

α -Toxin Facilitates the Generation of CXC Chemokine Gradients and Stimulates Neutrophil Homing in *Staphylococcus aureus* Pneumonia

Allison H. Bartlett,¹ Timothy J. Foster,³ Atsuko Hayashida,² and Pyong Woo Park²

¹Department of Pediatrics, Baylor College of Medicine, Houston, Texas; ²Division of Respiratory Diseases, Children's Hospital, Harvard Medical School, Boston, Massachusetts; ³Microbiology Department, Moyne Institute of Preventive Medicine, Trinity College, Dublin, Ireland

Background. *Staphylococcus aureus* α -toxin is a major virulence factor, but its mechanism of action in vivo is incompletely understood.

Methods. We examined the role of α -toxin in *S. aureus* pneumonia using the mouse model of intranasal lung infection with *S. aureus* strain 8325-4 (*hla*⁺ *S. aureus*) and an α -toxin-deficient mutant strain made on the 8325-4 background (*hla*⁻ *S. aureus*).

Results. Intranasal infection of mice with *hla*⁻ *S. aureus* resulted in substantially less lung injury and inflammation, pulmonary edema, and tissue bacterial burden than did infection with *hla*⁺ *S. aureus*. Furthermore, fewer mice infected with *hla*⁻ *S. aureus* died of the infection, compared with those infected with *hla*⁺ *S. aureus*. Levels of the CXC chemokines keratinocyte-derived chemokine and macrophage inflammatory protein-2 were significantly lower in the airways of mice infected with *hla*⁻ *S. aureus*, and this difference was the result of reduced secretion of newly synthesized chemokines into the airway. Consistent with these data, significantly fewer neutrophils were present in the airways and lungs of mice infected with *hla*⁻ *S. aureus*, compared with those infected with *hla*⁺ *S. aureus*.

Conclusions. These data suggest that α -toxin enhances virulence by facilitating the generation of CXC chemokine gradients and stimulating chemokine-induced neutrophil influx in *S. aureus* pneumonia.

Staphylococcus aureus is a major gram-positive bacterial pathogen that can cause a wide variety of infections and toxinoses, such as cellulitis, fasciitis, food poisoning, toxic shock syndrome, sepsis, endocarditis, osteomyelitis, and pneumonia [1]. Historically, *S. aureus* has been a leading cause of nosocomial infections, but it is also rapidly becoming an important community-acquired pathogen [2, 3]. Invasive *S. aureus* infections, particularly those caused by methicillin-resistant *S. aureus* (MRSA) strains, are a significant cause of mortality and morbidity [4]. Along with bacteremia, *S. aureus* pneu-

monia is one of the most prevalent invasive MRSA diseases [4]. *S. aureus* is the primary etiologic agent of ventilator-associated pneumonia (VAP) [5], and *S. aureus* VAP complicates the course of treatment in up to 47% of intubated patients [6] and can significantly increase the length and cost of hospital stays [7]. In addition, *S. aureus* pneumonia is a serious complication in individuals with cystic fibrosis [8], patients affected by immunosuppressive therapy or illness [1], and patients with other medical comorbidities, such as influenza infection [9]. One of the hallmarks of *S. aureus* pneumonia is the intense host inflammatory response characterized by a rapid and excessive recruitment of neutrophils [10–12]. In fact, accumulating evidence suggests that disease progression in bacterial pneumonia is largely mediated by the dysregulated and exaggerated host inflammatory response to infection. However, precisely how *S. aureus* dysregulates and exacerbates the neutrophil response in vivo is not understood.

Consistent with its capacity to cause a wide range of infectious diseases, *S. aureus* is armed with an impressive arsenal of virulence factors. Of these, α -toxin [13–15] and protein A [10, 13] have been established—and

Received 13 March 2008; accepted 3 June 2008; electronically published 29 September 2008.

Potential conflicts of interest: none reported.

Presented in part: Pediatric Academic Societies annual meeting, Toronto, Ontario, Canada, May 2007 (abstract 5735.1).

Financial support: GlaxoSmithKline (Pediatric Infectious Diseases Society Fellowship to A.H.B.); National Institutes of Health (grants HL69050 and HL81474 to P.W.P.).

Reprints or correspondence: Dr. Pyong Woo Park, Children's Hospital, Harvard Medical School, 320 Longwood Ave., Enders-144, Boston, MA 02115 (pyong.park@childrens.harvard.edu).

The Journal of Infectious Diseases 2008; 198:1529–35

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0022-1899/2008/19810-0015\$15.00

DOI: 10.1086/592758

Panton-Valentine leukocidin [16] has been proposed—as virulence factors in mouse models of *S. aureus* pneumonia. α -Toxin has also been shown to damage the air-blood barrier in a rat model of *S. aureus* lung infection [17]. Furthermore, challenge of isolated, perfused rabbit lungs with α -toxin alone was identified as a cause of ventilation-perfusion mismatch [18], and treatment of isolated mouse tracheas with α -toxin caused increased epithelial permeability [19]. *S. aureus* α -toxin is a major cytolytic toxin that is secreted as a soluble monomer and forms heptameric transmembrane pores in target cell membranes [20]. It has additional biological functions, such as binding to a putative glycoprotein receptor on host cells [20], activating intracellular signaling, and modulating several cellular processes [21–27]. However, exactly how these molecular and cellular functions of α -toxin contribute to *S. aureus* virulence in vivo remains to be fully elucidated.

The aim of this study was to investigate the underlying mechanism of how α -toxin contributes to lung injury and inflammation in *S. aureus* pneumonia. Our results suggest a previously unknown virulence mechanism involving α -toxin, in which the toxin facilitates the secretion of newly synthesized CXC chemokines into the airway and exaggerates neutrophil-mediated inflammatory lung injury.

METHODS

Bacteria. The *hla*⁺ *S. aureus* strain 8325-4 and the generation of *hla*[−] *S. aureus* on the 8325-4 background (DU1090) have been described elsewhere [28, 29]. Bacteria were grown overnight in tryptic soy broth (TSB) (Becton Dickinson) with aeration and agitation and then diluted in fresh TSB and regrown for 3 h. Bacteria were washed with TSB and resuspended in PBS to the desired concentration for intranasal infection.

Mouse model of intranasal lung infection. Wild-type C57BL/6J mice (Jackson Laboratory) were bred in the Baylor College of Medicine's specific pathogen-free barrier facility; all experimental procedures were approved by the local institutional animal care and use committee and complied with federal guidelines for research involving experimental animals. Mice (5–8 weeks old) were anesthetized intraperitoneally with 50 μ L of rodent III anesthetic (37.5 mg/mL ketamine, 1.9 mg/mL xylazine, and 0.37 mg/mL acepromazine) diluted to 100 μ L with saline solution. Mice were infected intranasally with varying doses of *hla*⁺ or *hla*[−] *S. aureus*, as indicated in the figure legends. The exact concentration of bacterial inoculum was determined by plating serial dilutions onto TSB agar (EMD Chemicals). The bacterial burden in the lungs and spleen was assessed at 20 h after infection. In brief, lungs and spleen were removed after mice were euthanized by anesthesia followed by cervical dislocation; tissues were weighed, strained through 7- μ m filters in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and incubated in TSB containing 0.1% Tween-20 for 30 min; and

serial dilutions of the lysate were plated onto TSB agar plates. Pulmonary edema, expressed as the lung wet-dry ratio, was determined at 10 h after infection by weighing the lung lobes immediately after isolation and again after 72 h in a 90°C convection oven.

Histopathology and immunohistochemistry. The lungs were perfused and inflated, and left lung lobes were fixed in PBS with 4% paraformaldehyde for 48 h at 4°C and embedded in paraffin. Sections of lung tissue (5 μ m) were stained with hematoxylin-eosin to determine the overall extent of lung injury and inflammation and with Gram stain to assess the bacterial burden visually. Lung tissue sections were also immunostained with rat anti-mouse Ly-6G/Ly-6C (GR-1) (Biolegend) and Alexa 594 donkey anti-rat secondary antibodies (Invitrogen Molecular Probes) to assess neutrophil accumulation.

Bronchoalveolar lavage (BAL) fluid collection and analyses. BAL fluid was collected after infection at the times indicated in the figure legends by intubating the trachea and performing lavage with 2 mL of ice-cold sterile PBS (1 mL $\times$ 2). Red blood cells were lysed with red blood cell-lysing buffer (Sigma-Aldrich), and total cells were counted using a hemacytometer. To differentially enumerate BAL fluid leukocytes, 150 μ L of BAL fluid was cytocentrifuged and stained with Hema-3 (Fisher Diagnostics), and cells were counted. BAL fluid concentrations of tumor necrosis factor (TNF)- α , interferon (IFN)- γ , keratinocyte-derived chemokine (KC), and macrophage inflammatory protein (MIP)-2 were measured by ELISA in accordance with the manufacturer's instructions (R&D systems).

Reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA (200 ng) was isolated from lungs at 0, 3, or 8 h after infection with the RNeasy Midi Kit (Qiagen), reverse transcribed into cDNA, and amplified using the SuperScript 1-step RT-PCR kit (Invitrogen). The primers used were 5'-TTCTCTGTGCAGCGCTGCTG-3' (sense) and 5'-GGAGCTTCAGGGTCAAGGCAA-3' (antisense) for KC, 5'-TGCCGGCTCCTCAGTGC-3' (sense) and 5'-TTAGCCTTGCCTTTGTTCAGTATC-3' (antisense) for MIP-2, and 5'-GTGGGCCGCTCTAGGCACCAA-3' (sense) and 5'-CTCTTTGATGCTACGCACGATTTC-3' (antisense) for β -actin. The predicted sizes of the resulting bands are 222, 200, and 540 bp for KC, MIP-2, and β -actin, respectively.

Statistical analysis. Data, expressed as mean $\pm$ SE or SD, were analyzed by Student's *t* test to determine the significance of differences in bacterial burden, lung wet-dry ratio, neutrophil percentage, and cytokine concentrations. Differences were considered statistically significant at *P* < .05.

RESULTS

α -Toxin in the recruitment of neutrophils, lung injury and inflammation, and pulmonary edema during *S. aureus* pneumonia. To determine the physiological significance of α -toxin in *S. aureus* pneumonia, we initially compared the in-

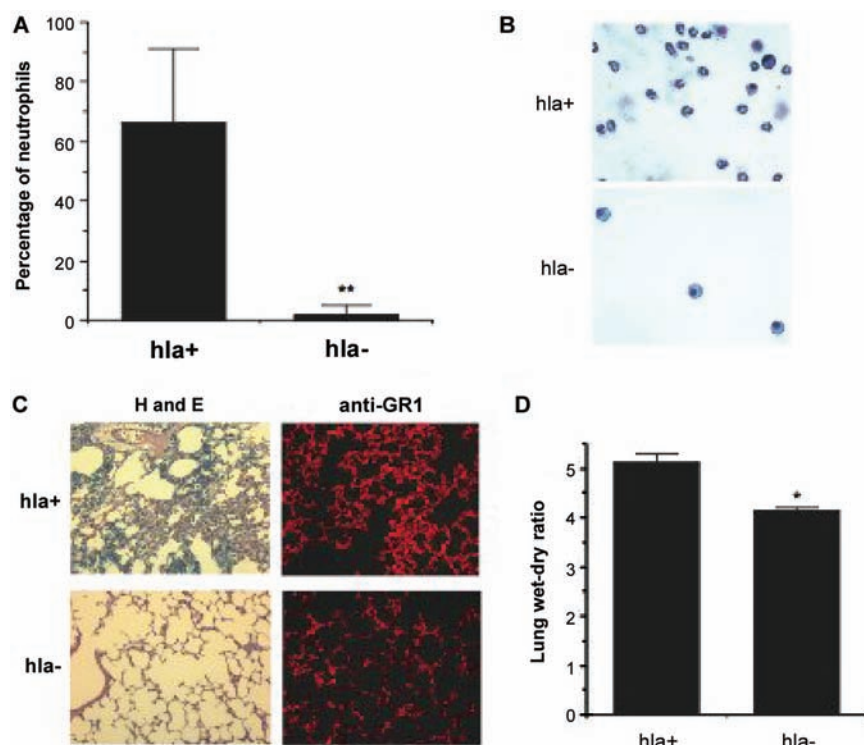


Figure 1. Influence of α -toxin on *Staphylococcus aureus* pneumonia. *A*, Mice were infected intranasally with 2×10^7 cfu of *hla*⁺ or *hla*⁻ *S. aureus* (4 mice with *hla*⁺ bacteria and 7 with *hla*⁻ bacteria) and bronchoalveolar lavage (BAL) fluid was collected 6 h after infection. Slides of cytocentrifuged BAL fluid were stained with Hema-3, and leukocytes were counted. Data are shown as the percentage of neutrophils among total BAL cells (data are mean $\pm$ SD; ** $P < .005$). No neutrophils were detected in BAL fluid at $t = 0$, and the actual number of neutrophils that infiltrated into the airways of mice infected with *hla*⁺ *S. aureus* at 6 h after infection was ~ 7500 (not shown). *B*, Representative photomicrographs of stained, cytocentrifuged slides (original magnification, $\times 100$). *C*, Mice were infected with 2×10^8 cfu of *hla*⁺ or *hla*⁻ *S. aureus*, and lungs were harvested at 10 h after infection, embedded in paraffin, and sectioned. Sections of lung tissue (5 μ m) were stained with hematoxylin-eosin (H and E) or anti-GR1 antibodies and Alexa 594 secondary antibodies (original magnification, $\times 100$). *D*, Mice ($n = 8$) were infected with 1×10^8 cfu of bacteria, and pulmonary edema was assessed by measuring the wet and dry weights of lungs harvested at 10 h after infection (data are mean $\pm$ SE; * $P < .05$).

flammatory responses in wild-type C57BL/6J mice infected intranasally with *hla*⁺ or *hla*⁻ *S. aureus*. Intranasal infection of mice with *hla*⁺ *S. aureus* led to significant neutrophil influx into the airway within 6 h after infection, with neutrophils comprising $>60\%$ of BAL leukocytes (figure 1*A* and 1*B*). In contrast, BAL fluid from mice infected with *hla*⁻ *S. aureus* contained essentially no neutrophils (figure 1*A* and 1*B*). The total numbers of macrophages in BAL fluid were similar in mice infected with *hla*⁺ and those infected with *hla*⁻ *S. aureus* (figure 1*B*), suggesting that α -toxin specifically induces the influx of neutrophils into the airway in *S. aureus* pneumonia.

We next assessed the extent of pulmonary injury and edema. Consistent with the BAL data, histopathological analyses revealed evidence of severe pneumonia in mice infected intranasally with *hla*⁺ *S. aureus*. There were significant accumulations of hematoxylin-positive inflammatory cells (dark blue or purple) in lung parenchyma and alveolar compartments, some hemorrhage, and loss of alveolar architecture in the group infected with *hla*⁺ *S. aureus* (figure 1*C*). Mice infected with *hla*⁻ *S. aureus* showed some isolated areas of intense inflammation and alveolar wall thickening (not shown) but mostly minimal, patchy lung

inflammation and normal alveolar morphology (figure 1*C*). The majority of inflammatory cells in *hla*⁺ *S. aureus*-infected lungs were determined to be neutrophils by anti-GR1 immunostaining of lung tissue sections (figure 1*C*). Furthermore, the lung wet-dry ratio was significantly increased in mice infected with *hla*⁺ *S. aureus*, compared with those infected with *hla*⁻ *S. aureus* (figure 1*D*). Overall, these data indicate that the rate of neutrophil recruitment to the lung, lung inflammation and injury, and pulmonary edema were significantly dampened when mice are infected with *S. aureus* deficient in α -toxin.

α -Toxin in the generation of CXC chemokine gradients.

The directional migration of neutrophils to sites of infection or injury is guided by chemotactic signals. The primary neutrophil chemoattractants in mice are KC and MIP-2, which are CXC chemokines with a Glu-Leu-Arg (ELR) motif near the N-terminus. Mice do not express interleukin (IL)-8, and KC and MIP-2 serve as functional homologues of human IL-8 in inducing neutrophil migration in mice. Both KC and MIP-2, and their receptor CXCR2, have been shown to be critical in the recruitment of neutrophils to the lung in several models of both infectious and noninfectious lung inflammation [30–35]. Fur-

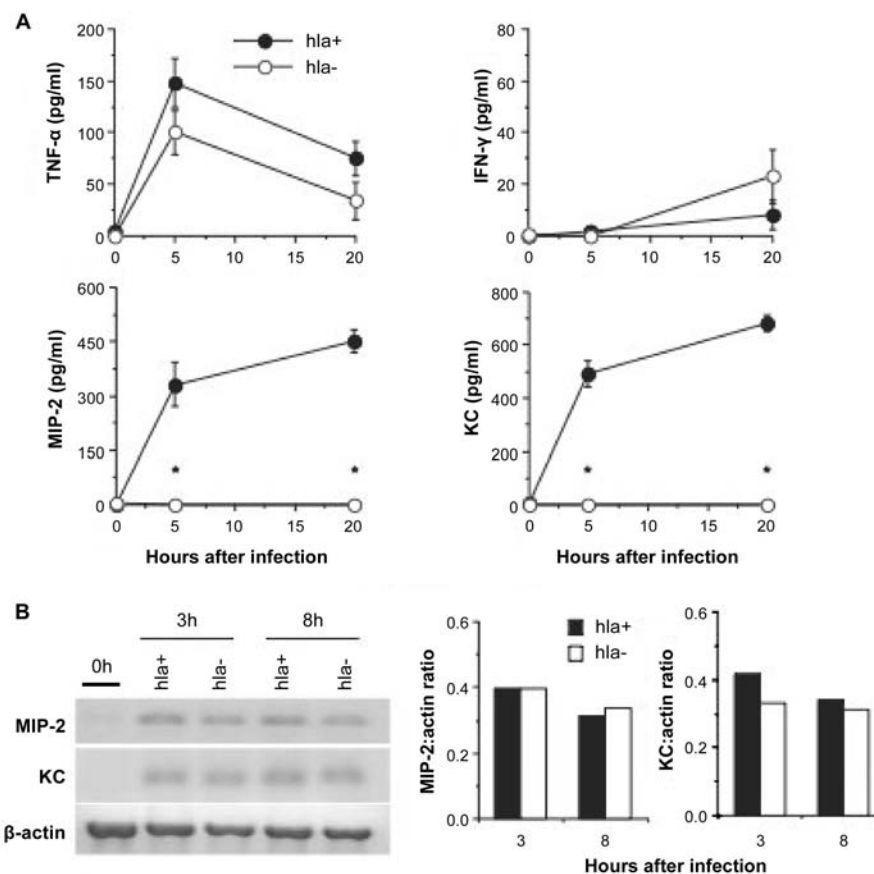


Figure 2. Concentrations of keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 in the airways of mice infected with *hla*⁻ *Staphylococcus aureus*. *A*, Mice ($n = 5$) were infected intranasally with 2×10^8 cfu of *hla*⁺ or *hla*⁻ *S. aureus*, and bronchoalveolar lavage (BAL) fluid was collected at the indicated times after infection. The BAL fluid concentrations of tumor necrosis factor (TNF)- α , interferon (IFN)- γ , KC, and MIP-2 were determined by ELISA (data are mean $\pm$ SE; * $P < .05$). *B*, MIP-2, KC, and β -actin mRNA in the lung were measured by reverse-transcriptase polymerase chain reaction (RT-PCR) before infection and at 3 and 8 h after infection with either *hla*⁺ or *hla*⁻ *S. aureus*. The panel on the left shows a representative RT-PCR gel, and the graph on the right shows the data as chemokine-actin ratios from this particular experiment, which was repeated once with similar results (not shown).

thermore, in addition to IL-8, *S. aureus* infection induces the expression of KC and MIP-2 [10, 27, 36]. Thus, to investigate the molecular basis of the observed difference in neutrophil influx induced by *hla*⁺ and *hla*⁻ *S. aureus*, we measured levels of KC, MIP-2, TNF- α , and IFN- γ in BAL fluid from infected mice.

Mice infected with either *hla*⁺ or *hla*⁻ *S. aureus* showed a significant increase in BAL fluid TNF- α concentrations at 5 and 20 h after infection (figure 2A). The IFN- γ concentration increased slightly at 20 h after infection in mice infected with either *hla*⁺ or *hla*⁻ *S. aureus* but was, for the most part, near baseline at the dose tested (figure 2A). In contrast, BAL fluid KC and MIP-2 levels were significantly higher in mice infected with *hla*⁺ *S. aureus* than in those infected with *hla*⁻ *S. aureus*. BAL fluid from mice infected with *hla*⁺ *S. aureus* had KC concentrations of approximately 500 and 700 pg/mL at 5 and 20 h after infection, whereas BAL fluid from mice infected with *hla*⁻ *S. aureus* contained essentially no KC at both time points (figure 2A). Similar results were observed for MIP-2; BAL fluid MIP-2 levels in *hla*⁺ *S. aureus*-infected mice increased >300 pg/mL by 5 h after in-

fection, and high levels were sustained at 20 h after infection, but BAL fluid from mice infected with *hla*⁻ bacteria contained little or no MIP-2 (figure 2A).

To investigate the specific difference in airway CXC chemokine concentrations, we used RT-PCR to determine the mRNA levels of KC and MIP-2 during the course of intranasal *S. aureus* pneumonia. At both 3 and 8 h after infection, lung samples from mice infected with either *hla*⁺ or *hla*⁻ *S. aureus* showed similar significant spikes in KC and MIP-2 mRNA (figure 2B). KC and MIP-2 mRNA levels were also similar in total BAL cells obtained from mice infected with either *hla*⁺ or *hla*⁻ *S. aureus* (not shown). Along with the similar induction of TNF- α by *hla*⁺ and *hla*⁻ *S. aureus* (figure 2A), these results indicate that the host inflammatory response was similarly mounted in response to *S. aureus* infection, regardless of the presence or absence of α -toxin. More importantly, these data indicate that the reduced BAL fluid levels of KC and MIP-2 proteins in mice infected with *hla*⁻ *S. aureus* is not the result of decreased gene expression. Instead, these results suggest that α -toxin is necessary for the

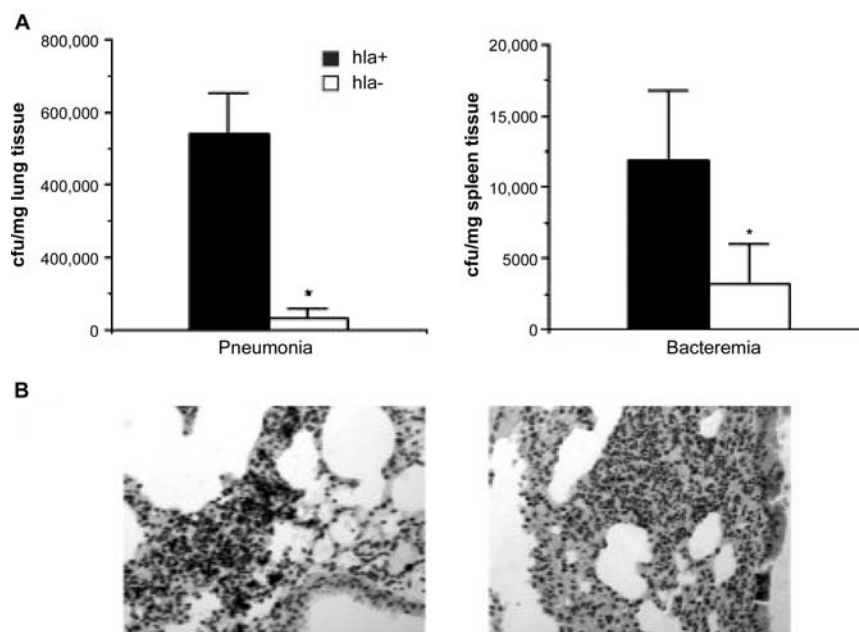


Figure 3. α -Toxin as a virulence factor in intranasal *Staphylococcus aureus* lung infection. Mice ($n = 6$) were infected with 2×10^8 cfu of either *hla*⁺ or *hla*⁻ *S. aureus*, and the extent of infection was assessed at 20 h after infection by quantifying the bacterial burdens in lung and spleen tissue (data are mean $\pm$ SE; * $P < .05$) (A) and gram-stained, paraffin-embedded sections of lung tissue (original magnification, $\times 200$) (B, left panel, *hla*⁺ *S. aureus*-infected lung; right panel, *hla*⁻ *S. aureus*-infected lung).

secretion of newly synthesized KC and MIP-2 proteins into the airway to generate CXC chemokine gradients that induce neutrophil migration.

α -Toxin as major virulence factor in the mouse model of intranasal *S. aureus* pneumonia. We next determined whether α -toxin is a virulence factor in intranasal *S. aureus* pneumonia. While we were performing the current studies, Wardenburg et al. reported that (1) *S. aureus* mutant strains deficient in *hla* are defective in their ability to cause pneumonia-related mortality, (2) the level of α -toxin expression by independent *S. aureus* strains directly correlates with their capacity to kill mice, (3) active immunization with mutant α -toxin deficient in its capacity to form pores protects mice from *S. aureus* pneumonia, and (4) passive transfer of rabbit anti- α -toxin antibodies protects naive mice from *S. aureus* challenge [13–15], but the effects of deleting *hla* on bacterial colonization and invasion were not directly examined. Thus, we measured the primary and secondary tissue bacterial burden in mice infected with *hla*⁺ or *hla*⁻ *S. aureus*.

Mice were infected intranasally with *hla*⁺ or *hla*⁻ *S. aureus*, and the extent of infection was assessed by quantifying the bacterial burden in the lungs and spleen. The bacterial load in the spleen was used as a surrogate marker of systemic invasion and bacteremia [37]. Mice infected with 2×10^8 cfu of *hla*⁺ *S. aureus* showed markedly increased numbers of bacteria in their lungs and spleens at 20 h after infection, consistent with the clinical observation that $\sim 40\%$ of *S. aureus* pneumonia cases are associated with bacteremia [38]. However, the bacterial burden in

the lungs and spleen was significantly lower in mice infected with the same dose of *hla*⁻ *S. aureus* (figure 3A). Furthermore, Gram staining showed very few bacteria in lungs of mice infected with *hla*⁻ *S. aureus*, even in localized areas of significant inflammation, whereas gram-stained lung sections of mice infected with *hla*⁺ *S. aureus* showed numerous intense foci of gram-positive bacteria (figure 3B). The cumulative mortality rate in mice infected with 2×10^8 – 8×10^8 cfu of *hla*⁺ or *hla*⁻ *S. aureus* in 3 separate experiments was 17 of 21 (81%) and 1 of 21 (5%), respectively. Altogether, these data indicate that α -toxin promotes *S. aureus* colonization in the lung and invasion into the bloodstream to cause bacteremia and infection of secondary organs.

DISCUSSION

S. aureus α -toxin is an important virulence factor in a wide variety of staphylococcal diseases, as evidenced by its essential role in causing fulminant infection in animal models of pneumonia, meningitis, septic arthritis, mastitis, and many other diseases [14, 20, 39–42]. The fact that most clinical isolates of *S. aureus* express α -toxin further underscores the importance of this toxin in pathogenesis. The pore-forming cytotoxic activity of α -toxin has been established using cell-based systems [20]. However, α -toxin has other biological effects on host cells in vitro, such as activation of intracellular signaling pathways, induction of cytokines and chemokines, activation of apoptosis, and stimulation of cell proliferation in some cell types [21–27], and there is a gap in our understanding of how α -toxin actually enhances viru-

lence in vivo. Our current study indicates that in *S. aureus* pneumonia, α -toxin facilitates the generation of CXC chemokine gradients in a posttranscriptional manner and potentiates CXC chemokine-induced homing of neutrophils into the lung and airway. Subsequently, neutrophil-mediated inflammatory lung injury, a requisite for bacterial attachment and invasion, is augmented, and bacterial burden in the lungs, bloodstream, and secondary organs is increased. Consistent with this pathogenic mechanism, mice infected with *hla*⁻ *S. aureus* show significantly reduced CXC chemokines in the airway, fewer neutrophils in the lung and airway, attenuated pulmonary injury and edema, and lower bacterial burden in the lungs and spleen. Based on these data, we propose that one of the primary mechanisms through which α -toxin enhances *S. aureus* virulence in the lung is by dysregulating and exaggerating CXC chemokine-induced neutrophil recruitment and subsequent inflammatory lung injury.

Precisely how α -toxin facilitates the formation of CXC chemokine gradients is not fully understood. Rose et al. have shown that α -toxin up-regulates IL-8 in cultured human airway epithelial cells [27]. However, our studies indicate that the presence or absence of α -toxin does not affect the expression of mouse functional homologues of human IL-8, KC and MIP-2, in the mouse model of intranasal *S. aureus* pneumonia. Furthermore, several other *S. aureus* virulence factors, such as lipoteichoic acid, peptidoglycan, and protein A, have been shown to induce the expression of IL-8, both in vitro and in vivo [10, 43–45]. Thus, it is likely that the combined actions of several virulence factors up-regulate KC and MIP-2 during *S. aureus* pneumonia in mice but that α -toxin is specifically required for the secretion of newly synthesized KC and MIP-2 proteins into the airway to generate CXC chemokine gradients that guide the directional migration of neutrophils.

How α -toxin contributes to the secretion of KC and MIP-2 proteins into the airway remains to be determined. To our knowledge, α -toxin does not interact directly with these CXC chemokines, suggesting that α -toxin affects a critical host factor and its processes to accomplish this task. Based on several criteria, we postulate that the host factor is syndecan-1, a major cell surface heparan sulfate proteoglycan, and the process affected is syndecan-1 ectodomain shedding. First, α -toxin specifically induces the shedding of syndecan-1 ectodomains from the cell surface of cultured epithelial cells [25]. α -Toxin does not directly shed syndecan-1 ectodomains but, rather, stimulates an endogenous mechanism in which α -toxin activates a protein tyrosine kinase-dependent signaling pathway that potentiates cleavage of syndecan-1 ectodomains at the cell surface by host cell metalloproteinases. Second, all chemokines, including KC and MIP-2, bind to heparan sulfate proteoglycans, such as syndecan-1, and this interaction is considered necessary for chemokine activity under physiological conditions [46]. Moreover, in a mouse model of bleomycin-induced acute lung injury, newly synthesized KC binds to cell surface syndecan-1, and

shedding of the syndecan-1 ectodomain–KC complex by matrix metalloproteinase-7 translocates KC into the alveolar compartment, generating a KC gradient that directs the transepithelial migration of neutrophils into the airway [33]. Thus, it is plausible that α -toxin facilitates the secretion of KC and MIP-2 into the airway by inducing the shedding of syndecan-1 ectodomains bound to these CXC chemokines in *S. aureus* pneumonia. Future studies directed at determining the role of syndecan-1 in α -toxin-induced neutrophil recruitment in vivo should shed additional mechanistic insights into how α -toxin potentiates neutrophil homing and neutrophil-mediated lung injury during *S. aureus* pneumonia.

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