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Mucosal Immunization with a *Staphylococcus aureus* IsdA-Cholera Toxin A_2/B Chimera Induces Antigen-Specific Th2-Type Responses in Mice

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Abstract

Staphylococcus aureus is a leading cause of opportunistic infection worldwide and a significant public health threat. The iron-regulated surface determinant A (IsdA) adhesin is essential for *S. aureus* colonization on human nasal epithelial cells and plays an important role in iron acquisition and resistance to human skin defenses. Here we investigated the murine immune response to intranasal administration of a cholera toxin (CT) A_2/B chimera containing IsdA. Plasmids were constructed to express the IsdA-CTA_2/B chimera and control proteins in *E. coli*. Proper construction of the chimera was verified by SDS-PAGE, western blot, GM1 ELISA, and confocal microscopy. Groups of female BALB/c mice were immunized with IsdA-CTA_2/B, IsdA mixed with CTA_2/B, IsdA alone, or mock, followed by one booster immunization 10 days post-priming. Analysis of serum IgG and nasal, intestinal, and vaginal IgA suggested that mucosal immunization with IsdA-CTA_2/B induces significant IsdA-specific humoral immunity. Functional *in vitro* assays revealed that α-IsdA immune serum significantly blocks the adherence of *S. aureus* to human epithelial cells. Splenocytes from mice immunized with IsdA-CTA_2/B showed specific cellular proliferation and production of IL-4 after *in vitro* stimulation. Immunization with IsdA-CTA_2/B drove isotype switching to IgG1, indicative of a Th2-type response. Our results suggest that the immunogenicity of the *S. aureus* IsdA-CTA_2/B chimera merits further investigation as a potential mucosal vaccine candidate.

Keywords: cholera toxin, *Staphylococcus aureus*, vaccine, mucosal adjuvant

Introduction

Staphylococcus aureus causes nosocomial and community-acquired infections, including: impetigo, cellulitis, food poisoning, and toxic shock syndrome, as well as invasive necrotizing pneumonia and endocarditis. Infection with this opportunistic pathogen causes an estimated 477,927 hospitalizations per year in the United States and, of these, more than 58% are from methicillin-resistant *S. aureus* (MRSA) (32). Invasive MRSA infections, which were previously considered a threat only in healthcare facilities, are now the known cause of life-threatening community-acquired multidrug resistant infection (13). While more recently, education and awareness has promoted decreases in infection rates in the U.S., vaccination continues to be of great interest to prevent *S. aureus* disease and support limited therapeutic options (29).

The anterior nares of humans are an important niche for *S. aureus*, and nasal carriage is the major risk factor for invasive infection (33, 61). One approach for staphylococcal vaccine development is to target the adhesins of *S. aureus* that mediate colonization. One such adhesin, iron-regulated surface determinant A (IsdA), binds to human desquamated nasal epithelial cells *in vitro* and is required for nasal colonization in cotton rats (7, 8). The N-terminal near iron transporters (NEAT) domain of IsdA binds to a broad spectrum of human ligands, including: transferrin, heme, fibrinogen, fibronectin, and corneocyte envelope proteins, to mediate adherence and dissemination of *S. aureus* (7, 19, 54, 60). In iron-limited human niches, IsdA functions with other Isd proteins to free iron from host heme for metabolism (37, 41). The C-terminal domain of IsdA defends *S. aureus* against human skin bactericidal fatty acids and antimicrobial peptides by making the cell surface hydrophilic (9). IsdA is known to be immunogenic in humans because antibodies against IsdA can be found in healthy individuals, more so in non-carriers than in commensal carriers, and patients with *S. aureus* disease (8, 59). Intramuscular injection with IsdA protected mice in an invasive model, and passive immunization with IsdA protected mice against abscess formation and intravenous challenge (31, 50). These reports support the premise that disruption of IsdA-mediated adhesion and iron uptake is a promising target for the development of a mucosal vaccine against *S. aureus.*

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A vaccine that prevents human staphylococcal disease is currently not available, and poorer than expected results from several vaccine candidates tested in pre-clinical and clinical trials indicate that vaccine development against *S. aureus* will be complex. The successful candidate will likely require a combination of antigens and the use of novel adjuvants. Priming of antigen-specific IgA-B lymphocytes in the mucosa-associated lymphoid tissue by means of mucosal immunization may reduce or prevent *S. aureus* adherence to nasal mucosa and eliminate carriage. The bacterial enterotoxins, including *Vibrio cholerae* cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LTI) have long been recognized as potent immunostimulatory molecules that can bind to and target immune effector cells at mucosal sites. Although not completely understood, CT immunomodulation is believed to involve the activation of antigen presenting cells, promotion of B-cell isotype switching, and upregulation of co-stimulatory and MHC class II expression (10, 21, 26, 44). Many of these responses result from the interaction of the CTB subunit with the ganglioside GM1 receptor on effector cells, such as dendritic cells, that promote antigen uptake, presentation, and cellular activation (16). A number of studies have reported that non-toxic CTB by itself can act as an antigen carrier and is highly immunostimulatory (17, 27, 47). The ability of non-toxic CTB to effectively block oral tolerance in the absence of enzymatic activity from CTA remains controversial however, and toxigenic CT is clearly a potent adjuvant even in the absence of CTB (1, 4, 38). Toxigenic CT however is unsuitable for use in humans and thus there has been much effort to separate the toxigenicity and adjuvanticity of this molecule. Stable holotoxin-like CTA_2/B chimeras where the toxic A_1 domain is replaced with an antigen of interest possess a number of advantages for use as mucosal vaccines, including: the absence of the toxic domain, non-covalent association of the vaccine antigen to a functional CTB subunit, and maintenance of the ER-targeting KDEL motif contained within the CTA_2 domain (22, 28). Evidence suggests that CTA_2/B genetic fusions can activate long term humoral responses, stimulate protection, and block the promotion of oral tolerance (18, 21, 23, 34).

Here we investigated the murine immune response to intranasal administration of a CTA_2/B chimera containing IsdA from *Staphylococcus aureus*. We hypothesized that a stable IsdA-CTA_2/B chimeric protein with GM1 binding properties could be purified from *E. coli* and would be immunogenic at the mucosal and systemic level. Our results support this theory and indicate that IsdA-CTA_2/B can induce significant IsdA-specific Th2-type humoral and cellular responses when delivered intranasally to mice.

Materials and Methods

Bacterial strains. MRSA252 was used for *isdA* isolation (25). MRSA USA300 (*pvl*-) was also used in adhesion assays (14). *Escherichia coli* TE1, a Δ*endA* derivative of TX1, and BL21(DE3)pLysS (Invitrogen, Carlsbad, CA) were used for protein expression (56). All strains were cultured using Luria-Bertani (LB) agar or broth at 37ºC with chloramphenicol (35 μg/ml), ampicillin (100 μg/ml), and/or kanamycin (50 μg/ml).

Plasmids. To construct pBA001 for the expression of IsdA-CTA_2/B, *isdA* was PCR-amplified from MRSA252, with primers that add 5'-*Sph*I (gctactg**gcatgc**ggcaacagaagctacgaac) and 3'-*Cla*I (gtgcatg**atcgat**tttggtaattctttagc) sites, and cloned into pARLDR19 (kindly provided by R.K. Holmes, UCHSC, Denver, CO) between the LTIIb leader sequence and $ctxA_2$. CTB is also expressed from this vector that has been described previously (55, 57). To make His_6-IsdA, *isdA* was amplified from MRSA252, with primers that add 5'-*Bam*HI (gctact**ggatcc**gcggcaacagaagctacgaac or gtgcat**aagctt**tcaagtttttggtaattctttagc) and 3'-*Hind*III (gtgcatg**atcgat**tttggtaattctttagc) sites, and cloned into pTrcHisA (Invitrogen, Carlsbad, CA) or pET-40b+ (Novagen, Madison, WI), yielding pBA009A and pBA015. pARLDR19 was used to express CTA_2/B for the mixed preparation. Plasmids were transformed into *E. coli* TE1 (pBA001, pBA009A, and pARLDR19) or BL21(DE3)pLysS (pBA015) and sequenced through junctions to verify correct composition.

Protein expression and purification. To express IsdA-CTA_2/B and CTA_2/B, cultures with pBA001 or pARLDR19 were grown to OD_{600} 0.9 and induced for 15 hours with 0.2% L-arabinose. Proteins were purified from the periplasmic extract using immobilized D-galactose as described previously (55). For mock, *E. coli* TE1 without plasmid was induced and the periplasmic extract was purified as above. IsdA was isolated from the cytosol of cultures containing pBA009A and purified by cobalt affinity chromatography (Talon Metal Affinity Resin, Clontech Laboratories, Mountain View, CA) under denaturing conditions. IsdA was also purified from periplasmic extracts of cultures containing pBA015 over Talon resin under native conditions. All proteins were dialyzed against PBS, reduced to <0.125 EU/ml lipopolysaccharide by passage through an endotoxin removal column, and quantified by bicinchoninic acid assay (Pierce, Rockford, IL) prior to the addition of 5% glycerol.

Electrophoresis and immunoblotting. Proteins resolved by SDS-12%PAGE were stained with coomassie or transferred to nitrocellulose membranes. Membranes were blocked overnight with 5% skim milk in PBS plus 0.05% tween-20 (PBS-T), incubated with polyclonal α-CTA (1:2500; kindly supplied by R.K. Holmes, UCHSC) and α-CTB (1:5000; Abcam, Cambridge, MA) or α-His_6 (1:2500; Abcam, Cambridge, MA) followed by HRP-conjugated α-rabbit IgG (1:5000; Promega, Madison, WI), and developed with Immobilon Western HRP Substrate (Millipore, Billerica, MA).

GM1 ELISA. As described, micro-titer plates were coated with 0.15 μM GM1 for 15 hours at 20ºC, blocked with 10% bovine serum albumin, and incubated with IsdA-CTA_2/B or CT for 1 hour at 37ºC (46). Plates were washed with PBS-T and incubated with α-CTA (1:2000) or α-CTB (1:5000) followed by HRP-conjugated α-rabbit IgG (Promega, Madison, WI), both for 1 hour at 37ºC. The reaction was developed with o-phenylenediamine dihydrochloride (*A*450 nm).

Tissue culture and confocal microscopy. Internalization assays were performed as described (55). Briefly, Vero epithelial (ATCC, Manassas, VA) and C57B1/6 murine dendritic (DC2.4; kindly provided by K.L. Rock, DFCI, Boston, MA) cells were grown to subconfluence on uncoated coverslips at 37°C with 5% CO_2 (48). Vero cells were maintained in DMEM with 4 mM L-glutamine, 4500 mg/L glucose, 10% bovine growth serum (BGS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (DMEM+10). DC2.4 cells were maintained in RPMI 1640 medium with 2 mM L-glutamine, 10% BGS, 10 mM HEPES, 55 μM 2-mercaptoethanol, 1x non-essential amino acids, and pen-strep. Cells were incubated with IsdA-CTA_2/B or CT for 15 minutes at 4°C and shifted to 37°C for 45 minutes. Cells were then washed with PBS-T, fixed, permeabilized, and blocked prior to incubation with polyclonal α-CT (Sigma, St. Louis, MO) and FITC-conjugated α-rabbit IgG (Sigma, St. Louis, MO). Coverslips were mounted with hard-set medium with DAPI (Vector Laboratories, Burlingame, CA) and visualized using a Zeiss LSM 510 META laser scanning confocal microscope running LSM 510 META software. Images were acquired using a 100× Alpha Plan-Fluar 1.45oil DIC objective and factory set diode (405 nm for DAPI) and argon (488 nm for FITC) lasers.

Mouse immunizations and sample collection. Female BALB/c mice, 7 to 9 weeks old, were purchased from Taconic (Oxnard, CA). On days 0 and 10, groups of 8 mice received IsdA-CTA_2/B (50 μg/20 μl), IsdA + CTA_2/B (17+33 μg/20 μl), IsdA (17 μg/20 μl; from pBA009A), or mock (20 μl) in 10 μl applied to each external nare by pipette while under light anesthesia. Doses were based upon pilot studies with 12.5, 25, and 50 μg and calculated so mice received equimolar concentrations of IsdA. Blood samples were obtained by lateral tail vein nicking on days 0, 10, and 14 and open-chest cardiac puncture on day 45. Blood was allowed to clot for 1 hour at 20ºC before centrifugation at 4000 x*g* for 10 minutes. Serum was drawn off and diluted 10X in inhibitor buffer (1x Halt Protease Inhibitor Cocktail, Thermo Scientific, Rockford, IL; and 5% glycerol in PBS). Spleens were removed (day 14, $n = 2$ per group; day 45, $n = 6$ per group) and stored in DMEM+10. Nasal fluids were collected from nares after ligating the inferior trachea and injecting the superior trachea with 1 ml of lavage solution (1x Halt Protease Inhibitor, 5 mM EDTA, and 0.02% NaN_3 in PBS). Intestinal fluids were collected from the inferior ileum after ligating the duodenum and flushing 3 ml of lavage solution through the intestine. Vaginal fluids were collected by pipetting 30 μl of lavage solution in and out of the vaginal vault three times, in triplicate. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Medical Center in Boise, Idaho.

IgG and IgA ELISAs. IsdA-specific antibodies in sera and mucosal fluids were approximated by ELISA. Micro-titer plates (Nunc 439454, Rochester, NY) were coated with 0.5 μg of IsdA (from pBA015) per well in PBS, blocked with 5% BGS, and incubated with pooled sera or mucosal fluids for 12 hours at 4ºC followed by washes with PBS-T. To quantify total IgA, 0.005 μg of α-mouse IgA (Thermo Scientific, Rockford, IL) per well was used to coat plates prior to incubation with pooled sera or mucosal fluids. Plates were then incubated with HRP-conjugated α-mouse IgG (1:10000; Thermo Scientific, Rockford, IL), IgG1 or IgG2a (1:10000; MP Biomedicals, Solon, OH), or IgA (1:1500; Southern Biotech, Birmingham, AL) in 5% BGS for 1 hour at 37ºC, washed with PBS-T, and developed with tetramethylbenzidine (TMB One; *A*370 nm; Promega, Madison, WI). IgG titers, IgG1 and IgG2a titrations, and IsdA-specific IgA/total IgA ratios were calculated after background values (protocol minus samples) were subtracted. Endpoint titers were defined as the $\log_2$ of the reciprocal of the dilution that delineates the intersection point with day 0 serum.

Splenocyte isolation and proliferation assays. Spleens were mashed through 70 μm strainers in serum-free DMEM+10. Pelleted cells were resuspended in erythrocyte lysis buffer (144 mM NH_4Cl, 17 mM Tris pH 7.4, and pen-strep) and washed in serum-free DMEM+10. Splenocytes in PBS plus 5% BGS were incubated with 5 μM

carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 minutes at 37ºC and washed after the reaction was quenched with ten volumes of cold DMEM+10 (43). Splenocytes (10 x 10^6 per well) were cultured in 6-well plates with IsdA (10 μg/ml; from pBA009A), concanavalin A (ConA; 2 μg/ml), or PBS in DMEM+10 plus 10 mM HEPES and 50 μM 2-mercaptoethanol (Complete DMEM) for 84 hours at 37ºC with 5% CO_2. Additional media plus IsdA, ConA, or PBS was added 36 hours into the assay. Washed cells were blocked with rat IgG (Invitrogen, Camarillo, CA) and α-mouse CD16/32 in staining buffer (5% BGS and 0.1% NaN_3 in PBS). Lymphocytes were identified by characteristic size and granularity, in combination with PE-Cy5 α-mouse CD3 and isotype control (BD Biosciences, San Diego, CA) staining. CFSE gates were set at the undivided peak of non-stimulated cells to determine the percent of proliferating T lymphocytes ($CFSE_{low}CD3+$) per mouse. Cell viability was higher than 65% as measured by propidium iodide staining. A minimum of 10,000 $CFSE_{low}CD3+$ cells were collected per sample and analyzed using a C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI) running CFlow Plus software. For resazurin assays, splenocytes (5 x 10^5 cells per well) were cultured in Complete DMEM with IsdA (10 μg/ml; from pBA009A), ConA (2 μg/ml), or PBS in 96-well plates at 37ºC with 5% CO_2. At 84 hours, resazurin (AbD Serotec, Oxford, UK) in 0.1 M KH_2PO_4 was added and fluorescence (530/590 nm) was measured 2 (ConA-stimulated) or 4 hours (IsdA-stimulated) later. The stimulation index (ratio of mean fluorescence from stimulated to non-stimulated cells) was calculated for each group.

Cytokine ELISAs. Levels of interleukin-4 (IL-4) and interferon-gamma (IFN-γ) in supernatants from splenocytes (5 x 10^5 per well) cultured *in vitro* for 84 hours with IsdA (10 μg/ml; from pBA009A) were determined by ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA). The assay sensitivity for IL-4 and IFN-γ was 4-500 pg/ml and 15-2000 pg/ml, respectively.

***In vitro* functional assays.** Human epithelial cells (HeLa; ATCC, Manassas, VA; 5 x 10^3 per well) were grown in 96-well plates with DMEM+10 at 37ºC with 5% CO_2. Day 45 sera (1:100), pooled by immunization group, was incubated with PBS-washed MRSA252 (5 x 10^7 CFU) or MRSA USA300 (5 x 10^9 CFU) and then added to confluent HeLa cells, both for 1 hour at 37ºC. HeLa cells were washed extensively with PBS, treated with 0.25% trypsin-1 mM EDTA, and lysed with 0.025% Triton X-100. Cell lysates were plated on LB agar and CFU/ml was determined.

Statistical analysis. All assays were performed in triplicate. Results are expressed as the mean ± the standard error of the mean (SEM) between triplicates unless otherwise noted. Comparisons between immunization groups were made using the unpaired student's t-test. A p-value < 0.05 was considered a statistically significant difference. Graphing and statistical analyses were performed using SigmaPlot 8.0 (Systat Software).

Results

Expression and purification of IsdA-CTA_2/CTB and control proteins. To direct the IsdA-CTA_2 and CTB peptides of the chimera to the *E. coli* periplasm for proper holotoxin assembly, pBA001 (Figure 1A) was constructed from pARLDR19 which utilizes the *E. coli* LTIIb N-terminal leader sequence (57). Induction of pBA001 and purification from the periplasm of *E. coli* resulted in efficient IsdA-CTA_2/B production (3-4 mg from 1 L of starting culture). SDS-PAGE analysis of the purification of IsdA-CTA_2/B, and immunoblotting using antibodies against CTA and CTB (Figure 1B), confirm that IsdA-CTA_2 (~38 kDa) was co-purified with CTB (~11 kDa) on D-galactose agarose which is indicative of proper chimera folding. IsdA alone was also purified using a 6X histidine tag, and Figure 1C shows an SDS-polyacrylamide gel of all resulting proteins used in animal studies, as well as immunoblotting of purified IsdA with α-His_6 (~37 kDa).

Binding and internalization of IsdA-CTA_2/B into Vero and DC2.4 cells. To compare the receptor binding affinity of the purified IsdA-CTA_2/B chimera with native CT, we performed a ganglioside GM1 ELISA using α-CTA and α-CTB antibodies. Results indicate that the B subunit of IsdA-CTA_2/B has GM1 binding affinity similar to CT (Figure 2A). Low α-CTA response from IsdA-CTA_2/B was an expected result from this fusion that contains only 46 bps of full length CTA. Confocal microscopy was used to confirm receptor binding and assess the transport of IsdA-CTA_2/B into epithelial and dendritic cells *in vitro*. Figure 2B shows α-CT-FITC labeled IsdA-CTA_2/B bound to the surface of the cells at 4°C and internalization after 45 minutes at 37°C, indicating that, at minimum, the CTB subunit of the chimera is efficiently imported into the cell. This transport is consistent with our previously reported trafficking of native CT into Vero cells and macrophages (55).

IsdA-specific humoral response. BALB/c mice were immunized intranasally with IsdA-CTA_2/B, IsdA + CTA_2/B, IsdA, or mock on day 0 and boosted on day 10 (Table 1). Sera collected on days 0, 10, 14, and 45 were pooled by treatment group at each time point and tested for recognition of IsdA by IgG ELISA. IsdA-specific serum IgG endpoint titers from mice immunized with IsdA-CTA_2/B were significantly higher as compared with mock-immunized on day 10, all control groups on day 14, and IsdA alone and mock-immunized on day 45 (Figure 3). Nasal, intestinal, and vaginal washes were collected on day 45, pooled by treatment group, and tested for recognition of IsdA by IgA ELISA. The percent of IsdA-IgA out of total IgA was significantly higher in nasal and vaginal washes from mice immunized with IsdA-CTA_2/B in comparison to IsdA + CTA_2/B, IsdA, and mock-immunized mice (Figure 4). In addition, intestinal IsdA-IgA was significantly higher in IsdA-CTA_2/B-immunized mice compared to IsdA and mock (Figure 4). Together these results demonstrate that IsdA-specific systemic and mucosal humoral immunity can be stimulated after intranasal vaccination with the IsdA-CTA_2/B chimera.

Proliferation of IsdA-stimulated splenocytes. Cellular proliferation was assessed using flow cytometry and resazurin-based fluorescent dye assay. CFSE-based flow cytometric results suggest that day 45 splenocytes derived from mice immunized with IsdA-CTA_2/B showed a modest, but significant, proliferation of IsdA-specific CD3+ T lymphocytes compared with mixed and IsdA control groups (Figures 5A, B). Mock samples contained low numbers of CD3+ T lymphocytes and could not accurately be quantitated by this assay (data not shown). Similarly, resazurin assays revealed that *in vitro* stimulation of splenocytes from IsdA-CTA_2/B-immunized mice induced significant proliferation compared with IsdA + CTA_2/B, IsdA, and mock groups on day 45 (Figure 5C). With the low sample size (n = 2 per group) on day 14, no significance was observed between groups. Stimulation was observed for the positive control, ConA. Collectively, these results suggest that intranasal administration of IsdA-CTA_2/B can induce a cellular activation response.

Induction of a Th2-type bias. The levels of IL-4 and IFN-γ in supernatants of splenocytes stimulated with IsdA *in vitro* were determined by ELISA. Splenocytes obtained from mice immunized with IsdA-CTA_2/B secreted high levels of IL-4, and these levels were significantly higher compared to all controls (Figure 6A). Although the level of IFN-γ was slightly higher in IsdA-CTA_2/B-immune splenocytes, low levels of IFN-γ, near the detection limit for the assay, were found in all control groups (Figure 6A). Titrations of IgG1 and IgG2a (Figure 6B) revealed that immunization with IsdA-CTA_2/B drove isotype switching primarily to the IgG1 subclass, although minute IgG2a levels were also detected. These results support the premise that immunization with IsdA-CTA_2/B promotes a Th2-type immune response.

***In vitro* functional assays.** Pooled sera from commonly immunized mice were used to investigate the ability of α-IsdA to functionally block adherence of *S. aureus* to human epithelial cells (HeLa). Pre-incubation of the *S. aureus* strain used for vaccination (MRSA252) with day 45 sera from IsdA-CTA_2/B-immunized mice significantly reduced bacterial adhesion to epithelial cells when compared to all control groups (Figure 7A). In addition, there was a significant reduction in bacterial adhesion to human epithelial cells after a different strain of *S. aureus* (MRSA USA300) was pre-incubated with day 45 sera from mice immunized with IsdA-CTA_2/B (Figure 7B).

Discussion

The aim of the present study was to purify a holotoxin-like IsdA-CTA_2/B chimera and characterize its immunogenicity after intranasal administration to mice. Our results demonstrate that IsdA-CTA_2/B can be expressed efficiently in *E. coli* and bind to ganglioside GM1 *in vitro*. GM1 is found ubiquitously on mammalian cells, but immune effector cells, such as dendritic cells, have a uniquely high affinity for CT and non-toxic CTB (2, 30). The binding and transport of IsdA-CTA_2/B into epithelial and dendritic cells was consistent with the uptake of native CT involving retrograde movement to the perinuclear domain of the Golgi apparatus and endoplasmic reticulum (5, 39, 55). We propose that the ability of IsdA-CTA_2/B to bind to GM1 and trigger internalization leads to the activation of immune effector cells by the CTB subunit and promotes antigen presentation on MHC molecules.

ELISA analysis of IsdA-specific responses from the sera and nasal, intestinal, and vaginal fluids of intranasally immunized mice verifies that the IsdA-CTA_2/B chimera can induce antigen-specific systemic and mucosal immunity in mice. As expected, IgG titers were highest on day 14 after the boost and began to diminish by day 45. A previous vaccine study in our laboratory comparing IsdA-CTA_2/B to IsdA and mock yielded comparable serum IgG titers for chimera on days 14 and 45 (unpublished data). The IgG titers reported here for IsdA-CTA_2/B are significant after only one booster, and alternative doses and schedules will continue to be explored to improve responses. Previous

reports suggest that higher serum IsdA-specific IgG titers (4.6×10^4) are required to significantly reduce bacterial load after invasive staphylococcal challenge, however even low titers (1.5×10^3) of passively administered IsdA-specific IgG can increase survival against invasive disease and abscess formation (31, 50). The results presented in this report are consistent with the characteristic ability of CT to induce systemic IgG to antigens co-administered with CT at mucosal sites. The presence of IsdA-specific IgA in nasal, intestinal, and vaginal fluids after intranasal immunization with IsdA-CTA_2/B suggests that IgA blasts migrated from the nasal-associated lymphoid tissue into distal mucosal effector sites in the nasal passage and gastrointestinal and genital tracts. Induction of T lymphocyte proliferation by IsdA-CTA_2/B is consistent with previous studies showing that CT and CTA_2/B chimeras can induce significant cellular responses (16, 18, 40, 62). These reports also support our findings that CT and CT derivatives promote more of a Th2-type response. We identified characteristic Th2 responses; secretion of IL-4 leading to induction of antibody class switching to noncomplement-activating IgG1. *In vitro* functional assays of antibodies revealed a significant reduction in internalized and cell-bound bacteria on human epithelial cells after pre-incubation of IsdA-CTA_2/B immune sera with the *S. aureus* isolate used for vaccination; MRSA252. In addition, antibodies were able to prevent adhesion of MRSA USA300. As described below, IsdA from MRSA252 and MRSA USA300 has 92% amino acid identity with the majority of differences present within the C-terminus. These results suggest that antibodies against IsdA are functional *in vitro* and may protect against multiple serotypes *in vivo.* Lastly, this report supports the hypothesis that the humoral and cellular response induced by IsdA-CTA_2/B is superior to that stimulated by a mixed preparation of antigen and adjuvant (IsdA + CTA_2/B). Thus the structure of the IsdA-CTA_2/B chimera is optimal for the induction of antigen-specific humoral responses and potentially for presentation on MHC molecules, which is consistent with previous reports of chimeric molecules using distinct antigens (18, 36, 51).

CT and the closely related LTI are gold standard mucosal adjuvants with a long history of use in animals and more recent use of non-toxic derivatives in humans. However, the safety of mucosal administration of these enterotoxins has been questioned due to reports that these molecules can redirect antigens to the central nervous system through GM1-dependent binding to olfactory epithelium (20, 58). Despite the elimination of the toxic domain in A_2/B chimeras, safety concerns may still remain. A brief licensure of an influenza vaccine and more recent clinical study of non-toxic LTI support a connection to facial nerve paralysis after intranasal delivery (35, 42). Oral vaccination with CTB however, does not target olfactory neurons, and does not raise the same safety concerns. CTB is a component of the current oral *V. cholerae* vaccine WC-rBS (Dukoral, Crucell) that is licensed in over 60 countries and is well tolerated with a good safety record (53). As proof of principle in this report, we have chosen the intranasal route that is well characterized and requires a lower concentration of antigen, however, safe and effective administration of CT and LTI-based vaccines by oral, sublingual, and transcutaneous routes has proved promising in mice (11, 12, 23, 24). Thus, alternative mucosal routes of IsdA-CTA_2/B delivery will be explored for the development of a potential human vaccine. In addition, *S. aureus* is the major cause of bovine mastitis in dairy cattle, and significant doses of CTB have been intranasally administered to cows without signs of neuronal toxicity (3, 45). The potential of the IsdA-CTA_2/B chimera for use as an intranasal veterinary vaccine to prevent bovine mastitis is also under investigation.

Continued development of IsdA-CTA_2/B will necessarily involve protection studies in animals against colonization as well as invasive disease. IsdA from MRSA252 is 92% identical to other clinical isolates of MRSA (MRSA USA300 NCBI#YP 001574962 and MRSA177 EMBL-CDS#EFW34902.1) and 100% identical to other *S. aureus* isolates (MN8 NCBI#ZP-06950067 and TCH60 NCBI#ADQ77565). Conserved regions are largely contained within the N-terminal binding domain, suggesting that IsdA may confer protection against multiple *S. aureus* serotypes. However, while our results suggest protection against colonization with multiple serotypes may be possible with an IsdA-based vaccine, incorporation of a single subunit is unlikely to provide sufficient protection against invasive disease. A number of additional surface proteins from *S. aureus* have been characterized as vaccine antigens, and the use of an enterotoxin A_2/B platform makes the incorporation of multiple antigens and/or epitopes into one vaccine relatively straightforward. Another important issue that will be addressed in future protection studies is the potential for the induction of immune unresponsiveness or tolerance by IsdA-CTA_2/B. While previous studies suggest that CTA_2/B chimeras can abrogate oral tolerance for up to 6 months, many studies have also shown that chemical conjugation or genetic fusion of antigens directly to CTB induces oral tolerance (18, 52). As the induction of tolerance to staphylococcal antigens may be quite detrimental in the case of invasive disease, careful long term studies that specifically address the induction of tolerance will be required before this vaccine could advance into humans.

While CT has been used previously in potential staphylococcal vaccines to enhance immunogenicity, to our knowledge, the construction of an A_2/B chimera is a novel approach for development of a mucosal vaccine against *S. aureus* (6, 15, 49). Our results suggest that the IsdA-CTA_2/B chimera induces an IsdA-specific systemic IgG, mucosal IgA, and cell-mediated response when administered intranasally to mice, and its immunogenicity warrants further investigation as a potential mucosal *S. aureus* vaccine.

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Figure Legends

Figure 1. Expression and purification of the IsdA-CTA_2/B chimera and control proteins. **A**. Structure and operon organization of pBA001 for IsdA-CTA_2/B expression. *isdA* was amplified from MRSA252 and cloned into pARLDR19 which directs the IsdA-CTA_2 and CTB peptides to the periplasm of *E. coli* for holotoxin assembly. **B**. SDS-PAGE analysis of flow through (FT), washes (W), and elution (E) of IsdA-CTA_2/B from D-galactose affinity purification and α-CTA/B western blot of purified IsdA-CTA_2/B (~38 and 11 kDa). **C**. SDS-PAGE analysis of proteins used in vaccination studies; IsdA-CTA_2/B chimera (G1), IsdA + CTA_2/B mixed (G2), IsdA (G3), and α-His_6 western blot of purified IsdA used in G2 and G3 (~37 kDa).

Figure 2. Characterization of IsdA-CTA_2/B *in vitro*. **A**. Ganglioside GM1 ELISA using α-CTA and α-CTB comparing the receptor binding of IsdA-CTA_2/B chimera and native CT. Error bars are based on triplicates, and results are representative of three independent assays. **B**. Confocal microscopy of IsdA-CTA_2/B binding and transport into Vero and DC2.4 cells using polyclonal α-CT and α-rabbit-FITC with DAPI. Cells were incubated with IsdA-CTA_2/B for 45 min at 4°C to inhibit, or 37°C to promote, cellular uptake. FITC/DAPI channel overlay is shown, and results are representative of three independent experiments.

Figure 3. Systemic antibody response to IsdA-CTA_2/B *in vivo*. IsdA-specific IgG ELISA endpoint titers from days 10, 14, and 45 sera pooled by immunization group (n = 6). Significance ($p < 0.05$) between mice immunized with IsdA-CTA_2/B versus controls (*) is shown, and error bars are based on assays performed in triplicate.

Figure 4. Mucosal antibody response to IsdA-CTA_2/B *in vivo*. Percent IsdA-specific IgA out of total IgA in day 45 nasal (1:2), intestinal (1:8), and vaginal (1:16) wash pooled by immunization group (n = 6). Significance ($p < 0.05$) between mice immunized with IsdA-CTA_2/B versus controls (*) is shown, and error bars are based on assays performed in triplicate.

Figure 5. Analysis of IsdA-specific cellular proliferation in mice immunized with IsdA-CTA_2/B chimera, IsdA + CTA_2/B mixed, IsdA, or mock. **A**. CFSE-labeled splenocytes were cultured *in vitro* for 84 hours with IsdA, stained with α-CD3-PE-Cy5, and analyzed by flow cytometry. The plots show a representative experiment; CFSE gates were set at the undivided peak of non-stimulated cells to determine proliferation of stimulated CD3+ cells. **B**. Percent proliferation of IsdA-specific CD3+ T lymphocytes from individual mice on day 45 as determined by flow cytometry. Error bars are based on n = 6. **C**. Resazurin assay of splenocytes from days 14 and 45 cultured *in vitro* for 84 hours with IsdA. The stimulation index equals the ratio of fluorescence from stimulated to non-stimulated cells. Error bars are based on n = 2 (day 14) or n = 6 (day 45). Significance ($p < 0.05$) between mice immunized with IsdA-CTA_2/B versus controls (*) is shown.

Figure 6. Cytokine and IgG subclass profiles of mice immunized with IsdA-CTA_2/B chimera, IsdA + CTA_2/B mixed, IsdA, or mock. **A**. IL-4 and IFN-γ levels in culture supernatants from splenocytes, pooled by immunization group (n = 6), stimulated *in vitro* for 84 hours with IsdA were measured by ELISA. Significance ($p < 0.05$) between mice immunized with IsdA-CTA_2/B chimera versus controls (*) is shown. Error bars are based on assays performed in triplicate, and results are representative of two independent assays. **B**. IsdA-specific IgG1 and IgG2a ELISA titrations from day 45 sera pooled by immunization group (n = 6). Significance ($p < 0.05$) between mice immunized with IsdA-CTA_2/B chimera versus IsdA (+) and mock (#) is shown, and error bars are based on assays performed in triplicate.

Figure 7. Effect of α-IsdA immune sera on *S. aureus* adhesion to human epithelial cells *in vitro*. Sera (1:100; day 45) was pooled by immunization group and incubated with **A**. MRSA252 (5×10^7 CFU) or **B.** MRSA USA300 (5×10^9 CFU) for 1 hour at 37ºC and then added to confluent HeLa cells. After washing and lysis, the number of internalized and cell-bound bacteria was enumerated. Error bars are based on three (A) or two (B) independent experiments. Significance ($p < 0.05$) between mice immunized with IsdA-CTA_2/B chimera versus controls (*) is shown.

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Tables

Table 1. Immunization strategy and sample collection schedule.

Antigen/ adjuvant	**Dose per vaccination (µg)**	***n***	**Intranasal vaccination**	**Sampling: sera**	**Sampling: mucosal secretions spleens**
IsdA-CTA_2/B	50	8	Days 0 & 10	Days 0, 10, 14, & 45	Days 14 (*n*=2) & 45 (*n*=6)
IsdA + CTA_2/B	17 + 33[a]	8	Days 0 & 10	Days 0, 10, 14, & 45	Days 14 (*n*=2) & 45 (*n*=6)
IsdA	17	8	Days 0 & 10	Days 0, 10, 14, & 45	Days 14 (*n*=2) & 45 (*n*=6)
Mock	N/A	8	Days 0 & 10	Days 0, 10, 14, & 45	Days 14 (*n*=2) & 45 (*n*=6)

[a]concentrations according to equimolar amounts of IsdA