Staphylococcus aureus Beta-Toxin Induces Lung Injury through Syndecan-1

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In pneumonia caused by the bacterium *Staphylococcus aureus*, the intense inflammatory response that is triggered by this infection can lead to the development of lung injury. Little is known, however, about the impact of specific virulence factors on this inflammatory disorder, which causes both significant mortality and morbidity. In this study, we examined the role of β -toxin, a neutral sphingomyelinase, in *S. aureus*-induced lung injury. Our results showed that the central features of lung injury—specifically, increased neutrophilic inflammation, vascular leakage of serum proteins into the lung tissue, and exudation of proteins into the airway—are significantly attenuated in mice infected intranasally with *S. aureus* deficient in β -toxin compared with mice infected with *S. aureus* expressing β -toxin. In addition, intranasal administration of β -toxin evoked the characteristic features of lung injury in wild-type mice whereas neutropenic mice were protected from such injury. However, mutant β -toxin mice deficient in sphingomyelinase activity failed to trigger features of lung injury. Ablation of sphingomyelinase activity also interfered with the ability of β -toxin to stimulate ectodomain shedding of syndecan-1, a major heparan sulfate proteoglycan found in epithelial cells. Moreover, syndecan-1-null mice were significantly protected from β -toxin-induced lung injury relative to wild-type mice. These data indicate that *S. aureus* β -toxin is a critical virulence factor that induces neutrophil-mediated lung injury through both its sphingomyelinase activity and syndecan-1. (*Am J Pathol* 2009, 174:509–518; DOI: 10.2353/ajpath.2009.080394)

problem in individuals with cystic fibrosis,² patients at the extremes of ages with predisposing diseases,^{3,4} and patients with immunosuppressive therapy or illness.⁵ Further, next to bacteremia, pneumonia is the second most prevalent condition during invasive methicillin-resistant *S. aureus* (MRSA) disease,⁶ and the incidence of severe pneumonia caused by MRSA strains is rising.^{6,7} A characteristic feature of *S. aureus* pneumonia is the intense host inflammatory response with a rapid and excessive recruitment of neutrophils.^{8–11} However, the underlying mechanisms of how *S. aureus* dysregulates the host inflammatory response and causes lung injury are incompletely understood.

Recent animal studies have identified Panton-Valentine leukocidin,¹² α -toxin,¹¹ and protein A⁸ as virulence factors of *S. aureus* pneumonia. Gomez et al showed that protein A promotes lung injury and inflammation through activating tumor necrosis factor receptor-1 signaling and inducing tumor necrosis factor α -like responses.⁸ Both α -toxin and Panton-Valentine leukocidin are pore-forming toxins and they can exaggerate the host inflammatory response by inducing the expression of pro-inflammatory cytokines and lysing inflammatory cells to release additional pro-inflammatory mediators,^{12–15} suggesting that these toxins possess both direct and indirect means of causing lung damage. Altogether, these observations suggest that the combined actions of several virulence factors may coordinately or synergistically enhance the characteristic neutrophil-mediated lung injury and edema seen in *S. aureus* pneumonia.

In addition to α -toxin and Panton-Valentine leukocidin, *S. aureus* expresses several other exotoxins and enterotoxins that have the potential of causing tissue injury and inflammation, such as β -toxin, γ -toxin, staphylococcal enterotoxin A, and toxic shock syndrome toxin-1. However, little is known about the physiological significance of these toxins in *S. aureus*-induced lung injury. *S. aureus* β -toxin is a Mg²⁺-dependent neutral sphingomyelinase

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Staphylococcus aureus pneumonia is a common complication among ventilated patients¹ and also a serious

that hydrolyzes membrane sphingomyelin of host cells to generate phosphorylcholine and the bioactive secondary messenger, ceramide.^{16–18} β -toxin does not lyse most types of host cells, but leaves them vulnerable to a number of other lytic agents, such as α -toxin and Panton-Valentine leukocidin. In fact, the cytotoxic effect of β -toxin is highly cell type- and species-specific, suggesting that its primary virulence activity is to modulate host processes that affect pathogenesis rather than to directly kill host cells.

Among the *S. aureus* toxins, the least is known about the functions of β -toxin *in vivo*. This is partly because relative to other virulence factors, which are expressed by the majority of clinical isolates, β -toxin was found to be expressed in 13% of septicemia and 11% of nasal carrier isolates.¹⁹ However, there is epidemiological evidence that β -toxin expression is strongly associated with recurrent *S. aureus* furunculosis and chronic osteomyelitis despite its overall low occurrence in clinical isolates.²⁰ Animal studies have also established that β -toxin is a virulence factor for *S. aureus* keratitis²¹ and mastitis.²² Further, a recent study showed that the β -toxin gene (*hlyb*) is present in the majority of MRSA strains²³ and suggested an association between *hlyb* carriage and the capacity of *S. aureus* to infect the respiratory tract. MRSA strains USA200 and USA1100, belonging to the same clonal complex (CC30), possess highly similar virulence gene repertoires. However, the majority of USA200, but not USA1100, isolates tested positive for the genes for β -toxin, toxic shock syndrome toxin-1 and staphylococcal enterotoxin A and, more importantly, 30.4% of USA200, compared with only 5.3% of USA1100, were isolated from the respiratory tract.²³ These observations suggest that β -toxin, along with toxic shock syndrome toxin-1 and staphylococcal enterotoxin A, may impart a survival advantage for USA200 in the pulmonary environment.

The present study was designed to examine the role of β -toxin in *S. aureus*-induced lung injury. Our data show that the major determinants of lung injury are significantly attenuated in mice infected intranasally with *S. aureus* deficient in β -toxin compared with those infected with *S. aureus* expressing β -toxin. Further, intranasal administration of β -toxin alone induces neutrophil influx into the lung and alveolar space, and increases vascular permeability and pulmonary edema. Our results also indicate that β -toxin achieves these pathological effects through syndecan-1, a major heparan sulfate proteoglycan of epithelial cells, because syndecan-1 null mice are significantly protected from β -toxin-induced lung injury. These data reveal a previously unknown *in vivo* function of β -toxin and establish that β -toxin is an important virulence factor in *S. aureus*-induced lung injury.

Materials and Methods

Materials

Polymyxin B-agarose, dithiothreitol, trinitrophenylaminolauroyl-sphingomyelin, red blood cell lysis buffer, and

Evans blue dye were purchased from Sigma (St. Louis, MO). Highly purified native β -toxin was obtained from Toxin Technology (Sarasota, FL). Hema-3 leukocyte staining solutions and lysozyme were from Fisher (Pittsburgh, PA) and EMD Biosciences (Gibbstown, NJ), respectively. The cationic nylon membrane, Immobilon Ny+, was from Millipore (Bedford, MA). Dulbecco's Modified Eagle's Medium, fetal calf serum, and other tissue culture supplements were purchased from Mediatech (Herndon, VA). Enhanced chemiluminescence reagents, bulk glutathione S-transferase (GST) purification module, and pGEX 4T-1 were from GE Amersham Biosciences (Piscataway, NJ). Rat anti-mouse syndecan-1 ectodomain monoclonal antibody (clone 281-2) was from BD Biosciences Pharmingen (San Jose, CA), mouse anti-human syndecan-1 monoclonal antibody (clone B-B4) was from Serotec (Raleigh, NC), horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA), and rat anti-mouse GR-1 (clone RB6-8C5) and rat anti-mouse CD3 (clone 17A2) monoclonal antibodies were purchased from Biolegend (San Diego, CA). A549 lung epithelial cells and low passage normal murine mammary gland (NMuMG) epithelial cells were from our culture collection and cultured as described previously.^{24–27} All other reagents and materials were obtained from either Fisher or VWR (Arlington Heights, IL).

S. aureus Strains

The parent *S. aureus* strain 8325-4 (*hlyb*+ *S. aureus*) and generation of the *hlyb*– *S. aureus* strain on the 8325-4 background (DU5719) have been described.^{28–30} The parent 8325-4 strain produces large amounts of exotoxin virulence factors, including α -toxin, β -toxin, γ -toxin, and δ -toxin.³¹ *S. aureus* strains were grown overnight in tryptic soy broth, then diluted in fresh tryptic soy broth and re-grown for 3 hours. The bacterial concentration was estimated by turbidity measurements, and bacteria were washed and resuspended in PBS to approximately 10^{10} cfu/ml. The exact concentration in the inoculum was determined by plating out serial dilutions onto tryptic soy broth agar and counting colonies the following day.

Expression of Recombinant β -Toxin Proteins

Genomic DNA of *S. aureus* 8325-4 was isolated as described previously³² and served as a template for PCR amplification of the region containing the sphingomyelinase domain of β -toxin (amino acid residues 44 to 331). The amplified fragment was cloned into the BamHI and XhoI sites of pGEX-4T1, a GST-tag expression vector, to generate *hlyb*/pGEX-4T1. To produce point mutant β -toxin constructs, various single nucleotide substitutions were introduced into *hlyb*/pGEX-4T1 by site-directed mutagenesis using sequential PCR, and the product was cloned into pGEX-4T1 and confirmed by DNA sequencing. The oligonucleotide primers used in this study are shown in Table 1. *E. coli* BL21(DE3) was transformed with mutant β -toxin constructs, grown to logarithmic growth phase,

Table 1. Oligonucleotide Primers Used in the Generation of Recombinant β -Toxin Proteins

		Primer sequence*
Wild-type β -toxin	Forward	5'-CGCGGATCCGCGTTGAAGTTAGTTAGTCATAA-3'
	Reverse	5'-CGAGCCTCGAGTGAGCACTATTTACTATAG-3'
Mutant β -toxin H184L	Forward	5'-TCGGTACACTTACACAATCT-3'
	Reverse	5'-AGATTGTGTAAGTGTACCGA-3'
H196L	Forward	5'-GTGCTGGACTTGATCGAAAA-3'
	Reverse	5'-TTTTCGATCAAGTCCAGCAC-3'
H255L	Forward	5'-TATGCAGGTCCTAATAGCAC-3'
	Reverse	5'-GTGCTATTAAAGACCTGCATA-3'
H278L	Forward	5'-GGTAAACCAGAACTTTTAGACT-3'
	Reverse	5'-AGTCTAAAAGTTCTGGTTTACC-3'
H323L	Forward	5'-TTTTCAGATCTTTACCCAAT-3'
	Reverse	5'-ATTGGGTAAAGATCTGAAAA-3'

*Mutated nucleotides are underlined.

and induced with 0.5 mmol/L isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4 hours. Cells were lysed with PBS containing 1 mmol/L dithiothreitol and 1 mg/ml lysozyme. Cell lysates were incubated with glutathione-Sepharose beads for 1 hour at 4°C, bound proteins were eluted with 10 mmol/L reduced glutathione, and the eluted recombinant β -toxin proteins were dialyzed extensively against PBS and absorbed to polymyxin B-agarose to remove residual LPS. The LPS level of recombinant β -toxin preparations was less than 1 endotoxin unit/mg as determined by the E-Toxate kit (Sigma).

Mice

The generation of syndecan-1 null (*Sdc1*^{-/-}) mice on the C57BL/6J background has been described.³³⁻³⁵ Wild-type C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Mice were bred and maintained in a specific pathogen-free barrier facility at the Baylor College of Medicine (Houston, TX). *Sdc1*^{-/-} mice backcrossed at least eight times onto the C57BL/6J background and wild-type C57BL/6J mice were used at 8 to 10 weeks old (both female and male). In some experiments, mice were made neutropenic by intraperitoneal injection of anti-GR1 antibody (30 μ g/mouse) or T lymphocytopenic by injection of anti-CD3 antibody (30 μ g/mouse) 24 hours before β -toxin inoculation. These treatments resulted in the reduction of circulating neutrophils from 1.2 to $1.9 \times 10^3/\mu$ l to 0.0 to $0.1 \times 10^3/\mu$ l and of circulating lymphocytes from 7.0 to $8.7 \times 10^3/\mu$ l to 1.0 to $1.3 \times 10^3/\mu$ l within 20 hours postinjection (CBC analyses). Mouse experiments were pre-approved by the local Institutional Animal Care and Use Committee and complied with federal guidelines for research with experimental animals.

Mouse Model of Lung Injury

The course of disease in the mouse model of intranasal *S. aureus* pneumonia closely parallels the clinical and histopathological features observed in human disease.¹¹ Mice were anesthetized intraperitoneally with 50 μ l of rodent III anesthesia (37.5 mg/ml ketamine, 1.9 mg/ml xylazine, and 0.37 mg/ml acepromazine) diluted into 100

μ l with saline, and infected intranasally with 10^8 cfu of *h1b*+ or *h1b*- *S. aureus* or inoculated intranasally with the indicated amount of wild-type or mutant β -toxin. At various times postinoculation, mice were processed for analyses of neutrophil infiltration, vascular leakage of serum proteins into lung tissues, and protein exudation into the airway. For collection of bronchoalveolar lavage (BAL) fluids, mice were anesthetized, intubated via the trachea, and lavaged with 2 ml (1 ml $\times$ 2) of ice-cold PBS. Total BAL cells were counted with a hemacytometer and 150 μ l of BAL was cytocentrifuged. BAL cytospin slides were stained with Hema-3 and leukocytes were differentially enumerated. For measurement of total BAL protein, cells were spun down and protein concentration in the supernatant was measured by Bradford. BAL fluids were also used to measure levels of shed syndecan-1 by dot immunoblotting as described below.

For assessment of vascular leakage of serum proteins into lung tissues, mice were injected intravenously with Evans blue dye (50 mg/kg in 100 μ l PBS) 30 to 40 minutes before processing. Mice were euthanized and lungs were perfused with PBS to remove intravascular dye, isolated, homogenized, and extracted in formamide for 24 hours at 60°C. The extract was centrifuged at $5,000 \times g$ for 30 minutes and the Evans blue dye concentration in the supernatant was quantified by measuring $A_{620 \text{ nm}}$ with a correction for heme ($A_{620} \text{ corrected} = A_{620} - (1.426 \times A_{740} + 0.03)$) and comparing with standard absorbance curves measured in parallel.

For histopathological analyses, lungs were perfused, inflated, isolated, and fixed in 4% paraformaldehyde in PBS for 48 hours. Fixed lungs were embedded in paraffin and lung sections (5 μ m) were stained with hematoxylin-eosin or immunostained with rat anti-mouse GR1 or 281-2 rat anti-mouse syndecan-1 ectodomain monoclonal antibodies to qualitatively assess the accumulation of neutrophils and cell surface expression of syndecan-1, respectively.

Syndecan-1 Shedding Assay

Syndecan-1 shedding was assessed as described previously.^{24-26,36} Briefly, confluent NMuMG epithelial cells in 96-well plates or confluent A549 epithelial cells in

24-well plates were incubated with wild-type or mutant β -toxin for 4 hours at 37°C. The conditioned medium was collected, spun down, and acidified. The acidified samples were dot blotted onto Immobilon Ny+ membranes, probed with 281-2 anti-mouse syndecan-1 or B-B4 anti-human syndecan-1 and horseradish peroxidase-conjugated secondary antibodies, and developed by enhanced chemiluminescence. Blots were scanned and quantified using NIH Image software. The concentration of syndecan-1 ectodomains in test samples was determined using purified NMuMG syndecan-1 ectodomains as standards.^{27,36,37}

Statistical Analysis

All data are expressed as mean $\pm$ SE. The statistical difference between control and experimental group was analyzed by Student's *t*-test. Comparisons between control and multiple experimental groups were analyzed by one-way analysis of variance, followed by Dunnett's test. All statistical analyses were performed using the STATVIEW 4.51 software (Abacus Concept, Berkeley, CA). *P* values <0.05 (*) and <0.01 (**) were considered significant and highly significant, respectively.

Results

β -Toxin Is Essential for *S. aureus*-Induced Lung Injury

To begin defining the physiological function of β -toxin in *S. aureus*-induced lung injury, we initially compared the inflammatory response of wild-type C57BL/6J mice infected intranasally with *hly+* or *hly-* *S. aureus*. Mice infected with *hly+* *S. aureus* showed a significant increase in the accumulation of Evans blue dye in lung tissues and total BAL protein concentration at 6 hours postinfection, whereas these parameters of vascular permeability and edema were significantly lower in mice infected with *hly-* *S. aureus* (Figure 1A and B). Intranasal infection with *hly+* *S. aureus* also led to a significant influx of neutrophils into the alveolar space as neutrophils comprised over 75% of total BAL cells (Figure 1C). Infiltrated neutrophils were identified by their typical segmented ring-shaped nuclei and pale staining cytoplasm (Figure 1D). However, substantially fewer neutrophils were found in BAL fluids of mice infected with *hly-* *S. aureus* (Figure 1, C and D). Because the total number of BAL macrophages was similar between mice infected with either *hly+* or *hly-* *S. aureus* (Figure 1D), these results indicate that β -toxin specifically augments neutrophil influx into the alveolar compartment. Consistent with the BAL data, histopathological analyses revealed evidence of markedly enhanced lung inflammation in mice infected with *hly+* *S. aureus* compared with those infected with *hly-* *S. aureus* (Figure 1E). Mice infected with *hly-* *S. aureus* showed some isolated areas of inflammation but mostly minimal, patchy lung inflammation and normal alveolar morphology (Figure 1E). Immunostaining also showed that syndecan-1 is expressed abundantly by alveolar

Figure 1. β -toxin is essential in the induction of lung injury by *S. aureus*. Wild-type C57BL/6J mice were anesthetized and inoculated intranasally with 14 μ l PBS or infected intranasally with PBS containing 1.2×10^8 cfu of *hly+* or *hly-* *S. aureus* and evaluated for determinants of lung injury at 6 hours postchallenge. **A:** Vascular leakage of serum proteins was assessed by measuring the accumulation of Evans blue dye in lung tissues as described in the Materials and Methods. Data shown are means of Evans blue dye (μ g) per lung weight (g) $\pm$ SE, *n* = 9. **B:** Total BAL protein concentration was measured by Bradford assay (mean $\pm$ SE, *n* = 8 to 9). **C:** BAL cytospin slides were stained with Hema-3 and leukocytes were counted. Data shown are percent neutrophils of total BAL cells (mean $\pm$ SE, *n* = 5). **P* < 0.05 between mice infected with *hly+* *S. aureus* and *hly-* *S. aureus*, ***P* < 0.01 between mice inoculated with PBS and infected with *hly+* *S. aureus*. **D:** Representative pictures of Hema-3-stained cytospin slides (magnification = original $\times 100$). Neutrophils are identifiable by their pale staining cytoplasm and segmented ring-shaped nuclei. **E:** Lung sections were stained with hematoxylin-eosin or immunostained with 281-2 anti-syndecan-1 monoclonal antibodies (magnification = original $\times 200$).

epithelial cells, primarily cuboidal type II cells, in mice treated with PBS, whereas syndecan-1 expression was substantially diminished in mice infected with *hly+* *S. aureus* (Figure 1E). Collectively, these data show that *S. aureus*-induced lung injury is dampened in the absence of β -toxin and that β -toxin reduces the expression of syndecan-1 on the surface of alveolar epithelial cells during *S. aureus* pneumonia.

We next examined if the administration of β -toxin alone can induce lung injury in mice. Wild-type recombinant β -toxin containing the sphingomyelinase domain was expressed as a GST fusion protein and its sphingomyelinase activity was confirmed by measuring the hydrolysis of trinitrophenylaminolauroyl-sphingomyelin (not shown). Mice were inoculated intranasally with 15 μ g recombinant β -toxin and parameters of lung injury were assessed at 7 hours postinoculation. Similar to the effects seen in mice infected with *hly+* *S. aureus*, intranasal administration of β -toxin significantly increased the accumulation of Evans blue dye in lung tissues and total BAL protein concentration (Figure 2, A and B). Further, greater than 80% of inflammatory cells that migrated into the airway in response to β -toxin challenge were neutrophils (Figure 2, C and D). Consistent with these data, there was significant accumulation of hematoxylin-positive inflammatory cells (dark blue/purple) in the lung parenchyma and alveolar compartments, and loss of alveolar architecture in the group inoculated with β -toxin relative to those treated with PBS (Figure 2E). Further, immunostaining showed that levels of cell surface syndecan-1 is markedly decreased in mice inoculated with β -toxin (Figure 2E). Altogether, these data indicate that β -toxin is a critical factor that induces lung injury and reduces cell surface expression of syndecan-1 in alveolar epithelial cells during *S. aureus* pneumonia.

β -Toxin-Induced Lung Injury Is Mediated by Neutrophils

To determine whether the increased influx of neutrophils was essential for β -toxin-induced lung injury, we assessed the response of neutropenic mice to intranasal β -toxin challenge. Mice made neutropenic by anti-GR1 immunodepletion were inoculated intranasally with β -toxin and the accumulation of Evans blue dye in lung tissues, total BAL protein, and % BAL neutrophils were measured. Lung sections were also stained with hematoxylin-eosin and immunostained with anti-GR-1 conjugated to Alexa 594 to assess neutrophilic lung inflammation. Neutropenic mice challenged with β -toxin showed a significant reduction in Evans blue dye accumulation (Figure 3A), total BAL protein (Figure 3B), % BAL neutrophils (Figure 3, C and D), and neutrophil accumulation in lung tissues (Figure 3E) compared with PBS pre-treated mice inoculated with β -toxin. However, T lymphocyte-penic mice challenged with β -toxin showed similar levels of Evans blue dye accumulation compared with PBS pre-treated mice inoculated with β -toxin (not shown). These data indicate that neutrophils are required for β -toxin to induce lung injury.

Figure 2. Intranasal administration of β -toxin causes lung injury. Wild-type mice were inoculated intranasally with PBS or 15 μ g β -toxin, and lung injury was assessed at 7 hours postinoculation. **A:** The accumulation of Evans blue dye in lung tissues is shown ($n = 4$ to 5). **B:** Total BAL protein concentration was measured by Bradford assay ($n = 8$). **C:** Leukocytes that have migrated into the alveolar space were enumerated by counting Hema-3-stained BAL cytosin slides. Data are shown as % BAL neutrophils ($n = 4$). ($*P < 0.05$; $**P < 0.01$) **D:** Representative pictures of BAL cytosin slides (magnification = original $\times 100$). **E:** Lung sections were stained with H&E or immunostained with 281-2 anti-syndecan-1 monoclonal antibodies (magnification = original $\times 200$).

β -Toxin Induces Syndecan-1 Shedding in Vivo Through its Sphingomyelinase Activity

To begin defining the underlying mechanism of how β -toxin enhances neutrophil-mediated lung injury, we examined if β -toxin functions through syndecan-1 shedding. β -toxin induces syndecan-1 shedding in cultured epithelial cells²⁷ and reduces the cell surface expression

Figure 3. Neutropenic mice are protected from β -toxin-induced lung injury. Wild-type mice were injected intraperitoneally with 30 μ g/mouse anti-GR1 antibodies or PBS 24 hours before intranasal inoculation of 15 μ g β -toxin, and lung injury was assessed at 7 hours post- β -toxin inoculation. **A:** Evans blue dye accumulation in lung tissues is shown ($n = 3$ to 5). **B:** Total BAL protein concentration was measured by Bradford assay ($n = 5$). **C:** % BAL neutrophils was enumerated by differentially counting leukocytes in Hema-3-stained BAL cytopsin slides ($n = 5$). $*P < 0.05$; $**P < 0.01$. **D:** Representative pictures of BAL cytopsin slides (magnification = original $\times 100$). **E:** Lung sections were stained with hematoxylin-eosin or immunostained with anti-GR1 antibodies directly conjugated to Alexa 594 (magnification = original $\times 200$).

of syndecan-1 in alveolar epithelial cells of mice inoculated intranasally with β -toxin (Figures 1E and 2E). Moreover, syndecan-1 shedding facilitates the migration of neutrophils into the alveolar space in a mouse model of bleomycin-induced acute lung injury.³⁸ Based on these data, we first examined if β -toxin indeed induces syndecan-1 shedding *in vivo*.

Figure 4. β -toxin induces syndecan-1 shedding *in vivo* through its sphingomyelinase activity. **A:** Wild-type mice were inoculated intranasally with PBS or β -toxin (15 μ g/mouse) and BAL was collected 7 hours later. BAL syndecan-1 ectodomain levels were determined by dot immunoblotting ($n = 7$ to 8). **B:** Confluent NMuMG epithelial cells were incubated with wild-type or mutant β -toxin (both at 30 μ g/ml) for 4 hours at 37°C, and the concentration of syndecan-1 ectodomains in the conditioned medium was quantified by dot immunoblotting ($n = 6$ to 8). **C:** Confluent A549 cells were incubated with wild-type or mutant β -toxin, and the concentration of syndecan-1 ectodomains in the conditioned medium was quantified ($n = 6$). **D:** Wild-type mice were inoculated intranasally with wild-type or mutant β -toxin (15 μ g/mouse) and BAL was collected 7 hours later. BAL syndecan-1 ectodomain levels were determined by dot immunoblotting ($n = 4$ to 7). $**P < 0.01$.

Mice were inoculated intranasally with PBS or β -toxin, and syndecan-1 shedding was assessed by measuring BAL syndecan-1 ectodomains. BAL syndecan-1 ectodomain levels were about 16-fold higher in mice challenged with β -toxin compared with mice inoculated with PBS, confirming that β -toxin induces syndecan-1 shedding *in vivo* (Figure 4A). We then examined the structural features of how β -toxin enhances syndecan-1 shedding. β -toxin is thought to induce syndecan-1 shedding through its sphingomyelinase activity²⁷ because its bioactive product, ceramide, stimulates syndecan-1 shedding.³⁹ To test this, we determined the effects of mutant β -toxin proteins deficient in its sphingomyelinase activity on syndecan-1 shedding. Based on the fact that ¹⁸⁴His and ³²³His are essential for β -toxin's sphingomyelinase activity,^{18,40} we generated mutant β -toxin constructs with a H184L or H323L substitution, and several other His $\rightarrow$ Leu point mutant constructs as controls by sequential PCR and expressed them as GST fusion proteins (Table 1). As expected, the trinitrophenylaminolauroyl-sphingomyelin hydrolysis assay confirmed that the H184L and H323L mutations ablate β -toxin's sphingomyelinase activity, whereas other His $\rightarrow$ Leu point mutations had no effect (not shown). More importantly, we found that the H184L and H323L mutations, but not other His substitutions, interfere with β -toxin's capacity to induce syndecan-1 shedding *in vitro* from the surface of cultured NMuMG (Figure 4B) and A549 epithelial cells (Figure 4C), and *in vivo* when administered intranasally into mice (Figure 4D). At the concentration tested, both wild-type and mutant β -toxin were not cytotoxic to NMuMG and

Figure 5. ¹⁸⁴His and ³²³His are critical in β -toxin-induced lung injury. Wild-type mice were administered intranasally with PBS or 15 μ g wild-type, H184L or H323L β -toxin and the extent of lung injury was assessed at 7 hours postinoculation by measuring: (A) the accumulation of Evans blue dye in lung tissues ($n = 4$ to 5) and (B) total BAL protein concentration ($n = 4$ to 7); and (C) assessing neutrophil influx into the alveolar space by Hema-3 staining of BAL cytopsin slides (magnification = original $\times 100$). * $P < 0.05$; ** $P < 0.01$.

A549 cells as determined by the MTT assay (not shown). These data establish that β -toxin induces syndecan-1 shedding through its sphingomyelinase activity.

β -Toxin Induces Lung Injury through Syndecan-1

To determine the role of syndecan-1 shedding in β -toxin-induced lung injury, we examined the capacity of the β -toxin mutant proteins deficient in syndecan-1 shedding activity to induce lung injury. Mice were inoculated intranasally with wild-type, H184L, or H323L β -toxin, and the extent of lung injury was assessed at 7 hours postinoculation. Wild-type β -toxin significantly increased the accumulation of Evans blue dye in lung tissues and total BAL protein concentration (Figure 5, A and B). However, these determinants of lung injury were significantly lower and near baseline levels in mice challenged with H184L or H323L β -toxin (Figure 5, A and B). Furthermore, BAL fluids of mice inoculated with H184L or H323L β -toxin contained substantially fewer neutrophils compared with those of mice challenged with wild-type β -toxin (Figure 5C). These data indicate that ¹⁸⁴His and ³²³His are essential for β -toxin to induce lung injury, and suggest that the capacity to enhance syndecan-1 shedding is critical.

However, because ¹⁸⁴His and ³²³His are essential for both β -toxin's sphingomyelinase and syndecan-1 shedding activities, the above studies did not clearly establish the importance of syndecan-1 shedding in β -toxin-induced lung injury. To address this issue, we examined the response of Sdc1^{-/-} mice, which are unable to shed their syndecan-1 ectodomains, to β -toxin challenge. Wild-type and Sdc1^{-/-} mice were inoculated intranasally with β -toxin, and the extent of lung injury and inflammation was assessed. Relative to wild-type mice, Sdc1^{-/-} mice showed significantly reduced accumulation of Evans blue dye in lung tissues and substantially fewer neutrophils in BAL fluids (Figure 6, A and B). Histopathological analyses of lung sections from wild-type mice inoculated with β -toxin showed increased accumulation of hematoxylin-positive inflammatory cells in the lung parenchyma compared with β -toxin-inoculated Sdc1^{-/-} mice, which showed minimal lung inflammation and normal alveolar morphology (Figure 6C). Further, anti-GR1 immunostaining showed that the majority of in-

flammatory cells in the lungs of wild-type mice challenged with β -toxin were neutrophils (Figure 6C). These data indicate that β -toxin induces lung injury through syndecan-1.

Discussion

S. aureus is a significant cause of bacterial pneumonia, but little is known about the impact of specific virulence factors on lung injury caused by the infection. Our current

Figure 6. β -toxin induces lung injury through syndecan-1. Wild-type and Sdc1^{-/-} mice were inoculated intranasally with β -toxin and lung injury was assessed at 7 hours postinoculation. **A:** Evans blue dye accumulation in lung tissues ($n = 10$ –11; * $P < 0.05$). **B:** Representative pictures of Hema-3-stained BAL cytopsin slides (magnification = original $\times 100$). **C:** H&E staining and anti-GR1 immunostaining of wild-type and Sdc1^{-/-} lung sections (magnification = original $\times 200$).

study indicates that β -toxin promotes lung injury during *S. aureus* pneumonia by enhancing the influx of neutrophils into the lung and alveolar space, vascular leakage of serum proteins into the lung parenchyma, and exudation of protein-rich edema fluid into the airway. Our studies showed that intranasal inoculation of β -toxin alone amplifies these major determinants of lung injury. However, mice made neutropenic by immunodepletion were protected, indicating that neutrophils are essential in β -toxin-induced lung injury. Furthermore, neutrophilic lung inflammation, Evans blue dye accumulation, and total BAL protein were significantly attenuated in mice infected with *hlyB*⁻ *S. aureus* relative to those infected with *hlyB*⁺ *S. aureus*, underscoring the physiological significance of β -toxin in lung injury caused during *S. aureus* pneumonia. In addition, β -toxin induced syndecan-1 shedding *in vivo* and caused substantially less lung inflammation, injury, and edema in *Sdc1*^{-/-} mice compared with wild-type mice. Altogether, these data indicate that *S. aureus* β -toxin is a critical virulence factor that induces lung injury through syndecan-1.

Precisely how β -toxin evokes lung injury through syndecan-1 remains to be determined. Our data showed that β -toxin augments syndecan-1 shedding through its sphingomyelinase activity *in vitro* and *in vivo*, and intranasal administration of mutant β -toxin lacking the capacity to shed syndecan-1 ectodomain does not cause lung injury in mice. These data suggest that β -toxin induces lung injury by enhancing syndecan-1 shedding. How β -toxin stimulates syndecan-1 shedding through its sphingomyelinase activity has yet to be clearly defined, but ceramide, the primary bioactive product of sphingomyelinase, has been shown to enhance syndecan-1 shedding in a cell-based assay through activating an endogenous shedding mechanism dependent on protein tyrosine kinase and metalloproteinase activities.³⁹ Furthermore, Goggel et al showed that platelet activating factor-induced acute lung injury is caused by ceramide generated by endogenous acid sphingomyelinase as mice deficient in acid sphingomyelinase were resistant to platelet activating factor-induced pulmonary edema, and administration of C2 ceramide mimicked the effects of platelet activating factor in inducing edema in rat lungs.⁴¹ Based on these findings, it is plausible that β -toxin induces lung injury by hydrolyzing membrane sphingomyelin of alveolar epithelial cells and generating ceramide, which in turn enhances syndecan-1 shedding in the alveoli.

Several independent lines of evidence suggest that the primary function of syndecan-1 shedding in β -toxin-induced lung injury is to facilitate neutrophil infiltration through the generation of chemotactic signals. First, β -toxin does not directly stimulate the migration of neutrophils,⁴² suggesting that β -toxin enhances neutrophil influx through modulating a host factor and its processes that are essential for neutrophil migration *in vivo*. Second, β -toxin up-regulates several pro-inflammatory cytokines and exaggerates the host inflammatory response,¹⁸ and activation of syndecan-1 shedding is an innate response to tissue injury and inflammation. Third, in a mouse model of bleomycin-induced acute lung injury, syndecan-1

shedding potentiates neutrophil influx into the alveolar space.³⁸ Here, intratracheal instillation of bleomycin induces expression of the neutrophil-chemotactic CXC chemokine KC, newly synthesized KC binds to cell surface syndecan-1 on alveolar epithelial cells, and shedding of the syndecan-1 ectodomain-KC complex by MMP-7 generates a KC gradient that guides the trans-epithelial migration of neutrophils into the alveolar compartment. Further, the majority of neutrophil-chemotactic inflammatory factors bind to heparan sulfate proteoglycans, such as syndecans,^{43,44} and heparan sulfate and heparin have been shown to potentiate the capacity of IL-8 to induce neutrophil migration.⁴⁵⁻⁴⁷ In addition, *S. aureus* expresses several virulence factors, such as lipoteichoic acid, peptidoglycan, protein A and α -toxin, that can induce the expression of KC and MIP-2 in mice, and IL-8 in humans.^{8,15,48,49} Collectively, these findings suggest a new mechanism in *S. aureus*-induced lung injury where several virulence factors up-regulate neutrophil chemotactic factors that bind to the heparan sulfate chains of cell surface syndecan-1. β -toxin induces shedding of the neutrophil chemotactic factor-syndecan-1 ectodomain complex into the alveolar compartment, generating a chemotactic signal that potentiates neutrophil migration into the alveolar space. This mechanism is also consistent with the paradigm that several virulence factors coordinate the pathogenesis of *S. aureus* diseases, and perhaps explains why such dramatic phenotypes are seen in mouse models of *S. aureus* pneumonia with single mutant strains lacking β -toxin, α -toxin, Panton-Valentine leukocidin, or protein A.

However, neutrophil influx and other determinants of lung injury were significantly, yet incompletely, attenuated in *Sdc1*^{-/-} mice challenged with β -toxin. These observations suggest that in addition to syndecan-1, β -toxin modulates other host factors and their processes that contribute to the potentiation of neutrophil infiltration. Consistent with this view, β -toxin can also stimulate the ectodomain shedding of protein receptors expressed on neutrophils, such as IL-6R, CD14 and syndecan-4.^{18,27} Syndecan-4 is an heparan sulfate proteoglycan related to syndecan-1 that is expressed by alveolar epithelial cells, albeit at a lower level than that of syndecan-1. However, syndecan-4 is thought to be up-regulated in *Sdc1*^{-/-} mice to compensate for the loss of syndecan-1, and syndecan-4 likely binds to various neutrophil-chemotactic factors through its heparan sulfate chains.^{43,50} In fact, syndecan-4 binds to antithrombin III and regulates the migration of human neutrophils induced by this serpin.⁵¹ Thus, the increased expression of syndecan-4 in compensation for the loss of syndecan-1 may have contributed to the small induction of neutrophil infiltration and pulmonary edema in β -toxin-inoculated *Sdc1*^{-/-} mice. This mechanism suggests that mice lacking both syndecan-1 and -4 will be completely protected from β -toxin-induced lung injury, but this remains to be tested.

In sum, our studies have uncovered a previously unknown *in vivo* function of β -toxin in enhancing lung injury during *S. aureus* pneumonia. Our data suggest that β -toxin exaggerates lung injury not through its cytotoxic activity, but through its capacity to enhance neutrophil

infiltration in a syndecan-1-dependent manner. Because dysregulated neutrophilic inflammation is important in the pathogenesis of many acute diseases, the pro-inflammatory mechanism mediated by sphingomyelinase, ceramide, and syndecan-1 may be critical in a broad range of acute inflammatory disorders.

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