Human mAbs to *Staphylococcus aureus* IsdA Provide Protection Through Both Heme-Blocking and Fc-Mediated Mechanisms

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The nutrient metal iron plays a key role in the survival of microorganisms. The iron-regulated surface determinant (Isd) system scavenges heme-iron from the human host, enabling acquisition of iron in iron-deplete conditions in *Staphylococcus aureus* during infection. The cell surface receptors IsdB and IsdH bind hemoproteins and transfer heme to IsdA, the final surface protein before heme-iron is transported through the peptidoglycan. To define the human B-cell response to IsdA, we isolated human monoclonal antibodies (mAbs) specific to the surface Isd proteins and determined their mechanism of action. We describe the first isolation of fully human IsdA and IsdH mAbs, as well as cross-reactive Isd mAbs. Two of the identified IsdA mAbs worked in a murine septic model of infection to reduce bacterial burden during staphylococcal infection. Their protection was a result of both heme-blocking and Fc-mediated effector functions, underscoring the importance of targeting *S. aureus* using diverse mechanisms.

Keywords. antibodies; *Staphylococcus aureus*; iron-regulated surface determinant system; NEAr iron transporter; IsdA; human.

Microorganisms require acquisition of nutrient metals to fulfill basic metabolic needs. In response, vertebrates produce metal-binding proteins that sequester nutrients, preventing their accessibility to pathogens, a process termed nutritional immunity [1]. To overcome this barrier, bacterial pathogens have evolved mechanisms to acquire necessary nutrient metals from their hosts. One such metal is iron, which plays a key role in cellular metabolism, respiration, and catalysis [1, 2]. Heme is both the most abundant and preferred source of iron for *Staphylococcus aureus* within the human host [3]. In order to obtain iron, staphylococcal toxins lyse host red blood cells, releasing hemoglobin, allowing the bacterial cell to import heme using the iron-regulated surface determinant system (Isd) system [4]. The Isd system is classified by the presence of a conserved NEAr iron transporter (NEAT) domain that is important for the capture of heme and hemoglobin [5, 6]. The surface receptor protein of the Isd system, IsdB, binds free hemoglobin, and another receptor, IsdH, binds haptoglobin hemoglobin [7]. Once bound, these proteins remove the heme cofactor and transport free heme to a third surface-exposed protein, IsdA, before heme is transferred to IsdC, which is anchored within

the peptidoglycan [8–11]. IsdC transfers heme to IsdDEF, a membrane-transporter that transports heme into the cytoplasm for degradation [12, 13]. These hemoproteins are necessary for the growth and virulence of *S. aureus* because of their specialized ability to capture and transport heme-iron [8, 14, 15].

Targeting bacterial mechanisms for heme acquisition has been a subject of intense study in the *S. aureus* field in an effort to identify novel candidate therapeutics [8, 15–18]. One way to target heme acquisition is through antibodies that bind to Isd proteins, as antibody-mediated therapy could be a promising alternative to traditional antibiotics. Therapies using monoclonal antibodies (mAbs) are both versatile and specific due to inherent molecular functions of antibodies that aid in the clearance of microbes, such as opsonization and antibody-dependent cellular cytotoxicity (ADCC) [19]. *S. aureus* expresses many surface antigens, that contribute to virulence and may be potential candidates for antibody-mediated therapies like passive immunization. Anti-*S. aureus* antibodies also may be useful for informing vaccine design by elucidating new epitopes and highlighting the humoral immune response to staphylococcal surface antibodies.

Much attention has been paid to the role of IsdB in *S. aureus* infection, as *isdB* mutants exhibit reduced virulence in murine models and have a decreased ability to utilize hemoglobin as a sole iron source [17, 20]. While previous studies reported isolation of murine or human mAbs to IsdB that led to the production of an IsdB vaccine candidate (Merck V710, based on recognition of CS-D7) [21–23], this vaccine ultimately proved to be ineffective in human trials [24, 25]. In contrast, little is

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known about the role of antibodies to the related *S. aureus* IsdA protein in protective immunity. However, increased IsdA antibody levels have been identified in patients infected with methicillin-resistant *S. aureus* (MRSA), correlating with similar findings in the serum antibody response to IsdB [26–29]. IsdA contains a NEAT domain that is responsible for heme binding, but the role of IsdA in nutrient acquisition and pathogenesis is less well defined. Furthermore, human mAbs to IsdA have not been described, particularly in response to invasive *S. aureus* disease. We describe here the isolation and characterization of the first panel of IsdA-specific human mAbs, in addition to novel IsdA/B and IsdA/B/H cross-reactive antibodies. We also show that IsdA-specific antibodies can reduce bacterial replication in a murine model of *S. aureus* septicemia. We found that this protection was the result of 2 distinct mechanisms: heme-iron blockage and Fc-mediated function.

MATERIALS AND METHODS

Human Subjects

Blood was collected at Vanderbilt University Medical Center (VUMC) from subjects with prior documented blood culture-confirmed history of invasive MRSA or methicillin-sensitive *S. aureus* infection. Heparinized peripheral blood was collected after informed parent consent and subject assent. The studies were approved by the Institutional Review Board of VUMC.

Generation of Human Monoclonal Antibodies

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation. Human hybridomas were generated by transforming PBMCs with Epstein-Barr virus (EBV). Cells were plated in 384-well plates and grown for 7 days before expanding to 96-well plates containing irradiated heterologous human PBMCs. Cells were screened via enzyme-linked immunosorbent assay (ELISA) using *S. aureus* surface antigens to identify wells containing B cells secreting antigen-specific antibodies. Reactant cell culture wells were fused with HMM2.5 myeloma cells via electrofusion. After fusion, hybridoma cell lines were sorted using single-cell fluorescence-activated cell sorting to achieve monoclonal hybridoma lines.

Bacterial Strains

The *S. aureus* strain Newman was grown at 37°C for 12–18 hours on tryptic soy agar (TSA) and in tryptic soy broth (TSB). Isogenic mutants were made by allelic recombination [4, 8, 30, 31] (Supplementary Methods).

Antigen Generation

The DNA sequence encoding IsdA, IsdB, and IsdH excluding the sorting signal and signal peptide was cloned into the pET15b plasmid vector and expressed using the BL21 (DE3) *Escherichia coli* expression system. Cultures were grown for

36 hours in Luria-Bertani broth and induced after 6 hours at 30°C with 1 mM isopropyl β -D-1-thiogalactopyranoside. Cells were harvested by centrifugation and resuspended in 50 mM Na₂HPO₄ plus 500 mM NaCl before disruption by sonication. The soluble fraction was purified using a HiTrap TALON column. Purified antigen was used at a concentration of 2 μ g/mL when screening PBMCs and supernatants for reactivity.

Hemoglobin Growth Curves

S. aureus strain Newman was grown overnight in RPMI medium plus 1 μ M ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid) (EDDHA). The optical density (OD)₆₀₀ was normalized to 1 and subcultured into RPMI plus EDDHA with 20 nM of human hemoglobin and 2 μ g/mL antibody. Tubes were grown at 37°C and OD₆₀₀ values were recorded at 12, 24, and 36 hours using pathlength correction in the Gen5 microtiter plate software (Bio-Tek). Human hemoglobin was purified from hemolysate by high performance liquid chromatography, as previously described [20].

Biolayer Interferometry Competition-Binding Assay

Competition-binding studies using biotinylated IsdA, IsdB, or IsdH (5 μ g/mL) was performed on an Octet RED biosensor (Pall FortéBio). The antigen was immobilized onto streptavidin-coated biosensor tips. Biosensor tips were first washed then immersed into wells containing a first antibody (100 μ g/mL) and then into the wells containing a second mAb (100 μ g/mL). Competition groups were determined by calculating the percent binding of the second mAb in the presence of the first mAb, compared to the maximal signal of the second mAb alone (the value of uncompleted binding was normalized to 100%).

Mouse Experiments

A mouse septic model of *S. aureus* infection was used. Overnight cultures of *S. aureus* strain Newman were subcultured 1:100 and grown to midexponential phase before collection by centrifugation and a wash with phosphate-buffered saline (PBS), with resuspension to a final concentration of 10⁷ colony forming units (CFU)/100 μ L for injection. Mice were weighed and injected with 10 mg/kg antibody via the intraperitoneal route. Seven-week-old female BALB/cJ (n = 20 for all experimental groups except STAU-239, n = 15) were anesthetized prior to retro-orbital injection with *S. aureus*. After 96 hours, the mice were euthanized using CO₂ inhalation. Organs were homogenized in PBS and serially diluted before plating on TSA for colony enumeration. Mouse experiments were approved and performed according to the guidelines of the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Hydrogen-Deuterium Exchange Mass Spectrometry

IsdA and Fabs were prepared at 10 pmol/ μ L. Labeling occurred in PBS pH 7.4 in D₂O at 20°C for 30 seconds or 4 hours. The reaction was quenched in PBS, 4 M guanidinium/HCl, 100 mM

tris (2-carboxyethyl) phosphine to a pH of 2.3, 0°C. Samples were injected into a nano-ACQUITY UPLC system with HDX technology. Online digestion was performed at 15°C and a flow of 134 µL/min of 0.1% formic acid using an immobilized-pepsin column and simultaneously trapped at 0°C on a VanGuard BEH C18 1.7 µm column. Peptides were eluted using 5%–35% acetonitrile, 0.1% formic acid in H₂O, and separated on a ACQUITY UPLC BEH C18 1.7 µm, 1 mm × 100 mm column and analyzed using Xevo G2-XS in MS^E-mode. Peptide identification was performed using Waters ProteinLynx Global Server 3.0.3 software and deuterium uptake was calculated using DynamX 3.0 software. Results were averaged across replicate analyses, at a given time point and the standard deviation determined.

Mapping *isdA* Genetic Variation Using *S. aureus* Genomes

A BLAST+ [32] database was created from the predicted protein sequences of 42 949 public *S. aureus* sequencing projects [33]. The IsdA protein sequence from the *S. aureus* strain Newman

(NCBI Reference Sequence NZ_CP023391) was queried against the BLAST + database (v2.7.1, blastp -db \$BLASTDB -query \$ISDA -outfmt 15). Proteins that had at least 90% sequence coverage and 80% identity to the IsdA protein were extracted and clustered at 100% identity using CD-HIT (v4.6, cdhit -i \$PROTEINS -o \$OUTPUT -c 1.0) [34, 35]. Each cluster sequence was compared against the Newman IsdA protein and changes were extracted from the BLAST alignment.

Statistical Analysis

All data were analyzed in Prism v 7.0 (GraphPad Software Inc.). Individual statistical analysis is described in the legend of each figure.

RESULTS

Isolation of Isd-Reactive Human mAbs

To define the human B-cell response to staphylococcal surface Isd proteins, we isolated a panel of human mAbs reactive with

Table 1. Characterization of Isd-Reactive Antibodies

Isd Binding Pattern of Antibody ^a	STAU Antibody	Subject No.	IgG Subclass	Light Chain	EC ₅₀ ^d (µg/mL) of Indicated mAb		
					IsdA	IsdB	IsdH
A/B/H	307	1	1	λ	0.01	0.00028	0.00001
	399	1	2	λ	8.7	0.53	0.95
	37	2	1	κ	3.8	16.1	17.5
A/H	147	1	2	λ	0.01	>	0.8
	407	1	1	λ	1.6		3.1
B/H	281	3	1	κ	> ^c	0.002	0.0027
	328	1	1	λ		0.08	0.06
	389	1	ND ^b	λ		0.13	0.34
A/B	239	1	1	λ	0.07	0.001	>
	229	1	3	λ	0.7	0.01	
	221	1	1	λ	0.03	1.9	
	218	1	1	κ	0.08	4.1	
	201	1	1	λ	0.26	9.5	
	299	1	ND	λ	0.22	12.7	
	402	1	1	λ	8.2	3.4	
	138	4	1	ND	5.9	9.5	
	401	1	1	λ	25.1	3.7	
	280	1	1	κ	52.1	7.6	
H	139	4	1	λ	>	>	2.7
B	75	5	1	κ	>	0.02	>
	64	5	1	κ		0.14	
	141	4	1	κ		10.2	
A	158	1	1	κ	0.01	>	
	231	1	1	λ	0.9		
	181	1	ND	κ	0.7		
	245	1	1	κ	0.2		
	22	6	1	κ	0.3		
	228	1	1	κ	3.2		
	149	1	1	κ	0.2		

^aIndicates the binding pattern of each antibody based on IsdA, IsdB, or IsdH protein binding.

^bND indicates the experiment was repeated but the subclass or light chain could not be determined.

^c>symbol indicates binding was not detected at the maximal concentration tested of 20 µg/mL.

^dEC₅₀ denotes the half-maximal binding concentration of antibody to the antigen.

IsdA, IsdB, or IsdH recombinant proteins. Antibodies were isolated from the circulating memory B cells of human subjects with varying severity of *S. aureus* invasive disease (Supplementary Table 1). Peripheral blood was drawn from these individuals 4–6 weeks following infection. PBMCs were isolated from the donor blood samples and immortalized by EBV before fusing with myeloma cells and single-cell flow cytometric sorting to isolate mAb-secreting hybridomas.

Antibodies were classified based on their binding pattern to IsdA, IsdB, or IsdH. We observed all possible cross-reactive binding patterns for clonal IgGs (Table 1). Antibodies specific to individual Isd proteins were identified; however, the majority of the mAbs bound to more than 1 Isd protein in ELISA. Three antibodies (STAU-307, -399, and -37) bound to all 3 Isd proteins. An ELISA was used to determine the immunoglobulin subclass of each mAb, with the majority of the panel being IgG1 (Table 1). The EC₅₀ values are the half-maximal binding concentrations of the antibodies to their antigen. Each antibody was tested for binding to IsdA, IsdB, or IsdH and any antibody that did not bind at the starting concentration of 20 µg/mL was classified as a nonbinder for that antigen. The cross-reactive nature of the identified IgGs was consistent with

the conservation of heme- and hemoglobin-binding functions in these 3 *S. aureus* proteins.

Competition-Binding Studies

We performed competition-binding studies using biolayer interferometry (BLI) to identify the major antigenic sites targeted by each mAb. Biotinylated recombinant IsdA or IsdB suspensions were used to coat streptavidin biosensor tips before association with a first and then a second mAb (Figure 1). Although all isolated antibodies were tested by BLI, not all antibodies bound to the biosensors with a high enough affinity to allow competition analysis. A recombinant form of the previously identified mAb CS-D7 [22, 36] was included in the IsdB competition-binding study for comparative purposes. Three competition-binding groups were identified for both IsdA and IsdB. Multiple cross-reactive antibodies bound to both IsdA and IsdB by ELISA, but in some cases these mAbs did not bind to both Isd antigens in the BLI assay. Two antibodies, STAU-239 and STAU-245, were members of a single competition-binding group to IsdA (Figure 1 outlined in blue), suggesting that they bound to a common major antigenic site that may be important for Isd function.

Figure 1. Competition-binding of IsdA- or IsdB-specific antibodies. Biolayer interferometry was used to perform competition-binding studies on the Isd-specific antibody panel. Either IsdA (left panel) or IsdB (right panel) was biotinylated and loaded onto streptavidin tips before testing of binding to a first and then second antibody. The percent binding of the second antibody to the biosensor tip in the presence of bound first antibody. Black boxes indicate the first antibody blocked binding of the second antibody by more than 70%. Grey boxes indicate the first antibody partially blocked binding of the second antibody, with 31%–75% residual binding of the second remaining. White boxes indicate the first antibody did not block binding of the second antibody to the biosensor tip. Assigned competition-binding groups are outlined in color. Certain cross-competing antibodies did not bind well to both IsdA and IsdB using biolayer interferometry, suggesting that they may be members of an additional competition-binding group that could not be tested in this way. Individual antibodies were competed in at least 2 independent experiments.

Human mAb Prophylaxis Against *S. aureus* in a Septic Model of Murine Infection

To determine the in vivo efficacy of the antibody panel, mAbs from each competition-binding group were tested in a septic mouse model of *S. aureus* infection. For initial testing, mAbs were combined at a 1:1 mixture and injected into mice via the intraperitoneal route before retro-orbital inoculation with *S. aureus*. An IsdA/IsdB and IsdA-specific mAb, STAU-239 + STAU-245, combined to reduce the bacterial burden by approximately 200-fold in the kidneys and liver compared to the isotype control and 135-fold in the heart (Figure 2A). To determine if either STAU-239 or STAU-245 alone was sufficient for this effect, each antibody was tested individually. STAU-239 did not reduce bacterial burden significantly in any organ tested, whereas STAU-245 reduced bacterial burden in the liver and kidneys alone. However, the combination of both antibodies led to a statistically significant reduction in every organ tested. The 12-fold reduction in bacterial burden in the kidneys caused by STAU-245 is also consistent with previously published data showing that a Δ isdA mutant *S. aureus* strain leads to a 10-fold reduction in bacterial burden [14]. This finding indicated that STAU-245 possesses inhibitory function and suggests that the addition of STAU-239 results in an additive effect not solely dependent on blocking IsdA. The weight loss for each mouse was monitored over the 96-hour infection, and percent weight loss is graphed. The STAU-239 + STAU-245 mouse group lost the least amount of weight, followed next by the STAU-245 treatment group (Figure 2B). These antibodies were tested further in a septic model of infection using a MRSA strain of *S. aureus*, USA300 (Supplementary Figure 1). These data were consistent with the infection data with strain Newman, as STAU-239 + STAU-245 reduced bacterial burden in the heart, kidneys, and livers of infected mice.

Inhibition of *S. aureus* Hemoglobin-Dependent Growth

To test the hypothesis that the efficacy of STAU-239 + STAU-245 in vivo was the result of inhibition of hemoglobin-dependent iron-acquisition, antibody-mediated blocking of hemoglobin as the sole iron source was tested in vitro with human hemoglobin (Figure 3). *S. aureus* was incubated overnight in iron-chelated conditions. The bacterial suspension was normalized for density by OD₆₀₀ and subcultured into fresh tubes to grow for 34 hours with the addition of: (1) no hemoglobin, (2) hemoglobin, (3) hemoglobin plus STAU-239, (4) hemoglobin plus STAU-245, or (5) hemoglobin plus the STAU-239 + STAU-245 combination. Hemoglobin was used at a concentration of 20 nM and antibody at a concentration of 2 µg/mL (molar ratio of Ab to Hb was 665 to 1).

Untreated samples without hemoglobin did not grow, due to a lack of available hemoglobin for use as an iron source [17]. When hemoglobin was added, samples grew to an OD₆₀₀

Figure 2. Isd-specific antibodies reduce bacterial burden in a systemic murine model of infection. **A**, Seven-week-old female BALB/c mice were inoculated retro-orbitally with an OD of 0.4 suspension (approximately 10^7 colony forming units [CFU]) *S. aureus* strain Newman. After 96 hours, the heart, livers, and kidneys of the infected mice were harvested in cold phosphate-buffered saline. CFU counts were determined by serial dilution plating on TSA. *P* values were determined by a Kruskal-Wallis nonparametric test and the Dunn Procedure for multiple comparisons. Data are expressed as the geometric mean $\pm$ geometric standard deviation. **B**, Weight loss over the course of 96 hours was calculated as a percentage of the starting weight. *P* values were determined by ANOVA. Experiments were performed 3 independent times and the pooled data are shown.

Figure 3. Partial inhibition of *Staphylococcus aureus* hemoglobin-dependent growth. *S. aureus* strain Newman was cultured overnight in RPMI with 1 μ M ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid) and normalized to OD₆₀₀ 1 before being subcultured and grown in broth for 34 hours. Statistically significant differences were determined by a Mann–Whitney test, and *P* values are shown where the hemoglobin-treated group was set as the comparator. Data shown are representative of 3 independent experiments.

greater than 1.0 as hemoglobin-dependent growth requires the Isd system [8, 37]. All antibody-treated groups led to a partial inhibition of *S. aureus* growth in comparison with hemoglobin treatment. The inability of the antibodies to completely reduce *S. aureus* growth in the presence of hemoglobin (similar to levels shown with no Hb and no mAb) suggested that hemoglobin blocking might not be the only mechanism by which the antibodies inhibited bacterial growth.

STAU-239 + STAU-245 Binding Site and Conservation on IsdA

Hydrogen-deuterium exchange mass spectrometry (HDX-MS) was performed to identify the binding sites of STAU-239 and STAU-245. HDX-MS identifies amino acids involved in protein-protein interactions by comparing the relative deuterium uptake in 2 different states. HDX-MS breaks the antigen into overlapping peptides and identifies peptides with decreased deuterium where the antibody binds the antigen. The Fab fragments of STAU-239 and STAU-245 were tested individually for binding to IsdA and both binding sites were found within the NEAT domain of IsdA (Figure 4A and Supplementary Figure 2A). The crystallized structure of the NEAT domain binding to heme was modeled with the NEAT domain (green) [5], heme (red), and binding site for STAU-239 (orange) and STAU-245 (blue) shown (Figure 4B). STAU-239 bound near the heme-binding pocket of IsdA-N1, to peptide residues H158–V161 and P162–Y166. STAU-245, however, bound to peptides (N120–L126, A127–T128) on the far region of IsdA-N1, away from the heme-binding site as modeled on the crystal structure in blue (Figure 4B and Supplementary Figure 2B and 2C).

Because STAU-239 binds both IsdA and IsdB (Table 1), we investigated genetic variation within the binding site of STAU-239. The conservation of each amino acid was determined by comparing the *S. aureus* strain Newman IsdA protein sequence against predicted protein sequences from 42 949 publicly

available *S. aureus* genomes sampled from over 2500 multilocus sequence types (STs) [33]. Genomes without a predicted full length IsdA protein owing to gaps in de novo assembly were excluded. The IsdA protein was extracted from the remaining 42 522 genomes and clustered based on 100% identity into 556 unique protein sequences. The most common sequence had 16 416 strains (including Newman) and the second most common had 7 420 strains. There were 29 strains (0.06%) that had predicted amino acid substitutions compared to the canonical Newman sequence in positions 159 and 167 (Figure 4C). At amino acid position 163, 18 ST22 strains shared a glutamine to arginine mutation (Q163R). Q163 is also a surface residue and the position farthest from heme in the binding site, which may explain the variability at this position (Supplementary Figure 3). The remaining mutations were shared in ≤ 4 strains. Five of the amino acid sites were invariant in the 42 522 strains, underscoring the conservation of this epitope across *S. aureus*.

Prophylaxis Against *S. aureus* in a Septic Model of Murine Infection With Antibodies Containing Fc Mutations

The Fc region of an IgG antibody binds to Fc receptors and mediates secondary functions, including phagocytosis and ADCC [19]. To test the contribution of Fc-mediated effector functions in vivo, variant STAU-239 + STAU-245 antibodies were made where the leucine residues at positions 234 and 235 of the CH2 domain were mutated to alanine (L234A/L235A or LALA). These substitutions prevent antibody binding to Fc γ RI, Fc γ RII, or Fc γ RIIIa [38–40]. The recombinant mAbs were tested by BLI to ensure the antibodies still bound to IsdA but lost binding to recombinant Fc γ R (Supplementary Figure 4). STAU-239 + STAU-245 LALA were tested next in the septic murine model of infection in comparison with STAU-239 + STAU-245 IgG (Figure 5). We did not detect a significant difference in bacterial burden between STAU-239 + STAU-245 LALA and the isotype control in the heart, liver,

Figure 4. Binding sites and conservation of STAU-239 and STAU-245 on iron-regulated surface determinant A-N1 (IsdA-N1). Hydrogen-deuterium exchange mass spectrometry (HDX-MS) was used to map the binding sites of STAU-239 and STAU-245 onto IsdA. *A*, The sequence of IsdA-N1 is shown and amino acids with the strongest decrease in deuterium uptake in the presence of STAU-239 (blue) or STAU-245 (orange) and IsdA are colored. *B*, The structure of the crystallized NEAT iron transporter (NEAT) domain of IsdA (Research Collaboratory for Structural Bioinformatics Protein Data Bank Identification [PDB ID]: 2ITF) is shown binding heme (red) with the predicted binding sites of STAU-239 (blue) and STAU-245 (orange). *C*, Amino acid changes in the *S. aureus* strain Newman IsdA protein were determined from 42 522 *S. aureus* genomes. Frequency distribution of all amino acid changes across the 350 residues of IsdA in 42 522 strains is shown (top). Amino acid changes observed specifically in the STAU-239 binding site (amino acid positions 159 and 167) are highlighted (bottom).

Figure 5. Monoclonal antibody (mAb) STAU-239 + STAU-245 Fc mutants do not reduce bacterial burden in systemic murine model of infection. **A**, Seven-week-old female BALB/c mice were inoculated retro-orbitally with OD₆₀₀ 0.4 suspension of *Staphylococcus aureus* strain Newman. Mice were given 1 of 2 mixes: (STAU-239 + STAU-245 IgG) or (STAU-239 + STAU-245 LALA [L234A, L235A] variant) by the intraperitoneal route 2 hours before infection. After 96 hours, the heart, livers, and kidneys of the infected mice were harvested in cold phosphate-buffered saline. Colony forming units (CFU) were determined by serial dilution plating on TSA. *P* values were determined by a Kruskal-Wallis nonparametric test and the Dunn Procedure for multiple comparisons. Data are expressed as the geometric mean $\pm$ geometric standard deviation. **B**, Weight loss over the 96-hour infection was calculated as a percentage of the starting weight. *P* values were determined by ANOVA. Experiments were performed 2 independent times and the pooled data are shown.

and kidneys. This finding indicates that these antibodies employ Fc-mediated functions to reduce bacterial burden in the systemic *S. aureus* model. These variant antibodies also were tested further in a septic model of infection using the MRSA strain of *S. aureus*, USA300 (Supplementary Figure 5). No significant difference in bacterial burden was detected between the LALA variant antibodies and the isotype control group.

DISCUSSION

S. aureus continues to grow as a public health threat, with no licensed vaccine for prevention. Diverse bacterial components have been proposed and studied as candidate *S. aureus* vaccines, including toxins, adherence factors, lipoproteins, and surface proteins [12, 27, 41, 42]. Inhibiting the capacity of bacterial surface proteins involved in the nutrient acquisition of iron is a decisive way to interrupt the pathogenesis of *S. aureus*. Indeed, previous studies have shown that passive transfer of anti-Isd antibodies or active immunization with IsdB, IsdA, or IsdH proteins alone or as a cocktail can reduce bacterial burden in multiple infection models including mice, rats, and macaques [15, 16, 36, 43–45].

Multiple studies following serum antibody response to *S. aureus* infection have identified a specific Isd protein of emerging importance, IsdA, as being an immunogenic protein in bacteremic patients [28, 29] or in young children with *S. aureus* colonization of the nares [46]. IsdA also has been identified as important for biofilm formation in epidermal models and on polystyrene surfaces [47]. IsdA-reactive antisera react with and opsonize both *S. aureus* and *S. agalactiae*, suggesting that IsdA is a conserved antigen across genera [48]. Previous antibody-based therapeutic strategies targeting IsdA and IsdB resulted in the identification of antibodies that mediate protection primarily through inhibition of heme-iron scavenging [49]. Herein we describe the identification of human antibodies that are capable of not only blocking heme-iron scavenging but also enhancing Fc-functions. These findings underscore the diverse functions of antibodies targeting *S. aureus* and reveal that IsdA could be a plausible component of a preventative vaccine strategy, not only due to its ability to interfere with heme-iron acquisition, but also by stimulating Fc-mediated protective effects.

In this study, we searched for naturally occurring human Isd-reactive antibodies after infection and identified the first panel of IsdA- and IsdH-specific human mAbs. This panel included antibodies that bound to all 3 Isd surface proteins, highlighting the human capacity to mount a broadly reactive humoral response to iron-binding proteins. Two of the identified IsdA mAbs showed a protective effect in vivo, which appeared to be due to 2 distinct mechanisms of action. STAU-239 partially inhibited the in vitro hemoglobin-dependent growth of *S. aureus* and bound to the heme-blocking site of IsdA; however, this only partially explained the in vivo phenotype observed. IgG variants of STAU-239 + STAU-245 containing the L234A, L235A variant mutations that functionally abrogate binding to Fc receptors lacked

the ability to protect mice from *S. aureus* infection. This finding suggests that antibody effector functions, especially Fc-mediated functions, play a critical role in the action of antibodies that reduce *S. aureus* infection. The combination of iron-blocking activity and Fc-mediated function combines to inhibit *S. aureus* through multiple mechanisms. This proof of principle suggests that blocking *S. aureus* pathogenesis via different pathways may be an effective approach to inhibit disease caused by this multifaceted pathogen.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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