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RESEARCH ARTICLE

Staphylococcal Protein A Contributes to Persistent Colonization of Mice with *Staphylococcus aureus*

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ABSTRACT *Staphylococcus aureus* persistently colonizes the nasopharynx in humans, which increases the risk for invasive diseases, such as skin infection and bacteremia. Nasal colonization triggers IgG responses against staphylococcal surface antigens; however, these antibodies cannot prevent subsequent colonization or disease. Here, we describe *S. aureus* WU1, a multilocus sequence type 88 (ST88) isolate that persistently colonizes the nasopharynx in mice. We report that staphylococcal protein A (SpA) is required for persistence of *S. aureus* WU1 in the nasopharynx. Compared to animals colonized by wild-type *S. aureus*, mice colonized with the Δ*spa* variant mount increased IgG responses against staphylococcal colonization determinants. Immunization of mice with a nontoxigenic SpA variant, which cannot cross-link B cell receptors and divert antibody responses, elicits protein A-neutralizing antibodies that promote IgG responses against colonizing *S. aureus* and diminish pathogen persistence.

IMPORTANCE *Staphylococcus aureus* persistently colonizes the nasopharynx in about one-third of the human population, thereby promoting community- and hospital-acquired infections. Antibiotics are currently used for decolonization of individuals at increased risk of infection. However, the efficacy of antibiotics is limited by recolonization and selection for drug-resistant strains. Here, we propose a model of how staphylococcal protein A (SpA), a B cell superantigen, modifies host immune responses during colonization to support continued persistence of *S. aureus* in the nasopharynx. We show that this mechanism can be thwarted by vaccine-induced anti-SpA antibodies that promote IgG responses against staphylococcal antigens and diminish colonization.

KEYWORDS *Staphylococcus aureus*, colonization, immune response, immunity, staphylococcal protein A

Staphylococcus aureus is a frequent cause of community- and hospital-acquired diseases, including skin and soft tissue infections, pneumonia, bacteremia, and endocarditis (1). Between 20 and 41% of the human population are persistently colonized by *S. aureus*, while others serve as intermittent carriers of the pathogen (2). *S. aureus* is predominately located in the anterior nasal vestibule and is also isolated from the oropharynx and gastrointestinal tract (3–5). Colonization with *S. aureus* constitutes a major risk for community- and hospital-acquired infections (6, 7). Antibiotic decolonization serves the dual purposes of reducing the risk of infection in individual carriers and preventing the spread of *S. aureus*, particularly in hospital settings (8). Mupirocin treatment is currently used for decolonization; however, its

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long-term success is low due to recolonization and selection for mupirocin-resistant strains (9).

S. aureus colonization occurs in the first weeks of life, as staphylococci can be readily isolated from the nasopharynx and perineum in 24 to 46% of infants (10). Colonization is associated with increases in serum IgG titers against secreted staphylococcal antigens, including sortase-anchored surface proteins and secreted toxins (11–13). Of note, *S. aureus* colonization, as well as invasive disease, increases the relative abundance of pathogen-specific IgG4 antibody responses compared to those of IgG1 subclass antibodies (12). However, serum IgG responses to *S. aureus* colonization or infection are not considered protective against either further colonization or subsequent invasive disease (7, 14, 15). No FDA-licensed vaccine capable of preventing *S. aureus* colonization or invasive disease is currently available (16).

Earlier work sought to identify *S. aureus* genes required for nasal colonization, using bacterial adherence to human desquamated nasal epithelial cells and *in vivo* colonization of cotton rats as models (17, 18). Another model system, nasal colonization of mice with human clinical isolates, typically requires prior antibiotic treatment to deplete the resident microbiota and to provide selection for colonization with antibiotic-resistant strains (19). These *in vitro* and *in vivo* model systems identified several surface components that are necessary for *S. aureus* colonization (20). Specifically, clumping factor B (ClfB) promotes staphylococcal adherence by binding to loricrin and cytokeratin 10 in nasal epithelia (21). Compared with wild-type *S. aureus*, an isogenic *clfB* mutant was cleared more rapidly from the nasal epithelia of human volunteers (7). Serine-aspartate repeat surface proteins C (SdrC) and D (SdrD), as well as iron-regulated surface determinant A (IsdA), also contribute to staphylococcal adherence to human nasal epithelial cells (17, 22). IsdA contributes to iron scavenging from host hemoproteins and also binds lactoferrin, which inhibits the antistaphylococcal activity of lactoferrin in human nasal secretions (23, 24). *S. aureus* surface protein G (SasG) mediates zinc-dependent adhesion between bacterial cells during biofilm formation and adherence to nasal tissue (25, 26). Finally, *S. aureus* synthesizes cell wall-linked wall teichoic acid (WTA), a polymer of ribitol-phosphate, with esterified D-alanyl (D-Ala) and α- and/or β-linked *N*-acetylglucosamine (GlcNAc) (20). D-Ala and GlcNAc modifications of WTA also contribute to staphylococcal adherence by binding the type F scavenger receptor (SREC-I) of human nasal epithelia and supporting nasal colonization in cotton rats (18, 27, 28).

The role of staphylococcal protein A (SpA) during *S. aureus* nasal colonization has been enigmatic (29). In contrast to many toxin and capsular polysaccharide genes and several other surface protein genes, *S. aureus* expresses *spa* during colonization of both humans and cotton rats (30, 31). Although the tandem-repeat structure of the *spa* gene promotes high-frequency recombination, human colonization selects for *spa* alleles whose products maintain five immunoglobulin binding domains (IgBDs), which endows staphylococci with potent B cell superantigen activity (32, 33). When analyzed in human volunteers who had been cleared of nasal carriage via mupirocin treatment, *S. aureus spa* expression was not required for bacterial adherence to human nasal tissue and for initial colonization, i.e., for a 10-day period following inoculation (34). In contrast, a human methicillin-resistant *S. aureus* (MRSA) multilocus sequence type 239 (ST239) isolate was reported to require *spa* expression for nasal adherence and 3-day colonization of mice that had been pretreated with ampicillin (35).

S. aureus JSNZ is a member of ST88, which is rare in human populations (36). Strain JSNZ was isolated from an outbreak of preputial gland abscesses among male C57BL/6 mice (36). Unlike human clinical isolates, *S. aureus* JSNZ persistently colonizes the nasopharynxes of mice without prior antibiotic treatment (36, 37). Here, we report the isolation of *S. aureus* WU1, another ST88/clonal complex 88 (CC88) isolate causing preputial gland abscess lesions in male mice. Similar to strain JSNZ, *S. aureus* WU1 persistently colonizes the nasopharynxes of mice and promotes serum IgG responses against staphylococcal surface molecules. Compared with wild-type *S. aureus* WU1, the Δ*spa* mutant displays a persistence defect during colonization and elicits increased IgG

responses against staphylococcal surface molecules. Immunization of mice with purified SpA_{KKAA}, a protein A variant that cannot bind IgG Fcγ or cross-link V_H3 idiotype B cell receptors, generates SpA-neutralizing antibodies that promote increased pathogen-specific IgG responses and decolonization of *S. aureus* ST88 isolates.

RESULTS

***S. aureus* WU1.** An outbreak of preputial gland infections of male C57BL/6 mice was observed in an animal breeding colony. Samples were collected from preputial gland adenitis (PGA) and from the nasopharynxes of male and female C75BL/6 mice and analyzed by growth on mannitol-salt agar (MSA [BBL; Becton Dickinson]) and Baird-Parker agar (BPA [Difco; Becton Dickinson]). Multilocus sequence typing (MLST) and *spa* genotyping revealed that the animals had been colonized with *S. aureus* ST88 *spa* genotype t186, which was also responsible for PGA in male mice. *S. aureus* CC88 with *spa* genotype t186 has been reported before as stably colonizing isolates from laboratory mice in the United States (37). Other *spa* genotypes include t325, t448, t690, t755, t786, t2085, t2815, t5562, t11285, and t12341 (37). The New Zealand JSNZ isolate carries the distinct *spa* genotype t729 (37). Nonetheless, both *S. aureus* JSNZ and WU1 share the type 8 capsular polysaccharide genes and lack the *mecA* gene, as well as mobile genetic element (MGE)-encoded T cell superantigens (37). Further, the *hlb*-converting phage that expresses human-specific immune evasion cluster 1 (IEC1) genes *sak* (staphylokinase), *chp* (CHIPS [chemotaxis-inhibitory protein of *S. aureus*]), and *scn* (SCIN-A [staphylococcal complement inhibitor A]) is absent in the genome of WU1, resulting in an intact β-hemolysin-encoding gene (*hlb*) (38). Of note, the WU1-encoded IEC2 carries the *scn* homologue *scb* or *scc* (SCIN-B/SCIN-C), along with *hla* (α-hemolysin) and *ssl12-14* (staphylococcal superantigen-like 12-14) (39). Unlike those of other CC88 isolates that stably colonize mice (37), the genome of WU1 harbors the *blaZ* gene. When analyzed for genes encoding sortase-anchored surface proteins, we observed that *S. aureus* WU1 carries genes for determinants previously associated with nasal colonization, including ClfB, IsdA, SdrC, SdrD, and SasG (Table 1).

S. aureus abscess formation has been linked to determinants of bacterial agglutination with fibrin (40, 41). Agglutination requires two *S. aureus*-secreted products that activate host prothrombin to convert fibrinogen into fibrin: coagulase (Coa) and von Willebrand factor binding protein (vWbp) (40). Clumping factor A (ClfA) binds fibrinogen and coats staphylococci with coagulase-generated fibrin fibrils, thereby interfering with *S. aureus* uptake and killing by host phagocytes (41, 42). The *clfA* genes in *S. aureus* WU1 and JSNZ are identical yet display allele-specific differences from *clfA* of *S. aureus* Newman (Table 1), a CC8 human clinical isolate that is used routinely for laboratory challenge experiments with mice (43). The observed differences in *clfA*, however, are clade specific, as they can be found in CC88 strains isolated either from human or from murine hosts (data not shown). The *coa* gene products of *S. aureus* WU1, JSNZ, and Newman are virtually identical (Table 1). In contrast, the product of the *vwb* genes of *S. aureus* WU1 and JSNZ differ significantly from that of *S. aureus* Newman, with the greatest sequence variation in the prothrombin-binding D1 and D2 domains (Fig. 1A). vWbp from WU1 and JSNZ was not recognized by polyclonal antibodies raised against Newman vWbp (Fig. 1B). Secreted vWbp proteins from the two CC88 strains could be recognized by a serum that had been raised against the conserved C-terminal domain of vWbp from strain USA300 (Fig. 1C). In contrast to *S. aureus* Newman, which secretes large amounts of Coa and rapidly agglutinates human and mouse plasma, *S. aureus* WU1 and JSNZ secrete less Coa and agglutinate mouse plasma more readily than human plasma (Fig. 1B, D, and E). The coagulase activity of *S. aureus* Newman is dependent on *coa* and *vwb* expression, as the corresponding Δ*coa*, Δ*vwb*, and Δ*coa* Δ*vwb* mutants displayed agglutination defects in mouse and human plasma (Fig. 1D and E). Taken together, these data suggest that the ST88 alleles of the *vwb* genes in *S. aureus* WU1 and JSNZ may promote efficient prothrombin-mediated coagulation and fibrin agglutination in mouse plasma, which may support the pathogenesis of invasive diseases, such as PGA.

TABLE 1 Conservation of protein products of select open reading frames in the genomes of *S. aureus* WU1, JSNZ, and Newman

	Amino acid identity (%) to WU1 gene product	
Protein	**JSNZ**	**Newman**
SpA	99	98
ClfA	100	93
ClfB	100	96
FnBPA	100	82
FnBPB	87	87
IsdA	100	100
IsdB	99	98
SdrC	100	100
SdrD	95	95
SdrE	100	98
EsxA	100	100
EsxB	100	100
SasA	100	99
SasD	100	99
SasF	100	98
SasI	99	100
SasG	100	69
SasK	100	93[a]
Coa	98	98
vWbp	100	71
Hla	100	99
SCIN	100	45[b]
Eap	100	99
Efb	100	99
Ebh	99	98
TarS	100	98

[a]Compared to *S. aureus* strain 04-02981.
[b]Compared to *S. aureus* strain USA300.

***S. aureus* WU1 persistently colonizes the nasopharynx in mice.** To analyze *S. aureus* WU1 for its ability to colonize mice, cohorts ($n = 10$) of female C57BL/6 animals were analyzed by spreading pharyngeal swabs and fecal material on BPA. Naive mice lacking bacterial growth on BPA were anesthetized and inoculated by pipetting 10-μl suspensions of 1×10^8 CFU *S. aureus* WU1 in phosphate-buffered saline (PBS) into the right nostril. The animals were analyzed for colonization by swabbing the oropharynx at weekly intervals, i.e., 7, 14, 21, 28, 35, and 42 days following inoculation. The swabs were spread on BPA, incubated for colony formation, and enumerated (Fig. 2A). Even without prior antibiotic treatment or antibiotic selection, *S. aureus* WU1 colonized experimental animals with a load ranging from 0.8 to 3.1 $\log_{10}$ CFU per swab over 42 days (Fig. 2A). To validate persistent colonization with *S. aureus* WU1, colonies obtained after 42 days were analyzed by MLST and *spa* genotyping. The data showed that the mice were still colonized with ST88 *spa* t186, indicating that *S. aureus* WU1 persistently colonizes the nasopharynxes of C57BL/6 mice. As a control, mock (PBS) inoculation of cohorts of C57BL/6 animals in separate cages that were maintained in the same animal facility room and the same cage racks as *S. aureus* WU1-colonized animals did not lead to staphylococcal colonization of the nasopharynx (Fig. 2A). Day 42 stool samples from the mice were homogenized in PBS and plated on MSA for CFU enumeration (Fig. 2B). Stool samples from *S. aureus* WU1-colonized mice harbored 5.1 to 7.3 $\log_{10}$ CFU g^{-1} feces, indicating that the gastrointestinal (GI) tract was also colonized with *S. aureus* strain WU1. As a control, mock (PBS)-inoculated mice did not harbor *S. aureus* in their stool samples (Fig. 2B).

***S. aureus* WU1 colonization triggers serum IgG response in mice.** Earlier work generated the *S. aureus* antigen matrix, which is comprised of 25 conserved secreted proteins. Each of the 25 recombinant affinity-tagged proteins was purified and immobilized on a membrane filter (44). To measure host immune responses during colonization, naive or *S. aureus* WU1-colonized animals were bled 15 days after inoculation,

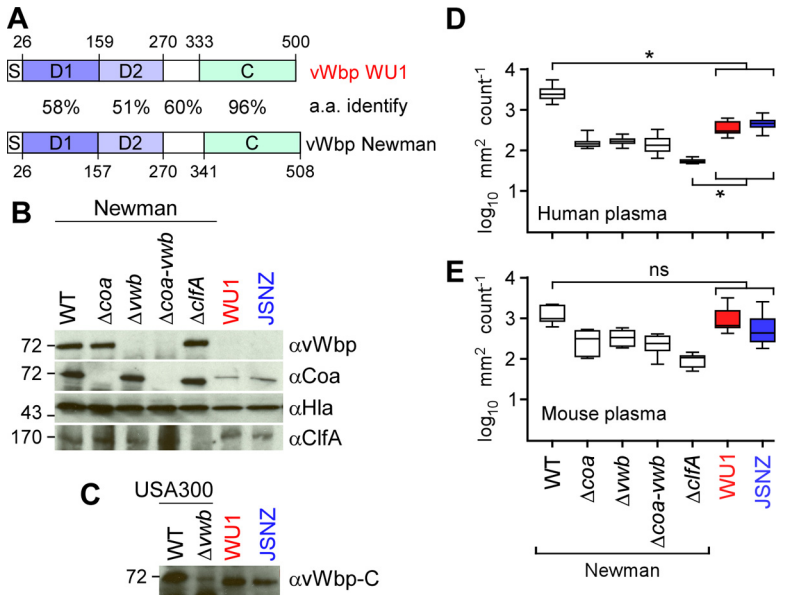

FIG 1 *S. aureus* ST88 isolate WU1, a mouse pathogen. (A) Domain structure and sequence homology of the *vwb* gene products from *S. aureus* WU1 and *S. aureus* Newman, a human clinical isolate. The percent amino acid (a.a.) identity of vWbp with its signal peptide (S), D1 and D2 domains (responsible for binding and activation of host prothrombin), linker (white box), and C-terminal fibrinogen binding domain (C) are displayed. (B) Immunoblots of *S. aureus* whole culture samples of strain Newman (wild type [WT]), as well as its Δ*coa*, Δ*vwb*, Δ*coa-vwb*, and Δ*clfA* variants, WU1, and JSNZ, were analyzed for the production of vWbp (αvWbp), Coa (αCoa), Hla (αHla), and ClfA (αClfA). Sizes are given on the left in kilodaltons. (C) Polyclonal antibodies against the vWbp C domain identify the vWbp allelic variants from strains JSNZ and WU1, as well as vWbp from strain USA300 LAC. (D and E) Agglutination of Syto-9-stained *S. aureus* strains in human (D) or mouse (E) plasma was measured as the average sizes and standard errors of the mean of clumped bacteria in 12 microscope fields of view, and statistical significance was assessed in pairwise comparisons with the WT using two-way ANOVA with Sidak multiple-comparison tests. *, $P < 0.05$; ns, not significant.

and serum IgG responses were analyzed by incubation with the *S. aureus* antigen matrix. IgG binding was detected with IRDye 680-conjugated goat anti-mouse IgG (Li-Cor) and quantified by infrared imaging. This experiment demonstrated that *S. aureus* WU1 colonization led to increases in serum IgG directed against the sortase-anchored surface proteins ClfA, ClfB, IsdA, and IsdB and the giant extracellular matrix-

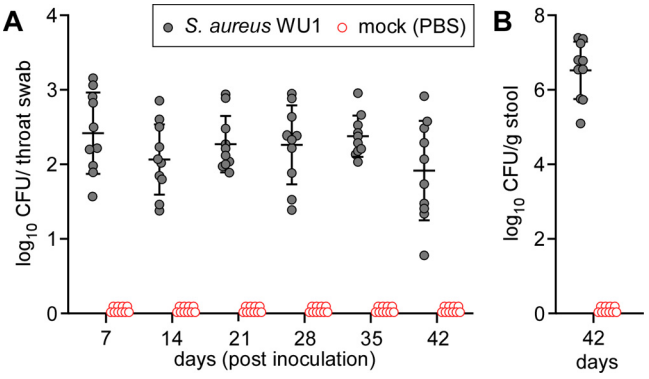

FIG 2 *S. aureus* WU1 persistently colonizes the nasopharynxes of C57BL/6 mice. Cohorts of C57BL/6 mice ($n = 10$) were inoculated intranasally with 1×10^8 CFU of the indicated *S. aureus* WU1 or PBS control. (A) Mice were swabbed in the throat weekly to enumerate the bacterial load. Each dot indicates the number of CFU in one mouse. (B) Stool samples were collected on day 42 following inoculation to enumerate the bacterial load. Each dot indicates the number of CFU per milliliter per gram of stool. The median and standard deviation for each group of animals on a given day are indicated by the horizontal lines and error bars.

TABLE 2 Serum IgG responses in C57BL/6J mice colonized with *S. aureus* WU1 or its Δ*spa* variant[a]

		WU1 (colonized)		WU1 Δ*spa*			
				Colonized		Cleared	
Protein type	Antigen	Fold change[b]	*P*[c] (vs naive)	Fold change	*P* (vs WU1, colonized)	Fold change	*P* (vs WU1 Δ*spa*, colonized)
Cell wall anchored surface	SpA_{KKAA}	1.3 ± 0.08	NS	1.1 ± 0.06	NS	1.1 ± 0.49	NS
	ClfA	5.3 ± 2.77	<0.0001	4.3 ± 0.83	NS	3.5 ± 1.69	NS
	ClfB	4.8 ± 0.72	0.001	3.9 ± 1.28	NS	17.4 ± 4.70	<0.0001
	Ebh	3.7 ± 0.50	0.0454	2.8 ± 0.62	NS	3.9 ± 1.56	NS
	FnbpA	1.9 ± 0.89	NS	1.3 ± 0.79	NS	2.6 ± 0.96	NS
	FnbpB	2.6 ± 1.33	NS	2.3 ± 0.85	NS	4.3 ± 0.96	NS
	IsdA	4.5 ± 0.84	0.0036	2.1 ± 0.22	NS	13.0 ± 0.44	<0.0001
	IsdB	5.2 ± 1.43	0.0002	2.7 ± 0.83	NS	2.8 ± 1.18	NS
	SdrC	1.1 ± 0.14	NS	1.5 ± 0.45	NS	1.7 ± 0.69	NS
	SdrD	1.5 ± 1.08	NS	1.0 ± 0.25	NS	1.2 ± 0.35	NS
	SdrE	1.8 ± 0.52	NS	2.9 ± 0.65	NS	1.4 ± 0.60	NS
	SasA	3.0 ± 1.33	NS	1.1 ± 0.44	NS	3.3 ± 1.14	NS
	SasB	5.1 ± 2.22	NS	1.0 ± 0.34	NS	5.7 ± 4.42	NS
	SasD	2.7 ± 1.47	NS	0.7 ± 0.23	NS	1.3 ± 0.59	NS
	SasF	1.2 ± 0.61	NS	0.9 ± 0.63	NS	1.2 ± 0.32	NS
	SasG	2.1 ± 0.24	NS	1.2 ± 0.47	NS	10.3 ± 1.19	<0.0001
	SasI	1.4 ± 0.75	NS	1.2 ± 0.08	NS	1.4 ± 0.53	NS
	SasK	2.5 ± 0.26	NS	1.3 ± 0.30	NS	1.7 ± 1.09	NS
Secreted	Coa	2.7 ± 0.29	NS	1.2 ± 0.45	NS	1.5 ± 0.45	NS
	vWbp	2.0 ± 0.97	NS	1.4 ± 0.59	NS	1.7 ± 0.89	NS
	Hla	1.8 ± 0.65	NS	1.2 ± 0.46	NS	1.2 ± 0.34	NS
	SCIN	4.3 ± 1.23	0.0071	2.8 ± 1.80	NS	1.4 ± 0.49	NS
	Eap	1.3 ± 0.20	NS	0.8 ± 0.97	NS	1.2 ± 0.31	NS
	Efb	2.9 ± 1.68	NS	2.6 ± 1.63	NS	1.6 ± 0.52	NS
	EsxA	2.6 ± 1.73	NS	1.6 ± 1.00	NS	2.6 ± 0.35	NS
	EsxB	2.8 ± 0.28	NS	1.6 ± 0.19	NS	1.9 ± 0.21	NS

[a]Cohorts of C57BL/6J mice were inoculated intranasally with 10^8 CFU of the indicated *S. aureus* strains. Fifteen days following inoculation, the animals were bled, and serum samples were analyzed for antibody responses to staphylococcal antigens.
[b]Fold changes were calculated by dividing the average signal intensities derived from *S. aureus*-inoculated mice by the average signal intensities from mice that remained naive. The data are presented as means ± standard deviations.
[c]*P* values were calculated using two-way ANOVA with Tukey multiple-comparison tests. NS, not significant.

binding protein (Ebh), a cell size and peptidoglycan synthesis determinant of *S. aureus* (45) (Table 2).

***S. aureus* WU1 requires staphylococcal protein A for persistent colonization.** Similar to that of *S. aureus* Newman, the *spa* gene of *S. aureus* WU1 encodes the YSIRK/GXXS signal peptide with a signal peptide cleavage site, five IgBDs, a cell wall repeat region (X_r), and a C-terminal LPXTG motif sorting signal. Compared to *spa* from *S. aureus* Newman, the coding sequence for the five IgBDs of *S. aureus* WU1 *spa* carries a single nucleotide substitution at codon 157 (AAA instead of AAT), replacing asparagine (N) at position 4 in the IgBD-A repeat with lysine (K). Immunoblotting experiments revealed that *S. aureus* strains Newman and WU1 produced similar amounts of SpA (Fig. 3A). Using allelic recombination, we generated the Δ*spa* mutant of *S. aureus* WU1. As measured by immunoblotting, SpA production was abolished in the Δ*spa* mutant, and this defect was restored by plasmid-borne expression of wild-type *spa* (pSpA) (Fig. 3A). Immunoblotting with antibodies against sortase A (SrtA) was used as a loading control (Fig. 3A). When inoculated into the right nostrils of mice and analyzed for colonization by oropharyngeal swabbing on day 7, the Δ*spa* mutant initially colonized C57BL/6 animals in a manner similar to that of wild-type strain WU1 (Fig. 3B). However, at later time points, particularly on days 35 and 42, the Δ*spa* mutant colonized fewer animals than wild-type strain WU1 (Fig. 3B). During bacterial growth, *S. aureus* releases SpA linked to peptidoglycan fragments into the surrounding milieu (46). In a mouse model of intravenous *S. aureus* challenge, released SpA activated B cell proliferation and enhanced secretion of V_H3 idiotype IgM and IgG molecules (33). However, expanded V_H3 idiotype IgG does not recognize staphylococcal antigens (33). The molecular basis

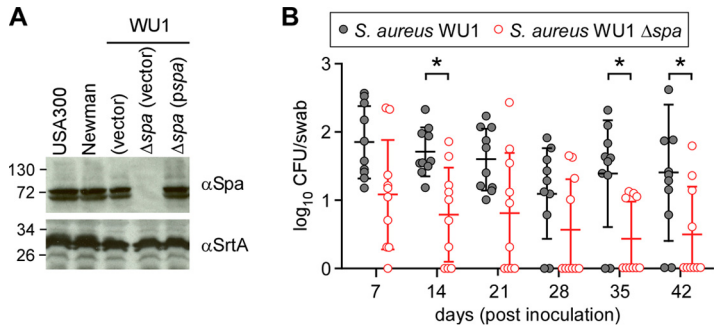

FIG 3 *S. aureus* WU1 expression of SpA is required for persistent colonization of C57BL/6 mice. (A) Immunoblots of *S. aureus* lysates derived from strains USA300 LAC, Newman, WU1, and the Δ*spa* variant of WU1 with and without a plasmid for *spa* expression (p*spa*) were probed with SpA-specific (αSpA) and sortase A-specific (αSrtA) antibodies. Sizes are shown on the left in kilodaltons. (B) Cohorts of C57BL/6 mice ($n = 10$) were inoculated intranasally with 1×10^8 CFU of *S. aureus* WU1 or its Δ*spa* variant, and the oropharynxes of the animals were swabbed at weekly intervals to enumerate the bacterial load. Each dot indicates the number of CFU per mouse. The median and standard deviation for each group of animals on a given day are indicated by the horizontal lines and error bars. The bacterial colonization data sets were analyzed with two-way ANOVA and Sidak multiple-comparison tests; statistically significant differences (*, $P < 0.05$) between the two groups of animals are indicated.

for this B cell superantigen activity is based on SpA-mediated cross-linking of V_H3 idiotype B cell receptors, which triggers B cell proliferation in a CD4 T helper cell and RIPK2 kinase-dependent manner (33, 47). Animals infected with Δ*spa* mutant staphylococci lack V_H3 idiotypic immunoglobulin expansion and exhibit an increased abundance of pathogen-specific IgG, thereby triggering immune responses that are protective against subsequent *S. aureus* infection (48). We wondered whether colonization with the Δ*spa* mutant of WU1 was associated with altered serum IgG responses. Sera from animals that had been colonized for 15 days were analyzed for IgG binding to components of the *S. aureus* antigen matrix (Table 2). This experiment revealed increases in antibodies against ClfB, IsdA, and SasG in animals that were subsequently decolonized but not in animals that remained colonized with the Δ*spa* mutant (Table 2). Taken together, these data suggest that nasopharyngeal colonization of C57BL/6 mice with Δ*spa* mutant staphylococci is associated with increased IgG responses against key colonization determinants, which appears to promote removal of Δ*spa* mutant *S. aureus* from the nasopharynx.

Protein A-neutralizing antibodies affect persistent colonization with *S. aureus*. Immunization of mice with wild-type protein A does not elicit IgG serum antibodies that bind and neutralize the capacity of the five IgBDs of SpA to bind either the Fcγ domain of IgG molecules or the variant heavy chain of V_H3 idiotype immunoglobulin (44). SpA_{KKAA} is a variant with 20 amino acid substitutions throughout the five IgBDs of SpA that abolish Fcγ binding and also diminish association with V_H3 idiotype immunoglobulin (44). Nevertheless, SpA_{KKAA} retains the overall α-helical content and antigen structure of protein A. As a result, immunization of mice with adjuvanted SpA_{KKAA} elicits high-titer protein A-neutralizing IgG (44). These antibodies block the antiopsonic and B cell superantigen activities of protein A during *S. aureus* infection, broadly enhancing IgG responses against staphylococcal antigens and promoting the development of protective immunity (44). To test whether protein A-neutralizing antibodies affect *S. aureus* colonization, C57BL/6 mice were immunized with adjuvanted SpA_{KKAA} or with adjuvant alone. Compared to mock-immunized animals, SpA_{KKAA}-treated animals elicited high-titer protein A-neutralizing antibodies (Table 3). When inoculated with *S. aureus* WU1, both mock- and SpA_{KKAA}-immunized animals were initially colonized in similar manners, as oropharyngeal swabs revealed average colonizing loads that were not significantly different on days 7 and 14 following inoculation (Fig. 4). However, beginning on day 21, SpA_{KKAA}-immunized mice were more frequently decolonized than mock-immunized animals (Fig. 4). Examination for serum IgG responses and comparison to naive mice showed that *S. aureus* WU1 colonization in mock-treated animals led

TABLE 3 Impact of SpA_{KKAA} immunization on serum IgG responses in *S. aureus* WU1-colonized C57BL/6 mice[a]

		SpA_{KKAA} immunized				PBS mock immunized (colonized)	
		Colonized		Cleared			
Protein type	Antigen	Fold change[b]	*P*[c] (vs PBS mock immunized)	Fold change	*P* (vs SpA_{KKAA} immunized, colonized)	Fold change	*P* (vs naive)
Cell wall anchored surface	SpA_{KKAA}	121.3 ± 64.98	<0.0001	126.3 ± 13.35	<0.0001	0.9 ± 0.16	NS
	ClfA	3.8 ± 0.49	<0.0001	5.7 ± 2.28	0.0069	1.3 ± 0.65	NS
	ClfB	1.1 ± 0.28	NS	14.8 ± 1.12	<0.0001	4.3 ± 1.49	<0.0001
	Ebh	1.0 ± 0.15	NS	1.3 ± 0.57	NS	1.3 ± 0.43	NS
	FnbpA	1.1 ± 0.34	NS	6.4 ± 1.86	<0.0001	1.1 ± 0.29	NS
	FnbpB	1.5 ± 0.33	NS	10.6 ± 1.0	<0.0001	1.2 ± 0.72	NS
	IsdA	1.8 ± 0.46	NS	2.8 ± 0.59	NS	2.0 ± 0.43	NS
	IsdB	1.7 ± 0.37	NS	5.8 ± 2.75	<0.0001	2.1 ± 0.96	NS
	SdrC	1.4 ± 0.67	NS	1.5 ± 0.61	NS	1.2 ± 0.45	NS
	SdrD	1.1 ± 0.39	NS	1.5 ± 0.36	NS	1.2 ± 0.23	NS
	SdrE	1.2 ± 0.36	NS	1.8 ± 0.94	NS	1.2 ± 0.22	NS
	SasA	1.8 ± 0.36	NS	1.6 ± 0.28	NS	0.8 ± 0.80	NS
	SasB	1.9 ± 0.90	NS	1.1 ± 0.42	NS	1.0 ± 0.24	NS
	SasD	1.3 ± 0.46	NS	1.0 ± 0.44	NS	2.4 ± 0.53	0.0023
	SasF	2.4 ± 0.34	NS	1.7 ± 0.55	NS	2.6 ± 1.59	0.004
	SasG	0.9 ± 0.15	NS	5.5 ± 1.04	<0.0001	1.1 ± 0.32	NS
	SasI	2.1 ± 0.46	NS	1.8 ± 0.02	NS	1.3 ± 0.22	NS
	SasK	2.3 ± 0.62	NS	2.7 ± 0.38	NS	1.1 ± 0.02	NS
Secreted	Coa	3.0 ± 1.31	0.0049	5.8 ± 0.87	<0.0001	1.2 ± 0.43	NS
	vWbp	5.7 ± 1.34	<0.0001	6.6 ± 2.82	NS	1.4 ± 0.65	NS
	Hla	2.9 ± 0.08	0.0070	3.6 ± 0.36	NS	1.1 ± 0.58	NS
	SCIN	2.1 ± 0.77	NS	1.4 ± 0.21	NS	1.0 ± 0.37	NS
	Eap	1.7 ± 0.38	NS	1.1 ± 0.22	NS	0.9 ± 0.23	NS
	Efb	1.5 ± 0.47	NS	1.49 ± 0.25	NS	0.98 ± 0.27	NS
	EsxA	2.4 ± 0.65	NS	3.22 ± 1.81	NS	0.82 ± 0.26	NS
	EsxB	2.5 ± 0.35	NS	3.75 ± 1.08	NS	1.46 ± 0.25	NS

[a]Cohorts of C57BL/6J mice were immunized with 50 μg of recombinant SpA_{KKAA} emulsified with CFA or mock immunized with PBS in CFA and boosted on day 11 with 50 μg of recombinant SpA_{KKAA} emulsified with IFA or mock boosted with PBS in IFA. On day 24, the mice were inoculated intranasally with 10^8 CFU of the indicated *S. aureus* strains and swabbed in the throat weekly to enumerate the bacterial load. Fifteen days following inoculation, the animals were bled, and serum samples were analyzed for antibody responses to staphylococcal antigens.

[b]Fold changes were calculated by dividing the average signal intensities derived from SpA_{KKAA}-immunized mice by the average signal intensities of PBS mock-immunized mice. The data are presented as means ± standard deviations.

[c]*P* values were calculated using two-way ANOVA with Tukey multiple-comparison tests. NS, not significant.

to antibody responses against ClfB, IsdA, IsdB, SasD, and SasF (Table 3). In animals that maintained *S. aureus* WU1 colonization, SpA_{KKAA} immunization led to antibody responses against ClfA, Coa, vWbp, and Hla (Table 3). SpA_{KKAA}-vaccinated C57BL/J mice that were subsequently decolonized exhibited elevated serum IgG against ClfA, ClfB, fibronectin binding proteins A (FnBPA) and B (FnBPB), IsdB, Coa, and SasG (Table 3). Together, these data indicate that SpA_{KKAA} vaccination elicited enhanced serum IgG responses in mice that had been colonized with *S. aureus*. Further, SpA_{KKAA} vaccine induced antibodies against many different staphylococcal antigens, including known colonization factors (ClfB, IsdA, and SasG). Thus, SpA_{KKAA} vaccine-induced IgG responses against colonizing staphylococci appear to promote decolonization of the nasopharynx.

***S. aureus* WU1 colonization of BALB/c mice.** To test whether *S. aureus* WU1 colonization was restricted to C57BL/6 mice, we inoculated cohorts ($n = 20$) of naive BALB/c mice with 1×10^8 CFU *S. aureus* WU1 into the right nostril and measured nasopharyngeal colonization with swab cultures. Similar to C57BL/6 mice, *S. aureus* WU1 persistently colonized BALB/c mice (Fig. 5). Immunization of BALB/c mice with SpA_{KKAA} did not affect the initial colonization with *S. aureus* WU1. However, compared to mock-immunized animals, vaccination with SpA_{KKAA} promoted decolonization of BALB/c mice (Fig. 5).

SpA_{KKAA} vaccine affects mouse colonization with *S. aureus* JSNZ. We wondered whether protein A-neutralizing antibodies also affect mouse colonization with *S. aureus*

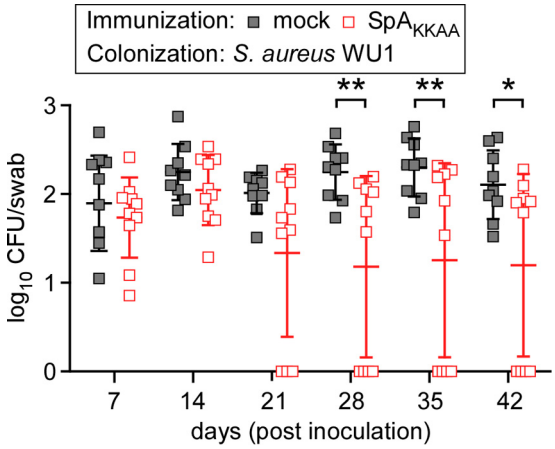

FIG 4 Immunization of C57BL/6 mice with SpA$_{KKAA}$ promotes decolonization of *S. aureus* WU1. C57BL/6 mice were immunized with 50 μg of purified recombinant SpA$_{KKAA}$ emulsified with CFA or mock immunized with PBS in CFA, boosted after 11 days with 50 μg of recombinant SpA$_{KKAA}$ emulsified with IFA or mock boosted with PBS in IFA, and inoculated with *S. aureus* 24 days following the initial immunization. On day 0 of the colonization experiment, cohorts of C57BL/6 mice ($n = 10$) were inoculated intranasally with 1×10^8 CFU of *S. aureus* WU1. The oropharynxes of the animals were swabbed at weekly intervals to enumerate the bacterial load. Each square indicates the number of CFU for one mouse. The median and standard deviation for each group of animals on a given day are indicated by the horizontal lines and error bars. Bacterial colonization data sets were analyzed with two-way ANOVA and Sidak multiple-comparison tests; statistically significant differences (*, $P < 0.05$; **, $P < 0.01$) between the two groups of animals are indicated.

JSNZ. Unlike those of strains Newman and WU1, the *spa* gene product of *S. aureus* JSNZ comprises only four IgBDs (37). Previous work demonstrated that SpA variants with four IgBDs are associated with diminished B cell superantigen activity compared to the five IgBDs generally associated with *S. aureus* colonization of the human nasopharynx (33). When inoculated into the right nostrils of anesthetized mice, *S. aureus* JSNZ effectively colonized the nasopharynxes of BALB/c mice over 42 days (Fig. 6). SpA$_{KKAA}$ vaccination did not affect initial colonization with *S. aureus* JSNZ. However, compared to mock-immunized mice, BALB/c mice with serum neutralizing protein A antibodies more frequently decolonized *S. aureus* JSNZ starting on day 21 (Fig. 6). Together, these data

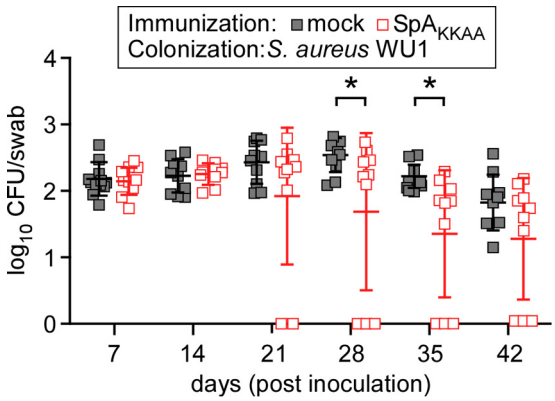

FIG 5 Immunization of BALB/c mice with SpA$_{KKAA}$ promotes decolonization of *S. aureus* WU1. BALB/c mice were immunized with 50 μg of purified recombinant SpA$_{KKAA}$ emulsified with CFA or mock immunized with PBS in CFA, boosted after 11 days with 50 μg of recombinant SpA$_{KKAA}$ emulsified with IFA or mock boosted with PBS in IFA, and inoculated with *S. aureus* 24 days following the initial immunization. On day 0 of the colonization experiment, cohorts of BALB/c mice ($n = 20$) were inoculated intranasally with 1×10^8 CFU of *S. aureus* WU1. The oropharynxes of the animals were swabbed at weekly intervals to enumerate the bacterial load. Each square indicates the number of CFU for one mouse. The median and standard deviation for each group of animals on a given day are indicated by the horizontal lines and error bars. Bacterial colonization data sets were analyzed with two-way ANOVA and Sidak multiple-comparison tests; statistically significant differences (*, $P < 0.05$) between groups of animals are indicated.

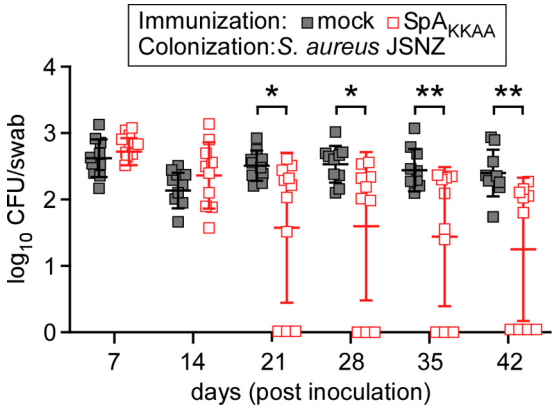

FIG 6 Immunization of BALB/c mice with SpA_{KKAA} promotes *S. aureus* JSNZ clearance from the nasopharynx. BALB/c mice were immunized with 50 μg of purified recombinant SpA_{KKAA} emulsified with CFA or mock immunized with PBS in CFA, boosted after 11 days with 50 μg of recombinant SpA_{KKAA} emulsified with IFA or mock boosted with PBS in IFA, and inoculated with *S. aureus* 24 days following the initial immunization. On day 0 of the colonization experiment, cohorts of BALB/c mice ($n = 10$) were inoculated intranasally with 1×10^8 CFU of *S. aureus* JSNZ. The oropharynxes of the animals were swabbed at weekly intervals to enumerate the bacterial load. Each square indicates the number of CFU for one mouse. The median and standard deviation for each group of animals on a given day are indicated by the horizontal lines and error bars. The bacterial colonization data sets were analyzed with two-way ANOVA with Sidak multiple-comparison tests; statistically significant differences (*, $P < 0.05$; **, $P < 0.01$) between the two groups of animals are indicated.

suggest that *S. aureus* JSNZ also requires protein A-mediated B cell superantigen activity for persistent colonization of mice.

DISCUSSION

In the United States, *S. aureus* causes skin and soft tissue infections (SSTI) that result in 14.2 million outpatient visits per year and in more than 850,000 hospital admissions (49–51). *S. aureus* SSTI are associated with complications such as bacteremia, the need for surgical intervention, and death (52, 53). *S. aureus* also causes more than 380,000 hospital-acquired infections per year (54). At increased risk of *S. aureus* infection are patients with surgical procedures, trauma, implantation of foreign bodies, endotracheal intubation, indwelling catheters, or hemodialysis, as well as immunosuppressive or cancer therapies (55). Even with antibiotic and surgical therapy, recurrent disease is a hallmark of *S. aureus* infection and manifests either as relapse with the index strain or as reinfection with another isolate (56). Relapses occur about four times more frequently than reinfections (57). *S. aureus* colonization predisposes individuals to both community- and hospital-acquired infections (6, 14, 58).

S. aureus vaccine efficacy trials enrolled hospital patients at high risk of infection, including patients with hemodialysis and patients undergoing open heart surgery (59, 60). Both trials failed to achieve their study endpoints: to diminish the incidence of *S. aureus* infection (59, 60). Further, immunization with one of the two vaccine candidates, a capsular polysaccharide conjugate, did not affect human colonization with *S. aureus* (61, 62). In contrast, licensed capsular polysaccharide vaccines that are effective at preventing bacterial meningitis also provide protection from nasopharyngeal colonization with *Neisseria meningitidis*, which is a key risk factor for invasive disease (63, 64). Moreover, *N. meningitidis* colonization eventually leads to the development of natural immunity via bactericidal, serotype-specific capsular polysaccharide antibodies that prevent further colonization with strains of the same serotype (65). In contrast, *S. aureus* colonization of humans is not associated with the development of bactericidal or opsonizing IgG antibodies that can prevent further colonization or invasive disease (2).

Our work has been focused on characterizing the *S. aureus* determinant responsible for suppressing adaptive immune responses. We hypothesized that the underlying mechanism may support persistent colonization of humans and also prevent the

development of immunity to invasive disease (66, 67). SpA, a molecule that impacts B cell development and antibody production, is expressed by all clinical *S. aureus* isolates (29, 32). SpA is secreted and anchored to the bacterial wall so that its N-terminal IgBDs (58- to 62-residue E, D, B, C, and A domains with a triple-helical fold) are displayed on the bacterial surface while the C-terminal end is linked to peptidoglycan (68–70). During growth, *S. aureus* murein hydrolases cut peptidoglycan and release SpA into the extracellular milieu to modify host B cell responses (33, 46). The IgBDs of SpA molecules on the staphylococcal surface bind to the Fcγ portion of human immunoglobulin (IgG1, IgG2, IgG4, and IgA) and block the effector functions of antibodies, which otherwise would engage Fcγ and complement receptors on phagocytes (71, 72). Released SpA binds and cross-links the variant heavy chain of V_H3 idiotype B cell receptors (IgM) (73, 74). SpA cross-linking promotes proliferation of V_H3 idiotype B cells and secretion of IgG and IgA molecules that are affinity matured for SpA binding yet lack specificity for *S. aureus* antigens (33, 75). SpA B cell superantigen activity diverts adaptive immune responses during invasive disease in a mouse bacteremia model with naive animals (48). In contrast, mice infected with an isogenic Δ*spa* mutant exhibit increased adaptive immune responses to staphylococcal disease and protection against subsequent infection (48). During *S. aureus* infection in humans, mice, or guinea pigs, the unique functional attributes of SpA also prevent the development of antibodies that can bind and neutralize its IgBDs (44, 48, 76). Here, we asked whether *spa* expression is required for *S. aureus* colonization, using a mouse model with an ST88 isolate that has evolved to persistently colonize laboratory mice and cause invasive disease.

Earlier work used *S. aureus* Newman, specifically, a streptomycin-resistant variant, to analyze nasal colonization in streptomycin-treated mice (19). In this model, *S. aureus* Newman adheres to mouse nasal epithelia and replicates for 14 days but is cleared by 21 days (21). The importance of surface proteins for attachment to nasal epithelia and for colonization was demonstrated with a sortase mutant (Δ*srtA*) that cannot anchor surface proteins with LPXTG-sorting signals (SpA, ClfA, ClfB, FnBPA, FnBPB, SdrC, SdrD, SdrE, IsdA, IsdB, SasG, etc.) to the bacterial envelope (77). The Δ*srtA* variant of *S. aureus* Newman exhibited a colonization defect 14 days after inoculation (78). A phenotype of diminished bacterial load in the nasal cavities of mice was also observed with the *S. aureus* Newman Δ*clfB* mutant (78). Further, ClfB-specific IgG antibodies, whether elicited by active immunization or passively transferred to naive mice, reduced staphylococcal loads in mouse nasal tissue but did not diminish colonization (78). ClfB binds to the α-chain of human and mouse fibrinogen and to the $Y(GS)_nY$ quasirepeats of loricrin and cytokeratin K10 (21, 79, 80). Whereas wild-type FVB mice were effectively colonized by *S. aureus* Newman over 10 days, loricrin knockout mice were partially decolonized over the same period (21). The contributions of IsdA and SasG to *S. aureus* colonization have not yet been studied in mice. In the cotton rat model, IsdA certainly contributed to *S. aureus* nasal colonization, and IsdA-specific antibodies provided partial protection against colonization (81). Thus, *S. aureus* colonization of the nasopharynxes of mice and humans likely requires the functions of multiple surface proteins, and IgG antibodies against the molecules likely impact the bacterial load and/or persistence of staphylococci in these tissues.

The isolation of *S. aureus* strains that persistently colonize the nasopharynx and gastrointestinal tract in laboratory mice offers unique research opportunities to systematically identify staphylococcal determinants of nasal attachment, colonization, and persistence in the host (36, 37). Further, animal models of persistent colonization can be used for vaccine studies by analyzing long-term immune responses during colonization and invasive disease, the contributions of specific immune-evasive factors, and interference of vaccines with either colonization or disease (36). Here, we contribute to this burgeoning field by isolating the mouse-adapted strain *S. aureus* WU1. Similar to isolates reported previously, *S. aureus* WU1 belongs to the CC88 clade. We noted the presence of *vwb* alleles that appeared to promote enhanced agglutination of mouse plasma by *S. aureus* WU1 and JSNZ. Earlier work highlighted the contributions of host-specific *vwb* alleles to *S. aureus* adaptation to ruminant and equine hosts (82).

Thus, host-specific adaptation of *vwb* appears to be a universal feature of *S. aureus* evolution, supporting the unique pathogenesis of its abscess lesions (83). We show further that *spa* is not required for the initial colonization of mice with *S. aureus* WU1, indicating that protein A is not a colonization factor *sensu stricto*. However, starting 14 days after the initial inoculation, mice inoculated with *spa* mutants are more frequently decolonized than control animals carrying wild-type staphylococci. Decolonization coincides with the development of serum IgG against *S. aureus* colonization factors. SpA-neutralizing antibodies, generated via SpA_{KKAA} immunization, achieve similar decolonization in mice. Such decolonization was also associated with the development of IgG specific for *S. aureus* colonization factors (ClfB, IsdA, and SasG). Together, these data suggest that the B cell superantigen activity of protein A may prevent adaptive immune responses against bacterial secreted products, thereby enabling persistent colonization with *S. aureus*. We note that some, but not all, animals were decolonized in a protein A-dependent manner over the 42-day time course of our experiments. It seems plausible that prolonged colonization (for 60 or more days) with *spa* mutant staphylococci or SpA-neutralizing antibodies may generate further increases in serum IgG responses that achieve increased decolonization. Alternatively, some hosts may require episodes of invasive disease to formulate adaptive immune responses that result in decolonization of the pathogen. Nevertheless, to our knowledge, these data provide the first experimental evidence in support of the hypothesis that SpA B cell superantigen activity contributes to the persistent *S. aureus* colonization of mice.

Unlike subunit vaccines that are comprised of individual or combinations of protective antigens, SpA_{KKAA} is a vaccine that activates antibody responses against all antigens of *S. aureus* that are recognizable by the immune system. This effect, however, can be achieved only when individuals with SpA-neutralizing antibodies encounter the pathogen (44, 84). Previous work demonstrated the vaccine effects of SpA-neutralizing antibodies in mice and guinea pigs with *S. aureus* bloodstream infections (44, 76). Here, we demonstrate that the vaccine effect of SpA_{KKAA} immunization can also be implemented in individuals that are colonized with *S. aureus*. The superantigen activity and immune-evasive attributes of SpA are confined to B cells with V_H3 idiotype IgM receptors, which, owing to the germ line repertoire of immunoglobulin variable heavy chain genes, are highly abundant in humans (>50%) but much less abundant in guinea pigs (20 to 25% of blood B cells) and mice (10% of blood B cells) (48, 73). We therefore speculate that the vaccine efficacy of nontoxigenic protein A vaccines for *S. aureus* colonization may be greater in humans than in mice.

MATERIALS AND METHODS

Media and bacterial growth conditions. *S. aureus* strain WU1 was isolated in the animal facility at Washington University, St. Louis, MO. *S. aureus* strains were propagated in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 37°C. For experiments investigating mouse nasopharyngeal colonization, throat swab samples were grown on Baird-Parker agar at 37°C as indicated. For experiments investigating *S. aureus* GI tract colonization, stool samples were grown on mannitol salt agar at 37°C as indicated. *Escherichia coli* strains DH5α and BL21(DE3) were grown in Luria broth (LB) or agar at 37°C. Ampicillin (100 μg/ml for *E. coli*) and chloramphenicol (10 μg/ml for *S. aureus*) were used for plasmid selection.

***S. aureus* genotyping.** *S. aureus* isolate WU1 was obtained from the nasopharynxes and preputial gland abscess lesions of mice in our animal facility. Mouse *S. aureus* strain JSNZ was provided by Siouxsie Wiles (36). Staphylococcal genomic DNA was isolated with the Wizard genomic DNA purification kit (Promega). *Spa* genotyping and MLST were performed as previously described (85). Briefly, for *spa* typing, the genomic DNA of *S. aureus* strain WU1 was PCR amplified with primers 1095F (5′-AGACGATCCTTCG GTGAGC-3′) and 1517R (5′-GCTTTTGCAATGTCATTTACTG-3′) (86). The PCR product was purified with the Nucleospin gel and PCR cleanup kit, sequenced with primers 1095F and 1517R, and analyzed with Ridom software (http://spaserver.ridom.de/spa-t186.shtml). For MLST, the genomic DNA of *S. aureus* strain WU1 was PCR amplified with primers *arc*-up (5′-TTGATTCACCAGCGCGTATTGTC-3′), *arc*-dn (5′-AGGTATCTGC TTCAATCAGCG-3′), *aro*-up (5′-ATCGGAAATCCTATTTCACATTC-3′), *aro*-dn (5′-GGTGTTGTATTAATAACGAT ATC-3′), *glp*-up (5′-CTAGGAACTGCAATCTTAATCC-3′), *glp*-dn (5′-TGGTAAAATCGCATGTCCAATTC-3′), *gmk*-up (5′-ATCGTTTTATCGGGACCATC-3′), *gmk*-dn (5′-TCATTAACTACAACGTAATCGTA-3′), *pta*-up (5′-G TTAAAATCGTATTACCTGAAGG-3′), *pta*-dn (5′-GACCCTTTTGTTGAAAAGCTTAA-3′), *tpi*-up (5′-TCGTTCATTC TGAACGTCGTGA-3′), *tpi*-dn (5′-TTTGCACCTTCTAACAATTGTAC-3′), *yqi*-up (5′-CAGCATACAGGACACCTAT TGGC-3′), and *yqi*-dn (5′-CGTTGAGGAATCGATACTGGAAC-3′) (http://saureus.mlst.net/misc/info.asp). The PCR product was purified with the Nucleospin gel and PCR cleanup kit, PCR amplified, and sequenced and analyzed with on-line software (http://saureus.mlst.net/sql/multiplelocus.asp). Whole-genome se-

quence files for *S. aureus* strain JSNZ were provided by Silva Holtfreter. TruSeq DNA-seq library preparation Illumina MiSeq sequencing was performed with the genomic DNA of *S. aureus* WU1 by the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory. Sequences were analyzed using Geneious software.

***S. aureus* mutants.** Allelic recombination with the plasmid pKOR1 was used to delete the *spa* gene of *S. aureus* WU1 (87). To construct the Δ*spa* mutant, two 1-kb DNA fragments upstream and downstream of the *spa* gene were amplified from the chromosome of *S. aureus* WU1 with primers ext1F (5′-GGGG ACCACTTTGTACAAGAAAGCTGGGTCATTTAAGAAGATTGTTTCAGATTTATG-3′), ext1R (5′-ATTTGTAAAGTCA TCATAATATAACGAATTATGTATTGCAATACTAAAATC-3′), ext2F (5′-CGTCGCGAACTATAATAAAAACAAACA ATACACAACGATAGATATC-3′), and ext2R (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCAACGAACGCCTAA AGAAATTGTCTTTGC-3′). The two flanking regions were fused in a subsequent PCR, and the final PCR product was cloned into pKOR1 using the BP Clonase II kit (Invitrogen). The resulting plasmids were consecutively transferred into *E. coli* DH5α, *S. aureus* strain RN4220, and finally *S. aureus* strain WU1 and temperature shifted to 40°C, blocking replication of the plasmids and promoting their insertion into the chromosome (87). Growth at 30°C was used to promote allelic replacement. Mutations in the *spa* genes were verified by DNA sequencing of the PCR amplification products.

Agglutination assay. Agglutination assays were performed as previously described (88). Briefly, overnight cultures of *S. aureus* strains were diluted 1:100 in fresh TSB and grown at 37°C for 6 h. Bacteria from 1 ml of culture (normalized to an optical density at 600 nm [OD_{600}] of 4.0) were incubated with Syto 9 (1:500; Invitrogen) for 15 min, washed twice with 1 ml PBS, and suspended in 1 ml PBS. The bacteria were mixed 1:1 with citrate-treated human plasma or mouse plasma on glass microscope slides and incubated for 30 min. Samples were viewed and images were captured on an IX81 live-cell total internal reflection fluorescence microscope using a 20× objective (Olympus). At least 10 images were acquired for each sample. The areas of agglutination complexes in each image were measured and quantified using ImageJ software.

Immunoblotting. Overnight cultures of *S. aureus* strains were diluted 1:100 in fresh TSB (with chloramphenicol in the presence of plasmids) and grown at 37°C to an OD_{600} of 0.5 to 1.0. Cells from 1 ml of culture were centrifuged, suspended in PBS, and incubated with 20 μg/ml lysostaphin (AMBI) at 37°C for 1 h. Proteins in the whole-cell lysate were precipitated with 10% trichloroacetic acid and 10 μg deoxycholic acid, washed with ice-cold acetone, air dried, suspended in 100 μl 0.5 M Tris·HCl (pH 6.8) and 100 μl SDS-PAGE sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 200 mM dithiothreitol), and boiled for 10 min. The proteins were separated on SDS-12% PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked with 5% milk in Tris-buffered saline with Tween 20 (TBST) (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween 20). Mouse anti-ClfA 2A12.12 monoclonal antibody (1:2,000 dilution) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cell Signaling; 1:10,000 dilution) were used to detect ClfA. Rabbit anti-Coa polyclonal antibody (1:1,000 dilution) and HRP-conjugated anti-rabbit IgG (1:10,000 dilution) were used to detect Coa. Two different rabbit anti-vWbp polyclonal antibodies (1:1,000 dilution), which recognize full-length vWbp from *S. aureus* Newman or the C-terminal domain of vWbp, respectively, and HRP-conjugated anti-rabbit IgG (1:10,000 dilution) were used to detect vWbp. HRP-conjugated human IgM in TBST (1:10,000 dilution) was used to detect SpA. Rabbit anti-SrtA polyclonal antibodies (1:10,000 dilution) and HRP-conjugated anti-rabbit IgG (1:10,000 dilution) were used to detect SrtA. Antibody-stained membranes were washed with TBST, incubated with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific), and developed on Amersham Hyperfilm ECL high-performance chemiluminescence films (GE Healthcare).

Purification of recombinant proteins. *E. coli* BL21(DE3) harboring pET15b+ plasmids for the expression of His-tagged SpA_{KKAA}, as well as 25 staphylococcal antigens (ClfA, ClfB, FnBPA, FnBPB, IsdA, IsdB, SasA, SasB, SasD, SasF, SasG, SasI, SasK, SdrC, SdrD, SdrE, EsxA, EsxB, SCIN, Eap, Efb, Hla, Coa, vWbp, and Ebh), was grown overnight, diluted 1:100 in fresh medium, and grown at 37°C to an OD_{600} of ~0.5. The cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and grown for an additional 3 h. The cells were pelleted, resuspended 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. The lysates were cleared of membrane and insoluble components by ultracentrifugation at 40,000 × *g*. The cleared lysates were subjected to Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography, and proteins were eluted in column buffer containing successively higher concentrations of imidazole (100 to 500 mM). The eluates were dialyzed with PBS, and the protein purity was verified by Coomassie-stained SDS-PAGE. Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific).

Mouse nasopharyngeal colonization. Overnight cultures of *S. aureus* strain WU1 and its Δ*spa* mutant were diluted 1:100 in fresh TSB and grown for 2 h at 37°C. The cells were centrifuged, washed, and suspended in PBS. Seven-week-old female BALB/c, C57BL/6J, or B6.129S2-*Ighm*^{tm1Cgn}/J mice (Jackson Laboratory) were anesthetized by intraperitoneal injection with 100 mg/ml ketamine and 20 mg/ml xylazine per kilogram of body weight, and 1×10^8 CFU of *S. aureus* (in a 10-μl volume) was pipetted into the right nostril of each mouse. On days 7, 14, 21, 28, 35, and 42 following inoculation, the oropharynxes of the mice were swabbed, and swab samples were spread on BPA and incubated for bacterial enumeration. On day 15 following inoculation, the mice were bled via periorbital vein puncture to obtain sera for antibody response analyses using the staphylococcal antigen matrix. On day 42 following inoculation, stool samples were collected and homogenized in PBS. The homogenates were plated on MSA and incubated for bacterial enumeration. All mouse experiments were performed 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. Animal experiments were repeated at least once to ensure reproducibility of the data.

Active immunization. Four-week-old mice were immunized by subcutaneous injection with 50 μg of SpA_{KKAA} emulsified in complete Freund's adjuvant (CFA) (Difco) and boosted with 50 μg of the same antigen emulsified in incomplete Freund's adjuvant (IFA) 11 days following the initial immunization. On day 21, the immunized mice were bled via periorbital vein puncture to obtain sera for enzyme-linked immunosorbent assay (ELISA). On day 24, the mice were inoculated intranasally with 1×10^8 CFU of *S. aureus* strain WU1 or JSNZ and monitored for nasopharyngeal colonization.

Staphylococcal antigen matrix. Nitrocellulose membranes were blotted with 2 μg affinity-purified staphylococcal antigens. The membranes were blocked with 5% degranulated milk and incubated with diluted mouse sera (1:10,000 dilution) and IRDye 680-conjugated goat anti-mouse IgG (Li-Cor). Signal intensities were quantified using the Odyssey infrared imaging system (Li-Cor).

Statistical analysis. Two-way analysis of variance (ANOVA) with Sidak multiple-comparison tests (GraphPad Software) was performed to analyze the statistical significance of nasopharyngeal colonization, ELISA, and antigen matrix data.

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Y.S., C.E., D.M., and O.S. declare competing interests as inventors of patents and intellectual property filings for vaccines that promote *S. aureus* decolonization.

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