Proteomic Identification of *saeRS*-Dependent Targets Critical for Protective Humoral Immunity against *Staphylococcus aureus* Skin Infection

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Recurrent *Staphylococcus aureus* skin and soft tissue infections (SSTIs) are common despite detectable antibody responses, leading to the belief that the immune response elicited by these infections is not protective. We recently reported that *S. aureus* USA300 SSTI elicits antibodies that protect against recurrent SSTI in BALB/c but not C57BL/6 mice, and in this study, we aimed to uncover the specificity of the protective antibodies. Using a proteomic approach, we found that *S. aureus* SSTI elicited broad polyclonal antibody responses in both BALB/c and C57BL/6 mice and identified 10 *S. aureus* antigens against which antibody levels were significantly higher in immune BALB/c serum. Four of the 10 antigens identified are regulated by the *saeRS* operon, suggesting a dominant role for *saeRS* in protection. Indeed, infection with USA300Δ*sae* failed to protect against secondary SSTI with USA300, despite eliciting a strong polyclonal antibody response against antigens whose expression is not regulated by *saeRS*. Moreover, the antibody repertoire after infection with USA300Δ*sae* lacked antibodies specific for 10 *saeRS*-regulated antigens, suggesting that all or a subset of these antigens are necessary to elicit protective immunity. Infection with USA300Δ*hla* elicited modest protection against secondary SSTI, and complementation of USA300Δ*sae* with *hla* restored protection but incompletely. Together, these findings support a role for both Hla and other *saeRS*-regulated antigens in eliciting protection and suggest that host differences in immune responses to *saeRS*-regulated antigens may determine whether *S. aureus* infection elicits protective or nonprotective immunity against recurrent infection.

Staphylococcus aureus is the most common cause of skin and soft tissue infections (SSTIs) in the United States (1, 2). Recurrent SSTIs are common, leading to the belief that they do not elicit immune responses that protect against subsequent infection. Because of the substantial morbidity and mortality associated with *S. aureus* infections, as well as increasing resistance of *S. aureus* isolates to antimicrobials, developing a vaccine to prevent these infections is a public health priority (3). Unfortunately, several vaccines comprising single *S. aureus* antigens have failed in phase III trials, most recently Merck's V710 (4).

S. aureus has a wide array of factors that contribute to its virulence and survival in host tissues (5). The redundancy in the function of many of the virulence factors, as well as the myriad ways in which *S. aureus* evades protective immune responses, complicates the selection of antigens to incorporate into prospective vaccines. For example, most *S. aureus* isolates have multiple factors that bind IgG (6), have superantigen activity (7), inhibit complement activity (8), or are toxic to leukocytes or other immune cells (9, 10). Although the cellular mechanisms by which many of these molecules interact with the host immune system have been defined, how they function in concert during *S. aureus* infection is less well understood. In particular, the role of microbial virulence factors in eliciting or preventing a protective adaptive immune response is not well understood.

The expression of virulence factors in *S. aureus* is tightly controlled and coordinated (11). One important global regulatory operon is the *S. aureus* accessory element (*saeRS*), which encodes a two-component system (SaeS and SaeR) and two upstream genes whose functions are less well defined (*saeP* and *saeQ*) (12). SaeR binds a consensus sequence upstream of a number of genes encoding virulence factors (13). Although a role for *saeRS* in the virulence of USA300, the dominant *S. aureus* genetic background

in the United States, has been established (13, 14), it is not known if *saeRS* is important in eliciting protective immunity.

We described a new mouse model of protective immunity against recurrent *S. aureus* SSTI (15). We found that *S. aureus* SSTI elicited protective antibody-mediated and Th17/interleukin-17A (IL-17A)-mediated immunity that resulted in smaller skin lesions and enhanced bacterial clearance in BALB/c but not C57BL/6 mice. Importantly, adoptive transfer of serum from previously infected BALB/c but not C57BL/6 mice into naive mice of either background was sufficient to confer protection, demonstrating that protective antibodies developed in BALB/c but not C57BL/6 mice. In the present study, to determine the antibody specificity associated with protection, we used a proteomic approach to identify the *S. aureus* antigens for which antibody levels were significantly higher in the serum of BALB/c mice than in that

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of C57BL/6 mice. Of the 10 identified antigens associated with superior protection, 4 were regulated by *saeRS*, suggesting a role for this operon in eliciting protection. In support of this hypothesis, expression of *saeRS* during primary SSTI was necessary to elicit antibody-mediated protective immunity against secondary SSTI, and complementation with *hla* resulted in only partial restoration of protection.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strains 923 (USA300) and 923 Δ *sae*, an isogenic deletion mutant of 923 in which a gene encoding resistance to spectinomycin (*aad9*) was inserted by allelic exchange in place of *saeR* and *saeS*, have been previously described (14). Strains 923 Δ *sae/psae* and 923 Δ *sae/hla* were constructed by inserting *saeRS* and *hla*, respectively, into multicopy plasmid pCN48 (16) modified by the addition of the P_{spac} promoter and transforming the resulting plasmids into 923 Δ *sae*. LAC Δ *hla* was a gift from Juliane Bubeck Wardenburg (University of Chicago) (17). Strain 923 Δ *hla* was subsequently generated by phage transduction of *hla::erm* into strain 923.

Mouse model of *S. aureus* SSTI. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago (protocols 71694 and 72405). Our mouse model of SSTI has been previously described (15). Seven-week-old female BALB/c and C57BL/6 mice were purchased from Jackson (BALB/c versus C57BL/6 study) or Taconic (*saeRS* study) and allowed to acclimatize for 1 week prior to inoculation. The mice were allowed free access to food and water throughout the experiments. On the morning of inoculation, an overnight culture was diluted 1:100 in fresh tryptic soy broth and grown to the exponential phase (3 h, optical density at 600 nm of 1.8). The bacteria were then washed in sterile phosphate-buffered saline (PBS) and resuspended to a concentration of 1.5×10^7 CFU/50 μ l. The mice were sedated, and the flank was shaved and cleansed with ethanol, after which inoculation was performed subcutaneously with 50 μ l of *S. aureus* or PBS. The size of each skin lesion was measured by digital photography as described previously (15). For the secondary SSTI, the mice were reinfected 8 weeks later on the opposite flank.

Serum transfer. Four weeks after the secondary SSTI, mice were sacrificed by forced CO₂ inhalation and blood was obtained by intracardiac puncture. Serum was prepared with serum separator tubes (Becton Dickinson). Within each group, serum samples from pairs of mice were pooled and used for adoptive transfer and subsequent antibody quantification (six to eight mice pooled, resulting in three or four samples per group). Adoptive transfer of serum from naive and immune mice (100 μ l/day for 2 days prior to infection) was performed by retroorbital injection.

Antibody quantification by enzyme-linked immunosorbent assay (ELISA). LukE and HlgC were generously provided by the Center for Structural Genomics for Infectious Diseases (Wayne Anderson, Northwestern University). To purify Map/Eap, its truncated open reading frame (ORF) was PCR amplified from strain 923, restriction digested, and cloned in frame with a His tag into pET28a (Novagen). The resulting plasmid was expressed in *E. coli* strain BL21(DE3) (Invitrogen). The proteins were chromatography purified from *E. coli* with the His-Bind kit (Novagen).

To quantify antigen-specific antibodies by ELISA, 96-well plates (Costar; Corning Inc.) were coated with 25 μ g/ml iron surface determinant B (IsdB; Merck), 5 μ g/ml alpha-hemolysin (Hla; Sigma-Aldrich), or 5 μ g/ml of the purified antigens. The serum was diluted 1:200 in PBS and added to the antigen-containing wells. For IgG, detection was performed with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:5,000, AffiniPure; Jackson ImmunoResearch) and the AP substrate *p*-nitrophenylphosphate (Sigma-Aldrich) in accordance with the manufacturer's recommendations. For the IgG subclasses, incubation was performed with goat anti-mouse IgG1, Ig2a, IgG2b, or IgG3 (1:1,000; Sigma-Aldrich), followed by incubation with the horseradish peroxidase-conjugated rabbit anti-goat IgG detection antibody (1:5,000; Jackson Im-

munoResearch). Absorbance was measured with a GENios spectrophotometer (Tecan).

Assessment of antibody responses by a proteomic approach. The *S. aureus* proteome-wide microarray was developed by Antigen Discovery, Inc. (18–21). Briefly, *S. aureus* USA300 ORFs were amplified with ORF-specific primers and an adapter sequence allowing cloning via homologous recombination into *E. coli* DH5 α cells. Plasmid DNA was prepared from the recombinants (Qiagen), and a subset of the clone collection was verified by PCR. A nonoverlapping subset was verified by DNA sequencing. Transcription and translation of the ORF-specific proteins were performed as described previously (18). The proteins were then printed on nitrocellulose-coated film slides with a microarray printer. To confirm the presence of the expressed protein, microarrays were probed with anti-polyhistidine and rat antihemagglutinin high-affinity monoclonal antibodies and bound antibodies were detected with a goat anti-mouse or goat anti-rat biotin secondary antibody. By these methods, over 90% of the translated proteins were confirmed.

For sample staining, the slides were blocked with protein array blocking buffer, followed by incubation with the serum samples (diluted 1:100) overnight. Antibodies were visualized with a goat anti-mouse IgG- or IgG1-specific biotinylated secondary antibody (Jackson ImmunoResearch Labs), followed by hybridization with streptavidin-PBXL3. Fluorescence intensities were determined in a ScanArray Express HT and quantified with ScanArray software (PerkinElmer). Raw data were normalized and transformed by variation stabilization normalization. A reactive antigen was defined as a protein for which the mean signal intensity was greater than the mean of the negative controls plus 2 standard deviations. The results are presented as retransformed intensities. For each experimental condition, there were three biologic replicates.

Data analysis. For analysis of skin lesion size, the area under the curve (AUC) for each individual animal was calculated. The AUC was then compared between or among groups by Student's *t* test or one-way analysis of variance with Tukey's posttest for multiple comparisons, as appropriate. The antibody levels determined by ELISA were compared by Student's *t* test or one-way analysis of variance with Tukey's posttest for multiple comparisons, as appropriate. For the proteomic analyses, normalized antigen intensities were compared between groups with Student's *t* test. For all analyses, differences were considered significant when the *P* value was <0.05. All statistical analyses were performed with GraphPad Prism.

RESULTS

Protective immunity in BALB/c mice was associated with higher levels of antibodies directed against *saeRS*-regulated antigens. We reported that protective humoral immunity in BALB/c mice was associated with significantly higher levels of IgG and IgG1 against alpha-hemolysin (Hla), whereas there were no significant differences in IgG or IgG1 levels against iron surface determinant B (IsdB), compared with C57BL/6 mice (15). These observations suggested that although protection was elicited only in BALB/c mice, both BALB/c and C57BL/6 mice could mount an antibody response to *S. aureus* infection. Moreover, they prompted the hypothesis that BALB/c mice mounted a more potent humoral response than C57BL/6 mice against a subset of *S. aureus* antigens and that these specific antibodies may confer protection against secondary SSTI. To test this hypothesis, we used an *S. aureus* proteome-wide array (18) to identify the bacterial antigens recognized by serum IgG1 from BALB/c versus C57BL/6 mice 4 weeks after secondary *S. aureus* SSTI, because we found that measuring total IgG sometimes obscured differences in antigen-specific IgG1. We elected to assess antibody responses after secondary infection in order to compare the strongest possible antistaphylococcal antibody repertoire that was protective (i.e., BALB/c mice) versus not protective (i.e., C57BL/6 mice). Indeed, adoptive trans-

TABLE 1 Differences in antigen-specific IgG1 levels between C57BL/6 and BALB/c mice after *S. aureus* SSTI^a

			IgG1		
Antigen	Description	<i>saeRS</i> regulated?	C57BL/6	BALB/c	<i>P</i> value
Higher in BALB/c than in C57BL/6					
SAUSA300_2364	IgG-binding protein Sbi	Yes	729	58,808	<0.01
SAUSA300_0883	Putative surface protein	No	302	55,329	<0.01
SAUSA300_0395	Exotoxin	No	267	53,439	<0.01
SAUSA300_2366	Gamma-hemolysin component C, HlgC	Yes	392	15,737	<0.01
SAUSA300_1058	Alpha-hemolysin precursor	Yes	265	3,651	<0.05
SAUSA300_0758	Triosephosphate isomerase (TpiA)	No	296	1,435	<0.05
SAUSA300_2441	Fibronectin-binding protein A (FnbA)	Yes	136	693	<0.05
SAUSA300_0552	Conserved hypothetical protein	No	381	637	<0.05
SAUSA300_0231	ABC transporter	No	368	523	<0.05
SAUSA300_0207	Conserved hypothetical protein	No	361	501	<0.05
Higher in C57BL/6 than in BALB/c					
SAUSA300_1030	Iron transport-associated domain protein	No	8,140	421	<0.05
SAUSA300_1756	Serine protease SplC	Yes	1,958	442	<0.05
SAUSA300_2540	Fructose-bisphosphate aldolase	No	925	458	<0.05

^a Data are presented as the mean normalized intensity (arbitrary units) of three biologic replicates.

fer of serum from C57BL/6 mice after secondary infection failed to protect, whereas serum from BALB/c mice at the same time point was protective (15). This comparison strengthens the translational application, because nonprotective antistaphylococcal antibodies are common in patients. Overall, we detected 446 antigens against which antibodies in the serum of BALB/c mice were reactive and 373 antigens in C57BL/6 mouse serum, demonstrating that *S. aureus* SSTI elicited broad polyclonal antibody responses in both mouse backgrounds.

To identify the *S. aureus* antigens against which IgG levels were significantly higher in infected BALB/c mice than in C57BL/6 mice, we set a stringent threshold by using a normalized mean intensity of >500 within any group (see Table S1 in the supplemental material) and a *P* value of <0.05. Using these criteria, we found 10 antigens that were bound by significantly higher levels of IgG1 (Table 1) in BALB/c mice. In contrast, there were higher levels of IgG1 against three antigens in C57BL/6 mice (Table 1). Therefore, we identified 10 unique antigens against which BALB/c mice developed a significantly more intense antibody response than C57BL/6 mice, raising the possibility that the protection we observed was mediated by high titers of one or more of these antibodies. These results were confirmed by ELISA for Hla, LukE, HlgC, and Eap/Map (Fig. 1). Interestingly, among the 10 antigens for which higher levels of IgG1 were observed in BALB/c serum, 4 are positively regulated by the *saeRS* operon in USA300 isolates (13) (Table 1).

SaeRS expression during primary SSTI was necessary to elicit protective immunity against secondary SSTI. The strong responses to *saeRS*-regulated antigens in BALB/c, but not C57BL/6, mice suggested that *saeRS*-regulated genes are important for eliciting protective immunity. To test this, a primary SSTI was performed with one of three strains of *S. aureus*: 923 (wild-type [WT] USA300), 923Δ*sae* (an isogenic *saeRS* deletion mutant), or 923Δ*sae*/psae (*saeRS* deletion mutant complemented with *saeRS* on a multicopy plasmid) (see Fig. S1 in the supplemental material). Consistent with previous findings (14), we found that infection with strain 923 resulted in dermonecrotic lesions that resolved within 2 weeks; however, no dermonecrosis was observed

after infection with strain 923Δ*sae* (Fig. 2A). Infection with strain 923Δ*sae*/psae resulted in lesions that were similar to those of mice infected with strain 923 (Fig. 2A).

Secondary SSTI with strain 923 was performed 8 weeks after the primary SSTI. As we previously reported (15), a primary SSTI with strain 923 elicited protective immunity against secondary SSTI (WT→WT) in BALB/c mice, with lesions after secondary infection being significantly smaller than those after primary infection (PBS→WT) (*P* < 0.01) (Fig. 2B and C). Remarkably, primary infection with strain 923Δ*sae* did not elicit protective immunity; there was no difference in the lesion size after a secondary SSTI with strain 923 (Δ*sae*→WT) compared with that seen after the primary infection (PBS→WT) (*P* = 0.3) (Fig. 2B and C). In contrast, primary SSTI with strain 923Δ*sae*/psae (923Δ*sae*/psae→WT) elicited protection similar to that elicited by strain 923 (*P* = 0.3 versus WT→WT; *P* < 0.01 versus Δ*sae*→WT) (Fig. 2B and C). We previously found that similar marked differences in lesion size were associated with a modest decrease in the number of bacteria recovered from the lesions (15); however, it should be noted that the difference in lesion size is out of proportion to the decrease in the bacterial burden. This finding has been reported by several groups (14, 22, 23) and may reflect a role for immunopathology in lesion size.

To confirm that infection with strain 923Δ*sae* was unable to elicit a protective antibody response, we generated cohorts of BALB/c mice that had primary and secondary SSTIs with strain 923 or 923Δ*sae*. Four weeks after the secondary SSTI, we adoptively transferred serum into naive BALB/c mice prior to a primary SSTI with strain 923. As previously reported (15), transfer of immune serum from mice infected with strain 923 was protective, in contrast to naive-mouse serum (*P* < 0.01) (Fig. 3A). In contrast, the skin lesions of recipients of immune serum from mice infected with strain 923Δ*sae* were only modestly smaller early after infection and not thereafter (Fig. 3A). Recipients of serum from strain 923-infected mice had skin lesions significantly smaller than those of recipients of serum from strain 923Δ*sae*-infected mice (*P* < 0.01) (Fig. 3A). These findings were also confirmed after adoptive transfer into C57BL/6 mice (Fig. 3B). Therefore, expression of

FIG 1 Confirmation of antigen-specific IgG subclass levels by ELISA. (A) There were higher levels of anti-Hla IgG, IgG1, and IgG3 antibodies after *S. aureus* SSTI in the serum of BALB/c mice than in that of C57BL/6 mice. The higher levels of anti-Hla IgG (B) and IgG1 (C) antibodies in BALB/c mice were confirmed by determining antibody titers. (D) There were higher levels of anti-HlgC IgG1 antibody but not other isotypes after *S. aureus* SSTI in the serum of BALB/c mice than in that of C57BL/6 mice. Antibody titers confirmed that there were no significant differences in anti-HlgC IgG antibody titers (E) but higher anti-HlgC IgG1 antibody titers (F) in BALB/c mice. There were no significant differences in the levels of anti-LukE (G to I) or anti-Eap (J to L) IgG isotypes after *S. aureus* SSTI in the serum of BALB/c versus C57BL/6 mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. There were four serum samples per group. Data are reported as the mean $\pm$ the standard error of the mean. Each experiment was performed twice. OD, optical density.

saeRS during a primary SSTI was necessary to elicit protective humoral immunity against a secondary SSTI.

Infection with strain 923 Δ *sae* elicits a nonprotective polyclonal antibody response. The antibody repertoires of mice in-

fectected with strain 923 or 923 Δ *sae* were compared to distinguish between two divergent explanations for the inability of strain 923 Δ *sae* to elicit protective antibody responses, i.e., that (i) strain 923 Δ *sae* infection is unable to elicit any *S. aureus* anti-

FIG 2 Role of *saeRS* expression during a primary *S. aureus* SSTI in eliciting protection against a secondary SSTI. (A) Infection of BALB/c mice with strain 923 (WT), but not strain 923 Δsae , resulted in dermonecrosis. Infection of mice with strain 923 $\Delta sae/psae$ resulted in lesions that were similar to those of recipients of strain 923. **, $P < 0.001$ versus the WT. (B) A secondary SSTI with strain 923 (WT→WT) resulted in significantly smaller skin lesions than a primary SSTI (PBS→WT). In contrast, mice that received strain 923 Δsae as the primary inoculum (Δsae →WT) were not protected against a secondary SSTI. Complementation of *saeRS* restored protective immunity to a secondary SSTI ($\Delta sae/psae$ →WT). **, $P < 0.01$ versus PBS→WT. (C) Representative lesions from each of the groups. Scale bars represent 1 cm. There were four or five mice per group. Data are reported as the mean $\pm$ the standard error of the mean. Each experiment was performed twice.

body response or (ii) infection with strain 923 Δsae fails to elicit antibodies specific for *saeRS*-regulated antigens but the antibody response to other *S. aureus* antigens is normal. In an initial screening, we quantified antibody levels against five antigens, four of which are *saeRS* regulated (Hla, LukE, HlgC, Eap/Map) and one of which is not (IsdB) (13). Infection with strain 923 Δsae elicited lower levels of anti-Hla IgG isotypes, compared with infection with strain 923 (Fig. 3C). In contrast, there were no significant differences in anti-IsdB IgG levels, regardless of isotype, between strain 923- and 923 Δsae -infected mice (Fig. 3D). Moreover, infection with strain 923 Δsae elicited lower levels of total IgG against the *saeRS*-regulated antigens Hla, LukE, HlgC, and Eap/Map, but not against IsdB, compared with infection with strain 923 (Fig. 3E). This suggested that infection with strain 923 Δsae was able to elicit an antibody response to *S. aureus* but not to *saeRS*-regulated antigens.

To more fully test this conclusion, we used the proteome-wide array to compare the serum IgG specificities of mice infected with strain 923 or 923 Δsae . Because the relative differences in antibody levels between BALB/c mice infected with strain 923 or 923 Δsae were similar for all of the IgG isotypes assessed, total reactive IgG was analyzed. There were 28 antigens identified as reactive with serum from naive mice, 248 with serum from mice infected with strain 923, and 229 with serum from mice infected with strain 923 Δsae . These data support the conclusion that infection with strain 923 Δsae elicits a broad polyclonal but nonprotective antibody response, as was the case for C57BL/6 mice infected with strain 923.

To further define the antibody specificities that are associated with protection, we compared the antibody levels of BALB/c mice

infected with strain 923 with those of mice infected with strain 923 Δsae . Using thresholds of a mean intensity of >500 (see Table S2 in the supplemental material) and a P value of <0.05 , we found 10 antigens for which antibody levels were significantly higher in serum from mice infected with strain 923 than in serum from mice infected with strain 923 Δsae (Table 2). Each of the 10 antigens is positively regulated by *saeRS*, and 4 were also associated with protection in BALB/c mice, in contrast to C57BL/6 mice. There were also two antigens (LukS-PV and LukE) for which antibody levels were higher in serum from strain 923 recipients than in that from strain 923 Δsae recipients the levels of which showed a strong trend toward being higher in BALB/c mice than in C57BL/6 mice ($P < 0.1$). We also found two antigens for which antibody levels were significantly higher in serum from mice infected with strain 923 Δsae than in that of mice infected with strain 923 (Table 2). Taken together, these findings provide strong support for the conclusion that *saeRS*-regulated genes are critical targets for protective antibody responses against *S. aureus* SSTI in BALB/c mice.

Role of *hla* expression in eliciting protective immunity. One possible explanation for the critical role of *saeRS* in eliciting protection is that it controls *hla* expression (13), and Hla-directed vaccine strategies have been demonstrated to protect against *S. aureus* SSTI in mice (24). To determine whether the absence of *hla* expression was the sole reason for the failure of strain 923 Δsae to elicit protective humoral immunity, BALB/c mice were infected with strain 923 Δhla , followed by secondary SSTI with strain 923. We found that primary SSTI with strain 923 Δhla resulted in modest protection against a secondary SSTI with strain 923; the lesions were smaller than those seen after a primary SSTI with strain 923

FIG 3 Role of *saeRS* expression in eliciting protective humoral immunity against recurrent *S. aureus* SSTI. (A) Adoptive transfer of serum from BALB/c mice infected with strain 923 (WT) protected naive mice against a primary SSTI. Transfer of serum from strain 923 Δ sae-infected mice resulted in modest protection of BALB/c mice early after infection, but this was inferior to the protection conferred by WT serum. (B) Adoptive transfer of serum from strain 923 Δ sae-infected BALB/c mice did not protect C57BL/6 mice against SSTI. *, $P < 0.05$ versus naive-mouse serum; **, $P < 0.01$ versus naive-mouse serum; ##, $P < 0.01$ versus strain 923 Δ sae-infected mouse serum. (C) There were no differences in the antibody levels against iron surface determinant B (IsdB) between mice infected with strains 923 and 923 Δ sae. (D) Infection with strain 923 elicited higher levels of IgG isotype antibodies against alpha-hemolysin (Hla) than infection with strain 923 Δ sae. (E) Infection with strain 923 elicited higher levels of total IgG against *saeRS*-regulated antigens (Hla, HlgC, LukE, and Eap), but not against a *saeRS*-independent antigen (IsdB), than did infection with strain 923 Δ sae. For ELISA results: *, $P < 0.05$; ***, $P < 0.001$; NS, not significant (all versus infection with strain 923 Δ sae). There were four or five mice per group. Data are reported as the mean $\pm$ the standard error of the mean. Each experiment was performed twice.

($P < 0.05$) (Fig. 4A). However, the lesions of mice that were primarily infected with strain 923 Δ hla were larger after a secondary SSTI than those of mice that were primarily infected with strain 923 ($P < 0.01$) (Fig. 4A). In a complementary approach, we performed a primary SSTI with strain 923 Δ sae/phla, which was obtained by complementing *hla* expression in strain 923 Δ sae (see

Fig. S1 in the supplemental material). Consistent with this hypothesis, mice that received strain 923 Δ sae/phla, followed by strain 923, had smaller lesions than those seen after primary infection or in mice that received strain 923 Δ sae, followed by strain 923 ($P < 0.05$), but larger lesions than mice that received strain 923, followed by strain 923 ($P < 0.05$) (Fig. 4B). Taken together, these

TABLE 2 Differences in antigen-specific IgG levels after infection of BALB/c mice with strain 923 (WT) or 923 Δ sae^a

Gene ID	Description	Higher in BALB/c than in C57BL/6?	<i>saeRS</i> regulated?	IgG			<i>P</i> value for WT vs 923Δ <i>sae</i>
				Naive	WT	Δ <i>sae</i>	
Higher in WT than in 923Δ <i>sae</i>							
SAUSA300_1757	Serine protease SplB	No	Yes	90	51,329	115	<0.001
SAUSA300_2364	IgG-binding protein Sbi	Yes	Yes	1,471	50,681	1,654	<0.001
SAUSA300_1917	Map/Eap protein, programmed frameshift	No	Yes	102	27,295	71	<0.001
SAUSA300_0693	Putative lipoprotein	No	Yes	104	23,521	93	<0.001
SAUSA300_1382	PVL, LukS-PV	No ^b	Yes	61	23,259	195	<0.05
SAUSA300_1769	Leukotoxin LukE	No ^b	Yes	77	16,678	138	<0.001
SAUSA300_0776	Thermonuclease precursor (Nuc)	No	Yes	125	6,325	132	<0.001
SAUSA300_2366	Gamma-hemolysin component C, HlgC	Yes	Yes	67	4,707	74	<0.05
SAUSA300_1058	Alpha-hemolysin precursor	Yes	Yes	62	2,816	81	<0.001
SAUSA300_2441-s1	Fibronectin-binding protein A (FnBA)	Yes	Yes	317	532	319	<0.05
Higher in 923Δ <i>sae</i> than in WT							
SAUSA300_0798	ABC transporter, substrate-binding protein	No	No	100	179	22,456	<0.001
SAUSA300_0995	Dihydrolipoamide acetyltransferase	ND ^c	No	82	1,302	16,702	<0.05

^a The naive group received sham inoculation with PBS. Data are presented as the mean normalized intensity (arbitrary units) of three biologic replicates. All comparisons are between mice infected with the WT and mice infected with strain 923 Δ sae.

^b This antigen had a *P* value between 0.05 and 0.1 in the BALB/c-versus-C57BL/6 comparison.

^c ND, not detected. The intensity was below the limit of detection in the BALB/c-versus-C57BL/6 study.

FIG 4 Role of *hla* expression in *saeRS*-dependent protective immunity against recurrent SSTI. (A) Primary SSTI with strain 923 Δ *hla* resulted in modest protection against secondary SSTI with strain 923 (WT), but primary SSTI with strain 923 (WT) elicited superior protection. *, $P < 0.05$ (PBS $\rightarrow$ WT versus Δ *hla* $\rightarrow$ WT); #, $P < 0.01$ (WT $\rightarrow$ WT versus Δ *hla* $\rightarrow$ WT). (B) Primary SSTI with strain 923 Δ *sae*/*phla* elicited modest protection against secondary SSTI with strain 923, but primary SSTI with strain 923 elicited superior protection. *, $P < 0.05$ (Δ *sae*/*phla* $\rightarrow$ WT versus PBS $\rightarrow$ WT and Δ *sae* $\rightarrow$ WT); #, $P < 0.05$ (WT $\rightarrow$ WT versus Δ *sae*/*phla* $\rightarrow$ WT). There were five mice per group. Data are reported as the mean $\pm$ the standard error of the mean. Each experiment was performed at least twice.

results suggest that *hla* expression during a primary SSTI was important for antibody responses to a secondary SSTI but that other factors were necessary for optimal protection.

DISCUSSION

The finding that *S. aureus* SSTI elicited a polyclonal antibody response in C57BL/6 mice that is not protective but a similarly broad antibody response in BALB/c that was protective has important implications in understanding protective immunity to recurrent *S. aureus* infections. Specifically, this suggests that the protective immune response is restricted to a small subset of *S. aureus* antigens and that antibodies to the majority of *S. aureus* antigens are not protective. These findings bode well for the design of an *S. aureus* vaccine and confirm that the immunogenicity of most *S. aureus* antigens is uncoupled from the protective properties of the elicited antibodies.

We found that infection with strain 923 Δ *sae* elicited a polyclonal antibody response that was largely similar to that elicited by infection with strain 923, demonstrating that the inability to elicit protective antibodies was due to the absence of a critical repertoire of antibodies specific for antigens regulated by *saeRS*. Importantly, higher levels of antibody recognizing the same four *saeRS*-regulated antigens (and four additional) were associated with protective immunity in both the BALB/c-versus-C57BL/6 mouse and strain 923-versus-strain 923 Δ *sae* studies. Five of these antigens are known to have defined interactions with the host immune system. Hla promotes epithelial damage by binding to ADAM10 (25), Panton-Valentine leukocidin (PVL) binds complement receptors C5a and C5L2 (26), Sbi binds immunoglobulin and interferes with complement activity (27), LukED binds CCR5 on T lymphocytes and CXCR1 and CXCR2 on leukocytes (10, 28), and FnBPA is important in platelet activation and adherence to and activation of host cells (29, 30). Therefore, our study clearly indicates that antibodies recognizing *saeRS*-regulated antigens are critical for protection, whereas strong IgG reactivity to 229 *S. aureus* antigens was insufficient for protection.

We also found that a protective immune response to *saeRS*-regulated antigens is elicited in the context of a specific mouse genetic background. One possible explanation is that the different adaptive immune phenotypes reflect differences between the innate immune responses of BALB/c and C57BL/6 mice. *saeRS*-regulated genes are expressed early in *S. aureus* exposed to neutrophils and in a mouse model of SSTI, consistent with an important

role for the operon in the evasion of innate immunity (31). However, the lack of significant differences in primary SSTI between BALB/c and C57BL/6 mice and the broad antibody responses elicited in both mouse strains suggest that innate immunity is not the explanation. Instead, there is a specific defect in humoral responses to *saeRS*-dependent antigens in C57BL/6 mice. While T and B cell receptor repertoires may explain these differences, we favor the hypothesis that major histocompatibility complex class II antigens (I-A^d and I-E^d) in BALB/c mice, but not in C57BL/6 mice (I-A^b), are able to present *saeRS*-regulated antigens to generate the subset of follicular T helper cells necessary to drive *saeRS*-specific IgG responses. While studies are ongoing to test this hypothesis, the observation that host genetics determine whether protective immune responses can be elicited has important implications for prospective vaccines in populations with nonprotective genotypes. In such situations, passive immunization may be necessary. Alternatively, vaccination strategies may have to be personalized to induce protective immune responses.

Taken together, our findings may reconcile speculation that the broad polyclonal antibody repertoire in patients is not associated with protective immunity with observations that high titers of antibodies to certain *S. aureus* antigens can be at least partially protective. The presence of antibodies to selected staphylococcal antigens is nearly ubiquitous in humans starting in childhood (32); however, an *S. aureus* proteome-wide antibody repertoire has not been defined in large populations. Furthermore, although antibody levels against selected *S. aureus* antigens increase with infection (33), a protective role for these acquired antibodies following *S. aureus* infection has not been established. In support of a role for selected *S. aureus* antibodies, the rate of adults with *S. aureus* bacteremia developing sepsis was inversely correlated with antibodies against Hla, PVL, delta-hemolysin, phenol-soluble modulins, and the enterotoxin SEC-1 (34). Higher levels of antibodies against Hla were also correlated with a lower rate of recurrent infection in children (35). Interestingly, not all antistaphylococcal antibody responses are protective. For example, antibodies against PVL were not associated with protection in children with SSTI (36). Anti-clumping factor A antibodies elicited by vaccination were functional, but not those elicited by natural infection (37), suggesting that antibody function may be uncoupled from immunogenicity. Therefore, it is likely that protective immunity in patients also requires a specific repertoire of functional anti-

bodies, and our studies point to the need for high titers of IgG recognizing *saeRS*-regulated antigens as being critical for protection.

Our results may also have important implications in the selection of antigens to include in a prospective staphylococcal vaccine. For example, we found no association between anti-IsdB antibody levels and protection. This negative finding is supported by the failure of the Merck V710 IsdB-based vaccine, despite high antibody levels among vaccine recipients (4). Among the *saeRS*-regulated antigens that were associated with protection in our mouse model, several are currently in clinical or preclinical vaccine trials. For example, active or passive vaccination with an Hla mutant protected mice against *S. aureus* pneumonia and SSTI (24, 38, 39). Our findings that deletion of *hla* partially, but not fully, abrogated protection and that complementation of strain 923Δ*sae* with *hla* partially restored protection further support an important role for anti-Hla antibodies in protective immunity. We cannot rule out a dose-dependent role for Hla, because *hla* expression in strain 923Δ*sae*/phla was slightly lower than that in strain 923 or 923Δ*sae*/psae. The protective role of anti-Hla antibodies in our studies contrast with the report by Sampedro et al. that Hla interfered with protective immunity to a secondary SSTI in a mouse model of recurrent infection (23). However, there are important differences between that model and ours, including the time interval between infections (3 versus 8 weeks) and the mouse genetic background (C57BL/6 versus BALB/c), both of which may impact whether Hla is protective or not.

The role of another *S. aureus* antigen, LukSF-PV, is controversial, in part because of the species specificity of host cell susceptibility to the toxin (9). Vaccination with LukSF-PV protected mice against bacteremia and against pneumonia and SSTI (40, 41). Other *saeRS*-regulated antigens identified in our study have also been reported as part of multicomponent vaccines in animal models, including HlgC, FnBP, and Eap (42–44). In contrast, the protective roles of Sbi, LukE, and Nuc in vaccines has not been reported. It should be noted that USA300 isolates do not express capsule (45, 46), limiting conclusions on the role of capsule in eliciting immune responses in this model.

We recognize some limitations to this study. First, we studied mice with SSTI, and future work will compare the immune responses elicited by and required to protect against different *S. aureus* infectious syndromes. Second, we chose to study protective immunity with a USA300 isolate because this is by far the most common cause of *S. aureus* SSTIs in the United States (47). Future studies will determine whether our findings can be extrapolated to other *S. aureus* genetic backgrounds. Third, we focused on the protective antibody response and did not assess T cell-dependent immunity in this work, even though our previous findings support a role for Th17/IL-17A responses working in concert with humoral immunity (15). Future investigations will explore quantitative and qualitative differences in T cell responses required to help B cell/antibody responses between mouse strains. Fourth, it is unclear if the findings from the mouse model will be applicable in the clinical setting, in which patients typically have high titers of antibodies against many *S. aureus* antigens. Therefore, future work will define the antigen-specific antibody repertoires of patients with *S. aureus* infections. Nevertheless, our study provides clear evidence that *saeRS*-dependent antigens elicit transferable humoral protection in BALB/c mice.

In summary, in a mouse model of *S. aureus* SSTI, we used a

proteomic approach to discriminate between protective and non-protective broad polyclonal antibody responses. We demonstrated that protective immunity was associated with high levels of IgG that recognize only 10 *S. aureus* antigens. The expression of many of these antigens was dependent on the global regulatory operon *saeRS*, and in the absence of *saeRS*, a nonprotective polyclonal antibody response was elicited but lacked antibodies recognizing 10 *saeRS*-regulated antigens. Finally, we confirmed an important role for *hla* expression in eliciting protection, but our findings suggest that responses to other *saeRS*-regulated antigens are also important. Taken together, these findings identify a key role for *saeRS* in eliciting protective humoral immunity and validate the mouse model as a tractable approach for defining protective immunity and for identifying antigens that could be incorporated into an *S. aureus* vaccine.

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