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 A2/B (CTA2/B) chimera containing IsdA. Plasmids were constructed to express the IsdA-CTA2/B chimera and control proteins in Escherichia coli. Proper construction of the chimera was verified by SDS-PAGE, Western blotting, GM1 enzyme-linked immunosorbent assay (ELISA), and confocal microscopy. Groups of female BALB/c mice were mock immunized or immunized with IsdA-CTA2/B, IsdA mixed with CTA2/B, or IsdA alone, followed by one booster immunization at 10 days postpriming. Analysis of serum IgG and nasal, intestinal, and vaginal IgA suggested that mucosal immunization with IsdA-CTA2/B induces significant IsdA-specific humoral immunity. Functional in vitro assays revealed that immune serum significantly blocks the adherence of S. aureus to human epithelial cells. Splenocytes from mice immunized with IsdA-CTA2/B showed specific cellular proliferation and production of interleukin-4 (IL-4) after in vitro stimulation. Immunization with IsdA-CTA2/B drove isotype switching to IgG1, indicative of a Th2-type response. Our results suggest that the immunogenicity of the S. aureus IsdA-CTA2/B chimera merits further investigation as a potential mucosal vaccine candidate.

Received 5 May 2011/Returned for modification 2 June 2011/Accepted 28 June 2011

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 health care facilities, are now the known cause of life-threatening community-acquired multidrug-resistant infections (13).

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 adhesion, 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 transporter (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 noncarriers than in commensal carriers, and in 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 of iron uptake is a promising target for the development of a mucosal vaccine against S. aureus.

A vaccine that prevents human staphylococcal disease is currently not available, and poorer than expected results from several vaccine candidates tested in preclinical 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 costimulatory and major histocompatibility complex (MHC) class II expression (10, 21, 26, 44). Many of these responses result from the interaction of...
the cholera toxin B (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 nontoxic CTB by itself can act as an antigen carrier and is highly immunostimulatory (17, 27, 47). The ability of nontoxic CTB to effectively block oral tolerance in the absence of enzymatic activity from CTB 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 holotoltoxin-like CTA2/B chimeras, where the tox A 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, noncovalent association of the vaccine antigen to a functional CTB subunit, and maintenance of the endoplasmic reticulum (ER)-targeting KDEL motif contained within the CTAB domain (22, 28). Evidence suggests that CTAB 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 CTA2/B chimera containing IsdA from S. aureus. We hypothesized that a stable IsdA-CTA2/B chimeric protein with GM1 binding properties could be purified from E. coli and would be immunogenic at the mucosal and systemic levels. Our results support this theory and indicate that IsdA-CTA2/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 mutant) was also used in adhesion assays (14). E. coli TE1, a Δmda4 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 37°C with chloramphenicol (35 g/ml). MRSA252 was used for isdA isolation (25). MRSA USA300 (pvl mutant) was also used in adhesion assays (14). E. coli TE1, a Δmda4 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 37°C with chloramphenicol (35 g/ml). MRSA252 was used for isdA isolation (25). MRSA USA300 (pvl mutant) was also used in adhesion assays (14). E. coli TE1, a Δmda4 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 37°C with chloramphenicol (35 g/ml).

Plasmids. To construct pBA001 for the expression of IsdA-CTA2/B, IsdA was PCR amplified from MRS252 with primers that add 5’ SphI (GCTACTGCG ATTCGGCAACAGAACCTAGGAAAG) and 3’ Clal (GTCATGATGATGA TTGTTGTAATTCTTTAGC) sites (in boldface) and cloned into pTriEx3-A (Invitrogen, Carlsbad, CA) or pET41 (Novagen, Madison, WI), yielding pBA009A and pBA015. pARLDR19 was used to express CTA2/B for the mixed preparation. Plasmids were transformed into E. coli TE1 (pBA001, pBA009A, and pARLDR19) or BL21(DE3)pLyS (pBA015) and sequenced through junctions to verify correct composition.

Protein expression and purification. To express IsdA-CTA2/B and CTAB, cultures with pBA001 or pARLDR19 were grown to an optical density at 600 nm (OD600) of 0.9 and induced for 15 h with 0.2% l-arabinose. Proteins were purified from the periplasmic extract using immobilized d-galactose as described previously (55). For mock cultures, E. coli TE1 without plasmid was induced, and the periplasmic extract was purified as described above. IsdA was isolated from the cytosol of cultures containing pBA009A and purified by cation affinity chromotography (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 phosphate-buffered saline (PBS), reduced to <0.125 endotoxin units (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% glycero1.

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 anti-CTA (1:2,500; kindly supplied by R. K. Holmes, UCHSC) and anti-CTB (1:5,000; Abcam, Cambridge, MA) or anti-His (1:2,500; Abcam, Cambridge, MA), followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5,000; Promega, Madison, WI) and developed with Immobilon Western HRP Substrate (Millipore, Billerica, MA).

GM1 enzyme-linked immunosorbent assay (ELISA). As described previously, microtiter plates were coated with 0.15 µg GM1 for 1 h at 37°C, blocked with 10% serum albumin, and incubated with IsdA-CTA2/B or CT for 1 h at 37°C (46). Plates were washed with PBS-T and incubated with anti-CTA (1:2,000) or anti-CTB (1:5,000) followed by HRP-conjugated anti-rabbit IgG (Promega, Madison, WI), both for 1 h at 37°C. The reaction was developed with o-phenylenediamine dihydrochloride (A405).

Tissue culture and confocal microscopy. Internalization assays were performed as described previously (55). Briefly, Vero epithelial (ATCC, Manassas, VA) and C57BL/6 mouse dendritic (DC2.4; kindly provided by K. L. Rock, Dana-Farber Cancer Institute, Boston, MA) cells were grown to subconfluence on 8-well coverslips at 37°C in 5% CO2 (48). Vero cells were grown to subconfluence on 12-well coverslips in Dulbecco's modified Eagle's medium (DMEM) with 4 mM l-glutamine, 4,500 mg/liter glucose, 10% bovine serum growth (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, 1% nonessential amino acids, and penicillin-streptomycin (Pen-Strep). Cells were incubated with IsdA-CTA2/B or CT for 15 min at 37°C and shifted to 37°C for 45 min. Cells were then washed with PBS-T, fixed, permeabilized, and blocked prior to incubation with polyclonal anti-CT (Sigma, St. Louis, MO) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma, St. Louis, MO). Coverslips were mounted with hard-set medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and visualized using a Zeiss LSM 510 META laser scanning confocal microscope running LSM 510 META software. Images were required using a 100× Alpha Plan-Fluar (1.45 numerical aperture) oil differential interference contrast (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-CTA2/B (50 µg/20 µl), IsdA plus CTAB/B mixed (17 and 33 µg/20 µl, respectively), IsdA (17 µg/20 µl; from pBA009A), or a mock immunization (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 h at 20°C before centrifugation at 4,000 × g for 10 min. Serum was drawn off and diluted 10-fold in inhibitor buffer (1% Halt Protease Inhibitor Cocktail [Thermo Scientific, Rockford, IL]) into PBS. Serial 5-fold dilutions up to 1:512 were made in 5% BGS, and incubated with pooled sera or mucosal fluids for 12 h at 4°C. Samples were approximated by ELISA. Microtiter plates (439454; Nunc, Rochester, NY) were coated with IsdA (from pBA015) per well in PBS, blocked with 5% BGS, and incubated with pooled sera or mucosal fluids for 12 h at 4°C followed by washes with PBS-T. To quantify total IgA, 0.005 µg of anti-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 anti-mouse IgG (1:10,000; Thermo Scientific, Rockford, IL), IgG1 or IgG2a (1:10,000; MP Biomedicals, Solon, OH), or IgA (1:1,500; Southern Biotech, Birmingham, AL) in 5% BGS for 1 h at 37°C, washed with PBS-T, and developed with tetramethylbenzidine (TMB One: A102; 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 log2 of the reciprocal of the dilution that delineates the intersection point with day 0 serum.

Downloaded from http://cvi.asm.org on July 20, 2018 by guest
Splenocyte isolation and proliferation assays. Spleens were mashed through 70-µm-pore-size strainers in serum-free DMEM +10. Pelleted cells were resuspended in erythrocyte lysis buffer (144 mM NH₄Cl, 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 min at 37°C and washed after the reaction was quenched with 10 volumes of cold DMEM +10 (43). Splenocytes (10 × 10⁶ per well) were cultured in six-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 h at 37°C with 5% CO₂. Additional medium plus IsdA, ConA, or PBS was added 36 h into the assay. Washed cells were blocked with rat IgG (Invitrogen, Camarillo, CA) and anti-mouse CD16/CD32 in staining buffer (5% BGS and 0.1% NaN₃ in PBS). Lymphocytes were identified by characteristic size and granularity, in combination with phycoerythrin (PE)-Cy5 anti-mouse CD3 and isotype control (BD Biosciences, San Diego, CA) staining. CFSE gates were set at the undivided peak of nonstimulated cells to determine the percentage of proliferating T lymphocytes (CFSElow CD3⁺) per mouse. Cell viability was higher than 65% as measured by propidium iodide staining. A minimum of 10,000 CFSElow 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 × 10⁵ 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 37°C with 5% CO₂. At 84 h, resazurin (AbD Serotec, Oxford, United Kingdom) in 0.1% BSA was added, and fluorescence (530/590 nm) was measured 2 h (ConA-stimulated) or 4 h (IsdA-stimulated) later. The stimulation index (ratio of mean fluorescence of stimulated to nonstimulated cells) was calculated for each group.

Expression and purification of IsdA-CTA₂/B. To direct the IsdA-CTA₂ and CTB peptides of the chimera to the E. coli periplasm for proper holotoxin assembly, pBA001 (Fig. 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-CTA₂/B production (3 to 4 mg from 1 liter of starting culture). SDS-PAGE analysis of the purification of IsdA-CTA₂/B and immunoblotting using antibodies against CTA and CTB (Fig. 1B) confirm that IsdA-CTA₂ (∼38 kDa) was copurified with CTB (∼11 kDa) on α-galactose agarose, which is indicative of proper chimera folding. IsdA alone was also purified using a six-histidine tag, and Fig. 1C shows an SDS-polyacrylamide gel of all resulting proteins used in animal studies, as well as immunoblotting of purified IsdA with anti-His₆ (37 kDa).

Receptor binding and internalization of IsdA-CTA₂/B into Vero and DC2.4 cells. To compare the receptor binding affinity of the purified IsdA-CTA₂/B chimera with native CT, we per-
formed a ganglioside GM1 ELISA using anti-CTA and anti-CTB antibodies. Results indicate that the B subunit of IsdA-CTA2/B has GM1 binding affinity similar to that of CT (Fig. 2A). Low anti-CTA response from IsdA-CTA2/B was an expected result from this fusion that contains only 46 bp of full-length CTA. Confocal microscopy was used to confirm receptor binding and assess the transport of IsdA-CTA2/B into epithelial and dendritic cells in vitro. Figure 2B shows anti-CT FITC-labeled IsdA-CTA2/B bound to the surface of the cells at 4°C and internalization after 45 min at 37°C, indicating that, at a 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 mock immunized or immunized intranasally with IsdA-CTA2/B, IsdA plus CTA2/B mixed, or IsdA 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-CTA2/B were significantly higher than those of mock-immunized mice on day 10, than those of all control groups on day 14, and than those of mice immunized with IsdA alone and mock-immunized mice on day 45 (Fig. 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 percentage of IsdA-IgA out of total IgA was significantly higher in nasal and vaginal washes from mice immunized with IsdA-CTA2/B than from mock-immunized mice, mice immunized with IsdA plus CTA2/B, or mice immunized with IsdA alone (Fig. 4). In addition, intestinal IsdA-IgA was significantly higher in IsdA-CTA2/B-immunized mice than in IsdA- and mock-immunized mice (Fig. 4). Together, these results demonstrate that IsdA-specific systemic and mucosal humoral immunity can be stimulated after intranasal vaccination with the IsdA-CTA2/B chimera.

Proliferation of IsdA-stimulated splenocytes. Cellular proliferation was assessed using flow cytometry and a resazurin-based fluorescent dye assay. CFSE-based flow cytometric results suggest that day 45 splenocytes derived from mice immunized with IsdA-CTA2/B showed a modest, but significant, proliferation of IsdA-specific CD3+ T lymphocytes compared with mixed and IsdA control groups (Fig. 5A and 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 stim-

### TABLE 1. Immunization strategy and sample collection schedule

| Antigen/adjuvant                  | Dose per vaccination (μg) | n | Days of intranasal vaccination | Days of sampling Sera | Mucosal secretions and spleen (n) |
|-----------------------------------|---------------------------|---|-------------------------------|-----------------------|-----------------------------------|
| IsdA-CTA2/B chimera               | 50                        | 8 | 0, 10                         | 0, 10, 14, 45         | 14 (2), 45 (6)                    |
| IsdA + CTA2/B                     | 17 + 33a                  | 8 | 0, 10                         | 0, 10, 14, 45         | 14 (2), 45 (6)                    |
| IsdA                              | 17                        | 8 | 0, 10                         | 0, 10, 14, 45         | 14 (2), 45 (6)                    |
| Mock                              | NAa                       | 8 | 0, 10                         | 0, 10, 14, 45         | 14 (2), 45 (6)                    |

*a Concentrations (respectively) are according to equimolar amounts of IsdA.

*b n, number of mice.

c NA, not applicable.
ulation of splenocytes from IsdA-CTA2/B-immunized mice induced significant proliferation compared with IsdA plus CTA2/B, IsdA, and mock groups on day 45 (Fig. 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-CTA2/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-CTA2/B secreted high levels of IL-4, and these levels were significantly higher than levels of all controls (Fig. 6A). Although the level of IFN-γ was slightly higher in IsdA-CTA2/B-immune splenocytes, low levels of IFN-γ, near the detection limit for the assay, were found in all groups (Fig. 6A). Titrations of IgG1 and IgG2a (Fig. 6B) revealed that immunization with IsdA-CTA2/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-CTA2/B promotes a Th2-type immune response.

**In vitro functional assays.** Pooled sera from commonly immunized mice were used to investigate the ability of immune serum to functionally block adherence of *S. aureus* to human epithelial cells (HeLa). Preincubation of the *S. aureus* strain used for vaccination (MRSA252) with day 45 sera from IsdA-CTA2/B-immunized mice significantly reduced bacterial adhesion to epithelial cells compared to all control groups (Fig. 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 preincubated with day 45 sera from mice immunized with IsdA-CTA2/B (Fig. 7B).

**DISCUSSION**

The aim of the present study was to purify a holotoxin-like IsdA-CTA2/B chimera and characterize its immunogenicity after intranasal administration to mice. Our results demonstrate that IsdA-CTA2/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 nontoxic CTB (2, 30). The binding and transport of IsdA-CTA2/B into epithelial and dendritic cells were 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-CTA2/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.

ELISAs of IsdA-specific responses from the sera and nasal, intestinal, and vaginal fluids of intranasally immunized mice verifies that the IsdA-CTA2/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-CTA2/B to IsdA and mock immunization yielded comparable serum IgG titers for chimera on days 14 and 45 (unpublished data). The IgG titers reported here for IsdA-CTA2/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 × 10^6) are required to significantly reduce bacterial load after invasive staphylococcal challenge; however, even low titers (1.4 × 10^5) 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 coadministered with CT at mucosal sites. The presence of IsdA-specific IgA in nasal, intestinal, and vaginal fluids after intranasal immunization with IsdA-CTA2/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-CTA2/B is consistent with previous studies showing that CT and CTB
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 a characteristic Th2 response: secretion of IL-4 leading to induction of antibody class switching to noncomplement-acti-

FIG. 5. Analysis of IsdA-specific cellular proliferation in mock-immunized mice or mice immunized with IsdA-CTA<sub>2</sub>/B chimera, IsdA plus CTA<sub>2</sub>/B mixed, or IsdA. (A) IL-4 and IFN-γ levels in culture supernatants from splenocytes, pooled by immunization group (n = 6), stimulated in vitro for 84 h with IsdA were measured by ELISA. Significance (P < 0.05) between mice immunized with IsdA-CTA<sub>2</sub>/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<sub>2</sub>/B chimera versus IsdA (+) and mock (#) is shown, and error bars are based on assays performed in triplicate.

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 a characteristic Th2 response: secretion of IL-4 leading to induction of antibody class switching to noncomplement-acti-

FIG. 6. Cytokine and IgG subclass profiles of mock-immunized mice or mice immunized with IsdA-CTA<sub>2</sub>/B chimera, IsdA plus CTA<sub>2</sub>/B mixed, or IsdA. (A) IL-4 and IFN-γ levels in culture supernatants from splenocytes, pooled by immunization group (n = 6), stimulated in vitro for 84 h with IsdA were measured by ELISA. Significance (P < 0.05) between mice immunized with IsdA-CTA<sub>2</sub>/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<sub>2</sub>/B chimera versus IsdA (+) and mock (#) is shown, and error bars are based on assays performed in triplicate.

proliferation of IsdA-specific CD<sup>3</sup> T lymphocytes from individual mice on day 45 as determined by flow cytometry. Significance is based on n = 6. (C) Resazurin assay of splenocytes from days 14 and 45 cultured in vitro for 84 h with IsdA. The stimulation index equals the ratio of fluorescence of stimulated to nonstimulated 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<sub>2</sub>/B versus controls (+) is shown.
responses induced by IsdA-CTA2/B are superior to those stim- 
ulated by a mixed preparation of antigen and adjuvant (IsdA 
plus CTA2/B). Thus, the structure of the IsdA-CTA2/B chi- 
mera 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 nontoxic derivatives in humans. However, the safety of 
mucosal administration of these enterotoxins has been ques- 
tioned due to reports that these molecules can redirect anti- 
gens to the central nervous system through GM1-dependent 
binding to olfactory epithelium (20, 58). Despite the elimina- 
tion of the toxic domain in A2/B chimeras, safety concerns may 
still remain. A brief licensure of an influenza virus vaccine and 
more recent clinical study of nontoxic LTI support a connec- 
tion 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-CTA2/B delivery will be 
examined due to reports that these molecules can redirect anti-

vating IgG1. In vitro functional assays of antibodies revealed a 
significant reduction in internalized and cell-bound bacteria on 
human epithelial cells after preincubation of IsdA-CTA2/B 
imune serum with the S. aureus isolate used for vaccination, 
MRSA252. In addition, antibodies were able to prevent adhe- 
sion of MRSA USA300. IsdA from MRSA252 and MRSA 
USA300 has 92% amino acid identity with the majority of 
differences present within the C terminus. These results sug- 
gest 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 
responses induced by IsdA-CTA2/B are superior to those stimu-
lated by a mixed preparation of antigen and adjuvant (IsdA 
plus CTA2/B). Thus, the structure of the IsdA-CTA2/B chi- 
mera 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).

Our results suggest that the IsdA-CTA2/B chimera induces an 
IsdA-specific systemic IgG, mucosal IgA, and cell-mediated 
response when administered intranasally to mice, and its im-
munogenicity warrants further investigation as a potential mu- 
cosal S. aureus vaccine.
ACKNOWLEDGMENTS

Much appreciation goes to Randall K. Holmes and Mike Jobling (UCHSC) for use of the pARLDR19 construct and continued support. We also thank the following people at Boise State University: Jie Yan for assistance with the intranasal immunizations and sample collection; Ken Cornell, Kristen Mitchell, and Chris Horras for technical support; and Raquel Brown for confocal microscopy training. In addition, we thank Donna MacDonald at the Boise VA Medical Center Boise, ID, for animal training.

This material is the work supported by resources from the Boise VA Medical Center Boise, ID, and by a 2008 WWAMI ISTM small grant (to J.K.T.), USDA CRIS project 65-36501-051-100, and an NSF Major Research Instrumentation grant (0619739; coprincipal investigator, J.K.T.).

REFERENCES

1. Agren, L. C., L. Ekman, B. Lowenadler, and N. Y. Lycke. 1997. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. J. Immunol. 158:3936–3946.
2. Anosova, N. G., et al. 2008. Cholera toxin, E.coli heat-labile toxin, and non-toxic derivatives induce dendritic cell migration into the follicle-associated epithelium of Peyer’s patches. Mucosal Immunol. 1:59–67.
3. Ayalew, S., D. L. Step, M. Montelongo, and A. W. Confer. 2009. Intranasal vaccination of calves with Mannheimia haemolytica chimeric protein containing the major surface epitope of outer membrane lipoprotein PIlP, the neutralizing epitope of leukoan, and cholera toxin subunit B. Vet. Immunol. Immunopathol. 132:295–302.
4. Bagley, K. C., S. F. Abdulwahab, R. G. Tuskan, and G. K. Lewis. 2003. An enzymatically active domain is required for cholera-like enterotoxins to induce a long-lived blockade on the induction of oral tolerance: new method for screening mucosal adjuvants. Infect. Immun. 71:6850–6856.
5. Bastiaens, P. L. I. V. Majoul, P. J. Vermeer, H. D. Soling, and T. M. Jovin. 1996. Imaging the intracellular trafficking and state of the ABS quaternary structure of cholera toxin. EMBO J. 15:4231–4235.
6. Brown, E. L., et al. 2009. The Panton-Valentine leukocidin vaccine protects mice against lung and skin infections caused by Staphylococcus aureus USA300. Clin. Microbiol. Infect. 15:156–164.
7. Clarke, S. R., et al. 2011. Identification of in vivo-expressed antigens of Staphylococcus aureus and their use in vaccinations for protection against nasal carriage. J. Infect. Dis. 203:1098–1108.
8. Clarke, S. R., et al. 2006. Intranasal administration of cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. Infect. Immun. 74:2408–2416.
9. Clarke, S. R., et al. 2011. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and enterotoxin. Vaccine 29:6461–6468.
10. Kawamura, Y. I., et al. 2003. Cholera toxin activates dendritic cells through dependence on GM1-ganglioside which is mediated by NF-κB translocation. Eur. J. Immunol. 33:3205–3212.
11. Klein, E., D. L. Smith, and R. Laxminarayan. 2007. Hospitalizations and deaths caused by mephitin-resistant Staphylococcus aureus, United States, 1999–2005. Emerg. Infect. Dis. 13:1840–1846.
12. Kleytmans, J. A., and H. F. Wertheim. 2005. Nasal carriage of Staphylococcus aureus and prevention of nosocomial infections. Infection 33:3–8.
13. Lee, S. F., S. A. Halperin, D. F. Salloum, A. MacMillan, and A. Morris. 2003. Mucosal immunization with a genetically engineered pertussis toxin SI fragment-cholera toxin subunit B chimeric protein. Infect. Immun. 71:2272–2277.
14. Li, X., et al. 2004. Use of the untranslated fusion of the MrpH fimbrial adhesin-binding domain with the cholera toxin A2 domain, coexpressed with the cholera toxin B subunit, as an intranasal vaccine to prevent experimental urinary tract infection by Proteus mirabilis. Infect. Immun. 72:7306–7310.
15. Liu, J. M., et al. 2008. Bacteriophage-induced IsdA to IsdB in the iron-regulated surface determinant (Isd) heme acquisition system of Staphylococcus aureus. J. Biol. Chem. 283:665–6676.
16. Lycke, N., T. Tsujii, and J. Holmgren. 1992. The adjuvant effect of Vibrio cholerae and Eschericia coli heat-labile enterotoxins is linked to their ADP-ribose transferase activity. Eur. J. Immunol. 22:2277–2281.
17. Majoul, I. V., P. I. Bastiaens, and H. D. Soling. 1996. Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells. J. Biol. Chem. 271:777–789.
18. Marinaro, M., et al. 1995. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J. Immunol. 155:4621–4629.
19. Mazmanian, S. K., H. Ton-That, K. Su, and O. Schneewind. 2002. An iron-regulated sortase anchors a class of surface protein during Staphylococcus aureus pathogenesis. Proc. Natl. Acad. Sci. U. S. A. 99:2293–2298.
20. Mutsch, M., et al. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell’s palsy in Switzerland. N. Engl. J. Med. 350:896–903.
21. Parish, C. R., M. H. Glidden, B. J. Quah, and H. S. Warren. 2009. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. Curr. Protoc. Immunol., chapter 4, unit 4.9. http://onlinelibrary.wiley.com/doi/10.1002/0471142735.im0409s84/full.
22. Pizza, M., et al. 2001. Mucosal vaccines: nontoxic derivatives of LT and CT as mucosal adjuvants. Vaccine 19:2534–2541.
23. Rebolatto, M. C., L. Siger, and H. Hogerse. 2001. Kinetics and type of immune response following intranasal and subcutaneous immunisation of calves. Res. Vet. Sci. 78:19–51.
24. Sanchez, J., J. Holmgren, and A. M. Svennerholm. 1990. Recombinant

Downloaded from http://cvl.asm.org/ on July 20, 2018 by guest
fusion protein for simple detection of *Escherichia coli* heat-stable enterotoxin by GM1 enzyme-linked immunosorbent assay. J. Clin. Microbiol. 28: 2175–2177.

47. Schnitzler, A. C., J. M. Burke, and L. M. Wetzler. 2007. Induction of cell signaling events by the cholera toxin B subunit in antigen-presenting cells. Infect. Immun. 75: 3150–3159.

48. Shen, Z., G. Reznikoff, G. Dranoff, and K. L. Rock. 1997. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J. Immunol. 158: 2723–2730.

49. Stiles, B. G., A. R. Garza, R. G. Ulrich, and J. W. Boles. 2001. Mucosal vaccination with recombinantly attenuated staphylococcal enterotoxin B and protection in a murine model. Infect. Immun. 69: 2031–2036.

50. Stranger-Jones, Y. K., T. Bae, and O. Schneewind. 2006. Vaccine assembly from surface proteins of *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U. S. A. 103: 16942–16947.

51. Sultan, F., L. L. Jin, M. G. Jobling, R. K. Holmes, and S. L. Stanley, Jr. 1998. Mucosal immunogenicity of a holotoxin-like molecule containing the serine-rich *Entamoeba histolytica* protein (SREHP) fused to the A2 domain of cholera toxin. Infect. Immun. 66: 462–468.

52. Sun, J. B., C. Czerkinsky, and J. Holmgren. 2010. Mucosally induced immunological tolerance, regulatory T cells and the adjuvant effect by cholera toxin B subunit. Scand. J. Immunol. 71: 1–11.

53. Verkaik, N. J., et al. 2010. Heterogeneity of the humoral immune response following *Staphylococcus aureus* bacteremia. Eur. J. Clin. Microbiol. Infect. Dis. 29: 509–518.

54. Vermeiren, C. L., M. Pluym, J. Mack, D. E. Heinrichs, and M. J. Stillman. 2006. Characterization of the heme binding properties of *Staphylococcus aureus* IsdA. Biochemistry 45: 12867–12875.

55. Wertheim, H. F., et al. 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteremia in nasal carriers versus non-carriers. Lancet 364: 703–705.

56. Xu-Amano, J., et al. 1994. Helper Th1 and Th2 cell responses following mucosal or systemic immunization with cholera toxin. Vaccine 12: 903–911.