RESEARCH ARTICLE

Protein A Suppresses Immune Responses during *Staphylococcus aureus* Bloodstream Infection in Guinea Pigs

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ABSTRACT ***Staphylococcus aureus*** **infection is not associated with the development of protective immunity, and disease relapses occur frequently. We hypothesize that protein A, a factor that binds immunoglobulin Fcγ and cross-links V_H3 clan B cell receptors (IgM), is the staphylococcal determinant for host immune suppression. To test this, vertebrate IgM was examined for protein A cross-linking. High V_H3 binding activity occurred with human and guinea immunoglobulin, whereas mouse and rabbit immunoglobulins displayed little and no binding, respectively. Establishing a guinea pig model of *S. aureus* bloodstream infection, we show that protein A functions as a virulence determinant and suppresses host B cell responses. Immunization with SpA_{KKAA}, which cannot bind immunoglobulin, elicits neutralizing antibodies that enable guinea pigs to develop protective immunity.**

IMPORTANCE ***Staphylococcus aureus*** **is the leading cause of soft tissue and bloodstream infections; however, a vaccine with clinical efficacy is not available. Using mice to model staphylococcal infection, earlier work identified protective antigens; however, corresponding human clinical trials did not reach their endpoints. We show that B cell receptor (IgM) cross-linking by protein A is an important immune evasion strategy of *S. aureus* that can be monitored in a guinea pig model of bloodstream infection. Further, immunization with nontoxigenic protein A enables infected guinea pigs to elicit antibody responses that are protective against *S. aureus*. Thus, the guinea pig model may support preclinical development of staphylococcal vaccines.**

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Staphylococcus aureus is a commensal of human skin and an invasive pathogen causing skin and soft tissue infections (SSTIs), bacteremia, sepsis, and endocarditis (1). *S. aureus* is responsible for more than 70% of the SSTIs in the United States (2). Even with antibiotic and surgical therapy, staphylococcal SSTIs can relapse, and recurrent disease is associated with bloodstream infection (3). An annual mortality rate of approximately 20,000 is attributed to *S. aureus* bloodstream infection, exceeding the combined deaths caused by influenza, viral hepatitis, and HIV/AIDS (4). Individuals at high risk for *S. aureus* bloodstream infection include patients with indwelling catheters, medical implants, surgical wounds, trauma, diabetes, old age, and low birth weight (5). The emergence and spread of drug-resistant strains, designated MRSA (methicillin-resistant *S. aureus*), has caused increased therapeutic failure and mortality rates due to staphylococcal infections (6). To address this public health crisis, significant effort has been directed toward developing vaccines that protect high-risk individuals against *S. aureus* infection (7).

Work on staphylococcal vaccines commenced more than a century ago (8). Clinical trials with whole-cell killed or subunit vaccines derived from secreted exotoxins, for example, α-hemolysin (Hla) and coagulase, failed to protect against recurrent SSTI (9, 10). Immunotherapy with polyclonal antibodies that neutralize Hla or clumping factor A (ClfA), a staphylococcal surface protein that binds fibrinogen (11), also did not protect against *S. aureus* infection (12, 13). Conjugates of *S. aureus* type 5/8 capsular polysaccharide (CP5/CP8) with *Pseudomonas* exotoxin A raised antibodies that promoted opsonophagocytic killing (OPK) of staphylococci (14). Nevertheless, the CP5/CP8 conjugate vaccine failed to protect hemodialysis patients against *S. aureus* infection (15). The clinical trial for V710, a vaccine composed of IsdB, a staphylococcal surface protein that binds heme and hemoglobin (16), was terminated (17); multiorgan dysfunction and death following *S. aureus* infection occurred more frequently in individuals receiving V710 than in control cohorts, and V710 immunization did not show a clinical benefit (17). Vaccine development has been hindered by the fact that *S. aureus* infection does not generate protective immunity in either humans or animals (18). Further, vaccines that elicit antibody responses against bacterial envelope components with the intent of promoting OPK of staphylococci (CP5/CP8, ClfA, or IsdB) may have failed because human immunoglobulin (Ig) effector functions are modified by staphylococcal protein A (SpA) (19).

Earlier work studied human serum IgG against staphylococcal antigens and sought to identify correlates with *S. aureus* disease susceptibility; these efforts identified at least eight different antigens where high-titer antibodies were associated with reduced occurrence of *S. aureus* infection (20–22). It is not clear, however,

whether antibodies against any one antigen are sufficient for protection of humans against *S. aureus* disease. Alternatively, the sum of many different antibody responses against a wide spectrum of antigens may be required for the development of protective immunity (18). The latter model may explain the conservation of the gene for SpA among *S. aureus* strains (23) and its function in modulating B cell and antibody responses in infected human hosts (24).

SpA is expressed by virtually all clinical *S. aureus* isolates (23, 25). The immunosuppressive attributes of SpA have been ascribed to two distinct binding activities for human and animal Igs, i.e., association with the Fcγ and Fab domains of antibodies (26–30). SpA binding to the Fcγ domain of IgG blocks OPK of staphylococci (31, 32), whereas SpA binding to Fab and cross-linking of IgM promotes B cell superantigen activity (27, 33). For V_H3 clonal B cells, SpA cross-linking of B cell receptors triggers proliferation and apoptotic collapse of the expanded lymphocyte populations (34). Nontoxigenic SpA, designated SpA_{KKAA}, was engineered by replacing 20 amino acid residues essential for its association with Ig Fcγ and Fab (35). Although SpA_{KKAA} harbors 20 amino acid substitutions, when it is adjuvanted and injected into mice or rabbits, immunization with this antigen elicits antibodies that neutralize SpA (35). The SpA_{KKAA}-derived polyclonal antibodies promote OPK of staphylococci and display adjuvant attributes by suppressing staphylococcal B cell superantigen activity and promoting humoral immune responses against a wide spectrum of antigens (35). Studies with mouse monoclonal antibodies (SpA_{KKAA} MAbs) corroborate this concept (36). SpA_{KKAA} MAb-mediated neutralization of SpA promotes OPK of *S. aureus* and the development of antibody responses against many different antigens (36). Together, the broad spectrum of humoral immune responses can prevent the pathogenesis of staphylococcal infections (18). When tested in mouse bloodstream infections, *S. aureus* lacking protein A (Δ*spa*) or expressing the nontoxigenic variant (spa_{KKAA}) displays defects in abscess formation and cannot suppress the adaptive immune responses of infected animals (37).

Earlier work on the preclinical development of staphylococcal vaccines involved efficacy studies with mice (14, 38). However, there is concern that protective immunity against *S. aureus* disease in mice is not predictive of vaccine success during clinical trials (39). In contrast to mice, guinea pigs have been successfully used as models for bacterial vaccine development, which includes vaccines licensed to prevent diphtheria and tetanus (40). Arne Forsgren used guinea pigs to model staphylococcal infections and to describe the pathophysiological attributes of SpA on the immune system (41–44). We show here that guinea pigs elaborate V_H3 clan IgG and IgM with high abundance, similar to the human immune system and unlike mice. Further, guinea pigs elaborate two IgG subclasses (IgG1 and IgG2) and represent a physiological host for *S. aureus* infection following transmission from humans (45). We therefore examined whether guinea pigs can be used to model *S. aureus* bloodstream infection, respond to SpA superantigen activity, and support the development of staphylococcal vaccines.

RESULTS

Guinea pig model of *S. aureus* bloodstream infection. Guinea pigs were anesthetized, and their fur was shaved in a triangular area defined by three anatomical positions, the right caudal ramus of the mandible, the greater tubercle of the humerus, and the manubrium. The exposed skin was sterilized by alternating applications of iodine and alcohol. A small surgical incision was used to expose the right jugular vein, into which a suspension of *S. aureus* was injected. The surgical site was closed by sterile suture and observed over the course of experiments. Guinea pigs did not suffer from local skin or soft tissue infections at the site of injection.

Cohorts of guinea pigs were infected with increasing doses of *S. aureus* Newman, a methicillin-sensitive *S. aureus* clinical isolate (46), or with USA300 LAC, the American epidemic MRSA clone (47). At challenge doses of 1×10^7 to 5×10^7 CFU of *S. aureus* Newman or MRSA USA300, guinea pigs displayed signs of acute disease, which was recorded as weight loss over a period of 4 to 5 days, amounting to an initial 10 to 20% drop in body weight (Fig. 1A). Mock-infected (phosphate-buffered saline [PBS]-treated) control animals did not display weight loss but gained body weight in a steady manner, similar to guinea pigs unencumbered by surgical procedures. Guinea pigs with a 5×10^7-CFU *S. aureus* Newman bloodstream infection had not regained the lost body weight by day 10 and even thereafter displayed only modest weight gains (mock infection versus *S. aureus* Newman bloodstream infection [5×10^7 CFU], $P < 0.05$ [days 4 to 17 postinfection]) (Fig. 1A). Guinea pigs infected with 1×10^7 CFU of MRSA isolate USA300 LAC displayed similar disease symptoms. Although these animals initially also lost 10 to 20% of their body weight, their loss was recovered by day 10 and thereafter they were able to gain weight in a steady manner (mock infection versus *S. aureus* USA300 LAC bloodstream infection, $P < 0.05$ [days 6 to 12 postinfection]) (Fig. 1A).

To analyze the pathological features of *S. aureus* bloodstream infection, cohorts of guinea pigs were euthanized on day 5 or 17 following the intravenous challenge. Their kidneys were removed during necropsy, and one of the two organs was examined for staphylococcal load by spreading tissue homogenates onto agar plates, incubating them at 37°C, and enumerating the *S. aureus* CFU. Bacteria were not found in kidney tissues of mock-treated animals (data not shown). On day 5 following the intravenous challenge, *S. aureus* Newman-infected guinea pigs harbored an average staphylococcal load of 5.80 $\log_{10}$ CFU per kidney, whereas animals challenged with USA300 harbored 6.24 $\log_{10}$ CFU (Fig. 1B). The bacterial loads in renal tissues declined by day 17 to 4.34 $\log_{10}$ CFU and 2.85 $\log_{10}$ CFU for *S. aureus* Newman and MRSA strain USA300, respectively (Fig. 1B).

The other kidney of necropsied guinea pigs was analyzed for histopathology features (Fig. 1C and D). In both *S. aureus* Newman- and USA300-infected animals, severe inflammatory responses with infiltrates of granulocytes, macrophages, and lymphocytes (red arrows), as well as necrosis of the glomerular capsules (blue arrows), were observed (Fig. 1C and D). *S. aureus* Newman-infected animals harbored an average of 3.8 ± 1.1 abscess lesions per section of kidney at day 5 postinfection. Similarly, *S. aureus* USA300-infected animals displayed an average of 3.4 ± 1.5 lesions per kidney section (Fig. 1C). Although the bacterial load decreased by day 17 in renal tissues, the numbers of infectious lesions remained similar for both *S. aureus* Newman- and USA300-infected animals (Fig. 1C). Taken together, these data suggest that an intravenous challenge with *S. aureus* Newman or MRSA USA300 causes bloodstream infection and disseminated infectious lesions in many different organ systems that can be clinically monitored as weight loss. Although disease is severe at an inoculum of 1×10^7 to 5×10^7 CFU, bloodstream infection is not

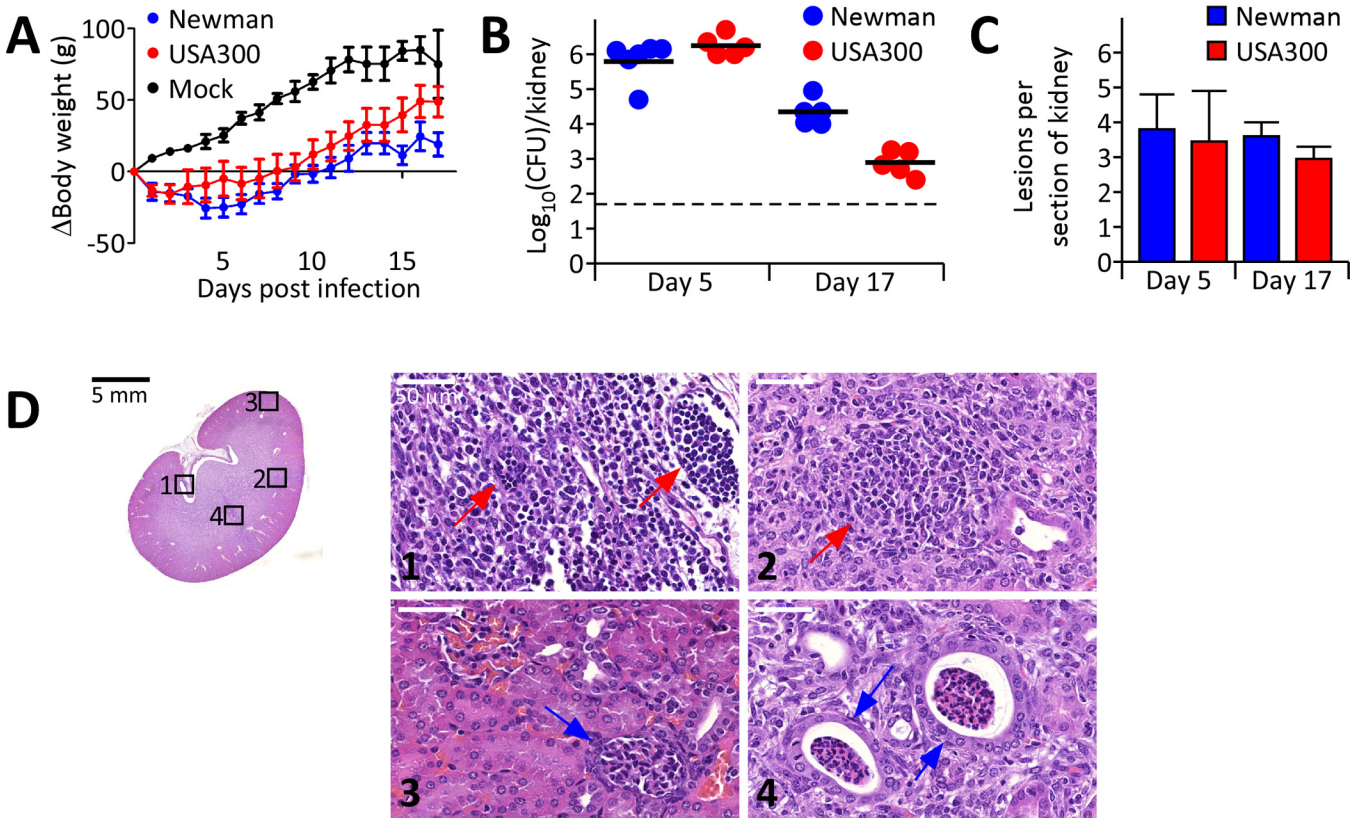

FIG 1 Guinea pig model of *S. aureus* bloodstream infection. (A) Cohorts of female Hartley guinea pigs ($n = 5$ or 6) were challenged by intravenous injection with *S. aureus* Newman (5×10^7 CFU) or USA300 (1×10^7 CFU), and body weight was recorded over 17 days. (B) On days 5 and 17 postinfection, guinea pigs were euthanized and necropsied; bacterial loads ($\log_{10}$ CFU) in renal tissues were quantified. (C) Hematoxylin-eosin-stained, thin-sectioned renal tissues of infected guinea pigs were examined by light microscopy to quantify the number of infectious lesions. (D) Representative images of infectious lesions. Red arrows identify immune cell infiltrates, including polymorphonuclear leukocytes, macrophages, and lymphocytes, whereas blue arrows identify necrosis of the glomerular capsule. The data are mean values, and error bars represent the standard errors of the means. Results are representative of two independent analyses.

lethal for guinea pigs and infected animals somewhat diminish the load of the bacterial invader.

Virulence defect of the *S. aureus strA* mutant. Sortase A (SrtA) is a transpeptidase that anchors surface proteins with LPXTG motif sorting signals to the cell wall envelope of Gram-positive bacteria (48, 49). An *S. aureus* Newman variant with a deletion of the *srtA* gene cannot anchor any of 19 different surface proteins in the bacterial envelope (50, 51). When tested in the mouse intravenous-challenge model, the *srtA* mutant cannot form abscess lesions or cause lethal disease (52, 53). To evaluate the contribution of SrtA to staphylococcal disease in guinea pigs, cohorts of animals were infected with 5×10^7 CFU of *S. aureus* Newman or the *srtA* mutant and monitored for 17 days (see Fig. S1 in the supplemental material). Compared to guinea pigs infected with wild-type *S. aureus*, animals infected with the *srtA* mutant quickly recovered from moderate body weight loss by day 6 and gained body weight at a rate similar to that of mock-treated animals (see Fig. S1A). Guinea pigs infected with wild-type staphylococci harbored an average bacterial load of 5.21 $\log_{10}$ CFU per kidney on day 5 and 4.40 $\log_{10}$ CFU on day 17 following infection. In contrast, the bacterial load of the *srtA* mutant was diminished, at 2.21 $\log_{10}$ CFU on day 5 of infection and 2.17 $\log_{10}$ CFU on day 17 (wild type versus *srtA* mutant, $P < 0.01$). Histopathology analysis revealed that the number of infectious lesions in renal tissues was reduced in guinea pigs infected with the *srtA* mutant: day 17 lesions, 8.1 ± 1.4 for wild-type *S. aureus* versus 1.5 ± 1.3 for the *srtA* mutant ($P < 0.05$; see Fig. S1C). Persistent infection with wild-type staphylococci caused severe inflammation and infiltration of large numbers of granulocytes in renal tissues that could even be detected in day 17 samples by histopathology analysis. In contrast, inflammatory responses to the *srtA* mutant were mostly resolved by day 17 (see Fig. S1D). These data suggest that SrtA and its anchored surface proteins are important contributors to the pathogenesis of *S. aureus* bloodstream infection in guinea pigs.

Association of guinea pig Ig with protein A. Genome sequencing revealed that the guinea pig IgH gene locus is similar in diversity to the human locus, whose V_H gene segments are responsible for generating diversity through a large spectrum of V_H3 idiotype B cells and are targeted by protein A (24, 54). To test whether protein A indeed associates with the heavy chains of guinea pig V_H3 clan antibodies, polyhistidine-tagged SpA and its variants were purified by affinity chromatography on Ni-nitrilotriacetic acid (NTA) Sepharose and used as bait for the binding of guinea pig, mouse, rabbit, and human IgG or IgM (Fig. 2A). As expected, SpA bound IgG from all four species and interacted with IgM from all of the species examined, except rabbit IgM (55, 56) (Fig. 2B and C). On the other hand, SpA_{KKAA} failed to associate with Igs from all four species (Fig. 2D to F).

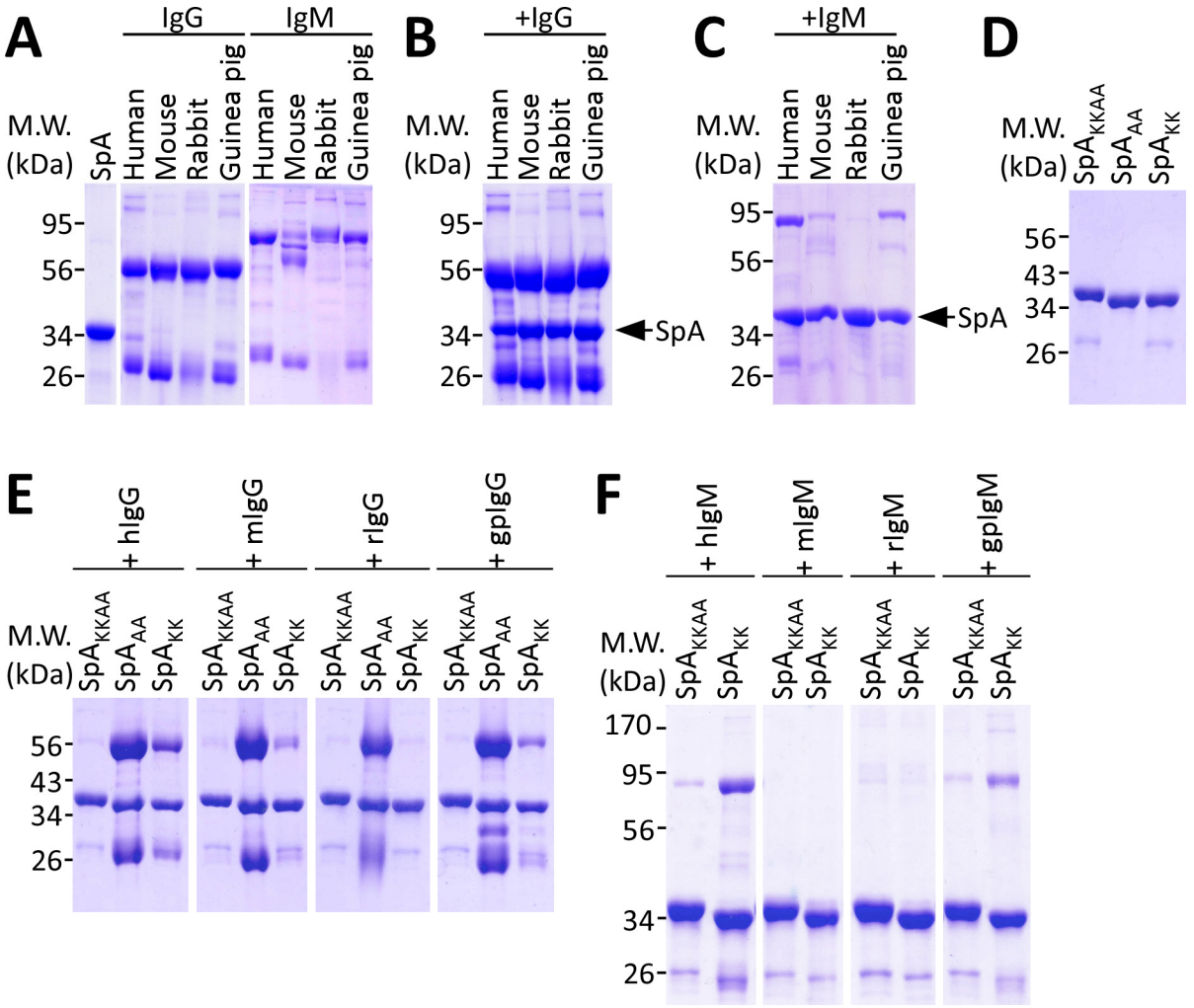

FIG 2 Protein A association with human, mouse, rabbit, and guinea pig Igs. (A) Protein A (SpA) was purified by affinity chromatography on Ni-NTA Sepharose and analyzed by Coomassie-stained SDS-PAGE along with human, mouse, rabbit, and guinea pig IgG or IgM. (B) SpA Ni-NTA Sepharose was subjected to IgG chromatography, and eluted complexes were analyzed by Coomassie-stained SDS-PAGE. (C) SpA Ni-NTA Sepharose was subjected to IgM chromatography, and eluted complexes were analyzed by Coomassie-stained SDS-PAGE. (D) SpA_{KK}, SpA_{AA}, and SpA_{KKAA} were purified on Ni-NTA Sepharose and analyzed by Coomassie-stained SDS-PAGE. (E) SpA_{KK}, SpA_{AA}, or SpA_{KKAA} Ni-NTA Sepharose was subjected to IgG chromatography, and eluted complexes were analyzed by Coomassie-stained SDS-PAGE. (F) SpA_{KK} or SpA_{KKAA} Ni-NTA Sepharose was subjected to IgM chromatography, and eluted complexes were analyzed by Coomassie-stained SDS-PAGE. M.W., molecular mass.

SpA_{AA}, which cannot bind V_H3 clan antibodies but associates with the Fcγ domain of IgG (37), retained similar amounts of human, mouse, rabbit, or guinea pig IgG (Fig. 2E). SpA_{KK}, a variant unable to associate with Fcγ yet unaffected in V_H3 clan Fab binding (37), retained part of human and guinea pig IgG and small amounts of mouse IgG; SpA_{KK} did not associate with rabbit IgG (Fig. 2E). When analyzed with IgM, SpA_{KK} retained large amounts of human and guinea pig IgM but not mouse or rabbit IgM (Fig. 2C). These data suggest that protein A associates with guinea pig Ig by binding the Fcγ domain of IgG molecules and the heavy chains of V_H3 clan IgG and IgM. Further, V_H3 clan IgG and IgM appear to be more abundant in guinea pig than mouse Ig, albeit that V_H3 clan abundance is not equivalent to that observed in human Ig.

Antigenicity of protein A and its variants in animals. B cell populations expressing V_H3 idiotype IgM receptors are thought to impact adaptive immune responses to protein A and other antigens presented by staphylococci during host invasion (24, 37). If this is so, we would predict an inverse correlation between the development of antibodies that recognize protein A as an antigen and the abundance of V_H3 IgM among mammalian species. To test this, we used purified SpA, SpA_{KK}, SpA_{AA}, and SpA_{KKAA} as antigens in mice, rabbits, and guinea pigs. The development of IgG antibodies in response to prime-boost immunization that specifically recognize protein A was quantified by enzyme-linked immunosorbent assay (ELISA) with SpA_{KKAA} as bait for immune serum. As control, immunization of animals with the adjuvant alone (PBS) did not raise protein A-specific antibodies in animals (see Fig. S2 in the supplemental material). Aluminum hydroxide-adsorbed SpA_{KKAA} failed to raise IgG responses in BALB/c mice, whereas SpA_{KKAA} emulsified in complete or incomplete Freund's adjuvant (CFA or IFA, respectively) elicited moderate amounts of SpA_{KKAA}-specific IgG (see Fig. S2A). Rabbits, which altogether lack V_H3-B cells, mounted a robust immune response against CFA- or IFA-emulsified SpA (see Fig. S2B). Mice elaborated low-

TABLE 1 Staphylococcal loads and abscess formation in renal tissue after passive immunization of mice with guinea pig immune serum

Immune serum[a]	$\log_{10}$ CFU g^{-1}[b]	*P* value[c]	Fold reduction[d]	IgG titer[e]	No. of lesions[f]	*P* value[c]
Naive	6.82 ± 0.50			<100	3.44 ± 1.01	
SpA	6.66 ± 0.32	0.9024	0.16	<100	2.50 ± 0.82	0.5338
SpA_{KK}	6.09 ± 0.31	0.2524	0.73	2245 ± 397	3.00 ± 0.91	0.8689
SpA_{AA}	5.37 ± 0.36	0.0537	1.45	2125 ± 84	0.80 ± 0.29	0.0520
SpA_{KKAA}	5.05 ± 0.33	0.0113	1.77	2699 ± 212	0.60 ± 0.34	0.0242

[a] Mice (6-week-old female BALB/c, $n = 10$) were passively immunized with 200 μl of guinea pig hyperimmune serum collected from naive animals or from animals immunized with SpA, SpA_{KK}, SpA_{AA}, or SpA_{KKAA} at 24 and 48 h prior to infection with 1×10^7 CFU of *S. aureus* Newman. At 5 days postinfection, animals were necropsied and bacterial loads and abscess formation were measured in the kidneys of infected animals.

[b] Mean staphylococcal loads ± the standard errors of the means calculated as $\log_{10}$ CFU g^{-1} in homogenized renal tissues 5 days following infection with a limit of detection of 1.99 $\log_{10}$ CFU g^{-1}.

[c] Statistical significance was calculated with the unpaired two-tailed Mann-Whitney test, and *P* values were recorded.

[d] Reductions in bacterial loads were calculated as $\log_{10}$ CFU g^{-1}.

[e] Mean half-maximal titers of antibody against SpA_{KKAA} ± the standard errors of the means in passively immunized mice as determined by ELISA at 5 days postinfection.

[f] Histopathology analysis of hematoxylin-eosin-stained, thin-sectioned kidneys. The number of abscesses per kidney was recorded and averaged for the final mean value ± the standard error of the mean.

titer antibodies that recognized protein A, whereas guinea pigs did not develop a SpA-specific antibody response (see Fig. S2B). As predicted, immunization with protein A variants that cannot cross-link V_H3 clan B cell receptors, SpA_{KKAA} and SpA_{AA}, resulted in increased antibody responses in mice and guinea pigs (see Fig. S2B). Surprisingly, SpA_{KK}, which is defective for Fcγ binding yet retains the attribute of V_H3 clan IgM cross-linking (37), could also elicit protein A-specific antibody responses in mice and guinea pigs, albeit not to the same level as SpA_{KKAA} and SpA_{AA} (see Fig. S2). Presumably, protein A association with the Fcγ domain of IgG molecules diminishes the immune system's ability to recognize this antigen. Together, these results support the hypothesis that protein A cross-linking of V_H3 clan B cell receptors interferes with the development of adaptive immune responses. Further, the severity of protein A-mediated interference is correlated with the abundance of V_H3 clan IgM and B cell receptors. Thus, on the basis of these data, we predict that the immunosuppressive attributes of protein A are most significant in humans, followed by guinea pigs, and are diminished in mice or rabbits.

Protein A-specific guinea pig antibodies prevent staphylococcal disease in mice. Previous work demonstrated that affinity-purified protein A-neutralizing antibodies from rabbits, when transferred to naive mice, protect against staphylococcal abscess formation (35). Here we asked whether serum of guinea pigs that were immunized with SpA or its variants can confer protection against staphylococcal disease. Cohorts of mice were immunized by intraperitoneal injection with guinea pig serum. Twenty-four hours following guinea pig serum transfer, animal cohorts were infected by intravenous injection of 1×10^7 CFU of *S. aureus* Newman. On day 5, animals were euthanized, their blood was sampled via cardiac puncture, and their kidneys were removed during necropsy. Protein A-specific antibody titers in blood samples were determined by ELISA with SpA_{KKAA} antigen (Table 1). Mice that had received SpA_{KKAA} guinea pig serum retained the highest SpA_{KKAA}-specific antibody titer, followed by those that had received SpA_{KK} and SpA_{AA} guinea pig serum. Renal tissues were either homogenized to determine their bacterial loads or subjected to histopathology analysis to quantify abscess lesions. Mice that had received guinea pig serum from mock-immunized animals harbored a bacterial load of 6.82 $\log_{10}$ CFU·g^{-1}, as well as an average of 3.44 ± 1.01 abscess lesions, in their renal tissues (Table 1). Serum from guinea pigs that had been immunized with either SpA or SpA_{KK} did not protect against this disease (Table 1). Animals that had been treated with SpA_{AA}-immune serum harbored reduced bacterial loads and fewer abscess lesions; however, these reductions were not statistically significant (Table 1). Mice that received SpA_{KKAA}-immune serum from guinea pigs displayed reduced staphylococcal loads (mock versus SpA_{KKAA} treatment, $P = 0.0113$) and diminished abscess formation (mock versus SpA_{KKAA} treatment, $P = 0.0242$) (Table 1). These data suggest that SpA_{KKAA} immunization of guinea pigs and passive transfer of serum into mice generate high titers of protein A-specific antibodies and protection against staphylococcal abscess formation.

B cell superantigen activity of protein A in guinea pigs. Previous work determined that SpA_{KKAA}-elicited antibodies from mice block the association of Ig with SpA and that these antibodies also neutralize B cell superantigen activity during *S. aureus* bloodstream infection in mice (35, 36). On the basis of earlier work with mice, where SpA_{KKAA}-specific antibody titers correlated with the ability of neutralizing protein A, we hypothesized that antibodies in guinea pig SpA_{KKAA}-immune serum would display the most potent neutralizing attributes, followed by antibodies in SpA_{KK}- or SpA_{AA}-immune serum. In contrast, antibodies in guinea pig SpA-immune serum did not bind to SpA_{KKAA} and we did not expect these antibodies to interfere with protein A binding to human IgG or IgM. To test this, the binding of SpA to either human IgG or IgM was analyzed in the absence or presence of guinea pig immune serum. Surprisingly, SpA binding to human IgG and IgM was perturbed to similarly high degrees by SpA-, SpA_{KK}-, and SpA_{KKAA}-immune serum, whereas SpA_{AA}-immune serum had a moderate effect and serum from mock (PBS)-immunized control guinea pigs did not interfere (see Fig. S3A and B in the supplemental material).

Humans possess large populations of V_H3^+ B cells but cannot develop protein A-neutralizing antibodies during infection (24). Nevertheless, *S. aureus* infection triggers large expansions of V_H3 idiotypic plasmablasts whose antibodies (B cell receptors) bind both SpA and SpA_{KK} but not SpA_{KKAA} (24). If this is so, does immunization of guinea pigs with SpA or SpA_{KK}—i.e., with B cell superantigen molecules—trigger dramatic increases in the abundance of V_H3-type antibodies in guinea pig serum, as occurs for human plasmablasts? Further, may guinea pig V_H3-type antibodies interfere with the association of protein A and human IgG or IgM? These predictions were tested, and V_H3-type IgG in guinea

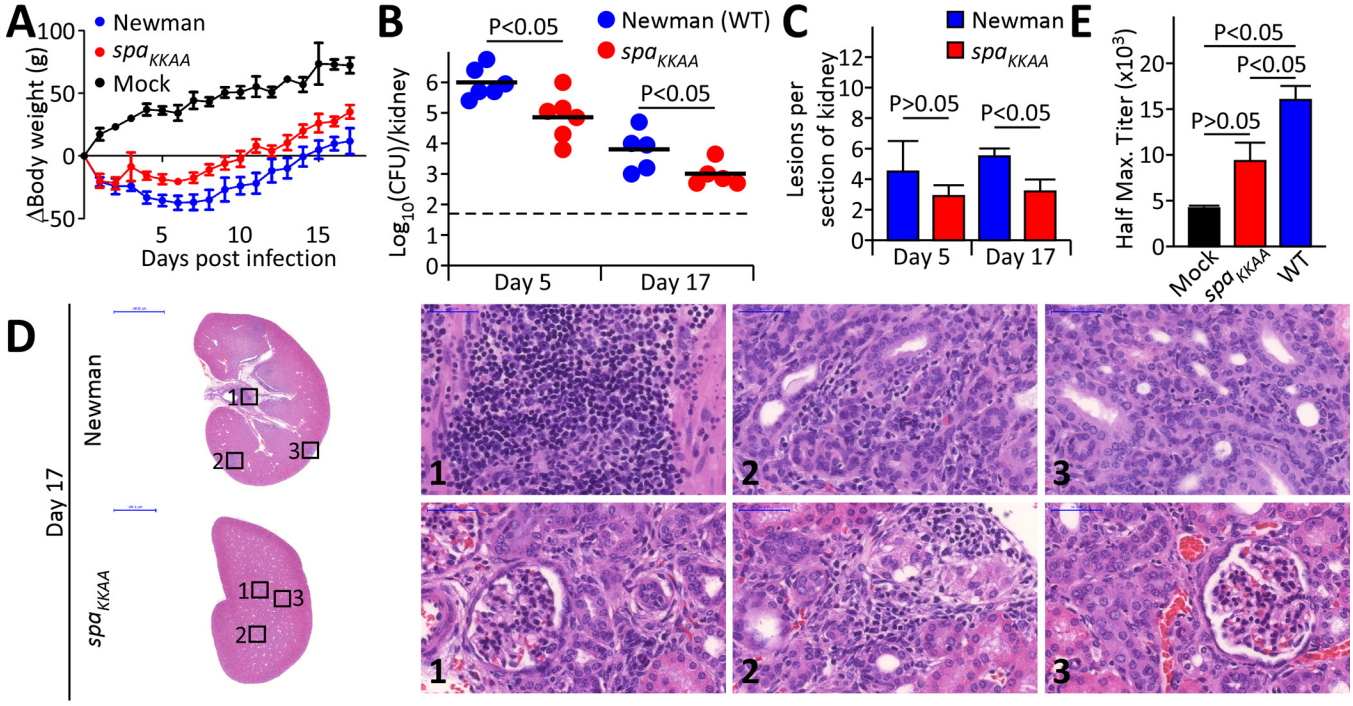

FIG 3 Guinea pig bloodstream infection with the *S. aureus* spa_{KKAA} mutant. (A) Guinea pigs ($n = 5$ or 6) were infected with 5×10^7 CFU of wild-type (WT) *S. aureus* Newman or its spa_{KKAA} mutant. Body weights of infected animals, as well as naive control animals (mock), were measured for 17 days. (B and C) On days 5 and 17 postinfection, animals were euthanized and necropsied and bacterial loads ($\log_{10}$ CFU per kidney) (B) and numbers of infectious lesions (C) in renal tissues were determined. (D) Representative images of thin-sectioned, hematoxylin-eosin-stained renal tissues on day 17 postinfection. (E) ELISA was performed to determine the abundance of SpA_{KK}-specific (V_H3^+ clonal) antibodies in serum collected on day 17 postinfection. The data are mean values, and error bars represent the standard errors of the means. Results are representative of two independent analyses.

pig serum was quantified by comparing Ig binding to SpA_{KK} and SpA_{KKAA}. Indeed, the abundance of V_H3 clonal IgG was strongly elevated in SpA-immune serum (SpA against SpA_{KKAA} versus SpA against SpA_{KK}, $P < 0.01$; see Fig. S3C) and was also moderately increased in SpA_{KK}-immune serum (SpA_{KK} against SpA_{KKAA} versus SpA_{KK} against SpA_{KK}, $P > 0.05$; see Fig. S3C). Of note, SpA_{AA} and SpA_{KKAA} immunizations did not trigger expansions of V_H3 clonal IgG antibodies in guinea pig serum (see Fig. S3C). No significant changes in V_H3 clonal IgM antibodies were observed in serum samples from SpA-, SpA_{KK}-, SpA_{AA}-, or SpA_{KKAA}-immunized guinea pigs; this was explained by the prime-two-boost schedule for guinea pig immunizations, where IgM responses wane in the weeks following the first immunization (see Fig. S3C).

To examine whether guinea pig V_H3 clonal antibodies can indeed interfere with the binding of human Ig to SpA, V_H3 clonal antibodies were purified by affinity chromatography on SpA_{KK} columns, whereas SpA_{KKAA}-specific antibodies were purified on SpA_{KKAA} columns with SpA- and SpA_{KKAA}-immune sera, respectively (see Fig. S3D). Purified V_H3 clonal antibodies (anti-SpA_{KK}) recognized SpA_{KK} but lacked affinity for SpA_{KKAA} (see Fig. S3D). On the other hand, affinity-purified SpA_{KKAA}-specific antibodies (anti-SpA_{KKAA}) recognized both SpA_{KK} and SpA_{KKAA} (see Fig. S3D). When subjected to binding inhibition assays, both anti-SpA_{KK} and anti-SpA_{KKAA} antibodies blocked the association of human IgG or IgM with protein A. Of note, V_H3-clonal IgG (anti-SpA_{KK}) was more potent than SpA_{KKAA}-specific (anti-SpA_{KKAA}) antibodies in blocking human IgM binding to protein A, as these antibodies harbor the same ligand for association with the staphylococcal superantigen (see Fig. S3E and F). Thus, immunization of guinea pigs with SpA or SpA_{KK} causes expansions of V_H3 clonal IgG, which interfere with the binding of human Ig to protein A. These results corroborate recent observations with plasmablasts and their antibodies from humans infected with *S. aureus*, where protein A triggers an expansion of V_H3 idiotypic plasmablasts and the production of antibodies that associate with the superantigen without recognizing its antigen properties or those of another staphylococcal surface molecule (24).

Contribution of protein A to *S. aureus* bloodstream infection in guinea pigs. To assess the role of protein A in the guinea pig model, animals were infected with 5×10^7 CFU of either *S. aureus* Newman or its spa_{KKAA} variant expressing nontoxigenic protein A. Animals infected with wild-type *S. aureus* lost 10% of their body weight and required 15 days to recover from this weight loss (Fig. 3A). In contrast, guinea pigs infected with the spa_{KKAA} variant lost 5% of their body weight and regained this lost weight within 10 days (wild type versus spa_{KKAA} variant on day 11, $P < 0.05$) (Fig. 3A). The ability of staphylococci to replicate and form abscess lesions in renal tissues was examined 5 and 17 days after infection (Fig. 3B and C). In *S. aureus* Newman-infected guinea pigs, average staphylococcal loads of 5.97 and 3.76 $\log_{10}$ CFU per kidney were recorded on days 5 and 17, respectively (Fig. 3B and C). Renal tissues of guinea pigs infected with the spa_{KKAA} variant harbored reduced staphylococcal loads and fewer abscess lesions (wild type versus spa_{KKAA}, $P < 0.05$) (Fig. 3B to D). In addition, the level of V_H3 clonal IgG was significantly higher in *S. aureus*

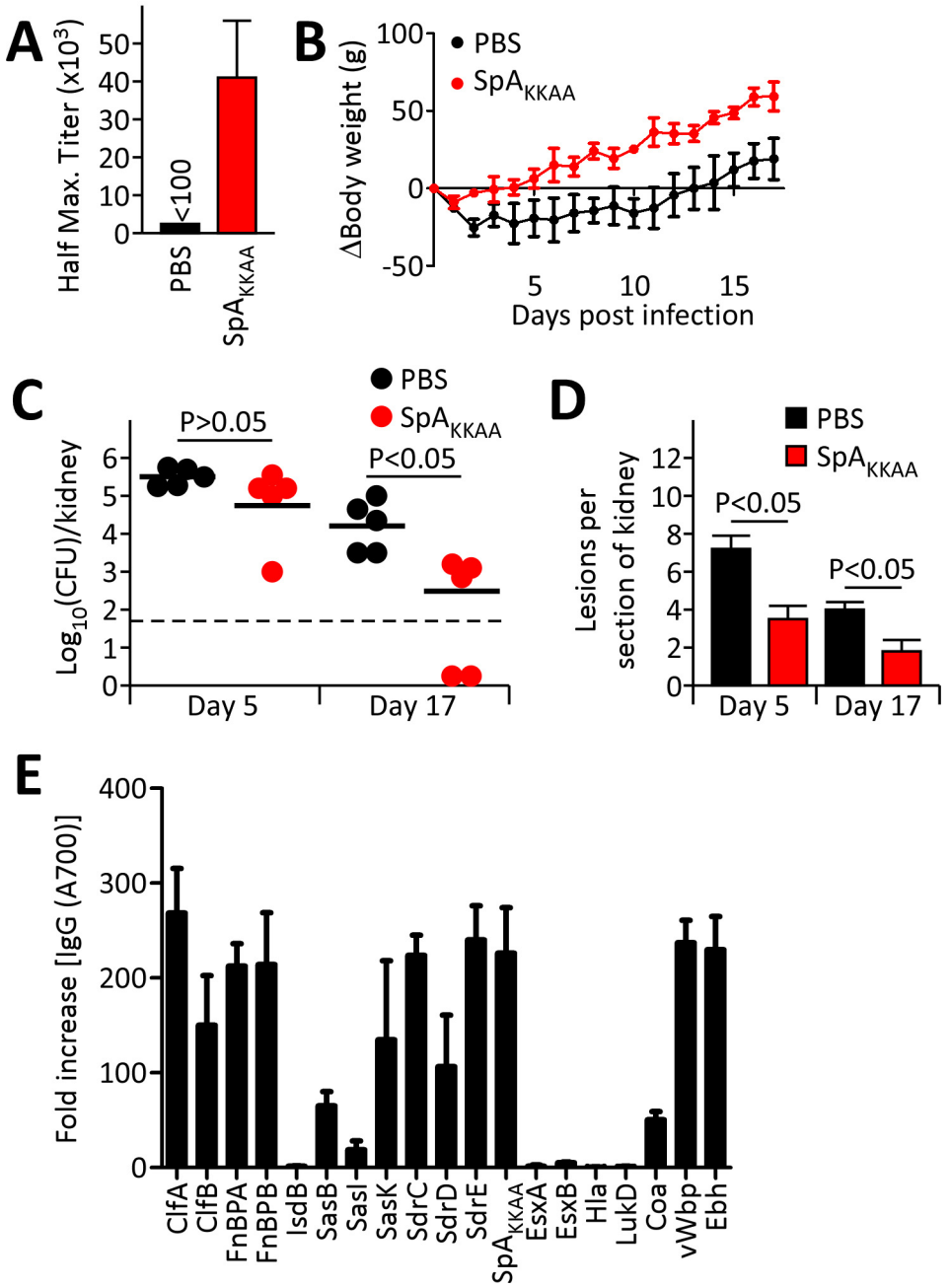

FIG 4 Immunization of guinea pigs with SpA_{KKAA} vaccine. (A) Guinea pigs ($n = 5$) were immunized with either adjuvant (PBS) alone or SpA_{KKAA} adsorbed to aluminum hydroxide and challenged by intravenous inoculation of *S. aureus* Newman (1×10^7 CFU). Their body weights were measured over a 17-day period. (B) SpA_{KKAA}-specific IgG titers of immunized guinea pigs were determined by ELISA. (C) On days 5 and 17 postinfection, guinea pigs were euthanized and necropsied and the bacterial loads in homogenized renal tissues (log_{10} CFU) were determined. (D) Numbers of infectious lesions in thin-sectioned, hematoxylin-eosin-stained renal tissues were determined on days 5 and 17 postinfection. (E) On day 17 postinfection, blood samples were obtained from infected guinea pigs and serum IgG responses to 19 staphylococcal secreted antigens were quantified by ELISA. Data are displayed as fold increases in the mean serum IgG titer of guinea pigs immunized with SpA_{KKAA} divided by the IgG titer of mock-immunized animals. The data are mean values, and error bars represent the standard errors of the means. Results are representative of two independent analyses.

Newman-infected guinea pigs than in mock- or spa_{KKAA} variant-infected animals (Fig. 3E). These data suggest that protein A serves as a virulence factor for the pathogenesis of *S. aureus* bloodstream infections in guinea pigs.

SpA_{KKAA} vaccine protects guinea pigs against staphylococcal disease. We asked whether vaccination with SpA_{KKAA} adsorbed to aluminum hydroxide protects guinea pigs against staphylococcal disease. Compared to guinea pigs treated with adjuvant alone, SpA_{KKAA}-vaccinated guinea pigs developed a robust protein A-specific antibody response (Fig. 4A). When challenged by intravenous injection with 1×10^7 CFU of *S. aureus* Newman, SpA_{KKAA}-vaccinated animals displayed less weight loss and recov-

ered this loss more quickly than mock-immunized animals (mock versus SpA_{KKAA} treatment on days 16 and 17, $P < 0.05$; Fig. 4B). When renal tissues were analyzed for pathogen burdens and histopathology and compared with mock-immunized animals, guinea pigs vaccinated with SpA_{KKAA} displayed reduced staphylococcal loads and numbers of abscess lesions (day 17 loads, mock versus SpA_{KKAA} treatment, $P < 0.05$; day 17 abscess lesions, mock versus SpA_{KKAA} treatment, $P < 0.05$; Fig. 4C and D). SpA_{KKAA} vaccination enabled guinea pigs to develop robust immune responses against many secreted virulence factors, including ClfA, ClfB, FnBPA, FnBPB, SasB, SasI, SasK, SdrC, SdrD, SdrE, Coa, vWbp, and Ebh (Fig. 4E). Of note, levels of IgG against IsdB, EsxA, EsxB, Hla, and LukD in serum were not increased in SpA_{KKAA}-vaccinated guinea pigs with *S. aureus* bloodstream infections (Fig. 4E). Taken together, these data suggest that guinea pig immunization with SpA_{KKAA} adsorbed to aluminum hydroxide raises high levels of protein A-specific antibodies that protect against *S. aureus* disease and promote the development of a broad spectrum of antibodies against many different staphylococcal antigens.

DISCUSSION

Recent work isolating activated B cells (plasmablasts) from blood samples of individuals with active *S. aureus* disease reported nonspecific activation and affinity maturation of V_H3 idiotype B cells against superantigenic epitopes of SpA and a lack of B cell responses against a wide spectrum of antigens that are secreted by the pathogen (24). The immunodominance of SpA, which targets large populations of V_H3 idiotype B cells, appears to prevent infected individuals from developing immune responses to the many different virulence factors of *S. aureus*, whose inhibition may be necessary for protection and memory formation (24). These results suggest that SpA neutralization is likely critical to the development of an effective vaccine by eliciting potent broad-spectrum immune responses against *S. aureus*.

The immune modifying attributes of protein A were heretofore examined in mice that had been challenged via bloodstream infection with either wild-type *S. aureus* or variants with specific defects in the protein A gene, i.e., Δ*spa*, spa_{KK}, spa_{AA}, and spa_{KKAA} (37, 57). The Δ*spa* mutant displayed defects in escape from OPK, which diminished the bacterial load during infection (57). Also, animals infected with the Δ*spa* mutant mount increased immune responses against *S. aureus* antigens, albeit that these antibodies cannot neutralize wild-type protein A (57). Thus, when subjected to a repeat challenge with wild-type *S. aureus*, animals with the Δ*spa* mutant memory response remain subject to protein A-mediated modification of B cell responses and are not protected against *S. aureus* disease (57). The spa_{KK} and spa_{AA} variants display intermediate phenotypes during bloodstream infection of mice. The spa_{KK} mutant cannot escape OPK and, its bacterial load is diminished, whereas the B cell superantigen activity of protein A remains intact (37). The spa_{AA} variant, on the other hand, has lost the B cell superantigen activity and elicits SpA-specific immune responses, although the functional Fcγ binding activity supported some disease establishment and partial suppression of immune responses (37). The most significant defect was observed in mice that had been infected with the spa_{KKAA} variant. Here, staphylococci neither escaped from OPK nor exerted B cell superantigen activity, which resulted in a broad spectrum of immune responses against secreted antigens, including protein A-neutralizing antibodies (37). Importantly, mice with a spa_{KKAA} mutant memory response were not affected by the immune modifying attributes of protein A and were protected against a repeat challenge with wild-type *S. aureus* (37).

The development of two *S. aureus* subunit vaccines has relied on mouse models for preclinical efficacy studies, which include intraperitoneal (lethal peritonitis, CP5/CP8 conjugate vaccine) (58, 59) and intravenous challenge models (bloodstream/renal abscess, IsdB vaccine) (38, 60). As the corresponding efficacy trials failed to reach their endpoints (15, 17), there has been concern about whether or not mouse models can predict the success of clinical trials (39). In support of this conjecture, Seok et al. reported that transcriptional responses of mice with inflammatory diseases displayed fundamental differences from human responses (61). We therefore considered other animal species as models for *S. aureus* vaccine development.

Historically, guinea pigs were a preferred model for bacterial pathogenesis and vaccine development, as these animals are susceptible to human infectious diseases, including tuberculosis (62), tetanus, diphtheria (63), and streptococcal and staphylococcal infections (40, 45). Further, several elements of the guinea pig immune system, including Ig, the major histocompatibility complex, complement, cytokines, and clusters of differentiation, are more closely related to the human immune system than the corresponding elements of mice are (40, 54, 64). Earlier work, for example, studies by Arne Forsgren, used guinea pigs as a model to study *S. aureus* pathogenesis and the immunomodulatory attributes of protein A (42, 65). Guinea pigs have also been used as models for *S. aureus* SSTIs (66), surgical site infection (67), disseminated intravascular coagulation (68), and infective endocarditis (69). Building on this body of work, we sought to develop an *S. aureus* bloodstream infection model that can be used for vaccine development.

Bloodstream infection of guinea pigs with *S. aureus* produced clinical disease that could be quantified as a rapid loss of body weight and establishment of infectious lesions in renal tissues detectable via histopathology analysis and bacterial load assessments. The pathogenesis of *S. aureus* bloodstream infection required SrtA and staphylococcal surface proteins, including protein A. Unlike rabbits, which mount SpA-specific immune responses and lack V_H3 idiotype B cells and Igs, guinea pig IgG and IgM encompass large amounts of V_H3 clan Ig and cannot mount SpA-specific immune responses. Thus, our studies indicate that the guinea pig immune system is modified by the B cell superantigen (V_H3 heavy chain) and Fcγ binding attributes of protein A. Further, SpA_{KKAA} adsorbed to aluminum hydroxide elicited specific antibodies that neutralized protein A superantigen activity and promoted immune responses against a broad spectrum of staphylococcal antigens, as well as protection against *S. aureus* disease. When transferred to mice, SpA_{KKAA}-immune serum also protected against staphylococcal disease. Together, these studies establish the guinea pig as a model for *S. aureus* bloodstream infection that may be used for vaccine development and preclinical efficacy studies.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strain Newman (46), its *srtA* (50) and protein A variant (spa_{KKAA}) (37), or MRSA isolate USA300 LAC (47) was grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 37°C. *Escherichia coli* strains DH5α and BL21(DE3) harboring the expression vector

pET15b or its clonal derivatives were grown in Luria broth or on Luria agar with ampicillin (100 $\mu g \cdot ml^{-1}$) at 37°C (70, 71).

Active and passive immunizations. BALB/c mice (3 weeks old, female; Charles River Laboratories) were immunized with 50 μg of purified protein emulsified in CFA (Difco) and boosted with 50 μg of protein emulsified in IFA (Difco) 11 days following the first immunization. Alternatively, mice were immunized with 50 μg of protein adsorbed to aluminum hydroxide (Alhydrogel "85"; Brenntag) and boosted with the same formulation. On day 21, mice were bled and serum was recovered for ELISAs. Six-month-old New Zealand White female rabbits (Harlan Laboratories) were immunized with 500 μg of protein emulsified in CFA and boosted with 500 μg of protein emulsified in IFA on days 21 and 42. On day 63, rabbits were bled and serum was recovered for ELISAs. Female guinea pigs (250-g body weight; Charles River Laboratories) were immunized with 100 μg of protein adsorbed to aluminum hydroxide (Alhydrogel "85"; Brenntag) and boosted on days 14 and 28. On day 35, guinea pigs were bled and serum was recovered for ELISAs. For passive-transfer immunization studies, guinea pig immune serum was filter sterilized and injected into the peritoneal cavities of BALB/c mice (6 weeks old, female; Charles River Laboratories) twice at 24 and 48 h prior to a challenge with *S. aureus* Newman. To determine antigen-specific IgG titers by ELISA, mice were bled by cardiac puncture.

Guinea pig model of *S. aureus* infection. Overnight cultures of *S. aureus* Newman or USA300 were diluted 1:100 in fresh TSB and grown for 2 h at 37°C. Staphylococci were sedimented by centrifugation at 8,000 × *g* and washed and suspended in PBS to the desired bacterial concentration. Inocula were quantified by spreading sample aliquots on TSA and enumerating the CFU. Guinea pigs (approximately 350- to 450-g body weight; Charles River Laboratories) were anesthetized via intraperitoneal injection with 100 $mg \cdot kg^{-1}$ ketamine and 6 $mg \cdot kg^{-1}$ xylazine. Bupivicaine (0.125% solution, no more than 2 $mg \cdot kg^{-1}$) was administered subcutaneously as an analgesic near the site of the surgical incision. Animals were regularly monitored for depth of anesthesia by studying their pinna reflex, heartbeat, and respiratory rate via pulseoximeter during both surgery and recovery. After the fur was shaved from the animal's neck, the skin was cleaned with three alternating scrubs with iodine and 70% ethanol prior to incision. A 2-cm aseptic incision was made along the neck to expose the jugular vein in the subcutaneous tissue. Guinea pigs were infected by injection of 1×10^7 to 5×10^7 CFU of *S. aureus* Newman or USA300 into the jugular vein. Sterile surgical sutures were used to close the surgical wound. Body weights of infected animals, as well as naive control animals, were measured daily. On day 5 or 17 following infection, guinea pigs were euthanized by CO_2 inhalation and thoracotomy. Both kidneys were removed during necropsy, 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 plates were incubated for colony formation. The remaining organ was examined by histopathology analysis. 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 analyzed via light microscopy (3DHistech; Integrated Microscopy Core Facility at the University of Chicago) to enumerate pathological lesions. Serum samples collected at 17 days postinfection were examined for antibodies against the staphylococcal antigen matrix. All guinea pig experiments were performed at least twice.

Mouse renal abscess model. Bacterial samples were prepared as explained above. BALB/c mice (6 weeks old, female; Charles River Laboratories) were anesthetized via intraperitoneal injection with 65 $mg \cdot kg^{-1}$ ketamine and 6 $mg \cdot kg^{-1}$ xylazine. Mice were infected by the injection of 1×10^7 CFU of *S. aureus* Newman into the periorbital venous plexus. On day 5 following the challenge, 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 analysis. All animal experiments were performed in accordance with institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at the University of Chicago.

Protein purification. *E. coli* BL21(DE3) harboring pET15b+ plasmids for the expression of His-tagged wild-type SpA, SpA_{KK}, SpA_{AA}, and SpA_{KKAA}, as well as 18 staphylococcal antigens (ClfA, ClfB, FnBPA, FnBPB, IsdB, SasB, SasI, SasK, SdrC, SdrD, SdrE, EsxA, EsxB, Hla, LukD, Coa, vWbp, and Ebh) (35), was grown overnight, diluted 1:100 in fresh medium, and grown at 37°C to an A_{600} of 0.5. Cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside 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 × *g*. Cleared lysates were subjected to 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 PBS, treated with Triton X-114 to remove endotoxin, and again dialyzed with PBS. Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific). Purity was verified by Coomassie-stained SDS-PAGE.

ELISA. To analyze protein A-specific serum IgG or IgM, recombinant purified SpA_{KKAA} was used to coat ELISA plates (Nunc MaxiSorp) at 1 $\mu g \cdot ml^{-1}$ in 0.1 M carbonate buffer (pH 9.5) at 4°C overnight. The following day, plates were blocked and incubated with serially diluted serum. Plates were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) specific to mouse IgG, rabbit IgG, or guinea pig IgG or IgM and developed with OptEIA reagent. To determine V_H3 clonal IgG or IgM in serum, recombinant purified SpA_{KK} was used to coat ELISA plates. For human IgG or IgM binding inhibition assays, ELISA plates were coated with recombinant purified SpA at 1 $\mu g \cdot ml^{-1}$ in 0.1 M carbonate buffer (pH 9.5) at 4°C overnight. The following day, plates were blocked and incubated with serially diluted guinea pig hyperimmune serum or affinity-purified guinea pig polyclonal antibodies. Plates were incubated with HRP-conjugated human IgG or IgM (1:10,000) and developed with OptEIA reagent.

Staphylococcal antigen matrix. Affinity-purified staphylococcal antigens (2 μg) were blotted onto nitrocellulose membrane. Membranes were blocked with 5% whole milk and then incubated with guinea pig serum diluted 1:10,000. IRDye 680-conjugated, affinity-purified anti-guinea pig IgG (Rockland) was used to quantify signal intensities with the Odyssey infrared imaging system (LI-COR). Fold increases in antibody signal intensity were calculated by dividing the A_{700} values of SpA_{KKAA}-immunized immune serum the A_{700} values of mock (PBS)-immunized immune serum.

Affinity chromatography of Ig. Purified, His_6-tagged SpA, SpA_{AA}, SpA_{KK}, and SpA_{KKAA} were immobilized on Ni-NTA Sepharose, washed, and incubated with human, mouse, rabbit, or guinea pig IgG or IgM in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl buffer. After washing, proteins were eluted with 500 mM imidazole and analyzed by SDS-PAGE.

Antibody purification. Recombinant purified SpA_{KK} or SpA_{KKAA} (5 mg) was covalently linked to a HITRAP NHS-activated HP column. Guinea pig SpA- or SpA_{KKAA}-immune serum was circulated through SpA_{KK} or SpA_{KKAA} columns to purify SpA_{KK}-specific (V_H3^+ clonal) or SpA_{KKAA}-specific antibodies, respectively. Antibodies were eluted with 1 M glycine (pH 2.5)–0.5 M NaCl, neutralized with 1 M Tris-HCl (pH 8.5), dialyzed overnight against PBS at 4°C, subjected to SDS-PAGE, and visualized with Coomassie blue.

Statistical analysis. Bacterial loads in the experimental-animal infection model were analyzed with the two-tailed Mann-Whitney test to measure statistical significance. The unpaired two-tailed Student *t* test or one-way analysis of variance (ANOVA) with Bonferroni's posttest was performed to analyze the statistical significance of ELISA data. Statistical

significance of abscess formation in experimental-animal models was determined with the unpaired two-tailed Student *t* test with Welch's correction. Two-way ANOVA with Bonferroni's posttest was used to calculate the statistical significance of body weight data. All data were analyzed by Prism (GraphPad Software, Inc.), and P values of <0.05 were deemed significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02369-14/-/DCSupplemental.

Figure S1, PDF file, 0.2 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.2 MB.

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