The neutrophil serine protease inhibitor *serpinb1* preserves lung defense functions in *Pseudomonas aeruginosa* infection

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Neutrophil serine proteases (NSPs; elastase, cathepsin G, and proteinase-3) directly kill invading microbes. However, excess NSPs in the lungs play a central role in the pathology of inflammatory pulmonary disease. We show that serpinb1, an efficient inhibitor of the three NSPs, preserves cell and molecular components responsible for host defense against *Pseudomonas aeruginosa*. On infection, wild-type (WT) and *serpinb1*-deficient mice mount similar early responses, including robust production of cytokines and chemokines, recruitment of neutrophils, and initial containment of bacteria. However, *serpinb1*$^{-/-}$ mice have considerably increased mortality relative to WT mice in association with late-onset failed bacterial clearance. We found that *serpinb1*-deficient neutrophils recruited to the lungs have an intrinsic defect in survival accompanied by release of neutrophil protease activity, sustained inflammatory cytokine production, and proteolysis of the collectin surfactant protein–D (SP-D). Coadministration of recombinant SERPINB1 with the *P. aeruginosa* inoculum normalized bacterial clearance in *serpinb1*$^{-/-}$ mice. Thus, regulation of pulmonary innate immunity by serpinb1 is nonredundant and is required to protect two key components, the neutrophil and SP-D, from NSP damage during the host response to infection.

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Abbreviations used: AnV, annexin V; BAL, bronchoalveolar; CG, cathepsin G; ES, embryonic stem; FSC, forward scatter; hpf, high power field; KC, keratinocyte-derived chemokine; MPO, myeloperoxidase; NE, neutrophil elastase; NSP, neutrophil serine protease; PI, propidium iodide; rrSP-D, recombinant rat SP-D; SP-D, surfactant protein–D; TSA, tryptic soy agar.

Neutrophils are the first and most abundant phagocytes mobilized to clear pathogenic bacteria during acute lung infection. Prominent among their antimicrobial weapons, neutrophils carry high concentrations of a unique set of serine proteases in their granules, including neutrophil elastase (NE), cathepsin G (CG), and proteinase-3. These neutrophil serine proteases (NSPs) are required to kill phagocytosed bacteria and fungi (1, 2). Indeed, neutrophils lacking NE fail to kill phagocytosed pathogens, and mice deficient for NE and/or CG have increased mortality after infection with pulmonary pathogens (3, 4). However, NSPs in the lung airspace can have a detrimental effect in severe inflammatory lung disease through degradation of host defense and matrix proteins (5–7). Thus, understanding of the mechanisms that regulate NSP actions during lung infections associated with neutrophilia will help identify strategies to balance host defense and prevent infection-induced tissue injury.

The online version of this article contains supplemental material.

SERPINB1, also known as monocyte NE inhibitor (8), is an ancestral serpin super-family protein and one of the most efficient inhibitors of NE, CG, and proteinase-3 (9, 10). SERPINB1 is broadly expressed and is at particularly high levels in the cytoplasm of neutrophils (11, 12). SERPINB1 has been found complexed to neutrophil proteases in lung fluids of cystic fibrosis patients and in a baboon model of bronchopulmonary dysplasia (13, 14). Although these studies suggest a role for SERPINB1 in regulating NSP activity, it is unclear whether these complexes reflect an important physiological role for SERPINB1 in the lung air space.

RESULTS

To define the physiological importance of SERPINB1 in shaping the outcome of bacterial lung infection, we generated mice deficient for *serpinb1* (*serpinb1*$^{-/-}$) by targeted mutagenesis in embryonic stem (ES) cells (Fig. 1, A–C). Crossings of heterozygous mice produced WT (+/+), heterozygous (+/−), and KO (−/−) mice for *serpinb1* at expected Mendelian ratios

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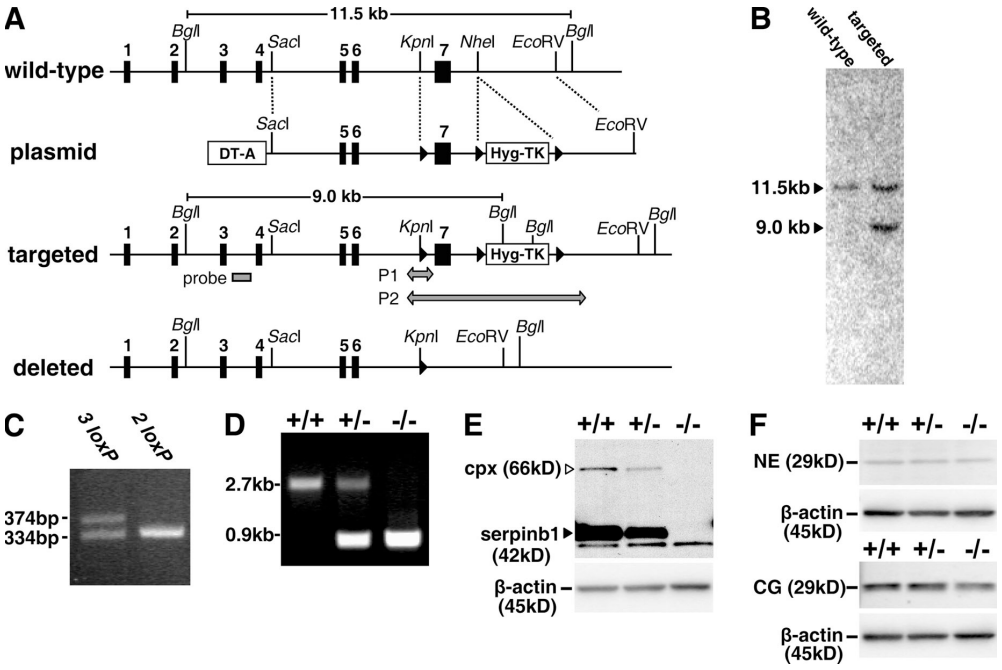

Figure 1. Generation of *serpinb1*$^{-/-}$ mice. (A) The WT *serpinb1* gene locus comprises seven exons (black rectangles). The targeting plasmid featured a negative selection cassette (DT-A) and a positive/negative selection cassette (Hyg-TK) flanked by two *loxP* sites (black triangles). The long homology arm also included a third *loxP* site in intron 6. Targeted ES clones selected for hygromycin resistance but not expressing DT-A were selected for homologous recombination by Southern blotting using an external probe in intron 3 (gray rectangle) after *Bgl* I restriction. Selected clones were further tested by PCR for the presence of the third *loxP* site in intron 6 (P1 arrow). ES clones with the deleted locus were identified by PCR (P2 arrow) after transient *Cre* expression and gancyclovir selection of targeted ES clones. (B) Southern blot of ES clones with WT or targeted loci. (C) PCR P1 was used to select homologous recombination ES clones that contained the *loxP* site in intron 6 (denoted as 3 *loxP*). (D) PCR P2 of mouse tail genomic DNA was used to genotype WT (+/+), heterozygous (+/−), and homozygous (−/−) deleted *serpinb1* mice. (E and F) Analysis of bone-marrow neutrophils. (E) Western blot analysis shows the native 42-kD serpinb1 (arrowhead) and the 66-kD serpinb1 complexed with neutrophil proteases (cpx). Protein molecular mass markers are indicated. Lower molecular mass nonspecific bands are also visible. (F) Western blot analysis show 29-kD NE (top) and CG (bottom). Blots were restained for β-actin to indicate equal protein loading.

(25% +/+, 51% +/−, and 24% −/−; $n = 225$; Fig. 1 D), indicating no embryonic lethality. Bone marrow neutrophils of *serpinb1*$^{-/-}$ mice lacked expression of the protein, whereas heterozygous *serpinb1*$^{+/-}$ mice had reduced levels compared with WT mice (Fig. 1 E). Importantly, levels of the cognate neutrophil proteases NE and CG, measured as antigenic units, were not altered by deletion of *serpinb1* (Fig. 1 F). When maintained in a specific pathogen-free environment, *serpinb1*$^{-/-}$ mice did not differ from WT littermates in growth, litter size, or life span (followed up to 12 mo), and no gross or histopathological defects were observed at necropsy in 8-wk-old mice.

6–8-wk-old animals were intranasally inoculated with the nonmucoid *Pseudomonas aeruginosa* strain PAO1. Using two infection doses (3×10^6 and 7×10^6 CFU/mouse), *serpinb1*$^{-/-}$ mice had a significantly lower survival probability and a shorter median survival time compared with WT mice (Fig. 2 A). Further groups of infected mice were used to evaluate bacterial clearance. At 6 h after infection, the bacteria were similarly restricted in mice of the two genotypes, suggesting that the *serpinb1*$^{-/-}$ mice have a normal initial response to infection. At 24 h, the median bacterial count in the lungs of *serpinb1*$^{-/-}$ mice was five logs higher than that of the WT mice ($P < 0.001$), and the infection had spread systemically in *serpinb1*$^{-/-}$ mice but not in WT mice, as shown by high median CFU counts in the spleen (Fig. 2 B). Histological examination at 24 h after infection revealed abundant neutrophil infiltration in the lungs of both WT and *serpinb1*$^{-/-}$ mice, and consistent with the bacteriological findings, numerous foci of bacterial colonies and large areas of alveolar exudates were found in *serpinb1*$^{-/-}$ mice only (Fig. 2 C). When challenged with the mucoid *P. aeruginosa* clinical strain PA M57-15 isolated from a cystic fibrosis patient, WT mice cleared >99.9% of the inoculum within 24 h, whereas serpinb1-deficient mice failed to clear the infection (Fig. 2 D). Thus, the NSP inhibitor serpinb1 is essential for maximal protection against pneumonia induced by mucoid and nonmucoid strains of *P. aeruginosa*.

To verify specificity of the gene deletion, we tested whether delivering rSERPINB1 would correct the defective phenotype. Indeed, intranasal instillation of rSERPINB1 to *serpinb1*$^{-/-}$ mice at the time of inoculation significantly improved clearance of *P. aeruginosa* PAO1 from the lungs assessed at 24 h and reduced bacteremia compared with infected

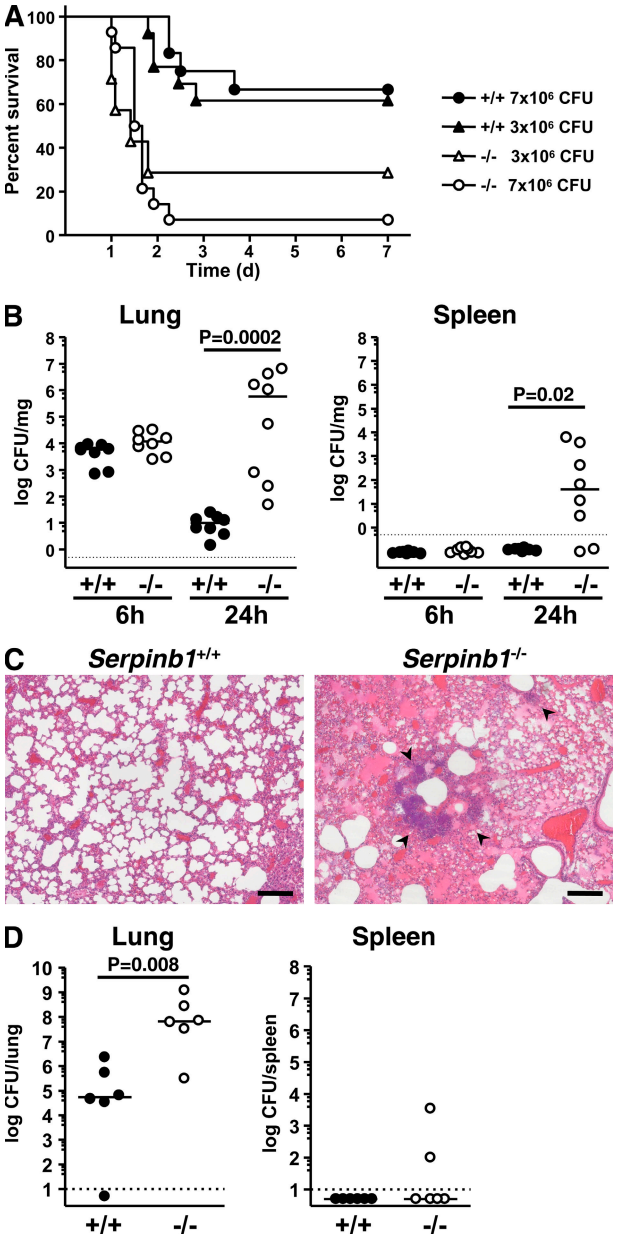

Figure 2. *Serpinb1*$^{-/-}$ mice fail to clear *P. aeruginosa* lung infection. (A) Kaplan-Meier survival curves of WT (+/+) and *serpinb1*-deficient (−/−) mice intranasally inoculated with nonmucoid *P. aeruginosa* strain PAO1. Increased mortality of *serpinb1*$^{-/-}$ mice was statistically significant (P = 0.03 at 3×10^6 CFU/mouse; P < 0.0001 at 7×10^6 CFU/mouse). (B) CFUs per milligram of lung (left) and splenic (right) tissue determined 6 and 24 h after inoculation with 3×10^6 CFU *P. aeruginosa* PAO1 in WT (+/+, filled circles) and *serpinb1*$^{-/-}$ (−/−, open circles) mice. Each symbol represents a value for an individual mouse. Differences between median values (horizontal lines) were analyzed by the Mann-Whitney U test. Data below the limit of detection (dotted line) are plotted as 0.5 CFU × dilution factor. (C) Lung sections stained with hematoxylin and eosin show bacterial colonies (arrowheads) and alveolar exudate in lungs of *serpinb1*$^{-/-}$ mice 24 h after infection with *P. aeruginosa* PAO1. Bars, 50 μm. (D) Total CFUs in the lung and spleen 24 h after inoculation with 2×10^8 CFU of the mucoid *P. aeruginosa* strain PA M57-15 in WT (+/+, filled circles) and *serpinb1*$^{-/-}$ (−/−, open circles) mice. Differences between median values (horizontal lines) were analyzed by the Mann-Whitney U test.

serpinb1$^{-/-}$ mice that received PBS instead of the recombinant protein (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20070494/DC1). We have previously demonstrated that rSERPINB1 has no effect on the growth of *P. aeruginosa* in vitro (15) and does not induce bacterial aggregation (16). Also, rSERPINB1 mixed with PAO1 had no effect on adherence of the bacteria to human bronchial epithelial and corneal epithelial cell lines (unpublished data). Therefore, the improved bacterial clearance in treated serpinb1$^{-/-}$ mice is not related to a direct antibacterial role for rSERPINB1 but rather to reducing injury induced by excess neutrophil proteases. In addition, previous in vivo studies in WT rats showed that rSERPINB1 can protect against elastase-induced lung injury (17) and accelerate bacterial clearance two- to threefold in the *Pseudomonas* agar bead model (15).

Evidence of excess NSP action was examined in the lungs of infected *serpinb1*$^{-/-}$ mice by measuring surfactant protein–D (SP-D). SP-D, a multimeric collagenous C-type lectin produced by alveolar epithelial cells, is highly relevant as a host defense molecule, because it functions as an opsonin in microbial clearance (18) and acts on alveolar macrophages to regulate pro- and antiinflammatory cytokine production (19). SP-D is also relevant as an NSP target because it is degraded in vitro by trace levels of each of the NSPs (16, 20). SP-D levels in lung homogenates of WT and *serpinb1*$^{-/-}$ mice were similar 6 h after *P. aeruginosa* infection. At 24 h, SP-D levels were reduced in the lungs of *serpinb1*$^{-/-}$ mice compared with WT mice, as indicated by immunoblots. A lower molecular mass band indicative of proteolytic degradation is also apparent (Fig. 3 A). Densitometry analysis of the 43-kD SP-D band relative to β-actin indicated that the reduction of SP-D level was statistically significant (+/+, 45 ± 6 [$n = 8$]; −/−, 10 ± 2 [$n = 8$]; $P < 0.0001$ according to the Student's t test). Furthermore, rSERPINB1 treatment of *P. aeruginosa*–infected *serpinb1*$^{-/-}$ mice partly prevented the degradation of SP-D in lung homogenates compared with nontreated mice (Fig. S1 B). As a further test of the impact of *serpinb1* deletion on NSP activity, isolated neutrophils of *serpinb1*$^{-/-}$ mice were treated with LPS and FMLP and tested for their ability to cleave recombinant rat SP-D (rrSP-D) in vitro. The extent of rrSP-D cleavage by *serpinb1*$^{-/-}$ neutrophils was fourfold greater than by WT neutrophils, as determined by densitometry. The cleavage was specific for NSPs because it was abrogated by rSERPINB1 and diisopropyl fluorophosphate (Fig. 3 B). Collectively, these findings indicate a direct role for serpinb1 in regulating NSP activity released by neutrophils and in preserving SP-D, an important-host defense molecule.

Efficient clearance of *P. aeruginosa* infection requires an early cytokine and chemokine response coordinated by both resident alveolar macrophages and lung parenchymal cells (21, 22). The IL-8 homologue keratinocyte-derived chemokine (KC) and the cytokines TNF-α, IL-1β, and G-CSF were measured in cell-free bronchoalveolar (BAL) samples. Although the tested cytokines were undetectable in sham-infected mice of both genotypes (unpublished data), comparable induction of these cytokines was observed in BAL of WT and

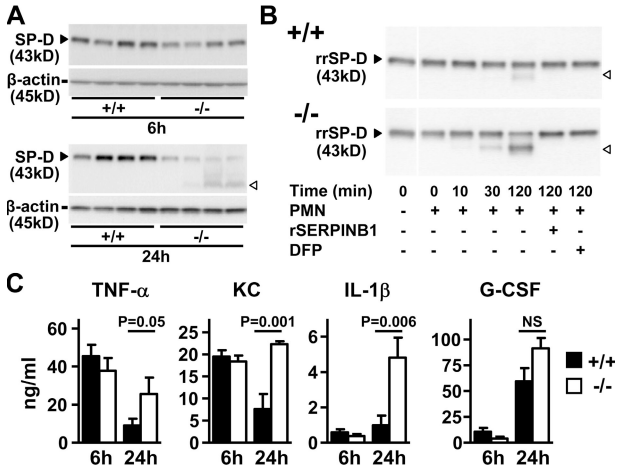

Figure 3. Proteolysis of SP-D and cytokine production in lungs of *P. aeruginosa* PAO1–infected mice. (A) Western blot analysis of SP-D (43 kD, black arrowheads) in disulfide-reduced lung homogenate samples at 6 and 24 h after infection. White arrowheads show the SP-D proteolytic cleavage product. Blots were restained for β-actin. (B) Increased in vitro proteolysis of rrSP-D by NSPs of *serpinb1*$^{-/-}$ PMNs compared with WT PMNs. (C) TNF-α, KC, IL-1β, and G-CSF were measured in BAL fluid of WT (+/+) and *serpinb1*-deficient (−/−) mice at 6 and 24 h after infection with *P. aeruginosa* (6 × 10^6 CFU/mouse; $n = 6$ per group). Data represent means ± SEM. Representative data from two or more independent experiments are shown (A–C). White lines indicate that intervening lanes have been spliced out.

serpinb1$^{-/-}$ mice at 6 h after infection, demonstrating that there is no early defect in cytokine production in *serpinb1*$^{-/-}$ mice. At 24 h, levels of TNF-α, KC, and IL-1β were sustained or increased in *serpinb1*$^{-/-}$ mice and significantly higher than cytokine levels in WT mice. G-CSF levels at 24 h were elevated to a similar extent in BAL of WT and KO mice (Fig. 3 C). However, G-CSF levels were significantly higher in the serum of serpinb1$^{-/-}$ mice (WT, 336 ± 80 ng/ml; KO, 601 ± 13 ng/ml; $n = 6$ of each genotype; $P < 0.01$). In addition, *serpinb1*$^{-/-}$ mice that were treated at the time of infection with rSERPINB1 had cytokine levels in 24-h lung homogenates that were indistinguishable from those of infected WT mice (Fig. S1 C). The increased cytokine production in the lungs of infected *serpinb1*$^{-/-}$ mice may be caused by failed bacterial clearance but also by excess NSPs, which directly induce cytokine and neutrophil chemokine production in pulmonary parenchymal cells and alveolar macrophages (23, 24).

Neutrophil recruitment to the lungs was next examined as a pivotal event of the response to *P. aeruginosa* infection (25). Lung homogenates were assayed for the neutrophil-specific enzyme myeloperoxidase (MPO) to quantify marginating, interstitial, and alveolar neutrophils. Neutrophils in BAL fluid were directly counted as a measure of neutrophil accumulation in the alveolar and airway lumen. MPO in lung homogenates was undetectable in uninfected mice and was comparably increased in mice of both genotypes at 6 h, suggesting normal early *serpinb1*$^{-/-}$ neutrophil margination and migration into the interstitium. However, by 24 h after infection, MPO levels in lung homogenates remained high in WT mice but were significantly decreased in *serpinb1*$^{-/-}$ mice (Fig. 4 A). Importantly, the content of MPO per cell was the same for isolated neutrophils of WT and *serpinb1*$^{-/-}$ mice (+/+, 369 ± 33 mU/10^6 cells; −/−, 396 ± 27 mU/10^6 cells). The numbers of neutrophils in BAL were negligible in uninfected mice and were similarly increased in WT and *serpinb1*$^{-/-}$ mice at 6 h after infection. Neutrophil counts in BAL further increased at 24 h, but the mean BAL neutrophil numbers were significantly lower in *serpinb1*$^{-/-}$ mice compared with WT mice (Fig. 4 B). The evidence from the 6-h quantitation of MPO in homogenates and neutrophils in BAL strongly suggests that neutrophil recruitment is not defective in infected *serpinb1*$^{-/-}$ mice. Moreover, the high levels of cytokines and neutrophil chemoattractant KC in *serpinb1*$^{-/-}$ mice at 24 h (Fig. 3 C) also suggest that, potentially, more neutrophils should be recruited. Therefore, to examine neutrophil recruitment in serpinb1$^{-/-}$ mice, we used a noninfectious model in which neutrophils are mobilized to migrate to the lung after intranasal delivery of *P. aeruginosa* LPS. MPO levels in lung homogenate and neutrophil numbers in BAL were not statistically different in WT and *serpinb1*$^{-/-}$ mice 24 h after LPS instillation (Fig. 4, C and D). Furthermore, the number of circulating blood neutrophils and recruited peritoneal neutrophils after injection of sterile irritants glycogen and thioglycollate did not differ in WT and *serpinb1*$^{-/-}$ mice (unpublished data). Alveolar macrophage numbers were similar in uninfected mice of both genotypes (~5 × 10^5 cells/mouse) and did not

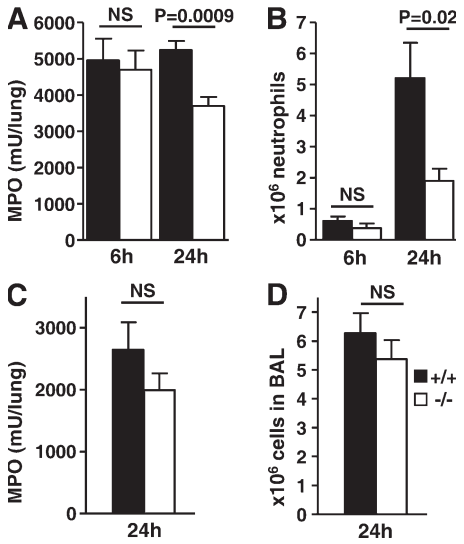

Figure 4. Neutrophil recruitment to the lungs upon challenge with *P. aeruginosa* and LPS. (A) Neutrophil (PMN) sequestration in lungs assessed by MPO activity in total-lung homogenate and (B) PMN counts in BAL were determined at 6 and 24 h after infection with *P. aeruginosa* PAO1 ($n = 6$–8). (C) PMN sequestration and (D) BAL PMN 24 h after intranasal instillation of 10 μg LPS ($n = 4$–9). Means ± SEM are shown, and data were analyzed by the unpaired *t* test.

substantially change upon infection. Collectively, these findings show that neutrophil recruitment to the lungs in response to *P. aeruginosa* infection is not defective in *serpinb1*$^{-/-}$ mice, and therefore, the recovery of lower numbers of *serpinb1*$^{-/-}$ neutrophils at 24 h after infection suggests their decreased survival.

To examine the putative increased death of *serpinb1*$^{-/-}$ neutrophils in the lungs after *P. aeruginosa* infection, lung sections were analyzed by immunohistochemistry. Caspase-3–positive leukocytes were more relevant in the alveolar space of *serpinb1*$^{-/-}$ mice compared with WT mice at 24 h after infection, suggesting increased neutrophil apoptosis (Fig. 5 A). The positive cells were counted in 50 high power fields (hpf's), and mean numbers of caspase-3–stained cells were increased in the lungs of *serpinb1*$^{-/-}$ mice (1.8 ± 0.2 cells/hpf) compared with WT mice (0.4 ± 0.1 cells/hpf; $P < 0.0001$). To characterize neutrophils in the alveoli and airways, neutrophils in BAL were identified in flow cytometry by forward scatter (FSC) and side scatter and were stained with annexin V (AnV) and propidium iodide (PI). At 24 h after infection, the proportion of late apoptotic/necrotic neutrophils (AnV$^+$PI$^+$) was increased at the expense of viable neutrophils (AnV$^-$PI$^-$) in the BAL of *serpinb1*$^{-/-}$ mice compared with WT mice (Fig. 5 B). Neutrophil fragments in BAL were also identified in flow cytometry by low FSC (FSC^{low}) within the neutrophil population defined by the neutrophil marker Gr-1. The number of neutrophil fragments (FSC^{low}, Gr-1$^+$) relative to intact neutrophils was increased two- to threefold at 24 h after infection for *serpinb1*$^{-/-}$ compared with WT mice (Fig. 5 C). Moreover, free MPO in BAL supernatants was increased in *serpinb1*$^{-/-}$ mice compared with WT mice at 24 h after infection, indicating increased PMN lysis or degranulation (Fig. 5 D).

Finally, we questioned whether the enhanced death of *serpinb1*$^{-/-}$ pulmonary neutrophils was a primary effect of gene deletion or a secondary effect caused by, for example, bacteria or components of inflammation. To address this, neutrophils were collected using the noninfectious LPS recruitment model and were cultured in vitro to allow for spontaneous cell death. After 24 h, the percentages of apoptotic and necrotic neutrophils evaluated by microscopy were increased in *serpinb1*$^{-/-}$ neutrophils compared with WT neutrophils (Fig. 6, A–C). A similar increase in apoptotic cells was observed using AnV/PI staining and measurements of hypodiploid DNA (unpublished data). Moreover, live cell numbers from *serpinb1*$^{-/-}$ mice remaining in culture after 24 h were significantly decreased compared with WT mice (Fig. 6 D). The in vitro findings indicate that enhanced death of pulmonary neutrophils of infected *serpinb1*$^{-/-}$ mice is at least in part a cell-autonomous defect likely mediated by unchecked NSP actions.

DISCUSSION

In this paper, we have demonstrated that serpinb1, an intracellular serpin family member, regulates the innate immune response and protects the host during lung bacterial infection. Serpinb1 is among the most potent inhibitors of NSPs and is carried at high levels within neutrophils. *Serpinb1*-deficient mice fail to clear *P. aeruginosa* PAO1 lung infection and succumb from systemic bacterial spreading. The defective

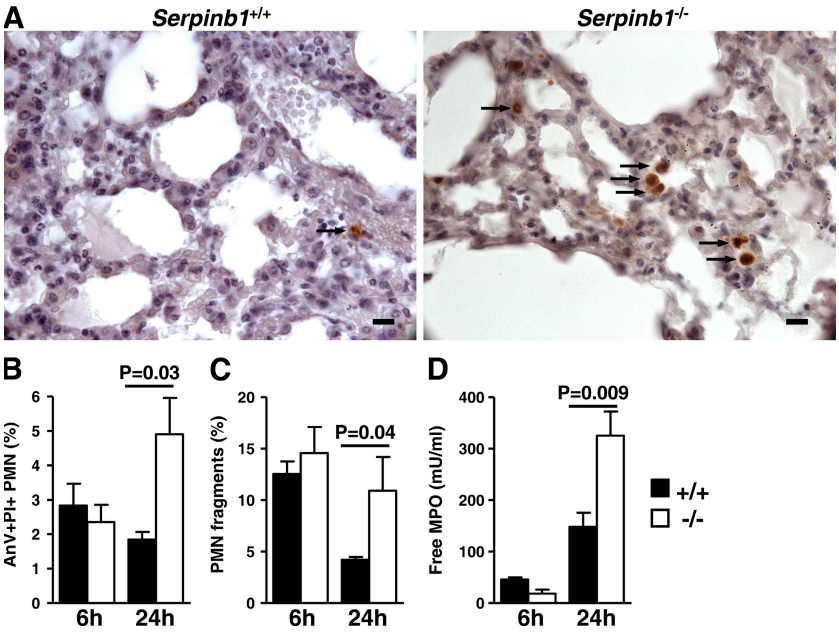

Figure 5. Increased death of recruited lung *serpinb1*$^{-/-}$ neutrophils in vivo. (A) Active caspase-3 staining of leukocytes (arrows) in alveolar space detected by immunohistochemistry at 24 h after infection with *P. aeruginosa* PAO1. Bars, 20 μm. (B) The percentage of late apoptotic neutrophils (PMNs; AnV$^+$PI$^+$) in BAL of *P. aeruginosa*–infected mice. (C) PMN fragments defined in flow cytometry by low FSC within the Gr1$^+$ population (Gr1$^+$FSC^{low}). (D) MPO in cell-free BAL supernatant. Means ± SEM are shown (n = 4–6), and the data were analyzed by the unpaired t test.

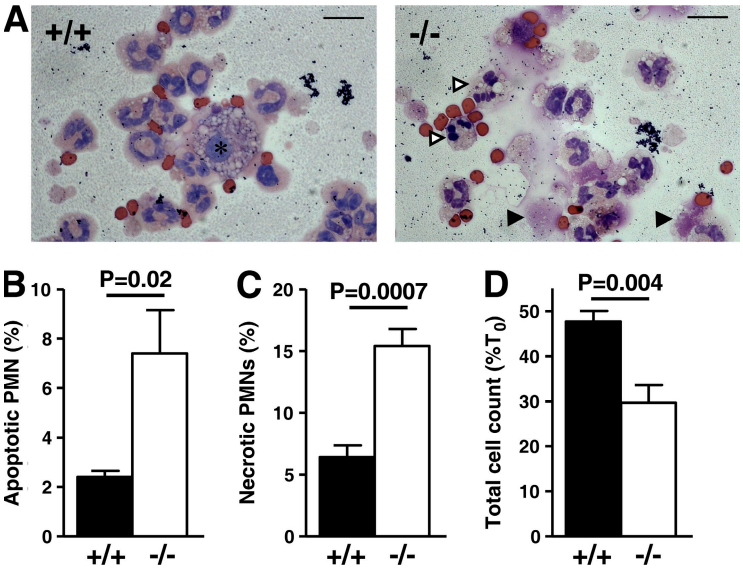

Figure 6. Increased apoptosis and necrosis of *serpinb1*$^{-/-}$ neutrophils in vitro. (A) Representative cytospins of LPS-recruited BAL cells after 24 h of in vitro culture. Macrophages are indicated by an asterisk, and apoptotic and lytic PMNs are indicated by white and black arrowheads, respectively. Bars, 10 μm. The percentage of PMNs with characteristic apoptotic (B) or lytic (C) morphologies relative to total PMNs after 24 h of in vitro culture. (D) The percentage of cells remaining after 24 h in culture. The means ± SEM of five experiments are shown, and data were analyzed by the unpaired *t* test (B–D).

immune function in serpinb1$^{-/-}$ mice stems at least in part from an increased rate of neutrophil necrosis, reducing the number of phagocytes and leading to increased NSP activity in the lungs with proteolysis of SP-D. In addition, serpinb1-deficient mice also have impaired clearance of the mucoid clinical strain PA M57-15. Interestingly, mucoid strains of *P. aeruginosa* are cleared with a very high efficiency from the lungs of WT and cystic fibrosis transmembrane conductance regulator–deficient mice (26). The phenotype of serpinb1$^{-/-}$ mice reproduces major pathologic features of human pulmonary diseases characterized by excessive inflammation, massive neutrophil recruitment to the air space, and destruction of cellular and molecular protective mechanisms. Importantly, *serpinb1* deficiency may be helpful as an alternative or additional model of the inflammatory lung pathology of cystic fibrosis.

The present study documents a key protective role for serpinb1 in regulating NSP actions in the lung. This role has previously been attributed to the NSP inhibitors α1-antitrypsin and secretory leukocyte protease inhibitor, which are found in the airway and alveolar lining fluid (27, 28). However, patients with α1-antitrypsin deficiency do not present with pulmonary infection secondary to innate immune defects despite increased NSP activity that leads to reduced lung elasticity and emphysema. Moreover, there is so far no evidence that deficiency in secretory leukocyte protease inhibitor results in failure to clear pulmonary infection. Because synthesis and storage of NSPs in granules is an event that exclusively takes place in bone marrow promyelocytes (29), the regulation of NSPs in the lung relies entirely on NSP inhibitors. Thus, the extent of the innate immune defect in *serpinb1*$^{-/-}$ mice and the normalization of bacterial clearance with topical rSERPINB1 treatment indicate that serpinb1 is required to regulate NSP activity in the airway fluids and that, during acute lung infection associated with high neutrophilic recruitment, there is insufficient compensation by other NSP inhibitors. The devastating effects of NSPs when released in the lungs by degranulating and necrotic neutrophils are well documented in human pulmonary diseases (5, 6, 30). Therefore, our findings clearly establish a physiological and nonredundant role for serpinb1 in regulating NSPs during pulmonary infection.

NSPs also cleave molecules involved in apoptotic cell clearance, including the surfactant protein SP-D and the phosphatidylserine receptor on macrophages (31, 32), thereby tipping the balance further toward a detrimental outcome. The increased numbers of leukocytes with active caspase-3 in the alveolar space of *P. aeruginosa*–infected *serpinb1*$^{-/-}$ mice suggest that the removal of apoptotic cells may be inadequate during infection. SP-D has been shown to stimulate phagocytosis of *P. aeruginosa* by alveolar macrophages in vitro (33), and SP-D–deficient mice were found to have defective early (6-h) clearance of *P. aeruginosa* from the lung (34). Although the destruction of SP-D alone may not entirely account for the defective phenotype of *serpinb1*$^{-/-}$ mice, loss of SP-D likely diminishes bacterial clearance and removal of apoptotic neutrophils.

Given that NSPs also mediate bacterial killing, why would NSP excess lead to a failed bacterial clearance? In the NE KO mice, the decreased killing activity of neutrophils is a direct consequence of the loss of the bactericidal activity of NE. The absence of an early bacterial clearance defect at 6 h after

infection in *serpinb1*$^{-/-}$ mice suggests that there is initially normal bacterial killing. The current understanding is that the compartmentalization of the NSPs is crucial to the outcome of their actions: on the one hand, NSPs are protective when killing microbes within phagosomes, and on the other hand, extracellular NSPs destroy innate immune defense molecules such as lung collectins, immunoglobulins, and complement receptors. We have shown that the regulation of NSP activity is essential and that cytoplasmic serpinb1 provides this crucial shield. Neutrophils undergoing cell death gradually transition from apoptosis, characterized by a nonpermeable plasma membrane, to necrosis and lysis, where cellular and granule contents, including NSPs, are released. The increased pace of *serpinb1*$^{-/-}$ neutrophil cell death strongly suggests that unopposed NSPs may precipitate neutrophil demise and, therefore, reduce the neutrophil numbers leading to a late-onset innate immune defect. High levels of G-CSF, a prosurvival cytokine for neutrophils, also indicate that increased cell death is likely independent or downstream of G-CSF.

In conclusion, serpinb1 deficiency unleashes unbridled proteolytic activity during inflammation and thereby disables two critical components of the host response to bacterial infection, the neutrophil and the collectin SP-D. The phenotype of the infected *serpinb1*-deficient mouse, characterized by a normal early antibacterial response that degenerates over time, highlights the delicate balance of protease–antiprotease systems that protect the host against its own defenses as well as invading microbes during infection-induced inflammation.

MATERIALS AND METHODS

Generation of *Serpinb1*-deficient mice. The targeting plasmid was designed to disrupt the *serpinb1* gene by deletion of exon 7, which encodes 35% of the protein, including the reactive center loop (11). The deletion strategy, schematized in Fig. 1 A, was based on a three *loxP* site construct and a two-step transfection and selection method (35), using a negative selection cassette (DT-A) (36) and a positive/negative selection cassette (Hyg-TK) (37), provided by J. Shen (Brigham and Women's Hospital, Boston, MA) and C. Beard (Whitehead Institute, Cambridge, MA), respectively. Mouse genomic DNA used in the targeting vector was from a P1-derived artificial chromosome (PAC) clone (A11) from a 129S6/SvEvTac (129S6) background provided by P. Bird (Monash University, Melbourne, Australia). The targeting plasmid was electroporated in 129S6/W4 ES cells (Taconic Farms). ES clones resistant for hygromycin but not expressing DT-A were selected for homologous recombination by Southern blotting using *Bgl* I restriction and an external probe in intron 3. Selected clones were further tested by PCR P1 for the presence of the *loxP* site in intron 6. ES clones with the deleted locus were generated from targeted ES clones with three *loxP* sites by transient *Cre* expression, including selection with gancyclovir and screening by PCR P2. Two independent clones with a deleted locus were injected into blastocysts, and each generated multiple germline chimeras, which were crossed to WT 129S6 to obtain two independent strains of *serpinb1*$^{-/-}$ in a pure 129S6 background. The two KO lines were phenotypically identical. All animal studies were approved by the Animal Care and Use Committee of the CBR Institute for Biomedical Research.

Inoculum preparation. *P. aeruginosa* strain PAO1, an LPS-smooth laboratory strain originally isolated from a burn wound, was provided by G. Pier (Brigham and Women's Hospital, Boston, MA). *P. aeruginosa* strain PA M57-15 is a mucoid strain isolated from a cystic fibrosis patient and was provided by A. van Heeckeren (Case Western Reserve University, Cleveland, OH). For each experiment, the bacterial inoculum was freshly prepared from a frozen glycerol stock and grown overnight on tryptic soy agar (TSA). The inoculum was washed in PBS, and the concentration was adjusted by spectrophotometry. The actual CFU in the inoculum was determined each time by plating serial dilutions on TSA. Mice were inoculated with 3–8 $\times$ 10^6 CFU/mouse for PAO1 and 2 $\times$ 10^8 CFU/mouse for PA M57-15. Purified human rSERPINB1 in PBS was prepared as previously described (38) and was premixed with the inoculum before intranasal instillation. Adherence assay of PAO1 in the presence of rSERPINB1 were performed as previously described (39).

Lung infection studies. Groups of mice were sedated with 100 mg/kg ketamine and 10 mg/kg xylazine and intranasally inoculated by applying 10 μl of inoculum onto each nare. In survival studies, mice were monitored at least every 6 h for 7 d. In other studies, mice were killed at the times indicated in the figures for tissue and body fluid harvest. The dissected spleen and right lung were homogenized, serially diluted in 1% proteose peptone, and plated in duplicate on TSA. Aliquots of lung homogenates were stored at −80°C until further analysis. Left lungs were fixed in Bouin's fixative and processed for immunohistochemistry.

LPS model. Alveolar recruitment of neutrophils was measured in response to 10 μg *P. aeruginosa* LPS (Sigma-Aldrich) intranasally instilled in sedated mice as described in the previous section.

BAL. Tracheas were canulated with an 18-gauge angiocath. Lungs were lavaged five times with 0.8 ml of cold sterile PBS. An analysis of resident and recruited cells in response to infection or LPS was performed on cells pooled from the five washes. For studies of cytokines and free MPO in BAL fluid, the first wash was collected separately and centrifuged, and the supernatant was stored at −80°C until analysis.

ELISA. Concentrations of cytokines and chemokines (TNF-α, IL-1β, KC, and G-CSF) in BAL or lung homogenate were measured in duplicate by ELISA (BioPlex; Bio-Rad Laboratories and R&D Systems).

MPO assay. MPO activity was measured in triplicate using a spectrophotometric assay in 50 mM potassium phosphate, pH 6, containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide.

Flow cytometry. Whole-blood, spleen, bone marrow, BAL, and peritoneal cells were stained with fluorochrome-labeled monoclonal antibodies and analyzed by a flow cytometer FACSCalibur (BD Biosciences). Phosphatidyl serine exposure and viability were assessed by AnV and PI staining.

Western blotting and SP-D proteolysis. Lung homogenates and BAL were resolved by SDS-PAGE under reducing conditions and immunoblotted using rabbit antiserum to NE (Calbiochem), CG (provided by T. Ley, Washington University, St. Louis, MO) (40), SERPINB1 (17), or SP-D (Chemicon). Blots were stripped and restained with rabbit anti–β-actin antibody (Cell Signaling Technology). rrSP-D dodecamers were provided by E. Crouch (Washington University, St. Louis, MO). In vitro activation of neutrophils and proteolysis of SP-D was performed as previously described (16).

Immunohistochemistry. Tissue sections of fixed and paraffin-embedded lungs were stained with a rabbit monoclonal antibody specific for active caspase-3 (clone 5A1; Cell Signaling Technology), using the manufacturer's recommendations, and detected with the ABC reagents (Vector Laboratories).

Neutrophil culture. LPS-recruited neutrophils were washed in IMDM containing 1% FCS, 2 mM L-glutamine, 20mM Hepes, and antibiotics. Neutrophils were 85–95% pure, and the remaining cells mainly consisted of alveolar macrophages and ~1% lymphocytes and eosinophils. Cells were seeded at 1–2 $\times$ 10^6 cells/ml in teflon beakers at 37°C. After 24 h, cells were counted, and cytospin preparations were stained with Wright-Giemsa.

Statistical analysis. All analyses were performed using Prism software (GraphPad). Kaplan-Meier survival curves were analyzed by the log-rank test. Differences in median bacterial CFUs per milligram of tissue were analyzed by the Mann-Whitney U test. Other data were expressed as means ± SEM and were analyzed by the unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 demonstrates the effect of delivering rSERPINB1 to *serpinb1*$^{-/-}$ mice inoculated with 8×10^6 CFU of *P. aeruginosa*. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070494/DC1.

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