung injury induced by bleomycin treatment.

Methods: Wild-type (WT) or SP-A deficient (SP-A^{-/-}) mice were challenged with bleomycin, and various indices of lung injury were analyzed.

Measurements and Main Results: On challenge with bleomycin, SP-A^{-/-} mice had a decreased survival rate as compared with WT mice. SP-A^{-/-} mice had a higher degree of neutrophil-dominant cell recruitment and the expression of the inflammatory cytokines in BAL fluid than did WT mice. In addition, SP-A^{-/-} mice had increased lung edema as assessed by the increased levels of intravenously injected Evans blue dye leaking into the lungs. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling and active caspase-3 staining suggested the increased apoptosis in the lung sections from SP-A^{-/-} mice challenged with bleomycin. SP-A also specifically reduced bleomycin-induced apoptosis in mouse lung epithelial 12 cells *in vitro*. Moreover, intratracheal administration of exogenous SP-A rescued the phenotype of SP-A^{-/-} mice *in vivo*.

Conclusions: These data suggest that SP-A plays important roles in modulating inflammation, apoptosis, and epithelial integrity in the lung in response to acute noninfectious challenges.

Keywords: collectin; noninfectious lung injury; apoptosis; surfactant replacement therapy

Acute lung injury (ALI) and its most severe manifestation, acute respiratory distress syndrome (ARDS), is a clinical syndrome characterized by significant hypoxemia with bilateral pulmonary infiltrates consistent with edema (1, 2). ALI has multiple causes, including noninfectious lung diseases, such as acute interstitial pneumonia and chemical pneumonia, as well as infectious diseases, such as microbial pneumonia and sepsis. Conventional treatment plans, such as high-dose glucocorticoid therapy, are not effective in many cases and overall 28-day mortality of ALI is fairly high (25–40%) (2). Despite decades of

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The expression level of surfactant protein A (SP-A), a pulmonary collectin with immunoregulatory function, is decreased in the lungs of patients with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). However, the role of SP-A in the development of noninfectious ALI is not known.

What This Study Adds to the Field

The results of this study provide *in vivo* and *in vitro* evidence that SP-A has a protective effect on noninfectious ALI and suggest that SP-A may have a role in the pathogenesis of ALI/ARDS.

intense investigation, the fundamental mechanisms that initiate and control ALI/ARDS have not been elucidated.

Surfactant protein A (SP-A) is a large multimeric protein found in the airways and alveoli of the lungs. SP-A is a member of the collectin family of proteins, characterized by NH₂-terminal collagen-like regions and COOH-terminal lectin domains. Although other surfactant proteins, such as SP-B, function to reduce surface tension in the lungs, SP-A (as well as SP-D) regulates the pulmonary immune response (3). By binding to a wide variety of pathogens, SP-A opsonizes and enhances pathogen uptake by phagocytes. *In vivo* studies using SP-A-deficient (SP-A^{-/-}) mice have shown that SP-A regulates responses involved in both initiation and potentiation of inflammation by decreasing production of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), in response to lipopolysaccharide (4) or by accelerating the clearance of a variety of pathogens (5–9). Thus, SP-A contributes to multiple aspects of pulmonary host defense.

Although SP-A has been shown to mediate host responses to a variety of microorganisms, the role of SP-A in the development of noninfectious ALI is not well understood. Previous studies have demonstrated that SP-A concentration in the bronchoalveolar lavage (BAL) fluid was significantly lower in both patients with established ARDS and patients at risk for ARDS, compared with healthy volunteers (10, 11). On the other hand, SP-A levels were increased in the serum of patients with ARDS (10). These findings led us to consider that SP-A might play an important role in the pathogenesis of noninfectious lung injury.

To evaluate the contribution of SP-A in the pathogenesis of noninfectious lung injury, we compared the development of bleomycin-induced ALI in wild-type (WT) and SP-A^{-/-} mice. Our findings suggest that SP-A not only regulates the inflammatory response induced by bleomycin but also regulates vascular permeability and apoptosis, which are considered to

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Correspondence and requests for reprints should be addressed to Jo Rae Wright, Ph.D., Department of Cell Biology, Duke University Medical Center, 438 Nana-line Duke Building Box 3709, Research Drive, Durham, NC 27710. E-mail: j.wright@cellbio.duke.edu

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be important factors in the development of ALI. Some of the results of this study have been previously reported in the form of an abstract (12).

METHODS

Detailed methods are described in the online supplement.

Reagents

Bleomycin was purchased from Bedford Laboratories (Bedford, OH). Evans blue dye was purchased from Sigma (St. Louis, MO).

SP-A Purification

SP-A was purified as previously described (13).

Animals

SP-A^{-/-} mice were generated as previously described (14) and back-crossed to C57BL/6 background for 11 generations. WT mice were obtained from Charles River Laboratories (Wilmington, MA). Nine- to 11-week-old female mice were used for all experiments.

Bleomycin Lung Injury Model and BAL

All *in vivo* bleomycin challenges were performed with 2.5 U/kg, or as otherwise specified. Control mice received saline intratracheally. Intratracheal bleomycin challenge and BAL analysis were performed as previously described (15). Experimental protocols were reviewed and approved by the Duke University Institutional Animal Care and Use Committee.

ELISA

ELISA for mouse TNF- α , IL-1 β (BD Biosciences, Franklin Lakes, NJ), KC (R&D Systems, Minneapolis, MN), and high mobility group box (HMGB) 1 protein (Shino-Test, Tokyo, Japan) were performed according to manufacturer's instructions using BAL samples from indicated time points.

Evans Blue Dye Leakage

Seven days after intratracheal instillation of bleomycin, WT or SP-A^{-/-} mice were injected via the tail vein with Evans blue dye (50 mg/kg). Three hours after injection, BAL was performed and then the lungs were perfused, removed, and immersed in formamide at 55°C. After 24 hours' incubation, formamide including eluted blue dye was collected. The absorbance at 620 nm was measured with BAL fluid and formamide samples.

Histology and Immunohistochemistry

The sections of snap frozen lung samples were stained with the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method by using TACS 2 TdT-Fluor *In Situ* Apoptosis Detection Kit following manufacturer's instruction (Trevigen, Gaithersburg, MD). TUNEL-positive index was calculated by dividing the TUNEL-positive by 4'-6-diamidino-2-phenylindole-positive (nucleated) cell numbers (magnification $\times 400$). For double staining of TUNEL and active caspase-3, staining with active caspase-3 antibody (Cell Signaling Technology, Danvers, MA) was performed after TUNEL staining. Sections of formalin-fixed, paraffin-embedded tissues were also stained with hematoxylin and eosin for histological examinations.

Exogenous SP-A Administration *In Vivo*

For some experiments, purified SP-A (25 μ g/50 μ l/dose) was administered intratracheally with oropharyngeal aspiration method as previously described (16) on Days 0, 1, 3, and 5 to rescue the bleomycin-induced lung injury on Day 7. For survival studies, SP-A was administered intratracheally on Days 0, 1, 3 and 5, followed by continuous administration every 4 days until Day 21.

Cell Culture

Mouse type II epithelial cell line MLE12 cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin. Cells were cultured at 37°C under 5% CO₂ in humidified air.

Figure 1. Surfactant protein A-deficient (SP-A^{-/-}) mice have significantly higher mortalities compared with wild-type (WT) mice in response to bleomycin challenge. WT and SP-A^{-/-} mice received intratracheal instillations of either (A) 2.5 U/kg, or (B) 5 U/kg bleomycin, and the percentages of surviving mice were plotted over a 21-day period. All saline-challenged mice (as a control) survived during the experimental period (data not shown). (A) $P < 0.05$; (B) $P < 0.01$; $n = 20$ in each group. (C) Representative histologic sections of lung tissue stained with hematoxylin and eosin. Increased cellularity, consolidation, and hemorrhage were seen in SP-A^{-/-} mice on Day 7 after bleomycin treatment (2.5 U/kg). Magnification $\times 100$.

In Vitro Apoptosis Detection

After culturing with various concentrations of SP-A and bleomycin, MLE 12 cells were stained with annexin V-fluorescein isothiocyanate and propidium iodide (PI) obtained from BD Biosciences (San Diego,

CA) according to manufacturer's protocol, and positive cells were analyzed by flow cytometry.

Statistics

Statistical analysis was performed using Student *t* test of unpaired samples. analysis of variance was used to analyze multiple comparisons. For survival studies, log-rank test was used to compare two groups.

RESULTS

SP-A^{-/-} Mice Are Susceptible to Bleomycin Challenge

For the first set of experiments, a survival study was performed to test whether SP-A^{-/-} mice are susceptible to intratracheal bleomycin challenge. After instillation of 2.5 or 5 U/kg of bleomycin, SP-A^{-/-} mice had significantly higher mortality rates than WT mice (Figures 1A and 1B). Microscopic analysis with hematoxylin and eosin staining revealed increased cell infiltration, consolidation, and hemorrhage in the lungs from SP-A^{-/-} mice on Day 7 (Figure 1C). No histological difference was seen in control (saline-treated) mice.

Bleomycin-challenged SP-A^{-/-} Mice Have Increased Inflammation

As histological findings suggested the increased cellularity in the lung of bleomycin-challenged SP-A^{-/-} mice, we examined the level of cell infiltration in BAL fluid. Seven days after bleomycin treatment, the total cell number in BAL fluid from SP-A^{-/-} mice was significantly higher than that in WT mice (Figure 2A). Total cell numbers in control group (saline-treated mice) were

similar between WT and SP-A^{-/-} mice. Differential cell counts revealed that an increase in polymorphonuclear neutrophils primarily contributed to the increase in total cell counts (Figure 2B). We also examined the inflammatory cytokine expression in BAL fluid to understand better the inflammatory phenotype in SP-A deficiency. Analysis of BAL fluid revealed a pronounced induction of TNF- α , KC, and IL-1 β cytokines 1 to 3 days after bleomycin instillation in SP-A^{-/-} mice relative to WT mice as assessed by ELISA (Figure 2C). These data suggest that SP-A regulates inflammation caused by bleomycin challenge.

HMGB1 Expression Is Increased in SP-A^{-/-} Mice in Response to Bleomycin

In addition to inflammatory cytokines, we also determined the level of HMGB1 protein as a marker of ALI. HMGB1, originally recognized as a transcription factor-like protein (17), has recently been proposed to be a cytokine-like molecule and one of the late mediators of lethality in sepsis (18–20). Recently, Ueno and colleagues showed that the concentrations of HMGB1 were increased in plasma and lung epithelial lining fluid of both patients and experimental mice with ALI (21). Therefore, HMGB1 appears to play an important role not only in sepsis lethality but also in the pathogenesis of ALI/ARDS. In our bleomycin-induced ALI model, HMGB1 expression in BAL fluid of WT mice was greatly increased beginning at 5 days after bleomycin challenge. SP-A^{-/-} mice had significantly higher levels of HMGB1 expression in BAL fluid compared with WT mice at 5 to 7 days after bleomycin challenge as assessed by ELISA (Figure 3). This finding suggests that SP-A

Figure 2. Increased inflammatory phenotype in surfactant protein A-deficient (SP-A^{-/-}) mice after bleomycin (bleo) challenge. After saline or bleomycin treatment, bronchoalveolar lavage (BAL) fluid was collected from wild-type (WT) and SP-A^{-/-} mice on Day 7. (A) Total cell and (B) differential cell counts were performed. SP-A^{-/-} mice had a higher degree of neutrophil-dominant cell recruitment. MNC = mononuclear cells; PMN = polymorphonuclear neutrophils. *n* = 9–12 in each group. ***P* < 0.05. (C) The levels of KC, tumor necrosis factor (TNF)- α , and IL-1 β protein in BAL fluid from WT and SP-A^{-/-} mice were measured by ELISA at the indicated time points after bleomycin challenge. *n* = 5 in each group. **P* < 0.01; ***P* < 0.05.

Figure 3. Increased high mobility group box (HMGB)1 protein expression in surfactant protein A-deficient (SP-A^{-/-}) mice after bleomycin challenge. After bleomycin challenge, bronchoalveolar lavage (BAL) was performed in wild-type (WT) and SP-A^{-/-} mice at the indicated time points. The levels of HMGB1 protein in BAL fluid were measured by ELISA. $n = 5-14$ in each group. $**P < 0.05$.

regulates both early and late mediators of ALI, such as the proinflammatory cytokines and HMGB1, respectively.

Bleomycin-challenged SP-A^{-/-} Mice Have Increased Evans Blue Dye Leakage

Histological analyses also revealed that SP-A^{-/-} mice had increased hemorrhage in response to bleomycin challenge (Figure 1C). This led us to consider that SP-A might regulate cell integrity and vascular permeability in the lung during acute noninfectious lung injury. To test this hypothesis, we injected Evans blue dye via the tail vein on Day 7 after bleomycin challenge and assessed the level of dye that was recovered in alveolar space or lung tissue. The representative pictures of the lungs show increased dye leakage when mice were treated with bleomycin (Figure 4A). On comparison with WT mice, the lungs from SP-A^{-/-} mice were much darker blue after bleomycin challenge, suggesting that SP-A^{-/-} mice have increased lung injury consistent with increased vascular permeability and decreased cell integrity. This was confirmed by measuring the levels of Evans blue dye that leaked into the lung by measuring the absorbance. In both BAL fluid and lung tissue, more Evans blue dye was recovered from BAL and lungs of SP-A^{-/-} mice than from WT mice (Figures 4B and 4C). No difference in absorbance was seen in saline treated mice. These results suggest that SP-A helps maintain cell integrity and regulates vascular permeability during acute noninfectious lung injury induced by bleomycin.

Apoptosis Is Increased in the Lung Tissue from Bleomycin-challenged SP-A^{-/-} Mice

Many studies have previously shown increased apoptosis in lung samples from patients with ARDS as well as bleomycin-challenged mice (22–25). Therefore, to further elucidate the role of SP-A in bleomycin-induced lung injury, we assessed the level of cell death in the lungs of WT and SP-A^{-/-} mice. First, we used TUNEL staining to assess the amount of cell death in the lung. Consistent with previous reports, the number of TUNEL-positive cells in the lung was increased after bleomycin challenge. In quantitative counts, significantly more TUNEL-positive cells were present in SP-A^{-/-} mice compared with WT mice (Figures 5A and 5B). Moreover, additional immunohistochemical staining was performed for active caspase-3, which is

Figure 4. Increased Evans blue dye leakage in the lungs of bleomycin-challenged surfactant protein A-deficient (SP-A^{-/-}) mice. Wild-type (WT) or SP-A^{-/-} mice were challenged with saline or bleomycin (bleo) intratracheally. On Day 7, Evans blue dye (50 mg/kg) was injected via the tail vein. Three hours after the injection, bronchoalveolar lavage (BAL) fluid and lung tissues were collected and the levels of Evans blue dye were assessed by measuring absorbance at 620 nm. (A) Representative pictures of lungs from the four treatment groups. The levels of Evans blue dye recovered in (B) BAL fluid and (C) lung tissue were increased in SP-A^{-/-} mice. $n = 5$ in each group. $*P < 0.01$; $**P < 0.05$.

a hallmark of ongoing apoptosis. Double staining of TUNEL and active caspase-3 revealed that some of the TUNEL-positive cells were caspase-3 positive, indicating that the number of cells that are undergoing apoptosis is increased in SP-A^{-/-} mice (Figure 6). These data suggest that SP-A plays an antiapoptotic role in response to noninfectious challenges such as bleomycin.

SP-A Inhibits Apoptosis Induced by Bleomycin in MLE12 Cells *In Vitro*

To determine if SP-A can directly inhibit apoptosis, an *in vitro* assay using the mouse lung epithelial cell line MLE12 cells was used. MLE12 cells were exposed to bleomycin with or without SP-A, and the cells were stained with annexin V–fluorescein isothiocyanate and PI to assess apoptosis. Representative results from multiple flow cytometric analyses with at least 15,000

Figure 5. Analysis of cell injury/death after bleomycin challenge by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. TUNEL staining was performed with lung tissue sections from wild-type (WT) and surfactant protein A-deficient (SP-A^{-/-}) mice on Day 7 after bleomycin treatment. (A) Representative pictures are shown. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). Magnification $\times 400$. (B) TUNEL-positive cells were counted from 20 random fields per section, and TUNEL-positive index was calculated as indicated in METHODS. Significant increase of TUNEL-positive cells was seen in SP-A^{-/-} mice in response to bleomycin. $n = 5$ in each group. * $P < 0.01$.

gated cells are shown in Figure 7A. Bleomycin increases the numbers of early (annexin V-positive, PI-negative) and mid to late (annexin V-positive, PI-positive) apoptotic cells. Interestingly, when cells were treated with both bleomycin and SP-A, only the population of early apoptotic cells was decreased (Figure 7A), suggesting that SP-A can only prevent early stages of apoptosis in these cells. The dose-dependent effects of SP-A and bleomycin are shown in Figure 7B. Bleomycin causes the increase of both early and mid to late apoptosis in MLE12 cells, and, as before, SP-A inhibits only early-stage apoptosis in a dose-dependent manner. We examined the specificity of SP-A-mediated inhibition of apoptosis in these cells by determining if other proteins with either structural or functional homology found in the alveolar space also had the ability to inhibit apoptosis. None of the proteins tested, which included SP-D, C1q, and IgG, significantly inhibit bleomycin-induced apoptosis in MLE12 cells (Table 1).

Exogenous SP-A Administration Reduces Bleomycin-induced Phenotype in SP-A^{-/-} Mice

After bleomycin challenge, mice were treated with exogenous SP-A as described in METHODS. When mice were treated with exogenous SP-A, total cell numbers in BAL fluid, Evans blue dye recovery, as well as mortality rate in SP-A^{-/-} mice were significantly reduced to approximately the levels of WT mice (Figures 8A–8C). Treatment of WT mice with exogenous SP-A did not change the baseline phenotype as assessed by the same experimental parameters. These experiments confirmed the critical protective effect of SP-A in bleomycin-induced lung injury.

DISCUSSION

The purpose of this study was to determine the role of SP-A in the development of noninfectious ALI. By assessing the acute phase of bleomycin-induced lung injury model, we showed that: (1) SP-A^{-/-} mice were more susceptible to bleomycin-induced death, (b) SP-A^{-/-} mice exhibited higher levels of inflammatory cytokine and HMGB1 expression as well as increased vascular permeability compared with WT mice, and (c) exogenous SP-A administration rescued the phenotype of SP-A^{-/-} mice. In combination with *in vitro* experiments, we have also shown that SP-A reduces apoptosis induced by bleomycin. These results suggest the importance of SP-A in the pathogenesis of noninfectious ALI.

Figure 6. Double staining of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and active caspase-3. TUNEL staining was performed in conjunction with intracellular staining for cleaved (active) caspase-3 using lung tissue sections from surfactant protein A-deficient (SP-A^{-/-}) mice on Day 7 after bleomycin treatment. Some active caspase-3 positive (apoptotic) cells are also TUNEL-positive. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). Magnification $\times 400$.

Figure 7. Surfactant protein A (SP-A) prevents bleomycin-induced apoptosis in MLE12 cells. MLE12 cells were treated with bleomycin (bleo) with or without SP-A. The apoptotic cells were assessed by flow cytometric analysis with annexin V and propidium iodide (PI). Annexin V-positive, PI-negative (A+/P-) population was considered as indicative of early apoptotic cells in the dot plots, and annexin V-positive, PI-positive (A+/P+) population was considered as mid to late apoptotic cells. (A) Representative flow cytometric analysis for the treatment groups. (B) Percentages of A+/P- and A+/P+ population are shown. Data are average of three independent experiments. ** $P < 0.05$.

Bleomycin-induced lung injury in rodents has both acute and chronic phases. Bleomycin treatment has been used as a model of pulmonary fibrosis (chronic lung injury), as histological features of the lungs are similar to those observed in human chronic lung diseases such as idiopathic pulmonary fibrosis (26). As we and others have shown (27, 28), the other key feature of this model is that it also has an acute phase of inflammatory lung injury. In our study, the expression of the inflammatory cytokines (TNF- α , IL-1 β , and KC) followed by HMGB1 was up-regulated before the cell recruitment in BAL fluid. This result seems reasonable, as inflammatory cytokines play important roles in recruiting cells to the site of lung injury. Histologically, it has been reported that, predominantly in early phase, alveolitis and interstitial infiltration of inflammatory cells with epithelial cell injury can be seen in bleomycin-treated rodent lungs (29, 30). In addition, the early stage of bleomycin-induced lung injury has been reported to have histologic similarities to the acute phase of ALI/ARDS or diffuse alveolar damage in humans (31, 32). Thus, the early phase of bleomycin-induced lung injury is one model for exploring the pathogenesis of

ALI, whereas the late phase is more analogous to chronic lung disease/pulmonary fibrosis. We analyzed hydroxyproline levels to verify the increase of collagen deposition in the lungs, and confirmed that the hydroxyproline content did increase after bleomycin treatment in both WT and SP-A^{-/-} mice. Moreover, lung hydroxyproline content in SP-A^{-/-} mice was significantly higher than in WT mice on Day 21 (see Figure E1 in the online supplement), suggesting that the enhanced inflammation and apoptosis preceded the subsequent fibrosis in SP-A^{-/-} mice.

We have shown that SP-A regulates not only the early mediators of lung injury (proinflammatory cytokines), but also late mediators, such as HMGB1. HMGB1 is reported to be passively released from damaged cells, and because HMGB1 is a ubiquitous protein and does not contain a signal sequence, the passive release of HMGB1 from dead cells could induce inflammation (33). Therefore, in the absence of SP-A, increased cell death in the lung in response to bleomycin challenge might induce passive release of HMGB1. On the other hand, in addition to the passive release from dead cells, Yang and colleagues have shown that HMGB1 can be actively released

TABLE 1. EFFECT OF SURFACTANT PROTEIN A AND OTHER HOMOLOGS ON BLEOMYCIN-INDUCED APOPTOSIS IN MLE12 CELLS

Bleomycin + Treatment	% Positive Cells	
	A+/P−	A+/P+
Bleomycin only	15.29 ± 1.24	9.08 ± 1.86
SP-A	7.81 ± 1.17*	6.48 ± 1.43
SP-D	14.31 ± 1.28	9.10 ± 2.04
C1q	12.24 ± 1.40	7.95 ± 1.82
IgG	12.29 ± 1.55	7.82 ± 1.70

Definition of abbreviations: A = annexin V; P = propidium iodide; SP = surfactant protein.

Apoptosis was induced in MLE12 cells with 100 μ g/ml of bleomycin in the presence of SP-A (50 μ g/ml) or other proteins (SP-D, 2 μ g/ml; C1q, 50 μ g/ml; mouse IgG, 50 μ g/ml). Percentages of A+/P− (early apoptotic) and A+/P+ (mid to late apoptotic) cells were evaluated. Values are expressed as means $\pm$ SE of three independent experiments.

* $P < 0.05$ compared with bleomycin only.

from macrophages/monocytes (34), although the precise mechanism is still unknown. Thus, the second possible mechanism by which SP-A could regulate HMGB1 expression is that active release of HMGB1 could be controlled by SP-A. Further study will be needed to explore the mechanism(s) whereby SP-A controls HMGB1 secretion from live and/or damaged cells.

In addition to the increased cell infiltration and enhanced production of early and late mediators of lung injury observed in bleomycin-treated SP-A^{−/−} mice, there was also increased vascular permeability and apoptosis in the lung *in vivo*. As seen in Figures 5 and 6, there are significantly more TUNEL-positive cells in SP-A^{−/−} mice, and some of them are active caspase-3 positive. Thus, SP-A appears to have a protective role in bleomycin-induced apoptosis. These findings are consistent with other reports demonstrating the protective effect of SP-A against type II pneumocyte apoptosis (35). In addition, *in vitro* experiments suggest that although SP-A cannot entirely prevent apoptosis in MLE12 cells, it does exert a protective effect against the initial onset or early stage of apoptosis, as evidenced by the reduced numbers of annexin V-positive, PI-negative cells. SP-A did not prevent apoptosis once cells are in mid to late apoptosis (annexin V-positive, PI-positive). Cells that are beyond this stage may also have a very low potential for recovery. Although the precise mechanism by which SP-A regulates the early stage of apoptosis needs to be further determined, this observation might also explain the increased vascular permeability and HMGB1 levels in SP-A^{−/−} mice compared with WT mice subject to bleomycin treatment. Maintaining the integrity of the airway epithelium is a crucial step in the regulation of ALI.

The mechanism of ALI and subsequent immune responses and tissue repair remains an open question. However, one key mechanism of bleomycin-induced lung injury and tissue repair was recently revealed by Jiang and colleagues (25). They have shown that TLR2 and TLR4 provide signals that initiate inflammatory responses, maintain epithelial cell integrity, and promote recovery from bleomycin-induced ALI by interacting with hyaluronan. Moreover, Park and colleagues have reported that TLR2 and TLR4 can serve as receptors for HMGB1, which appears to play a role in ALI/ARDS and murine bleomycin-induced lung injury model, in addition to its known receptor, the receptor for advanced glycation end products (36). These reports suggest that TLR2 and TLR4 play important roles in ALI induced by bleomycin, and SP-A may possibly be involved in this mechanism. It has been shown that SP-A regulates inflammatory responses induced by pathogen-derived products

Figure 8. Exogenous surfactant protein A (SP-A) rescued bleomycin-induced lung injury in SP-A-deficient (SP-A^{−/−}) mice. (A) Wild-type (WT) (shaded bars) and SP-A^{−/−} (solid bars) mice were challenged with intratracheal saline or bleomycin (bleo), and exogenous SP-A (or saline as a control) was administered intratracheally as indicated in METHODS. On Day 7 after bleomycin challenge, total cell count in bronchoalveolar lavage (BAL) was counted. SP-A administration to SP-A^{−/−} mice reduced the number of BAL total cell count to the level of WT mice. SP-A administration did not affect saline-challenged mice. $n = 8-9$. ** $P < 0.05$. (B) On Day 7 after bleomycin treatment, Evans blue dye leakage in BAL fluid was determined. SP-A treatment significantly reduced the Evans blue dye leakage in bleomycin-challenged SP-A^{−/−} mice, but not in WT mice. $n = 8-10$. * $P < 0.01$. (C) The mortality rate of SP-A-rescued SP-A^{−/−} mice (SP-A^{−/−} SP-A) was significantly lower than that of saline-rescued mice (SP-A^{−/−} Saline) in response to bleomycin challenge ($P < 0.05$). No significant difference in survival was seen in WT mice with SP-A rescue. $n = 18-23$ in each group.

such as peptidoglycan and lipopolysaccharide via TLRs (37–39). In addition to its role in TLR-mediated cellular responses induced by infectious challenges, it is very probable that SP-A regulates lung injury caused by noninfectious challenges (such as bleomycin-induced lung injury) through the interaction with TLRs. However, the interaction between SP-A and TLRs in the field of noninfectious lung injury is not well identified. Further study will be needed to understand the mechanisms of immune responses and TLR signaling in the context of SP-A and noninfectious ALI.

We also analyzed SP-A levels in BAL fluid after bleomycin treatment and confirmed that SP-A expression in BAL fluid was decreased in response to bleomycin challenge in WT mice (Figure E2). This finding is consistent with previous reports indicating that the surfactant expression was decreased in BAL fluid from patients with ALI/ARDS (40, 41). Based on these reports, many clinical studies have been performed to investigate the efficacy and the improvement in survival due to exogenous surfactant instillation in patients with ALI/ARDS (42, 43). So far, no significant effect of surfactant treatment has been shown on survival in phase III studies in adult patients. Two possible directions might be considered in the future to improve the outcome. First, subgroups of patient with ALI/ARDS should be defined. Because ALI/ARDS is caused by various background diseases and insults, therapeutic efficacy may be improved by selecting subpopulations that share biochemical and/or genetic homogeneity. Indeed, pooled analysis of clinical studies with recombinant SP-C surfactant treatment suggests that, although SP-C surfactant did not reduce mortality in the broad population of patients with ARDS, it may have a beneficial effect on the patients who have pneumonia or have aspirated gastric contents and have a severe deficiency of oxygenation (44). The second way is to develop the replacement surfactant therapies with different compositions. The surfactant therapies that have been used in the clinical studies to date do not contain SP-A. Although our current study does not support SP-A's beneficial effect in SP-A-competent animals or humans, the modification of the preparation may be the alternative way to improve the efficacy of surfactant replacement therapy.

In conclusion, we demonstrate that, in the absence of SP-A, the mice exhibit elevated inflammation and HMGB1 expression and decreased epithelial integrity possibly due to increased apoptosis in the lung. In addition, SP-A was able to depress induction of epithelial cell apoptosis *in vitro*. Furthermore, administration of SP-A into SP-A^{-/-} mice rescued the phenotype. Although the precise molecular mechanism of SP-A in regulating apoptosis remains to be verified, these results indicate that SP-A plays important protective role in the progression of noninfectious ALI.

Conflict of Interest Statement: H.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.G.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.M. has a pending patent application from UCSB (previous research lab). P.W.N. is employed by Intermune (received \$10,001–\$50,000), Actelion (received \$1,001–\$5,000), Gilead Sciences (received \$1,001–\$5,000), Stomedix (received \$1,001–\$5,000), and Almiral (received \$1,001–\$5,000) as a consultant, and received \$10,001–\$50,000 from Intermune Pharmaceuticals, \$5,001–\$10,000 from Actelion, \$5,001–\$10,000 from Gilead, \$5,001–\$10,000 from Novartis, and \$5,001–\$10,000 from Boehringer Ingelheim in consultancy fees. K.L.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.R.W. received \$1,001–\$5,000 from Ono Pharmaceuticals in lecture fees.

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