Natural Anti-Infective Pulmonary Proteins: In Vivo Cooperative Action of Surfactant Protein SP-A and the Lung Antimicrobial Peptide SP-B

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*J Immunol* published online 10 July 2015
http://www.jimmunol.org/content/early/2015/07/10/jimmunol.1500778

Supplementary Material  
http://www.jimmunol.org/content/suppl/2015/07/10/jimmunol.1500778.DCSupplemental

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Natural Anti-Infective Pulmonary Proteins: In Vivo Cooperative Action of Surfactant Protein SP-A and the Lung Antimicrobial Peptide SP-B<sup>N</sup>

Juan Manuel Coya,*† Henry T. Akinbi,‡ Alejandra Sáenz,*† Li Yang,‡ Timothy E. Weaver,‡ and Cristina Casals*†

The anionic antimicrobial peptide SP-B<sup>N</sup>, derived from the N-terminal saposin-like domain of the surfactant protein (SP)-B proprotein, and SP-A are lung anti-infective proteins. SP-A-deficient mice are more susceptible than wild-type mice to lung infections, and bacterial killing is enhanced in transgenic mice overexpressing SP-B<sup>N</sup>. Despite their potential anti-infective action, in vitro studies indicate that several microorganisms are resistant to SP-A and SP-B<sup>N</sup>. In this study, we test the hypothesis that these proteins act synergistically or cooperatively to strengthen each other’s microbicidal activity. The results indicate that the proteins acted synergistically in vitro against SP-A– and SP-B<sup>N</sup>–resistant capsulated K. pneumoniae (serotype K2) at neutral pH. SP-A and SP-B<sup>N</sup> were able to interact in solution (K<sub>d</sub> = 0.4 μM), which enabled their binding to bacteria with which SP-A or SP-B<sup>N</sup> alone could not interact. In vivo, we found that treatment of K. pneumoniae–infected mice with SP-A and SP-B<sup>N</sup> conferred more protection against K. pneumoniae infection than each protein individually. SP-A/SP-B<sup>N</sup>–treated infected mice showed significant reduction of bacterial burden, enhanced neutrophil recruitment, and ameliorated lung histopathology with respect to untreated infected mice. In addition, the concentrations of inflammatory mediators in lung homogenates increased early in infection in contrast with the weak inflammatory response of untreated K. pneumoniae–infected mice. Finally, we found that therapeutic treatment with SP-A and SP-B<sup>N</sup> 6 or 24 h after bacterial challenge conferred significant protection against K. pneumoniae infection. These studies show novel anti-infective pathways that could drive development of new strategies against pulmonary infections.

Klebsiella pneumoniae is a well-known opportunistic pathogen that may be responsible for as much as 8% of all hospital-acquired infections (1). The emergence of multidrug-resistant strains of K. pneumoniae has significantly complicated the management and treatment of infections involving this organism by contributing to increased bacterial drug resistance, toxic effects, and health care costs (1). The pipeline for new antibiotics is insufficient to match the health burden posed by lung infection, and the effectiveness of current therapeutic strategies against pneumonia is threatened (2).

Endogenous antimicrobial peptides possess desirable features of new classes of antibiotics, including broad-spectrum activity, neutralization of endotoxins, activity in vivo, and possible synergy with one another and with antibiotics (3). Consequently, a variety of host defense proteins are gaining considerable attention and clinical interest because of their importance in maintaining lung homeostasis in ambient conditions and their potential to kill multidrug-resistant microorganisms. Among these proteins, surfactant protein (SP)-A and the N-terminal segment of the SP B propeptide (SP-B<sup>N</sup>) have been shown to be potent innate immune molecules in the lung (4, 5). SP-A is a large protein, secreted into the alveolar fluid, which plays a key role in lung innate immunity. It enhances phagocytosis of microorganisms by opsonization or direct alveolar macrophage stimulation (4). It is able to regulate inflammatory mediator production depending upon the pathogen, the responding host cell, and the cytokine environment (4). In addition, it has been demonstrated that SP-A has potent macrophage-independent antibacterial activity in vivo (6), and SP-A deficiency in gene-targeted mice causes increased susceptibility to lung infections (4). This antimicrobial property is shared with antimicrobial peptides present in the alveolar space such as SP-B<sup>N</sup>, an ~80-aa saposin-like peptide, which is secreted into the air space together with surfactant components (5). In vitro, SP-B<sup>N</sup> indirectly promotes the phagocytosis of bacteria by macrophage cell lines, and it directly kills bacteria at acidic, but not at neutral, pH, consistent with a lysosomal antimicrobial function (5). Moreover, overexpression of SP-B<sup>N</sup> in the distal airway epithelium protects mice against infection with Pseudomonas aeruginosa and Streptococcus pneumoniae (5).

Despite the potential anti-infective action of SP-A and SP-B<sup>N</sup> in the lung, a number of questions remain unanswered, including the mechanism by which SP-B<sup>N</sup> might kill bacteria in the alveolar fluid at neutral pH or the factors that govern SP-A’s anti-microbial activity because in vitro studies indicate that several microorganisms are resistant to SP-A (7). It is possible that cooperative interactions of SP-A with other antimicrobial peptides or proteins present in the alveolar fluid enhance the microbicidal defense of the lungs, yet little is known about in vivo interactions between soluble factors and SP-A and their relevance in innate host defense.
In this study, we investigate the potential interaction of SP-A and SP-B\(^{\text{N}}\) in airway host defense. Results show the concerted antimalarial action of SP-A and SP-B\(^{\text{N}}\) in vitro and in vivo against \textit{K. pneumoniae} K2 infection and the therapeutic benefit of SP-A/SP-B\(^{\text{N}}\) treatment after bacterial challenge, suggesting that harnessing the host’s natural antimicrobial proteins might provide an adjunct to the current therapy for pneumonia.

**Materials and Methods**

**SP-B\(^{\text{N}}\) and SP-A**

Recombinant human SP-B\(^{\text{N}}\) (molecular mass, 8 kDa) was expressed in \textit{Escherichia coli} BL21 (DE3) and purified over a Ni-NTA agarose column (Novagen) as previously described (5). Human SP-A was isolated from bronchoalveolar lavage (BAL) of patients with alveolar proteinosis using \textit{K. pneumoniae} K2 (from Dr. Bengoechea). To minimize variability in virulence, we selected \textit{K. pneumoniae} K2 (5, 10, and 20 \(\mu\)g/ml) or combinations of both, and then washed twice with PBS before 1-h infection with \(3 \times 10^7\) CFU. In another experiment, the bacterial suspension was incubated for 90 min to kill extracellular bacteria. Cells were washed to remove gentamicin (300 \(\mu\)g/ml). SP-A, SP-B\(^{\text{N}}\), or human serum albumin (HSA) were added to the cultures for 1 h at room temperature. Binding protein detection was performed by adding 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma). The colorimetric reaction was halted with 4 M sulfuric acid, and the absorbance was read at 450 nm on an ELISA reader (DigiScan; Asys HiTech GmbH, Eugendorf, Austria). The results obtained were expressed as nanograms of bound protein/10\(^5\) bacteria.

**SP-B\(^{\text{N}}\) binding to SP-A.** To explore whether SP-B\(^{\text{N}}\) binds to immobilized SP-A (100 \(\mu\)g/ml) or SP-B\(^{\text{N}}\) in solution, BALF of patients with alveolar proteinosis using \textit{K. pneumoniae} K2 that caused lung inflammation with minimal mortality in WT FVB/N mice. Ten thousand CFUs of \(3 \times 10^7\) \textit{K. pneumoniae} K2 were grown at 37\(^\circ\)C and 5% CO\(_2\) in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FBS. Prophagecysis and bacterial killing assay in vitro

Bacteria were grown in LB at 37\(^\circ\)C with continuous shaking to exponential phase. Bacteria were then harvested, resuspended in PBS, and adjusted to the desired final concentration.

For phagocytosis assays, MH-S or RAW 264.7 cells were seeded in 24-well tissue culture plates at a density of \(3 \times 10^5\) cells/well 15 h before each experiment. Macrophages were subsequently preincubated 4 h with or without different concentrations of SP-A (25, 50, and 100 \(\mu\)g/ml) and SP-B\(^{\text{N}}\) (5, 10, and 20 \(\mu\)g/ml), or combinations of both, and then washed twice with PBS before 1-h infection with \(3 \times 10^7\) CFU. After 1 h of infection, monolayer cells were washed three times with PBS and incubated with RPMI 1640 containing 10% FCS, 10 mM Heps, gentamicin (300 \(\mu\)g/ml), and polymyxin B (15 \(\mu\)g/ml) for 90 min to kill extracellular bacteria. Cells were washed to remove gentamicin and polymyxin, lysed with 0.5% saponin, and resuspended in PBS. The lysates were diluted serially for CFU enumeration of viable bacteria.

**Binding assays**

SP-A and SP-B\(^{\text{N}}\) binding to bacteria. To explore the ability of biotinylated SP-A or biotinylated SP-B\(^{\text{N}}\) to bind to \(3 \times 10^7\) \textit{K. pneumoniae} K2 in the presence of SP-B\(^{\text{N}}\), respectively, we performed binding assays as previously described (12). In a slight modification, the stock solution of SP-A or SP-B\(^{\text{N}}\) was incubated with biotin (10 \(\mu\)g/ml) and then added to a 96-well plate MaxiSorp (Nunc, Rochester, NY) and allowed to bind 1 h at 37\(^\circ\)C. The plate was blocked with 5 mM Tris HCl containing 10% PBS for 1 h at 37\(^\circ\)C. After extensive washing, streptavidin-HRP was added to the wells and incubated for 1 h at room temperature. Biotinylated protein detection was performed by adding 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma). The colorimetric reaction was halted with 4 M sulfuric acid, and the absorbance was read at 450 nm on an ELISA reader (DigiScan; Asys HiTech GmbH, Eugendorf, Austria). The results obtained were expressed as nanograms of bound protein/10\(^5\) bacteria.

**SP-B\(^{\text{N}}\) binding to SP-A.** To explore whether SP-B\(^{\text{N}}\) binds to immobilized SP-A (100 \(\mu\)g/ml) or SP-B\(^{\text{N}}\) in solution were carried out as previously described (13), using an SLM-Aminco AB-2 spectrophotometer equipped with a thermostat-regulated cuvette holder (\(\pm 0.1\) \(\text{C}\)). Tryptophan fluorescence emission spectrum of SP-A (15 \(\mu\)g/ml) was recorded from 305 to 400 nm on excitation at 295 nm at 25\(^\circ\)C in 5 mM Tris HCl buffer (pH 7.4) containing 150 mM NaCl, 2 mM CaCl\(_2\), and 0.1% Tween 20 and blocked with washing buffer containing 2.5% nonfat dried milk for 2 h. Subsequently, biotinylated SP-B\(^{\text{N}}\) in concentrations from 0 to 6 \(\mu\)g/ml was added to the wells and incubated in the same buffer for 1 h at room temperature. To detect SP-A- and SP-B\(^{\text{N}}\)-bound biotinylated SP-B\(^{\text{N}}\), we added streptavidin-HRP as described earlier.

**Intrinsic fluorescence experiments.** Tryptophan SP-A fluorescence measurements to explore the binding between SP-A and SP-B\(^{\text{N}}\) in solution were carried out as previously described (13), using an SLM-Aminco AB-2 spectrophotometer equipped with a thermostat-regulated cuvette holder (\(\pm 0.1\) \(\text{C}\)). Tryptophan fluorescence emission spectrum of SP-A (15 \(\mu\)g/ml) was recorded from 305 to 400 nm on excitation at 295 nm at 25\(^\circ\)C in 5 mM Tris HCl buffer (pH 7.4) containing 150 mM NaCl. Subsequently, the titration experiment was started by adding increasing amounts of SP-B\(^{\text{N}}\) to the protein solution in the cuvette. The fluorescence intensity readings were corrected for the dilution caused by SP-B\(^{\text{N}}\) addition.

**Data from equilibrium dissociation constants of SP-A were analyzed by linear least-squares fitting to** Eq. 1:

\[
\Delta F = \Delta F_{\text{max}} \frac{[\text{peptide}]^n}{K_{d} + [\text{peptide}]} \tag{1}
\]

where \(\Delta F\) is the change in fluorescence emission intensity at 335 nm relative to the intensity of free SP-A, \(\Delta F_{\text{max}}\) is the change in fluorescence intensity at saturating SP-B\(^{\text{N}}\) concentrations, \(K_d\) is the apparent equilibrium dissociation constant, [peptide] is the molar concentration of free SP-B\(^{\text{N}}\), and \(n\) is the Hill coefficient.

**Dynamic light scattering.** The hydrodynamic diameters of SP-B\(^{\text{N}}\) and SP-A particles, as well as mixtures of these components, were measured at 25\(^\circ\)C in a Zetasizer Nano S from Malvern Instruments (Worcestershire, U.K.) equipped with a 633-nm HeNe laser, as previously described (9). The interfacial SP-A/SP-B\(^{\text{N}}\) was examined by monitoring the concentrations of SP-B\(^{\text{N}}\) (from 0 to 5.5 \(\mu\)M) at a fixed concentration of SP-A (10 \(\mu\)M) in 20 mM phosphate buffer, pH 7.4, with or without 150 mM NaCl. Experiments were also performed in 5 mM sodium acetate buffer, pH 5.5.

**Bacterial killing in vivo**

Preliminary experiments identified a dose of intratracheal \textit{K. pneumoniae} K2 that caused lung inflammation with minimal mortality in WT FVB/N mice. Ten thousand CFUs \textit{K. pneumoniae} K2 was administered intracheally as previously described (14). A combination of \textit{K. pneumoniae} K2 and either SP-A, SP-B\(^{\text{N}}\), or both proteins resuspended in 100 \(\mu\)l 5 mM Tris HCl, 150 mM NaCl, pH 7.4, was administered intratracheally to mice to assess the concerted action of SP-A and SP-B\(^{\text{N}}\) in vivo. Mice were sacrificed at 6 or 24 h postinfection. For therapeutic application of SP-A.SP-B\(^{\text{N}}\), mice infected with \textit{K. pneumoniae} were treated with SP-A/SP-B\(^{\text{N}}\) 6 or 24 h
after the bacterial challenge. Mice were sacrificed at 24 or 48 h postinfection, respectively. In both applications, lungs were harvested, weighed, and homogenized in 1 ml sterile PBS. For in vivo studies, an appropriate concentration of SP-A (150 nM) and SP-BN (2.5 μM) was used. Dilutions of lung homogenates were plated on LB agar plates, and the number of CFUs was determined after an overnight incubation at 37°C. Each experimental group included six mice and data were expressed as CFU ± SEM/mouse.

Lung histopathology

Mice were exsanguinated and their lungs were inflation-perfused with paraformaldehyde, excised, and further fixed with 4% paraformaldehyde for 24 h. Fixed tissues were embedded in paraffin, and 5-μm sections were prepared and stained with H&E, as previously described (14, 15).

BAL cell count

WT FVB/N-infected mice (untreated or treated with SP-A and/or SP-BN) were exsanguinated and their lungs were then lavaged three times with 1-ml aliquots of sterile PBS. BAL fluid was centrifuged at 2000 × g for 10 min, and the pellet was resuspended in 0.5 ml PBS as described previously (14). A 50-μl aliquot was stained with an equal volume of 0.4% trypan blue (Life Technologies) for total cell count on a hemocytometer. Differential cell counts were made on cytospin preparations stained with Diff-Quik (Scientific Products, McGraw Park, IL).

Cytokine levels in lung homogenates

At 6 or 24 h after bacterial inoculation, levels of cytokines associated with severe lung injury (mouse IL-1β, IL-6, IL-17a, TNF-α, and MIP-2) were assessed in lung homogenates by ELISA using MilliplexTM Multiplex kits (Millipore, Billerica, MA) according to manufacturer’s protocol.

Statistical analysis

Data are presented as mean ± SD (in vitro experiments) or SEM (in vivo experiments). Differences in mean between groups were evaluated by one-way ANOVA followed by the Bonferroni multiple-comparison test. For comparison of two groups, Student’s t test was used. An α level ≤5% (p ≤ 0.05) was considered significant.

Results

Synergic actions of SP-BN and SP-A against K. pneumoniae K2 at neutral pH

To assess potential synergic interaction between SP-BN and SP-A in K. pneumoniae killing, we incubated capsulated K2 (WT) and noncapsulated bacteria with increasing concentrations of SP-BN in the absence or presence of SP-A (100 μg/ml) at pH 7.4, and colony count assays were performed. Fig. 1A shows that neither SP-BN nor SP-A alone was able to kill capsulated or noncapsulated K. pneumoniae at neutral pH; however, in combination, SP-A and SP-BN effectively killed capsulated and noncapsulated K. pneumoniae at pH 7.4, indicating that the capsule polysaccharide did not protect bacteria from SP-BN/SP-A–mediated antimicrobial activity. Addition of 2 mM calcium did not enhance SP-BN/SP-A–mediated killing (data not shown), indicating that the presence of Ca2+ is not essential for bactericidal activity. Given that SP-BN is able to kill bacteria at acidic pH (5), we also performed killing experiments under these conditions. However, at this pH, the bactericidal action of SP-BN on K. pneumoniae K2 was not increased by the presence of SP-A (Supplemental Fig. 1A).

Because SP-A and SP-BN, individually, have been proved to enhance phagocytosis of several respiratory pathogens (4, 5), we investigated the potential cooperative action between SP-BN and SP-A to enhance uptake of K. pneumoniae K2 by mouse alveolar (MH-S) or peritoneal (Raw 264.7) macrophages. Preincubation of these macrophage cell lines with increasing concentrations of SP-BN (5–20 μg/ml) for 4 h before the addition of bacteria resulted in a significant increase in viable intracellular bacteria compared with untreated cells. In contrast, preincubation with SP-A (100 μg/ml, 0 μg/ml SP-BN) had no effect (Fig. 1B). However, preincubation of macrophages with SP-A in combination with increasing amounts of SP-BN resulted in a significant increase in bacterial uptake, compared with untreated cells and cells treated with SP-BN alone (Fig. 1B). These data suggest that SP-A and SP-BN act in conjunction to indirectly increase phagocytosis of K. pneumoniae by macrophages. In contrast, we found that bacterial phagocytosis did not increase after 2-h preincubation of K. pneumoniae K2 with SP-A+SP-BN or with each protein separately (data not shown). These experiments indicate that SP-A and/or SP-BN did not directly trigger K. pneumoniae phagocytosis by bacterial opsonization.

To determine the binding of SP-A to K. pneumoniae K2, we incubated bacteria with biotinylated SP-A (0–1.25 μg/ml) in the absence or presence of SP-BN (10 μg/ml) or HSA (10 μg/ml). SP-A alone was unable to bind K. pneumoniae K2, even in the presence of HSA (Fig. 1C). However, SP-A bound avidly to K. pneumoniae in a dose-dependent manner when SP-BN was added to the solution (Fig. 1C). Similarly, the binding of biotinylated SP-BN (0–5 μg/ml) was determined in the absence or presence of SP-A (100 μg/ml) or HSA (10 μg/ml) (Fig. 1D). Biotinylated SP-BN alone was able to bind to K. pneumoniae K2 at acidic but not neutral pH (Supplemental Fig. 1B). However, SP-BN bound avidly to this pathogen in a dose-dependent manner at neutral pH when SP-A was added to the solution (Fig. 1D). Thus, at neutral pH, SP-A and SP-BN effectively bound to (Figs. 1C, 1D) and killed (Fig. 1A) K. pneumoniae K2 only when presented to the bacteria in combination.

SP-BN binds directly to SP-A

To understand how SP-A and SP-BN jointly strengthen each other’s microbicidal activity against K. pneumoniae, we studied the potential interaction of SP-A and SP-BN in solution by monitoring changes in the intrinsic fluorescence of SP-A after SP-BN binding (Fig. 2A). The fluorescence of SP-A is dominated by the contribution of its two conserved tryptophan residues at the COOH-terminal end of the protein. Addition of increasing concentrations of SP-BN (0–5.5 μM) resulted in a significant SP-BN concentration-dependent decrease in the amplitude of the fluorescence emission spectrum of SP-A, without any shift in the wavelength of the emission maxima (Fig. 2A). These data suggest that the interaction of SP-BN with SP-A produces conformational changes in SP-A resulting in a higher level of tryptophan fluorescence quenching by polarizable groups in the proximity of these residues (9, 13). The estimated dissociation constant (Kd) for SP-A/SP-BN interaction was 0.39 ± 0.02 μM and the Hill coefficient (n) = 1.

The interaction between SP-BN and SP-A was also examined by solid-phase binding assay. Fig. 2B shows that SP-BN interacted with immobilized SP-A in a dose-dependent and saturable manner with a Kd of 0.4 ± 0.08 μM. SP-BN did not bind to wells coated with HSA (Fig. 2B, open circles) or wells containing buffer alone. The Kd obtained by this method was quite similar to that calculated for SP-A and SP-BN in solution (Fig. 2A).

In addition, we found that the binding of SP-BN to SP-A at neutral pH caused an SP-BN concentration-dependent increase of SP-A hydrodynamic size in the presence of salts, as determined by DLS (Fig. 2C), resulting in the formation of protein-protein aggregates of 950 ± 50 nm. SP-BN alone formed large aggregates at acidic but (Supplemental Fig. 1C) not at neutral pH. At acidic pH, SP-A/SP-BN interaction was not observed (Supplemental Fig. 1C).

Killing of K. pneumoniae in mice treated with SP-A and SP-BN

To determine an appropriate bacterial dose for in vivo studies, 103–105 CFUs K. pneumoniae K2 was inoculated intratracheally in wild-type FVB/N mice (six mice/group). Administration of Klebsiella was well tolerated, and all animals survived the 72-h study period at the 105 CFU dose. Alteration in activity or physical appearance of the animals was not detected until 72 h postinfection. K. pneumonia-
FIGURE 1. Synergic actions of SP-BN and SP-A against *K. pneumoniae* at neutral pH. (A) A total of $10^7$ CFUs/ml noncapsulated or capsulated *K. pneumoniae K2* (WT) was incubated for 1 h at 37°C with increasing concentrations of SP-BN in the absence or presence of 100 μg/ml SP-A, in 20 mM phosphate buffer, pH 7, 150 mM NaCl. Then bacteria were plated on LB agar for CFU count after 18 h of incubation at 37°C. Results are shown as % viable bacteria (percentage of live colony counts compared with untreated control) and are mean ± SD of four experiments, each duplicated. A p value <0.0001 was obtained for overall ANOVA (Bonferroni-corrected p values; **p < 0.01, ***p < 0.001) versus the corresponding group without SP-A). (B) RAW 264.7 macrophages were preincubated with SP-BN (0–20 μg/ml) with or without 100 μg/ml SP-A for 4 h. Cells were washed twice with PBS and infected 1 h with *K. pneumoniae K2* (5 × $10^7$ CFU). Postinfection, monolayer cells were washed three times with PBS, media were replaced, and 300 μg/ml gentamicin and 15 μg/ml polymyxin B were added to kill extracellular *K. pneumoniae*. Cells were lysed with 0.5% PBS-saponin, and the number of viable bacteria was assessed by quantitative culture of cell lysates. Similar results were found using MH-S macrophages. Results are mean ± SD of four experiments, each one duplicated. (C) Increasing concentrations of biotinylated SP-A were incubated alone (gray circles) or in the presence of SP-BN (100 μg/ml polymyxin B were added to kill extracellular *K. pneumoniae*. Cells were lysed with 0.5% PBS-saponin, and the number of viable bacteria was assessed by quantitative culture of cell lysates. Similar results were found using MH-S macrophages. Results are mean ± SD of four experiments, each one duplicated. (D) Increasing concentrations of biotinylated SP-BN were incubated alone (gray circles) or in the presence of SP-A 100 μg/ml (black circles) or HSA 10 μg/ml (white circles) with $10^7$ CFU *K. pneumoniae K2* at room temperature for 30 min. Total Klebsiella-associated SP-A was measured by solid-phase assay and expressed as total nanograms of SP-A/10^7 bacteria. Results are mean ± SD of four experiments, each one duplicated. **p < 0.01, ***p < 0.001; Fig. 3), indicating that at the doses used in this study, these proteins are more effective in slowing microbial growth when they are coadministered. At 72 h postinfection, there was no difference among the groups, suggesting that exogenous SP-A and SP-BN are consumed after 24 h and that retreatment and/or higher doses will be required to control infection. 

**Lung histopathology and inflammation in SP-A and SP-BN–treated mice after *K. pneumoniae* infection**

There was a modest increase in the total BAL cell counts in mice 24 h, but not 6 h, postinfection with *K. pneumoniae K2*, compared with uninjected mice treated with vehicle (Fig. 4A). Total cell numbers in BAL from SP-A– or SP-BN–treated groups were significantly increased at 24 h, but not 6 h, postinfection compared with untreated infected animals. Combined SP-A/SP-BN treatment further enhanced cell recruitment at 24 h postinfection compared with untreated infected animals (p < 0.001) and with infected mice treated with SP-A or SP-BN alone (p < 0.001). Administration of both proteins in uninfected mice did not produce significant changes in BAL cell counts (Fig. 4A).

Histological examination of lung sections showed mild leukocytic alveolitis in lungs of infected mice at 6 h postinfection, which was more pronounced in infected mice treated with SP-A+SP-BN (Fig. 4B), consistent with neutrophil recruitment (Fig. 5). At 24 h postinfection, alveolar and peribronchioronal infiltrates increased in both SP-A/SP-BN–treated and untreated infected mice. However, areas of lobular pneumonia were more intense in untreated mice (Fig. 4B, black arrows). No pathological changes were observed in saline-challenged (control) lungs from uninfected mice (Fig. 4B).
Analyses of cell types in BAL from infected mice treated with SP-A+SP-BN revealed that recruited cells consisted predominantly of neutrophils (Fig. 5). There was no difference in macrophages among the groups regardless of treatment regimen. However, neutrophil recruitment was significantly greater in infected mice treated with SP-A+SP-BN compared with infected mice treated with SP-A or SP-BN and untreated infected mice (Fig. 5). Control experiments indicate that neutrophil recruitment did not significantly increase in uninfected mice treated with SP-A+SP-BN compared with untreated uninfected mice (Fig. 5).

Early neutrophil recruitment was strongly associated with elevated levels of TNF-α, IL-1β, IL-6, MIP-2 \((p < 0.001)\), and IL-17α \((p < 0.05;\) Fig. 6). The cytokine response was detected only in animals treated with SP-A/SP-BN at 6 h postinfection and was transient, returning to baseline at 24 h postinfection. Untreated infected mice showed a weak inflammatory response both at 6 and at 24 h postinfection (Figs. 5, 6).

**Therapeutic effect of SP-A and SP-BN in mice with an established infection**

WT FVB/N mice were first infected with *K. pneumoniae* K2 and subsequently treated with exogenous proteins 6 or 24 h postinfection to assess the therapeutic potential of SP-A/SP-BN treatment. Lung bacterial burden and BAL cells were analyzed at either 24 h postinfection (for animals treated 6 h after pathogen inoculation) or 48 h postinfection (for animals treated 24 h after inoculation).

Fig. 7 shows that SP-A/SP-BN treatment 6 h after bacterial inoculation significantly reduced bacterial burden (67%; \(p < 0.001\)) compared with untreated infected mice. The bacterial burden was expressed as CFUs/mouse (Fig. 7). The p values 0.0009 (6-h infection) and 0.0002 (24-h infection) were obtained for overall ANOVA. Bonferroni-corrected p values: ***\(p < 0.001\), **\(p < 0.01\), *\(p < 0.05\) versus the untreated infected group.
at 24 h postinfection. Moreover, SP-A/SP-BN treatment after
24 h of pathogen inoculation also decreased bacterial CFUs
at 48 h postinfection (72%; p < 0.001). Reduced bacterial
burden was associated with increased neutrophil infiltration at
24 and 48 h postinfection in SP-A/SP-BN–treated mice com-
pared with untreated infected mice (p < 0.05). Therefore, our
results indicate that therapeutic treatment with SP-A and SP-B
conferred a significant protection against K. pneumoniae K2
infection.

Discussion

The purpose of this study was to evaluate the potential cooperative
action of two components of lung innate host defense: the
N-terminal saposin-like domain of the SP-B proprotein of ∼81 aa
residues, and the large oligomeric protein SP-A. Both are syn-
thesized and proteolytically processed by type II alveolar epithe-
lial cells. Once secreted to the alveolar fluid, the anionic peptide
SP-BN has been detected unassociated with surfactant membranes
(5) that are rich in anionic phospholipids and that sequester cat-

FIGURE 4. Effect of SP-A and SP-BN treatment on
total BAL cell count (A) and lung histopathology (B) in
K. pneumoniae K2–infected mice. WT FVB/N mice
were instilled with buffer or infected with 10^6 CFUs
K. pneumoniae K2 with or without SP-A (100 μg), SP-BN
(20 μg), or SP-A (100 μg) + SP-BN (20 μg). Mice were
sacrificed at 6 or 24 h postinfection. (A) Total cell count
in BAL was assessed (n = 6 mice for each group).
Results are mean ± SEM. A p value <0.0001 was ob-
tained for overall ANOVA. Bonferroni-corrected p values:
*p < 0.05, ***p < 0.001 when compared with the un-
treated infected group; #p < 0.01, ###p < 0.001 when
comparing SP-BN+SP-A–treated versus SP-B or SP-A–
treated infected groups; p < 0.05 when untreated infected
versus uninfected groups are compared. (B) Lung
sections from uninfected, infected/untreated, and infected/
treated (SP-A + SP-BN) were stained with H&E. These
are representative sections of six mice in each experiment
with similar results. Arrows indicate areas of lobular
pneumonia. Original magnification ×10.

FIGURE 5. SP-A and SP-BN treatment enhance early neutrophil recruitment in infected mice. WT FVB/N mice were instilled with buffer or infected
with 10^6 CFUs K. pneumoniae K2 with or without SP-A (100 μg), SP-BN (20 μg), or SP-A (100 μg) + SP-BN (20 μg). Mice were sacrificed at (A) 6 h or
(B) 24 h postinfection, and total alveolar macrophage and neutrophil count in BAL were assessed. Six mice were evaluated for each group at each time
point. Results are mean ± SEM. A p value <0.0001 was obtained for overall ANOVA (6 and 24 h postinfection). Bonferroni-corrected p values: *p < 0.05,
**p < 0.01, ***p < 0.001, when compared with the untreated infected group; p < 0.05, ***p < 0.001, when SP-BN+SP-A–treated versus SP-B or SP-A–
treated infected groups are compared.
ionic, but not anionic, antimicrobial peptides at neutral pH. The anionic peptide SP-B^N shows microbicidal activity only at acidic pH, which is incompatible with its potential extracellular antimicrobial activity. However, elevated SP-B^N concentrations in the airspaces of transgenic mice overexpressing SP-B proprotein were associated with decreased bacterial load and enhanced survival, post intranasal infection with P. aeruginosa (5). This suggests that other factors present in the alveolar fluid might act cooperatively with SP-B^N, reinforcing the microbicidal defense of the lungs.

We hypothesize that SP-B^N binding to SP-A, a versatile recognition protein that binds to a great variety of immune and nonimmune ligands (4), synergizes antimicrobial activity. Our results indicate that both proteins were able to interact at neutral pH in the presence of salts (K_a = 0.4 μM). This protein–protein interaction allowed SP-A/SP-B^N to bind to pathogenic K. pneumoniae K2 in vitro at pH 7.4 (whereas neither of these proteins bound to K. pneumoniae K2 alone) and kill bacteria at neutral pH. Moreover, SP-A/SP-B^N complexes indirectly increased phagocytosis of K. pneumoniae by macrophages. In addition, cooperative interaction between SP-A and SP-B^N against K. pneumoniae K2 was demonstrated in vivo. Importantly, the antimicrobial action of SP-A and SP-B^N, administered 6 or 24 h after pathogen inoculation, suggests that exogenous administration of recombinant SP-A and SP-B^N may be of therapeutic benefit.

Several reports have suggested the importance of cooperative interaction between mammalian antimicrobial defenses (16). However, little is known about the biological basis of interactions among several soluble factors and between soluble factors and pathogens in the airways and alveolar space. Soluble factors that do not interact with each other might act additively against pathogens, whereas molecular interactions between soluble factors might facilitate or impede their biological activities. In the alveolar fluid, lactoferrin and lysozyme have been proved to act synergistically, whereas hlgD-2 and human cathelicidin act additively against E. coli (17). Synergistic activity in triple combinations of lysozyme, lactoferrin, and the protease inhibitor secreted by leukocytes has also been reported (17). Moreover, SP-A and SP-B have been shown to have additive neutralizing activity with cathelicidin against influenza A virus (18). Our results suggest that synergistic interaction of SP-A and SP-B^N might be important to overcome Gram-negative bacterial infections in the alveolar airspaces.

With respect to structural characteristics of SP-B^N, this peptide has a B-type saposin module. Saposin B contains three strictly conserved intramolecular disulfide bridges that stabilize a saposin-fold, composed of amphipathic helices. The three-dimensional structure of saposin B reveals an unusual shell-like dimer consisting of a monolayer of α-helices enclosing a large hydrophobic cavity (19). Most of the hydrophobic residues that line the concave surface of the monomers remain exposed to a large interior cavity, and the protein does not have a packed hydrophobic core. The existence of the large hydrophobic cavity suggests extraction of target lipids from membranes with which saposins interact (19). SP-B^N, as well as saposin B, is negatively charged. Thus, its interaction with negatively charged bacterial capsules and membranes (such as LPS) is pH dependent. Low pH would trigger the protonation and neutralization of negatively charged residues of SP-B^N, which would otherwise avoid binding to anionic bacterial capsules or bacterial membranes. In addition, SP-B^N underwent pH-dependent aggregation that might be relevant for its antimicrobial activity at acidic pH (5).

In contrast, SP-A is a large extracellular protein structurally characterized by an N-terminal collagen-like domain connected by a neck segment with globular C-terminal domains that include a C-type carbohydrate recognition site (4). SP-A is assembled in multiples of three subunits because of its collagen domain, and its supratrimeric assembly has a bouquet-type structure similar to mannose binding protein or C1q (4, 8). We previously found that the binding of SP-B^N to SP-A at neutral pH caused SP-A–conformational changes and an increase of the protein hydrodynamic size that is dependent on SP-B^N concentration. SP-B^N binding to SP-A resulted in the formation of protein aggregates of 950 ± 50 nm. SP-A/SP-B^N complexes effectively killed K. pneumoniae K2 in vitro at neutral pH, in contrast with SP-A or SP-B^N alone. The mechanism by which SP-A and SP-B^N produce bacterial death synergistically remains to be determined. It is possible that NaCl-dependent binding of SP-B^N to SP-A would trigger the neutralization of negatively charged residues of SP-B^N, which would otherwise hinder SP-B^N binding to the anionic...
bacterial capsule and/or LPS. In addition, SP-A/SP-B N complexes could form a type of fiber that might destabilize the bacterial capsule and/or the outer bacterial membrane. Importantly, SP-B N alone also formed aggregates at acidic pH as determined by DLS, and SP-B N oligomers were able to kill K. pneumoniae K2. Significantly, a number of antimicrobial peptides (such as protegrin-1, dermaseptin S9, and temporins B and L) have been reported to form fibrillar aggregates (21, 22), suggesting a possible mechanistic connection between protein/peptide aggregation and antimicrobial activity.

Combined SP-A/SP-B N intratracheal administration to mice infected with K. pneumoniae K2 conferred more protection against bacterial infection than each protein individually, measured at 6 and 24 h postinfection. SP-A/SP-B N–treated infected mice showed significant reduction of bacterial burden in comparison with untreated infected mice. Exogenous SP-A or SP-B N, administered alone, also showed protection against infection even though K. pneumoniae K2 is resistant to either SP-A or SP-B N in vitro at neutral pH. This is likely due to the presence in the alveolar fluid of endogenous proteins/peptides that strengthen their antimicrobial actions.

The ability of SP-A and SP-B N to enhance clearance of K. pneumoniae K2 in vivo could be related to their capability to both kill bacteria and modulate host inflammatory response. Thus, the antibacterial activity of SP-A/SP-B N in K. pneumoniae K2–infected mice was accompanied by increased early neutrophil recruitment in BAL, which is consistent with the enhancement of an early inflammatory response. Untreated infected mice showed a weak neutrophil influx into the lung at both 6 and 24 h postinfection and a lack of an early inflammatory response in mice lungs. This finding is consistent with the fact that K. pneumoniae K2 antagonizes the activation of NF-κB to subvert the host inflammatory response for its own benefit (23, 24). Several reports have demonstrated that activation of host inflammatory response is essential to clear K. pneumoniae infection (25, 26), and we found that SP-A and SP-B N were able to enhance early inflammatory response to K. pneumoniae K2. SP-A/SP-B N boosted the expression of several proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17-α, and MIP-2) at 6 h postinfection. This is consistent with SP-A/SP-B N–induced recruitment of neutrophils to the alveolus, which can eliminate resistant pathogens by intracellular and extracellular mechanisms (27).

Results also indicate that exogenous SP-B N, but not SP-A, treatment was effective in neutrophil recruitment. Although data supporting the role of SP-B N in inflammation are sparse, anionic antimicrobial peptides have been shown to induce cytokine and chemokine production. Dermcidin is an antimicrobial peptide secreted into sweat, which shares some features with SP-B N such as anionicity, antimicrobial activity at acidic pH, and constitutive expression. Dermcidin-derived peptides have been shown to activate normal human keratinocytes by inducing proinflammatory and chemoattractive mediators (28). In addition, cationic antimicrobial peptides have been reported to promote chemotaxis, both indirectly by stimulating the expression of chemokines and directly by acting as chemokines themselves to recruit a variety of immune cells (29).

Interestingly, the early proinflammatory response induced by SP-A/SP-B N treatment to infected mice was transient, returning to baseline at 24 h postinfection. It is remarkable that the number of alveolar macrophages did not change in infected groups, regardless of treatment regimen, compared with uninfected mice. Alveolar macrophages ingest apoptotic neutrophils recruited in the alveolar space after pathogen challenge, and SP-A enhances the clearance of apoptotic neutrophils (30), promoting the resolution of inflammation. It is believed that SP-A plays a significant role in tipping the balance of inflammation to protect the alveolar epithelium while facilitating pathogen clearance, although the molecular mechanisms by which SP-A interacts with various immune cells are poorly understood.

In summary, we found that lung surfactant-derived components SP-A and SP-B N act in conjunction to protect the lungs against K. pneumoniae K2 infection. Our data indicate that SP-A and SP-B N in combination directly kill K. pneumoniae K2. SP-A/SP-B N treatment significantly reduces bacterial burden and enhances endogenous protective mechanisms to aid in the clearance of bacteria. In addition, SP-A/SP-B N treatment after 24 h of pathogen inoculation significantly decreases bacterial burden at 48 h postinfection. Therefore, our data indicate that therapeutic treatment with SP-A and SP-B N confers significant protection against K. pneumoniae K2 infection. Given the alarming increase in multidrug-resistant Gram-negative bacteria in the face of a paucity of new antibiotics, we have shown that naturally occurring lung proteins may provide a novel model of adjunctive therapy for Gram-negative bacterial infections.

Acknowledgments
We thank Alyssa Spores of Research Flow Cytometry at Cincinnati Children’s Hospital Medical Center for assistance. We also thank Dr. Korhagen (Cincinnati Children’s Hospital) for providing K. pneumoniae strain K2 and

FIGURE 7. Therapeutic administration of SP-A/SP-B N increases bacterial clearance and neutrophil recruitment in mice with an established K. pneumoniae K2 infection. WT FVB/N mice were instilled with buffer (control) or 10^5 CFUs K. pneumoniae K2. Infected mice were treated with buffer (white bars) or SP-A (100 μg) + SP-B N (20 μg) (black bars) 6 (A) or 24 h (B) after the bacterial challenge. Mice were sacrificed at 24 (A) or 48 h (B) postinfection, respectively, to assess lung bacterial burden in lungs. The numbers of viable bacteria were assessed by colony counting and expressed as CFUs/mouse. Results are mean ± SEM (n = 6 mice for each group). Differences in mean between SP-B N–treated and untreated infected groups were evaluated by Student t test (***p < 0.001 versus untreated group). In addition, total alveolar macrophage and neutrophil count in BAL were assessed. Results are mean ± SEM (n = 6). A p value <0.0001 was obtained for overall ANOVA (24 h and 48 h infection). Bonferroni-corrected p values: ***p < 0.001 when compared with the uninfected group; *p < 0.05 when comparing SP-B N + SP-A–treated versus untreated infected mice.

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Dr. Bengoechea (Queen’s University Belfast) for providing K. pneumoniae 52145 strains, which were used in this study.

Disclosures
The authors have no financial conflicts of interest.

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