Efficacy of Active Immunization With Attenuated α -Hemolysin and Panton-Valentine Leukocidin in a Rabbit Model of *Staphylococcus aureus* Necrotizing Pneumonia

Vuvi G. Tran,^{1,a} Arundhati Venkatasubramaniam,^{2,a} Rajan P. Adhikari,² Subramaniam Krishnan,² Xing Wang,¹ Vien T. M. Le,¹ Hoan N. Le,¹ Trang T. T. Vu,¹ Erika Schneider-Smith,¹ M. Javad Aman,² and Binh An Diep¹

¹Division of HIV, Infectious Diseases and Global Medicine, Department of Medicine, University of California, San Francisco; and ²Integrated Biotherapeutics, Inc, Rockville, Maryland

Staphylococcus aureus is a common pathogen causing infections in humans with various degrees of severity, with pneumonia being one of the most severe infections. In as much as staphylococcal pneumonia is a disease driven in large part by α -hemolysin (Hla) and Panton-Valentine leukocidin (PVL), we evaluated whether active immunization with attenuated forms of Hla (Hla_{H35L/H48L}) alone, PVL components (LukS-PV_{T28F/K97A/S209A} and LukF-PV_{K102A}) alone, or combination of all 3 toxoids could prevent lethal challenge in a rabbit model of necrotizing pneumonia caused by the USA300 community-associated methicillin-resistant *S. aureus* (MRSA). Rabbits vaccinated with Hla toxoid alone or PVL components alone were only partially protected against lethal pneumonia, whereas those vaccinated with all 3 toxoids had 100% protection against lethality. Vaccine-mediated protection correlated with induction of polyclonal antibody response that neutralized not only α -hemolysin and PVL, but also other related toxins, produced by USA300 and other epidemic MRSA clones.

Keywords. MRSA; pneumonia; α -toxin; PVL; antibodies; vaccine.

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes disease epidemics within communities and hospitals across the United States [1]. Development of an effective vaccine for staphylococcal infection is urgently needed to stem the emergence of antimicrobial resistance. Previous efforts toward vaccine development have focused on surface antigens including the capsular polysaccharides 5 and 8 (CP5/CP8) [2], the iron-regulated surface determinant B (IsdB) [3], or a combination of CP5/CP8 with clumping factor A (ClfA) and manganese transport protein C (MntC) [4]. However, all of these vaccines have failed to meet their clinical endpoints. Immunization with IsdB (Merck V710) in patients undergoing elective cardiothoracic surgery even led to an exacerbated disease after *S. aureus* infection, with 5-fold higher mortality compared to placebo [3].

The pathogenicity of *S. aureus* is dependent on numerous virulence factors, including cell surface proteins, polysaccharides, and pore-forming toxins (PFTs) [5, 6]. The latter causes tissue damage, promotes bacterial dissemination and metastatic

growth in distant organs, and allows the pathogen to thwart the host immune response [7]. Over the past decade, numerous studies have demonstrated the significance of PFTs in the pathogenesis of community-acquired MRSA and as vaccine and therapeutic targets. The β -barrel PFT α -hemolysin (Hla), also known as α -toxin, is produced by nearly all virulent strains and has been implicated in skin and soft tissue infections (SSTIs) as well as pneumonia [8, 9]. Hla intoxicates a variety of cell types including epithelial, endothelial [10, 11], keratinocytes [12], and various hematopoietic cells [13, 14]. Hla plays a key role in tissue injury and barrier disruption [15–18], platelet aggregation [18], and activation of inflammatory cellular responses [9, 10, 19]. The bicomponent PFTs are another major group of toxins produced by *S. aureus*, in which the S subunit (eg, LukS-PV, LukA, HlgA, HlgC, LukE) binds to specific host cell receptors and the F subunit (eg, LukF-PV, LukB, HlgB, LukD) causes oligomerization, resulting in ring-shaped pores on membranes of phagocytic cells [20]. Some of the bicomponent PFTs, Panton-Valentine leukocidin (PVL) (LukS-PV and LukF-PV) and Luke/LukD, are produced by some of the major MRSA clones, including USA300 epidemic clone. PVL and other bicomponent leukotoxins kill a wide range of immune cells, including neutrophils, macrophages, and lymphocytes, and trigger inflammatory responses [20–22]. PVL is epidemiologically [21, 23] and experimentally [24–27] implicated in the pathogenesis of community-associated (CA)–MRSA necrotizing pneumonia. Interspecies variation in the cellular receptors targeted by the S [21, 28] and F [29] subunits have been shown to determine

Received 19 May 2019; editorial decision 19 August 2019; accepted 21 August 2019; published online August 26, 2019.

Presented in part: 18th International Symposium on Staphylococci and Staphylococcal Infections, Copenhagen, Denmark, 2018.

^aV. G. T. and A. V. contributed equally to this work.

Correspondence: B. A. Diep, PhD, University of California, San Francisco, 1001 Potrero Ave, Bldg 80, Rm 707, San Francisco, CA 94110 (binh.diep@ucsf.edu).

The Journal of Infectious Diseases® 2020;221:267–75

© The Author(s) 2019. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com. DOI: 10.1093/infdis/jiz437

the differential susceptibilities of mammalian species to PFTs. Neutrophils from humans and rabbits are highly susceptible to bicomponent PFTs whereas those from monkeys and rodents are highly resistant to their cytotoxic effects [22, 25, 30], with the exception of murine neutrophils that are lysed by LukED at high concentrations [25, 31].

Considerable controversies exist as to whether neutralization of PFTs by active or passive immunization strategies could prevent or improve staphylococcal disease outcomes. Antibody-mediated neutralization of Hla conferred excellent protection in mouse pneumonia models [25, 32–34], but only partial protection in rabbit pneumonia models [24, 25, 35]. Full protection in the rabbit pneumonia model required neutralization of both Hla and PVL by specific antibodies because rabbits are highly susceptible to PVL [24, 25]. Of concern, however, is a single report showing that anti-PVL polyclonal antibodies can potentially produce detrimental outcomes in the mouse model [36].

We have previously identified an Hla vaccine candidate, AT-62aa, that exhibits strong immunogenicity in mice when used with 2 clinically tested adjuvants Aluminum phosphate and Glucopyranosyl Lipid Adjuvant-Stable Emulsion, with significant protection shown against intranasal and intraperitoneal challenge with *S. aureus* USA300 as well as surgical wound and subcutaneous abscess models in mice [37, 38]. We have also identified mutant forms of both PVL subunits, LukS_{T28F/K97A/S209A} and LukF_{K102A}, hereafter termed LukVax, as potential vaccine candidates as they conferred protection in a mouse bacteremia model [39]. As mice do not have receptors for PVL, the observed protective efficacy of this attenuated LukVax vaccine is most likely related to cross-reactivity with other leukotoxins that are toxic in mice [40].

Vaccination with full-length Hla toxoid harboring a single mutation at the position H35 (H35L) that rendered the mutant unable to properly oligomerize while maintaining receptor binding [41] was shown previously to protect against *S. aureus* pneumonia [32] and skin infection [42]. Because at least 22 second site mutations with the α -hemolysin gene could result in toxic reversion of the single H35 mutation [41], this could represent a safety concern as mutations could accumulate during manufacturing of the vaccine. Herein, we developed a new attenuated full-length Hla molecule, containing mutations in 2 histidine residues, H35 and H48, which are known to be critical for Hla oligomerization and toxicity [43], generating the double mutant Hla_{H35L/H48L}.

In this study, we evaluated efficacy of active immunization with Hla_{H35L/H48L} alone, LukVax alone, or the combination of Hla_{H35L/H48L}-LukVax in the rabbit pneumonia model. We also evaluated whether active immunization with these toxoids elicited neutralization titers to Hla, LukS, and LukF as well as cross-neutralizing titers to related bi-component toxins. Our results show that active immunization with the combination of Hla_{H35L/H48L}-LukVax conferred superior protection in the rabbit pneumonia model compared to vaccination with the individual components.

METHODS

Bacterial Strains

SF8300, a minimally passaged clinical strain representative of the epidemic clone USA300-0114, was used to establish necrotizing pneumonia in the rabbit model for evaluating efficacy of various vaccine candidates. The USA400 (MW2), USA1000, USA1100, and ST80 are clinical isolates representative of the major epidemic community-associated MRSA clones. The USA100, USA200, and MN8 are representative of hospital-associated MRSA clones.

Rabbit Model of Necrotizing Pneumonia

Outbred New Zealand White rabbits, 2.0–2.8 kg, were randomized for vaccination 3 times on days 0, 14, and 28 with 20 μ g each of Hla_{H35L/H48L}, LukS_{T28F/K97A/S209A}, or LukF_{K102A}, or a combination of Hla_{H35L/H48L}, LukS_{T28F/K97A/S209A}, LukF_{K102A} (20 μ g each antigen), or bovine serum albumin (BSA), in Alhydrogel. Vaccination was performed by intradermal injection into 4–5 different sites (20–25 μ L per site) in the dorsal lumbar region. On day 46 after the first vaccination, a 1.5-mL instillate containing approximately 6×10^9 colony-forming units (CFUs) of strain SF8300 USA300, shown previously to cause lethality in 80%–100% of infected rabbits, was delivered directly into the lungs of anesthetized rabbits through a 2.5-mm pediatric endotracheal tube as previously described for the rabbit MRSA necrotizing pneumonia model [22]. Just prior to bacterial challenge on day 46, blood was also drawn from each rabbit to be used for immunogenicity testing. Upon bacterial challenge, rabbits were monitored every 2 hours for the first 36 hours postinfection (hpi) and then 3 times daily thereafter, and survivors were euthanized at 96 hpi. Lungs, spleen, or kidneys were removed aseptically from euthanized rabbits or those that were found dead. Organs were cut into <0.5-cm pieces. Part of the organ sample was homogenized in 0.9% saline and CFU titers were generated by plating serial dilutions on blood agar. The rabbit experimental pneumonia protocol was reviewed and approved by the University of California, San Francisco Institutional Animal Care and Use Committee and conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Statistical Analysis

Survival curves were generated using the Kaplan–Meier method, and significance was assessed by means of the log-rank (Mantel–Cox) test, with Bonferroni correction for multiple comparisons (GraphPad version 6.0 software). Normal distribution was not assumed, so lung weight to body weight ratio (LW/BW) and log₁₀ CFU for different experimental groups were compared by 1-way analysis of variance with Kruskal–Wallis test followed by Dunn multiple comparisons post hoc test. Comparison of enzyme-linked immunosorbent assay (ELISA) or toxin neutralization assay (TNA) titers between groups of animals was performed by unpaired, 2-tailed

t test. In vitro toxin neutralization assay with bacterial supernatants was performed in triplicate and data are presented as mean $\pm$ standard error of the mean.

See the Supplementary Data for detailed information on in vitro methods, including preparations of toxins and antigens, ELISA for determination of total immunoglobulin G (IgG) titer in vaccinated rabbits, and TNAs.

RESULTS

Full-Length Toxoid Hla_{H35L/H48L}, but Not AT62, Elicited Highly Neutralizing Antibodies

In preliminary studies, we found that 4 of 4 rabbits vaccinated with AT62 in Alhydrogel, and 3 of 4 rabbits vaccinated with the combination of AT62 and LukVax, died of lethal challenge in the rabbit pneumonia model with the USA300 strain (data not shown), suggesting that vaccination with a small fragment of α -hemolysin may not confer protective immunity. Therefore, we developed a new attenuated full-length Hla_{H35L/H48L} that could confer greater protection. As expected, recombinant Hla_{H35L/H48L} exhibited no toxicity as determined in a standard rabbit red blood cell hemolytic assay (Figure 1A). While rabbits immunized with Hla_{H35L/H48L} and AT62 developed high IgG ELISA binding titers to wild-type Hla, the titers were much higher for the AT62-immunized rabbits (Figure 1B). Conversely, Hla_{H35L/H48L} immunization elicited significantly higher neutralizing titers to Hla ($P < .001$; unpaired, nonparametric *t* test) compared with AT62 immunization (Figure 1C), which may help explain the lack of protection from AT62 immunization in the rabbit model of MRSA necrotizing pneumonia in preliminary studies. Based on these findings, we decided to use Hla_{H35L/H48L} for further studies.

LukVax Elicited Cross-Neutralizing Antibodies

Rabbits immunized with LukS_{T28F/K97A/S209A} (formulated in Complete Freund's Adjuvant/Incomplete Freund's Adjuvant)

had high binding titers to LukS, HlgC, LukE and HlgA, whereas those immunized with LukF_{K102A} had high binding titers to LukF, HlgB, and LukD (Supplementary Figure 1A). Importantly, rabbit antisera against LukS_{T28F/K97A/S209A} or LukF_{K102A} exhibited high cross-neutralizing titers toward PVL, HlgAB, HlgCB, and LukED (Supplementary Figure 1B), indicating that each toxin component could contribute independently to the cross-neutralizing activity and that active immunization with both components may confer protection in vivo.

Efficacy and Immunogenicity of Hla_{H35L/H48L}-LukVax Vaccination

Four groups of rabbits were immunized with either BSA (as control), Hla_{H35L/H48L}, LukVax (LukS_{T28F/K97A/S209A} and LukF_{K102A}), or the combination of Hla_{H35L/H48L}-LukVax, before challenge in a rabbit pneumonia model. All BSA-vaccinated control rabbits died from infection, with a median survival of 12 hpi (Figure 2A). Although 11 of 12 Hla_{H35L/H48L}-vaccinated rabbits died, their median survival time of 18 hpi was significantly greater than the median survival time of BSA-vaccinated rabbits ($P < .001$ by log-rank test). LukVax-vaccinated rabbits had 50% survival ($P < .001$ vs BSA-vaccinated rabbits; median survival of 74 hpi), whereas rabbits vaccinated with Hla_{H35L/H48L}-LukVax exhibited 100% survival ($P < .001$ vs BSA-vaccinated rabbits). The LW/BW ratio, a quantitative marker of pulmonary edema and acute lung injury, was reduced in rabbits vaccinated with LukVax alone or the combination Hla_{H35L/H48L}-LukVax compared with those vaccinated with BSA or Hla_{H35L/H48L}; however, the difference was not statistically significant (Figure 2B). Representative lung images showed evidence of severe necrosis and hemorrhage for those vaccinated with BSA or Hla_{H35L/H48L}, which were largely absent in those vaccinated with LukVax or Hla_{H35L/H48L}-LukVax (Figure 2C). Log₁₀ CFU counts in lung, spleen, and kidneys at the time of death or when rabbits were euthanized at 96 hpi were significantly reduced in rabbits vaccinated with LukVax or Hla_{H35L/H48L}-LukVax compared with those vaccinated

Figure 1. Characterization of Hla_{H35L/H48L} toxicity and immunogenicity. A, Rabbit red blood cell (RBC) hemolytic assay shows lack of hemolytic activity by Hla_{H35L/H48L} in comparison to Hla wild-type (Hla_{WT}). Rabbits were immunized with 20 μ g of Hla_{H35L/H48L} or AT62 thrice every 2 weeks, and sera from 1 week after last immunization were used to test total immunoglobulin G reactivity (enzyme-linked immunosorbent assay) to Hla_{WT} (B) and total neutralizing titers to Hla by toxin neutralization assay (TNA) using rabbit RBCs (C). NT₅₀ is the serum dilution that affords 50% neutralization of Hla_{WT} toxin in TNA with rabbit RBCs. Bars represent mean. Abbreviations: EC₅₀, half maximal effective concentration gives the serum dilution that affords 50% antibody binding; ELISA, enzyme-linked immunosorbent assay; Hla_{WT}, Hla wild-type; NT₅₀, serum dilution that affords 50% neutralization; OD, optical density; TNA, toxin neutralization assay.

Figure 2. Protective efficacy of active immunization with Hla_{H35L/H48L} and/or LukVax in rabbit model of USA300 necrotizing pneumonia. Bovine Serum Albumin (BSA) was used as a control group for immunization. **A**, Kaplan–Meier survival curves for rabbits immunized with the indicated antigens (12 rabbits per experimental group) and then challenged by endobronchial instillation with 6.2×10^9 colony-forming units (CFU) of USA300 (SF8300) in the rabbit model of necrotizing pneumonia [22, 46]. Two-sided log-rank (Mantel–Cox) test was used to test multiple hypotheses regarding survival of animals in the different experimental groups, with $P < .0083$ (significance level of .05 divided by 6 different comparisons) being considered statistically significant to account for multiple comparisons using Bonferroni method. **B**, Lung weight to body weight (LW/BW) ratios for each rabbit in the 4 experimental groups were compared by nonparametric 1-way analysis of variance with Kruskal–Wallis test followed by Dunn multiple comparisons test. Filled symbols represent data from dead animals, and open symbols represent data from surviving animals that were euthanized at 96 hours postinfection (hpi). Because between-group differences in LW/BW ratios may be due to different sampling times (see also Discussion), we used filled symbols to represent data from dead animals and open symbols to represent data from surviving animals that were euthanized at 96 hpi. **C**, Photographs depict gross pathology of representative lungs; these lungs were selected because they have the median LW/BW ratios for their respective experimental groups as shown in (B).

with BSA or Hla_{H35L/H48L} (Figure 3), but this could be due in part to differences between rabbits that died early compared to those that survived to 96 hpi, allowing greater time to clear bacteria from infected organs [22, 24].

Serum antibody response at day 46 was evaluated by ELISA and TNA, showing that the antigens elicited the expected homologous and heterologous total IgG responses (Figure 4A). Rabbits immunized with Hla_{H35L/H48L} or Hla_{H35L/H48L}-LukVax

Figure 3. Bacterial burden in vital organs for the rabbit vaccine challenge study. Log₁₀ colony-forming units (CFU) in the lungs (A), spleen (B), and kidneys (C) were compared by nonparametric 1-way analysis of variance with Kruskal–Wallis test followed by Dunn multiple comparisons test. Because between-group differences in log CFU per organ may be due to different sampling times (see also Discussion), we used filled symbols to represent data from dead animals and open symbols to represent data from surviving animals that were euthanized at 96 hours postinfection.

Figure 4. Total immunoglobulin G titers and Hla neutralization titers for the rabbit vaccine challenge study. *A*, Binding titers to Hla, LukS, LukF, HlgB, HlgA, LukD, LukE, and HlgC for day 46 serum from rabbits immunized with Bovine Serum Albumin (BSA), Hla_{H35L/H48L}, LukVax, and Hla_{H35L/H48L}-LukVax, respectively, shown as half maximal effective concentration values. Data from 1 rabbit from Hla_{H35L/H48L}-LukVax was omitted due to loss of serum sample during study. *B*, Neutralizing titers to Hla for rabbits immunized with BSA or the indicated antigens. NT₅₀ is the serum dilution that affords 50% neutralization of Hla wild-type toxin in toxin neutralization assay with rabbit red blood cells. Bars represent means. Filled symbols represent data from dead animals, and open symbols represent data from surviving animals that were euthanized at 96 hours postinfection. Abbreviations: BSA, Bovine Serum Albumin; EC₅₀, half maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; NT₅₀, serum dilution that affords 50% neutralization; TNA, toxin neutralization assay.

developed high neutralizing titers against Hla (Figure 4B), whereas those immunized with LukVax or Hla_{H35L/H48L}-LukVax had high titer of neutralizing antibodies against PVL, HlgAB,

and HlgCB (Figure 5A, 5C, and 5D). Low cross-neutralizing titers against LukED were observed in only 26% (6/23) rabbits vaccinated with LukVax or LukVax-Hla_{H35L/H48L} (Figure 5B),

Figure 5. Pantone-Valentine leukocidin (PVL) (*A*), LukED (*B*), HlgAB (*C*), and HlgCB (*D*) neutralization titers for the rabbit vaccine challenge study. One rabbit serum sample from Hla_{H35L/H48L}-LukVax was omitted due to loss of serum sample during the study. NT₅₀ is the serum dilution that affords 50% neutralization of PVL, LukED, HlgAB, and HlgCB in toxin neutralization assay with human HL-60 neutrophil cell line. Bars represent mean. Filled symbols represent data from dead animals, and open symbols represent data from surviving animals that were euthanized at 96 hours postinfection. Abbreviations: BSA, Bovine Serum Albumin; NT₅₀, serum dilution that affords 50% neutralization; PVL, Pantone-Valentine leukocidin; TNA, toxin neutralization assay.

which may be due to the need to use very high concentration of 80 nM LukED toxin (vs 4.16 nM of PVL) in the HL60 assay.

In as much as prophylactic administration of affinity purified human polyclonal antibodies or monoclonal antibodies against PVL and/or Hla conferred significant protection in the same rabbit model of MRSA necrotizing pneumonia [24], we performed a post hoc analysis to determine whether there were differences in antibody response when comparing LukVax-vaccinated rabbits that survived or died (Figure 6). The vast majority (11 of 12) rabbits vaccinated with Hla_{H35L/H48L}-LukVax exhibited high titers to both PVL and Hla. Only 1 of 12 rabbits vaccinated with Hla_{H35L/H48L}-LukVax had low neutralizing titers against both Hla and PVL (Figure 6), but this rabbit survived challenge nonetheless. No significant differences in toxin neutralization or total IgG titers were observed between survivors and those that died in the LukVax immunized group (Figure 5), suggesting that mortality was not due to failure to elicit an antibody response. In the Hla_{H35L/H48L}-vaccinated group the only survivor had a log titer of 2.48, which is similar to the mean log titer of 2.45 among dead animals in this group (Figure 4B). Taken together, these data suggest that neutralization of both Hla and PVL were important for protection in the rabbit model of USA300 necrotizing pneumonia.

Neutralization of Hemolytic and Polymorphonuclear Leukocytes Toxins Produced by Various *S. aureus* Strains

Pooled sera from rabbits vaccinated with Hla_{H35L/H48L}-LukVax were tested for neutralizing activities against bacterial supernatant from a panel of clinically relevant and genetically unrelated MRSA strains. The results indicate that vaccination with Hla_{H35L/H48L}-LukVax elicited antibody responses that neutralized

the hemolytic and leukotoxic activities of a wide range of bacterial strains (Figure 7A and 7B). Highest neutralizing titers were observed for USA300 and USA1000 in the hemolytic assay and USA300, USA1100, and ST80 in the HL60 assay.

DISCUSSION

We described herein the development of nontoxic and immunogenic vaccine candidates, Hla_{H35L/H48L} and LukVax, for prevention of lethal pneumonia caused by *S. aureus*. The Hla residue H35 is important in formation of stable oligomers [44], and alanine substitution of H35 leads to loss of oligomerization and inability to activate ADAM10, the cellular receptor for Hla [45]. While Hla_{H35L} is fully attenuated and widely used as an experimental immunogen, Panchal et al identified second site revertants that can overcome the effect of H35L mutation [41]. Therefore, we introduced a second safety mutation in position H48, also known to be important for oligomerization [44], to generate Hla_{H35L/H48L} as a safe vaccine candidate. Hla_{H35L/H48L} was fully attenuated and elicited high levels of neutralizing antibodies (Figure 1). Using a rational structure-based approach, we had previously designed mutants of LukS and LukF, designated as LukS_{T28F/K97A/S209A} and LukF_{K102A}, respectively, that were defective in octamerization and highly attenuated, while retaining immunogenicity in mice [39]. This mutagenesis allowed us to include both subunits as vaccine and achieve a broader reactivity to bicomponent pore-forming toxins (Supplementary Figure 1).

Our previous study using monoclonal antibodies indicated that neutralization of both Hla and PVL are required for optimal protection against MRSA necrotizing pneumonia in rabbits, while neutralization of Hla alone was sufficient for protection in murine pneumonia models [25]. Therefore, we used the rabbit model to evaluate the ability of PVL and Hla vaccines to protect against MRSA necrotizing pneumonia. The Hla_{H35L/H48L}-LukVax vaccine was able to induce neutralizing antibodies to Hla, PVL, HlgAB, and HlgCB indicating lack of interference between the antigens when used in combination (Figures 4 and 5). Immunization with Hla_{H35L/H48L} or LukVax afforded 8.3% and 50% protection in rabbits challenged with USA300 MRSA (Figure 2A). The low but significant protective effect of Hla immunization is similar to that achieved by anti-Hla monoclonal antibodies in our prior studies [25, 35]. However, active immunization with Hla_{H35L/H48L}-LukVax provided 100% protection against necrotizing pneumonia (Figure 2A). Protection was associated with reduced LW/BW ratio and reduced bacterial burden in the lungs, spleen, and kidneys. However, since the bacterial burden was measured at the time of death or at the end of the study at 96 hpi for survivors, the CFUs may largely reflect the timing of sampling (Figure 3). Neutralization of the toxins is not expected to have a direct effect on bacterial burden, but rather to provide protection of targeted immune cells and vital organs, thus indirectly allowing time for host-mediated bacterial clearance from infected organs [22, 24].

Figure 6. Relationship between antibody titers and protection from necrotizing pneumonia. For the 12 rabbits vaccinated with Hla_{H35L/H48L}-LukVax, Hla and Pantone-Valentine leukocidin toxin neutralization assay (PVL TNA) titers are plotted against each other and stratified for survivors and dead animals in each group. Four quadrants around an arbitrary antibody cutoff of 1.5 logs are identified. Filled symbols represent data from dead animals, and open symbols represent data from surviving animals that were euthanized at 96 hours postinfection.

Figure 7. Neutralization of cytotoxic activity of various methicillin-resistant *Staphylococcus aureus* (MRSA) strains by antibodies elicited by Hla_{H35L/H48L}-LukVax. Antibodies elicited by Bovine Serum Albumin (BSA), a control antigen, were also measured. Reduction in percentage toxicity of bacterial supernatants as measured in rabbit red blood cell (RBC) hemolytic assay (A) or HL60 cell assay (B). For these toxin neutralization assays (TNAs), we first determined the dilution of the bacterial supernatants that induce about 70%–90% cytotoxicity against rabbit RBCs or HL-60 cells and then used that dilution in the presence and absence of a 1:40 (in rabbit RBC assay) or 1:60 (in induced HL60 cell assay) dilution of rabbit sera. Because USA100 and USA200 supernatants did not induce toxicity in HL60, these 2 strains were not included in the TNA presented in (B). Vertical bars represent mean and error bars represent standard error of the mean.

It was notable that all but 1 of the rabbits vaccinated with the Hla_{H35L/H48L}-LukVax vaccine had high neutralizing titers to both PVL and Hla (Figure 6). These data support the notion that neutralization of both toxins is required for robust protection, consistent with our studies with antitoxin antibodies [24, 25]. However, as survival in the Hla_{H35L/H48L}-LukVax vaccine group was 100%, it was not possible to evaluate correlation of survival with antibody titer.

Our study has limitations. The rabbit efficacy study in this report was performed only with SF8300, a USA300 MRSA isolate. USA300 is currently the most dominant circulating clone in the United States. Nonetheless, our in vitro data indicate that the antisera from rabbits vaccinated with LukVax-Hla_{H35L/H48L} protected red blood cells and HL60 Polymorphonuclear leukocytes from cytotoxic effects of supernatants from other clinically relevant strains, including Healthcare or Hospital-Acquired MRSA USA100, USA1000, USA1100, and the European CA-MRSA clone ST80 (Figure 7). Future studies should evaluate if this vaccine would also protect against these other clinically relevant clonal lineages in vivo.

Besides pneumonia, Hla and PVL are important virulence factors for SSTI. We are currently further exploring the efficacy of these toxoids along with additional antigens in the context of a heptavalent toxoid vaccine against SSTI and other syndromes of *S. aureus* disease.

In summary, these data provide evidence that active immunization against both Hla and PVL elicited broadly cross-neutralizing antibodies and protected against death in a rabbit model of USA300 necrotizing pneumonia. These data are significant in light of the high sensitivity of rabbits to staphylococcal PFTs and similarities of the disease pathology in humans

and rabbits [22, 24]. These data support further preclinical development of LukVax-Hla_{H35L/H48L} as part of a multivalent toxoid vaccine to prevent severe invasive diseases caused by *S. aureus*.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Financial support. This work was supported in part by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (grant number R01AI087674 to B. A. D. for preclinical development of Hla_{H35L/H48L} and grant number R01AI111205 to M. J. A. for preclinical development of multivalent Hla-LukVax vaccine).

Potential conflicts of interest. A. V., R. P. A., S. K., and M. J. A. are employees of Integrated Biotherapeutics. M. J. A. has stocks and R. P. A. has stock options in Integrated Biotherapeutics. All other authors report no potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Kong EF, Johnson JK, Jabra-Rizk MA. Community-associated methicillin-resistant *Staphylococcus aureus*: an enemy amidst us. *PLoS Pathog* 2016; 12:e1005837.

2. Spellberg B, Daum R. Development of a vaccine against *Staphylococcus aureus*. *Semin Immunopathol* **2012**; 34:335–48.
3. Fowler VG, Allen KB, Moreira ED, et al. Effect of an investigational vaccine for preventing *Staphylococcus aureus* infections after cardiothoracic surgery: a randomized trial. *JAMA* **2013**; 309:1368–78.
4. Begier E, Seiden DJ, Patton M, et al. SA4Ag, a 4-antigen *Staphylococcus aureus* vaccine, rapidly induces high levels of bacteria-killing antibodies. *Vaccine* **2017**; 35:1132–9.
5. Otto M. *Staphylococcus aureus* toxins. *Curr Opin Microbiol* **2014**; 17:32–7.
6. Archer GL. *Staphylococcus aureus*: a well-armed pathogen. *Clin Infect Dis* **1998**; 26:1179–81.
7. Powers ME, Bubeck Wardenburg J. Igniting the fire: *Staphylococcus aureus* virulence factors in the pathogenesis of sepsis. *PLoS Pathog* **2014**; 10:e1003871.
8. Bhakdi S, Tranum-Jensen J. Alpha-toxin of *Staphylococcus aureus*. *Microbiol Rev* **1991**; 55:733–51.
9. Berube BJ, Bubeck Wardenburg J. *Staphylococcus aureus* α -toxin: nearly a century of intrigue. *Toxins* **2013**; 5:1140–66.
10. Grimminger F, Rose F, Sibelius U, et al. Human endothelial cell activation and mediator release in response to the bacterial exotoxins *Escherichia coli* hemolysin and staphylococcal alpha-toxin. *J Immunol* **1997**; 159:1909–16.
11. Powers ME, Kim HK, Wang Y, Bubeck Wardenburg J. ADAM10 mediates vascular injury induced by *Staphylococcus aureus* α -hemolysin. *J Infect Dis* **2012**; 206:352–6.
12. Craven RR, Gao X, Allen IC, et al. *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* **2009**; 4:e7446.
13. Manohar M, Maheswaran SK, Frommes SP, Lindorfer RK. Platelet damaging factor, a fifth activity of staphylococcal alpha-toxin. *J Bacteriol* **1967**; 94:224–31.
14. Nygaard TK, Pallister KB, DuMont AL, et al. Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS One* **2012**; 7:e36532.
15. Kebaier C, Chamberland RR, Allen IC, et al. *Staphylococcus aureus* α -hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome. *J Infect Dis* **2012**; 205:807–17.
16. Kielian T, Cheung A, Hickey WF. Diminished virulence of an alpha-toxin mutant of *Staphylococcus aureus* in experimental brain abscesses. *Infect Immun* **2001**; 69:6902–11.
17. O'Callaghan RJ, Callegan MC, Moreau JM, et al. Specific roles of alpha-toxin and beta-toxin during *Staphylococcus aureus* corneal infection. *Infect Immun* **1997**; 65:1571–8.
18. Surewaard BGJ, Thanabalasuriar A, Zeng Z, et al. Alpha-toxin induces platelet aggregation and liver injury during *Staphylococcus aureus* sepsis. *Cell Host Microbe* **2018**; 24:271–84 e3.
19. Suttorp N, Fuhrmann M, Tannert-Otto S, Grimminger F, Bhadki S. Pore-forming bacterial toxins potently induce release of nitric oxide in porcine endothelial cells. *J Exp Med* **1993**; 178:337–41.
20. Aman MJ, Adhikari RP. Staphylococcal bicomponent pore-forming toxins: targets for prophylaxis and immunotherapy. *Toxins* **2014**; 6:950–72.
21. Spaan AN, van Strijp JAG, Torres VJ. Leukocidins: staphylococcal bi-component pore-forming toxins find their receptors. *Nat Rev Microbiol* **2017**; 15:435–47.
22. Diep BA, Chan L, Tattevin P, et al. Polymorphonuclear leukocytes mediate *Staphylococcus aureus* Panton-Valentine leukocidin-induced lung inflammation and injury. *Proc Natl Acad Sci U S A* **2010**; 107:5587–92.
23. Lo WT, Wang CC. Panton-Valentine leukocidin in the pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus* infection. *Pediatr Neonatol* **2011**; 52:59–65.
24. Diep BA, Le VT, Badiou C, et al. IVIG-mediated protection against necrotizing pneumonia caused by MRSA. *Sci Transl Med* **2016**; 8:357ra124.
25. Diep BA, Le VT, Visram ZC, et al. Improved protection in a rabbit model of community-associated methicillin-resistant *Staphylococcus aureus* necrotizing pneumonia upon neutralization of leukocidins in addition to alpha-hemolysin. *Antimicrob Agents Chemother* **2016**; 60:6333–40.
26. Prince A, Wang H, Kitur K, Parker D. Humanized mice exhibit increased susceptibility to *Staphylococcus aureus* pneumonia. *J Infect Dis* **2017**; 215:1386–95.
27. Tseng CW, Biancotti JC, Berg BL, et al. Increased susceptibility of humanized NSG mice to Panton-Valentine leukocidin and *Staphylococcus aureus* skin infection. *PLoS Pathog* **2015**; 11:e1005292.
28. Spaan AN, Henry T, van Rooijen WJM, et al. The staphylococcal toxin Panton-Valentine leukocidin targets human C5a receptors. *Cell Host Microbe* **2013**; 13:584–94.
29. Tromp AT, Van Gent M, Abrial P, et al. Human CD45 is an F-component-specific receptor for the staphylococcal toxin Panton-Valentine leukocidin. *Nat Microbiol* **2018**; 3:708–17.
30. Löffler B, Hussain M, Grundmeier M, et al. *Staphylococcus aureus* Panton-Valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog* **2010**; 6:e1000715.
31. Alonzo F 3rd, Benson MA, Chen J, Novick RP, Shopsin B, Torres VJ. *Staphylococcus aureus* leukocidin ED contributes to systemic infection by targeting neutrophils and

- promoting bacterial growth in vivo. *Mol Microbiol* **2012**; 83:423–35.
32. Bubeck Wardenburg J, Schneewind O. Vaccine protection against *Staphylococcus aureus* pneumonia. *J Exp Med* **2008**; 205:287–94.
 33. Hua L, Hilliard JJ, Shi Y, et al. Assessment of an anti-alpha-toxin monoclonal antibody for prevention and treatment of *Staphylococcus aureus*-induced pneumonia. *Antimicrob Agents Chemother* **2014**; 58:1108–17.
 34. Ragle BE, Bubeck Wardenburg J. Anti-alpha-hemolysin monoclonal antibodies mediate protection against *Staphylococcus aureus* pneumonia. *Infect Immun* **2009**; 77:2712–8.
 35. Diep BA, Hilliard JJ, Le VT, et al. Targeting alpha toxin to mitigate its lethal toxicity in ferret and rabbit models of *Staphylococcus aureus* necrotizing pneumonia. *Antimicrob Agents Chemother* **2017**; 61. doi:10.1128/AAC.02456-16.
 36. Yoong P, Pier GB. Antibody-mediated enhancement of community-acquired methicillin-resistant *Staphylococcus aureus* infection. *Proc Natl Acad Sci U S A* **2010**; 107:2241–6.
 37. Adhikari RP, Karauzum H, Sarwar J, et al. Novel structurally designed vaccine for *S. aureus* α -hemolysin: protection against bacteremia and pneumonia. *PLoS One* **2012**; 7:e38567.
 38. Adhikari RP, Thompson CD, Aman MJ, Lee JC. Protective efficacy of a novel alpha hemolysin subunit vaccine (AT62) against *Staphylococcus aureus* skin and soft tissue infections. *Vaccine* **2016**; 34:6402–7.
 39. Karauzum H, Adhikari RP, Sarwar J, et al. Structurally designed attenuated subunit vaccines for *S. aureus* LukS-PV and LukF-PV confer protection in a mouse bacteremia model. *PLoS One* **2013**; 8:e65384.
 40. Adhikari RP, Kort T, Shulenin S, et al. Antibodies to *S. aureus* LukS-PV attenuated subunit vaccine neutralize a broad spectrum of canonical and non-canonical bicomponent leukotoxin pairs. *PLoS One* **2015**; 10:e0137874.
 41. Panchal RG, Bayley H. Interactions between residues in staphylococcal alpha-hemolysin revealed by reversion mutagenesis. *J Biol Chem* **1995**; 270:23072–6.
 42. Kennedy AD, Bubeck Wardenburg J, Gardner DJ, et al. Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. *J Infect Dis* **2010**; 202:1050–8.
 43. Menzies BE, Kernodle DS. Site-directed mutagenesis of the alpha-toxin gene of *Staphylococcus aureus*: role of histidines in toxin activity in vitro and in a murine model. *Infect Immun* **1994**; 62:1843–7.
 44. Jursch R, Hildebrand A, Hobom G, et al. Histidine residues near the N terminus of staphylococcal alpha-toxin as reporters of regions that are critical for oligomerization and pore formation. *Infect Immun* **1994**; 62:2249–56.
 45. Wilke GA, Bubeck Wardenburg J. Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. *Proc Natl Acad Sci U S A* **2010**; 107:13473–8.
 46. Cohen TS, Hilliard JJ, Jones-Nelson O, et al. *Staphylococcus aureus* alpha toxin potentiates opportunistic bacterial lung infections. *Sci Transl Med* **2016**; 8:329ra31.