Increased Surfactant Protein D Fails to Improve Bacterial Clearance and Inflammation in *serpinB1*^{-/-} Mice

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Previously, we described the protective role of the neutrophil serine protease inhibitor serpinB1 in preventing early mortality of *Pseudomonas aeruginosa* lung infection by fostering bacterial clearance and limiting inflammatory cytokines and proteolytic damage. Surfactant protein D (SP-D), which maintains the antiinflammatory pulmonary environment and mediates bacterial removal, was degraded in infected *serpinB1*-deficient mice. Based on the hypothesis that increased SP-D would rescue or mitigate the pathological effects of *serpinB1* deletion, we generated two *serpinB1*^{-/-} lines overexpressing lung-specific rat SP-D and inoculated the mice with *P. aeruginosa*. Contrary to predictions, bacterial counts in the lungs of SP-D^{low} *serpinB1*^{-/-} and SP-D^{high} *serpinB1*^{-/-} mice were 4 logs higher than wild-type and not different from *serpinB1*^{-/-} mice. SP-D overexpression also failed to mitigate inflammation (TNF- α), lung injury (free protein, albumin), or excess neutrophil death (free myeloperoxidase, elastase). These pathological markers were higher for infected SP-D^{high} *serpinB1*^{-/-} mice than for *serpinB1*^{-/-} mice, although the differences were not significant after controlling for multiple comparisons. The failure of transgenic SP-D to rescue antibacterial defense of *serpinB1*-deficient mice occurred despite 5-fold or 20-fold increased expression levels, largely normal structure, and dose-dependent bacteria-aggregating activity. SP-D of infected wild-type mice was intact in 43-kD monomers by reducing SDS-PAGE. By contrast, proteolytic fragments of 35, 17, and 8 kD were found in infected SP-D^{low} *serpinB1*^{-/-}, SP-D^{high} *serpinB1*^{-/-} mice, and *serpinB1*^{-/-} mice. Thus, although therapies to increase lung concentration of SP-D may have beneficial applications, the findings suggest that therapy with SP-D may not be beneficial for lung inflammation or infection if the underlying clinical condition includes excess proteolysis.

Keywords: surfactant protein D; lung infection; inflammation; serpinB1; proteolysis

SerpinB1 (previously called MNEI) is an ancient and broadly expressed serine protease inhibitor increasingly recognized as a regulator of pulmonary innate immunity. At the biochemical level, SerpinB1 is a highly efficient cognate inhibitor of the neutrophil serine proteases (NSPs) elastase, cathepsin G, and proteinase-3 (1). These are glycoprotein enzymes with overlapping function laid down in primary granules during neutrophil generation in the bone marrow (2). In the lung, neutrophils are the first cells recruited to defend against an invading pathogen, and NSPs are an important component of their antimicrobial arsenal, in particular because NSPs

CLINICAL RELEVANCE

The presented studies use a transgenic approach to address the therapeutic potential of surfactant protein D (SP-D) in situations of bacterial infection where neutrophil proteases are in excess. For this, we used mice deficient for the serine protease inhibitor serpinB1, previously shown to replicate features of chronic pulmonary diseases and cystic fibrosis, including failed *Pseudomonas* clearance and increased proteolysis and inflammation in the lungs. We now show that overexpressed SP-D fails to rescue microbial clearance or inflammatory injury. Our work brings a message of caution that will be of broad interest because the findings can be transposed to other therapeutic interventions using recombinant proteins that are susceptible to inactivation by proteases.

kill microbes (3, 4). However, excess NSPs play a central role in the pathology of neutrophil-dominated inflammatory pulmonary diseases (5). To dissect the disparate roles of the NSPs and SerpinB1 in pulmonary antimicrobial defense, we deleted the murine *serpinB1* gene with the aim of coordinately altering NSP function (6).

On intranasal challenge of *serpinB1*^{-/-} mice with *Pseudomonas aeruginosa* or influenza virus, immune responses are initially normal but spiral out of control over time, leading to protracted inflammation and lung injury (6, 7). *SerpinB1*^{-/-} mice infected with these microbes died earlier and in greater numbers than wild-type (WT) mice. When examined 24 hours after infection with *P. aeruginosa*, bacterial numbers were several logs higher for *serpinB1*-deficient mice compared with WT mice, and infection had spread systemically in most of the gene-deleted mice (6). Inflammatory cytokine and chemokine production was similar in *serpinB1*^{-/-} and WT mice at 6 hours after infection; these levels returned toward baseline in WT mice by 24 hours but remained elevated in *serpinB1*^{-/-} mice. Lung injury was increased in infected *serpinB1*^{-/-} mice at 24 hours, as were neutrophil death and release of neutrophil myeloperoxidase (MPO), and protective mucosal proteins were proteolytically destroyed. These findings for infected *serpinB1*^{-/-} mice are consistent with prior studies showing efficacy of recombinant human SERPINB1 in rat models of lung injury and infection (8, 9). Recombinant SERPINB1 also largely rescued the defective phenotype of *serpinB1*^{-/-} mice with dramatically improved bacterial clearance, reduced inflammation, and reduced proteolytic activity in the lungs (6). Collectively, the findings demonstrate that serpinB1 protects lung antimicrobial defense capacity and protects against lung inflammation.

Surfactant protein-D (SP-D), a multimeric hydrophilic protein, is a critical component of innate pulmonary antimicrobial and antiinflammatory defense (10–12). SP-D is also a highly sensitive target of NSPs (13–15), and SP-D depletion has been documented in human inflammatory pulmonary diseases, including

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bacterial pneumonia and acute respiratory distress syndrome (16, 17), and is especially marked in cystic fibrosis (18, 19). Immunoblots revealed proteolysed SP-D in lungs of *P. aeruginosa*-infected *serpinB1*^{-/-} mice compared with intact SP-D in infected WT mice (6). SP-D is synthesized in the lung by alveolar type II cells and Clara cells. The subunit, a trimer, has a short hydrophobic N-terminal domain, a collagen domain, a coiled-coil hydrophobic neck domain, and three calcium-dependent lectin domains; the trimers are assembled via their N-termini into dodecamers in a cruciform arrangement or as higher-order structures (20). Mice with targeted disruption of the SP-D gene have increased baseline lung inflammation in association with enlarged airway macrophages that produce increased amounts of metalloproteases and oxidants; the mice spontaneously develop emphysema (21) and show defective responses to microbial infection (22, 23). SP-D functions as a pattern recognition molecule, binding via the lectin domains to surface oligosaccharides on microbes. By acting as an opsonin and aggregating agent, SP-D enhances phagocytic clearance of pathogens by alveolar macrophages and recruited neutrophils. SP-D also increases clearance of apoptotic neutrophils by the macrophage efferocytotic pathway (24, 25) and directly inhibits bacterial proliferation (26) and regulates pro- and antiinflammatory cytokine production by alveolar macrophages (27).

On the basis of this background, we surmised that proteolysis of SP-D by NSPs may be a central determining event leading to failure to clear *P. aeruginosa* and prolonged lung inflammation and injury of *serpinB1*^{-/-} mice. We hypothesized that increased expression of SP-D in lungs of these mice would restore antimicrobial protection and prevent inflammation and injury. To test this hypothesis, we generated *serpinB1*^{-/-} mice overexpressing SP-D and examined their response to *P. aeruginosa* infection.

MATERIALS AND METHODS

Animals

SerpinB1-deficient (*serpinB1*^{-/-}) mice were generated in 129S6/SvEv/Tac (129S6) background (6). Unchallenged *serpinB1*^{-/-} mice are healthy, with normal growth, reproduction, and tissue morphology. WT (129S6) mice from Taconic Labs were maintained with *serpinB1*^{-/-} mice in the animal facility of the Immune Disease Institute or Children's Hospital Boston for at least several weeks before the study or were bred in house.

To generate SP-D transgenic mice, a 1.3-kb fragment with full-length rat SP-D was excised with *EcoRI* from a pGEM4Z plasmid provided by Drs. J.H. Fisher and D.R. Voelker (28) and cloned into a unique *EcoRI* site between the human SP-C promoter and the SV40 polyadenylation sequence in the pUC18-based expression vector h3.7SP-C (29) kindly provided by Dr. Stephan W. Glasser (University of Cincinnati). One correctly oriented clone, identified by *SacI* digestion, was expanded and purified, and the promoter and SP-D sequences were confirmed by sequencing (Iowa State University). The plasmid was linearized with *NdeI* and *NotI*, and the 5.8-kb construct was separated from plasmid sequences by electrophoresis on 0.8% agarose, excised, and gel purified. Microinjection of the linearized vector into pronuclei from 129S6 female mice and implantation of the pronuclei into pseudo-pregnant mice were performed by the Brigham and Women's Hospital Transgenic Mouse Facility (Boston, MA).

Pups were screened by PCR with exon 3 forward primer GGTTCG CAGGACCTATG and exon 4 reverse primer GCCCAGCCTCT CCTTTAG. To verify transgene expression and to determine copy number, genomic DNA was digested with *NcoI*, and Southern blots were probed with digoxigenin-labeled exon 7 (500 bp) of rat SP-D (PCR DIG Probe Synthesis Kit I) and alkaline phosphatase-labeled antidigoxigenin antibody (Roche Applied Science, Indianapolis, IN). The bands were quantified by chemiluminescence (Quantity One; Bio-Rad Laboratories, Hercules, CA), and the transgene copy number was calculated. Two founder mice were selected: one expressing two transgene copies per genome (SP-D^{low}) and another expressing nine

transgene copies (SP-D^{high}). These were backcrossed twice with *serpinB1*^{-/-} mice to generate SP-D^{low} *serpinB1*^{-/-} mice and SP-D^{high} *serpinB1*^{-/-} mice. N2 backcross generation mice within each line were crossed with *serpinB1*^{-/-} mice to generate experimental mice.

Inoculum

P. aeruginosa was strain PAO1, a LPS-smooth strain isolated from a burn wound and provided by Gregory Priebe (Children's Hospital Boston). The inoculum was freshly prepared from frozen stock and grown overnight on tryptic soy agar, harvested from the plate, combined with PBS, and suspended by vortexing. The concentration was adjusted by OD_{650nm}, and the actual colony-forming units were determined by plating serial dilutions on tryptic soy agar.

Lung Infection

Groups of age-matched female mice were sedated with ketamine and xylazine (100 and 10 mg/kg, respectively) delivered intraperitoneally or with isoflurane (Baxter Healthcare Corp., Deerfield, IL) delivered in a Matrix VIP300 isoflurane vaporizer chamber. The mice were inoculated by applying 15 µl onto each nare. The Institutional Animal Care and Use Committee of the Immune Disease Institute and/or Children's Hospital Boston approved animal studies.

Bronchoalveolar Lavage Fluid and Lung Homogenates

To prepare bronchoalveolar lavage fluid (BALF), naive mice or mice infected 24 hours earlier were killed, and tracheas were exposed and cannulated with an 18-gauge angiocath. Lungs were lavaged once with 1.0 ml of cold PBS and twice with a second 1.0 ml of PBS. The lavage fluids were combined and centrifuged to generate cell-free BALF. To prepare lung homogenates in other groups of mice, dissected whole lungs (or right lungs) were homogenized in 1% proteose peptone. BALF or lung or spleen homogenates were serially diluted in 1% proteose peptone and plated in duplicate on tryptic soy agar. Aliquots of BALF or lung homogenates or postlavage homogenates were stored at -80°C until analyzed, except that BALF for cell-free MPO analysis was analyzed immediately or stored at 4°C.

ELISA

TNF-α and albumin were assayed in duplicate by ELISA using reagents from BioLegend (San Diego, CA) and Bethyl Laboratories (Montgomery TX), respectively. Protein was measured in duplicate by Bradford assay.

Immunoblots

Homogenates and BALF were resolved by SDS electrophoresis under reducing conditions and immunoblotted for SP-D. Unless otherwise indicated, the detecting antibody was AB3434 rabbit antiserum that was generated to purified mouse SP-D and reacts also with rat SP-D (Chemicon/Millipore, Billerica, MA). Bands were quantified by chemiluminescence. Native PAGE gels were run according to the supplier's instructions (Invitrogen, Carlsbad, CA). Briefly, BALF samples were combined with cold native sample buffer (four times) and run on a 4 to 16% NativePAGE gel (Invitrogen) for 2 hours at room temperature at 150V. Size standards for NativePAGE were run in parallel. Proteins were transferred onto PVDF using NuPAGE transfer buffer (without methanol) for 1 hour at room temperature at 30V. Native blots were stained with rabbit anti-SP-D antiserum CR-2011A, which was custom generated by Cocalico Biologicals (Reamstown, PA). The immunogen was the previously described trimeric fusion protein that includes the neck plus carbohydrate recognition domain (NCRD) of rat SP-D expressed in bacteria, isolated from inclusion bodies, purified by nickel chromatography, cut with enterokinase to remove all but a few amino acids of the fusion peptide, and purified by gel filtration (30). Recombinant rat and human SP-D dodecamers, used as standards, were expressed in Chinese hamster ovary cells and purified by maltosyl-agarose affinity chromatography and gel filtration (31); the NCRD standard was the trimeric fusion protein prepared as described above without the enterokinase treatment.

Maltosyl Affinity Chromatography

Pooled BALF from naive mice (15–50 μ l) was diluted to 450 μ l with 20 mM Tris HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween-20 (TBST) containing 15 mM CaCl_2 and was incubated for 2 hours at room temperature on a Ferris wheel together with 120 μ l packed maltose-agarose (M8896; Sigma-Aldrich, Saint Louis, MO) equilibrated with the same buffer. The suspension was centrifuged, and the resin was washed twice with TBST-2 mM CaCl_2 and eluted with TBST-4.5 mM EDTA.

Aggregation Assays

P. aeruginosa or *Escherichia coli* strain BL21 were prepared as described above for inoculations. The bacteria were pelleted; washed once with 10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.05% Tween-20 (HBST); and suspended in HBST with 10 mM CaCl_2 (HBST-Ca) and adjusted to $\text{OD}_{650\text{nm}}$ of 0.10 (*P. aeruginosa*) or 0.05 (*E. coli*). The bacterial suspensions (300 μ l) were plated in 96-well, flat-bottom tissue culture plates (Costar, Corning, NY) that had been blocked with 1% BSA in HBST-Ca for 1 hour at room temperature and washed five times with HBST-Ca. Pooled BALF (2 ml) of naive SP-D transgenic mice was supplemented with 15 mM CaCl_2 , and SP-D was purified by adherence to 1.5 ml packed maltose-agarose equilibrated with TBST-15 mM CaCl_2 for 18 hours at 4°C on a Ferris wheel. The resin was washed twice with TBST-2 mM CaCl_2 , and SP-D was eluted with TBST-4 mM EDTA. The eluate fraction or vehicle was added to the bacteria in a volume of 30 μ l and incubated at room temperature for 3 hours. Where indicated, EDTA was included at 20 mM. Bright field micrographs of the aggregates were taken at 10 $\times$ magnification using a Nikon Eclipse TS100 microscope (Nikon Instruments, Melville, NY).

MPO and Elastase

MPO activity was measured in triplicate by spectrophotometric assay in 50 mM potassium phosphate (pH 6) containing 0.17 mg/ml *o*-dianisidine and 0.0005% hydrogen peroxide using human leukocyte MPO (Sigma-Aldrich) as standard. Neutrophil elastase activity was measured by hydrolysis of N-methoxysuccinyl-Ala-Ala-Pro-Ala-*p*-nitroanilide (2 mM) (Sigma-Aldrich) in 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.05% Tween, and 4 mM DTT as change of $\text{OD}_{450\text{nm}}$ over time at 22°C with human neutrophil elastase (Elastin Products, Owensville, MO) as standard.

Statistical Analysis

For bacterial colony-forming units, individual data points are shown, and medians are indicated. All other data are presented as means $\pm$ SEM. Differences of medians (or means) among groups were analyzed by ANOVA followed by Holm-Sidak pairwise multiple comparisons. For data sets that failed the normality or equal variance tests, differences among groups were analyzed by Kruskal-Wallis ANOVA on Ranks followed by Dunn's multiple comparison. A *P* value < 0.05 was considered statistically significant. Analyses were performed in SigmaStat V3.5 (Systat Software, Inc., Port Richmond, CA).

RESULTS

Transgenic Lines

To generate mice overexpressing lung SP-D in the genetic background of the *serpinB1*^{-/-} line (129S6), we replicated a previous approach (32) by introducing a transgene-encoding rat SP-D under control of the human SP-C promoter. The resulting 18 pups were screened by PCR. Amplification from adjacent exons produced a 228-bp rat cDNA-derived band for seven mice in addition to the intron-spanning 470-bp endogenous band (Figure 1A). The intensity of the 228-bp product differed among the transgene-positive mice, suggesting a different copy number. The DNA was restricted with *NcoI*, which cuts once within the transgene, and Southern blots were probed with amplified exon 7, which is 93% identical in rat and mouse. Relative intensity of the transgene band compared with the endogenous band was used to estimate copy number. Two founders were chosen that had transgene copy

Figure 1. Generation of surfactant protein D (SP-D) transgenic mice and evaluation of SP-D expression. (A) PCR screening. Schematic showing the 228-bp band derived from rat cDNA and the endogenous 470-bp intron-spanning band. Wild-type (WT) mice (No. 496, 499, 504) produced only the larger band; transgenic mice (No. 497, 498, 505, and 506) produced both bands. The 228-bp transgenic band is more intense for No. 505 than for Nos. 497, 498, and 506, indicating higher transgene copy number. (B) Southern blot. Genomic DNA was digested with *NcoI*, and exon 7 of rat SP-D was used as probe. Tandemly integrated transgenes produced a 5.8-kb band and a unique band with size determined by location of the integration site; endogenous SP-D produces a 4.3-kb band. Copy number was estimated from the intensity ratio of the 5.8-kb band to the endogenous 4.3-kb band plus the number of integration sites. Shown from left are WT (no transgene), line SP-D^{low} founded by No. 498 with two tandem transgene copies, WT, and SP-D^{high} founded by No. 505 with nine transgene copies and three integration sites. (C, D) SP-D in bronchoalveolar lavage fluid (BALF) of WT (*serpinB1*^{+/+}, B1^{+/+}); *serpinB1*^{-/-} (B1^{-/-}), transgenic SP-D^{low} *serpinB1*^{-/-} (B1^{-/-}SPD^{low}), and transgenic SP-D^{high} *serpinB1*^{-/-} (B1^{-/-}SPD^{high}) mice examined by reducing SDS-PAGE and immunoblot. (C) Equal volumes of pooled BALF. (D) SP-D content determined by chemiluminescence of blots. BALF of individual mice was run undiluted (B1^{+/+}, B1^{-/-}) or diluted 1:3 (B1^{-/-}SPD^{low}) or 1:5 (B1^{-/-}SPD^{high}) and band intensities were quantified by densitometry. Shown are means $\pm$ SEM for four mice per genotype.

numbers of 2 and 9 (Figure 1B), and these were backcrossed with *serpinB1*^{-/-} mice. Selected pups were used to start the transgenic lines: SP-D^{low}*serpinB1*^{-/-} (*B1*^{-/-}SPD^{low}) and SP-D^{high}*serpinB1*^{-/-} (*B1*^{-/-}SPD^{high}).

Expression of SP-D in the airways and alveoli of the transgenic *serpinB1*^{-/-} mice was evaluated by immunoblotting of BALF samples stained with rabbit antibodies reactive with mouse and rat SP-D. Comparison of equal volumes showed increased content of SP-D in SP-D^{low}*serpinB1*^{-/-} mice and very high content in SP-D^{high}*serpinB1*^{-/-} mice compared with WT and *serpinB1*^{-/-} mice (Figure 1C). Quantitation by densitometry of additional blots in which BALF of transgenic mice was diluted before SDS-PAGE indicated that the SP-D content of SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} mice, respectively, are 5-fold and 20-fold that of WT mice (Figure 1D).

Structure and Function of Transgenic SP-D Protein

Several approaches were used to evaluate structure and function of the SP-D expressed in lungs of SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} mice. To examine tertiary structure, whole BALF from the different genotype mice were diluted to approximately equal SP-D concentration and subjected to nondenaturing electrophoresis and immunoblotting in parallel with standards. Recombinant rat NCRD, a trimeric fusion protein of 26-kD monomers, migrated at a position approximating its molecular weight (Figure 2A, left lane), and recombinant rat and human SP-D (lanes 2 and 8,

respectively) barely entered the 4 to 16% native gel, as anticipated for dodecamers. The major SP-D species in BALF of WT, *serpinB1*^{-/-}, SP-D^{low}*serpinB1*^{-/-}, and SP-D^{high}*serpinB1*^{-/-}, also barely entered the native gels (Figure 2A). These findings indicate that the major species of SP-D in BALF of the transgenic mice are dodecamers or larger structures. However, for SP-D^{high}*serpinB1*^{-/-} BALF, a small amount of faster migrating species suggests that SP-D had not completely formed dodecamers or that the dodecamers are less stable. To evaluate integrity of the lectin domain, BALF was supplemented with CaCl₂ and chromatographed on maltosyl-agarose. SP-D in the BALF, but not contaminating antibody-reactive proteins, bound to maltosyl-agarose and was quantitatively eluted on chelation of Ca²⁺, indicating that the lectin domain of SP-D of the transgenic mice is correctly folded (Figure 2B). Finally, we determined whether the transgenic protein in BALF supports bacterial aggregation, a property that requires correct folding and assembly. We found that SP-D from BALF of SP-D^{high}*serpinB1*^{-/-} mice aggregated *P. aeruginosa* and *E. coli* and that this aggregation was dose-dependent and inhibited by EDTA (Figures 2C and 2D).

P. aeruginosa Lung Infection of SP-D–Overexpressing *serpinB1*-Deficient Mice

Using the previously described infection model, groups of WT, *serpinB1*^{-/-}, and the two lines of SP-D–overexpressing *serpinB1*^{-/-} mice were intranasally inoculated with *P. aeruginosa* strain PAO1. After 24 hours, median lung bacterial counts of SP-D^{low}*serpinB1*^{-/-}

Figure 2. Structure–function characterization of SP-D in BALF of SP-D–overexpressing *serpinB1*^{-/-} mice. (A) Native gel electrophoresis of pooled whole BALF of the four genotype mice diluted to approximately equal SP-D concentration. The immunoblot shows recombinant rat neck plus carbohydrate recognition domain (NCRD) (200 ng); recombinant rat SP-D (200 ng); BALF of WT mice (*B1*^{+/+}); *serpinB1*^{-/-} (*B1*^{-/-}), SP-D^{low}*serpinB1*^{-/-} (*B1*^{-/-}SPD^{low}) (1:2 dilution), and SP-D^{high}*serpinB1*^{-/-} (*B1*^{-/-}SPD^{high}) (1:8 and 1:16 dilution) mice; and recombinant human SP-D (200 ng). Samples were electrophoresed on 4 to 16% nativePAGE gels, transferred, and stained with CR-2011A antiserum; marker positions are indicated. Arrows on the left indicate SP-D dodecamers (lanes 2–8) and NCRD (lane 1); the dashed lines indicate the top of the gel. (B) Maltosyl affinity chromatography. Shown is an immunoblot of (left to right) pooled SP-D^{high}*serpinB1*^{-/-} (*B1*^{-/-}SPD^{high}) BALF applied to the resin, nonadherent fraction, wash fraction, and EDTA eluate. Similar results were obtained for SP-D^{low}*serpinB1*^{-/-} BALF (not shown). (C, D) Aggregation of *Pseudomonas aeruginosa* (C) and *Escherichia coli* (D) by the indicated volumes of maltosyl-purified SP-D of SP-D^{high}*serpinB1*^{-/-} BALF. Shown are bright field images taken at 10× magnification.

and SP-D^{high}*serpinB1*^{-/-} mice were 4 logs higher than those of WT mice ($P < 0.05$) and were not different from median bacterial counts of *serpinB1*^{-/-} mice (Figure 3A). Moreover, infection had spread to the spleen in two of five SP-D^{low}*serpinB1*^{-/-}, two of four SP-D^{high}*serpinB1*^{-/-}, and two of four *serpinB1*^{-/-} mice but in none of five WT mice tested (Figure 3B). Although the differences of splenic CFU for this small subset of mice are not significant, the trend suggests that SP-D overexpression does not rescue the systemic spread of infection previously demonstrated for infected *serpinB1*^{-/-} mice (6). Production of the proinflammatory cytokine TNF- α was measured by ELISA of lung homogenates. TNF- α levels were increased for SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} mice compared with WT mice ($P < 0.05$) and were not different compared with *serpinB1*^{-/-} mice (Figure 3C). Immunoblots of proteins in lung homogenates resolved by SDS-PAGE in the presence of reducing agent revealed degradation of the 43-kD SP-D monomer in infected SP-D^{low}*serpinB1*^{-/-}, SP-D^{high}*serpinB1*^{-/-}, and *serpinB1*^{-/-} mice and generation of SP-D fragments of 35 and 17 kD (Figure 3D) and 8 kD (not shown); these fragments were absent or minimal in lung homogenates of infected WT mice (Figure 3D). Because of higher overall expression levels, the absolute amounts of SP-D fragments were higher in the SP-D-overexpressing mice compared with *serpinB1*^{-/-} mice. Residual 43-kD SP-D subunits were readily detectable in lungs of many of the SP-D^{low}*serpinB1*^{-/-} mice and in most of the SP-D^{high}*serpinB1*^{-/-} mice, indicating that proteolytic fragmentation of SP-D monomers was not complete.

Lung Injury and Inflammation of Infected SP-D-Overexpressing *serpinB1*-Deficient Mice

Further groups of mice were infected with *P. aeruginosa* so that lung injury parameters could be investigated 24 hours later. Preliminary comparison of bacterial load in BALF and post-lavage lung homogenates of a subgroup of the mice showed that 90% or more of the bacteria were harvested in BALF (means of

91–96%; four or five mice per genotype, four genotypes). In line with the findings shown in Figure 3A, CFU in BALF were several logs higher for SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} mice compared with WT mice ($P = 0.0001$) and were not different from *serpinB1*^{-/-} mice (Figure 4A). Protein and albumin content of BALF were evaluated as indicators of the integrity of the lung permeability barrier. Higher amounts of total protein and albumin were found for *serpinB1*^{-/-} mice and the two lines of SP-D overexpressing *serpinB1*^{-/-} mice than for WT mice (Figure 4B); however, after controlling for multiple comparisons, the differences were significant only for SP-D^{high}*serpinB1*^{-/-} versus WT (total protein) or for SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} versus WT (albumin). Enzyme activity was used to quantify free elastase and MPO, specific markers of neutrophils, and thus their content in cell-free BALF represents non-apoptotic neutrophil death. The activities of elastase and MPO were higher for *serpinB1*^{-/-} mice and the two lines of SP-D overexpressing *serpinB1*^{-/-} mice than for WT mice (Figure 4C); after controlling for multiple comparisons, the differences were significant only for SP-D^{high}*serpinB1*^{-/-} versus WT (elastase) or for SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} versus WT (MPO) (Figure 4C).

DISCUSSION

Transgenic SP-D-overexpressing mice were generated to determine whether increasing SP-D concentration in the lung would rescue antibacterial and antiinflammatory defense of *serpinB1* deficiency. The new mouse lines are deleted for *serpinB1* and express SP-D equivalent to 5-fold (SP-D^{low}*serpinB1*^{-/-}) or 20-fold (SP-D^{high}*serpinB1*^{-/-}) that of WT mice. The major finding of the study is that, contrary to predictions, overexpressed SP-D failed to rescue antibacterial defense of mice lacking *serpinB1*. SP-D overexpression also failed to mitigate inflammation, as judged by the 24-hour lung levels of TNF- α , to mitigate lung injury, as indicated by protein and albumin in cell-free lavage

Figure 3. *P. aeruginosa* lung infection of SP-D-overexpressing *serpinB1*^{-/-} mice. Matched groups of WT (B1^{+/+}), *serpinB1*^{-/-} (B1^{-/-}), SP-D^{low}*serpinB1*^{-/-} mice (B1^{-/-}SPD^{low}), and SP-D^{high}*serpinB1*^{-/-} (B1^{-/-}SPD^{high}) mice were intranasally inoculated with 3×10^6 colony-forming units (CFU) of strain PAO1. The mice were killed 24 hours later, and lung homogenates were prepared. (A) CFU per lung. Each symbol represents a value for an individual mouse; the horizontal lines show the medians, and the dotted line shows the limits of detection. (B) CFU per spleen determined for a (random) subset of mice. (C) TNF- α measured by ELISA. Means $\pm$ SEM are shown. (D) Immunoblots stained for SP-D of lung homogenates examined undiluted (B1^{+/+}, B1^{-/-}, and B1^{-/-}SPD^{low}) or diluted 1 to 5 (B1^{-/-}SPD^{high}). Shown are representative samples from individual mice. (A–D) Data are from two experiments. (A, C) Differences among median or mean values were analyzed by Kruskal-Wallis ANOVA on Ranks followed by Dunn's pairwise multiple comparisons.

Figure 4. Bronchoalveolar inflammation and injury of infected SP-D-overexpressing *serpinB1*^{-/-} mice. WT (*B1*^{+/+}), *serpinB1*^{-/-} (*B1*^{-/-}), and SP-D transgenic *serpinB1*^{-/-} mice (*B1*^{-/-}SPD^{low} and *B1*^{-/-}SPD^{high}) were intranasally inoculated with 10⁷ CFU *P. aeruginosa*. BALF were prepared 24 hours later. (A) CFU per ml BALF. Symbols represent values for individual mice, and horizontal lines indicate medians. (B) Protein (left) and albumin (right). (C) Free elastase (left) and MPO (right). Data are from two experiments. (B, C) Means ± SEM are shown. (A, C) Differences among median or mean values were analyzed by ANOVA followed by Holm-Sidak pairwise multiple comparisons except for albumin values, which were analyzed by Kruskal-Wallis ANOVA on Ranks followed by Dunn's pairwise multiple comparisons.

of infected lungs, or to mitigate excess neutrophil death, as indicated by the content of MPO and elastase. None of these measures of pathology was decreased for infected SP-D-overexpressing *serpinB1*^{-/-} mice compared with infected *serpinB1*^{-/-} mice. Thus, there was no detectable benefit from SP-D overexpression in *P. aeruginosa*-infected mice that lack *serpinB1*. Moreover, although

the differences were small and not statistically significant, bacterial burden, albumin, protein, MPO, and elastase values were higher in SP-D-overexpressing mice, especially SP-D^{high}*serpinB1*^{-/-} mice, compared with *serpinB1*^{-/-} mice, suggesting that overexpression of SP-D might enhance pathology in bacterial infections where neutrophil proteases are in excess.

Evaluation of SP-D in infected lungs by reducing SDS-PAGE showed primarily intact 43-kD monomers in WT mice and, by contrast, proteolysed SP-D including fragments of 35, 17, and 8 kD in SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} mice, and, as previously shown, in *serpinB1*^{-/-} mice. Similar reducing SDS-PAGE of BALF of uninfected mice revealed only intact 43-kD SP-D monomers for all four genotypes, indicating that proteolytic fragmentation is the result of infection and not a consequence of high level expression. The concentrations of SP-D in SP-D^{low}*serpinB1*^{-/-} and SP-D^{high}*serpinB1*^{-/-} mice at baseline (5- and 20-fold, respectively), relative to WT mice, should be considered approximate because quantitation relied on an antibody that may not react equally with mouse and rat SP-D. Recombinant SP-D recovered in lung lavage of the transgenic mice had the structural properties and activities established for authentic SP-D. Monomer size was normal, and dodecamers had formed as indicated by the comigration of the bulk of BALF molecules with recombinant SP-D dodecamers on native PAGE. Specific calcium ion-dependent lectin activity was demonstrated, as was calcium ion-dependent aggregation of *P. aeruginosa* and *E. coli*, indicating that the transgene product has normal secondary structure and multimerization. Adverse effects due to high expression levels are unlikely; they were not anticipated because lung morphology and levels of phospholipid and non-SP-D surfactant proteins were normal in transgenic mice that were previously generated by the same approach and that expressed even higher SP-D concentrations (20- to 50-fold increased) (32). Thus, the inability of transgenic SP-D to rescue antibacterial defense of *serpinB1*-deficient mice occurs despite high-level expression and despite normal or near-normal structural properties and despite the ability of the recombinant protein to aggregate *P. aeruginosa*.

In contrast to the outcome reported here, most previous studies of SP-D-transgenic mice reported beneficial effects. SP-D overexpression corrected lipid homeostasis abnormalities in lungs of SP-D^{-/-} mice (32). When compared with WT mice, SP-D-overexpressing mice were more resistant to bleomycin-induced mortality and morbidity (33) and were protected against hyperoxic lung injury through modulation of proinflammatory cytokines and antioxidant systems (34). Pharmacological delivery of recombinant forms of SP-D also produced favorable outcomes, decreasing airway inflammation and hypersensitivity in several models of allergen exposure (35-37; reviewed in Reference 38) and inhibiting lung inflammation when delivered together with Surfactant to ventilated premature newborn lambs during the first 5 hours of life (39).

Two prior studies evaluated SP-D transgene expression in the context of lung infection. SP-D overexpression in WT mice was not beneficial in a *Pneumocystis* model; rather, it facilitated development of infection and lung inflammation (40). These mice were immunocompromised (CD4 depleted) to produce a murine model relevant for human *Pneumocystis* infection. In a study in which comparison was made to immunosufficient WT mice, SP-D overexpression increased clearance of *E. coli* K12 measured 6 hours after inoculation (26). The disparate outcomes of this and the current infection study could be due to different mouse backgrounds or different bacteria or delivery by the intratracheal route versus intranasal or due to *serpinB1* deficiency versus sufficiency. However, the different timing of the models (6 versus 24 h) may be important. Although early times

were not evaluated in the current study, we found previously that anti-*Pseudomonas* defense properties, including colony counts and inflammatory cytokine levels, were normal in *serpinB1*^{-/-} mice at 6 hours after infection; all evidence of pathology develops in this model between 6 and 24 hours (6).

The likely explanation for the current nonbeneficial effect of SP-D overexpression is that posttranslational changes occurring in the infected lung alter the transgene protein. Posttranslational changes that interfere with SP-D structure and function have been previously characterized in association with lung inflammation. These include interference with multimerization due to S-nitrosylation of N-terminal cysteine residues by nitric oxide (41), nitration and nondisulfide cross-linking due to peroxynitrite (42), and abnormal cross-linking due to reactive species generated by the MPO-peroxide-halide system (43). These potential oxidative alterations were not investigated in the current model. The SP-D alteration that was well documented in the *P. aeruginosa*-infected SP-D transgenic *serpinB1*^{-/-} mice is proteolytic cleavage, which destroys the secondary structure required for SP-D to function in bacterial aggregation, opsonophagocytosis, and efferocytosis. In addition, neutrophils can kill bacteria extracellularly by producing neutrophil extracellular traps (NETs) (44), and it was recently shown that SP-D, which can simultaneously bind NETs and microbes, augments bacterial trapping by NETs (45). However, this protective effect of SP-D would also be destroyed by proteolysis. According to a well defined hypothesis (27), intact SP-D maintains the antiinflammatory environment of the lung by binding via the multiple C-terminal lectin "heads" exposed on assembled oligomers to SIRPα on macrophages, inducing signaling that blocks NF-κB activation. In contrast, SP-D structures or fragments with exposed N-termini ("tails") bind to macrophage calreticulin/CD91 to induce NF-κB activation and generate proinflammatory cytokines, including TNF-α. A putative contribution by this type of scenario is consistent with the trend toward increased lung inflammation and injury parameters in the infected SP-D transgenic *serpinB1*^{-/-} mice compared with *serpinB1*^{-/-} mice.

Although the exact mechanisms remain unresolved, the findings provide a cautionary note for the proposed therapeutic delivery of recombinant SP-D. Increased lung concentration of SP-D may be beneficial for preventing inflammation in some settings and supporting beneficial transition to adaptive immune responses. However, increased SP-D may lack beneficial effects and might even be counterproductive in microbial infections that recruit large numbers of neutrophils and generate free proteases that degrade SP-D. In these cases, protease inhibitors may be needed to prevent SP-D cleavage.

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