Differential Requirements for Protection against Mucosal Challenge with *Francisella tularensis* in the Presence versus Absence of Cholera Toxin B and Inactivated *F. tularensis*

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Differential Requirements for Protection against Mucosal Challenge with *Francisella tularensis* in the Presence versus Absence of Cholera Toxin B and Inactivated *F. tularensis*

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*Francisella tularensis* is a category A biothreat agent for which there is no approved vaccine and the correlates of protection are not well understood. In particular, the relationship between the humoral and cellular immune response to *F. tularensis* and the relative importance of each in protection is controversial. Yet, understanding this relationship will be crucial to the development of an effective vaccine against this organism. We demonstrate, for the first time, a differential requirement for humoral vs cellular immunity in vaccine-induced protection against *F. tularensis* infection, and that the requirement for Ab observed in some protection studies, may be overcome through the induction of enhanced cellular immunity. Specifically, following intranasal/mucosal immunization of mice with inactivated *F. tularensis* organisms plus the cholera toxin B subunit, we observe increased production of IgG2a/c vs IgG1 Ab, as well as IFN-γ, indicating induction of a Th1 response. In addition, the requirement for *F. tularensis*-specific IgA Ab production, observed in studies following immunization with inactivated *F. tularensis* alone, is eliminated. Thus, these data indicate that enhanced Th1 responses can supersede the requirement for anti-*F. tularensis*-specific IgA. This observation also has important ramifications for vaccine development against this organism. *The Journal of Immunology*, 2009, 182: 4899–4909.
cases (14, 16–18). In addition, there is no direct evidence that IgM alone can be protective against virulent *F. tularensis*. In fact, it is generally believed that the generation of immunogen-specific IgG is preferable in most vaccines, in part due to its high affinity, its ability to enhance opsonophagocytosis, and its ability to fix complement (19).

Importantly, recent studies from this laboratory have demonstrated that inactivated *F. tularensis* (ifT) can induce protection against subsequent challenge with *F. tularensis* biovar B, as well as biovar A, when targeted to Fc receptors, and that this protection requires both humoral and cellular immunity (10). Furthermore, these observations are consistent with those using OMP as immunogen (15). Thus, we sought to further enhance these protective responses and to generate a more effective vaccine by using a well-established mucosal adjuvant, cholera toxin B (CTB) (20–27). CTB is a potent mucosal adjuvant, in particular for the induction of protective Ab. In addition, it lacks the toxicity of cholera toxin itself due to the absence of the toxic A subunit (22–25, 28). In addition, CTB also enhances cellular immunity, although the precise impact on Th1 vs Th2 responses can vary significantly. For example, i.n. and oral administration of CTB tends to drive Th2-like responses (29–31), while transtracheal and intravaginal routes tend to elicit Th1 responses (32, 33). However, not only does the route of immunization influence the ability of CTB to stimulate cellular immunity, but also the type of Ab used (34). Thus, we considered the possibility that CTB may enhance both the humoral and cellular responses to ifT when administered i.n. In fact, when ifT is administered i.n. with CTB, it enhances both cellular (Th1) and humoral immune responses, while also enhancing protection against both the biovar A and B strains of *F. tularensis*. More significantly, we demonstrate for the first time, a differential requirement for protection in the presence and absence of CTB. Specifically, the requirement for anti-*F. tularensis*-specific IgA, demonstrated in previous protection studies following immunization with ifT alone (10), is eliminated when immunizing with ifT plus CTB. In contrast, the requirement for IFN-γ remains. Furthermore, ifT plus CTB concomitantly enhances the production of IgG2a/IgG2c and IFN-γ, characteristics of a Th1 response. Whether IgA is a Th1 or Th2 dominant isotype is unclear, although there is some evidence that IgA is produced in the mucosa under the influence of IL-4, a Th2 cytokine (35). Thus, a shift toward a Th1 response induced by CTB could potentially reduce the production of IgA in response to ifT. However, this was not observed. Alternatively, the increased level of the cellular immune response induced by CTB could simply overcome the need for Ab-mediated protection. Most importantly, these data demonstrate for the first time a correlation between the level of Th1 response induced and the requirement for IgA in vaccine-induced protection against *F. tularensis* challenge. This observation not only has significant ramifications for vaccine development, but may also help to resolve ongoing disagreement regarding the role of Ab in protection against *F. tularensis* infection.

### Materials and Methods

#### Mice

BALB/c and C57BL/6 mice were procured from Taconic Farms. IgA/−/− mice with a C57BL/6 × 129 background were provided by Dr. D. Metzger (Albany Medical College). The B6.129S7-Ifngtm1Ts/J (IFN-γ−/−) and the B10.129S2(B6)-Igh-6tm1Cgm/J (B cell-deficient) mice were obtained from The Jackson Laboratory. All mice were housed in the Animal Resources Facility at Albany Medical College. Mice were provided with ad libitum water and food during the course of each experiment. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee.

### Bacterial strains

*F. tularensis* live vaccine strain (LVS) organisms were provided by Dr. M. Forsman (Swedish Defense Research Agency, Umea, Sweden). *F. tularensis* SchuS4 organisms, originally isolated from a human case of tularemia (36), were obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Frederick, MD). All experiments using SchuS4 were conducted within a Centers for Disease Control-certified ABSL-3/BSL-3 facility at Albany Medical College. *F. tularensis* organisms were grown in Mueller-Hinton broth (Fisher Scientific) at 37°C to a density of ~0.5–1 × 10^9 CFU/ml. Live *F. tularensis* preparations were stored in liquid nitrogen at −80°C in PBS.

#### Immunogen

Inactivated GFP-expressing *F. tularensis* LVS (ifT) was used as immunogen. *F. tularensis* LVS organisms were grown in Mueller-Hinton to 37°C to a density of ~0.5–1 × 10^9 CFU/ml. To inactivate *F. tularensis* LVS, 1 × 10^10 CFU/ml live bacteria were incubated in 1 ml of sterile PBS (Mediatech) containing 2% paraformaldehyde (Sigma-Aldrich) for 2 h at room temperature. Fixed bacteria (ifT) were then washed with sterile PBS three times. Inactivation was verified by culturing a 100-μl sample (1 × 10^7 ifT organisms) on chocolate agar plates (Fisher Scientific) for 7 days. The ifT preparations were stored at −20°C in PBS.

#### Immunization and challenge experiments

Mice were divided into groups consisting of six to eight mice per group, 8–12 wk of age. Each mouse was anesthetized by i.p. injection of 20% ketamine plus 5% xylazine and subsequently administered i.n. either 20 μl of PBS (vehicle), 5 μg of CTB (Sigma-Aldrich) in 20 μl of PBS, 2 × 10^10 ifT organisms in 20 μl of PBS, or 2 × 10^5 ifT organisms combined with 5 μg of CTB in 20 μl of PBS. Before immunization, mice were bled and serum was isolated and tested for the presence of *F. tularensis*-specific Ab. Unless otherwise indicated, mice were immunized on day 0 and boosted on day 21. Immunized mice were then challenged on day 35 i.n. with 2–8 × 10^6 CFU of live *F. tularensis* LVS organisms or 20 CFU of *F. tularensis* SchuS4 organisms and subsequently monitored for survival for at least 21 days.

#### Quantification of bacterial burden

Following immunization and challenge, mice were euthanized at various time intervals and tissues such as lung, liver, and spleen were collected aseptically in PBS containing a protease inhibitor mixture (one tablet in 10 ml of sterile PBS) (Roche Diagnostics) and subjected to mechanical homogenization using a MiniBeadBeater-8 (BioSpec Products) using 1-mm zirconia/silica beads. This is a recently established protocol that has been successfully used in similar studies that involve bacterial quantification in tissues (37–39). Tissue homogenates were then diluted 10-fold in sterile saline and 10 μl of each dilution was spotted onto chocolate agar plates in duplicate and incubated at 37°C for 2 days. The number of colonies on the plates were counted and expressed as log_{10} CFU per ml for the respective tissue homogenate was also spun 14,000 g for 20 min and the clarified homogenate was stored at −20°C for cytokine analysis.

#### Cytokine measurement

Mice were divided into groups consisting of six mice each and were immunized i.n. on days 0 and 21. Mice received either: 20 μl of PBS (vehicle), 5 μg of CTB in 20 μl of PBS, 2 × 10^10 ifT organisms in 20 μl of PBS, or 2 × 10^5 ifT organisms combined with 5 μg of CTB in 20 μl of PBS. For in vivo cytokine measurements, mice were bled submandibularly 2 days after immunization boost (day 21) and the sera were obtained. For in vitro cytokine measurements, on day 35 after primary immunization, spleen cells were harvested from each mouse and incubated in either the presence of fixed *F. tularensis* LVS organisms in RPMI 1640 (Mediatech) plus 10% FBS (HyClone) or medium alone. More specifically, 4 × 10^6 spleen cells/ml were incubated with 4 × 10^10 CFU/ml fixed *F. tularensis* LVS organisms in a total volume of 1 ml of RPMI 1640/10% FBS per well and cultured for 3, 5, and 7 days at 37°C. Supernatants were then harvested at each time point and kept at −20°C until they were analyzed. Mouse Th1/Th2 cytokometric bead array (CBA) kits (BD Biosciences-BD Pharmingen) were used for measurement of multiple cytokines in the sera and in the collected spleen cell supernatants. Data were acquired on a FACSArray Instrument (BD Immunocytometry Systems) and analyzed using CBA software version 1.0.1 (BD Immunocytometry Systems).

#### Histological evaluation

Following immunization and challenge, mice were euthanized at various time intervals and tissues including lung, liver, and spleen were collected

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FIGURE 1. iFt plus CTB enhances iFt-induced protection against i.n. challenge with F. tularensis LVS. C57BL/6 (A) and BALB/c (B) mice were divided into four groups and immunized i.n. with 20 μl of either PBS, 5 μg of CTB in 20 μl of PBS, 2 × 10⁷ iFt organisms in 20 μl of PBS, or 2 × 10⁷ iFt organisms with 5 μg of CTB in 20 μl of PBS. Mice were immunized on day 0 and boosted on day 21. Mice were then challenged on day 35 i.n. with 2 × 10⁴ CFU (LD₅₀ × 4) (A and B) or 2–8 × 10⁴ CFU (C and D) of live F. tularensis LVS and subsequently monitored for 21 days for survival. Survival curves are presented. The survival data are representative of four individual experiments (a total of six mice per group in each experiment).

Measurement of F. tularensis-specific Ab production

Anti-F. tularensis Ab production in response to immunization with iFt was measured by ELISA. Briefly, ELISA plates (Nalge Nunc International) were coated with 100 μl of live F. tularensis LVS (5 × 10⁵ CFU/ml) in carbonate buffer (4.3 g/L sodium bicarbonate (Sigma-Aldrich) and 5.3 g/L sodium carbonate (Sigma-Aldrich) at pH 9.4) for 2 h at 37°C. The plates were washed with PBS containing 0.05% Tween 20 (Fisher Scientific) and blocked for 2 h with 200 μl of PBS containing 10% BSA (Baxter Healthcare). Serial 2-fold dilutions of sera (starting with 1/50) and bronchoalveolar lavage (BAL) fluids (starting with 1/1) were added to the plates (100 μl/well) and incubated for 90 min at 37°C. After washing, biotin-conjugated goat anti-mouse Ab specific for IgG, IgG1, IgG2a, IgG2c or IgA (Caltag Laboratories) were added and incubated for 1 h at 37°C. Plates were washed and then 100 μl/well of streptavidin conjugated to HRP (Caltag Laboratories) was added. The plates were incubated for 20 min followed by washes and addition of 100 μl of tetramethylbenzidine per-oxidase substrate (Kirkegaard & Perry Laboratories). All samples were read simultaneously using a kinetic microplate reader (Molecular Devices). Therefore, acid stop solution was not added, and the samples were read at 650 nm as instructed by the manufacturer when acid stop is not used. BSA-coated wells were used as specificity controls for F. tularensis LVS.

Statistical analysis

To compare survival curves, a regular Mann-Whitney U two-sample rank test was used. Statistical data for bacterial burden and cytokine analysis were generated using ANOVA on day 7, which is the peak of infection. Ab titers were assessed by using the unpaired two-tailed t test comparing the log values of each experimental group. GraphPad Prism 4 analysis software was used.

Results

Use of CTB as a mucosal adjuvant increases protection against lethal F. tularensis LVS challenge when using iFt as immunogen

Previous studies from our laboratory demonstrated that immunization with iFt provided only partial protection against lethal F. tularensis LVS challenge (10). Thus, we sought to improve this level of protection by using CTB as adjuvant. In the current study, coadministration of iFt with 5 μg of CTB i.n., followed
by a booster immunization 21 days later, provided 100% protection against lethal \( F. \) \( tularensis \) LVS challenge in both C57BL/6 (Fig. 1A) and BALB/c mice (Fig. 1B), while iFt alone continued to provide only partial protection. Furthermore, CTB in the absence of iFt provided no protection (Fig. 1A and B). Protective responses were also titratable, in that the protective effect of coadministering CTB with iFt could be reduced with increased challenge doses of \( F. \) \( tularensis \) LVS (Fig. 1, C and D).

**Use of CTB as an adjuvant reduces bacterial burden in the tissues of immunized mice**

Following our observation of increased protection against \( F. \) \( tularensis \) LVS challenge when coadministering CTB with iFt, we measured the bacterial burden in the tissues of immunized, then challenged animals. Indeed the use of CTB as an adjuvant significantly reduced bacterial burden in the lung (Fig. 2A), liver (Fig. 2B), and spleen (Fig. 2C) of immunized mice on day 7 after infection vs that of mice immunized with iFt alone. This time point was chosen based on our previous studies showing day 7 to be the peak of \( F. \) \( tularensis \) LVS infection (10).

**FIGURE 3.** Levels of proinflammatory cytokines are reduced in the tissues of iFt plus CTB-immunized mice. BALB/c mice were immunized and challenged as indicated in Fig. 1. On day 7 after challenge, the lung, spleen, and liver of mice were harvested and the cytokine levels were measured in the tissue homogenates by CBA. The levels of IFN-\( \gamma \) (A), TNF-\( \alpha \) (B), and MCP-1 (C) in the lungs of immunized mice are presented. The levels of these cytokines were significantly lower in mice immunized with iFt plus CTB (*, \( p < 0.05 \) and **, \( p < 0.01 \)). The data are a representative of two experiments.

**FIGURE 4.** Inflammation is reduced in the lungs, liver, and spleen of iFt plus CTB-immunized mice as compared with mice immunized with iFt alone: BALB/c mice were immunized and challenged as indicated in Fig. 1. Tissues were then harvested 7 days after challenge, fixed with paraformaldehyde, paraffin embedded, sectioned, and stained with H&E. In this figure, we show lung samples of a normal unimmunized and unchallenged mouse (A), a PBS-immunized and challenged mouse (B), a mouse immunized with iFt alone and challenged (C), and a mouse immunized with iFt plus CTB and challenged (D). All samples are at \( \times 20 \) magnification. Reduced tissue pathology was observed in the iFt plus CTB-immunized mice.

Levels of proinflammatory cytokines are reduced when CTB is used as an adjuvant

Severe tissue pathology during systemic and often lethal bacterial infection with \( F. \) \( tularensis \) has been previously demonstrated (10). Proinflammatory cytokines play a central role in triggering and driving these tissue-destructive responses. Thus, we chose to examine the generation of proinflammatory cytokines. In this study, we demonstrate that when CTB is used as an adjuvant and coadministered with iFt, a significant reduction of proinflammatory cytokines in the tissues of immunized and subsequently infected mice is observed at 7 days after infection. This is likely due to the fact that immunization with iFt plus CTB enables the mice to respond more rapidly and to control infection, resulting in lower bacterial counts and thus lower levels of inflammation and proinflammatory cytokines by day 7. Indeed, immunized mice had reduced levels of IFN-\( \gamma \) (Fig. 3A), TNF-\( \alpha \) (Fig. 3B), and MCP-1 (Fig. 3C) in their lungs compared with mice immunized with iFt alone. Measurement of these cytokines in the spleen and liver of immunized mice showed analogous differences. We also examined nonproinflammatory cytokines including IL-4, IL-5, and IL-2. However, the levels of these cytokines were similar among all immunized groups as well as unvaccinated animals (data not shown).

**Immunization of mice with iFt plus CTB reduces tissue pathology**

To determine whether reduced levels of proinflammatory cytokines observed following immunization with iFt plus CTB would be reflected in the tissue pathology of \( F. \) \( tularensis \) LVS-infected mice, tissues from immunized mice were harvested on day 7 after \( F. \) \( tularensis \) LVS infection, sectioned, and stained with H&E.
immunized i.n. with 20 μg of either PBS, 5 μg of CTB in 20 μl of PBS (only BALB/c), 2 × 10^7 iFt in 20 μl of PBS, or 2 × 10^7 iFt plus 5 μg of CTB in 20 μl of PBS. Mice were immunized on day 0 and boosted on day 21 as in challenge studies in Fig. 1. BAL fluid or sera was then collected by immunization with iFt plus CTB. Pathology was also reduced in mice immunized with iFt alone (Fig. 4C), however, not to the extent of their iFt plus CTB-immunized counterparts. Histopathology in the liver and spleen of immunized mice showed similar results (data not shown).

F. tularensis-specific IgA responses are enhanced when using CTB as adjuvant

The ability of CTB to enhance Ab responses is well established. In addition, studies by this laboratory and others have indicated that Ab, including IgA, can play a role in controlling F. tularensis infection, in particular when iFt is used as the protective immunogen (9, 10). For this reason, we examined the ability of CTB to enhance F. tularensis-specific IgA responses in the BAL fluid and sera of immunized mice. BAL fluid and blood were collected from mice 10–12 days after booster immunization. Indeed, we found that the levels of F. tularensis LVS-specific IgA were significantly elevated in BAL fluid and sera of iFt plus CTB-immunized mice as compared with mice immunized with iFt alone (Fig. 5, A and B). This was the case for both BALB/c and C57BL/6 mice (Fig. 5C).

F. tularensis-specific IgA is not required for protection in iFt plus CTB-immunized mice

Consistent with our previously published observations (10), we observed that immunization with iFt provides partial protection against F. tularensis LVS challenge (Fig. 6A). In addition, the role of IgA in mucosal immunity has been widely established (40–42). For this reason, we examined the ability of CTB to enhance F. tularensis-specific IgA responses in the BAL fluid and sera of immunized mice. BAL fluid and blood were collected from mice 10–12 days after booster immunization. Indeed, we found that the levels of F. tularensis LVS-specific IgA were significantly elevated in BAL fluid and sera of iFt plus CTB-immunized mice as compared with mice immunized with iFt alone (Fig. 5, A and B). This was the case for both BALB/c and C57BL/6 mice (Fig. 5C).

FIGURE 5. Immunization with iFt plus CTB enhances production of F. tularensis-specific IgA. BALB/c (A and B) and C57BL/6 (C) mice were divided into four and three groups, respectively (five mice per group) and immunized i.n. with 20 μl of either PBS, 5 μg of CTB in 20 μl of PBS (only BALB/c), 2 × 10^7 iFt in 20 μl of PBS, or 2 × 10^7 iFt plus 5 μg of CTB in 20 μl of PBS. Mice were immunized on day 0 and boosted on day 21 as in challenge studies in Fig. 1. BAL fluid or sera were then collected at 35 days and tested for the presence of anti-F. tularensis IgA Ab by ELISA as described in Materials and Methods. Ab data represent the mean of four to five mice ± SD. The levels of IgA were significantly higher in the BAL fluid and sera of iFt plus CTB-immunized mice vs mice immunized with iFt alone (p < 0.01 and p < 0.05, respectively in BALB/c mice and p < 0.01 in C57BL/6). These results are representative of two experiments.
**F. tularensis** cell-mediated immunity (in particular IFN-γ) was required. Numerous studies support the idea that induced protection, we sought to determine whether cell-mediated immunity, B cell-deficient mice were protected against *F. tularensis* LVS challenge when immunized with iFt plus CTB (B). PBS and subsequently infected with a lethal challenge of *F. tularensis* LVS. However, in contrast to our previous studies, in which IgA-deficient mice immunized with iFt alone did not survive (10), 100% protection was observed when iFt was coadministered with CTB in IgA-deficient mice (Fig. 6B).

*F. tularensis*-specific Ab is not required for protection in iFt plus CTB-immunized mice

It remained possible that CTB-enhanced *F. tularensis*-specific IgG production could compensate for the lack of IgA, and thus humoral immunity could still play a critical role in the observed iFt plus CTB-induced protection we observed. To investigate this possibility, B cell-deficient mice were immunized with either: iFt plus CTB, iFt, or PBS and boosted 21 days later. On day 35, the mice were challenged with a lethal dose of *F. tularensis* LVS and their survival was monitored. Although iFt alone protected 60% of normal mice (Fig. 7A), 90% of B cell-deficient mice died when immunized with iFt alone. In contrast, 100% of B cell-deficient mice immunized with iFt plus CTB were protected (Fig. 7B), indicating that Ab are not required for protection when immunizing with iFt plus CTB.

**IFN-γ is required for iFt plus CTB-induced protection**

Having demonstrated Ab is not necessary for iFt plus CTB-induced protection, we sought to determine whether cell-mediated immunity was required. Numerous studies support the idea that cell-mediated immunity (in particular IFN-γ) can protect against *F. tularensis* infection. Thus, we immunized IFN-γ-deficient mice, with either: iFt plus CTB, iFt, or PBS. Two weeks after the booster immunization, the mice were infected with a lethal dose of *F. tularensis* LVS. In fact, the use of CTB as a mucosal adjuvant with iFt did not overcome the previously demonstrated requirement for IFN-γ, demonstrated when using iFt alone as immunogen (10). Specifically, no protection was observed in IFN-γ-deficient mice whether or not CTB was added to iFt (Fig. 8B). This is also despite the fact that IFN-γ-deficient mice immunized with iFt plus CTB exhibited a 3-fold increase in *F. tularensis*-specific IgA levels as compared with iFt-immunized mice (Fig. 8C).

**iFt plus CTB induces a Th1-type response against F. tularensis**

We next sought to determine whether CTB enhances the Th1-type response, thereby explaining its protective activity. First, we focused on the specific IgG responses. Sera from mice immunized with PBS, CTB, iFt alone, or iFt plus CTB were obtained from IFN-γ-deficient mice immunized with iFt plus CTB (**Fig. 8**B). Sera from IFN-γ-deficient mice were obtained before the *F. tularensis* LVS challenge and the *F. tularensis*-specific IgA levels were measured by ELISA. The mice immunized with iFt plus CTB exhibited a 3-fold increase in IgA levels vs iFt-immunized mice (C). These data are representative of two separate experiments.

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**FIGURE 7.** B cells are not required for protection induced by iFt plus CTB immunization. C57BL/6 (A) and B cell-deficient (B) mice were divided into three groups, immunized, and infected with *F. tularensis* LVS as described in Fig. 1. Their survival was monitored for at least 21 days after infection. B cell-deficient mice were protected against *F. tularensis* LVS challenge when immunized with iFt plus CTB (B).

**FIGURE 8.** IFN-γ is required for iFt plus CTB-induced protection. C57BL/6 (A) and IFN-γ-deficient (B) mice were divided into three groups, immunized, and infected with *F. tularensis* LVS as described in Fig. 1. Survival was monitored for at least 21 days after infection. IFN-γ-deficient mice were not protected against *F. tularensis* LVS challenge when immunized with iFt plus CTB (B). Sera from IFN-γ-deficient mice were obtained before the *F. tularensis* LVS challenge and the *F. tularensis*-specific IgA levels were measured by ELISA. The mice immunized with iFt plus CTB exhibited a 3-fold increase in IgA levels vs iFt-immunized mice (C). These data are representative of two separate experiments.
both BALB/c and C57BL/6 mice (Fig. 9, A and D, respectively). Also, coadministering iFt plus CTB significantly increased the levels of IgG2a (BALB/c) and IgG2c (C57BL/6) in the sera of these mice, compared with mice immunized with iFt alone (Fig. 9, B and E, respectively). In contrast, the levels of IgG1 in the sera of these mice were comparable between iFt plus CTB and iFt-immunized groups (Fig. 9, C and E, respectively). This subclass Ab profile is indicative of the induction of a Th1-type response. Control (PBS) mice and mice immunized with CTB alone induced no significant F. tularensis-specific Ab responses (Fig. 9, A–C). Furthermore, the levels of IgG2c were also increased in the sera of IgA-deficient mice immunized with iFt plus CTB (Fig. 9F).

To provide additional evidence for the induction of a Th1-type response by iFt plus CTB, the cytokine profile in the sera of iFt plus CTB-immunized mice, as well as that secreted by splenocytes from these mice “in vitro” was analyzed by CBA and compared with that of PBS- and iFt-immunized mice. In the first instance, immunized mice were bled 2 days following booster immunization (day 23). Alternatively, in a separate experiment, but under the same immunization conditions, the spleens from immunized mice were harvested and the IFN-γ levels were measured in the tissue homogenate by CBA. The levels of IFN-γ in the lungs of immunized mice are significantly lower at day 7 in mice immunized with iFt plus CTB vs iFt alone (p < 0.01). In an additional experiment (C), C57BL/6 mice were immunized and challenged on day 35 after primary immunization with iFt plus CTB compared with mice immunized with iFt alone. No significant difference was detected in the case of IgG1 between the same groups of mice. These data are representative of two different experiments.

FIGURE 9. Enhanced production of Th1-type IgG isotypes in the sera of mice immunized with iFt plus CTB. BALB/c (A–C), C57BL/6 (D, E), and IgA-deficient (F) mice were divided into four or three groups (four to five mice per group, respectively, and immunized i.n. with 20 µl of either PBS, 5 µg of CTB in 20 µl of PBS (only BALB/c), 2 × 10⁷ iFt in 20 µl of PBS, or 2 × 10⁷ iFt plus 5 µg of CTB in 20 µl of PBS. Mice were immunized on day 0 and boosted on day 21 as in challenge studies in Fig. 1. Serum was then collected at 35 days and tested for the presence of anti-F. tularensis IgG2a/2c and IgG1 subclasses by ELISA as described in Materials and Methods. Ab data represent the mean of four to five mice ± SD. The levels of IgG2a/2c were significantly higher (p < 0.05) in BALB/c (A–C), C57BL/6 (D and E), and IgA-deficient (F) mice immunized with iFt plus CTB compared with mice immunized with iFt alone. No significant difference was detected in the case of IgG1 between the same groups of mice. These data are representative of two different experiments.

FIGURE 10. Enhanced Th1-type cytokine production in response to immunization with iFt plus CTB. Immunizations were done as indicated in Fig. 1. At 23 days after primary immunization, sera were obtained from mice and their cytokine content was measured by CBA (A). In a different experiment, at 35 days after primary immunization, spleen cells were harvested from each mouse and incubated in the presence or absence of iFt as described in Materials and Methods. Supernatants were then harvested and their cytokine content was measured by CBA (B). These data are representative of two experiments. Each bar represents the average of four to six individual mice ± SD. Both in vivo (A) and in vitro (B) results showed a significant increase of IFN-γ when mice were immunized with iFt plus CTB vs iFt alone (p < 0.01). In an additional experiment (C), C57BL/6 mice were immunized and challenged on day 35 after primary immunization as indicated in Fig. 1. On days 1, 3, and 7 after challenge, the lungs were harvested and the IFN-γ levels were measured in the tissue homogenate by CBA. The levels of IFN-γ in the lungs of immunized mice are presented. The levels of IFN-γ were significantly higher at days 1 and 3 after challenge in mice immunized with iFt plus CTB vs iFt alone, but significantly lower at day 7 in mice immunized with iFt plus CTB vs iFt alone (*, p < 0.05 and ***, p < 0.01).
FIGURE 11. Protection against mucosal F. tularensis SchuS4 challenge is also enhanced in mice immunized with iFt plus CTB: C57BL/6 mice were divided into four groups (eight mice per group) and immunized i.n. with 20 μl of PBS, 5 μg of CTB in 20 μl of PBS, 2 × 10^7 iFt in 20 μl of PBS, or 2 × 10^7 iFt with 5 μg of CTB in 20 μl of PBS. Mice subsequently received an immunization boost on days 14 and 28. Mice were then challenged on day 42 i.n. with 23 CFU (≈10–20 × LD50) of live F. tularensis SchuS4 and subsequently monitored for survival. The figure is representative of data from three different experiments.

were harvested on day 35 after primary immunization and cultured in complete medium in the presence or absence of iFt. On days 3, 5, and 7, supernatants were collected and analyzed by CBA. CBA analysis showed significantly elevated levels of IFN-γ in the sera of mice immunized with iFt plus CTB compared with the iFt alone (Fig. 10A). Also, levels of IL-4, a Th2-type cytokine, were similarly low in both groups (Fig. 10A). The cytokine profile present in the supernatants of the cultured splenocytes derived from iFt plus CTB-immunized mice also supported the induction of a Th1-type response, in that IFN-γ production was enhanced by splenocytes obtained from iFt plus CTB-immunized mice (Fig. 10B). Other cytokines tested included IL-2, IL-5, TNF-α, and MCP-1, with no significant differences among these experimental groups being observed (data not shown).

Despite the above observations, IFN-γ levels in the lungs of iFt plus CTB-immunized mice are actually lower than those of non-immunized mice 7 days after infection (Fig. 3), raising what would seem to be a paradox regarding the role of IFN-γ in protection. Thus, we sought to determine whether there was in fact an early burst of IFN-γ production shortly (1–3 days) after challenge, followed by the observed reduction in IFN-γ levels observed in Fig. 3. In fact, consistent with our observations in Fig. 3 and Fig. 10, A and B, iFt plus CTB-immunized animals had significantly higher IFN-γ levels in the first 3 days after challenge with F. tularensis LVS as compared with PBS- and iFt-immunized mice (Fig. 10C), followed by a significant reduction in IFN-γ levels in the iFt plus CTB-immunized group vs PBS and iFt-immunized groups 7 days after infection (Fig. 10C).

Immunization with iFt plus CTB induces partial protection against the highly virulent F. tularensis SchuS4 strain

To assess the potential efficiency of CTB as a mucosal adjuvant against the more virulent strain of F. tularensis SchuS4, we immunized C57BL/6 mice with either PBS (control), CTB alone, iFt alone, or iFt plus CTB. All mice were subjected to two immunization boosters on days 14 and 28 after initial administration. Two weeks after the final boost (day 42), all mice were infected with 23 CFUs of SchuS4. All of the mice from the PBS, CTB-alone, and iFt-alone immunized groups succumbed to the infection within 10 days after SchuS4 challenge. In the iFt plus CTB group, 50% protection against SchuS4 was observed (Fig. 11). This result indicates that the use of CTB as adjuvant also represents a viable adjuvant approach for use against the highly virulent biovar A strains of F. tularensis.

Discussion

Until recently, it was widely accepted that cellular immunity, in particular the production of IFN-γ and the development of a Th1-type T cell response, was the critical response in generating protection against F. tularensis infection (14, 43–45). However, more recent studies (9, 15, 46, 47), including studies from this laboratory (10), have indicated Ab may also be vital to the generation of protection against this infectious organism. Importantly, understanding the relationship between the humoral and cellular immune response to F. tularensis, and the relative importance of each in protection, is crucial to the development of an effective vaccine against this pathogen.

Given recent studies suggesting that humoral as well as cellular immunity may be critical for protection against F. tularensis infection, we sought to test the efficacy of a mucosal adjuvant, CTB, which is known for its ability to stimulate humoral immunity (24, 48–51). In addition, although the ability of CTB to stimulate Th1-type cellular immunity is less clear, evidence suggests such responses can also occur under appropriate circumstances (32, 33, 52).

In fact, when iFt plus CTB was administered i.n., enhanced protection against subsequent challenge with both F. tularensis biovar A and biovar B organisms was observed (Figs. 1 and 11). Survival of immunized mice was correlated with reduced bacterial burden (Fig. 2) and a subsequent reduction of proinflammatory cytokines (Fig. 3). The protection also correlated with enhanced F. tularensis-specific IgG and IgA Ab in the sera and BAL fluid of iFt plus CTB-immunized mice, as compared with iFt-immunized recipients (Figs. 9 and 5, respectively). In regard to enhanced IgG production, the levels of IgG2a/IgG2c vs IgG1 were also significantly increased (Fig. 9), suggesting the promotion of a Th1-type response. This conclusion was also supported by an observed increase in the production of IFN-γ, both in vitro and in vivo, by mice immunized with iFt plus CTB, as opposed to mice immunized with iFt only (Fig. 10). The CTB-mediated enhancement of the Th1-type response is of particular significance, since this has not been demonstrated previously following i.n. immunization with CTB-adjuvanted vaccine, while prior studies have demonstrated enhanced Th1-type responses correlate with protection against F. tularensis infection (53–55). Thus, this observation is consistent with the overall belief that the Th1-type response is key to protection against F. tularensis infection. Indeed, mice lacking IFN-γ were not protected following immunization with either iFt alone or iFt plus CTB (Fig. 8). Also consistent with the belief that IFN-γ plays a key role in CTB-induced protection, iFt plus CTB-immunized mice mounted a more rapid IFN-γ response in the first 3 days after infection with F. tularensis (Fig. 10C). The subsequent control of infection likely results in the significant reduction of IFN-γ levels in the iFt plus CTB-immunized animals as observed by day 7 after infection (Fig. 3).

It is also important to note that in previous studies using iFt as a protective immunogen a requirement for both IFN-γ and IgA was observed (9, 10), suggesting a critical role for IgA in the protective response when using iFt alone as immunogen. Thus, we fully expected this also to be the case in the presence of CTB. In fact, despite studies by others suggesting that a Th1-type response may suppress IgA production (35), enhanced F. tularensis-specific IgA production was observed in the presence of iFt plus CTB as compared with iFt alone. Surprisingly however, F. tularensis-specific IgA was not required for protection, as IgA-deficient mice
challenged with \textit{F. tularensis} LVS survived when immunized with iFt plus CTB, but not when immunized with iFt alone (Fig. 6). In fact, this is the first instance in which a differential role for Ab in vaccine-induced protection against \textit{F. tularensis} infection has been observed. The significance of this observation is further strengthened by recent studies, which have reexamined the role of Ab and questioned the belief that the Th1-type T cell response plays the primary role in protection against \textit{F. tularensis} infection (8–10, 39, 47, 56).

In fact, IgA is known to play a central role in immune defense, controlling the spread of mucosal pathogens. Indeed, in some models of \textit{F. tularensis} infection, IgA has been shown to play a key role in protection against challenge (10, 39). This was also evident in our current study, in that IgA-deficient mice vaccinated with iFt in the absence of CTB were not protected, whereas partial protection was observed in wild-type mice (Fig. 6). Somewhat surprisingly however, this requirement was overcome when mice were immunized with iFt plus CTB, in that IgA-deficient mice were protected (Fig. 6). The latter also correlated with an enhanced Th1-type response (Figs. 9 and 10), which was required, in that immunization with iFt plus CTB still failed to protect IFN-γ-deficient mice, even in the presence of enhanced production of \textit{F. tularensis}-specific IgA (Fig. 8C).

Furthermore, any requirement for Ab in general was eliminated by CTB, in that 100% of B cell-deficient mice immunized with iFt plus CTB and subsequently challenged with \textit{F. tularensis} LVS survived, while 90% of the same strain immunized with iFt alone did not. These results suggest that the requirement for IgA previously observed using iFt alone as immunogen can be overcome in the presence of a sufficiently high Th1-type T cell response, potentially explaining seeming contradictions between studies which question the role of humoral vs cell-mediated immunity in protection against infection with this pathogen.

Although we did not observe full protection (~50%) against SchuS4 challenge under the specific conditions of vaccination we used, we do anticipate that this can be improved upon with further optimization of the vaccine regimen, specifically, through enhancement of the cellular immune response via alterations in the dose of immunogen and/or CTB, timing and/or number of immunizations, or use of additional adjuvants in combination with CTB. It should also be noted that 50% protection is particularly significant in this case, given the fact that C57BL/6 mice and a challenge dose of 23 CFU (~20 × LD_{50}) were used. Specifically, the C57BL/6 mouse strain is particularly difficult to protect against Schu4 challenge (as opposed to BALB/c mice for example) (57, 58), and as little as 1 CFU administered i.n. is fatal without vaccination. To further enhance the cellular response to \textit{F. tularensis}, it may also be possible to combine other adjuvant strategies with CTB, such as the use of Fc receptor-targeted immunogens (10) or IL-12 (59). In fact, additional studies are currently under way using these approaches.

In regard to the use of CTB as a mucosal adjuvant against \textit{F. tularensis}, additional evaluation will be required. However, the observation that iFt plus CTB can enhance IgA, IgG, and Th1-type T cell responses, as well as protection against \textit{F. tularensis} SchuS4 challenge, is promising. In fact, CTB has been used successfully with various infectious disease models, both bacterial and viral. For example, coadministration of CTB with \textit{Mycobacterium bovis}-bacillus Calmette-Guérin was shown to enhance delayed-type hypersensitivity reactions to purified protein derivative (60). Also, mucosal administration of CTB with \textit{Streptococcus pneumoniae} surface Ags, such as PsaA and Rib, reduced bacterial colonization in the nasal passages and lungs of the immunized animals (61, 62). Comparable results were observed in an influenza study in which i.n. administration of the subunit vaccine with CTB induced influenza virus-specific IgG and IgA Ab in the nasal passages and sera of immunized mice and reduced viral load following subsequent influenza infection (63). Furthermore, in studies similar to our own, cholera toxin induced CD4+ T cell-mediated immunity to \textit{S. pneumoniae} infection, which was also Ab independent (64–66). Promising results have also been observed in human studies. In a study conducted by Bergquist et al. (49), i.n. administration of CTB to human subjects induced systemic and local Ab responses in the upper respiratory tract and the vagina. Despite these promising results however, some safety concerns do exist. Observed side effects from the use of CTB include: excessive nasal secretions, “bloody nose,” headaches, and migraines (67). In addition, CTB has affinity for GM1 ganglioside receptors in the brain, although studies also suggest potential problems associated with this can be avoided through appropriate dosing (68). In fact, numerous human trials have indicated minimal side effects in subjects following immunization with CTB (69, 70).

In summary, these are the first studies to demonstrate a differential requirement for Ab in vaccine-induced protection against \textit{F. tularensis} infection, dependent on the immunization strategy used. These studies are particularly timely, given recent disagreement regarding the importance of Ab vs cellular immunity in generating protection against this infection, and suggest that the requirement for Ab observed may be overcome when the level of cellular immunity generated is sufficient. Furthermore, we demonstrate for the first time, the ability of CTB to induce a Th1-type immune response when administered i.n. with an inactivated immunogen.

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\section*{Disclosures}
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\section*{References}


