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Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infections in a mouse model

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Abstract

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections are predominantly those affecting skin and soft tissues. Although progress has been made, our knowledge of the molecules that contribute to the pathogenesis of CA-MRSA skin infections is incomplete. Here we tested the hypothesis that alpha-hemolysin (Hla) contributes to severity of USA300 skin infections in mice and determined whether vaccination against Hla reduces disease severity. Compared with wild-type USA300 and Newman strains, isogenic *hla*-negative (Δ*hla*) strains caused significantly smaller skin lesions in a mouse infection model. Moreover, infection with wild-type strains produced dermonecrotic skin lesions, whereas there was little or no dermonecrosis in mice infected with Δ*hla* strains. Passive immunization with Hla-specific antisera or active immunization with a non-toxigenic form of Hla significantly reduced the size of skin lesions caused by USA300 and prevented dermonecrosis. We conclude Hla is a potential target for therapeutics or vaccines designed to moderate severe *S. aureus* skin infections.

Keywords

alpha-hemolysin; MRSA; skin infection; *Staphylococcus aureus*; vaccine

Introduction

S. aureus is a leading cause of infection in hospitals as well as in the community. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains typically cause infections in otherwise healthy individuals, and therefore, may be considered as highly virulent. This notion is supported by data from animal infection models, in which prominent CA-MRSA strains such as USA300 are more virulent than traditional hospital-associated strains [1,2]. The enhanced virulence phenotype of USA300 is likely due in part to relatively high expression of virulence factors such as phenol soluble modulins (PSMs) and alpha-hemolysin (α-hemolysin, α-toxin, Hla) [1]. Hla is a secreted pore-forming toxin that has cytolytic activity toward a variety of

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host cell types, including human keratinocytes, epithelial cells, and lymphocytes [3-8]. Expression of Hla is regulated at least in part by the two-component *agr* and *saeR/S* signal transduction systems [9].

Hla is lethal to animals, especially rodents and rabbits [10], and *S. aureus* strains deficient in *hla* have significantly reduced virulence in animal infection models [6,11-18]. Despite this previous work, the role of Hla in the pathogenesis of CA-MRSA infections was unknown until recently. Using USA300 and USA400 wild-type and isogenic *hla*-negative mutant (Δ*hla*) strains, Bubeck Wardenburg et al. demonstrated that Hla is essential for pathogenesis in a mouse model of CA-MRSA pneumonia [6,13]. Subsequent studies showed that vaccination against Hla protects mice from lethal USA300 or USA400 pneumonia [6,13]. More recent studies by Bartlett et al. demonstrated that Hla elicits production of CXC chemokines by host cells during experimental *S. aureus* pneumonia, thereby promoting severe lung inflammation [19].

Inasmuch as CA-MRSA infections are primarily those affecting the skin and soft tissues, we determined the role played by Hla in a mouse model of USA300 skin infection, and in turn, tested whether a vaccine approach directed at Hla moderated disease severity.

Methods

S. aureus strains and culture conditions

S. aureus strains used in this study were characterized previously [13]. To prepare inocula, *S. aureus* were cultured to mid-exponential phase of growth, washed twice in Dulbecco's phosphate buffered saline (DPBS), and diluted in DPBS to the appropriate concentration. Inocula were stored on ice until used.

Mouse skin infection model

We used a previously described mouse skin infection model [2,20,21]. Animal experiments were performed in accordance with a protocol approved by the IACUC at Rocky Mountain Laboratories, NIAID, NIH. Shaved female Balb/c mice were anesthetized with isoflurane and inoculated by subcutaneous injection in the right flank with 1×10^7 *S. aureus* in 50 μL of DPBS. Mice were weighed before inoculation and mass and abscess formation were monitored at 24-h intervals for 14 days. The size abscesses was calculated using a standard formula for area [$A = (\pi/2) \times l \times w$], as described previously [2,21,22]. Dermonecrosis was scored as present or absent (+ or −). Fifteen mice were used for each treatment (bacterial strain) or control group unless indicated otherwise.

Histological examination of mouse tissues

Mouse skin was harvested on day 1, 2, and 3 post inoculation and fixed in 10% neutral-buffered formalin for 48 h. Fixed tissues were dehydrated in a graded series of ethanol, cleared in xylene, infiltrated and embedded in Paraplast Extra (Thermo Fisher Scientific) following a routine 12-h schedule in a VIP Tissue Tek processor (Sakura). Tissue blocks were sectioned at 5 μm and slides stained with hematoxylin-eosin. Tissue samples were evaluated by an experienced veterinary pathologist (D.J.G.).

Passive immunization

Rabbit polyclonal Hla-specific antisera was generated using purified Hla_{H35L} as an immunogen as described previously [6]. Female Balb/c mice (~7 wks of age) received 100 μl of rabbit pre-immune serum or polyclonal Hla-specific rabbit antisera via i.p. injection 4 h before *S. aureus* challenge and 2 days after *S. aureus* challenge. Animal weight and abscess formation were monitored once per day for 14 days as noted above. Passive immunization with rabbit

anti-Hla sera typically results in circulating rabbit antibody titers of ~1:500, as detected by ELISA, which was shown previously to protect against the effects of staphylococcal alpha-hemolysin [6].

Active immunization

Female Balb/c mice (4 wks of age) were administered 20 μg of endotoxin free GST-Hla_{H35L} in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, MI) via intramuscular (i.m.) injection, followed by a boost with 20 μg of endotoxin free GST-Hla_{H35L} in incomplete Freund's adjuvant (IFA, Difco Laboratories) 10 days later. Animals were challenged with *S. aureus* 21 days after the initial immunization and monitored once per day for 14 days. Sera were collected on day 20 (24 hours prior to infection) to assess Hla-specific antibody titer by ELISA.

Anti-Hla ELISA

Blood was obtained from 5 control and 10 infected mice via submandibular bleeding as described by Golde et al. [23]. Mouse serum antibody titers were determined by ELISA using MaxiSorp microtiter plates (ThermoFisher Scientific) coated with 1 μg/ml Hla_{H35L} as described [6]. Antibody reactivity to Hla was detected with horseradish peroxidase-conjugated secondary antibodies and Opti-EIA (BD Biosciences) using a microplate spectrophotometer (GENios, Tecan).

Immunoblot analysis

S. aureus strains were cultured to early stationary phase of growth or overnight (late stationary phase of growth) and culture supernatants were harvested by centrifugation. Exoproteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with anti-Hla rabbit IgG (Sigma, St. Louis, MO), pre-immune rabbit sera or anti-Hla rabbit sera. Proteins were detected using donkey anti-rabbit secondary antibodies conjugated to horseradish peroxidase (GE Healthcare, Piscataway, NJ) and enhanced chemiluminescence (SuperSignal West Pico Detection kit, Pierce, Rockford, IL).

Statistical analyses

Statistics for abscess/lesion area were performed using a one-way analysis of variance (ANOVA) with a Dunnett's posttest (figure 1C) or 2-way ANOVA and Bonferroni's posttest as indicated. Student's t-test or one-way ANOVA and Dunnett's posttest were used to compare the number of mice with dermonecrosis over the course of 14 days (aggregate analysis). Results are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. Analyses were done using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, California) or SigmaPlot2001 for Windows version 7.0 (SPSS Inc., Chicago, IL).

Results

Expression of *hla* enhances virulence in skin and soft-tissue infections

As a first step toward understanding the role played by *hla* in USA300 skin infections, we compared skin abscess formation in mice following infection with LAC (USA300) or Newman (non-USA300) wild-type, isogenic *hla*-negative mutant (Δ*hla*), and Δ*hla*-complemented mutant strains (Δ*hla*::p*hla*) (figure 1). Infection of mice with LAC or Newman caused skin abscess or lesions that reached maximum size by day 5 or 6 (figure 1A and B). Mice infected with LACΔ*hla* had significantly smaller lesions than animals infected with the wild-type strain (figure 1A and C). Unexpectedly, there was no difference in lesion size between Newman wild-type or Δ*hla*-infected mice (figure 1B and C). Animals infected with the complemented mutant strain (LACΔ*hla*::p*hla*) had abscesses that were similar in appearance to those infected with

the wild-type strain, but lesions were significantly larger and took much longer to resolve by comparison (figure 1A-C). LACΔ*hla*::p*hla* and NewmanΔ*hla*::p*hla* expressed more Hla compared to the wild-type strains (reference [6] and data not shown), thereby providing an explanation for the larger abscess size observed in the mice infected with complemented mutant strains (figure 1). Consistent with differences in abscess size between wild-type and Δ*hla*-infected mice, animals infected with wild-type or complemented mutant strains in general lost more body mass over the course of the experiment than those infected with Δ*hla* strains (data not shown).

S. aureus skin infections in this animal model manifested as either raised abscesses/lesions or relatively flat dermonecrotic lesions (figure 2A and B). One of the more notable differences in lesion phenotype between wild-type and Δ*hla*-infected mice was in the formation of dermonecrotic skin lesions (figure 2A and B). Mice infected with wild-type strains had dermonecrotic lesions, whereas those infected with LACΔ*hla* and NewmanΔ*hla* had lesions with little or no dermonecrosis (figure 2B and C). Dermonecrosis was defined by necrosis of the epidermis and dermis, and significant numbers of PMNs and cell debris adjacent to this region (figure 2A). This finding was especially striking in mice infected with complemented mutant strains (figure 2B and C). Taken together, these data demonstrate that Hla contributes to the severity of USA300 skin infections.

Passive immunization with Hla-specific antisera reduces skin lesion size and severity

Inasmuch as Hla contributed to disease severity in the mouse skin infection model, we next determined if passive immunization with rabbit antisera directed against Hla would moderate severity of skin disease in infected mice. Previous studies demonstrated that intraperitoneal injection of Hla-specific rabbit antisera protects mice from lethal *S. aureus* pneumonia [6]. Therefore, we used this vaccination model to test whether passive immunization against Hla prevents or moderates severe skin infection. Skin lesions of mice infected with LAC or Newman were significantly smaller following passive immunization with Hla-specific rabbit antisera compared to those of mice that received pre-immune sera (figure 3A and B). Moreover, the clinical course of disease in immunized mice is altered, as the peak lesion size is reached by day 2, followed by gradual resolution of the abscess by days 12-14 (figure 3A and B). In contrast, mice treated with pre-immune sera prior to infection with LAC suffer progressive disease until day 5, after which resolution occurs by days 12-14 (figure 3A). Similar results were seen for mice infected with strain Newman (figure 3B). Immunized mice infected with LAC also lost less weight than those given pre-immune sera (data not shown).

Mice that received Hla-specific rabbit antisera either failed to develop dermonecrotic lesions following infection with either LAC or strain Newman or the area of dermonecrosis was limited (figure 4). Abscesses or lesions of wild-type infected mice given Hla-specific antisera developed skin lesions comparable in appearance and size to those infected with Δ*hla* strains in the absence of passive vaccination (compare figure 2 and figure 4). We note that rabbit pre-immune serum contains naturally occurring antibodies specific for *S. aureus*, and therefore, skin disease caused by wild-type strains in the presence of pre-immune serum was less severe compared to that in untreated animals (compare figure 2B and figure 4B).

Immunization with Hla_{H35L} moderates severity of USA300 skin infections

Previous studies have shown that a non-cytolytic mutant form of Hla (Hla_{H35L}) can be used as protective immunogen against *S. aureus* infections in animal infection models [6,17]. To determine whether active immunization with Hla_{H35L} protects mice from severe *S. aureus* skin infections, mice were immunized i.m. with Hla_{H35L} 21 days prior to infection with LAC or Newman. Mice immunized with Hla_{H35L} produced antibodies specific for the toxin as determined by ELISA (half-maximal anti-Hla antibody titers were 1:491 ± 49 and 1:596 ± 87

for each of two groups of 5 immunized mice tested, whereas anti-Hla was undetectable in 5 unimmunized mice). Correspondingly, *S. aureus* abscess size was reduced significantly in mice immunized with Hla_{H35L} (figure 5A and B). The modest or limited protective effect observed in mice infected with strain Newman may reflect a limited contribution of Hla to severity of skin disease caused by this strain (see figure 1B). In addition, there was little or no dermonecrosis in infected mice that had been immunized (figure 6), demonstrating that active immunization with Hla_{H35L} moderates severity of *S. aureus* skin infections.

Discussion

USA300 is the leading cause of community-associated bacterial infections in the United States [24]. In addition, USA300, which is epidemic in the United States, appears to have enhanced virulence compared to traditional hospital-associated MRSA strains [1,25]. Although the pathogen can cause severe or fatal invasive disease [26,27], the vast majority of USA300 infections are those of skin and soft tissue [28,29]. There has been an intense effort to better understand mechanisms of USA300 virulence and transmission (reviewed in refs. [30-33]). Recent work indicates Hla is produced by USA300 at relatively high levels in vitro [34], and studies in animal models revealed a prominent role for this secreted toxin in the pathogenesis of USA300 pneumonia [6,13]. However, the contribution of Hla to the severity of USA300 skin infections had not been evaluated until now.

Herein we demonstrate that Hla contributes significantly to the severity of USA300 skin infections in a mouse model. Compared with strain Newman, the contribution of Hla to disease severity was more pronounced in mice infected with USA300 (LAC) (figure 1 and figure 3). LAC is a widely used clinical isolate representative of the USA300 epidemic clone [12,25], whereas Newman is a methicillin-susceptible *S. aureus* strain originally isolated from a case of secondarily infected osteomyelitis in 1952 [35]. Newman expresses less Hla compared to LAC (ref. [6] and data not shown), and consistent with this observation, deletion of Hla from Newman minimally changed the size of skin lesions (figure 1B). These data are in accordance with the recent studies of Li et al. [1], which indicate that differential expression of virulence molecules rather than presence or absence in the core genome dictates (at least in part) differences in virulence among *S. aureus* strains. Thus, the findings here suggest factors other than Hla in strain Newman play a more prominent role in the pathogenesis of skin infections, whereas the toxin is a major determinant of severity in USA300 skin infections. It is also evident that multiple *S. aureus* molecules contribute to skin infections in general, since deletion or neutralization of Hla did not completely ablate formation of abscesses.

Previous studies have not attempted to correlate expression of Hla with severity of skin infections. However, production of Hla was recently shown to correlate with refractory *S. aureus* skin colonization in patients with atopic dermatitis [36]. Inasmuch as *hla* is present in the genome of many *S. aureus* strains, it is likely the toxin contributes to severity of human *S. aureus* infection, a notion that has been confirmed in animal infection models [6,11,13,14, 17,37]. Although the mouse skin infection model cannot mimic all of the features of human skin infection, such as the size of inoculum, the primary readouts of our mouse skin infection model (skin necrosis and abscess size) are two parameters used to classify severe human skin and soft tissue infections [38]. Moreover, USA300 is known to cause necrotizing skin and soft tissue infections in humans [26]. Therefore, it is reasonable to conclude that the mouse skin infection model described herein can be used as a general or rudimentary approximation of *S. aureus* skin infection in humans.

There is also little information on anti-Hla antibody titers in human skin infections. In 1962, Lack and Towers reported that only 48% of patients with proven *S. aureus* infection had relatively high anti-Hla antibody titers [39]. More notably, anti-Hla antibody titers did not

consistently increase in patients with *S. aureus* infections, and when they did increase following acute infection, antibody levels then decreased rapidly over time [39]. These early observations suggest humans may lack or have limited protection against Hla, albeit the anamnestic antibody response to Hla should be robust in individuals re-infected with Hla-producing *S. aureus*. The passive and active immunization data presented here suggest Hla is a potential target for vaccines or therapeutics designed to moderate the severity of *S. aureus* skin infections. Inasmuch as Hla is a core genome-encoded toxin present in virtually all CA-MRSA strains, a therapeutic approach directed at Hla would be relatively broad in scope.

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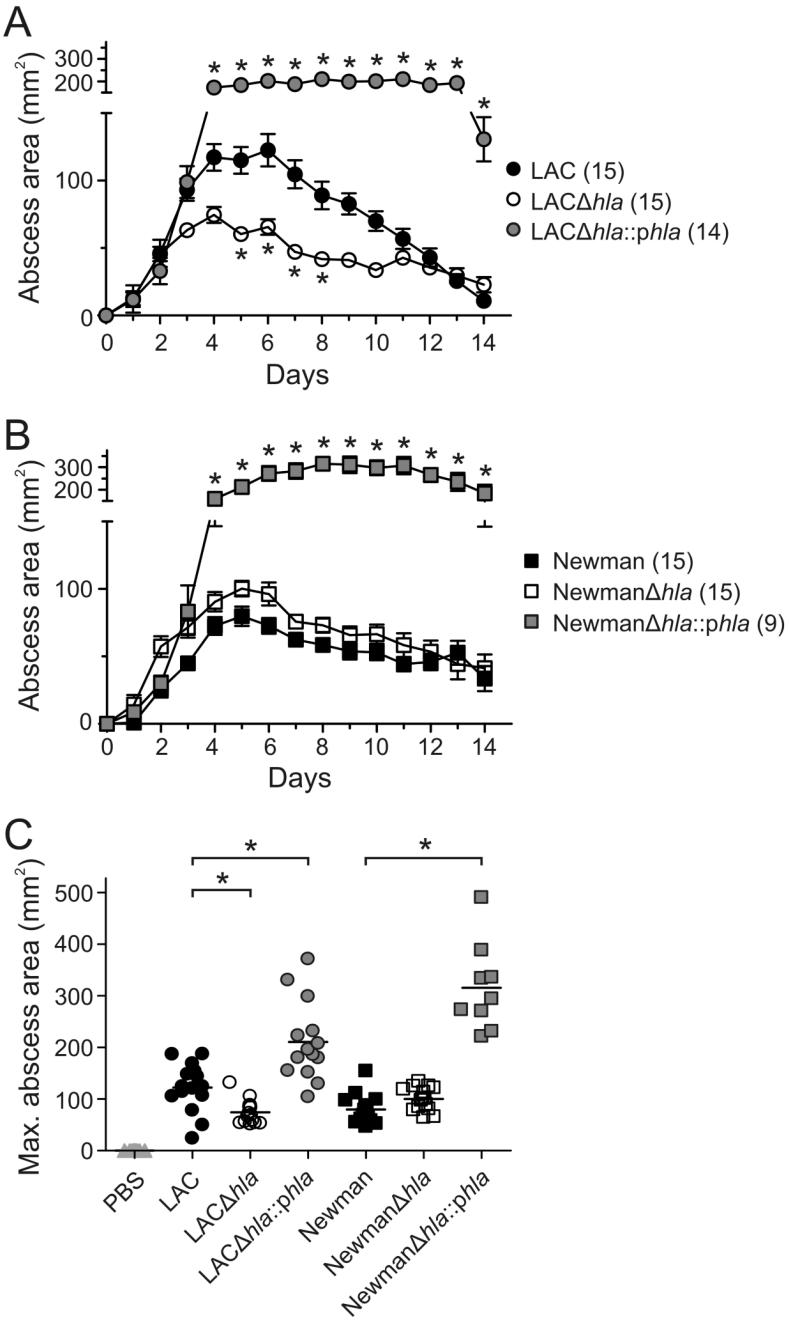

Figure 1.

hla contributes to the pathogenesis of USA300 skin infections. Results are the mean +/- SEM for all groups; (n) = number of mice per group. *A* and *B*, Mouse abscess size was monitored once per day after subcutaneous infection with 1×10^7 of the indicated bacteria. *, $p < 0.05$ versus wild-type LAC or Newman strains using a two-way ANOVA and Bonferroni's posttest. *C*, Size of abscesses at maximum. *, $p < 0.05$ using an ANOVA and Dunnett's posttest.

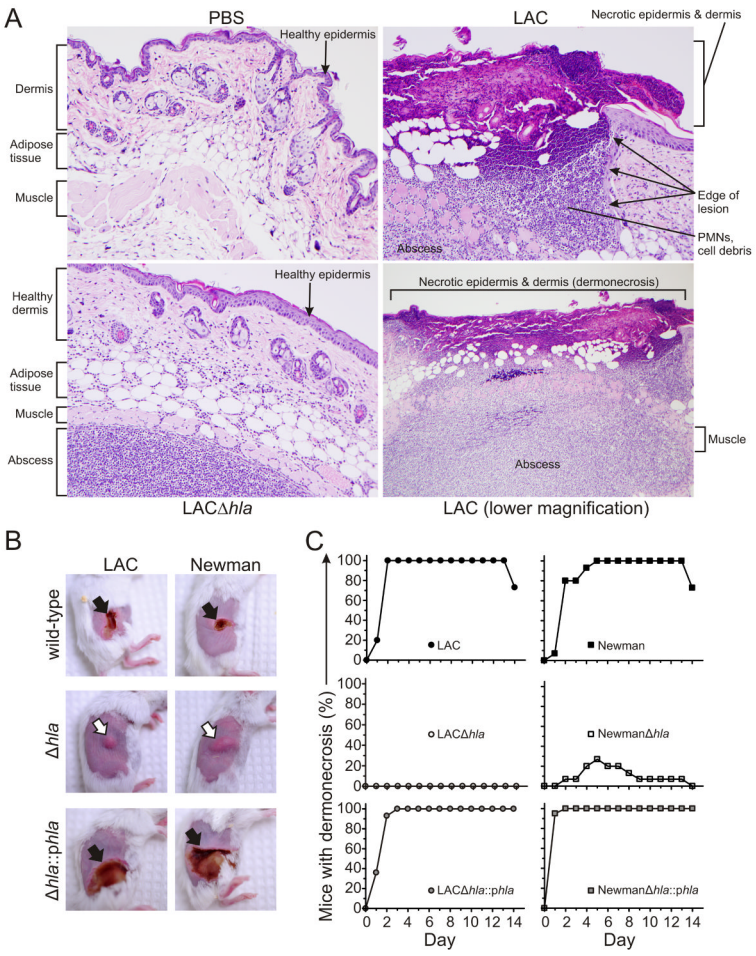

Figure 2.

hla promotes dermonecrosis. *A*, Representative histological sections showing normal mouse skin tissue (PBS), mouse USA300 abscess with dermonecrosis (LAC), and an *hla*-deficient USA300 abscess without dermonecrosis (LACΔ*hla*). Mice were inoculated s.c. with either PBS, LAC, or LACΔ*hla* as described in Methods and skin was harvested on day 3. Original magnification of images labeled as PBS, LAC and LACΔ*hla* is ×200; that for LAC (lower magnification) is ×100. *B*, Representative mouse skin lesions (day 5). Black arrows indicate dermonecrosis and white arrows indicate abscess formation without dermonecrosis. *C*, Percentage of mice per group that had dermonecrosis on each day. $p < 0.001$ for mice infected with either LAC or Newman wild-type or complemented mutant strains versus Δ*hla* strains over the 14-day time course. Data were analyzed using a one-way ANOVA and Dunnett's posttest.

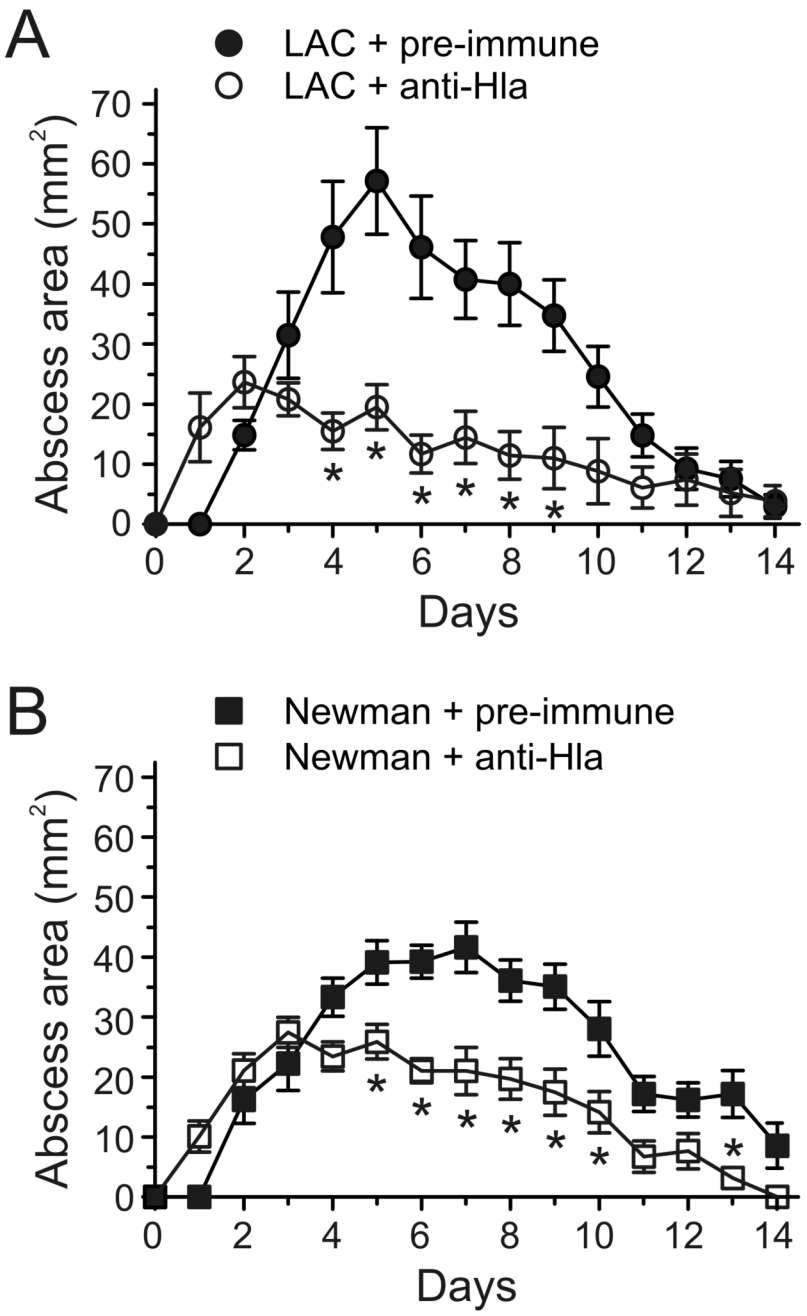

Figure 3.

Passive immunization with Hla-specific rabbit anti-sera (anti-Hla) reduces size of lesions caused by USA300 or Newman. *A* and *B*, Mice received 100 μl of pre-immune rabbit sera (Pre-immune) or Hla-specific rabbit anti-sera (anti-Hla) 4 h before subcutaneous infection with 1 × 10^7 with LAC or Newman and on day 2 post-infection. Results are the mean +/- SEM for all groups; n = 15 mice per group. *, $p < 0.05$ versus wild-type LAC or Newman strains using a two-way ANOVA and Bonferroni's posttest.

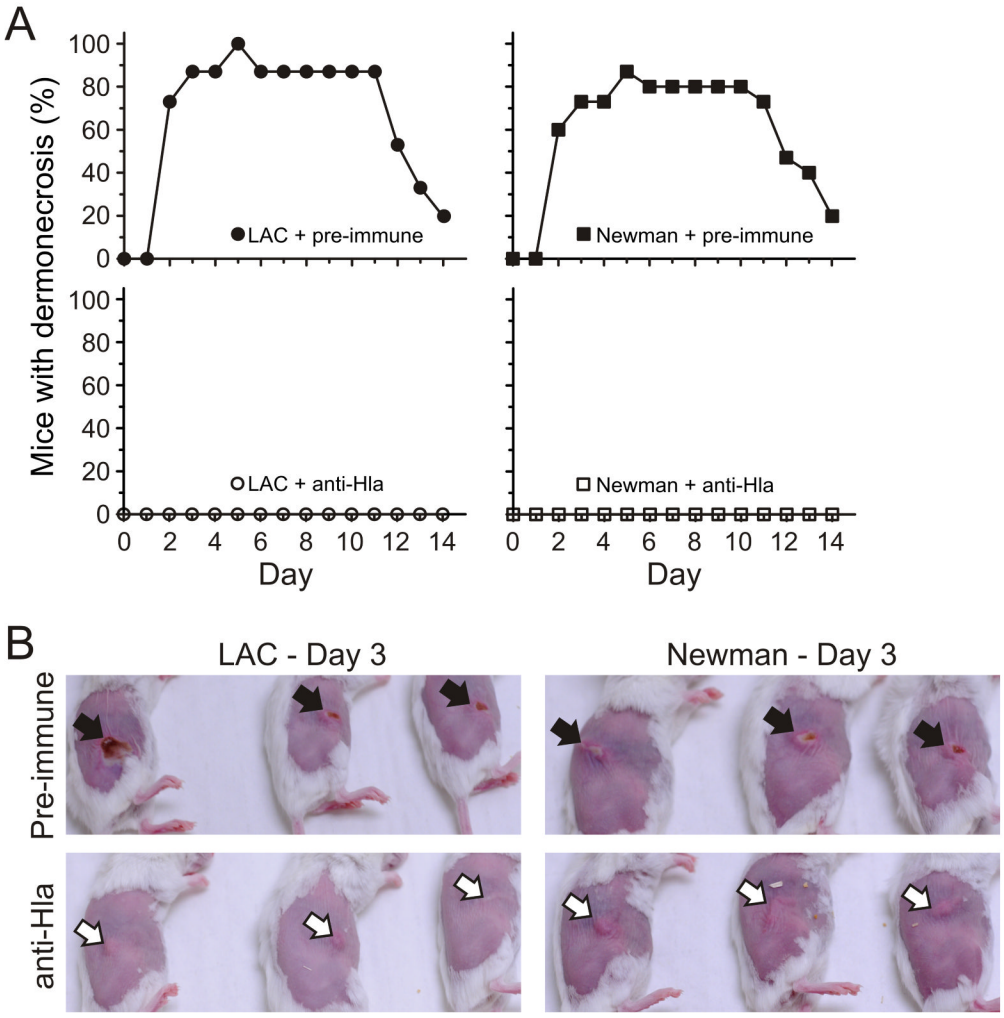

Figure 4.

Passive immunization with Hla-specific rabbit anti-sera prevents dermonecrosis. *A*, Percentage of mice per group that had dermonecrosis on each day. $p < 0.0001$ for mice administered pre-immune versus anti-Hla serum following infection with either LAC or Newman over the 14-day time course. *B*, Representative skin lesions of mice on day 3 for each of the treatment conditions. Passive immunization was performed as described in Methods and the legend of figure 3. Pre-immune rabbit sera (Pre-immune); Hla-specific rabbit anti-sera (anti-Hla). Black arrows indicate dermonecrosis and white arrows indicate abscess formation without dermonecrosis.

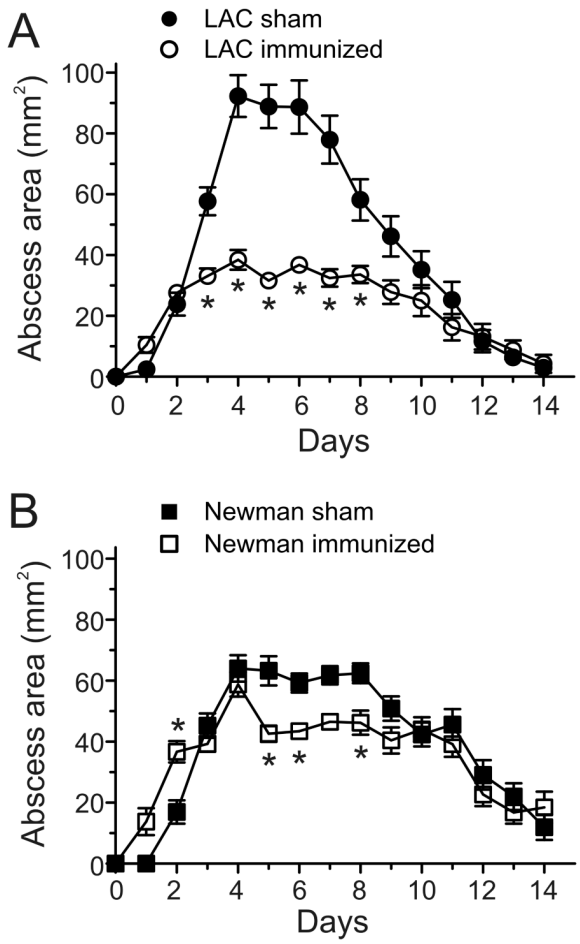

Figure 5.

Active immunization with Hla_{H35L} decreases size of abscesses caused by USA300 or Newman. Mice were injected i.m. with CFA + DPBS at 4 wks of age followed by IFA + DPBS 10 days later (sham) or CFA + Hla_{H35L} at 4 wks of age and IFA + Hla_{H35L} 10 days later (immunized). *A* and *B*, Abscess formation was monitored once per day after subcutaneous infection with 1×10^7 of the indicated bacteria 21 days after primary immunization. Results are the mean +/- SEM and n = 15 mice per group. *, $p < 0.05$ versus wild-type LAC or Newman strains using a two-way ANOVA and Bonferroni's posttest.

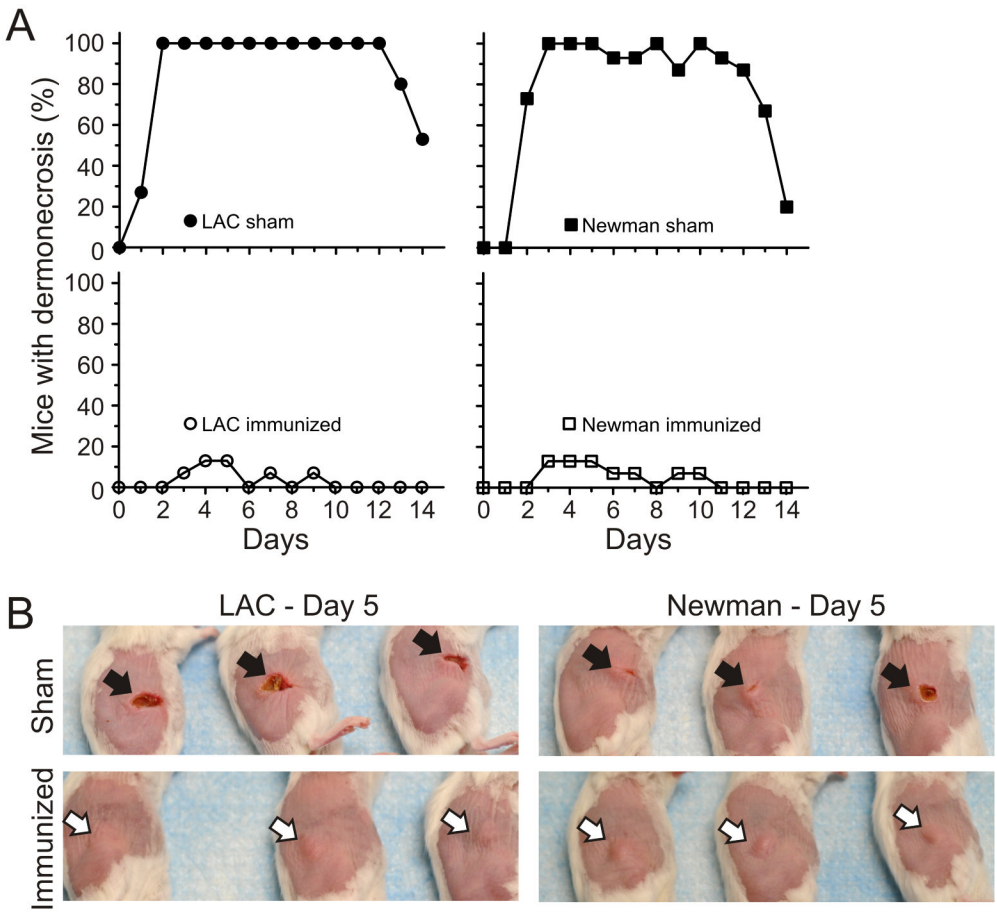

Figure 6.

Active immunization with Hla_{H35L} prevents dermonecrosis caused by USA300 or Newman skin infections. *A*, Percentage of mice per group that had dermonecrosis on each day. Immunization is described in the legend of figure 5. $p < 0.0001$ for sham versus immunized mice following infection with either LAC or Newman over the 14-day time course. *B*, Representative mouse skin lesions, day 5. Black arrows indicate dermonecrosis and white arrows indicate abscess formation without dermonecrosis.