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 Fcγ domain of immunoglobulin (Ig) and cross-links the Fab domain of V_H3-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 spa_{KKAA} 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 spa_{KKAA} 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_H3-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*.**

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Staphylococcus 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_H3 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_H3 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

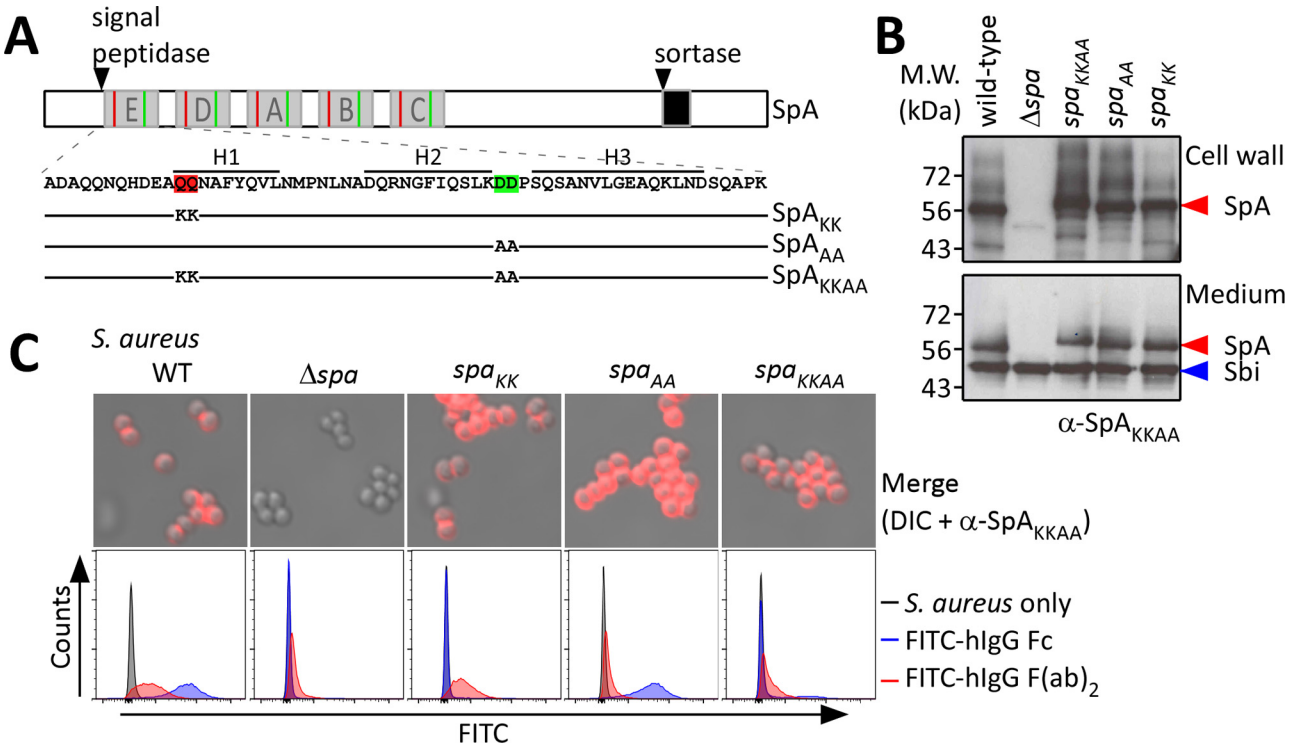

FIG 1 Amino acid substitutions in protein A (SpA) that abrogate *Staphylococcus aureus* binding to the Fcγ or F(ab)$_2$ domain of human IgG. (A) Diagram illustrating the binding sites in each of the five immunoglobulin-binding domains (IgBDs E, D, A, B, and C) of protein A and the positions of substitutions that affect its association with Fcγ (SpA$_{KK}$) or F(ab)$_2$ (SpA$_{AA}$); H1, H2, and H3 identify helices in the triple helical bundle structure of each IgBD. (B) Immunoblotting with rabbit α-SpA$_{KKAA}$ to detect SpA in the envelope of wild-type, Δ*spa*, *spa*$_{KKAA}$, *spa*$_{AA}$, or *spa*$_{KK}$ 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.

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 Fcγ 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 Fcγ or Fab (27, 28), we generated SpA variants with amino acid substitutions at residues 9 and 10 (Gln^9-Lys and Gln^{10}-Lys) and/or residues 36 and 37 (Asp^{36}-Ala and Asp^{37}-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 recombinant SpA with Fcγ (SpA$_{KK}$), Fab (SpA$_{AA}$), or Fcγ and Fab (SpA$_{KKAA}$) (26) (Fig. 2A). The corresponding mutations were introduced into the coding sequence for each of the five IgBDs of the *spa* gene (Fig. 1A). *S. aureus* wild-type strain Newman (29, 30) and its isogenic variants carrying a deletion of *spa* (Δ*spa*), the Gln^9-Lys and Gln^{10}-Lys substitutions (*spa*$_{KK}$), the Asp^{36}-Ala and Asp^{37}-Ala substitutions (*spa*$_{AA}$), or both sets of substitutions within the same protein A gene (*spa*$_{KKAA}$) were probed by immunoblotting with specific antibodies (Fig. 1B). Similar amounts wild-type and mutant SpA were detected in the bacterial envelope and in the extracellular medium of *S. aureus* cultures, indicating that the substitutions did not affect expression, surface display, or stability of mutant protein A molecules (Fig. 1BC). The secretion of Sbi, a second staphylococcal IgG binding protein with homology to SpA (31), was also not impacted by *spa* mutations (Fig. 1B). Binding of protein A on the surface of wild-type *S. aureus* to fluorophore-conjugated Fcγ or F(ab)$_2$ domains of human immunoglobulin was revealed via flow cytometry (Fig. 1C). In contrast to wild-type staphylococci, the binding to Fcγ was abolished in the *spa*$_{KK}$ and *spa*$_{KKAA}$ variants but not in the *spa*$_{AA}$ mutant (Fig. 1C). The binding of human F(ab)$_2$ fragments to *spa*$_{AA}$ and *spa*$_{KKAA}$ mutants was reduced, but it was not affected in the *spa*$_{KK}$ variant (Fig. 1C). The residual amount of F(ab)$_2$ fragment binding to the *spa*$_{KKAA}$ mutant is likely based on antibody recognition of staphylococcal surface antigens, as similar binding activities were observed for *S. aureus* mutants lacking the entire *spa* gene (Δ*spa*) (Fig. 1C).

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.

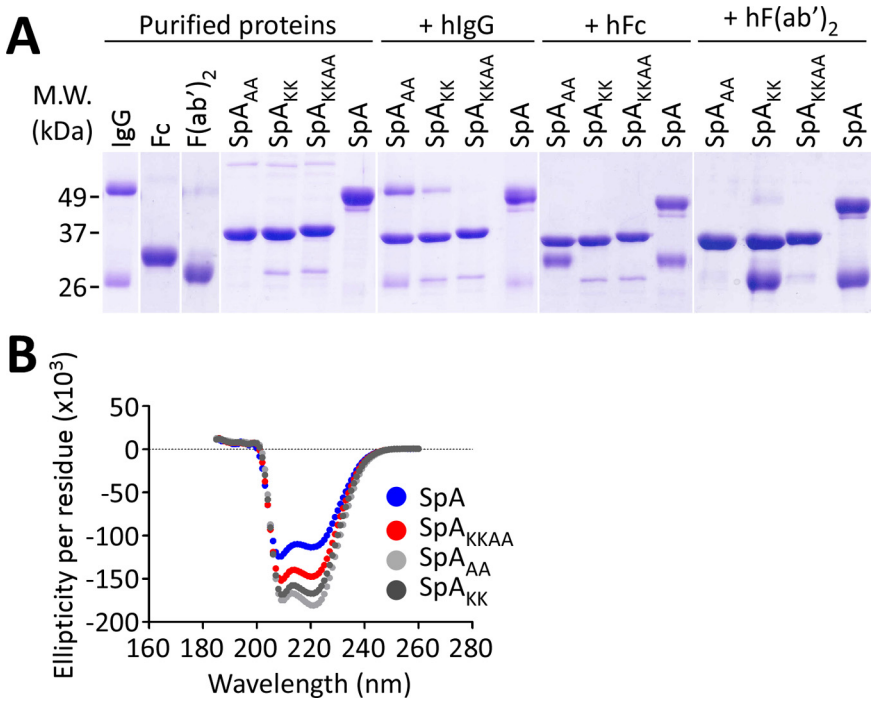

FIG 2 Binding of human immunoglobulin to protein A and its variants. (A) Human IgG, its Fcγ and $F(ab)_2$ fragments, recombinant affinity-purified SpA_{KK}, SpA_{AA}, SpA_{KKAA} (IgBDs E to C), and wild-type SpA (IgBDs E to C plus region X) were separated by SDS-PAGE and stained with Coomassie blue. 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.

Animals were euthanized 15 days after challenge and necropsied, and staphylococcal load and abscess formation in renal tissues were determined (Table 1). The spa_{KKAA} variant was attenuated for both abscess formation in renal tissues and staphylococcal load, similar to the Δ*spa* mutant (32) (Table 1). The spa_{AA} and spa_{KK} mutants displayed an intermediate phenotype for the staphylococcal load. Further, the spa_{KK} mutant was defective for abscess formation, whereas the spa_{AA} variant was not (Table 1). These data indicate that both biological activities of protein A, Ig Fcγ 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.

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 spa_{AA} mutant *S. aureus* survived in the bloodstream of naive mice, whereas reduced numbers of the Δ*spa*, spa_{KK}, and spa_{KKAA} 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 Δ*spa*, spa_{KK}, and spa_{KKAA} variants (Fig. 3B). Compared to that in wild-type C57BL/6 mice, the survival of wild-type *S. aureus* was re-

TABLE 1 Virulence defects of *S. aureus spa* variants[a]

S. aureus strain	No. of mice[b]	Staphylococcal load			Abscess formation	
		Log_{10} CFU g^{-1}[c]	*P* value[d]	Reduction[e]	No. of abscesses/kidney[f]	*P* value[g]
Wild-type	18	6.20 ± 0.43			8.50 ± 1.75	
Δ*spa*	20	4.49 ± 0.41	0.0017	1.71	2.25 ± 0.71	0.0015
spa_{KK}	20	5.29 ± 0.41	0.0924	0.91	2.50 ± 0.74	0.0023
spa_{AA}	19	4.70 ± 0.53	0.0528	1.50	5.11 ± 1.41	0.1383
spa_{KKAA}	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 Δ*spa*, spa_{KK}, spa_{AA}, or spa_{KKAA} 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 log_{10} CFU g^{-1}.
[d] Statistical significance was calculated with the unpaired two-tailed Mann-Whitney test.
[e] Reduction in bacterial load, calculated as log_{10} 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.

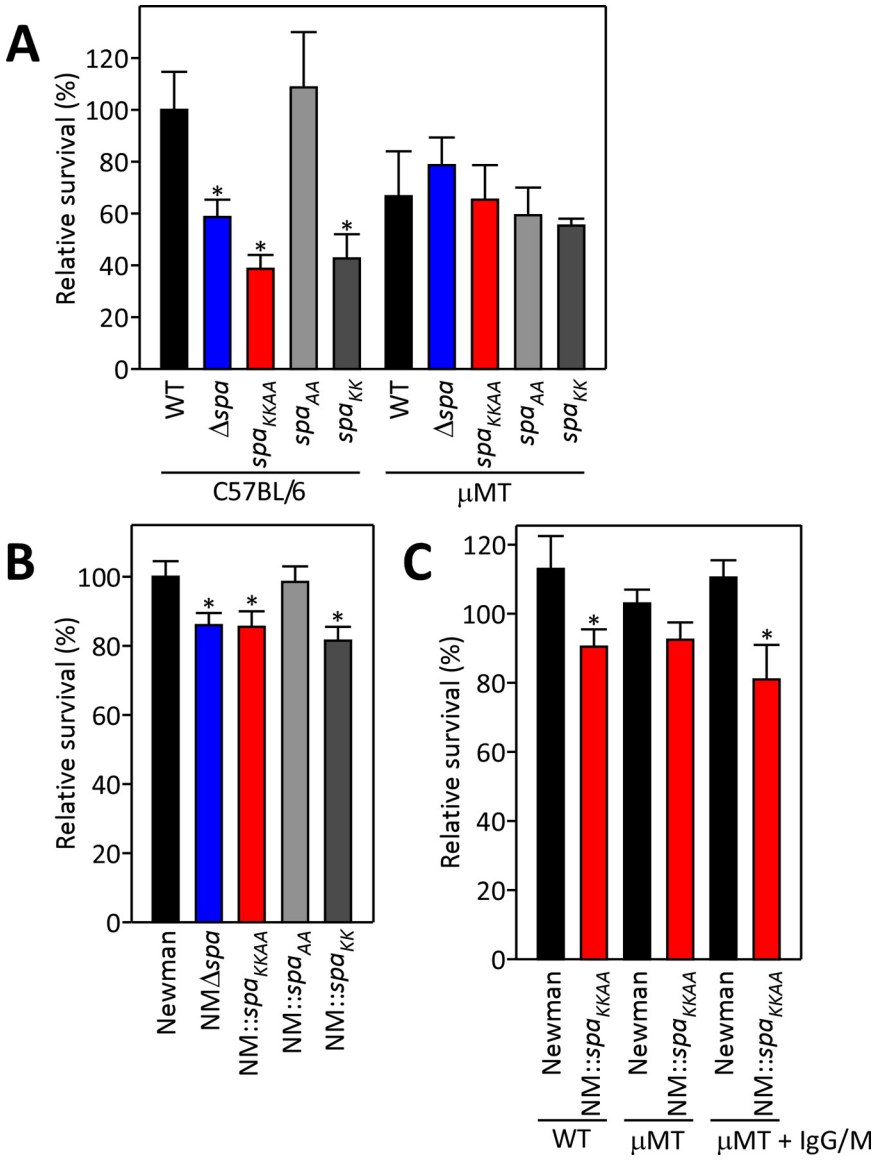

FIG 3 Protein A binding to immunoglobulin protects staphylococci from phagocytic killing. (A) Survival of wild-type and *spa* mutant *S. aureus* Newman injected into the bloodstream of wild-type C57BL/6 or μMT mice, lacking mature B cells and immunoglobulin ($n = 5$; data are means ± standard errors 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 Δ*spa*, spa_{KK}, spa_{AA}, and spa_{KKAA} 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 *S. aureus* 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_{KKAA} mutant *S. aureus* for 30 min, and bacterial survival was measured (*, $P < 0.05$).

duced in the bloodstream of μMT mice, which lack both mature B cells and immunoglobulin (Fig. 3A). Further, no significant difference in bloodstream survival in μMT mice was detected between wild-type and spa_{KKAA} mutant *S. aureus* (Fig. 3A). Interestingly, addition of affinity-purified immunoglobulin G and M to anti-coagulated μMT mouse blood restored protein A-mediated resistance against opsonophagocytosis (Fig. 3C). Mice infected with the spa_{AA} and spa_{KKAA} mutants (but not animals infected with the wild type or the Δ*spa* or spa_{KK} variant) developed IgG antibodies against protein A (SpA_{KKAA}) (Fig. 4C). Compared to naive mice or animals with a history of wild-type *S. aureus* infection, mice that had been infected with the spa_{KKAA} variant and treated with daptomycin acquired protection from lethal challenge with *S. aureus* LAC, the current epidemic MRSA (USA300) strain in the United States (33) (Fig. 4A). Mice infected with the spa_{KKAA} variant developed IgG antibodies against six secreted virulence factors that represent leading vaccine candidates: ClfA, FnBPB, IsdB, Coa, Hla, and SpA (Fig. 4B). These results suggest that prior infection of mice with the spa_{KKAA} variant elicits antibodies against *S. aureus* protective antigens and raises protective immunity in mice against highly virulent MRSA strains. The development of protective immunity by the spa_{KKAA} variant is due to the loss of protein A-dependent B cell superantigen activity. In support of this hypothesis, immunization of mice with purified SpA_{KKAA} elicited high-titer specific antibodies and IgG class switching (IgG1 and IgG2b) (Fig. 4D). This was not observed when mice were immunized with either wild-type SpA or SpA_{KK}. SpA_{AA} immunization elicited specific antibodies; however, IgG titers were lower and IgG class switching did not occur (Fig. 4D).

Staphylococcal escape from host immune surveillance requires protein A and immunoglobulin. We asked whether Sbi, which binds IgG Fcγ as well as complement factors H and C3b (34), contributes to *S. aureus* escape from host immune surveillance by generating the spa_{KKAA} *sbi* mutant (Fig. 5A). When subjected to flow cytometry with mouse IgM and IgG, antibodies of both Ig types bound to the surface of the spa_{KKAA} *sbi* mutant (Fig. 5B). These natural antibodies against *S. aureus* were detected in sera from naive BALB/c and C57BL/6 animals but not in μMT mice. To discern whether natural antibodies provide protection against *S. aureus*, C57BL/6 and μMT mice were infected with wild-type and spa_{KKAA} or spa_{KKAA} *sbi S. aureus* strains. As expected, the staphylococcal load and abscess formation in organ tissues from spa_{KKAA} infected animals were lower than those of mice infected with wild-type *S. aureus* (Table 2). Mice infected with the spa_{KKAA} *sbi* mutant did not display a further reduction in bacterial load and abscess formation (Table 2). The virulence defects associated with wild-type *spa* were not observed in μMT mice, as similarly low numbers of bacteria and abscesses were isolated from organ tissues of μMT

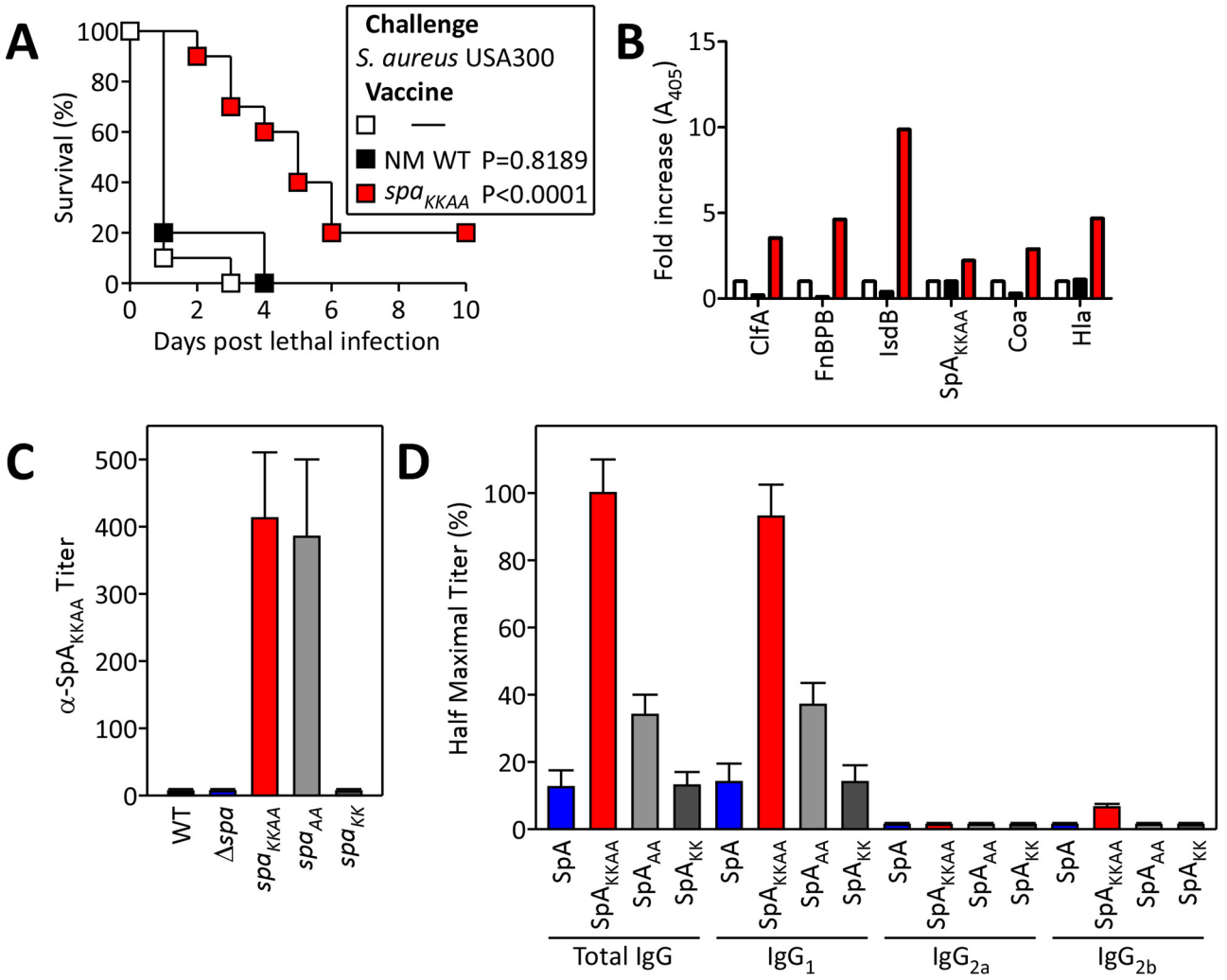

FIG 4 *S. aureus* requires protein A to escape host immune surveillances. (A) Kaplan-Meier study comparing the survival of mice ($n = 10$) challenged with a lethal dose of methicillin-resistant *S. aureus* USA300 LAC (intravenous injection of 5×10^7 CFU) without (naive) or with prior infection of wild-type (WT) or spa_{KKAA} mutant *S. aureus* Newman (NM). (B) IgG antibodies specific for staphylococcal protective antigens (ClfA, FnBPB, IsdB, SpA_{KKAA}, Coa, or Hla) in the serum of mice ($n = 10$) without (naive) or with previous infection of wild-type or spa_{KKAA} mutant *S. aureus*. (C) SpA_{KKAA}-specific IgG antibodies in the serum of mice ($n = 10$) infected with wild-type and *spa* mutant *S. aureus* (mean ± SEM). (D) Purified SpA, SpA_{KK}, SpA_{AA}, or SpA_{KKAA} emulsified with complete Freund's adjuvant was used for immunization of mice ($n = 10$) followed by a booster with the same antigen emulsified with incomplete Freund's adjuvant. The serum of immunized mice was examined for IgG antibodies against protein A (SpA_{KKAA}) and their IgG1, IgG2a, and IgG2b subclasses.

animals infected with wild-type, spa_{KKAA}, or spa_{KKAA} *sbi* strains (Table 2). Further, similarly low staphylococcal loads and abscess numbers were detected in C57BL/6 and μMT mice infected with either the spa_{KKAA} or spa_{KKAA} *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 Fcγ domain of human and animal immunoglobulins (35, 36). This biochemical activity allows exogenously supplied protein A to block complement-dependent (opsono)phagocytosis 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 (μMT) (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_H3 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

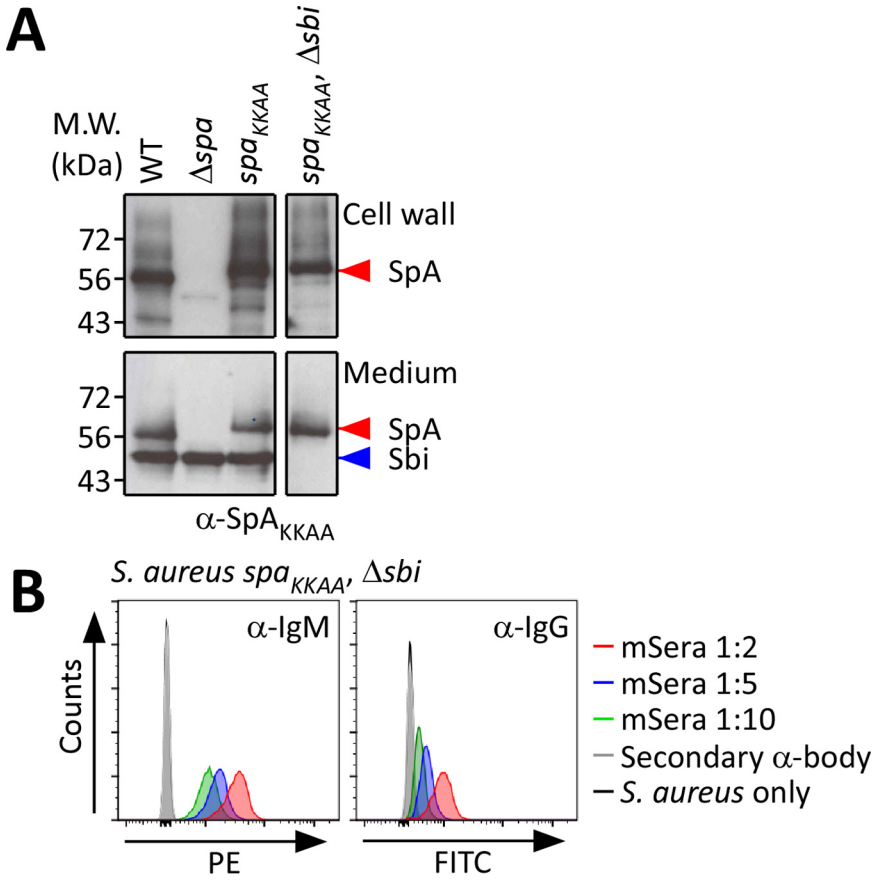

FIG 5 *S. aureus* escape from host immune surveillance requires protein A and immunoglobulin. (A) Immunoblotting reveals SpA in the envelope and in the extracellular medium of wild-type and spa_{KKAA} *sbi* mutant *S. aureus* cultures. (B) Natural IgG and IgM antibodies specific for spa_{KKAA} *sbi* mutant *S. aureus* in the serum of naive mice were detected by flow cytometry.

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_H3 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_{AA} and SpA_{KKAA}). These mutants harbor either 10 (spa_{KK} and spa_{AA}) 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 Δ*spa* 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_{AA} 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

TABLE 2 Virulence defects of *S. aureus spa* variants in μMT mice[a]

Mouse	*S. aureus* genotype	No. of mice[b]	Staphylococcal load			Abscess formation	
			Log_{10} CFU g^{-1}[c]	*P* value[d]	Reduction[e]	No. of abscesses/kidney[f]	*P* value[g]
C57BL/6	Wild type	12	6.32 ± 0.30			5.75 ± 0.73	
	spa_{KKAA}	10	4.07 ± 0.43	0.0009	2.25	3.17 ± 0.91	0.0442
	spa_{KKAA} Δ*sbi*	8	4.51 ± 0.48	0.0043	1.81	2.86 ± 0.89	0.0205
μMT	Wild type	14	5.28 ± 0.47			2.75 ± 1.06	
	spa_{KKAA}	14	4.87 ± 0.59	0.5051	0.41	2.33 ± 0.91	0.7692
	spa_{KKAA} Δ*sbi*	7	3.92 ± 0.66	0.2165	1.36	2.29 ± 1.17	0.7733

[a] Age and gender matched C57BL/6 and μMT mice were infected with 1×10^7 CFU of wild-type, spa_{KKAA}, or spa_{KKAA} Δ*sbi* mutant *S. aureus* Newman. At 28 days postinfection, animals were euthanized and necropsied, and bacterial load and numbers of abscesses in kidney tissues were determined.
[b] Number of 3-week-old female C57BL/6 and μMT mice per study.
[c] Staphylococcal load in homogenized renal tissues 28 days following infection. Values are means ± SEM; limit of detection, 1.99 log_{10} CFU g^{-1}.
[d] Statistical significance was calculated with the unpaired two-tailed Mann-Whitney test.
[e] Reduction in bacterial load, calculated as log_{10} 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.

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 nontoxigenic 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 SpA_{KKAA} 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 (Δ*spa*), and transposon selection (Δ*sbi*).

***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′ GGGGACCACTTTGTACAAGAAAGCTGGGTCATTTAAGAAGATTGTTTCAGATTTATG 3′), ext1R (5′ ATTTGTAAAGTCATCATAATATAACGAATTATGTATTGCAATACTAAAATC 3′), ext2F (5′ CGTCGCGAACTATAATAAAAACAAACAATACACAACGATAGATATC 3′), and ext2R (5′ GGGGACAAGTTTGTACAAAAAAGCAGGCAACGAACGCCTAAAGAAATTGTCTTTGC 3′). The DNA sequences of spa_{KKAA}, spa_{AA}, and spa_{KK} mutants were previously described (26). These sequences were amplified using the primers spaF (CATAATTCGTTATATTATGATGACTTTACAAATACATACAGGG) and spaR (GTATTGTTTGTTTTTATTATAGTTCGCGACGACGTCCA). 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, SpA_{KK}, SpA_{AA}, and SpA_{KKAA} (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-thiogalatopyranoside (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/in^2. Lysates were cleared of membrane and insoluble components by ultracentrifugation at 40,000 $\times$ *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, SpA_{AA}, SpA_{KK}, and SpA_{KKAA} in 10 mM phosphate buffer (pH 7.2), 50 mM Na_2SO_4 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 A_{600} 0.7. Bacteria were centrifuged, washed, fixed with glutaraldehyde and blocked. Cells were incubated with affinity purified anti-SpA_{KKAA} rabbit IgG for 1 h, washed, incubated with Alexafluor 647-conjugated goat anti-rabbit IgG (Invitrogen), and washed in PBS. Bacteria were settled in polylysine-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-scanner 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 Fcγ 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 His_6-tagged SpA, SpA_{AA}, SpA_{KK}, and SpA_{KKAA} 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 $\times$ 10^5 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 eukaryotic cells. 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_{KKAA}, ClfA, 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 A_{600} 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 $MgCl_2$ with 100 μg ml^{-1} lysostaphin] 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 electrotransferred to polyvinylidene difluoride (PDVF) membranes and analyzed by immunoblotting using affinity-purified rabbit anti-SpA_{KKAA} 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 Δ*spa*, spa_{AA}, spa_{KK}, and spa_{KKAA} 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 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 × 10^7 CFU of *S. aureus* Newman or its variants into the periorbital venous sinus of the right eye. On day 15 or 28 following infection, mice were euthanized by CO_2 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 spa_{KKAA} mutant for 15 days and treated with daptomycin at 10 mg kg of body weight^{-1} for 4 days. Three days after the last injection of daptomycin, animals were challenged with 5 × 10^7 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 Δ*spa*, spa_{AA}, spa_{KK}, or spa_{KKAA} 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/6J and B6.129S2-Ighm^{tm1Cgn}/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 × 10^6 CFU of *S. aureus* into the periorbital venous sinus of the right eye. At 30 min postinfection, mice were euthanized by CO_2 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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