BRIEF REPORT

Targeting *Staphylococcus aureus* α-Toxin as a Novel Approach to Reduce Severity of Recurrent Skin and Soft-Tissue Infections

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Staphyococcus aureus **frequently causes recurrent skin and soft-tissue infection (SSTI). In the pediatric population, elevated serum antibody targeting *S. aureus* α-toxin is correlated with a reduced incidence of recurrent SSTI. Using a novel model of recurrent SSTI, we demonstrated that expression of α-toxin during primary infection increases the severity of recurrent disease. Antagonism of α-toxin by either a dominant-negative toxin mutant or a small molecule inhibitor of the toxin receptor ADAM10 during primary infection reduces reinfection abscess severity. Early neutralization of α-toxin activity during *S. aureus* SSTI therefore offers a new therapeutic strategy to mitigate primary and recurrent disease.**

Keywords. *Staphylococcus aureus*; skin and soft-tissue infection; α-toxin; recurrent infection; novel therapeutics.

Staphylococcus aureus is the leading cause of skin and soft-tissue infection (SSTI), resulting in more than 10 million outpatient visits, approximately 500 000 hospital admissions per year in the United States and a substantial economic burden [1–3]. Although most SSTIs are successfully managed with surgical drainage and oral antimicrobial therapy, cutaneous infection can potentiate serious invasive disease and is associated with recurrent infection in up to 50% of patients [2]. Recurrent infection contributes to increased morbidity and exposes the patient to multiple antimicrobials, promoting drug resistance. Epidemic community-associated methicillin-resistant *S. aureus* strains that have circulated in the United States for more than a decade are adept at causing recurrent infection in healthy adults and children, suggesting that pathogen-associated traits may increase primary SSTI risk and simultaneously blunt the development of protective immunity. To date, however, there has not been a mechanistic link between specific *S. aureus* virulence factors and potentiation of reinfection, in part owing to a lack of suitable animal model systems of recurrent SSTI.

Host predictors of reinfection susceptibility have been ill defined, with the exception of immunodeficiency syndromes, including chronic granulomatous disease and hyper–immunoglobulin E syndrome, which are associated with innate immunity defects that predispose to *S. aureus* infection [4]. On this backdrop, a recent study of the pediatric serologic response to primary infection demonstrated that increased circulating antibody recognizing *S. aureus* α-toxin (α-hemolysin [Hla]) is correlated with protection against recurrent SSTI for up to 12 months after primary infection [5]. These findings suggest a potential role for this toxin in patterning the host response and highlight a specific virulence factor that may be targeted for intervention during primary SSTI.

S. aureus Hla is a small pore-forming cytotoxin expressed by almost all clinical isolates [6]. Increased Hla expression has been noted in community-associated methicillin-resistant *S. aureus* strain USA300 and in historic clinical isolates associated with epidemic human disease, correlating with increased severity of SSTI and pneumonia [7, 8]. Hla causes dermonecrotic skin injury by interacting with ADAM10, a zinc-dependent metalloprotease that cleaves E-cadherin and destabilizes the epithelial barrier on toxin binding [9, 10]. Supporting the role of the Hla-ADAM10 interaction in pathogenesis, primary SSTI is mitigated by immunization strategies targeting Hla as well as a small molecule ADAM10 inhibitor that blocks toxin binding [10–12]. Coupled with human clinical data on the anti-Hla response in protection against recurrent SSTI [5], these observations suggest that identification of a role for Hla in recurrent infection could accelerate the development of highly targeted interventions. To this end, we developed a tractable mouse model of *S. aureus* recurrent SSTI to examine the molecular contribution of Hla to reinfection.

METHODS

S. aureus strains USA300/LAC, its isogenic Δ*hla* mutant, and Δ*hla* strains harboring plasmids encoding wild-type (WT)

Received 12 November 2013; accepted 4 April 2014; electronically published 16 April 2014.
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The Journal of Infectious Diseases 2014;210:1012–8

DOI: 10.1093/infdis/jiu223

Hla (Δ*hla*/p*hla*) or the inactive Hla_{H35L} (Δ*hla*/p*hla*$_{H35L}$) mutant were prepared for infection as described elsewhere [10]. Bacterial stocks were adjusted and confirmed to yield $1–4 \times 10^7$ staphylococci per 50 μL inoculation. Recombinant Hla_{H35L} for therapy studies was prepared as described elsewhere [7].

Animal studies were conducted according to protocols approved by the University of Chicago Institutional Animal Care and Use Committee. For recurrent SSTI modeling, 4-week-old C57BL/6 male mice (Jackson Laboratory) were anesthetized using a intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) before right flank subcutaneous challenge with 1×10^7 colony-forming units *S. aureus* USA300 or its isogenic variants in 50 μL of phosphate-buffered saline [10]. Lesional abscess area (measured in square millimeters) was monitored at 24-hour intervals for 14 days, after which mice were observed for a 7-day recovery period before reinfection via left flank subcutaneous injection of $3\text{-}4 \times 10^7$ WT USA300. Secondary lesions were monitored for 14 days. Abscess size was determined according to the formula $A = (\pi/2)(\text{length}_{mm})(\text{width}_{mm})$ [10, 11].

We performed enzyme linked-immunosorbent assay (ELISA)–based determination of anti-Hla titers in naive serum on day 14 after primary infection and 14 days after secondary infection, as described elsewhere [7]. ELISA absorbance readings were used to generate 4-parameter log dose-response curves using Prism 5.0b software. End point titers were calculated as the reciprocal of the highest dilution yielding a positive reaction, using a cutoff value of 3 times the mean assay background value obtained in the absence of serum addition. For rabbit red cell hemolysis assays, preincubation of 1.5 nmol/L recombinant Hla with a 1:100 dilution of serum harvested 14 days after reinfection was performed for 30 minutes before the addition of 5×10^7 rabbit red blood cells. Assays were performed in phosphate-buffered saline in a 96-well plate format in which unlysed cells were sedimented after 45 minutes of incubation and supernatants subjected to absorbance measurements at 450 nm. The percentage of lysis was scored relative to 100% detergent lysis. Passive immune serum samples were obtained from mice 24 hours after intraperitoneal delivery of 10 mg/kg anti-Hla monoclonal antibody 7B8 [13].

Skin lesion punch biopsy specimens were obtained from anesthetized mice at the time points indicated after infection for determination of staphylococcal burden, histopathologic analysis, and cytokine analysis by ELISA (R&D Systems) according to the manufacturer's instructions, as described elsewhere [10, 14]. For therapy studies, mice received a single 30-μL intralesional injection of purified Hla_{H35L} (7.5 μg) or the ADAM10 inhibitor GI254023X (1 μg) [10]. Control treatment groups included a recombinant version of an irrelevant, genetically inactivated mutant protein toxin (*Bacteroides fragilis* fragilisyn) or dimethyl sulfoxide vehicle control, respectively.

Statistical analysis for 2-way comparisons was performed using the Student *t* test, with pair-wise comparisons performed using Bonferroni correction to adjust for multiplicity at a significance level of .05.

RESULTS

To investigate the role of Hla in recurrent *S. aureus* SSTI, we used established mouse models of staphylococcal skin infection as a framework to develop a model of recurrent SSTI (Figure 1*A*). We infected 4-week-old mice with either 1×10^7 WT or isogenic variant forms of *S. aureus* USA300 (primary infection). After a 1-week period of recovery following clearance of the primary lesion, mice were subjected to reinfection on the opposite flank with $3\text{-}4 \times 10^7$ staphylococci (secondary infection). We reasoned that the delivery of a low initial inoculum would allow pathogen modulation of host immune pathways without causing substantial tissue damage observed with higher inocula [10]. In this model, primary SSTI infection with either WT USA300 (Figure 1*B*) or an isogenic Hla- mutant (Δ*hla*) confirmed the previously observed virulence defect of the Δ*hla* strain [11].

Reinfection of these mice with WT *S. aureus* demonstrated larger lesions in mice initially infected with WT *S. aureus* than in those initially infected with the Δ*hla* strain (Figure 1*B*; statistical analysis in Supplementary Table 1*A*), suggesting that Hla expression during primary infection interferes with the development of host immunity against recurrent infection. *S. aureus* recovery from primary and secondary lesions harvested 1 or 3 days after reinfection did not differ depending on whether the primary infection was caused by WT or Δ*hla S. aureus* (Figure 1*C*). These findings are consistent with observations that Hla does not substantially modify bacterial load at early time points after infection and that lesion size is not solely determined by bacterial recovery [10, 12, 14].

Histopathologic analysis of WT- and Δ*hla*-infected primary lesions 3 days after infection revealed abscesses confined to the subcutaneous space without overlying epidermal injury (Figure 1*D*, *left panels*). The appearance of lesions harvested after secondary infection was notable for increased epidermal damage and lesion size relative to primary infection, consistent with the increased inoculum (Figure 1*D*, *right panels*). These findings, however, were less severe in mice initially infected with Δ*hla* than in those infected with WT *S. aureus* (Figure 1*D*, *right panels*). Although a significant inflammatory cell infiltrate is apparent at 3 day after infection in primary lesions, this appears less prominent at the same interval after reinfection. In contrast, the overall histologic appearance of primary and secondary lesions in both conditions appears similar 1 day after infection (Supplementary Figure 1*A*), again revealing the earlier onset of epidermal injury in mice that received primary infection with WT versus Δ*hla S. aureus*. Consistent with

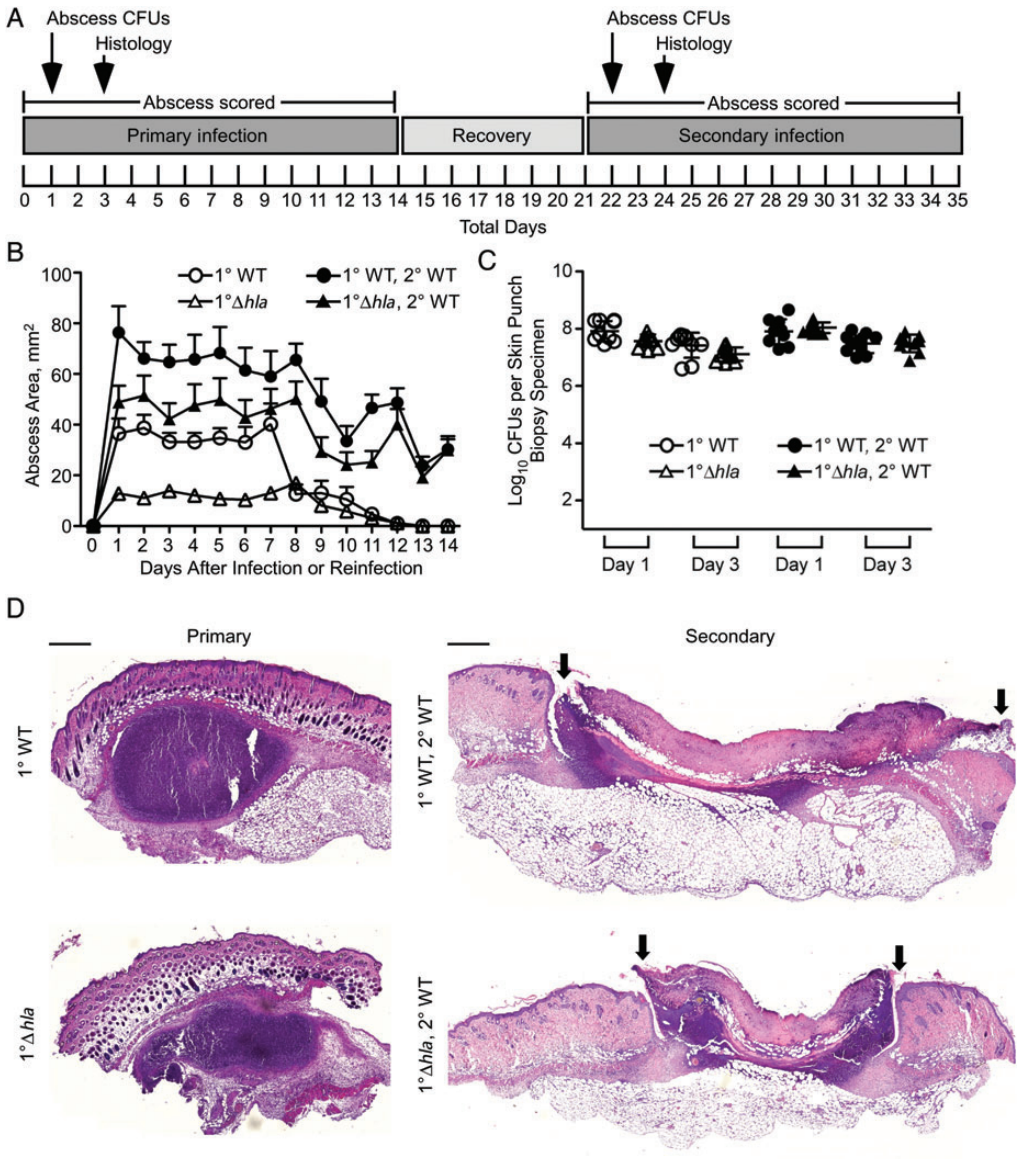

Figure 1. α-Toxin (Hla) modulates *Staphylococcus aureus* abscess formation during recurrent skin and soft-tissue infection (SSTI). *A,* Timeline for recurrent SSTI mouse model demonstrating primary infection of mice at 4 weeks of age, with abscess scoring and infection recovery, and secondary infection, with abscess scoring. CFU, colony-forming units. *B,* Abscess mean area recordings in mice after primary infection with wild-type (WT; *open circles*) or Hla-deficient (Δ*hla*; *open triangles*) *S. aureus* USA300 and in the same groups of mice then subjected to reinfection with WT *S. aureus* (*filled symbols*). Error bars represent standard error of the mean for each time point, calculated from recordings in groups of 20 mice. *C,* Recovery of WT *S. aureus* from the tissues of mice reinfected as in *B,* harvested 1 and 3 days after primary (*open symbols*) or reinfection (*closed symbols*); values are given as means with standard errors of the mean (*error bars*). *D,* Hematoxylin-eosin–stained sections of skin lesions harvested from mice after primary infection with WT or Δ*hla S. aureus* (*left panels*) or mice receiving primary infection with WT or Δ*hla S. aureus* followed by reinfection with WT *S. aureus* (*right panels*). Histopathologic samples were harvested 3 days after infection. Arrows demarcate extent of epidermal injury overlying the abscess lesion. All experiments were repeated for reproducibility, with displayed results representative of 2 or 3 independent analyses.

the findings of Tkaczyk et al [12], who observed that genetic deletion of Hla or immunotherapeutic neutralization of the toxin led to increased host interleukin 1β and 17 responses to skin infection, we observe these cytokine alterations after primary infection in our model system (Supplementary Figure 1*B*).

Because Hla pore formation is required for cytotoxicity to epithelial cells and toxin-mediated ADAM10 activation that results in epithelial barrier injury [9, 10, 15], we examined the requirement for toxin activity in susceptibility to recurrent infection. To this end, we used a Δ*hla* mutant USA300 strain complemented with plasmid-encoded WT Hla (Δ*hla*/p*hla*) or an

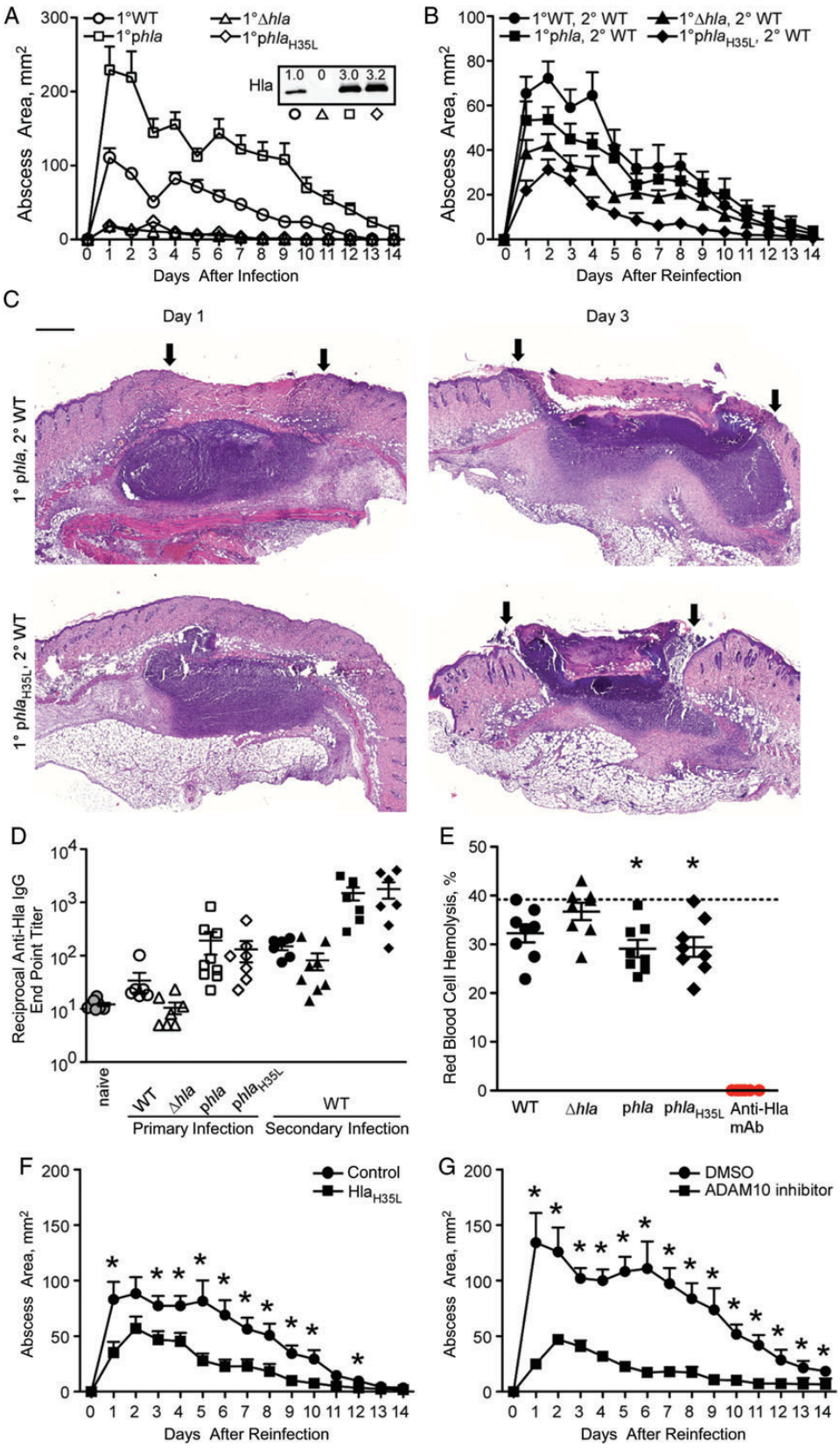

Figure 2. Contribution of active α-toxin (Hla) in modulating the host response to recurrent *Staphylococcus aureus* skin and soft-tissue infection (SSTI). *A, B,* Analysis of abscess formation in mice subjected to primary infection (1°) with wild-type (WT; *circles*), Δ*hla* (*triangles*), Δ*hla*/p*hla* (*squares*), or Δ*hla*/p*hla*$_{H35L}$ (*diamonds*) strains (*A*) or (*B*) after WT reinfection of mice infected as in *A*; 2°, secondary infection. Abscess mean area (n = 15) with plus standard error of the mean (SEM) over 14 days. For the inset in *A*, overnight culture supernatants of *S. aureus* WT, Δ*hla*, Δ*hla*/p*hla*, or Δ*hla*/p*hla*$_{H35L}$ were probed by

Hla variant containing a single amino acid substitution (Hla_{H35L}, Δ*hla*/p*hla*$_{H35L}$) that retains ADAM10 binding capability but is unable to form the injurious pore, thereby functioning as a dominant-negative toxin [9]. Complementation leads to approximately 3-fold overexpression of WT Hla or Hla_{H35L} (Figure 2*A*, *inset*) [7].

Primary infection of mice with WT, Δ*hla*, Δ*hla*/p*hla*, or Δ*hla*/p*hla*$_{H35L}$ demonstrated the effect of active Hla on infection severity, because active Hla overexpression by the Δ*hla*/p*hla* strain resulted in severe skin lesions, whereas the Δ*hla*/p*hla*$_{H35L}$ was associated with a minimal lesion similar to the Δ*hla* mutant (Figure 2*A*; statistical analysis in Supplementary Table 1*B*). Reinfection of these mice with WT USA300 demonstrated that the most significant degree of protection was afforded by primary infection with the Δ*hla*/p*hla*$_{H35L}$ strain (Figure 2*B*), followed by the Δ*hla* strain, indicating that the absence of toxin activity during primary infection is beneficial to the host in establishing a protective response to reinfection (statistical analysis in Supplementary Table 1*C*).

Primary infection with the Δ*hla*/p*hla* strain was significantly less protective than the Δ*hla*/p*hla*$_{H35L}$ strain in spite of the similar level of toxin expression by these strains. Histopathologic analysis of skin lesions from these 2 groups of mice revealed larger lesions and increased tissue injury in Δ*hla*/p*hla*-infected mice after reinfection than in those with primary infection caused by Δ*hla*/p*hla*$_{H35L}$ *S. aureus* (Figure 2*C*). The serum anti-Hla antibody titer after primary infection with Δ*hla*/p*hla* (Figure 2*D*) and Δ*hla*/p*hla*$_{H35L}$ was significantly elevated relative to Δ*hla* and similar in the 2 complemented strains (statistical analysis in Supplementary Table 1*D*). After secondary infection, anti-Hla titers increased in all conditions relative to the corresponding primary infection (Figure 2*D*). Titers in mice initially infected with Δ*hla*/p*hla* or Δ*hla*/p*hla*$_{H35L}$ were significantly elevated compared with both WT and Δ*hla* primary-infected mice (Figure 2*D*) but did not differ between mice initially infected with Δ*hla*/p*hla* or Δ*hla*/p*hla*$_{H35L}$. Examination of serum toxin-neutralizing activity 2 weeks after reinfection demonstrated that mice initially infected with Δ*hla*/p*hla* (Figure 2*E*) or Δ*hla*/p*hla*$_{H35L}$ (Figure 2*E*) mount a significant response relative to preinfected animals, whereas those with WT primary infection only demonstrated a trend toward neutralization. The magnitude of this response, however, was indistinguishable between reinfected groups and was significantly weaker than in serum samples harvested from mice passively immunized with a well-characterized, Hla-neutralizing monoclonal antibody (*red circles* in Figure 2*E*) [13].

The observation that primary infection with Δ*hla*/p*hla* and Δ*hla*/p*hla*$_{H35L}$ leads to the generation of quantitatively and qualitatively equivalent antibody responses, yet distinct reinfection outcomes, underscores the importance of the active toxin in modulating the host response. Importantly, these data suggest that an anti-toxin antibody response alone is insufficient to optimize protective immunity against recurrent SSTI. We hypothesized that reinfection severity may be reduced by deliberate antagonism of Hla activity, coupled with preservation of antigenic exposure during primary infection to stimulate the development of toxin-neutralizing antibodies. Because SSTI is primarily managed in the outpatient setting, a clinically effective therapeutic intervention must be cost-effective for delivery to a large number of otherwise healthy patients who present with primary SSTI and an indeterminate risk for recurrence. We therefore examined therapeutic delivery of either recombinant, dominant-negative Hla_{H35L} or a small molecule active-site inhibitor of ADAM10 that blocks Hla receptor binding (GI254023X) [15] to reduce reinfection severity. These novel strategies were predicted to maintain host exposure to Hla while abrogating toxin activity during primary infection. Therapy was delivered as a single intralesional dose 6–8 hours after primary WT *S. aureus* infection (Supplementary Figure 2*A*); these treatments blunted primary skin lesions (Supplementary Figure 2*B*). After reinfection, lesions size was substantially reduced in mice treated with either Hla_{H35L} (Figure 2*F*) or the ADAM10 inhibitor (Figure 2*G*), compared with the respective control mice.

DISCUSSION

Together, these results suggest that reinfection abscess severity is a functional integration of 2 distinct but related

Figure 2 continued. α-Hla quantitative immunoblotting with relative expression level noted in comparison with the WT strain. *C*, Hematoxylin-eosin–stained sections of WT-reinfected skin lesions harvested from mice subjected to primary infection with Δ*hla*/p*hla* (*upper panels*) or Δ*hla*/p*hla*$_{H35L}$ (*lower panels*). Histopathologic samples were harvested 1 and 3 days after infection. Arrows demarcate extent of epidermal injury overlying the abscess lesion. *D*, Reciprocal end point titer analysis of the anti-Hla response calculated from enzyme-linked immunosorbent assay (ELISA) measurements performed on serum samples collected from mice on day 14 after primary infections (*A*) and secondary infection (*B*); 6–9 mice were analyzed in each group. Error bars represent mean ± SEM. IgG, immunoglobulin G. *E*, Percentage of red blood cell hemolysis observed in an in vitro assay assessing the ability of serum harvested from reinfected mice to protect against Hla-mediated lysis of rabbit red blood cells. The percentage of hemolysis is calculated relative to 100% hemolysis obtained in detergent-lysed wells. Dotted line represent mean hemolysis in wells incubated with preinfection serum; error bars, mean + SEM; mAb, monoclonal antibody. *$P < .05$ (sample vs preinfected serum control). Red dots denote percent red blood cell hemolysis in the presence of serum derived from mice that received passive immunization with a neutralizing anti-Hla monoclonal antibody 24 hours prior to serum harvest. *F, G,* SSTI mean abscess areas in mice reinfected with WT *S. aureus* after being treated with either purified, recombinant Hla_{H35L} or a control protein 6–8 hours after primary infection (*F*) or being treated with either the ADAM10 inhibitor (GI254023X) or the dimethyl sulfoxide (DMSO) vehicle 6–8 hours after primary infection (*G*). *$P < .05$ (Student *t* test). All experiments were repeated for reproducibility; results are representative of 2–3 independent analyses.

processes: (1) the deleterious effects of active Hla on host cells during primary infection and (2) the degree of antigenic exposure to Hla. Although the presence of active Hla impairs host protective responses to reinfection (Figures 1*B*, 1*D*, 2*B*, and 2*C*), antigenic exposure in the context of toxin overexpression (Figure 2*E*) or inactive toxin (as with the Hla_{H35L} mutant or in the presence of the ADAM10 inhibitor; Figure 2*F* and 2*G*) seems to promote the generation of a toxin-neutralizing antibody response. Toxin-neutralizing approaches seem to afford the additional benefit of limiting the harmful effects of the active toxin on host immunity. Hla causes direct cytotoxicity to immune cells [16–18], also demonstrating the ability to modulate immune cell recruitment, inflammasome activation, host cytokine and chemokine responses, and bacterial killing [6].

To date, the impact of Hla on host immune responses has been investigated only during primary infection, with recent studies underscoring the complex effects of the toxin in the tissue microenvironment. Deletion of Hla or prophylactic passive immunization with an anti-Hla monoclonal antibody augments tissue expression of multiple cytokines and chemokines, correlated with priming of the Th1 and Th17 responses and improved SSTI outcome [12]. In contrast, loss of Hla sensitivity by the selective deletion of ADAM10 on myeloid lineage cells leads to exacerbated skin lesions and a dampened host interleukin 1β response to primary infection, indicative of a beneficial effect of toxin immune priming [14]. These observations indicate that detailed analysis of cell-type specific immune responses to Hla during primary infection is required to elucidate how the toxin manipulates immunity and predisposes to recurrent infection. Because toxin antagonism does not fully eliminate recurrent infection, our findings clearly indicate that virulence factors other than Hla modulate the host response to reinfection. It will be of interest to define these factors and the molecular mechanisms by which multiple factors act in concert to blunt the development of protective immunity.

These studies refine and extend our knowledge of the clinical approaches for protecting against *S. aureus* SSTI: (1) through active immunization before onset of initial infection, driving the generation of a preexisting and potent antibody response that neutralizes Hla, or (2) by therapeutic neutralization of toxin activity during primary infection, through novel strategies that preserve (in the case of ADAM10 inhibition) or even enhance (Hla_{H35L}) antigenic stimulation to develop a protective antibody response. These approaches may be most efficacious if implemented in the pediatric population, capitalizing on the earliest opportunity to direct host immunity. Future studies are required to more comprehensively define the precise mechanisms by which *S. aureus* modulates the host response during initial infection and to determine how age- and site-specific immunologic patterning may optimize interventional approaches for human application.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Michael Powers for assistance with animal experimentation.

Financial support. This work was supported by the National Institutes of Heath (NIH; award AI097434-01) and the Burroughs Wellcome Foundation (Investigators in the Pathogenesis of Infectious Disease Fellowship to J. B. W.). The authors are members in and receive support from the Region V "Great Lakes" Regional Center of Excellence (RCE) (RCE, NIH award 2-U54-AI-057153). R. E. N. B. is a trainee in the NIH Medical Scientist Training Program at the University of Chicago (grant GM007281). B. J. B. and M. J. G. were partially supported by the National Institutes of Health (grant T32 GM007183).

Potential conflicts of interest. J. B. W. can receive royalties from Novartis Vaccines and Diagnostics in relation to patents owned by the University of Chicago. All other authors report no potential conflicts.

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.

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