



Intradermal or Sublingual Delivery and Heat-Labile Enterotoxin Proteins Shape Immunologic Responses to a CFA/I Fimbria-Derived Subunit Antigen Vaccine against Enterotoxigenic *Escherichia coli*

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ABSTRACT Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of infectious diarrhea in children, travelers, and deployed military personnel. As such, development of a vaccine would be advantageous for public health. One strategy is to use subunits of colonization factors combined with antigen/adjuvant toxoids as an ETEC vaccine. Here, we investigated the intradermal (i.d.) or sublingual (s.l.) delivery of CFA/I fimbrial antigens, including CfaEB and a CfaE-heat-labile toxin B subunit (LTB) chimera admixed with double mutant heat-labile toxin (LT) LT-R192G/L211A (dmLT). In addition, we compared dmLT with other LT proteins to better understand the generation of adjuvanted fimbrial and toxoid immunity as well as the influence on any local skin reactivity. We demonstrate that immunization with dmLT admixed with CfaEB induces robust serum and fecal antibody responses to CFA/I fimbriae and LT but that i.d. formulations are not optimal for s.l. delivery. Improved s.l. vaccination outcomes were observed when higher doses of dmLT (1 to 5 μ g) were admixed with CfaEB or, even better, when a CfaE-LTB chimera antigen was used instead. Serum anti-CFA/I total antibodies, detected by enzyme-linked immunosorbent assay, were the best predictor of functional antibodies, based on the inhibition of red blood cell agglutination by ETEC. Immunization with other LT proteins or formulations with altered B-subunit binding during i.d. immunization (e.g., by addition of 5% lactose, LTA1, or LT-G33D) minimally altered the development of antibody responses and cytokine recall responses but reduced skin reactivity at the injection site. These results reveal how formulations and delivery parameters shape the adaptive immune responses to a toxoid and fimbria-derived subunit vaccine against ETEC.

KEYWORDS CFA/I, ETEC, LTA1, dmLT, intradermal, sublingual, vaccine

Diarrheal illness contributes to malnutrition, stunted growth, impaired cognitive development, and high morbidity rates, affecting 20% of children worldwide (1). In children under age 5 years, enterotoxigenic *Escherichia coli* (ETEC) accounts for 14,000 to 42,000 deaths annually (2). Both travelers and deployed military personnel are also populations vulnerable to ETEC-induced diarrhea and potential disease complications (3, 4). Thus, development of an ETEC vaccine would benefit multiple populations across the world.

ETEC induces diarrheal disease through heat-stable enterotoxin (ST) or heat-labile

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enterotoxin (LT), but attachment to small intestinal epithelial cells via expressed colonization factors (CFs) is also critical. One vaccine strategy is to target toxin proteins and CFs, such as adhesins and pili (5). Passive protection with antibodies against the CF CFA/I minor adhesin (CfaE) can protect human volunteers from diarrheal disease (6) and nursing mouse pups (7). Immunization with CfaE antigen also elicits functional antibodies able to disrupt bacterial adherence. Moreover, CfaE administered with the mutant LT LT-R192G (mLT) protected *Aotus nancymae* nonhuman primates from experimental diarrhea with CFA/I⁺ ETEC strain H10407, with intranasal (i.n.) immunization being better than oral immunization (8). Additionally, a newer antigen has been designed to expand antibody epitopes by fusing the major subunit of CFA/I (CfaB, pilin domain) to CfaE, creating CfaEB. CfaEB exhibits native folding properties and has a 1:1 ratio between the subunits (9), in contrast to the bacterial class 5 fimbriae, which have adhesin/pilus ratios closer to 1:1,100 (10, 11), and other fusion proteins, which can lack conformation integrity.

LT is an ADP-ribosylating protein similar to cholera toxin with an AB₅ structure that is expressed by ~60% of clinical ETEC strains and binds primarily to GM1 gangliosides on cells (12, 13). LT is both immunogenic and an adjuvant, inducing antibody responses to both itself and a codelivered antigen(s) (14). LT-producing ETEC strains (alone or in combination with ST- and LT-producing ETEC strains) exhibit important regional and seasonal contributions to the burden of diarrhea (15, 16). In studies with breast-feeding infants and natural exposure or adult experimental challenge models, anti-LT IgA or IgG antibodies are strongly associated with protection from ETEC (17, 18). In addition, the risk for recurrent ETEC diarrheal disease in areas of endemicity drops after 5 years of age (19, 20), concurrent with the development of anti-LT antibodies (21).

We and others have previously analyzed the role of LT subunits and found that the LT B subunit (LTB) is critical for protein uptake at gastrointestinal mucosal surfaces (22, 23) and promotes antitoxin antibodies (24, 25) but is not a good adjuvant by itself (14). In addition, chimera proteins consisting of genetic or chemical fusions of LTB or the cholera toxin B subunit to an ancillary antigen(s) can promote antigen uptake (26, 27). However, GM1 binding during intradermal (i.d.) immunizations in both mice and humans can contribute to minor skin reactogenicity, which is preventable through a G33D mutation in the B subunit (28, 29). In contrast, the enzymatic A subunit (LTA) and the A1 domain (LTA1) are good adjuvants when they are delivered by i.n. immunization but are weak toxoid antigens (22, 30). The development of adjuvanted immunity by LT proteins, including the induction of Th17, IgG2a, and IgA responses to a codelivered antigen, is A subunit dependent, although the magnitude of the postvaccination responses is maximized with admixed LTA and LTB or with AB₅ toxoids (22, 30). The most advanced candidate for an LT toxoid adjuvant/antigen is currently an AB₅ LT mutant with two mutations in the A subunit that alter the protease sensitivity while retaining the immunologic properties of native LT (referred to as the double mutant LT [dmLT]) (31, 32). This mutant LT replaces an earlier version of this molecule, mLT, since it is less enterotoxic when administered orally (32). dmLT provides both antigenic and adjuvant properties, has proved safe in oral and sublingual (s.l.) phase 1 safety studies (32, 33), and is currently being tested for i.d. delivery (ClinicalTrials.gov registration number NCT02531685).

Selection of the appropriate route for vaccine delivery is critical. While intramuscular injection is common, i.d. delivery has been suggested to be better due to the higher density of antigen-presenting cells in the skin than in muscle tissue (34) and the possibility of dose sparing (35–37). However, this strategy is not optimal in a resource-poor setting, since trained personnel to administer the injection may be limited and unsafe needle use can lead to disease outbreaks (38–41). Mucosal immunization is an attractive option, particularly given the gastrointestinal location of ETEC infection and disease; however, i.n. delivery is not a feasible option with an AB₅ LT toxoid (42), and oral delivery requires large doses of antigens and risks the degradation of subunit antigens by gastric proteases and low pH. One option is to use s.l. delivery (43). A recent publication describing dmLT and ETEC adhesion protein EtpA vaccination indicated

that either s.i. or oral immunization protected mice from the development of bacterial colonization in an antibiotic-ETEC challenge model, despite a 5 to 40 times lower antigen dose in the s.i. groups (44).

In the current study, we compared the use of the i.d. and s.i. delivery of two different fimbrial tip adhesin fusion proteins, CfaEB or a CfaE-CTA2/LTB chimera protein (referred to here as the chimera or CfaE-LTB), with dmLT in mouse models. In addition, we evaluated alternative forms of LT for antigenicity, adjuvanticity, and i.d. skin reactogenicity to better understand the contribution of an LT protein to immunization outcomes. Our findings have a direct impact for vaccine formulation strategies against ETEC and enhance our understanding of how LT-derived proteins interact with host cells at distinct tissue sites to promote immunologic responses.

RESULTS

The antigen/adjuvant dmLT admixed with CfaEB antigen improves i.d. immunization outcomes, but this formulation is not optimal for s.i. delivery. To test vaccination with CfaEB and dmLT, we immunized groups of BALB/c mice by the i.d. or s.i. route with 10 μ g CfaEB alone or in combination with 0.1 μ g dmLT. The animals were boosted after 4 weeks, and then serum and fecal samples were analyzed 2 weeks later for the development of anti-CFA/I or LT antibodies (Fig. 1A to E).

For the groups immunized i.d., mice immunized with CfaEB developed significant levels of anti-CFA/I serum total antibodies (total Ig) and IgG1 compared to naive mice ($P \leq 0.05$; Fig. 1B and D). However, as expected, mice immunized with CfaEB-dmLT developed significantly more robust and diverse responses than mice immunized with CfaEB alone, including serum IgG1, IgG2a, and fecal IgA antibody responses ($P \leq 0.05$; Fig. 1B and D). In contrast, mice receiving identical formulations by s.i. delivery did not develop a significant CFA/I antibody response (Fig. 1D). All mice immunized i.d. or s.i. with CfaEB-dmLT developed anti-LT fecal IgA, but only the group immunized i.d. developed significant circulating serum antibodies (e.g., anti-LT total Ig, IgG1, and IgG2a [$P \leq 0.001$]; Fig. 1C and E). Thus, as expected, mice immunized i.d. with CfaEB developed serum and fecal anti-CFA/I antibodies, and the inclusion of dmLT enhanced the magnitude of these responses. However, the same formulations were not ideal for s.i. delivery, indicating that the antigen composition, antigen dose, and/or adjuvant dose needs to be optimized to increase the antibody response elicited by this route.

Higher doses of dmLT combined with CfaEB or CfaE-LTB chimera antigen improve s.i. immunization outcomes. s.i. vaccination likely requires effective antigen transport to cross mucosal tissue and avoid salivary protease degradation. Older studies using dmLT in polio virus, *Shigella*, or EtpA ETEC vaccines utilized 0.2, 5, or 10 μ g dmLT (44–47). Since our s.i. immunization with 0.1 μ g dmLT failed (Fig. 1D), we next evaluated if 10 μ g CfaEB combined with higher doses of dmLT alone (1 or 5 μ g) or combined with excess LTB (10 μ g) would improve the vaccination outcomes. Concurrently, we also evaluated whether an LTB fusion antigen would be more effective by using a chimera protein containing the minor adhesin subunit CfaE linked to LTB through a cholera toxin A2 peptide sequence (full name, CfaE-CTA2/LTB, here abbreviated CfaE-LTB). Groups of mice were immunized by prime/boost s.i. and evaluated for the development of serum and fecal CFA/I and LT antibodies.

As before, mice immunized s.i. with CfaEB did not develop significant levels of serum or fecal anti-CFA/I antibodies compared to naive mice (Fig. 2A and B, top). However, mice immunized with CfaEB admixed with 1 μ g dmLT, 5 μ g dmLT, or 1 μ g dmLT and 10 μ g LTB developed similar levels of serum anti-CFA/I total Ig, IgG1, and IgG2a. The total Ig levels were statistically significantly different from the total Ig levels in naive mice ($P \leq 0.001$), but the levels of serum anti-CFA/I total Ig, IgG1, and IgG2a were not statistically significantly different from each other. No groups immunized with CfaEB antigen developed anti-CFA/I fecal IgA. In contrast, mice immunized with the CfaE-LTB chimera antigen developed robust anti-CFA/I antibodies compared to naive mice ($P \leq 0.01$), and with the exception of serum IgG1, these antibodies were significantly enhanced with the addition of 1 μ g dmLT ($P \leq 0.01$). This CfaE-LTB–dmLT group

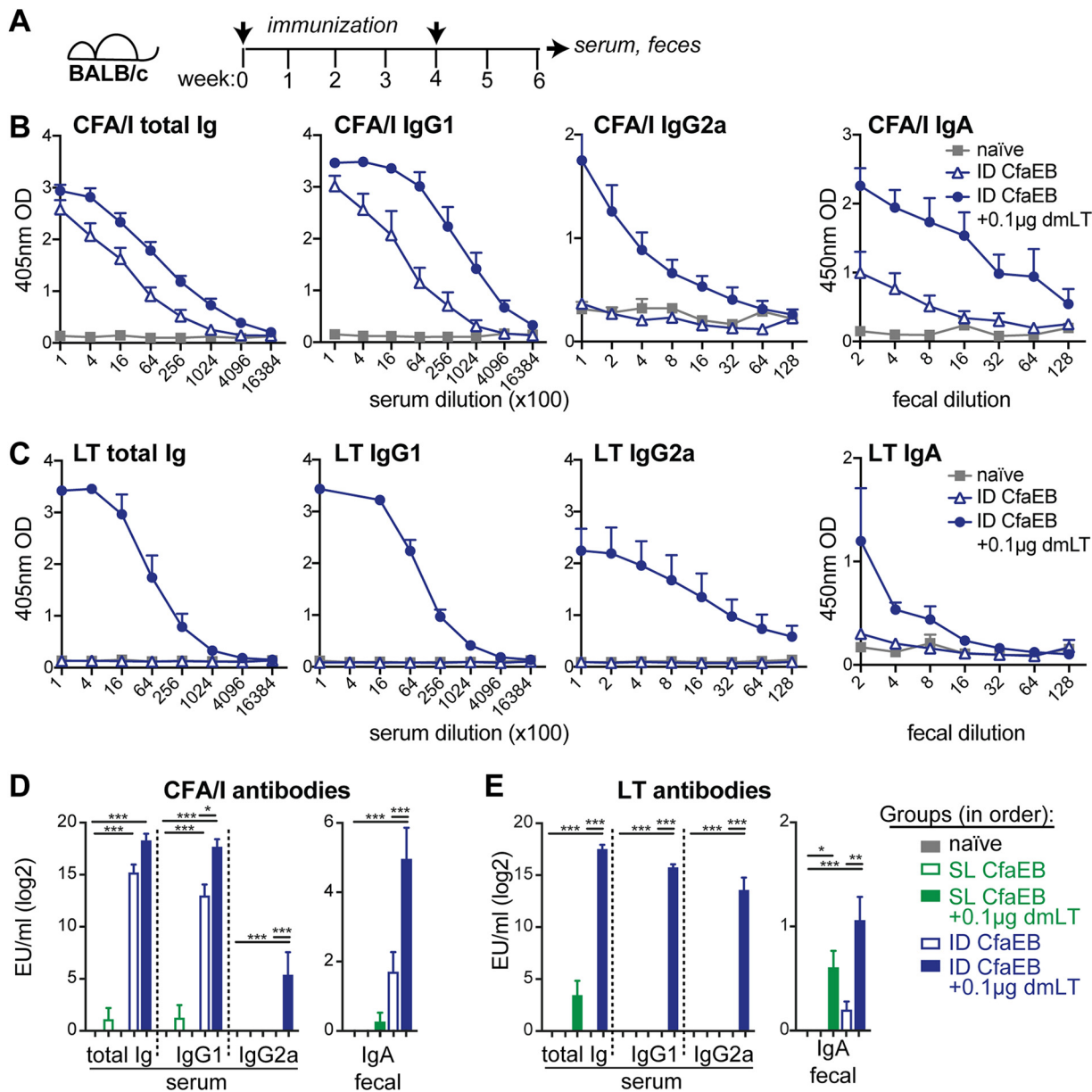


FIG 1 Immunization with dmlT admixed with CfaEB induces robust antibody responses to CFA/I fimbriae and LT, but i.d. formulations are not optimal for s.l. delivery. (A) Schematic of immunization experiments. BALB/c mice were left naive or immunized with 10 µg CfaEB with or without 0.1 µg dmlT by i.d. ($n = 5$) or s.l. ($n = 10$) delivery on weeks 0 and 4, and then samples were collected at week 6. (B) Raw ELISA data for anti-CFA/I antibodies, given as the optical density (OD), for the i.d. immunized groups. (C) Raw ELISA data for anti-LT antibodies for the i.d. immunized groups. (D) Compiled number of ELISA units (EU) per milliliter for anti-CFA/I serum and fecal antibodies after i.d. and s.l. immunizations. (E) Compiled numbers of EU per milliliter for anti-LT serum and fecal antibodies after i.d. and s.l. immunizations. All bars are the mean + SEM. Significance was determined using a one-way ANOVA with the Bonferroni posttest for all groups compared to the naive group and for the i.d. CfaEB group compared to the i.d. CfaEB plus 0.1 µg dmlT group. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

exhibited the highest anti-CFA/I serum total Ig and IgG2a levels and fecal IgA levels observed in any s.l. group, and the antibody levels were more similar to those achieved with i.d. immunization (Fig. 1). As expected, high levels of anti-LT serum and fecal antibodies were observed in all groups receiving dmlT, CfaE-LTB, and/or LT antigen ($P \leq 0.001$; Fig. 2A and B, bottom), with similar levels being observed between these groups, except for the group receiving CfaE-LTB alone. Thus, s.l. immunization with the fimbrial antigen and optimal doses of dmlT can induce robust circulating antibodies to CFA/I. However, the generation of fecal IgA responses required an alternative antigen form that included a mucosal binding component (i.e., LTb).

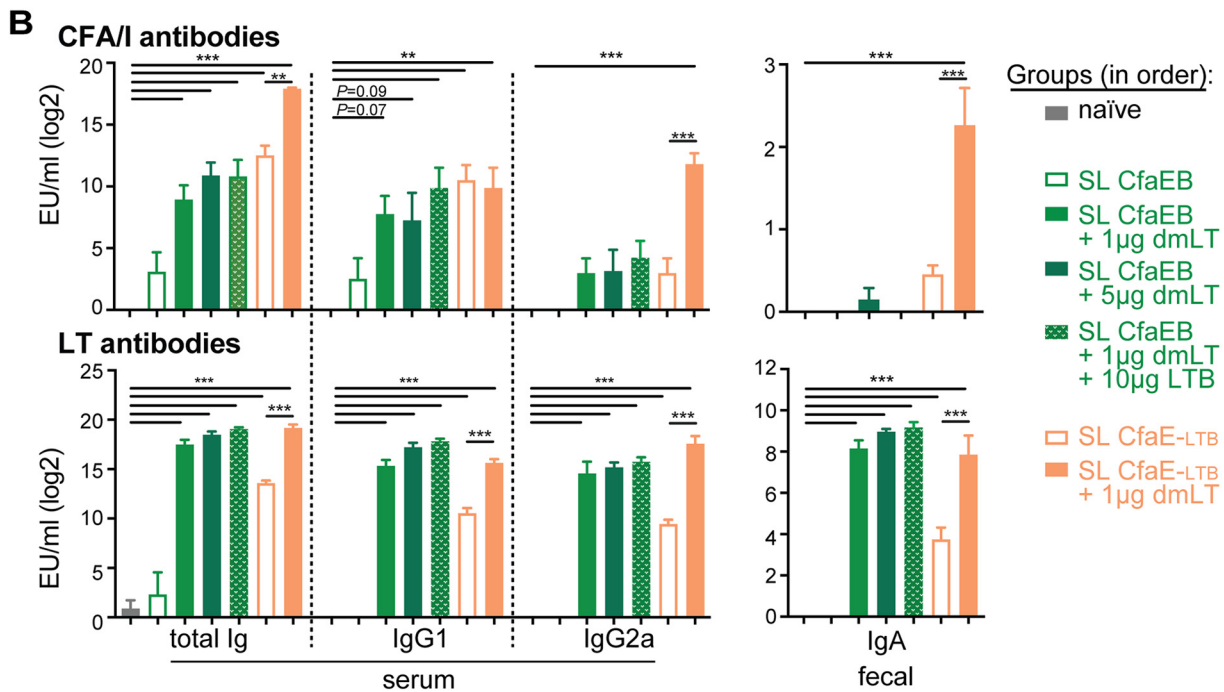
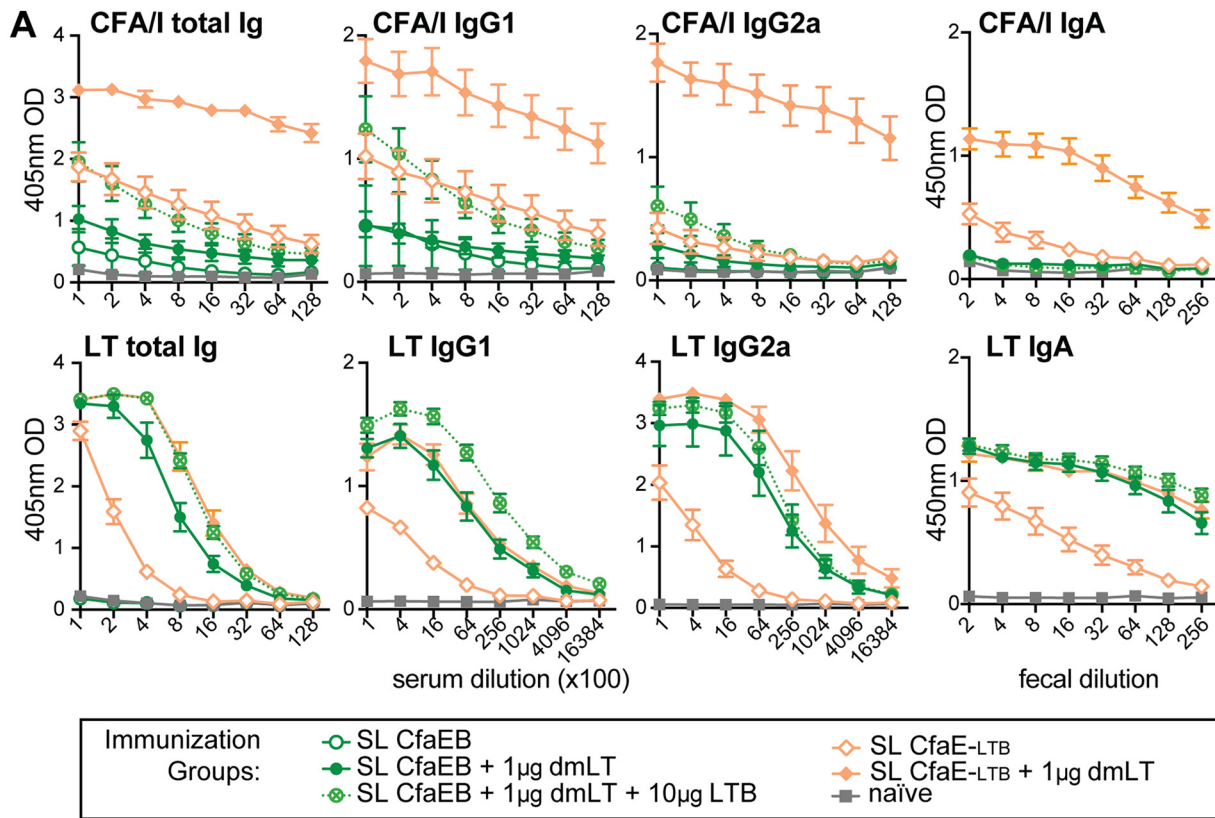


FIG 2 Higher doses (1 to 5 µg) of dmLT combined with CfaEB or the CfaE-LTB chimera improve s.i. vaccination outcomes. Mice were left naïve or immunized with 10 µg CfaEB or the CfaE-LTB chimera with or without the indicated doses of dmLT and LTB by s.i. delivery (n = 9 to 10 per group) on weeks 0 and 4, and then samples were collected at week 6. (A) Raw data from ELISA testing for anti-CFA/I or anti-LT antibodies, as shown after s.i. immunizations (data for the CfaEB plus 5 µg dmLT group are not shown). (B) Compiled numbers of EU per milliliter for anti-CFA/I and anti-LT serum and fecal antibodies after s.i. immunization given as the mean + SEM. Significance was determined using a one-way ANOVA with the Bonferroni posttest for all groups compared to the naïve group and the CfaE-LTB group compared to the CfaE-LTB plus dmLT group. **, P ≤ 0.01; ***, P ≤ 0.001.

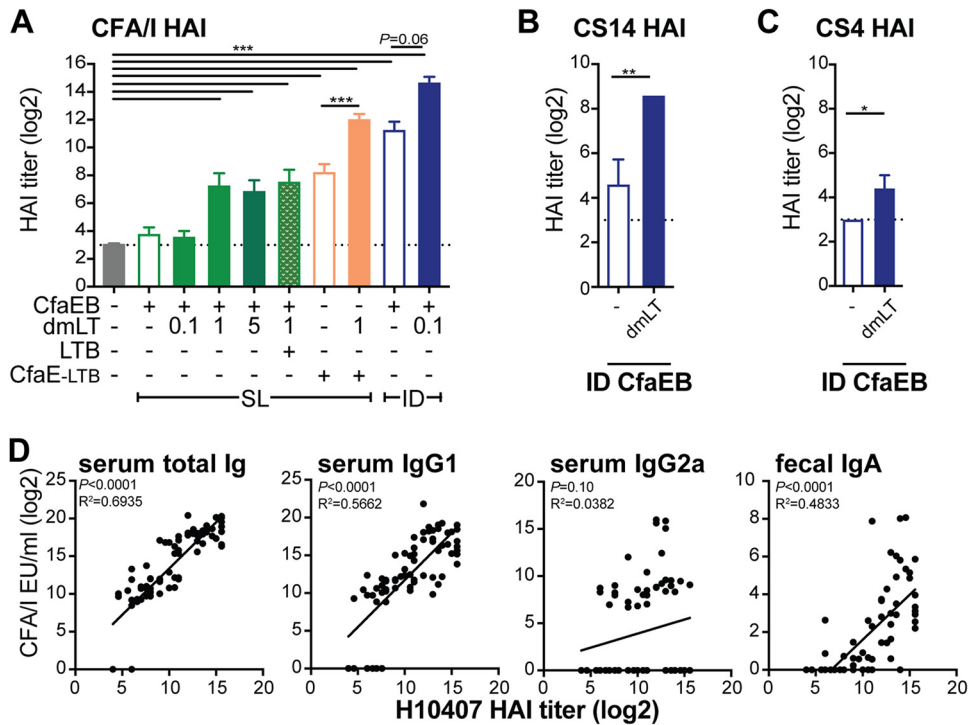


FIG 3 CFA/I Ig and IgG1 are the best predictors of strain H10407 HAI responses, which were the most strongly induced in the dmLT-adjuvanted i.d. or CfaE-LTB chimera group. Sera from all i.d. and s.i. studies were assessed for HAI titers of functional antibodies. (A) HAI titers in serum of functional antibodies against CFA/I⁺ ETEC. (B) Serum HAI titers of antibodies against CS14⁺ ETEC. (C) Serum HAI titers of antibodies against CS4⁺ ETEC. All bars are the mean + SEM, with the dotted line indicating the assay limit of detection (LOD). Significance was determined using one-way ANOVA with the Bonferroni posttest for all groups compared to the naive group or as shown (A) or using a two-tailed t test (B and C). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. (D) Linear regressions between H10407 HAI titers and serum CFA/I IgG, IgG1, and IgG2a and fecal IgA antibodies (log₂ transformed), with P values and R^2 (coefficient of determination) values indicated. Values were included in the regression analyses if the HAI titers were not below the limit of detection (which excluded 74 values).

CFA/I serum total antibody ELISA results are the best predictor of functional antibody responses, with CFA/I serum IgG1 and fecal IgA also being significant.

The goal of a fimbrial antigen is to elicit an antibody response to prevent ETEC bacterial attachment to mucosal tissue. We exploited the mannose-resistant hemagglutination property of the ETEC class 5 adhesins to assess the functionality of antiadhesin antibodies to inhibit the agglutination of bovine red blood cells (RBCs) using a hemagglutination inhibition (HAI) assay as a proxy for bacterial intestinal attachment (5). Using sera from all i.d. and s.i. experiments performed with a prime and a boost 4 weeks apart (Fig. 1 and 2; see also Fig. 7), we determined HAI titers using the CFA/I-expressing H10407 ETEC strain. Serum HAI titers mirrored those obtained by antibody enzyme-linked immunosorbent assays (ELISAs), with the highest levels being observed in the CfaEB-dmLT i.d. or the CfaE-LTB-dmLT s.i. group ($P \leq 0.01$; Fig. 3A). Only for the groups immunized i.d., we also tested sera for in-class heterologous HAI titers using CS14- and CS4-expressing ETEC strains. We observed a significant expansion in the ability of serum antibodies from the CfaEB-dmLT i.d. group compared with those from the CfaEB i.d. group to neutralize other ETEC class 5a strains ($P \leq 0.05$; Fig. 3B and C).

To determine the strength of the relationship of specific antibodies to H10407 HAI titers, we performed linear regressions between all collected data (Fig. 3D). Serum total Ig and IgG1 responses and fecal IgA responses were significantly related to HAI titers ($P \leq 0.001$), but serum IgG2a responses were not. R^2 values indicated that the variation in HAI titers could be explained by a linear relationship with serum total Ig (69%) and IgG1 (57%) responses and fecal IgA responses (48%). Thus, immunized animals displayed functional antibodies that inhibited bacterial binding, and these were more

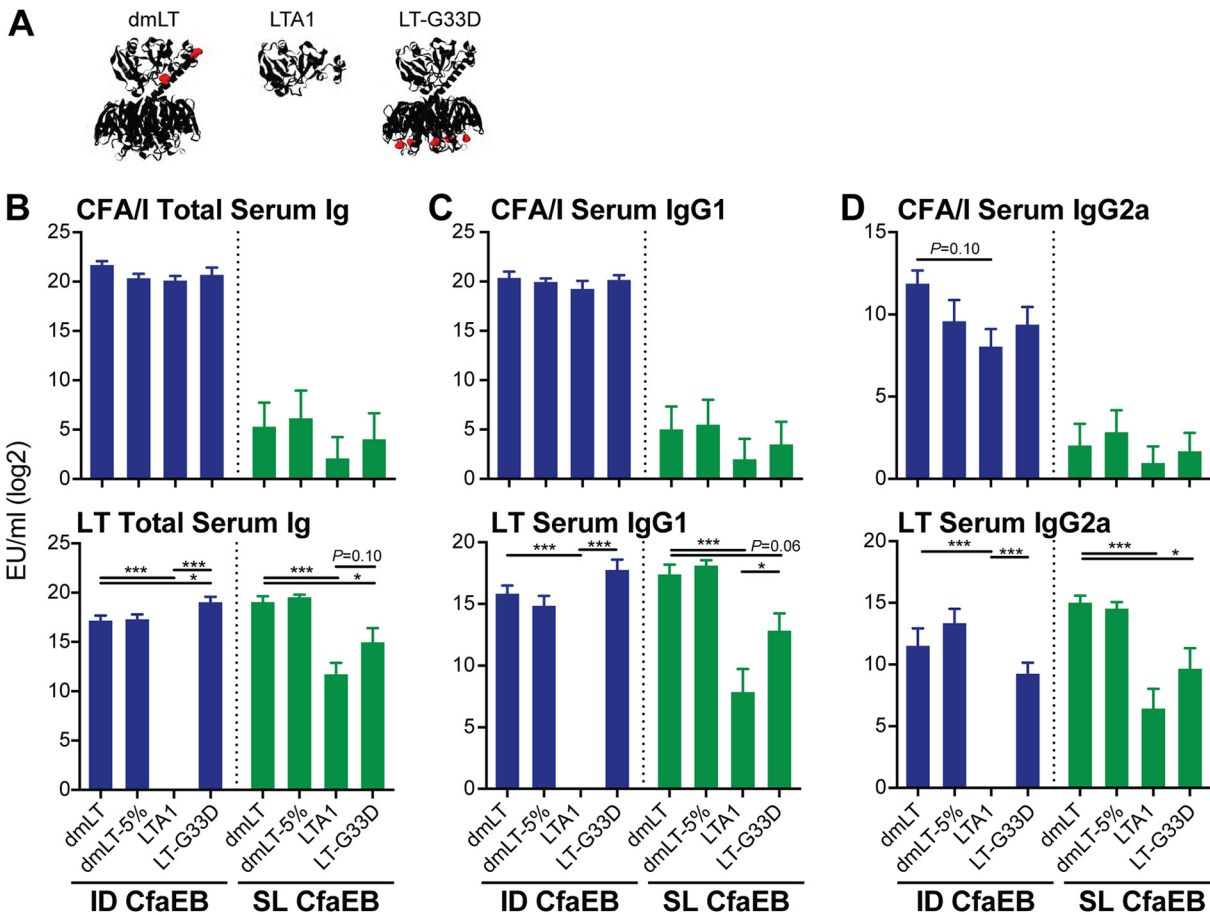


FIG 4 Altered binding groups (dmLT-5%, LTA1, LT-G33D) exhibit fewer changes by i.d. than by s.l. immunization. Mice were left naive or immunized with 10 μ g CfaEB alone or with 10 μ g CfaEB admixed with 0.1 μ g dmLT, 0.1 μ g dmLT in 5% lactose buffer (dmLT-5%), 1 μ g LTA1, or 0.1 μ g LT-G33D by the i.d. route or 5 μ g of protein (dmLT, LTA1, LT-G33D) by s.l. delivery on weeks 0 and 3, and then samples were collected at week 5 ($n = 4$ to 6 per group). (A) Ribbon diagram of dmLT, LTA1, and LT-G33D, with mutations indicated in red. (B) Total number of serum Ig ELISA units (EU) per milliliter for anti-CFA/I (top) or anti-LT (bottom). (C) Serum IgG1 for anti-CFA/I (top) or anti-LT (bottom). (D) Serum IgG2a for anti-CFA/I (top) or anti-LT (bottom). All bars are the mean + SEM. Significance was determined by a one-way ANOVA with the Bonferroni posttest for all adjuvanted groups compared to the CfaEB-dmLT group. *, $P \leq 0.05$; ***, $P \leq 0.001$.

highly related to anti-CFA/I total antibodies than serum IgG1 and fecal IgA. This indicates that total serum antibodies detected by ELISA can act as a proxy for expected functional HAI antibodies, at least for our experimental conditions.

Alteration of LT protein B-subunit binding within the i.d. or s.l. vaccine formulation modestly impacts the development of fimbrial humoral and cellular immunity but can reduce toxoid immunity.

It remains an important consideration to understand how LT proteins contribute to vaccination outcomes, including efficacy and safety. We and others have compared dmLT with alternative LT protein antigens or formulations (22, 31, 48, 49), but this has yet to be performed with fimbrial adhesin antigens and by i.d. delivery. We were particularly interested in the role of B-subunit binding, since this played such an important role in s.l. vaccination responses (Fig. 2) and is known to affect skin reactogenicity after i.d. immunization (and, as such, can be prevented by the G33D mutation) (28). In addition, dmLT for clinical studies (e.g., a good manufacturing practice [GMP] product) is consistently packaged in vials with 5% lactose, which may impact dmLT function, as the B subunit is known to bind to galactose-containing residues (50). To investigate this, we immunized groups of mice by either the i.d. or s.l. route with CfaEB alone or CfaEB admixed with dmLT, dmLT in 5% lactose buffer (dmLT-5%), LTA1, or LT-G33D (Fig. 4A) in a prime/boost immunization as before and analyzed the changes to antibodies, antigen-recall cytokine responses,

and skin at the injection site (the groups receiving i.d. immunizations only). All LT-derived proteins except LTA1 were delivered i.d. and at the same dose as dmLT (0.1 μ g), since we have found that, without a B subunit, LTA1 requires a slightly higher dose than dmLT when delivered parenterally (22, 30; E. Valli, A. J. Harriett, M. K. Nowakowska, R. L. Baudier, W. B. Provosty, L. B. Lawson, Y. Nakanishi, and E. B. Norton, submitted for publication).

i.d. immunizations with CfaEB and admixed LT proteins resulted in comparable levels of serum anti-CFA/I total Ig, IgG1, and IgG2a antibodies, although there was a nonsignificant reduction in antibody levels in the dmLT-5%, LTA1, and LT-G33D groups compared with the dmLT group (Fig. 4B to D, blue bars). Similar formulations delivered s.i. with CfaEB elicited modest or low anti-CFA/I responses, with no clear difference being observed between the groups (Fig. 4B to D, green bars), similar to the results of the previous experiments (Fig. 2). High levels of anti-LT serum total Ig and IgG1 antibodies were observed in the dmLT-5% and LT-G33D i.d. groups, similar to the findings for the dmLT i.d. group, but these responses were markedly absent in the LTA1 i.d. group ($P \leq 0.001$; Fig. 4). Relatedly, both the LTA1 s.i. group and the LT-G33D s.i. group exhibited reduced anti-LT serum responses compared with the dmLT s.i. group ($P \leq 0.06$). Interestingly, the anti-LT IgG2a level was lower after both the i.d. and the s.i. immunizations with the GM1-binding deficient-mutant protein LT-G33D. Altogether, these results point to the importance of the B subunit in the generation of maximal anti-LT serum responses by both the s.i. and i.d. routes and indicate that the addition of 5% lactose did not appreciably alter these responses. In addition, B-subunit GM1 binding was critical for the generation of LT serum antibodies by the s.i. route or IgG2a antibodies by any route.

For these experiments, we also evaluated cellular immunity and memory CD4 T-cell responses by measuring cytokine secretion in splenocytes recovered from immunized animals and stimulated *ex vivo* with protein antigens (e.g., CFA/I, dmLT) or 15-amino-acid long peptides (e.g., CfaE or LTB peptide pools). We observed that similar cytokine levels were secreted within each immunization group in response to either protein antigens or related peptide pools, indicating that memory CD4 T cells were induced with vaccination ($P \leq 0.05$; Fig. 5A to D; see also Fig. S1 in the supplemental material). In addition, the delivery route and antigen strongly influenced the generation of these responses, with the highest levels of IL-17 and IL-2 secretion being observed after s.i. immunizations compared with the levels observed after i.d. immunizations, and higher levels of IL-17 secretion were observed with dmLT or LTB peptide restimulation than with CFA/I or CfaE peptide restimulation. The highest levels of these cellular responses were observed in the groups immunized with dmLT or dmLT-5% admixed with CfaEB, although both LTA1 and LT-G33D also induced significant cytokine secretion ($P \leq 0.05$). As expected, unlike the dmLT, dmLT-5%, and LT-G33D i.d. groups, the LTA1 i.d. group exhibited low levels of cytokine secretion in response to the LTB peptide; however, curiously, this was not as apparent in the LTA1 s.i. group for the interleukin-2 (IL-2), IL-17A, and IL-6 cytokines. These results reveal the surprising finding that cytokine recall responses indicative of CD4 T-cell memory responses (e.g., consistently equivalent levels of expression of IL-17 after s.i. delivery and after i.d. delivery or higher levels of expression of IL-17 after s.i. delivery than after i.d. delivery) are distinct from the observed serum antibody responses. In addition, the addition of 5% lactose, the absence of a B subunit, or GM1 binding did not inhibit the generation of CFA/I-specific cellular responses and only minimally altered LT-specific cellular responses.

Alteration of LT protein B-subunit binding within i.d. vaccine formulations reduces skin reactogenicity. Skin reactogenicity is an important consideration of any i.d. vaccine. To examine how altered B-subunit binding or LT proteins may impact this, we repeated the prime/boost experiments and examined the responses at 2 or 45 days postimmunization. Acute reactogenicity at 2 days was selected based on previous observations that local induration and swelling peak at this time with mLT or dmLT delivered i.d. (M. Maciel, Jr., unpublished data). Skin samples from the site of immunization were evaluated for changes, including pathology scoring on a scale ranging from

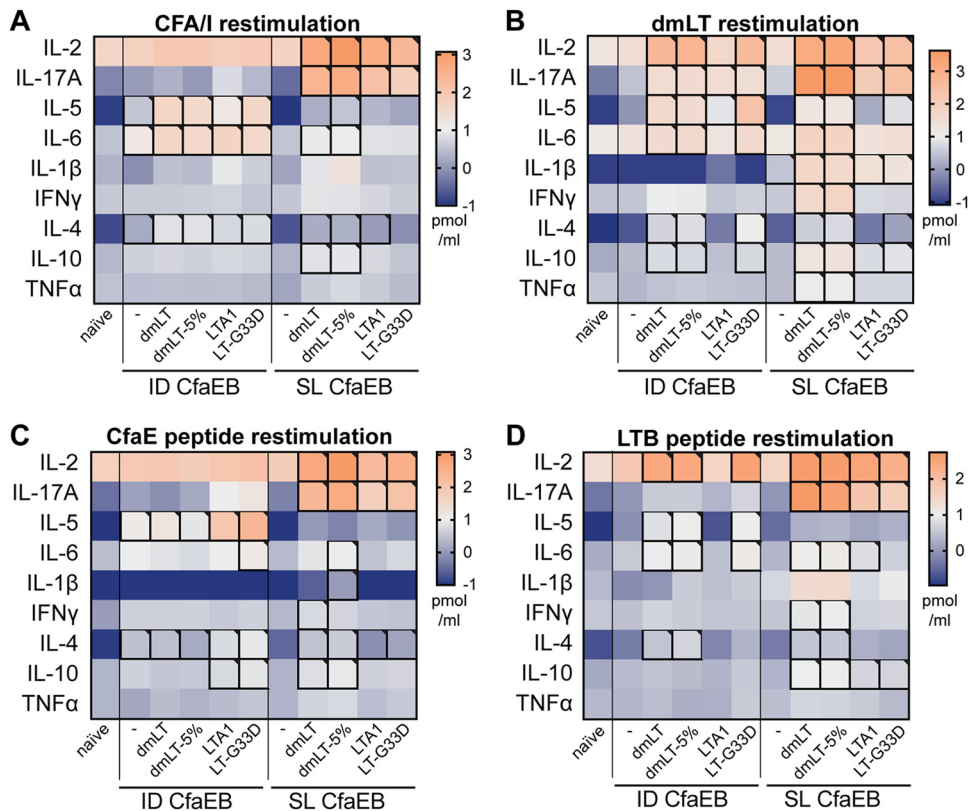


FIG 5 Recall cytokine responses after i.d. and s.l. vaccination with CFA/I or dmLT and peptide restimulation. The results obtained after *ex vivo* restimulation of splenocytes from naive or immunized mice 2 weeks after receiving a prime/boost of 10 μ g CfaEB with or without dmLT, dmLT in 5% lactose buffer, LTA1, or LT-G33D by i.d. or s.l. delivery on weeks 0 and 3 ($n = 4$ to 6 per group) are shown. Cytokines were detected in culture supernatants by a Bio-Plex assay. (A) Heat map of \log_{10} cytokine levels after CFA/I protein stimulation. (B) Heat map of \log_{10} cytokine levels after dmLT protein stimulation. (C) Heat map of \log_{10} cytokine levels after CfaE peptide pool stimulation. (D) Heat map of \log_{10} cytokine levels after LTB peptide pool stimulation. Significant values ($P \leq 0.05$) obtained by one-way ANOVA with Dunnett's posttest for all groups compared to the naive group are indicated by outlining with a black box. IFN- γ , gamma interferon; TNF α , tumor necrosis factor alpha.

0 to 5 for inflammation or skin lesions. A score of 3 (moderate dermatitis) or below is noted as an acceptable level of skin reactivity based on this scoring system (although any acceptable level would need to consider the severity, duration, and character of inflammation). There was a significant difference in the day 2 postimmunization skin gross thickness or histology scores between both the dmLT and dmLT-5% i.d. groups and the CfaEB-only i.d. group ($P \leq 0.05$) but not the LTA1 or LT-G33D i.d. group (Fig. 6A and B). This included significant inflammation (histology median score = 4) and edema in the dmLT or dmLT-5% i.d. group, characterized by neutrophil infiltration in the dermis (i.e., dermatitis) and subcutis (i.e., panniculitis) with mild epidermal hyperplasia. The LT-G33D or LTA1 i.d. group exhibited moderate inflammation (histology median score = 3) and edema with dilated lymphatics, as well as neutrophil infiltrates admixed with macrophages and a few lymphocytes. Mice immunized with CfaEB alone (histology median score = 1.5) had no edema and only mild focal aggregates of a few neutrophils, macrophages, and lymphocytes. No dermatitis or panniculitis was observed in the mock-immunized mice (mice receiving phosphate-buffered saline [PBS] buffer only).

In animals prime/boost immunized and analyzed on day 45, we did not observe any changes in gross skin thickness at the primary or secondary site of immunization from that at adjacent skin samples or in naive animals. Thus, in order to more carefully examine skin changes, the thickness of the skin was measured using hematoxylin-eosin (H&E)-stained sections that were also scored as described above (Fig. 7A). We primarily

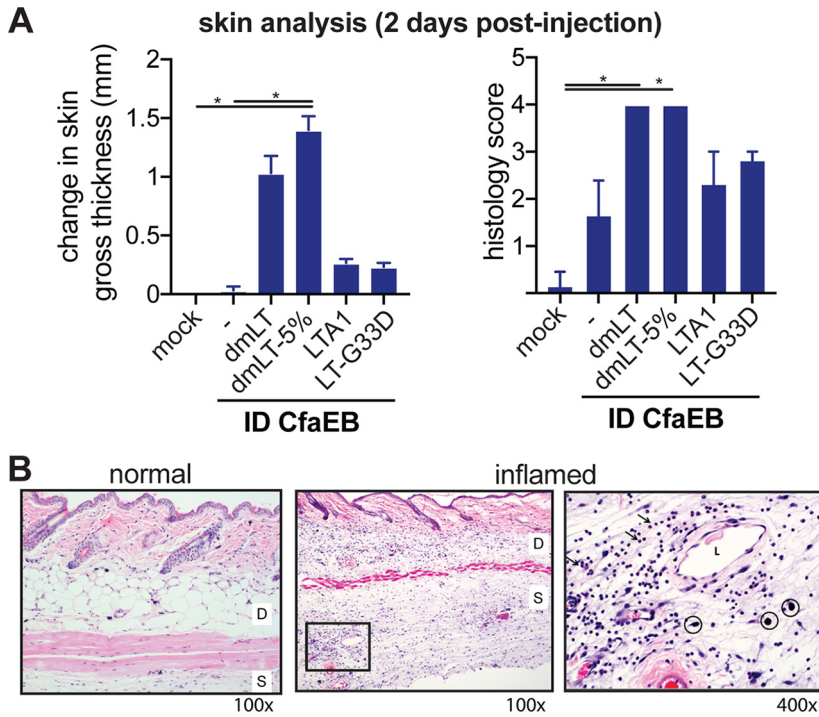


FIG 6 Acute skin reactivity is altered by LT antigen after intradermal immunization. Mice ($n = 3$ per group) were immunized with PBS buffer alone (mock) or with PBS buffer containing $10 \mu\text{g}$ CfaEB with or without dmLT, dmLT in 5% lactose buffer, LTA1, or LT-G33D by i.d. delivery (all at $0.1 \mu\text{g}$, except for $1 \mu\text{g}$ for LTA1). Skin samples were collected at 2 days postimmunization. (A) Changes to skin gross thickness, measured by the use of digital calipers, between the injection site and the adjacent back skin. The histology score for the severity of inflammation or tissue lesion is for H&E-stained skin sections and ranges from 0 to 5. Significance compares all groups to the naive or CfaEB i.d. groups and was determined using one-way ANOVA with the Kruskal-Wallis test with Dunn's posttest. *, $P \leq 0.05$. (B) Representative normal and inflamed injection sites are shown at magnifications of $\times 100$ and $\times 400$. D, dermis; S, subcutis; L, dilated lymphatics; arrows, neutrophil infiltrate; circles, mast cells. There were no differences in mast cells noted between groups.

observed significant skin reactions only in the dmLT group ($P \leq 0.01$; histology median score = 3), but these were clearly reduced from those seen at day 2 (Fig. 6A) and were characterized by infiltrates of plasma cells and lymphocytes in the dermis with few neutrophils and minimal to no edema (Fig. 7B). Lower levels of inflammation were also present in the dmLT-5% (histology median score = 2), LTA1 (histology median score = 1), and LT-G33D (histology median score = 0.33) groups. Similar to the antibody responses observed in previous experiments (Fig. 4B), all i.d. vaccinated groups were significantly different from naive animals for the development of CFA/I total Ig and CFA/I HAI ($P \leq 0.001$), but for CFA/I fecal IgA, only the responses in the dmLT ($P \leq 0.01$), dmLT-5% ($P \leq 0.001$), and LTA1 ($P \leq 0.01$) groups were significantly different from those in the naive animals. To determine if these vaccination outcomes correlated to skin reactivity, we performed correlation analyses with all data from immunized animals. Except for the histology score and anti-CFA/I serum total antibodies and fecal IgA (Fig. 7F and G), most of these relationships were not significant (Fig. 7H to K). In addition, the correlation coefficients were all closer to 0 than to 1, indicative of disagreement between values. Thus, alteration of B-subunit binding reduced skin reactivity. Furthermore, the level of skin reactivity did not strongly correlate with the development of antigen-specific antibodies. Thus, our results indicate that manipulation of B-subunit binding through either buffer formulation (e.g., addition of 5% lactose) or inclusion of a different LT antigen/adjuvant (e.g., LTA1, LT-G33D) can benefit site-specific effects without compromising vaccination outcomes.

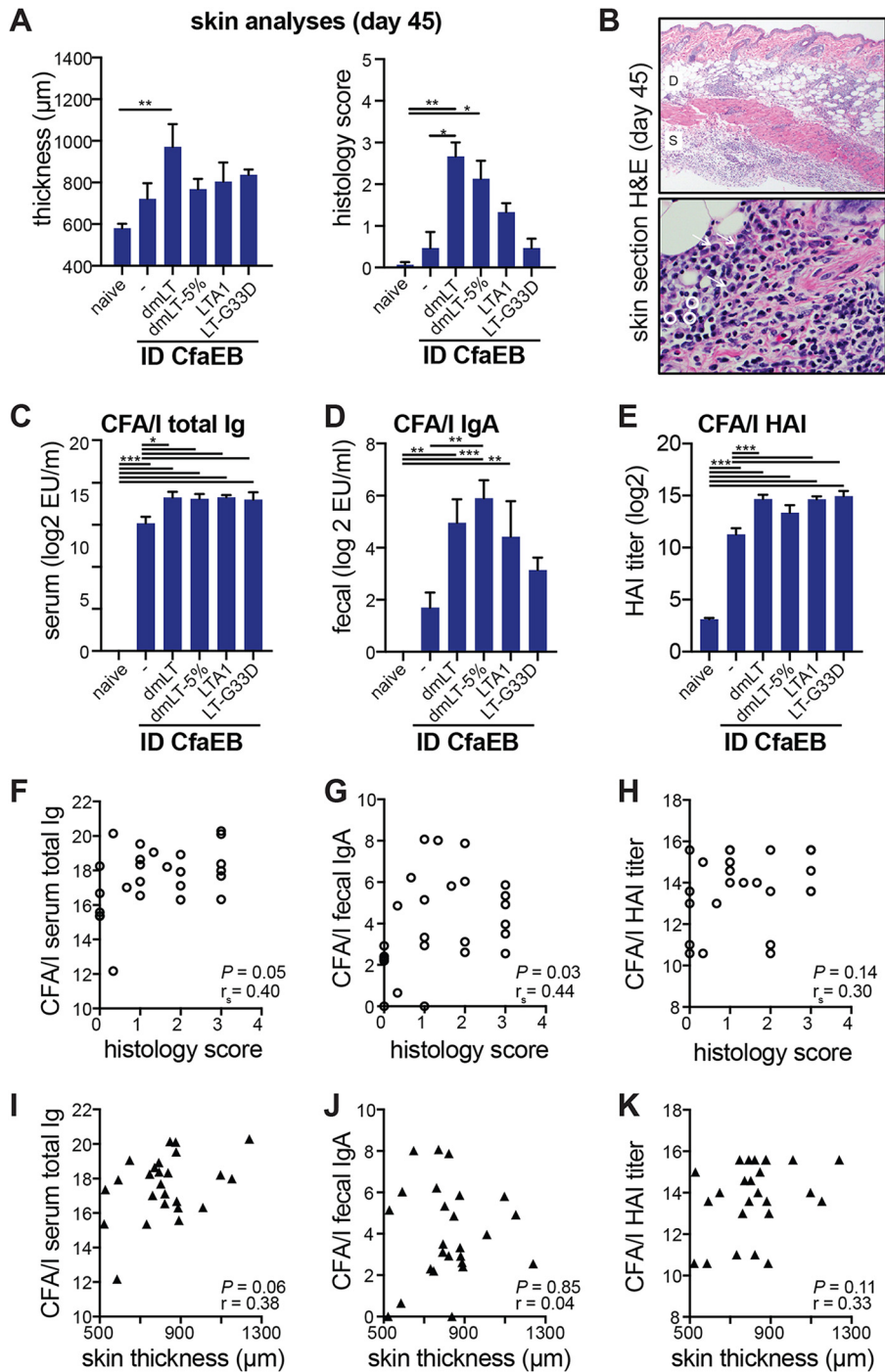


FIG 7 Chronic skin reactivity is altered by LT antigen after intradermal immunization and partially correlates to vaccination outcomes. BALB/c mice were immunized with 10 µg CfaEB with or without 0.1 µg dmlLT by the i.d. route on weeks 0 and 4, and skin samples from the injection sites were collected on week 6 (or day 45). (A) Histological scores are for the severity of inflammation or skin lesion on a scale of from 0 to 5 for H&E-stained skin sections and the thickness of the tissue samples. (B) Representative images of H&E-stained skin inflammation, identifying cellular infiltration of plasma cells (arrows) and lymphocytes (circles) but little edema. (C to E) Antibody analyses, including anti-CFA/I total serum Ig determined by ELISA (C), anti-CFA/I fecal IgA determined by ELISA (D), or serum HAI titers for functional antibodies against CFA/I⁺ ETEC (E). All bars are the mean + SEM. Significance was determined using one-way ANOVA with either the Bonferroni posttest or the Kruskal-Wallis test with Dunn's posttest (histology score only), comparing all groups to the naive or CfaEB i.d. group. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. (F to H) Spearman correlations to histology scores, with P values and correlation coefficient (r_s) indicated, using anti-CFA/I serum total Ig (F), anti-CFA/I fecal IgA (G), or CFA/I HAI titer (H). (I to K) Pearson correlation to skin thickness, with P values and correlation coefficient (r) indicated, using anti-CFA/I serum total Ig (I), anti-CFA/I fecal IgA (J), or CFA/I HAI titer (K).

DISCUSSION

An effective ETEC vaccine to protect children, travelers, and U.S. military personnel should cover about 80% of the epidemiologically relevant ETEC CFs and toxins (12). CfaEB is a fusion protein with the minor (CfaE) and major (CfaB) subunits of CFA/I, which are representative CFs from the ETEC class 5 fimbria family and which can also extend coverage against CS4 and CS14 colonization factors. Subunit protein vaccine candidates, such as CfaEB and CfaE-LTB, offer several advantages over strategies based on attenuated or killed whole-cell vaccines or purified whole fimbriae (e.g., CFA/I): (i) optimized immunogenicity, (ii) well-defined manufacturing processes, (iii) easy parenteral administration, and (iv) the possibility of combination with other vaccines on the Expanded Program on Immunization schedule. However, contrary to whole cells, which can retain their natural adjuvanticity due to pathogen-associated molecular patterns, protein-based subunit vaccines likely present low antigenicity and usually require the use of exogenous adjuvants. The use of LT-based adjuvants for a subunit-based ETEC vaccine aims at enhancing the antigenicity of the vaccine antigens as well as eliciting antitoxin immunity.

In the experiments presented here, we have shown that i.d. delivery of dmLT combined with CfaEB elicited high levels of fimbrial and toxin immunity (Fig. 1, 3, 4, 5, and 7). This is the first time that this fimbrial antigen has been evaluated with dmLT in a mouse model for the antibody and cellular response, updating older studies that utilized CfaE and mLT (8, 32, 51). We also report the first evaluation of vaccination with this combination of antigens by the s.l. route (Fig. 2 to 5), an attractive needle-free mucosal delivery route. Of note, our studies analyzed fecal IgA postimmunization. While we have previously found that fecal IgA correlates with antibody-secreting lymphocytes in intestinal tissue after parenteral immunization with the dmLT adjuvant (52), in this study, we did not specifically confirm the presence of antibody-secreting cells in the intestines. We did find that antibody responses similar to those achieved with i.d. vaccination were achieved only with the s.l. delivery of a CfaE-LTB chimera antigen in lieu of CfaEB, although as reported previously, higher levels of dmLT in s.l. formulations improve vaccine responses (44–47). This would indicate that while dmLT improves s.l. vaccines, another strategy to induce robust antibody responses is to engineer proteins to have their own mucosal binding mechanisms (e.g., by creation of an LTB fusion protein). Addition of free LTB had only a modest effect very similar to that of admixed dmLT. There is an oral ETEC vaccine in clinical trials that admixes a whole-cell killed vaccine with an LTB subunit-related toxoid (LCTBA) and small amounts of admixed dmLT (25). Our results do not show a clear advantage to this approach, at least for a subunit antigen s.l. vaccine. In contrast to our antibody responses, we observed robust cellular responses, including cytokine secretion from memory CD4⁺ T cells in the CfaEB-dmLT s.l. immunization group. High levels of IL-17, IL-2, and even gamma interferon in response to fimbrial and toxin antigen restimulation were observed by s.l. delivery, indicative of a Th17 or Th1 memory response. Many published ETEC vaccine studies have focused on the development of long-lasting antibody responses, but there has been less evaluation of memory T-cell responses, particularly at mucosal tissue. However, these may play a critical role in protection. A recently published study on a live-attenuated oral immunization with admixed dmLT (ACE527-dmLT) as an ETEC vaccine demonstrated significant clinical protection from moderate to severe diarrhea after oral challenge with the CFA/I-positive (CFA/I⁺) H10407 ETEC strain 6 months after vaccination (32, 53). However, there were no appreciable differences in serum or fecal antibody responses detected from subjects receiving ACE527 or ACE527-dmLT, despite poor disease protection in subjects receiving only ACE527. Thus, both humoral and cellular responses may play an additional, critical role in orchestrating immunity to ETEC diarrheal disease and warrant further evaluation.

There is no widely accepted challenge model for ETEC infection in rodents, as infection does not induce diarrheal disease. This is likely due to differences in attachment to intestinal cells or *in vivo* toxin expression, as direct LT or ST oral administration

induces measurable fluid secretion in the intestines (31, 54). CfaE, the minor subunit of CFA/I, is essential to the attachment of CFA/I⁺ ETEC to the human small intestine. We know that the adhesive capacity of CFA/I can be assessed *in vitro* with differentiated Caco2 cells as well as with mammalian RBCs through HAI (55). Moreover, beads coated with CfaE but not beads coated with CfaB (the major subunit of CFA/I) are able to agglutinate RBCs. The binding site of CfaE is located in the upper pole of the molecule and consists of conserved residues, including arginine 181 (56). In fact, experimental mutagenesis of that residue and its replacement by alanine led to a loss of function (9). The HAI assay can measure the *in vitro* inhibition of bacterial binding by neutralizing antibodies targeting CfaE. Contrary to the evaluation of functional neutralizing antibodies with Caco2 cells, the HAI assay is high throughput and more reproducible, although the neutralization titers measured by this assay have not yet been correlated to protection in human studies. We found that the neutralization titers obtained by the HAI assay correlated to the ELISA results, particularly the serum anti-CFA/I total antibody responses (Fig. 3). Curiously, serum anti-CFA/I IgG2a responses, often related to a Th1 or Th17 response (57), did not seem to be involved in this response, although care should be taken in extrapolating these results to other models or vaccination formulations/routes.

We also report a critical evaluation of the role of the B subunit in a subunit ETEC vaccine, with in-depth analyses being performed particularly with i.d. vaccination using a 5% lactose formulation, LTA1, or LT-G33D (Fig. 4 to 7). To our knowledge, the effect of lactose on the immunologic profile of dmLT has not been previously evaluated, even though the B subunit has a low affinity of binding to blood sugars like lactose and GMP material is included in the buffer in vials with dmLT-5% (58, 59). In previous studies, we evaluated the adjuvanticity and antigenicity of LTA1 given by the i.n. route (22, 30), and others have published information on the ability of LT-G33D to reduce skin reactogenicity while maintaining i.d. adjuvanticity (28). Our major finding was that a simple change to the buffer composition by inclusion of 5% lactose in the formulation with dmLT prevented higher skin histology scores and thicknesses after the prime/boost vaccination without any changes to serum or fecal antibodies or memory T-cell responses. This is likely due to altered or delayed binding to the GM1 on cells upon immunization and would be interesting to explore further. Substitution of altered LT proteins, including LTA1 (with no B subunit) or LT-G33D (lacking GM1 binding), for dmLT further attenuated skin reactogenicity as well as acute skin changes 2 days after the prime, with no major trade-offs in CFA/I serum antibodies or memory T-cell responses being seen. However, these immunization groups did exhibit a lower magnitude of induced CFA/I fecal antibodies and LT immunity, particularly when LTA1 was used instead of dmLT. This was anticipated, as while natural infection induces A-subunit antibodies, the i.n. or s.l. delivery of LTA1 in mice has not previously induced strong anti-LT antibodies (22). It was surprising, then, that we detected cytokine recall responses with LTA1 and LT-G33D after s.l. vaccination, indicating a deficit in the movement of intact antigens across mucosal surfaces but not the prevention of immune stimulation. This will need to be investigated further.

In conclusion, we found that the i.d. or s.l. delivery route, the form of LT antigen, or the use of an LTB fusion protein played an important role in vaccination outcomes. Our findings help reveal how formulations and delivery parameters shape the adaptive immune responses to a toxoid- and fimbria-derived subunit vaccine against ETEC. In addition, these strategies can benefit vaccine design when using dmLT as an antigen or adjuvant in vaccines targeting ETEC or other pathogens, with notable findings including methods to achieve robust humoral or cellular responses by s.l. vaccination as well as approaches to i.d. vaccination to promote immunity without skin reactogenicity.

MATERIALS AND METHODS

Purification of proteins. LT, LTB, and dmLT were produced from *E. coli* clones expressing recombinant protein derived from the human ETEC isolate *E. coli* H10407 as previously described (31, 60, 61). The LT-G33D mutation was created by site-directed mutagenesis of LT expression plasmid pCS96 using a mutagenic oligonucleotide (5'-GGAATCGATGGCAGACAAAAGAGAAATGGTTATCA-3') to make a single

nucleotide substitution (the underlined nucleotide in the sequence given above) at amino acid position 33 (the boldface amino acid in the sequence given above) from the N terminus of LTb. Briefly, organisms were cultured overnight in Casamino Acids yeast extract medium, the cells were lysed, the lysate was clarified by centrifugation, and LT-related proteins were purified by galactose affinity chromatography. His-tagged LTA and His-tagged LTA1 were prepared from solubilized inclusion bodies by high-performance liquid chromatography with a nickel-affinity column as previously described (30). The composition and purity of each protein were confirmed by SDS-PAGE and a *Limulus* amoebocyte lysate assay (Lonza, Inc.). The endotoxin content of the final products was <1 endotoxin unit/mg. The CfaEB protein was expressed and purified from a BL21(DE3) expression host using previously described methods (62). Briefly, the donor strand complement of CfaE (dscCfaEB) was purified from cell lysates using a HisTrap FF column, in which the protein was eluted with a linear imidazole gradient (5 to 300 mM imidazole over 20 column volumes) in 20 mM phosphate buffer, pH 7.4, followed by a HiTrap SP HP column, in which the protein was eluted with a linear salt gradient (0 to 1 M sodium chloride) in a 20 mM MES (morpholineethanesulfonic acid) buffer, pH 5.5. The dscCfaE-CTA2/LTb chimera protein was engineered through the replacement of the A1 subunit of CT with a donor strand complement of CfaE (dsc14CfaE-sCTA2). Briefly, the fusion protein was coexpressed with LTb in an *E. coli* BL21 expression system, forming a noncovalently associated dsc14CfaE-sCTA2/LTb chimera (here referred to as CfaE-LTb) protein purified to $\geq 85\%$ purity with endotoxin levels of <2,000 endotoxin units/mg using metal-affinity chromatography (Talon Superflow column; Clontech) and cation exchange chromatography (SP HP column; GE Healthcare). The proteins were stored lyophilized and freshly resuspended prior to use (LT, LTb, LT-G33D, dmLT) or were kept frozen at -80°C until use (LTA1, CfaEB, CfaE-LTb).

Animals, immunizations, and sample collections. Female BALB/c mice 6 to 8 weeks of age were purchased from Charles River Laboratories and housed in sterile cages. All animal studies were approved by the Tulane University Institutional Animal Care and Use Committee. The study protocol was reviewed and approved by the Tulane University Institutional Animal Care and Use Committee and was in compliance with all applicable federal regulations governing the protection of animals in research. Immunization formulations were prepared immediately before administration by admixing antigen with or without adjuvant in sterile PBS alone or, where specified, in sterile PBS containing 5% (wt/vol) beta-lactose (MilliporeSigma). For i.d. immunizations, hind flank skin was shaved and 20 μl was injected using the Manteaux technique with a 28-gauge insulin syringe. For s.i. immunization, the mice were intraperitoneally injected with ketamine-xylazine and then held upright for 1 min while 10 μl formulation was pipetted underneath the tongue and allowed to absorb. Immunizations were performed 2 times at 3- to 4-week intervals prior to CO_2 euthanasia for sample collection. Blood was collected by cardiac puncture and processed for serum. Fecal material was collected from the colon, weighed, and homogenized in 1 ml PBS containing 0.05% Tween 20 (MilliporeSigma), 1 mM EDTA (MilliporeSigma), and a protease inhibitor cocktail (Roche), followed by centrifugation and collection of supernatants. Spleens were homogenized in 3 ml of PBS containing 2% bovine serum albumin (MilliporeSigma) and 1 mM EDTA using gentleMACS C tubes and tissue dissociator (Miltenyi Biotec). RBCs were lysed using ACK lysis buffer (Gibco), and splenocytes were filtered and counted.

Antibody ELISAs. Serum total Ig, IgG1, and IgG2a and fecal IgA antibody ELISAs were performed using CoStar 96-well flat-bottom plates as previously described (30, 31). Briefly, the wells of the plates were coated with 2 $\mu\text{g}/\text{ml}$ of CFA/I (purified from wild-type ETEC strain WS1933D, as previously reported [8]), 1 $\mu\text{g}/\text{ml}$ of LT, or an external recombinant mouse IgG1 or IgG2a standard (MilliporeSigma) or IgA standard (Southern Biotech), incubated with dilutions of individual serum or fecal samples, and detected using alkaline phosphatase-conjugated anti-mouse IgG (which binds nonspecifically to IgG, IgA, and IgM; MilliporeSigma) or IgG1 or IgG2a (BD Biosciences) or using horseradish peroxidase-conjugated anti-mouse IgA (MilliporeSigma). The absorbance was read at 405 nm, and the data were analyzed using a sigmoidal dose-response with least-squares fit. The results were \log_2 transformed after adding 1 and quantitated as the number of ELISA units (EU) per milliliter using the average for two sample dilutions closest to the midpoint of the standard curve.

ETEC HAI assay. The hemagglutination inhibition (HAI) assay was adapted from previous studies (55). Briefly, ETEC class 5a bacterial strains expressing different CFs, namely, CFA/I (strain H10407), CS4 (strain Bang-10 SP), and CS14 (strain WS3294A), were grown overnight on colonization factor antigen (CFA) agar plates with bile salts, harvested, and resuspended in 0.5% D-mannose (MilliporeSigma) in PBS (PBS-M) to a final solution with an optical density at 650 nm (OD_{650}) of 0.2 ± 0.02 . Serum samples were initially diluted at 1:8 in PBS-M followed by 2-fold serial dilutions, for a final volume of 25 μl per well. Twenty-five microliters of the bacterial suspension was added to each well, doubling the final sample dilution, and the plate was shaken at 500 rpm for 30 min at room temperature. After the incubation, 25 μl of 1.5% bovine RBCs was added per well, the plate was shaken at 500 rpm for 30 min at 4°C , and the presence or absence of agglutination was recorded immediately after. Each sample was tested in duplicate, and the average HAI titer that completely inhibited mannose-resistant hemagglutination was calculated and \log_2 transformed.

Skin reactivity analyses. Skin samples from the injection sites were collected at day 2 after the prime or week 2 after the boost (day 45). For acute-phase analysis, the injection site was marked at the time of immunization with a permanent marker. The gross skin thickness was directly measured from skin excised from euthanized animals using a digital caliper. For histopathological analysis, skin samples were placed in 10% neutral buffered formalin and fixed for a minimum of 24 h at room temperature. The fixed tissue was processed by ethanol dehydration and embedded in paraffin, cut into 5- μm -thick sections, and stained with hematoxylin and eosin (H&E). H&E-stained sections were scored in a blind manner by a board-certified veterinary pathologist for the severity of inflammation (noting the immune cell type)

and skin thickness (in micrometers). The severity scores ranged from 0 to 5 and was classified as follows: 0, no lesions; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe.

Memory cell cytokine secretion. The 30 15-amino-acid peptides (overlapping by 10 amino acids) spanning the whole LTB protein were purchased (GenScript) to create the LTB peptide pool. The 13 highest-ranked peptides from a CfaE pool created using epitope predication software (RankPep) were similarly purchased to create the CfaE pool. Splenocytes were treated at 1×10^6 cells/200 μ l/well in RPMI-10% fetal bovine serum with antibiotic-antimycotic (Gibco) and 5 μ g/ml CFA/I, 1 μ g/ml dmLT, 4 μ g/ml CfaEB, or LTB peptide pools. The cells were incubated at 37°C with 5% CO₂ for 72 h, and the supernatants were collected and stored at -20°C. Freshly thawed samples were tested using a Bio-Plex Pro Th17 6-plex assay and IL-2, IL-4, and IL-5 mouse single-plex beads (Bio-Rad) and evaluated with a Bio-Plex 200 system (Bio-Rad) following the kit instructions.

Statistical analysis. Statistical analyses were performed using Prism (v7; GraphPad Software) and SAS (v9.4; SAS Institute Inc.) software. Parametric data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's posttest for all groups compared to a control group or the Bonferroni correction for comparison of selected pairs. Nonparametric data were analyzed by the Kruskal-Wallis test with Dunn's posttest. CfaEB and LT antibodies were tested for a linear relationship with strain H10407 HAI responses, and significant models were determined to be adequate by nonsignificant results using lack-of-fit test results. Serum total Ig and fecal IgA titers compared with HAI titers were nonsignificant for a lack of fit, indicating that the serum IgG1 titer alone does not adequately explain the variance in HAI titers. Correlations to parametric data were performed using a Pearson correlation, and correlations to nonparametric data were performed using a Spearman correlation.

Data availability. Any materials and data that are reasonably requested by others will be made available in a timely fashion, at reasonable cost, to members of the scientific community for noncommercial purposes.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00460-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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S.J.S. and M.A.S. served as military service members over the course of this work, which was prepared as part of their official duties.

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