Role of Protein A in the Evasion of Host Adaptive Immune Responses by *Staphylococcus aureus*

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**ABSTRACT** Heritable defects in human B cell/antibody development are not associated with increased susceptibility to *Staphylococcus aureus* infection. Protein A (SpA), a surface molecule of *S. aureus*, binds the Fc domain of immunoglobulin (Ig) and cross-links the Fab domain of V_{H}3-type B cell receptors (IgM). Here we generated *S. aureus spa* variants harboring amino acid substitutions at four key residues in each of the five Ig-binding domains of SpA. Wild-type *S. aureus* required SpA binding to Ig to resist phagocytosis and SpA-mediated B cell receptor cross-linking to block antibody development in mice. The spaKKAA mutant, which cannot bind Ig or IgM, was phagocytosed and elicited B cell responses to key virulence antigens that protected animals against lethal *S. aureus* challenge. The immune evasive attributes of *S. aureus* SpA were abolished in μMT mice lacking mature B cells and antibodies. Thus, while wild-type *S. aureus* escapes host immune surveillance, the spaKKAA variant elicits adaptive responses that protect against recurrent infection.

**IMPORTANCE** *Staphylococcus aureus* causes recurrent skin and bloodstream infections without eliciting immunity. Heritable defects in neutrophil and T cell function, but not B cell or antibody development, are associated with increased incidence of *S. aureus* infection, and efforts to develop antibody-based *S. aureus* vaccines have thus far been unsuccessful. We show here that the Fcγ and V_{H}3-type Fab binding activities of staphylococcal protein A (SpA) are essential for *S. aureus* escape from host immune surveillance in mice. The virulence attributes of SpA in mice required mature B cells and immunoglobulin. These results suggest that antibodies and B cells play a key role in the pathogenesis of staphylococcal infections and provide insights into the development of a vaccine against *S. aureus*.

*S. aureus* is an invasive pathogen that causes skin and soft tissue infections (SSTI), bacteremia, sepsis and endocarditis (1). In the United States, an annual mortality of more than 20,000 is attributed to *S. aureus* infection, exceeding deaths caused by influenza, viral hepatitis, and HIV/AIDS (2). Of particular concern are patients with recurrent invasive infections, which occur in up to 13% of individuals with surgical and antibiotic therapy (3). Recurrent infection leads to invasive *S. aureus* disease with bacteremia but is not associated with the development of immunity (4). Antibiotic-resistant strains, designated MRSA (methicillin-resistant *S. aureus*), cause community- as well as hospital-acquired infections and represent a major public health problem (5). Although there is a clear need for a vaccine against *S. aureus* (6), past clinical trials with whole-cell vaccines and with purified subunits have failed (7, 8).

Intravenous *S. aureus* infection of mice leads to disseminated, persistent abscess formation and to lethal disease (9). Similar to humans, infected animals raise antibodies against some of the secreted products of *S. aureus* but generally fail to develop protective immunity (10, 11). The contributions of several virulence mechanisms for staphylococcal infection have been revealed, including blood coagulation (12), agglutination with fibrin cables (13, 14), adenosine synthesis (15), heme iron scavenging (16), toxin-mediated dissemination (17), and escape from complement activation (18, 19). These mechanisms are important for the establishment of disease; however, they are not known to be required for staphylococcal escape from host adaptive immune responses (20). Recent work implemented protein A (SpA) as a vaccine antigen (11), and this prompted us to investigate its contribution to staphylococcal escape from protective immune responses. SpA is anchored in the bacterial cell wall envelope and released during staphylococcal growth (21). Each of its five immunoglobulin-binding domains (IgBDs) captures the Fcγ domain of human or mouse IgG (22) as well as the Fab domain of V_{H}3 clan IgG and IgM (23). Fcγ binding to SpA is thought to protect staphylococci from opsonophagocytic killing (24). Moreover, purified SpA triggers B cell superantigen activity through cross-linking of V_{H}3 type B cell receptors (surface IgM), resulting in proliferative supraclonal expansion as well as apoptotic collapse of the activated B cells (25).

When used as a subunit vaccine, SpA variants that are unable to capture IgG Fcγ and cross-link B cell receptors elicit protein...
A-neutralizing antibodies in mice, which protect these animals against S. aureus abscess formation (11). SpA-neutralizing antibodies also enable infected animals to mount antibody responses against many different staphylococcal antigens (11). Similar protective effects are achieved when protein A-neutralizing monoclonal antibodies are passively transferred into naive mice (26). Here we asked whether protein A represents the key immune-evasive determinant of S. aureus by infecting mice with spa variants lacking the ability to capture IgG Fc/H9253 and/or to cross-link B cell receptors.

RESULTS

S. aureus spa variants defective for immunoglobulin binding. Guided by the structural analysis of protein A cocrystallized with Fc/H9253 or Fab (27, 28), we generated SpA variants with amino acid substitutions at residues 9 and 10 (Gln9-Lys and Gln10-Lys) and/or residues 36 and 37 (Asp36-Ala and Asp37-Ala) of all five IgBDs, which retained the alpha-helical fold of the immunoglobulin-binding domains (Fig. 1A and 2B). When substitutions were introduced into recombinant protein A and the resulting mutant proteins were analyzed for the binding of purified polypeptides to human immunoglobulin, it was found that specific substitutions abolished the association of Fcγ (SpAKK) or Fab (SpAAA) with SpA, spaKK, and/or spaKAA mutant S. aureus Newman as well as SpA and Sbi (staphylococcal binder of immunoglobulin) in the extracellular medium of staphylococcal cultures. (C) (Top) Merged differential interference contrast (DIC) and anti-SpA fluorescence microscopy images of wild-type and mutant S. aureus. Bars, 10 μm. (Bottom) Flow cytometry analysis of S. aureus strains with FITC-labeled Fcγ or F(ab)2 fragments of human IgG.

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FIG 1 Amino acid substitutions in protein A (SpA) that abrogate Staphylococcus aureus binding to the Fcγ or F(ab)2 domain of human IgG. (A) Diagram illustrating the binding sites in each of the five immunoglobulin-binding domains (IgBDs E, D, A, B, and C) of protein A and the positions of substitutions that affect its association with Fcγ (SpAKK) or F(ab)2 (SpAAA); H1, H2, and H3 identify helices in the triple helical bundle structure of each IgBD. (B) Immunoblotting with rabbit α-SpA to detect SpA in the envelope of wild-type, Δspa, spaKK, spaAA, or spaKKAA mutant S. aureus Newman as well as SpA and Sbi (staphylococcal binder of immunoglobulin) in the extracellular medium of staphylococcal cultures. (C) (Top) Merged differential interference contrast (DIC) and anti-SpA fluorescence microscopy images of wild-type and mutant S. aureus. Bars, 10 μm. (Bottom) Flow cytometry analysis of S. aureus strains with FITC-labeled Fcγ or F(ab)2 fragments of human IgG.

Contribution of protein A to staphylococcal virulence. The virulence of wild-type and spa mutant staphylococci was assessed by intravenous injection of 1 × 10^7 CFU into naive BALB/c mice.
Animals were euthanized 15 days after challenge and necropsied, and staphylococcal load and abscess formation in renal tissues were determined (Table 1). The spaKKAA variant was attenuated for both abscess formation in renal tissues and staphylococcal load, similar to the /H9004 spa mutant (32) (Table 1). The spaAA and spaKK mutants displayed an intermediate phenotype for the staphylococcal load. Further, the spaKK mutant was defective for abscess formation, whereas the spaAA variant was not (Table 1). These data indicate that both biological activities of protein A, Ig Fc binding and Fab cross-linking, contribute to the pathogenesis of S. aureus infections in mice. Moreover, protein A-dependent B cell superantigen activity is not required for the formation of staphylococcal abscess lesions in naive mice.

TABLE 1  Virulence defects of S. aureus spa variants

| S. aureus strain | No. of miceb | Staphylococcal load | Abcess formation |
|------------------|--------------|---------------------|-----------------|
|                  |              | Log10 CFU g−1c      | P valued | Reductione | No. of abscesses/kidneyf | P valueg |
| Wild-type        | 18           | 6.20 ± 0.43         |          |            | 8.50 ± 1.75               |         |
| Δspa             | 20           | 4.49 ± 0.41         | 0.0017   | 1.71       | 2.25 ± 0.71               | 0.0015   |
| spaKK            | 20           | 5.29 ± 0.41         | 0.0924   | 0.91       | 2.50 ± 0.74               | 0.0023   |
| spaAA            | 19           | 4.70 ± 0.53         | 0.0528   | 1.50       | 5.11 ± 1.41               | 0.1383   |
| spaKKAA          | 20           | 4.24 ± 0.47         | 0.0069   | 1.96       | 2.85 ± 0.98               | 0.0065   |

a BALB/c mice were infected with 1 × 10^7 CFU of wild-type or Δspa, spaKK, spaAA, or spaKKAA mutant S. aureus Newman. At 15 days postinfection, animals were euthanized and necropsied, and bacterial load and numbers of abscesses in kidney tissues were determined.
b Number of 6-week-old female BALB/c mice per study.
c Staphylococcal load in homogenized renal tissues 15 days following infection. Values are means ± SEM; limit of detection, 1.99 log10 CFU g−1.
d Statistical significance was calculated with the unpaired two-tailed Mann-Whitney test.
e Reduction in bacterial load, calculated as log10 CFU g−1.
f Determined by histopathology of hematoxylin-eosin-stained, thin-sectioned kidneys. Values are means ± SEM.
g Statistical significance was calculated with the unpaired two-tailed Student’s t test.

Immunoevasive attributes of protein A during S. aureus infection of mice. To further explore the contributions of protein A to S. aureus disease, we infected mice by intravenous inoculation into the retroorbital plexus, removed blood samples after 30 min by cardiac puncture, and enumerated staphylococcal CFU. Wild-type and spaAA mutant S. aureus survived in the bloodstream of naive mice, whereas reduced numbers of the Δspa, spaKK, and spaKKAA variants were isolated from blood (Fig. 3A). The reduced bacterial load in blood is likely due to increased killing by phagocytes, as an in vitro opsonophagocytosis assay with anticoagulated mouse blood revealed increased killing of the Δspa, spaKK, and spaKKAA variants (Fig. 3B). Compared to that in wild-type C57BL/6 mice, the survival of wild-type S. aureus was re-

FIG 2  Binding of human immunoglobulin to protein A and its variants. (A) Human IgG, its Fcγ and F(ab)_2 fragments, recombinant affinity-purified SpA_KK, SpA_AA, SpA_KKAA (IgBDs E to C), and wild-type SpA (IgBDs E to C plus region X) were separated by SDS-PAGE and stained with Coomassie blue. Ni-NTA Sepharose beads were charged with SpA_KK, SpA_AA, SpA_KKAA, or SpA, and human IgG or its Fcγ and F(ab)_2 fragments were loaded on the column. The eluate was analyzed by Coomassie-stained SDS-PAGE. (B) Circular dichroism spectroscopic analysis of SpA_KK, SpA_AA, SpA_KKAA, and SpA revealed the α-helical character of protein A and its variants.
Protein A binding to immunoglobulin protects staphylococci from phagocytic killing. (A) Survival of wild-type and spa mutant S. aureus Newman injected into the bloodstream of wild-type C57BL/6 or μMT mice, lacking mature B cells and immunoglobulin (n = 5; data are means ± standard errors of the means [SEM]; *, P < 0.05). (B) Anti-coagulated mouse blood (n = 3) was incubated with 5 × 10^5 CFU of S. aureus Newman (wild-type) and its Δspa, spa_ΔKK, spa_ΔAA, and spa_ΔKAAA variants for 30 min. Staphylococcal escape from phagocytic killing was measured by enumerating CFU in lysed blood samples. Average survival of staphylococci was calculated from three independent experimental determinations and analyzed by assigning the survival of wild-type as 100%. Survival of each mutant was analyzed in pairwise comparison with wild-type S. aureus (*, P < 0.05). (C) Anti-coagulated mouse blood (n = 3) from C57BL/6 or μMT mice with or without exogenous addition of purified mouse IgG and IgM (2 mg ml⁻¹ and 0.4 mg ml⁻¹, respectively) was incubated with 5 × 10^5 CFU of wild-type or spa_ΔKAAA mutant S. aureus for 30 min, and bacterial survival was measured (*, P < 0.05).

FIG 3 Protein A binding to immunoglobulin protects staphylococci from phagocytic killing. (A) Survival of wild-type and spa mutant S. aureus Newman injected into the bloodstream of wild-type C57BL/6 or μMT mice, lacking mature B cells and immunoglobulin (n = 5; data are means ± standard errors of the means [SEM]; *, P < 0.05). (B) Anti-coagulated mouse blood (n = 3) was incubated with 5 × 10^5 CFU of S. aureus Newman (wild-type) and its Δspa, spa_ΔKK, spa_ΔAA, and spa_ΔKAAA variants for 30 min. Staphylococcal escape from phagocytic killing was measured by enumerating CFU in lysed blood samples. Average survival of staphylococci was calculated from three independent experimental determinations and analyzed by assigning the survival of wild-type as 100%. Survival of each mutant was analyzed in pairwise comparison with wild-type S. aureus (*, P < 0.05). (C) Anti-coagulated mouse blood (n = 3) from C57BL/6 or μMT mice with or without exogenous addition of purified mouse IgG and IgM (2 mg ml⁻¹ and 0.4 mg ml⁻¹, respectively) was incubated with 5 × 10^5 CFU of wild-type or spa_ΔKAAA mutant S. aureus for 30 min, and bacterial survival was measured (*, P < 0.05).
animals infected with wild-type, \(spa_{KAA}\), or \(spa_{KAA}\ sbi\) strains (Table 2). Further, similarly low staphylococcal loads and abscess numbers were detected in C57BL/6 and \(\mu\)MT mice infected with either the \(spa_{KAA}\) or \(spa_{KAA}\ sbi\) mutant (Table 2). These data therefore suggest that natural IgG and IgM antibodies of mice do not provide protection against staphylococci and that Sbi binding to IgG does not contribute to \(S.\ aureus\) virulence.

**DISCUSSION**

Following the discovery of \(S.\ aureus\) binding to immunoglobulin, Sjöquist and colleagues purified protein A and revealed its binding to the Fcy domain of human and animal immunoglobulins (35, 36). This biochemical activity allows exogenously supplied protein A to block complement-dependent (opsonophagocytosis of \(S.\ aureus\) or \(Escherichia\ coli\) by human neutrophils (37). Purified protein A was also shown to exert mitogen activity for human B cells (24). Nevertheless, mice with heritable B cell deficiencies, for example, a mutation in Bruton’s tyrosine kinase (X-linked immunodeficiency) (38) or gene-targeted B-cell ablation (\(\mu\)MT) (39), display similar or reduced disease progression in \(S.\ aureus\) sepsis or septic arthritis models as wild-type animals (40, 41). These findings were interpreted as evidence that, unlike neutrophils or Th17 T cells (42–45), neither antibodies nor B cells contribute to the pathogenesis of \(S.\ aureus\) infections or affect host susceptibility to staphylococcal infection (40).

More recently, it was discovered that protein A binds also to the Fab domain of immunoglobulin heavy chains (IgG, IgA, IgM, and IgE) (46), specifically to the Fab domains of human and mouse \(V_{\mu}\)3 clan antibodies (47–49). Protein A binding to IgM triggers cross-linking of B cell receptors, proliferative supraclonal expansion, and apoptotic collapse of activated B cells (25). This B cell superantigen activity can be demonstrated in mice injected with purified protein A as prolonged ablation-adaptive B cell responses (50). Following intravenous inoculation of mice with
staphylococci, wild-type *S. aureus* replicates in abscess lesions in many different tissues, including the kidneys (51), and also causes septic arthritis (52). Compared to wild-type staphylococci, *S. aureus* Δspa mutants display defects in renal abscess formation, septic arthritis, and lethal sepsis (9, 32, 52). Further, *S. aureus* Δspa mutants fail to produce increased amounts of V_{1,3} clan IgG and IgM antibodies, as occurs following infection with wild-type staphylococci (52). Nevertheless, the *in vivo* contributions of protein A toward *S. aureus* escape from opsonophagocytic killing and the prevention of adaptive immune responses that protect animals against recurrent infection were heretofore not known.

To test the possibility that protein A blocks the development of protective B cell responses during infection, we generated *S. aureus* strains that express SpA variants with specific defects in the capture of Igs via the Fcγ domain (SpA_{KK}) or/and the cross-linking of B cell receptors via Fab binding (SpA_{LAA} and SpA_{KK}). These mutants harbor either 10 (SpA_{KK} and SpA_{LAA}) or 20 codon substitutions (SpA_{KKAA}) in the spa gene yet display similar amounts of protein A on the bacterial surface. Similar to variants with a deletion of the entire protein A gene, the *S. aureus* spa_{KKAA} mutant cannot escape opsonophagocytic killing in blood, fails to suppress adaptive immune responses, and is defective in the establishment of staphylococcal abscess lesions. In contrast to infection with the Δspa mutant, *S. aureus* spa_{KKAA} infection elicits specific antibody responses against protein A, measured as IgG binding to SpA_{KKAA}. The virulence contributions of protein A are dependent on its binding to both the Fcγ and Fab domains of Ig, as *S. aureus* spa_{KK} and spa_{LAA} variants displayed incremental defects in the pathogenesis of systemic infections. Furthermore, the virulence contributions of *S. aureus* protein A are not observed in μMT mice, which lack both mature B cells and their antibody products (39). Unlike infection with wild-type staphylococci, infection with *S. aureus* spa_{KKAA} elicits immune responses that partially protect mice from...
a lethal challenge with the highly virulent strain LAC (USA300), which is responsible for the current epidemic of community-associated MRSA infections (33).

Similar to *S. aureus*, Mycobacterium tuberculosis infection of humans or animals causes distinctive lesions, i.e., granulomas, and is also not associated with the development of protective immunity against disease (tuberculosis) (53). Bacillus Calmette-Guérin (BCG) infection, which is not associated with granuloma formation among other defects, raises protective T cell immune responses against tuberculosis (54, 55). At least in mice, BCG-derived immunity is impaired in mice with a defect in Bruton’s tyrosine kinase and X-linked immune deficiency (XID), a disease with impaired B lymphocyte function (56). XID mice lack the activity of B cells to down-regulate neutrophil motility in order to promote macrophage-mediated phagocytosis, antigen presentation as well as clearance of mycobacteria (56). In view of these findings, we cannot exclude the possibility that defects in *S. aureus* pathogenesis in μMT mice not only may be due to the absence of immunoglobulin (as shown in Fig. 3BC) but also may be affected by B cell–dependent neutrophil motility at sites of staphylococcal infection.

In summary, the data reported here explain the seeming paradox that protein A, an Ig-binding protein and B cell superantigen, functions as a key virulence factor for the pathogenesis of *S. aureus* infections, whereas heritable B cell and/or antibody deficiencies are not associated with defects in the development of immunity to staphylococcal infection. We propose that *S. aureus* expression of protein A and its binding to Ig ameliorate B cell responses during infection, thereby interfering with the development of protective immunity. This model may explain why heritable defects in humoral immune responses cannot affect host susceptibility to staphylococcal infection—protein A effectively disables humoral immune responses. In contrast, perturbation of staphylococcal virulence via mutations in protein A, immunization with nontoxicogenic protein A or administration of protein A-neutralizing monoclonal antibodies each can elicit protective antibodies in mice against highly virulent MRSA strains (11, 26). We therefore propose that SpAKKAA represents a protective antigen for the development of a staphylococcal vaccine.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. aureus* strains Newman and its variants or USA300 LAC were grown in tryptic soy broth (TSB) or agar at 37°C. *E. coli* strains DH5α and BL21(DE3) were grown in Luria broth (LB) or agar at 37°C. Ampicillin (100 μg ml⁻¹ for *E. coli*), spectinomycin (200 μg ml⁻¹ for *S. aureus*), and erythromycin (20 μg ml⁻¹ for *S. aureus*) were used for plasmid selection (pET15b+), mutant allele selection (Δspa), and transposon selection (Δifi).

**S. aureus spa mutants.** Two 1-kb DNA sequence segments upstream and downstream of the *spa* gene were amplified from the chromosome of *S. aureus* Newman (30) with primers ext1F (5’GGGGACCATTTGTA CAAAGAAGTTGCTGACATTTAAGAAGATTGTTCAGATTAT3’), ext1R (5’ATTGTGAAATGCAATCATATATAAAGATTATTTG TCAATATTCAATT3’), ext2F (5’CGTCCGGAACATATAAATAAAAACA ACAATACAGCAGGATTAT3’), and ext2R (5’GGGGACCAAGTT GTGACAAAAAGCGACAGCAGCTTAAAGAATTGTTCAGATTAT TGC C3’). The DNA sequences of *spaKKAA*, *spaAA*, and *spaKK* mutants were previously described (26). These sequences were amplified using the primers spaF (CATAATCTGGATATATGACATTTACAAATATA TACAGGG) and spaR (GTAATGTGTTTTTTATATGAATTCGCGAC GACGTCCA). For each construct, mutant *spa* genes and their two flanking regions were fused together in a subsequent PCR. The final PCR products were cloned into pKOR1 using the BP Clonase II kit (Invitrogen). Plasmids were electroporated into the *S. aureus* Δspa variant and temperature shifted to 42°C, blocking replication of plasmids and promoting their insertion into the chromosome. Growth at 30°C was used to promote allelic replacement. Mutations in the *spa* genes were verified by DNA sequencing of PCR amplification products.

**Purification of protein A.** *E. coli* BL21(DE3) harboring pET15b+ plasmids for the expression of His-tagged wild-type SpA, SpAAK, SpAAKAA, and SpAKKAA (26) was grown overnight, diluted 1:100 into fresh medium, and grown at 37°C to an *A*₆₀₀ of 0.5. Cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for an additional 3 h. Bacterial cells were sedimented by centrifugation, suspended in column buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) and disrupted with a French pressure cell at 14,000 lb/in². Lysates were cleared of membrane and insoluble components by ultracentrifugation at 40,000 × g. Cleared lysates were subjected to nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, and proteins were eluted in column buffer containing successively higher concentrations of imidazole (100 to 500 mM). Eluates were dialyzed with phosphate-buffered saline (PBS), treated with Triton-X114 to remove endotoxin, and again dialyzed with PBS. Protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Scientific). Purity was verified by Coomassie-stained SDS-PAGE.

**Circular dichroism (CD) spectroscopy.** Far ultraviolet (UV) CD, spectra of purified SpA, SpAA, SpAK, and SpAKAA in 10 mM phosphate buffer (pH 7.2), 50 mM Na₂SO₄ were recorded on an AVIV 202 CD spectrometer (University of Chicago Biophysics Core Facility) at room temperature.

**Immunofluorescence microscopy.** Overnight cultures of staphylococci were diluted 1:100 and grown at 37°C with shaking to an *A*₆₀₀ of 0.7. Bacteria were centrifuged, washed, fixed with glutaraldehyde and blocked. Cells were incubated with affinity purified anti-SpAKKAA, rabbit IgG for 1 h, washed, incubated with Alexafluor 647-conjugated goat anti-rabbit IgG (Invitrogen), and washed in PBS. Bacteria were settled in polylines-treated glass coverslips and then applied to glass coverslips containing a drop of SlowFade anti-fading reagent (Invitrogen). Images were captured on a Leica SP5 tandem-scorer spectral two-photon confocal microscope at the University of Chicago Light Microscopy Core Facility.

**Flow cytometry.** Overnight cultures of staphylococci grown in TSB were diluted 1:100 and grown at 37°C with shaking to an *A*₆₀₀ of 0.6. Bacteria were centrifuged, washed, fixed, and blocked. To analyze immunoglobulin binding to staphylococci, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated Fcy or F(ab)₂ fragments of human IgG (1:250) and washed in 1% BSA–PBS. To examine the presence of natural antibodies against *S. aureus* in naive mouse serum, staphylococci were incubated with dilutions of naive mouse sera (C57BL/6 and BALB/c; Taconic) for 30 min at room temperature with slow rotation. Cells were washed, incubated with phycoerythrin-conjugated goat anti-mouse IgM or FITC-conjugated goat anti-mouse IgG (1:250), and washed in 1% BSA–PBS.

**Affinity chromatography of immunoglobulin.** Purified His₅-tagged SpA, SpAA, SpAK, and SpAKAA were immobilized on nickel-nitrilotriacetic acid (Ni-NTA) Sepharose, washed, and incubated with human IgG or Fc or F(ab)₂ fragments in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl buffer. After being washed, proteins were eluted with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 500 mM imidazole and analyzed by SDS-PAGE.

**Staphylococcal survival in blood in vitro.** Whole blood was collected from mice by cardiac puncture, and coagulation was inhibited with 10 μg ml⁻¹ lepirudin. *S. aureus* Newman or its variants (50 μl, 5 × 10⁵ CFU ml⁻¹) were mixed with 400 to 950 μl of mouse blood in the presence or absence of affinity-purified mouse IgG and IgM (2 mg ml⁻¹ and 0.4 mg ml⁻¹, respectively). Samples were incubated at 37°C with slow rotation for 30 min and then incubated on ice with 1% saponin–PBS to lyse erythrocytes. Dilutions of staphylococci were plated on agar for colony formation.
Enzyme-linked immunosorbent assay. To determine antigen-specific serum IgG, recombinant purified staphylococcal antigens (SpA, KAA, Cia, FnBPB, IsdB, Coa, and Hla) (11) were used to coat enzyme-linked immunosorbent assay (ELISA) plates at 1 µg ml⁻¹ in 0.1 M carbonate buffer (pH 9.5 at 4°C overnight). The following day, plates were blocked and incubated with serially diluted sera. Plates were incubated with horseradish peroxidase-conjugated secondary antibody specific to mouse IgG (or isotype specific antibodies) and developed using OptEIA reagent.

Protein A expression in S. aureus. Overnight cultures of staphylococci were diluted 1:100 and grown at 37°C with shaking to an A600 of 2. For fractionation of staphylococci, cultures were centrifuged, and the extracellular medium in supernatant was precipitated with 5% trichloroacetic acid (TCA). The pellet was suspended in TSM [50 mM Tris (pH 7.5), 500 mM sucrose, and 10 mM MgCl₂] with 100 µg ml⁻¹ lysozyme and incubated at 37°C to solubilize the cell wall envelope. The resulting protoplasts were sedimented by centrifugation, and the supernatant was precipitated with TCA (cell wall fraction). TCA-precipitated proteins were washed in acetone, dried, solubilized in sample buffer, and separated by SDS-PAGE. Proteins were electrophoresed to polyvinylidene difluoride (PVDF) membranes and analyzed by immunoblotting using affinity-purified rabbit anti-SpA, KAA antibody (11).

Active immunization. Three-week-old female BALB/c mice (Charles River Laboratories) were immunized with 50 µg of SpA or its variants emulsified in complete Freund’s adjuvant (CFA; Difco) and given boosters of 50 µg of the same antigen emulsified in incomplete Freund’s adjuvant (IFA) 11 days following the first immunization. On day 21, mice were bled, and serum was recovered for ELISAs.

Mouse renal abscess model. Overnight cultures of S. aureus Newman (wild-type) and its Δspa, spaKAA, spaKK, and spaKKAA variants were diluted 1:100 into fresh TSB and grown for 2 h at 37°C. Staphylococci were sedimented, washed, and suspended in PBS to the desired bacterial concentration. Inocula were quantified by spreading sample aliquots on TSA and enumerating CFU. BALB/c mice were anesthetized using anesthesia and administered by intramuscular injection with anti-SpA, KAA antibody (11).

For fractionation of staphylococci, cultures were centrifuged, and the extracellular medium in supernatant was precipitated with 5% trichloroacetic acid (TCA). The pellet was suspended in TSM [50 mM Tris (pH 7.5), 500 mM sucrose, and 10 mM MgCl₂] with 100 µg ml⁻¹ lysozyme and incubated at 37°C to solubilize the cell wall envelope. The resulting protoplasts were sedimented by centrifugation, and the supernatant was precipitated with TCA (cell wall fraction). TCA-precipitated proteins were washed in acetone, dried, solubilized in sample buffer, and separated by SDS-PAGE. Proteins were electrophoresed to polyvinylidene difluoride (PVDF) membranes and analyzed by immunoblotting using affinity-purified rabbit anti-SpA, KAA antibody (11).

Mice were infected with 5 × 10⁷ CFU of S. aureus Newman or its variants into the periportal venous sinus of the right eye. On day 15 or 28 following infection, mice were euthanized by CO₂ inhalation and cervical dislocation. Both kidneys were removed, and the staphylococcal load in one organ was analyzed by homogenizing renal tissue with PBS, 0.1% Triton X-100. Serial dilutions of homogenate were spread on TSA and incubated for colony formation. The remaining organ was examined by histopathology. Briefly, kidneys were fixed in 10% formalin for 24 h at room temperature. Tissues were embedded in paraffin, thin-sectioned, stained with hematoxylin-eosin, and inspected by light microscopy to enumerate abscess lesions. Immune serum samples collected at 15 days postinfection were examined by ELISA against the staphylococcal antigen matrix. To examine whether attenuated strains elicit protective efficacy, animals were infected with the spaKKAA mutant for 15 days and treated with daptomycin at 10 mg kg⁻¹ of body weight⁻¹ for 4 days. Three days after the last injection of daptomycin, animals were challenged with 5 × 10⁷ CFU of S. aureus USA300 and monitored for 10 days. All mouse experiments were performed at least twice and conducted in accordance with the institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Staphylococcal survival in blood in vivo. Overnight cultures of S. aureus Newman and its Δspa, spaKAA, spaKK, or spaKKAA variants were diluted 1:100 in fresh medium and grown for 2 h at 37°C. Staphylococci were sedimented by centrifugation, washed, and suspended in PBS to the desired bacterial concentration. Inocula were quantified by spreading sample aliquots on TSA and enumerating the colonies that formed upon incubation. C57BL/6 and B6.129S2-Lgmb101/J (µMT) mice (Jackson Laboratory) were anesthetized via intraperitoneal injection with 65 mg ml⁻¹ ketamine and 6 mg ml⁻¹ xylazine per kilogram of body weight. Mice were infected by injection with 1 × 10⁶ CFU of S. aureus into the periorbital venous sinus of the right eye. At 30 min postinfection, mice were euthanized by CO₂ inhalation. Blood was collected by cardiac puncture and mixed with 2% saponin–PBS in 1:1. Dilutions of staphylococci were plated on agar for colony formation.

Statistical analysis. Bacterial loads in the experimental animal infection model were analyzed with the two-tailed Mann-Whitney test to measure statistical significance. Unpaired two-tailed Student’s t tests were performed to analyze the statistical significance of ELISA data, blood survival data, and abscess formation in the experimental animal infection model. All data were analyzed by Prism (GraphPad Software, Inc.), and P values less than 0.05 were deemed significant.

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