Nontoxigenic protein A vaccine for methicillin-resistant \textit{Staphylococcus aureus} infections in mice

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The current epidemic of hospital- and community-acquired methicillin-resistant \textit{Staphylococcus aureus} (MRSA) infections has caused significant human morbidity, but a protective vaccine is not yet available. Prior infection with \textit{S. aureus} is not associated with protective immunity. This phenomenon involves staphylococcal protein A (SpA), an \textit{S. aureus} surface molecule that binds to Fcγ of immunoglobulin (Ig) and to the Fab portion of \(V_{\gamma3}\)-type B cell receptors, thereby interfering with opsonophagocytic clearance of the pathogen and abating adaptive immune responses. We show that mutation of each of the five Ig-binding domains of SpA with amino acid substitutions abolished the ability of the resulting variant SpAKKAA to bind Fcγ or Fab \(V_{\gamma3}\) and promote B cell apoptosis. Immunization of mice with SpAKKAA raised antibodies that blocked the virulence of staphylococci, promoted opsonophagocytic clearance, and protected mice against challenge with highly virulent MRSA strains. Furthermore, SpAKKAA immunization enabled MRSA-challenged mice to mount antibody responses to many different staphylococcal antigens.

\textit{Staphylococcus aureus} is the leading cause of bloodstream, lower respiratory tract, skin, and soft tissue infections in the United States (Klevens et al., 2007). Methicillin-resistant \textit{S. aureus} (MRSA) strains are isolated in more than half of all community and hospital infections (Klevens et al., 2008). MRSA strains harbor methicillin resistance genes, rendering the entire class of \(\beta\)-lactam antimicrobials obsolete as therapeutic agents (Berger-Bächi, 1994). Some MRSA isolates also acquired resistance to vancomycin, the antibiotic of last resort. These strains threaten a return to the preantibiotic era (Chang et al., 2003). Thus, there is an urgent need for vaccines that protect against staphylococcal infection.

\textit{S. aureus} infection in humans is not associated with the generation of protective immunity, as patients often suffer recurrent bouts of skin and soft tissue infections (Lowy, 1998). Recent advances described several mechanisms for staphylococcal escape from innate host defenses (de Haas et al., 2004; Rooijakkers et al., 2005; Thammavongsa et al., 2009); however, the molecular events underlying the escape from adaptive immune responses during staphylococcal infection are not known. Human diseases caused by \textit{S. aureus} can be recapitulated in animals. In particular, experimental infections of the lung, skin, or soft tissues and internal organs have been established in mice (Bubeck Wardenburg et al., 2008; Cheng et al., 2009). Using these models and molecular genetics approaches, staphylococcal protein A (SpA), a cell wall–anchored surface protein (Sjöquist et al., 1972), was identified as a crucial virulence factor for lung infections, sepsis, and abscess development (Palmqvist et al., 2002; Gómez et al., 2004; Cheng et al., 2009).

The vast majority of clinical \textit{S. aureus} isolates express SpA (Forsgren, 1970; Shopsin et al., 1999), which binds to the Fcγ portion of most Ig subclasses (Jensen, 1958; Lindmark et al., 1983), \(V_{\gamma3}\) type B cell receptors (Sasso et al., 1989), von Willebrand factor (vWF; Harleib et al., 2000), and TNFR1 (Gómez et al., 2004). Interaction of SpA with B cell receptors (IgM) leads to clonal expansion and subsequent cell death of B cell populations with effects on adaptive and innate immune responses (Forsgren and Quie, 1974; Forsgren et al., 1976; Goodyear and Silverman, 2004;
RESULTS AND DISCUSSION

SpA is a virulence factor for lethal S. aureus infections

The contribution of the spa gene toward lethal S. aureus challenge has thus far not been appreciated. To address this, we generated the isogenic spa deletion variant S. aureus NewmanΔspa. After intraperitoneal challenge with 2 × 10^8 CFU of wild-type S. aureus Newman, 60% of animals succumbed to challenge. In contrast, animals infected with the isogenic mutant resulted in only 25% mortality (Fig. S1 A). In addition, the spa mutant displayed a consistent survival defect when examined in naive mouse blood (see Fig. 3 D). These results suggest that SpA is a crucial virulence factor for lethal infections of S. aureus in mice.

SpA-DKKAA cannot bind to immunoglobulin and trigger B cell apoptosis

Guided by amino acid homology, the triple α-helical bundle structure of Ig binding domains (Deisenhofer, 1981), and their atomic interactions with Fab V\textsubscript{H}3 (Graille et al., 2000) or Fc\textgamma (Gouda et al., 1998), we selected glutamine 9 and 10, as well as aspartate 36 and 37, as critical for the association of SpA with immunoglobulin (Fig. 1, A and B; and Fig. S2, A and B). To test this, substitutions Gln 9Lys, Gln10Lys, Asp36Ala, and Asp37Ala were introduced into the D domain to generate SpA-DKKAA (Fig. 1 B). The ability of isolated SpA-D or SpA-DKKAA to bind human IgG or IgM was analyzed by affinity chromatography and ELISA (Fig. 1, C and D). Polyhistidine-tagged SpA-D, as well as full-length SpA, retained human IgG on Ni-NTA, whereas SpA-DKKAA or a negative control (sortase A; Mazmanian et al., 1999) did not (Fig. 1, C and D). A similar result was observed with vWF (Hartleib et al., 2000), which, along with TNFR1 (Gómez et al., 2004), can also bind SpA via glutamines 9 and 10 (Gómez et al., 2006; Fig. 1 D). Human Ig encompasses ~50% V\textsubscript{H}3-type IgG (Cook and Tomlinson, 1995). Human Fc\textgamma and F(ab\textsubscript{2}) fragments, as well as IgM, all...
Table I. Active immunization of mice with SpA vaccines

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<th>Antigen</th>
<th>Staphylococcal load and abscess formation in renal tissue</th>
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<td></td>
<td>Log10 CFU g⁻¹a</td>
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<td><strong>S. aureus Newman challenge</strong></td>
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<tr>
<td>Mock</td>
<td>6.46 ± 0.25</td>
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<tr>
<td>SpA</td>
<td>3.95 ± 0.56</td>
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<tr>
<td>SpA-D</td>
<td>4.43 ± 0.41</td>
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<tr>
<td>SpA-D KKAA</td>
<td>3.39 ± 0.50</td>
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<tr>
<td><strong>S. aureus USA300 (LAC) challenge</strong></td>
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<tr>
<td>Mock</td>
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<tr>
<td>SpA</td>
<td>6.81 ± 0.26</td>
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<tr>
<td>SpA-D</td>
<td>6.34 ± 0.52</td>
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<tr>
<td>SpA-D KKAA</td>
<td>6.00 ± 0.42</td>
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<tr>
<td>SpA-D GGSS</td>
<td>3.66 ± 0.76</td>
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aMeans ± SEM of staphylococcal load calculated as log10 CFU g⁻¹ in homogenized renal tissues 4 d after infection in cohorts of 15–20 BALB/c mice per immunization. A representative of three independent and reproducible animal experiments is shown.
bStatistical significance was calculated with the unpaired two-tailed Students t test and p-values were recorded.
cReduction in bacterial load calculated as log10 CFU g⁻¹.
dMeans ± SEM of five randomly chosen serum IgG titers were measured before staphylococcal infection by ELISA using SpA-D KKAA or SpA-D GGSS as antigens.
eHistopathology of hematoxylin-eosin–stained thin-sectioned kidneys from 10 animals. The mean number of abscesses per kidney was recorded and averaged again for the final mean ± SEM.

Table I

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Antibodies against SpA-D KKAA protect against MSSA and MRSA infections

Naive BALB/c mice were injected with 50 µg each of purified SpA, SpA-D, or SpA-D KKAA emulsified in CFA and boosted with the same antigen emulsified in IFA. IgG responses to immunization were examined by ELISA with SpA-D GGSS and SpA-D KKAA. SpA-D GGSS harbors amino acid substitutions at the same positions as SpA-D KKAA, however, glutamines 9 and 10 were each replaced with glycine and aspartic acids 36 and 37 with serine. Similar to SpA-D KKAA, SpA-D GGSS does not interact with human IgG (unpublished data). It is of note that similar antibody titers were measured with SpA-D GGSS and SpA-D KKAA antigen, indicating that the four amino acid substitutions do not diminish the reactivity of antibodies raised with heterologous antigens (IgG titers against SpA-D KKAA vs. SpA-D GGSS, P = 0.8315). After immunization of mice with either SpA-D or SpA-D KKAA, we observed a 10-fold higher titer of SpA-specific antibodies for the nontoxicogenic variant as compared with the B cell superantigen (P < 0.0001; Table I). Antibody titers raised by immunization with full-length SpA were higher than those elicited by SpA-D (P = 0.0022), which is likely a result of the larger size and iterative domain structure of this antigen (Table I). Nevertheless, even SpA elicited lower antibody titers than SpA-D KKAA (P = 0.0003), which encompasses only 50 aa of the mature 520-residue protein. Immunized mice were challenged by intravenous inoculation with *S. aureus* Newman, and the ability of staphylococci to seed abscesses in renal tissues was examined by necropsy 4 d after challenge (Cheng et al., 2009). In homogenized renal tissue of mock (PBS/adjuvant) immunized mice, a mean staphylococcal load of 6.46 log10 CFU g⁻¹ was enumerated (Table I). Immunization of mice with SpA or SpA-D led to a reduction in staphylococcal load; however, SpA-D KKAA–vaccinated animals displayed an even greater 3.07 log10 CFU g⁻¹ reduction of *S. aureus* Newman in renal tissues (P < 0.0001; Table I). Abscess formation in kidneys was analyzed by histopathology (Fig. S3 A–H). Mock immunized animals harbored a mean of 3.7 (±1.2) abscesses per kidney (Table I). Vaccination with SpA-D KKAA reduced the mean number of abscesses to 0.5 (±0.4; P = 0.0204), whereas immunization with SpA or SpA-D did not cause a significant reduction in the number of abscess lesions (Table I). Lesions from SpA-D KKAA–vaccinated animals were smaller in size, with fewer infiltrating PMNs, and characteristically lacked staphylococcal abscess communities (Cheng et al., 2009; Fig. S3, A–H). Abscesses in animals that had been immunized with SpA or SpA-D displayed the same overall structure of lesions in mock immunized animals (Fig. S3, A–H).

We wondered whether SpA-D KKAA immunization could protect mice against MRSA strains and selected the USA300...
SpA-DKKAA antibodies neutralize immunoglobulin binding activities of SpA

Rabbits were immunized with SpA-DKKAA, and specific antibodies were purified on SpA-DKKAA affinity column and analyzed by Coomassie blue-stained SDS-PAGE under reducing conditions (lane 1, α-SpA-DKKAA). SpA-DKKAA antibodies were treated with pepsin to separate Fc and F(ab)2 (lane 2). The resulting F(ab)2 fragments were repurified by affinity chromatography on SpA-DKKAA (lane 3). (B and C) SpA-DKKAA specific F(ab)2 was added to wild-type SpA, SpA-D, or SpA-DKKAA, and the association with human IgG (B; n = 3) or vWF (C; n = 3) was measured. Data are the means and error bars represent ±SEM. Results in A–C are representative of three independent analyses. *, P < 0.01.

Figure 3. Full-length nontoxigenic SpA elicits antibodies that stimulate opsonophagocytic clearance of staphylococci. (A) Full-length SpA-KAA was purified on Ni-NTA sepharose and analyzed by Coomassie blue-stained SDS-PAGE. (B) ELISA examining the association of immobilized SpA or SpA-DKKAA with human IgM, IgG, and its Fc or F(ab)2, fragments or vWF (n = 3). *, P < 0.01. (C) CD19+ B lymphocytes in splenic tissue of 6-wk-old BALB/c mice (n = 3) that had been mock immunized or treated with SpA or SpA-DKKAA were quantified by FACS. (D, Left) Anticoagulated mouse whole blood mouse whole blood (lepirudin) was incubated with 5 × 10^6 CFU S. aureus Newman or its isogenic spa variant and survival measured (n = 3). (D, Right) Opsonophagocytic clearance of staphylococci (5 × 10^4, 5 × 10^5, or 5 × 10^6 CFU) was measured in the presence of affinity-purified V10 (αV10) or SpA-DKKAA-specific antibodies in naive mouse whole blood whole blood (n = 3). *, P < 0.05. Data are the means and error bars represent ±SEM. Results in A–D are representative of three independent analyses.

SpA-DKKAA antibodies neutralize immunoglobulin binding activities of SpA

Rabbits were immunized with SpA-DKKAA, and specific antibodies were purified on SpA-DKKAA affinity column and analyzed by SDS-PAGE (Fig. 2 A, lane 1). SpA-DKKAA-specific IgG was cleaved with pepsin to generate Fc and F(ab)2 fragments (Fig. 2 A, lane 2), the latter of which were repurified by affinity chromatography on SpA-DKKAA column (Fig. 2 A, lane 3). Binding of human IgG or vWF to SpA or SpA-D was perturbed by SpA-DKKAA-specific F(ab)2, indicating that SpA-DKKAA-derived antibodies can block the Fcγ- and vWF-binding properties of SpA (Fig. 2 B and C).

SpA-KAA generates improved protective immune responses

To further improve the vaccine properties for nontoxicogenous SpA, we generated SpA-DKKAA, which includes all five Ig binding domains with four amino acid substitutions—Gln18Lys, Gln19Lys, Asp38Ala, and Asp39Ala—in each of its five domains (E, D,
A representative of three independent and reproducible animal experiments is shown.

Means ± SEM of five randomly chosen serum IgG titers were measured before staphylococcal infection by ELISA.

Reduction in bacterial load calculated as log10 CFU g⁻¹.

Statistical significance was calculated with the unpaired two-tailed Students t test and p-values were recorded.

Affinity-purified antibodies were injected into the peritoneal cavity of BALB/c mice at a concentration of 5 mg × kg⁻¹ body weight into the peritoneal cavity of BALB/c mice (Table II). 24 h later, antibody titers specific for SpA-DKKAA/SpAKKAA were determined in serum and animals challenged by intravenous inoculation with S. aureus Newman. Passive transfer reduced the staphylococcal load in kidney tissues for SpA-DKKAA⁻ (P = 0.0016) or SpAKKAA⁻ (P = 0.0005)-specific antibodies. On histopathology examination, both antibodies reduced the abundance of abscess lesions in kidneys of mice challenged with S. aureus Newman (Table II). Compared with control cohorts treated with nonspecific antibody (α-V10), animals that had been injected with SpA-specific antibodies were protected against the B cell superantigen activity of SpA (Fig. S2, C and D). In addition, SpA-specific antibodies (2 µg × ml⁻¹) induced opsonophagocytic clearance of S. aureus Newman inoculated into naive mouse blood (Fig. 3 D) and reduced the mortality associated with lethal staphylococcal challenge (Fig. S1 C). Together these data reveal that disease protection after immunization with SpA-DKKAA⁻ or SpAKKAA⁻ is conferred by antibodies that bind SpA and neutralize its ability to bind Ig.

SpA-DKKAA⁻ immunization promotes host antibody response to staphylococcal infection

After infection with virulent S. aureus Newman and clearance of the pathogen with antibiotic treatment, mice do not develop protective immunity against subsequent infection with the same strain (Fig. S3 S). The mean abundance of SpA-DKKAA⁻–specific IgG in these animals was determined by dot blot at 0.20 µg ml⁻¹ (±0.04) and 0.14 µg ml⁻¹ (±0.01) for infections caused by S. aureus strains Newman and USA300 LAC, respectively (Fig. 4 A). A concentration of 4.05 µg ml⁻¹ (±0.88) for SpA–specific IgG was estimated to confer disease protection in SpA-DKKAA⁻ or SpA-DKKAA⁻–immunized mice (P ≤ 0.05 log₁₀ reduction in staphylococcal CFU g⁻¹ renal tissue; unpublished data). The mean serum concentration of SpA–specific IgG in adult healthy human volunteers (n = 16) was 0.21 µg ml⁻¹ (±0.02). Such antibody concentration may not be sufficient to generate protection against staphylococcal infection.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Staphylococcal load and abscess formation in renal tissue</th>
<th>Log₁₀ CFU g⁻¹</th>
<th>P value</th>
<th>Reduction (log₁₀ CFU g⁻¹)</th>
<th>IgG titer</th>
<th>Number of abscesses</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Mock (α-V10)</td>
<td></td>
<td>7.10 ± 0.14</td>
<td>N/A</td>
<td>N/A</td>
<td>&lt;100</td>
<td>4.5 ± 0.8</td>
<td>N/A</td>
</tr>
<tr>
<td>α-SpA⁻DKKAA</td>
<td></td>
<td>5.53 ± 0.43</td>
<td>0.0016</td>
<td>1.57</td>
<td>466 ± 114</td>
<td>1.9 ± 0.7</td>
<td>0.0235</td>
</tr>
<tr>
<td>α-SpAKKAA</td>
<td></td>
<td>5.69 ± 0.34</td>
<td>0.0005</td>
<td>1.41</td>
<td>1,575 ± 152</td>
<td>1.6 ± 0.5</td>
<td>0.0062</td>
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A, B, and C; Sjödahl, 1977. Polyhistidine-tagged SpA⁻KKA was purified by affinity chromatography and analyzed by Coomassie blue-stained SDS-PAGE (Fig. 3 A). Unlike full-length SpA, SpA⁻KKA did not bind human IgG, Fc and F(ab)₂, IgM, or vWF (Fig. 3 B). SpA⁻KKA failed to display B cell superantigen activity, as injection of the variant into BALB/c or C57BL/6 mice did not cause a depletion of both CD19⁺ and CD45R⁺ B cells in splenic tissue (Fig. 3 C; Fig. S2, C and D; and not depicted). SpA⁻KKA immunization generated higher specific antibody titers than SpA⁻DKKA⁻ and provided mice with elevated protection against S. aureus USA300 (Table I) or Mu50 challenge (Fig. S3 U; Mu50 is a Japanese MRSA isolate). 4 d after challenge, SpA⁻KKA⁻–vaccinated animals harbored 3.54 log₁₀ CFU g⁻¹ fewer staphylococci in renal tissues (P = 0.0001) and also caused a greater reduction in the number of abscess lesions (P = 0.0199; Table I; and Fig. S3, F–R). Furthermore, SpA⁻KKA⁻ immunization reduced the mortality of mice that received a lethal S. aureus challenge dose (Fig. S1 B).

Staphylococci persist in mouse organ tissues (Cheng et al., 2009). Over time, abscesses harboring communities of the infectious agent increase in size and eventually rupture, thereby releasing staphylococci into circulation. This initiates the formation of new abscesses and precipitates lethal outcomes 30–60 d after challenge (Cheng et al., 2009). We asked whether SpA⁻DKKA⁻ or SpA⁻KKA⁻ immunization prevents staphylococcal replication over a longer period of time. 15 d after challenge with S. aureus USA300, immunization with SpA did not generate significant protection of animals compared with the mock control (P = 0.5817; Table S1). In contrast, immunization with SpA⁻DKKA⁻ caused a 1.56 log₁₀ CFU g⁻¹ reduction (P = 0.0183), whereas SpA⁻KKA⁻ vaccination reduced the load by 2.7 log₁₀ CFU g⁻¹ (P = 0.0059). These data suggest that antibodies against SpA, generated via active immunization using nontoxigenic SpA⁻KKA, can interfere with bacterial persistence in host tissues.

SpA⁻KKA⁻ immunization promotes host antibody response to staphylococcal infection

After infection with virulent S. aureus Newman and clearance of the pathogen with antibiotic treatment, mice do not develop protective immunity against subsequent infection with the same strain (Fig. S3 S). The mean abundance of SpA⁻DKKA⁻–specific IgG in these animals was determined by dot blot at 0.20 µg ml⁻¹ (±0.04) and 0.14 µg ml⁻¹ (±0.01) for infections caused by S. aureus strains Newman and USA300 LAC, respectively (Fig. 4 A). A concentration of 4.05 µg ml⁻¹ (±0.88) for SpA–specific IgG was estimated to confer disease protection in SpA⁻KKA⁻ or SpA⁻DKKA⁻–immunized mice (P ≤ 0.05 log₁₀ reduction in staphylococcal CFU g⁻¹ renal tissue; unpublished data). The mean serum concentration of SpA–specific IgG in adult healthy human volunteers (n = 16) was 0.21 µg ml⁻¹ (±0.02). Such antibody concentration may not be sufficient to generate protection against staphylococcal infection.

SpA⁻KKA was used to immunize rabbits. Rabbit antibodies specific for SpA⁻DKKA⁻ or SpA⁻KKA⁻ were affinity purified on matrices with immobilized cognate antigen and injected at a concentration of 5 mg × kg⁻¹ body weight into the peritoneal cavity of BALB/c mice (Table II). 24 h later, antibody titers specific for SpA⁻DKKA⁻/SpA⁻KKA⁻ were determined in serum and animals challenged by intravenous inoculation with S. aureus Newman. Passive transfer reduced the staphylococcal load in kidney tissues for SpA⁻DKKA⁻ (P = 0.0016) or SpA⁻KKA⁻ (P = 0.0005)-specific antibodies. On histopathology examination, both antibodies reduced the abundance of abscess lesions in kidneys of mice challenged with S. aureus Newman (Table II). Compared with control cohorts treated with nonspecific antibody (α-V10), animals that had been injected with SpA–specific antibodies were protected against the B cell superantigen activity of SpA (Fig. S2, C and D). In addition, SpA–specific antibodies (2 µg × ml⁻¹) induced opsonophagocytic clearance of S. aureus Newman inoculated into naive mouse blood (Fig. 3 D) and reduced the mortality associated with lethal staphylococcal challenge (Fig. S1 C). Together these data reveal that disease protection after immunization with SpA⁻DKKA⁻ or SpA⁻KKA⁻ is conferred by antibodies that bind SpA and neutralize its ability to bind Ig.
infection. By comparison, the mean serum concentration of IgG specific for diphtheria toxin in human volunteers, 0.68 µg ml⁻¹ (± 0.20), is thought to be within range for protective immunity against diphtheria (Lagergård et al., 1992).

These results are in agreement with a model of immune evasion during S. aureus infection. Cell wall–anchored or secreted SpA (e.g., 20% of peptidoglycan and attached surface protein is released during bacterial division; Ton-That et al., 1999) activate B cells via IgM receptor cross-linking. Without stimuli from specific antigens, activated B cells undergo apoptosis, thereby hindering the production of antibody against staphylococcal antigens. If so, neutralizing antibodies directed against SpA may enable humoral immune responses against many different staphylococcal antigens. This was tested by immunizing BALB/c mice with SpA or an adjuvant (aluminum hydroxide) control, followed by intravenous challenge with a sublethal dose of MRSA strain USA300. Serum samples were withdrawn 30 d after MRSA challenge and then analyzed by immunoblotting with 27 staphylococcal antigens immobilized on a membrane filter (Fig. 4 B). Naive mice, which had not been infected with the MRSA strain USA300 LAC, did not harbor antibodies against staphylococcal antigens (unpublished data). Mock immunized mice (adjuvant only) that had been subjected to USA300 infection developed high-titer antibodies against the Eap protein as well as low-titer antibodies against Hla, IsdA, IsdB, LukD, LukE, and LukF (Fig. 4 B). In response to USA300 challenge, animals that had been immunized with SpA or an adjuvant (aluminum hydroxide) control mounted humoral immune responses against every antigen examined (Fig. 4 B). With the exception of Eap, IsdA, and IsdB antibodies, the serum of SpA-immunized animals harbored higher antibody titers against staphylococcal antigens as compared with mice that had been naive at the time of challenge (Fig. 4 B).

In summary, S. aureus isolates express SpA, an essential virulence factor whose B cell superantigen activity and evasive attributes toward opsonophagocytic clearance are required for staphylococcal abscess formation and the establishment of lethal disease (Cheng et al., 2009). SpA can be thought of as a toxin that is essential for pathogenesis and whose molecular attributes must be neutralized to achieve protective immunity.

By generating nontoxigenic variants unable to bind Igs via Fcy or Fv3-Fab domains, we identified SpA-neutralizing immune responses as a correlate for protective immunity against S. aureus infection. In contrast to many methicillin-sensitive strains, the CA-MRSA isolate USA300 LAC is significantly more virulent (Cheng et al., 2009). For example, immunization of experimental animals...
with the surface protein IsdB (Kuklin et al., 2006) raises antibodies that confer protection against S. aureus Newman (Kuklin et al., 2006) but not against USA300 challenge (Fig. S3 T). In contrast, neutralizing SpA antibody responses generate protection against strains of the current MRSA epidemic. These antibodies exert at least two functions. As shown in Fig. 3, the SpA antibodies enable phagocytic killing of staphylococci in blood. Moreover, by neutralizing B cell superantigen activity, SpA antibodies enable the development of humoral immune responses to many different antigens that, assuming synergism, may together contribute toward the establishment of immunity. In agreement with this, the SpA{\text{KKAA}} vaccine elicited greater protection against abscess formation, which monitors infected animals over a prolonged period of time (Fig. 4), as compared with the lethal challenge, when most animals die within 1–2 d (Fig. S1).

**MATERIALS AND METHODS**

**Antibody isolation.** 5 mg of protein was covalently linked to HiTrap NHS-activated HP and loaded with rabbit serum. Antibodies were eluted with 1 M glycine, pH 2.5, and 0.5 M NaCl, neutralized with 1 M Tris-HCl, pH 8.5. F(ab\text{\textsubscript{2}}) fragments were again affinity purified, dialyzed against PBS at 4°C, with 3 mg pepsin at 37°C for 30 min and quenched with 1 M Tris-HCl, pH 8.5. F(\text{ab\textsubscript{2}}) fragments were again affinity purified, dialyzed against PBS at 4°C, separated by 15% SDS-PAGE, and visualized with Coomassie Blue.

**Active and passive immunization.** The coding sequence for SpA was PCR amplified with two primers, 5'-GCTGCACATGCGCAACAGAT-GAAGCTCAAAC-3' and 5'-ATAGAAGGCGCTTTTTTTATCTTTGTTGAACATATG-3', using S. aureus Newman DNA. SpA-D was PCR amplified with two primers: 5'-AACTATGTTCACCAAAAGCATAACAAGG-3' and 5'-AAGGACTCCAGTTGTGGAATTTTTC-3'. The sequence for SpA-D{\text{KAA}} was mutated with two sets of primers: 5'-CATATGTTCACAACAAAGATAAAAAAGCGCCTTCTATGAA-3' and 5'-GATTCCTGTAGGATCCAAAGGATGG-3'. The sequence for SpA-D{\text{KAA}} was amplified with two primers: 5'-AACATATGTTCACCAAAAGCATAACAAGG-3' and 5'-AAGGACTCCAGTTGTGGAATTTTTC-3'. The sequence for SpA-D{\text{KAA}} was synthesized by Integrated DNA Technologies, Inc. PCR products were cloned into pET-15b generating N-terminal His\textsubscript{\text{6}}-tagged recombinant protein. BALB/c mice were immunized by intramuscular injection and boosted with the same antigen after 11 d. On day 20, mice were bled to obtain serum for specific antibody titers. Affinity-purified antibodies were injected into the peritoneal cavity of BALB/c mice at 4–24 h, and blood was collected for specific antibody titer before challenge.

**Mouse infection.** Staphylococci were used to infect anesthetized mice by retroorbital injection (1 × 10\textsuperscript{7} CFU S. aureus Newman, 5 × 10\textsuperscript{7} CFU S. aureus USA300, or 3 × 10\textsuperscript{7} CFU S. aureus Mu50). On day 4, 15, or 30, mice were killed, kidneys removed, and homogenized tissue spread on agar for colony formation. Animal experiments were performed in accordance with the institutional guidelines according to experimental protocol review and approval by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of Chicago.

**Bacterial survival in blood.** As previously described (Thammavongsa et al., 2009), whole blood was collected from BALB/c mice with 5 µg ml\textsuperscript{−1} of oleprudin anticoagulant. 50 µl of 5 × 10\textsuperscript{8} CFU ml\textsuperscript{−1} S. aureus Newman were mixed with 950 µl of mouse blood. Samples were incubated at 37°C with slow rotation for 30 min and then incubated on ice with 1% saponin/PBS. Dilutions of staphylococci were plated on agar for colony formation.

**SpA ligands.** 1 µg ml\textsuperscript{−1} of purified SpA and its variants were coated onto ELISA plates in 0.1 M carbonate buffer, pH 9.5. Plates were incubated with peroxidase-conjugated human IgG, Fe, or F(\text{ab\textsubscript{2}}), fragments, IgM (The Jackson Laboratory), and vWF (Thermo Fisher Scientific) and developed using OptEIA reagent. For inhibition, plates were incubated with either naive rabbit F(\text{ab\textsubscript{2}}) fragments (The Jackson Laboratory) or 10 µg ml\textsuperscript{−1} of affinity-purified F(\text{ab\textsubscript{2}})\textsubscript{2} fragments before ligand binding.

**B cell apoptosis.** 150 µg of purified protein was injected into the peritoneum of 6-wk-old BALB/c 4 h after injection, animals were killed and spleens removed and homogenized. Red blood cells were lysed in ACK buffer. White blood cells were stained with R-PE–conjugated anti-CD19 (eBioscience). Cells were washed and fixed with formalin and analyzed by FACSCanto (BD).

**Antibody quantification.** Nitrocellulose membrane was blotted with human/mouse IgG (The Jackson Laboratory), SpA{\text{KKAA}} and CRM197, blocked, and incubated with either human or mouse sera. IRDye 700DX–conjugated anti-human/mouse IgG (Rockland Immunochemicals, Inc.) was used to quantify signal intensities from healthy human volunteers or mice using the Odyssey infrared imaging system (LI-COR, Biosciences). Experiments with blood from human volunteers involved protocols that were reviewed, approved, and performed under regulatory supervision of The University of Chicago’s Institutional Review Board. For the staphylococcal antigen matrix, nitrocellulose membrane was blotted with 2 µg of a collection of Ni-NTA affinity-purified recombinant His\textsubscript{\text{6}}-tagged staphylococcal proteins. Signal intensities in mouse sera were quantified and normalized using anti-His, antibody with the Odyssey.

**Statistical Analysis.** Unpaired two-tailed Student’s t tests were performed to analyze the statistical significance of renal abscess, ELISA, and B cell superantigen data.

**Online supplemental material.** Fig. S1 shows that SpA is a virulence factor for lethal infection after intraperitoneal injection of S. aureus Newman into BALB/c mice and that active or passive immunization with antibodies raised against SpA{\text{KKAA}} can protect against this disease. Fig. S2 shows that SpA{\text{KKAA}} unlike wild-type SpA, does not induce B cell apoptosis in mice and that antibodies raised against SpA{\text{KKAA}} neutralize the B cell superantigen attributes of SpA. Fig. S3 shows that immunization of mice with SpA{\text{KKAA}} prevents multiple S. aureus strains from inducing renal abscess formation in mice and that prior infection with S. aureus does not elicit immunity to subsequent infection with the same strain. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092514/DC1.

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**REFERENCES**


SUPPLEMENTAL MATERIAL

Kim et al., http://www.jem.org/cgi/content/full/jem.20092514/DC1
Figure S1. SpA is a virulence factor for lethal *S. aureus* infections and the nontoxigenic SpA vaccine protects mice against this disease. (A) BALB/c mice (*n* = 30) were infected via intraperitoneal infection with 2 × 10^8^ CFU *S. aureus* Newman or its isogenic spa deletion variant (Δspa). (B) BALB/c mice (*n* = 30) were prime booster immunized with SpA^KKAA^ or PBS/adjuvant control (mock). Each animal was subsequently infected by intraperitoneal injection with 6 × 10^8^ CFU *S. aureus* Newman. (C) BALB/c mice (*n* = 30) were injected with 5 mg x kg^−1^ affinity-purified rabbit IgG specific for SpA^KKAA^ (α-SpA^KKAA^) or for the plague vaccine antigen rV10 (mock). 4 h later, each animal was infected by intraperitoneal injection with 3 × 10^8^ CFU *S. aureus* Newman. Animals were monitored for a period of 10 d after infection. Statistical significance was analyzed with the unpaired two-tailed log-rank test (GraphPad Software, Inc.). Data in A–C are representative of three independent experiments.
Figure S2. Nontoxigenic SpA vaccine does not induce B cell apoptosis and neutralizes the ability of SpA to bind immunoglobulin. (A) Ribbon structure of the Fc portion of human IgG (gray) complexed with SpA (orange). SpA residues Gin 9 and 10 (presented in red as stick structures at the beginning of helix I) interact with Met, Ile, and His of IgG (blue stick structures). Amino groups are printed in red, carbonyl groups in blue, and sulfur atoms in orange. (B) Asp 36 and 37 (green) of SpA (orange) reside between helix II and III and interact with Lys, Thr, Ser, and Tyr (magenta stick structures) of human IgM V,3 Fab (gray). Structures were constructed using PyMol. (C and D) 6-wk-old C57BL/6 mice (n = 5 per group) were passively immunized with affinity-purified V10 (α-V10) control antibodies or with SpA-specific antibodies (α-SpAKKAA). 150 µg of purified SpA or SpAKKAA was injected into the peritoneum of immunized animals and, after 4 h, animals were killed and their spleens were removed and homogenized. Red blood cells were lysed in ACK buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA). White blood cells were washed and suspended in PBS and stained with R-PE–conjugated anti-CD19 or anti-CD45R monoclonal antibody, as well as APC-conjugated anti-CD3e monoclonal antibody (eBioscience). Cells were washed and fixed with 4% formalin and analyzed in a FACS Canto. (C) The abundance of CD45R⁺ B lymphocytes was determined. (D) The ratio between CD45R⁺ to CD3e⁺ cells in the spleen was quantified. Data in C and D are the means and error bars represent ±SEM. Data are representative of three independent analyses. (E) 4 h after injection of PBS (mock; E), SpA-D (F), or SpA-D KKAA (G; n = 4 per group) in the peritoneal cavity of BALB/c mice, spleen tissues were removed during necropsy, thin sectioned, and analyzed by TUNEL staining for the presence of apoptotic B cells. Arrowheads identify large clusters of apoptotic cells.
Figure S3. **Nontoxigenic SpA vaccine prevents abscess formation.** Histopathology of hematoxylin-eosin–stained renal tissue isolated during necropsy of BALB/c mice that had been mock immunized (PBS) or vaccinated with SpA, SpA-D, SpA-D_{KKAA}, or SpA_{KKAA} and challenged with *S. aureus* Newman (A–H) or *S. aureus* USA300 (I–R). White arrowheads identify polymorphonuclear leukocyte (PMN) infiltrates. Blue arrowheads identify staphylococcal abscess communities. Each histopathology slide is one representative sample of a cohort of 10 animals whose organs were also analyzed for histopathology. (S) BALB/c mice (*n* = 10) were infected with *S. aureus* Newman or mock challenged (PBS) for 18 d and infection cleared with chloramphenicol treatment. Both cohorts of animals were then challenged with *S. aureus* Newman and bacterial load (CFU) in kidney tissue homogenate analyzed after necropsy on day 4. See Fig. 4 D for an analysis of the humoral immune response to SpA. (T) BALB/c mice (*n* = 10) were immunized with IsdB (100 µg IsdB emulsified in CFA followed by IFA/IsdB booster on day 11) and challenged with 5 × 10^6 CFU *S. aureus* USA300 (LAC) and bacterial load (CFU) in kidney tissue homogenate analyzed after necropsy on day 4. Compared with mock immunized (PBS/adjuvant) animals with 6.93 (±0.24) log_{10} CFU g^{-1}, IsdB vaccination was associated with 6.25 (±0.46) log_{10} CFU g^{-1} and did not generate statistically significant protection (*P* = 0.2138) from USA300 (LAC) challenge. (U) Cohorts of BALB/c mice (*n* = 15) were prime booster immunized with SpA_{KKAA} or PBS/adjuvant control (mock) and subsequently infected with 3 × 10^7 CFU *S. aureus* Mu50 to measure CFU in kidney tissue homogenate analyzed after necropsy on day 4. Animal data are representative of three independent experimental trials. Statistical significance was calculated with the unpaired two-tailed Student’s *t* test and *p*-value was recorded. Horizontal bars indicate the means of staphylococcal load calculated as log_{10} CFU g^{-1} in homogenized renal tissues.
Table S1. SpA vaccines prevent abscess formation over 15 d

<table>
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<tr>
<th>Antigen</th>
<th>Staphylococcal load and abscess formation in renal tissue</th>
<th>Log_{10} CFU g^{-1}</th>
<th>P-value</th>
<th>Reduction (log_{10} CFU g^{-1})</th>
<th>IgG titer</th>
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<td>S. aureus USA300 (LAC) challenge</td>
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<td>Mock</td>
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<td>SpA</td>
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<td>0.0059</td>
<td>2.70</td>
<td>7,080 ± 1467</td>
</tr>
</tbody>
</table>

*Means ± SEM of staphylococcal load calculated as log_{10} CFU g^{-1} in homogenized renal tissues 15 d after infection in cohorts of 15 BALB/c mice per immunization. Two animals in each group died during the course of infection. A representative of three independent and reproducible animal experiments is shown. ± SEM is indicated.

bStatistical significance was calculated with the unpaired two-tailed Students t test and p-values were recorded. P-values <0.05 were deemed significant.

cReduction in bacterial load calculated as log_{10} CFU g^{-1}.

dMeans ± SEM of five randomly chosen serum IgG titers were measured before staphylococcal infection by ELISA.