Nontoxigenic protein A vaccine for methicillin-resistant *Staphylococcus aureus* infections in mice

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The current epidemic of hospital- and community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infections has caused significant human morbidity, but a protective vaccine is not yet available. Prior infection with *S. aureus* is not associated with protective immunity. This phenomenon involves staphylococcal protein A (SpA), an *S. aureus* surface molecule that binds to Fcγ of immunoglobulin (Ig) and to the Fab portion of V_H3-type B cell receptors, thereby interfering with opsonophagocytic clearance of the pathogen and ablating 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 SpA_{KKAA} to bind Fcγ or Fab V_H3 and promote B cell apoptosis. Immunization of mice with SpA_{KKAA} raised antibodies that blocked the virulence of staphylococci, promoted opsonophagocytic clearance, and protected mice against challenge with highly virulent MRSA strains. Furthermore, SpA_{KKAA} immunization enabled MRSA-challenged mice to mount antibody responses to many different staphylococcal antigens.

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Abbreviations used: MRSA, methicillin-resistant *S. aureus*; SpA, staphylococcal protein A; vWF, von Willebrand factor.

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 *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 β-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.

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 *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, septicemia, and abscess development (Palmqvist et al., 2002; Gómez et al., 2004; Cheng et al., 2009).

The vast majority of clinical *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_H3 type B cell receptors (Sasso et al., 1989), von Willebrand factor (vWF; Hartleib 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;

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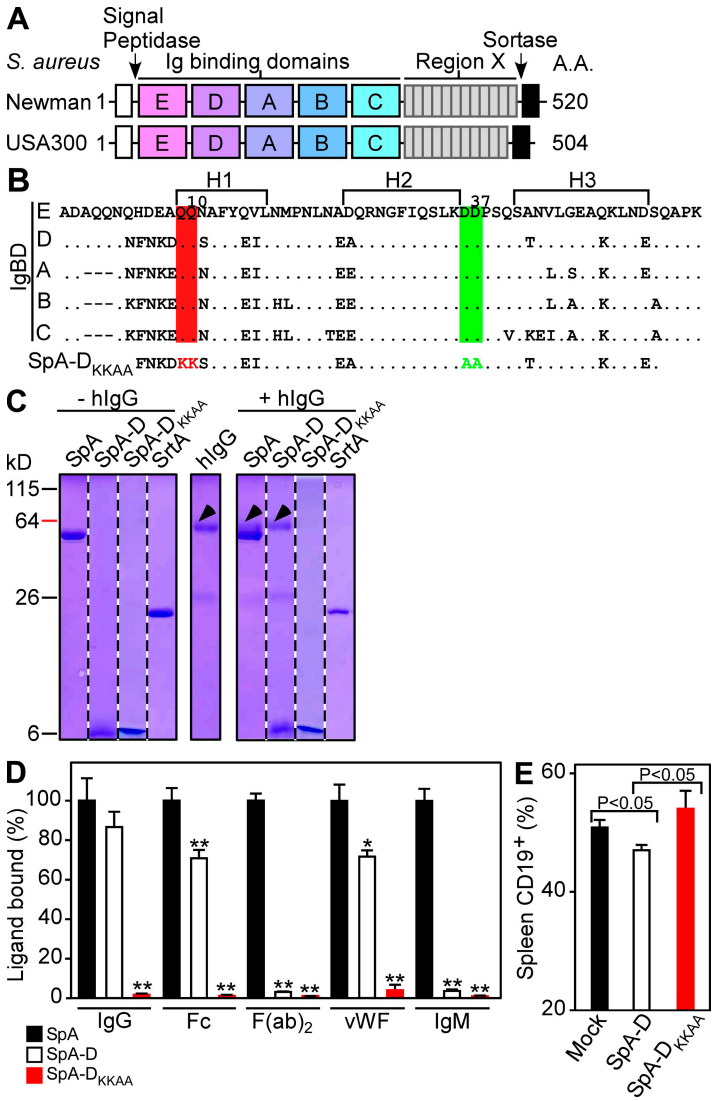

Figure 1. Generation of a nontoxigenic SpA vaccine. (A) SpA of *S. aureus* Newman and USA300 LAC harbors an N-terminal signal peptide (white boxes), five Ig binding domains (E, D, A, B, and C), variable region X, and C-terminal sorting signal (black boxes). (B) Amino acid sequence of the five Ig binding domains, as well as nontoxigenic SpA-D$_{KKAA}$, with the positions of triple α-helical bundles (H1, H2, and H3), as well as glutamine (Q, red) 9 and 10 and aspartate (D, green) 36 and 37 as indicated. (C) Coomassie blue–stained SDS-PAGE of SpA, SpA-D, SpA-D$_{KKAA}$, or sortase A purified on Ni-NTA sepharose in the presence or absence of human immunoglobulin (hIgG). (D) ELISA examining the association of immobilized SpA, SpA-D, or SpA-D$_{KKAA}$ with human IgG, as well as its Fc or F(ab)$_2$ fragments, vWF and IgM. Statistical significance of SpA-D$_{KKAA}$ binding to each ligand was compared against SpA-D, and SpA-D binding was compared against SpA ($n = 3$). *, $P < 0.05$; **, $P < 0.01$. (E) CD19$^+$ B lymphocytes in splenic tissue of 6-wk-old BALB/c mice ($n = 6$) that had been mock immunized or treated with SpA-D or SpA-D$_{KKAA}$ were quantified by FACS. Data are the means and error bars represent ±SEM. Results in C–E are representative of three independent analyses.

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* Newman *Δspa*. 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-D$_{KKAA}$ 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_H3 (Graille et al., 2000) or Fcγ (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^{9}Lys, Gln^{10}Lys, Asp^{36}Ala, and Asp^{37}Ala were introduced into the D domain to generate SpA-D$_{KKAA}$ (Fig. 1 B). The ability of isolated SpA-D or SpA-D$_{KKAA}$ 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-D$_{KKAA}$ 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_H3-type IgG (Cook and Tomlinson, 1995). Human Fcγ and F(ab)$_2$ fragments, as well as IgM, all

Silverman and Goodyear, 2006). In contrast, SpA binding to the Fcγ of Ig interferes with opsonophagocytic clearance of staphylococci by polymorphonuclear leukocytes (Peterson et al., 1977). SpA is synthesized as a precursor with an N-terminal signal peptide and a C-terminal sorting signal for covalent anchoring to the cell wall (Schneewind et al., 1992). The N-terminal part of mature SpA is comprised of four or five 56–61-residue Ig binding domains (Sjödahl, 1977), which fold into triple helical bundles connected by short linkers (Deisenhofer, 1981). The C-terminal region X is comprised of Xr, a highly repetitive yet variable octapeptide, and Xc, a domain of unique sequence which abuts the cell wall anchor structure of SpA (Guss et al., 1984; Schneewind et al., 1995).

As a result of the attribute of simultaneously binding Fcγ and Fab, SpA vaccines with neutralizing antibodies and protective immunity have hitherto not been reported (Greenberg et al., 1989). We wondered whether antibodies that neutralize the immunosuppressive properties of SpA could affect the outcome of *S. aureus* infections.

Table I. Active immunization of mice with SpA vaccines

Antigen	Staphylococcal load and abscess formation in renal tissue					
	Log_{10} CFU g^{-1}[a]	P value[b]	Reduction (log_{10} CFU g^{-1})[c]	IgG titer[d]	Number of abscesses[e]	P-value[b]
S. aureus **Newman challenge**						
Mock	6.46 ± 0.25	N/A	N/A	<100	3.7 ± 1.2	N/A
SpA	3.95 ± 0.56	0.0003	2.51	1,706 ± 370	2.1 ± 1.2	0.3581
SpA-D	4.43 ± 0.41	0.0001	2.03	381 ± 27	1.5 ± 0.8	0.1480
SpA D_{KKAA}	3.39 ± 0.50	<0.0001	3.07	5,600 ± 801	0.5 ± 0.4	0.0204
S. aureus **USA300 (LAC) challenge**						
Mock	7.20 ± 0.24	N/A	N/A	<100	4.0 ± 0.8	N/A
SpA	6.81 ± 0.26	0.2819	0.39	476 ± 60	3.3 ± 1.0	0.5969
SpA-D	6.34 ± 0.52	0.1249	0.86	358 ± 19	2.2 ± 0.6	0.0912
SpA-D_{KKAA}	6.00 ± 0.42	0.0189	1.20	3,710 ± 1147	1.6 ± 0.6	0.0277
SpA_{KKAA}	3.66 ± 0.76	0.0001	3.54	10,200 ± 2476	1.2 ± 0.5	0.0109

[a]Means ± SEM of staphylococcal load calculated as log_{10} 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.
[b]Statistical significance was calculated with the unpaired two-tailed Students *t* test and p-values were recorded.
[c]Reduction in bacterial load calculated as log_{10} CFU g^{-1}.
[d]Means ± 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.
[e]Histopathology 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.

bound to full-length SpA or SpA-D but not to SpA-D_{KKAA} (Fig. 1 D). Injection of SpA-D into the peritoneal cavity of mice resulted in B cell expansion followed by apoptotic collapse of $CD19^+$ (or $CD45R^+$) lymphocytes in the spleen tissue of BALB/c or C57BL/6 mice (Goodyear and Silverman, 2003; Fig. 1 E). B cell superantigen activity was not observed after injection with SpA-D_{KKAA}, and TUNEL-staining of splenic tissue failed to detect the increase in apoptotic cells that follows injection of SpA or SpA-D (Fig. 1 E; and Fig. S2, E–G).

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 nontoxigenic 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 reiterative 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 log_{10} 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 log_{10} 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

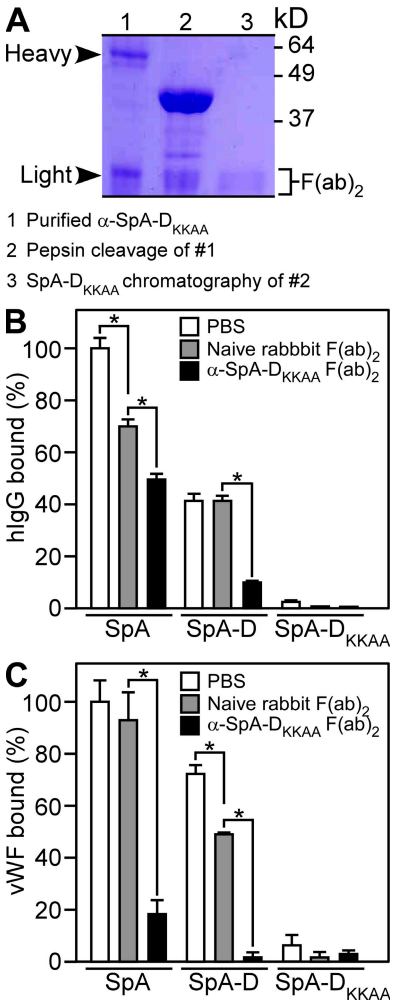

Figure 2. Antibodies raised by the nontoxigenic SpA vaccine block the association of immunoglobulin and vWF with SpA. (A) Rabbit antibodies raised against SpA-D_{KKAA} were affinity purified on a matrix with immobilized antigen and analyzed by Coomassie blue–stained SDS-PAGE under reducing conditions (lane 1, α-SpA-D_{KKAA}). SpA-D_{KKAA} 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-D_{KKAA} (lane 3). (B and C) SpA-D_{KKAA} specific $F(ab)_2$ was added to wild-type SpA, SpA-D, or SpA-D_{KKAA}, 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. *, $P < 0.01$.

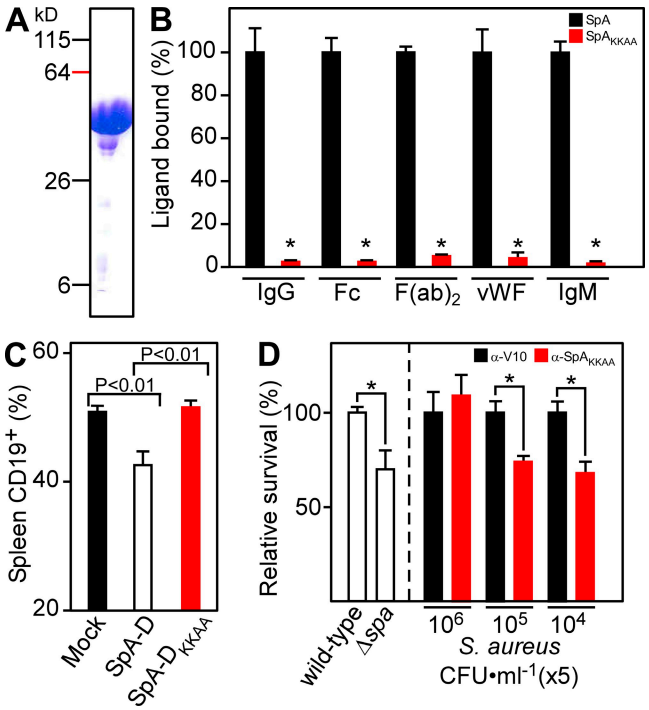

Figure 3. Full-length nontoxigenic SpA elicits antibodies that stimulate opsonophagocytic clearance of staphylococci. (A) Full-length SpA_{KKAA} was purified on Ni-NTA sepharose and analyzed by Coomassie blue–stained SDS-PAGE. (B) ELISA examining the association of immobilized SpA or SpA_{KKAA} with 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 ($n = 5$) that had been mock immunized or treated with SpA or SpA_{KKAA} were quantified by FACS. (D, Left) Anticoagulated mouse whole blood (lepirudin) was incubated with 5×10^5 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_{KKAA}-specific antibodies in naive mouse 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.

LAC isolate for animal challenge (Diep et al., 2006). This highly virulent CA-MRSA strain spread rapidly throughout the United States, causing significant human morbidity and mortality. Compared with adjuvant control mice, SpA-D_{KKAA}–immunized animals harbored a 1.20 $\log_{10}$ CFU g^{-1} reduction in bacterial load of infected kidney tissues. Histopathology examination of renal tissue after *S. aureus* USA300 challenge revealed that the mean number of abscesses was reduced from 4.0 (±0.8) to 1.6 (±0.6; $P = 0.0277$). In contrast, SpA or SpA-D immunization did not cause a significant reduction in bacterial load or abscess formation (Table I; and Fig. S3, I–L and N–Q).

SpA-D_{KKAA} antibodies neutralize immunoglobulin binding activities of SpA

Rabbits were immunized with SpA-D_{KKAA}, and specific antibodies were purified on SpA-D_{KKAA} affinity column and analyzed by SDS-PAGE (Fig. 2 A, lane 1). SpA-D_{KKAA}–specific IgG was cleaved with pepsin to generate Fcγ and $F(ab)_2$ fragments (Fig. 2 A, lane 2), the latter of which were repurified by affinity chromatography on SpA-D_{KKAA} column (Fig. 2 A, lane 3). Binding of human IgG or vWF to SpA or SpA-D was perturbed by SpA-D_{KKAA}–specific $F(ab)_2$, indicating that SpA-D_{KKAA}–derived antibodies can block the Fcγ- and vWF-binding properties of SpA (Fig. 2, B and C).

SpA_{KKAA} generates improved protective immune responses

To further improve the vaccine properties for nontoxigenic SpA, we generated SpA_{KKAA}, which includes all five Ig binding domains with four amino acid substitutions—Gln^9Lys, $Gln^{10}Lys$, $Asp^{36}Ala$, and $Asp^{37}Ala$—in each of its five domains (E, D,

Table II. Passive immunization of mice with antibodies against SpA

Antibody[a]	Staphylococcal load and abscess formation in renal tissue					
	Log_{10} CFU g^{-1}[b]	P value[c]	Reduction (log_{10} CFU g^{-1})[d]	IgG titer[e]	Number of abscesses[f]	P-value[c]
Mock (α-V10)	7.10 ± 0.14	N/A	N/A	<100	4.5 ± 0.8	N/A
α-SpA-D_{KKAA}	5.53 ± 0.43	0.0016	1.57	466 ± 114	1.9 ± 0.7	0.0235
α-SpA_{KKAA}	5.69 ± 0.34	0.0005	1.41	1,575 ± 152	1.6 ± 0.5	0.0062

[a]Affinity-purified antibodies were injected into the peritoneal cavity of BALB/c mice at a concentration of 5 mg × kg^{-1} 24 h before intravenous challenge with 1 × 10^7 CFU *S. aureus* Newman.

[b]Means ± SEM of staphylococcal load calculated as log_{10} CFU g^{-1} in homogenized renal tissues 4 d after infection in cohorts of 15 BALB/c mice per immunization. A representative of three independent and reproducible animal experiments is shown.

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

[d]Reduction in bacterial load calculated as log_{10} CFU g^{-1}.

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

[f]Histopathology 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.

A, B, and C; Sjödahl, 1977). Polyhistidine-tagged SpA_{KKAA} was purified by affinity chromatography and analyzed by Coomassie blue–stained SDS-PAGE (Fig. 3 A). Unlike full-length SpA, SpA_{KKAA} did not bind human IgG, Fc and $F(ab)_2$, IgM, or vWF (Fig. 3 B). SpA_{KKAA} 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_{KKAA} immunization generated higher specific antibody titers than SpA-D_{KKAA} 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_{KKAA}-vaccinated animals harbored 3.54 log_{10} CFU g^{-1} fewer staphylococci in renal tissues ($P = 0.0001$) and also caused a greater reduction in the number of abscess lesions ($P = 0.0109$; Table I; and Fig. S3, I–R). Furthermore, SpA_{KKAA} 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-D_{KKAA} or SpA_{KKAA} 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-D_{KKAA} caused a 1.56 log_{10} CFU g^{-1} reduction ($P = 0.0183$), whereas SpA_{KKAA} vaccination reduced the load by 2.7 log_{10} CFU g^{-1} ($P = 0.0059$). These data suggest that antibodies against SpA, generated via active immunization using nontoxigenic SpA_{KKAA}, can interfere with bacterial persistence in host tissues.

SpA-specific antibodies generate protective immunity

SpA_{KKAA} was used to immunize rabbits. Rabbit antibodies specific for SpA-D_{KKAA} or SpA_{KKAA} 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-D_{KKAA}/SpA_{KKAA} 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-D_{KKAA}- ($P = 0.0016$) or SpA_{KKAA} ($P = 0.0005$)-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-D_{KKAA} or SpA_{KKAA} is conferred by antibodies that bind SpA and neutralize its ability to bind Ig.

SpA_{KKAA} 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-D_{KKAA}–specific IgG in these animals was determined by dot blot as 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_{KKAA}- or SpA-D_{KKAA}–immunized mice ($P \leq 0.05$ log_{10} 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. 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_{KKAA} 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_{KKAA} (IgG titer 2,907 [±357]; $P < 0.001$, SpA_{KKAA} vs. mock) mounted humoral immune responses against every antigen examined (Fig. 4 B). With the exception of Eap, IsdA, and IsdB antibodies, the serum of SpA_{KKAA}-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 Fcγ or V_H3-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

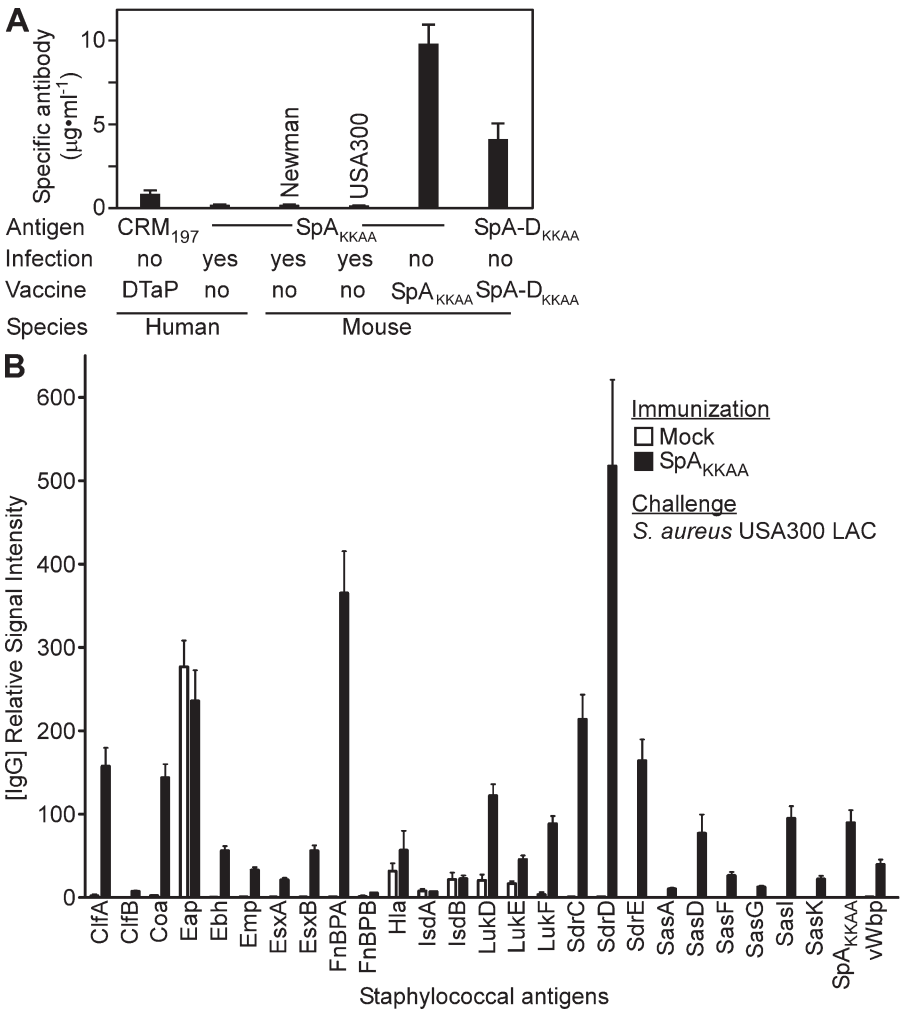

Figure 4. SpA_{KKAA} immunization modifies host immune responses to staphylococcal infection. (A) Human or mouse serum antibody titers to diphtheria toxoid (CRM197) and nontoxigenic SpA_{KKAA} or SpA-D_{KKAA}. Human volunteers with a history of diphtheria (DTaP) immunization and staphylococcal infection ($n = 16$), as well as mice ($n = 20$) that had been infected with *S. aureus* Newman or USA 300 LAC or immunized with SpA_{KKAA} or SpA-D_{KKAA}, were examined by quantitative immunoblot. (B) Cohorts of BALB/c mice ($n = 15$) were immunized with SpA_{KKAA} or with PBS/adjuvant control (mock) and then challenged by intravenous inoculation with 5×10^6 CFU *S. aureus* USA300 LAC. 30 d after infection, animals were bled and serum samples were analyzed for antibody responses to staphylococcal antigens. 27 recombinant His_6-tagged staphylococcal proteins were purified by Ni-NTA affinity chromatography and immobilized on nitrocellulose membrane at 2 μg. Signal intensities in sera from mice were quantified and normalized by infrared imaging. Data are the means and error bars represent ±SEM. Results in A and B are representative of three independent analyses.

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_{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, and dialyzed against PBS. Affinity-purified antibodies were mixed with 3 mg pepsin at 37°C for 30 min and quenched with 1 M Tris-HCl, pH 8.5. $F(ab)_2$ 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′-GCTGCACATATGGCGCAACACGATGAAGCTCAAC-3′ and 5′-AGTGGATCCTTATGCTTGAGCTTTGTTAGCATCTGC-3′, using *S. aureus* Newman DNA. SpA-D was PCR amplified with two primers: 5′-AACATATGTTCAACAAAGATCAACAAAGC-3′ and 5′-AAGGATCCAGATTCGTTTAATTTTTTAGC-3′. The sequence for $SpA\text{-}D_{KKAA}$ was mutagenized with two sets of primers: 5′-CATATGTTCAACAAAGATAAAAAAAGCGCCTTCTATGAAATC-3′ and 5′-GATTTCATAGAAGGCGCTTTTTTTATCTTTGTTGAACATATG-3′ for Q9K and Q10K, and 5′-CTTCATTCAAAGTCTTAAAGCCGCCCCAAGCCAAAGCACTAAC-3′ and 5′-GTTAGTGCTTTGGCTTGGGGCGGCTTTAAGACTTTGAATGAAG-3′ for D36A and D37A. The sequence of SpA_{KKAA} was synthesized by Integrated DNA Technologies, Inc. PCR products were cloned into pET-15b generating N-terminal His_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 titers before challenge.

Mouse infection. Staphylococci were used to infect anesthetized mice by retroorbital injection (1×10^7 CFU *S. aureus* Newman, 5×10^6 CFU *S. aureus* USA300, or 3×10^7 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 agreement 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^{-1} of lepirudin anticoagulant. 50 μl of $5 \times 10^{4-6}$ CFU ml^{-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^{-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, Fc, or $F(ab)_2$ fragments, IgM (The Jackson Laboratory), and vWF (Thermo Fisher Scientific) and developed using OptEIA reagent. For inhibition, plates were incubated with either naive rabbit $F(ab)_2$ fragments (The Jackson Laboratory) or 10 μg ml^{-1} of affinity-purified $F(ab)_2$ 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_{KKAA}, and CRM_{197}, 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_6-tagged staphylococcal proteins. Signal intensities in mouse sera were quantified and normalized using anti-His_6 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_{KKAA} can protect against this disease. Fig. S2 shows that SpA_{KKAA}, unlike wild-type SpA, does not induce B cell apoptosis in mice and that antibodies raised against SpA_{KKAA} neutralize the B cell superantigen attributes of SpA. Fig. S3 shows that immunization of mice with SpA_{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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