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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*¹

Constantine Bitsaktsis,²* Deepak B. Rawool,²† Ying Li,* Nitin V. Kurkure,† Bibiana Iglesias,* and Edmund J. Gosselin³*

*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 observed increased production of IgG2a/2c 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.

E lucidating the immune mechanisms involved in protection against microbial pathogens is key to successful vaccine development. In fact, there is a pressing need for development of vaccines against mucosal pathogens. In these studies, we examine the immunological requirements for protection against the intracellular bacterium *Francisella tularensis*. The two subspecies of *F. tularensis*, *tularensis* (biovar A) and *holarctica* (biovar B), are the causative agents of pneumonic tularemia (1). The subspecies biovar A is the most virulent, as inhalation of fewer than 25 organisms can cause fulminate disease in humans (2). The disease appears abruptly 3–5 days after exposure, at which point, it can progress to severe pneumonia, respiratory failure, and even death (3). In addition, *F. tularensis* has been designated a category A biothreat agent due not only to its virulence, but also due to the potential for it to be developed as a bioterrorism agent, which can be dispersed within heavily populated areas (3).

Although it is currently believed that cellular immunity plays the key role in protection against *F. tularensis* infection (4–7), the precise role played by *F. tularensis*-specific Ab is controversial. In regard to cellular immunity, protection studies in mice using either attenuated or inactivated *F. tularensis* organisms as immunogens have demonstrated the requirement for IFN-γ and/or induction of robust cellular immune responses to these immunogens, as indicated by increased IFN-γ, IL-2, and/or IL-12 production, evidence of a Th1-type response (7–10). In regard to humoral immunity, immunization with *F. tularensis* LPS generated Ab-dependent protection against intradermal and i.p. challenges with *F. tularensis* subspecies *holarctica* (biovar B), but not against the more virulent biovar A strain (11–15). More specifically, passive immunization of naive mice with sera from LPS-immunized mice protected recipients against *F. tularensis* live vaccine strain (LVS)⁴ challenge, highlighting the importance of Ab in this instance. Furthermore, depletion of CD4⁺ and CD8⁺ T cells from immunized mice did not affect protection significantly (12). However, more recent studies using outer membrane proteins (OMP), from *F. tularensis* administered intranasally (i.n.) provided partial protection against the highly virulent biovar A strain SchuS4 and appeared to involve the generation of both anti-OMP-specific Ab, as well as IL-2 and TNF-α (15). As a result, it was suggested that the mechanism of protection in this case is complex and that cellular and humoral immunity, both correlates of protection in this instance, likely play a role in protection against *F. tularensis* infection. In addition, a more significant role for Ab in generating protection against *F. tularensis* challenge has been supported in other recent studies (9, 10). However, despite evidence favoring a role for Ab in protection against *F. tularensis* infection, the role of specific Ab isotypes is unclear. Although *F. tularensis*-specific IgM Ab is generated in response to vaccination with *F. tularensis* LPS and *F. tularensis* infection, *F. tularensis*-specific IgG Ab is also generated in these studies.

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⁵ Abbreviations used in this paper: LVS, *F. tularensis* live vaccine strain; iFt, inactivated *F. tularensis*; OMP, outer membrane protein; i.n., intranasally; CTA, cholera toxin A; BAL, bronchial alveolar lavage; CBA, cytometric bead array.

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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 transcutaneous 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 Ig 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 and absence of CTB. Furthermore, we considered the possibility that CTB may enhance both the humoral and cellular responses to iFt when administered i.n.

In addition to its mucosal adjuvant properties, CTB also enhances cellular immunity, although the mechanism of action is not fully understood (32, 33). However, it is known that CTB can enhance the production of Th1 cytokines, which are important for protective immunity against F. tularensis infection. 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).

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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-Ifngtm1Tyj/J (IFN-γ−/−) and the B10.129S2(B6)-Igh−6mrcypm/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 × 10^8 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 at 37°C to a density of ~0.5–1 × 10^8 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 × 10^8 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. 20 µl of PBS (vehicle), 5 µg of CTB (Sigma-Aldrich) in 20 µl of PBS, 2 × 10^7 IFI organisms in 20 µl of PBS, or 2 × 10^7 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 to 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 tissues. Tissue homogenate was also spun at 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 (Sigma-Aldrich) in 20 µl of PBS, 2 × 10^7 IFI organisms in 20 µl of PBS, or 2 × 10^7 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 23) 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 × 10^6 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 FACSAria Instrument (BD Immunocytometry Systems) and analyzed using CBA software version 1.01 (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.
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 coadministrating 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 coadministrating 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).

### 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-γ (Fig. 3A), TNF-α (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.
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 × 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. BAL fluid or sera was 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.

Control (PBS) mice demonstrated severe pathology in the lungs with extended tissue necrosis, thickened alveolar septa, alveolar congestion, and extensive cellular infiltrates compared with normal naive mice (Fig. 4, B and A, respectively). In contrast, mice immunized with iFt plus CTB showed minimal tissue destruction on day 7 after infection (Fig. 4D), most likely due to the reduced bacterial loads observed in these mice as a consequence of a more rapid and effective immune response primed 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 × 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. BAL fluid or sera was 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.

FIGURE 6. IgA is not required for protection induced by iFt plus CTB immunization. IgA-deficient mice were divided into three groups and immunized, and challenged with F. tularensis LVS as described in Fig. 1. Survival was monitored for at least 21 days after infection. IgA-deficient mice (B), as well as control (C57BL/6) mice (A), were protected against F. tularensis LVS challenge when immunized with iFt plus CTB. These data are representative of three separate experiments.
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 mice 10–12 days after boost. *F. tularensis*-specific total IgG, IgG2a (BALB/c), IgG2c (C57BL/6), and IgG1 Ab titers were measured by ELISA. Use of iFt plus CTB enhanced total IgG levels in
PBS, 5

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,

**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 × 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. Serum was then collected at 35 days and tested for the presence of 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.

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

**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 measured by CBA. The levels of IFN-γ 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 (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 (p < 0.01).

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**In vivo**

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**In vitro**

<table>
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<tr>
<th>PBS</th>
<th>CTB</th>
<th>iFt</th>
<th>iFt+CTB</th>
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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 *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 *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 *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 *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 *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 *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 seemingly 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 × LD50) were used. Specifically, the C57BL/6 mouse strain is particularly difficult to protect against *F. tularensis* (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 *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 *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 *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 *Mycobacterium bovis*-bacillus Calmette-Guérin was shown to enhance delayed-type hypersensitivity reactions to purified protein derivative (60). Also, mucosal administration of CTB with *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 *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 *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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**Disclosures**

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**References**

21. Tochikubo, K., M. Isaka, Y. Yasuda, S. Kozuka, K. Matano, Y. Miura, and
27. Isaka, M., Y. Zhao, E. Nobusawa, S. Nakajima, K. Nakajima, Y. Yasuda,
20: 3465–3471.


