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Cholera toxin B induced activation of murine macrophages exposed to a fixed bacterial immunogen

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Abstract

Objectives: Previous studies have demonstrated that intranasal administration of inactivated (fixed) *Francisella tularensis* (*iFt*) live vaccine strain (LVS) in conjunction with the mucosal adjuvant, cholera toxin B (CTB), provides full protection against subsequent lethal challenge with *Ft* LVS and partial protection against the more virulent *Ft* SchuS4 strain. Understanding the mechanisms of CTB-induced immune stimulation that confer protection against *Ft* will be valuable to the development of an effective vaccine against this highly virulent fatal pathogen. In this study, an *in vitro* system was utilized to further elucidate the immunologic adjuvant effect of CTB when administered with the fixed bacterial immunogen *iFt*.

Methods: The murine macrophage cell line (RAW264.7) was treated with combinations of *iFt* and CTB. The treated RAW264.7 cells and their supernatants were collected and assessed for cell surface marker expression and cytokine secretion. In addition, the ability of RAW264.7 cells to present bacterial antigens (*iFt* or LVS) to an *Ft*-specific T-cell hybridoma cell line, following exposure to CTB, was analyzed.

Results: We found that RAW264.7 cells responded to treatment with *iFt* + CTB by an increased secretion of the proinflammatory cytokines interleukin 6 and tumor necrosis factor α and upregulation of the surface expression of toll-like receptor 4 and the costimulatory molecules CD80 and CD86. Furthermore, the experimental vaccine treatment *iFt* + CTB enhanced the ability of macrophages to present *iFt* antigens to an *FT*-specific T-cell hybridoma cell line, although they failed to do so with LVS.

Conclusion: The adjuvant CTB administered in conjunction with *iFt* showed evidence of enhancing an antigen-specific proinflammatory response *in vitro.* These observations allow us to define, in part, the mechanisms of immune activation conferred by mucosal administration of *iFt* + CTB against lethal *F. tularensis* challenge.

Keywords: cholera toxin B, immunologic adjuvants, vaccines

Introduction

Francisella tularensis (*Ft*) is a Gram-negative intracellular pathogen that results in the potentially lethal disease tularemia. Less than 10 colony-forming units (CFU) of the highly pathogenic type A strain are required to cause life-threatening illness, with mortality rates between 30% and 60% when left untreated [Pechous *et al.* 2009]. Due to the highly infectious nature and potential for aerosol dissemination, *Ft* has been classified as a category A biological threat with no US Food and Drug Administration (FDA) approved vaccine available [Oyston *et al.* 2004]. A major contributor to the bacterium's pathogenicity is its capacity for intracellular replication and evasion of the host immune system. Several mechanisms of immune subversion have been described, including expression of poorly immunogenic lipopolysaccharide (LPS), downregulation of inflammatory cytokine secretion, and prevention of phagosome acidification and maturation in host macrophages [Hajjar *et al.* 2006; Telepnev *et al.* 2003; Clemens *et al.* 2004; Steiner *et al.* 2014]. A successful vaccination strategy against *Ft* will likely rely on both

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the innate and adaptive response to overcome the immune evading mechanisms that contribute to the highly virulent nature of *Francisella*. In addition, research has demonstrated intranasal immunization with the *Ft* live vaccine strain (LVS) confers superior protection against subsequent exposure to the virulent biovar A strain, suggesting that mucosal immunization may provide optimal protection against *Francisella* [Wu *et al.* 2005]. Stimulating both a cellular and humoral response through intranasal delivery will likely require the use of a mucosal adjuvant, such as cholera toxin B (CTB), to enhance the immune response against *Ft*. Administration of CTB has been reported to increase mucosal epithelium permeability, enhance antigen presentation, and drive T-cell proliferation, B-cell differentiation, and B-cell isotype switching [Lavelle *et al.* 2004; Gagliardi *et al.* 2002; Kim *et al.* 1998; Maeyama *et al.* 2001; Holmgren *et al.* 2003]. Studies conducted by Bitsaktsis and colleagues have demonstrated CTB does act as an effective adjuvant against lethal *Ft* challenge when administered with inactivated *Ft* LVS (*iFt*) [Bitsaktsis *et al.* 2009]. To further elucidate the role of CTB as an adjuvant, an *in vitro* system was utilized to determine the effects of *iFt* + CTB mixture on murine macrophages, which are the preferred host tissue of *Ft*. Our results suggest an important role for CTB in modulating the immune response in macrophage by increasing proinflammatory cytokine secretion and upregulating the expression of costimulatory receptors CD80 and CD86 which contribute to robust antigen presentation, leading to T-cell activation and expansion. Indeed, experimental vaccine treatments of *iFt* and CTB increase the ability of the fixed immunogen, *iFt*, to be presented to an *Ft*-specific T-cell hybridoma by the murine macrophage cell line, RAW 264.7. However, despite the CTB-driven macrophage activation observed *in vitro*, antigen presentation of LVS to the same Ft-specific T-cell hybridoma was not significant, suggesting that cytokine secretion is the contributing factor by macrophages in the protection against *Ft* challenge following the use of CTB as a mucosal adjuvant.

Materials and methods

Bacteria

The *Ft* LVS was kindly provided by Dr Edmund Gosselin (Albany Medical College, Albany, NY, USA). The bacterium was cultured at 37°C in Muller–Hinton broth supplemented with 2% IsovitaleX (Becton Dickinson, Franklin Lakes, NJ). Cultures were grown to a density of 2×10^9 CFU/ml and stored in liquid nitrogen. *iFt* was then washed three times in sterile phosphate-buffered saline (PBS).

Generation of immunogen

To generate *iFt* LVS, live bacteria grown to a concentration of 2×10^9 CFU/ml were incubated in 25 ml of sterile PBS containing 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 90 min at room temperature while shaking. *iFt* was then washed three times in sterile PBS. Inactivation was confirmed by culturing 100 µl of the fixed *iFt* on chocolate agar plates (Fisher Scientific, Waltham, MA) and monitoring for 7 days. The *iFt* preparation were stored in PBS at −20°C until use.

Cell culture

The murine myeloid cell line RAW 264.7 was kindly provided by Dr Allan Blake (Seton Hall University, South Orange, NJ, USA). Cells were grown in RPMI 1640 medium with 2 mM L-glutamine (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (Lonza) and 100 U/ml penicillin with 100 µg/ml streptomycin (ATCC, Manasas, VA). The *Ft*-specific T-cell hybridoma FT256D10 was obtained from Dr Edmund Gosselin (Albany Medical College). These cells were grown in RPMI 1640 medium with 2mM L-glutamine (Lonza) containing 10% fetal bovine serum (Lonza) and 100 U/ml penicillin, 100 µg/ml streptomycin (ATCC) supplemented with 1 mM sodium pyruvate (Sigma-Aldrich), 1% minimal essential medium (MEM) nonessential amino acids (Sigma-Aldrich), 50 µM 2-mercaptoethanol (Sigma-Aldrich), and 0.02 mg/ml Hygromycin B (Sigma-Aldrich). Cells were maintained at 37°C and 5% CO_2.

Cytokine measurement

Raw 264.7 (5×10^5 cells/well) were treated with CTB (1 and 5 µg/ml; Sigma-Aldrich), *iFt* (1×10^5 CFU/well), or a combination of *iFt* and CTB at the previous concentrations. Supernatants were harvested at 2, 12, 24, and 48 h, and stored at −20°C until cytokine analysis. Supernatants were assayed for interferon γ (IFNγ), tumor necrosis factor α (TNFα), interleukin (IL)-10, IL-4, and IL-6 using enzyme-linked immunosorbent assay (ELISA) Max Kits (Biolegend, San Diego, CA) according to manufacturers' instructions.

Flow cytometry

Raw 264.7 (5×10^5 cells/well) were treated with CTB (1 or 5 μg/ml), *iFt* (1×10^5 CFU/well), or a combination of *iFt* and CTB at the previous concentrations. After 24 h cells were collected and resuspended in flow cytometry staining buffer (FACS) buffer (PBS with 10% fetal bovine serum (FBS) and 0.02% sodium azide). Cells were stained for murine cell surface markers using the following antibodies purchased from Biolegend: CD86 (Pacific Blue), I-A/I-E (Alexa fluor 488), CD80 (PE), CD282 (Alexa fluor 647), CD284 (PE), and CD11b (Pacific Blue, PE, or Alexa Fluor 488). Following staining, cells were washed with PBS and resuspended in 2% paraformaldehyde and analyzed on a MACS Quant flow cytometer (Miltenyi Biotech, San Diego, CA). Cells were gated using FlowJo software according to forward and side scatter properties (FlowJo, Ashland, OR).

Antigen presentation assay

Raw 264.7 cells (5×10^5 cells/well) were pretreated with 1 or 5 μg/ml of CTB, *iFt* (1×10^5 CFU/well), or a combination of CTB and *iFt* at the concentrations previously listed. Cells were then incubated for 24 h at 37°C in 5% CO_2 and then exposed to *Ft* LVS or *iFt*. The *Ft* LVS or *iFt* (1×10^6 CFU/well) was added to the pretreated Raw 264.7 cells, which were then cocultured with the Ft256D10 T-cell hybridoma (2.5×10^5) for a period of 24 h. The supernatants were collected and analyzed for IL-5 as an indicator of activation using ELISA (Biolegend).

Statistical analyses

Experimental conditions for cytokine analysis were performed in triplicate and statistical data were generated using Student's t test to compare treatments with PBS controls. Statistical analysis and graphs were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Flow cytometry density plots were generated using FlowJo software.

Results

CTB enhances the proinflammatory cytokine response to the iFt *immunogen in RAW 264.7 macrophages*

To evaluate the potential of CTB as an immune-stimulating adjuvant in combination with the fixed bacterial immunogen *iFt*, we assessed the cytokine secretion by the RAW 264.7 cells, treated with CTB or *iFt* alone, or CTB in combination with *iFt* (Figure 1). Cells treated with both 1 and 5 μg of CTB in combination with *iFt* showed increased secretion of IL-6 at both 24 and 48 h compared with cells treated with the adjuvant or immunogen alone [Figure 1(a, b)]. While cells treated with CTB alone produced elevated amounts of IL-6 compared with the control (PBS), the addition of *iFt* in conjunction with CTB significantly increased IL-6 production by 20-fold, suggesting that the adjuvant enhanced the ability of the *iFt* immunogen to elicit a proinflammatory response. IL-6 is a proinflammatory cytokine which enhances the innate immune system through the induction of acute phase proteins and stimulation of T and B lymphocytes [Heinrich *et al.* 2003]. In addition, a 15-fold increase in the proinflammatory cytokine TNFα was observed at 2 and 24 h in cells treated with CTB compared with groups not exposed to the adjuvant [Figure 1(c, d)]. TNFα is primarily produced by activated macrophages and works in concert with IL-6 to orchestrate the local inflammatory response through leukocyte recruitment and activation. While CTB treatment increased the secretion of TNFα, the addition of *iFt* did not significantly alter the cytokine production, suggesting that CTB has a direct immunogenic effect on murine macrophages in terms of TNFα production. In addition, anti-inflammatory cytokines IL-4 and IL-10 were not detected in any of the treatment groups (data not shown).

CTB treatment in combination with iFt *increases expression of toll-like receptor 4 on RAW 264.7 cells*

Pattern recognition receptors, such as toll-like receptors (TLRs), identify conserved pathogen associated molecules and initiate signaling cascades that are essential to the innate immune response. Since TLR4 is the major ligand for LPS, a molecule present in the cell wall of all Gram-negative bacteria, we sought to determine whether treatment with CTB and *iFt* would increase the expression of this pattern recognition receptor. For this purpose, expression of TLR4 on Raw 264.7 cells following treatment was analyzed by flow cytometry. Cells treated with CTB and *iFt* had a 37.2% increase in TLR4 expression at 24 h compared with the untreated control [Figure 2(a, c)]. After 48 h of treatment, TLR4 expression was 59.2% greater than the expression observed in the untreated cells [Figure 2(b, c)]. At both time points, the mean fluorescent intensity

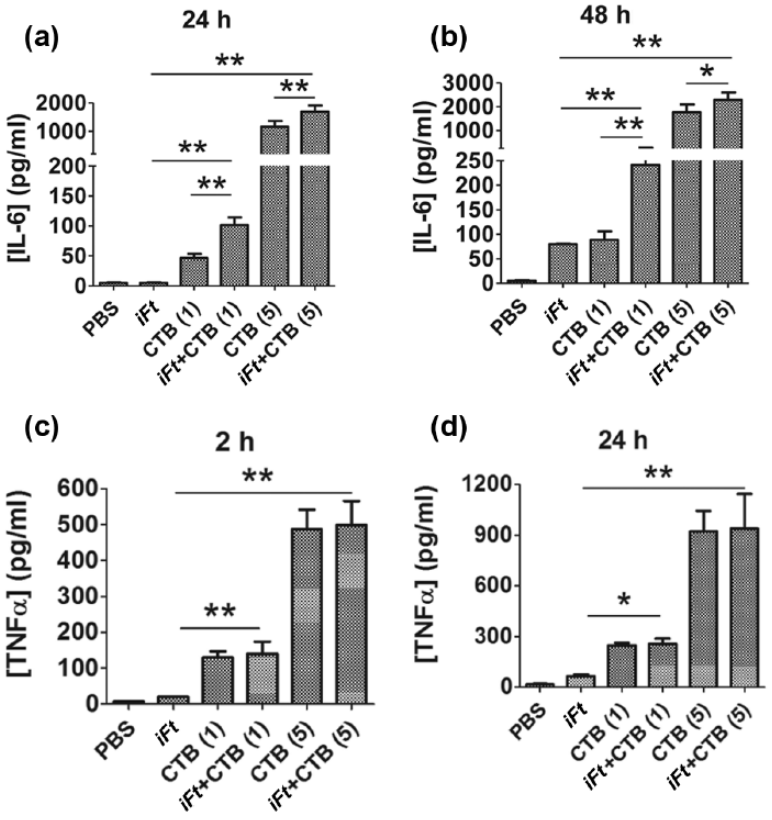

Figure 1. Increased production of proinflammatory cytokines by RAW 264.7 cells treated with CTB and *iFt*. RAW 264.7 cells were treated with *iFt* (1×10^5 CFU/well) and CTB (1 or 5 μg) or a combination of CTB and *iFt*. Supernatants were collected at 2, 24, and 48 h and the levels of IL-6 (a, b) and TNFα (c, d) were measured by ELISA (*$p < 0.05$ and **$p < 0.01$, $n = 3$).
CTB, cholera toxin B; ELISA, enzyme-linked immunosorbent assay; *iFt*, inactivated (fixed) *Francisella tularensis*; IL, interleukin; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

(MFI) of TLR4 on RAW 264.7 cells treated with *iFt* + CTB was threefold higher than when treated with *iFt* alone [Figure 2(c)]. In fact, it is of interest that TLR4 expression decreased following treatment of cells with *iFt* alone, perhaps suggesting that this may be an alternate mechanism of immune evasion by the bacterium [Figure 2(a–c)]. TLR4 expression was unaffected by CTB alone (data not shown).

CTB treatment enhances expression of the costimulatory molecules CD80 and CD86 on RAW 264.7 cells

Costimulatory molecules CD80 and CD86 are expressed at low levels on unstimulated macrophages. These ligands provide the necessary secondary signal, along with major histocompatibility complex (MHC) complexed antigenic peptide, to induce T-cell activation allowing for clonal expansion and cytokine production [Crawford *et al.* 2006). Therefore, the effect of CTB on the expression levels of CD80, CD86 and MHC class II on RAW cells was determined *in vitro*. For this purpose, RAW 264.7 cells were incubated for 24 and 48 h with the fixed bacterial immunogen *iFt* in the presence or absence of the CTB adjuvant, and the expression levels of costimulatory molecules was determined by flow cytometry (Figure 3). Cells treated with *iFt* and CTB had a concentration-dependent increase in CD80 [Figure 3(a)] and CD86 [Figure 3(b)] on their surface. Treatment of cells with *iFt* or CTB alone did not affect the expression of these costimulatory molecules, suggesting that the increase observed requires the synergism between the bacterial immunogen and CTB (Figure 3 and data not shown).

Antigen presentation in vitro *is enhanced by pretreatment of RAW 264.7 cells with* iFt *plus CTB*

Unstimulated macrophages express low levels of MHC class II and are generally poor activators of naïve T cells. The enhanced MHC class II and

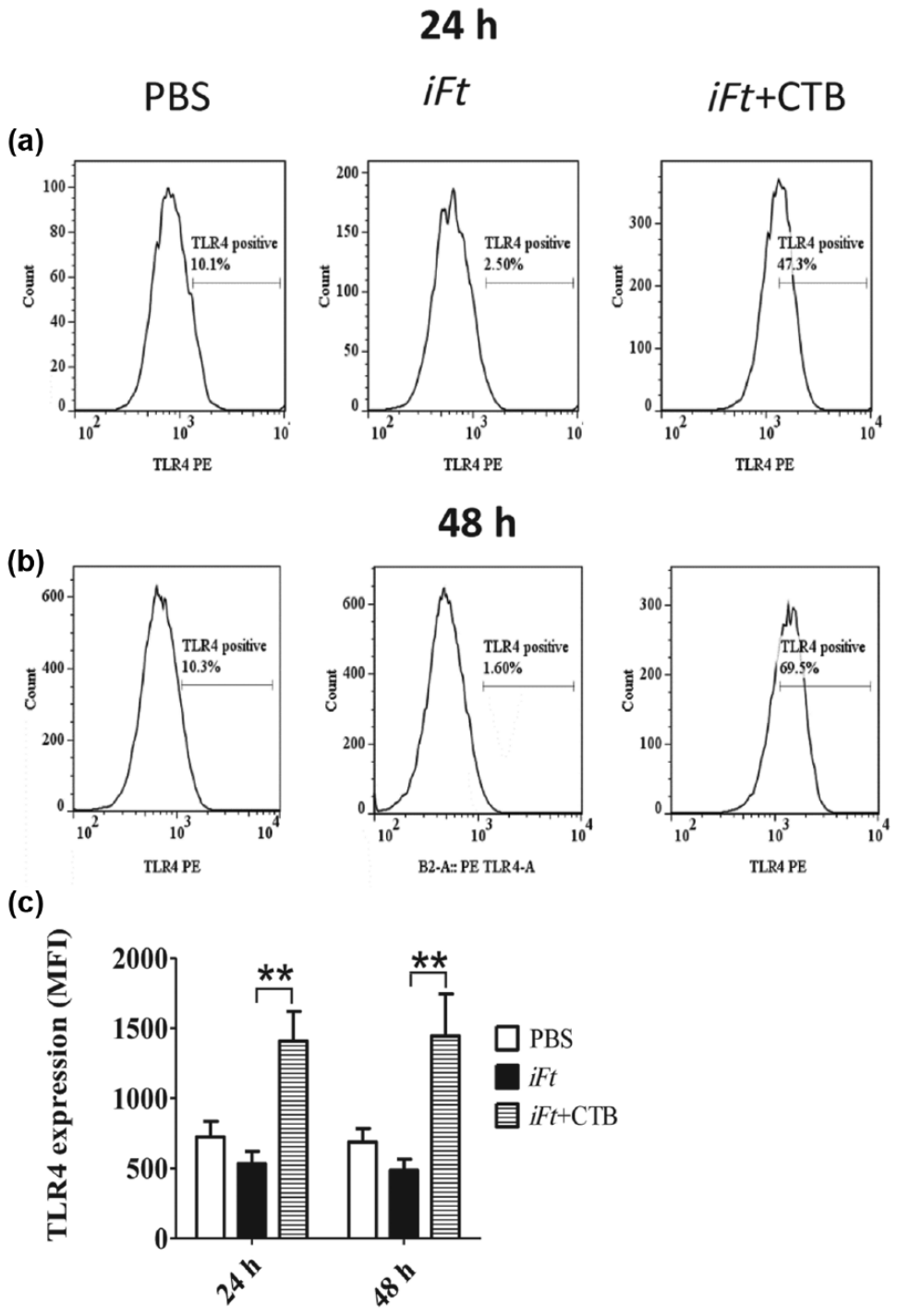

Figure 2. TLR4 expression is increased on RAW 264.7 cells treated with CTB and *iFt*. RAW 264.7 cells were treated with PBS, *iFt* or *iFt* + CTB for 24 or 48 h. The levels of TLR4 were detected by flow cytometry (a, b). The mean fluorescent intensity (MFI) of TLR4 is also presented for each treatment (c) (**$p < 0.01$). CTB, cholera toxin B; *iFt*, inactivated (fixed) *Francisella tularensis*; IL, interleukin; PBS, phosphate-buffered saline; TLR, toll-like receptor; PE, phycoerythrin.

costimulatory molecule expression by RAW 264.7 cells treated with *iFt* + CTB suggests that the adjuvant may increase the capacity of these cells to present antigen *in vitro*. In order to assess antigen presentation, the RAW 264.7 cells were primed with *iFt* + CTB and cultured with either *iFt* or *FT* LVS and the *Ft*-specific T-cell hybridoma (FT256D10). Presentation of *Ft* antigens

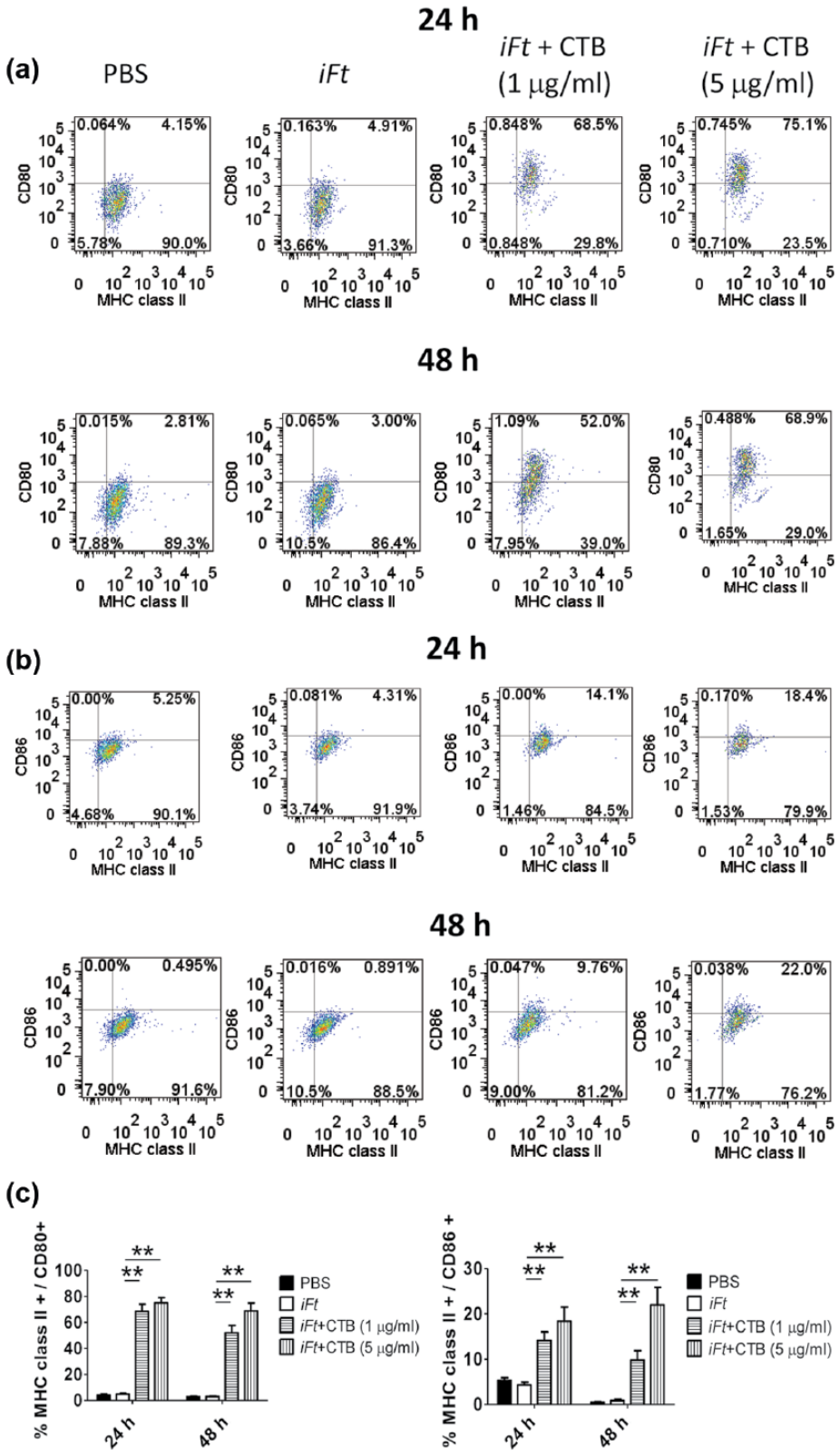

Figure 3. Costimulatory molecules CD80 and CD86 are upregulated with *iFt* + CTB treatment. RAW 264.7 cells were cultured with *iFt* in the presence or absence of CTB for 24 and 48 h. Expression of MHC class II, CD80 (a) and CD86 (b) was assessed by flow cytometry. Results are also presented as a bar graph (c). These data are representative of three experiments (*$p < 0.05$ and **$p < 0.01$, $n = 2$). CTB, cholera toxin B; *iFt*, inactivated (fixed) *Francisella tularensis*; IL, interleukin; MHC, major histocompatibility class; PBS, phosphate-buffered saline.

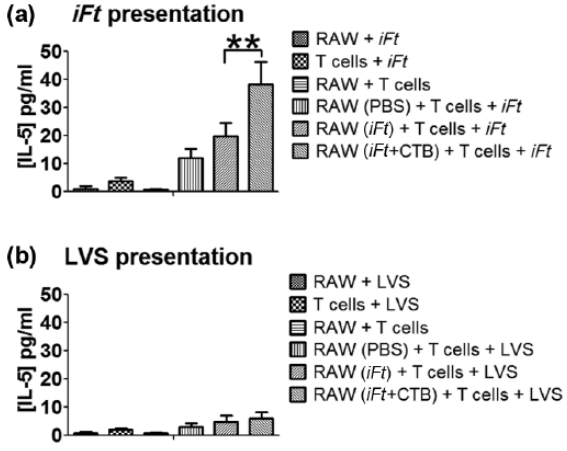

Figure 4. Experimental vaccine treatment of *iFt* and CTB enhances antigen presentation of fixed bacterial antigens by RAW 264.7 cells. RAW 264.7 cells were treated as previously described and subsequently cocultured with the *Ft*-specific T-cell hybridoma, FT256D10, for 24 h together with either *iFt* (a) or LVS (b) as a source of bacterial antigen. Antigen presentation was assessed by the levels of IL-5 secreted by the activated T-cell hybridoma. IL-5 in the culture supernatants was measured by ELISA (*$p < 0.05$ and **$p < 0.01$, $n = 3$). CTB, cholera toxin B; ELISA, enzyme-linked immunosorbent assay; *iFt*, inactivated (fixed) *Francisella tularensis*; IL, interleukin; PBS, phosphate-buffered saline.

and subsequent activation of the T-cell hybridoma was assessed through IL-5 secretion. Cells treated with *iFt* + CTB and further exposed to *iFt* antigens had a significant increase in IL-5 secretion compared with cells treated with *iFt* alone [Figure 4(a)], indicating improved antigen presentation from the macrophage cells. Conversely, when pretreated macrophages were exposed to *FT* LVS there was little evidence of T-cell activation in all groups [Figure 4(b)]. The difference in IL-5 secretion observed between the live and fixed antigen may be attributed to the ability of live *Ft* to subvert innate antimicrobial effector functions and proliferate in the intracellular niche of RAW 264.7.

Discussion

Studies suggest the primary immune response to *Ft* infection requires IFNγ- and TNF-mediated activation of macrophages and neutrophil recruitment, followed by a secondary response from CD4 and CD8 T cells required to overcome the infection and produce a long-lasting memory response [Elkins *et al.* 2003]. In the current study we observed an increase in macrophage mediated proinflammatory cytokines IL-6 and TNFα, suggesting enhancement of the acute inflammatory response when CTB is administered with *iFt*. Harnessing a healthy acute response through adjuvant addition would provide crucial support in bacterial clearance and antigen presenting cell (APC) recruitment during the initial phase of infection. Following the acute response, activated macrophages and other professional APCs present antigen to T cells which require a costimulatory signal provided by CD80 and CD86 on the APCs binding CD28 on T cells. The increase of costimulatory molecules in response to CTB and *iFt* could indicate the activated macrophages are more efficient at costimulation, limiting the occurrence of antigen presentation resulting in tolerance or anergy. Moreover, the abundance of costimulatory molecules on macrophages could enhance the proliferation of effector T cells and the generation of an *Ft* specific response.

In addition to increased costimulatory molecules, higher levels of the pathogen recognition receptor TLR4 were detected by flow cytometry. LPS is found on the surface of Gram-negative bacteria like *Ft* and is usually a potent activator of TLR4. The LPS expressed by *Ft* has limited antigenic activity compared with LPS produced by other Gram-negative bacteria such as *Escherichia coli* and *Salmonella*. As a result, *Ft* LPS has been shown to have limited binding to TLR4 and signals primarily through TLR2. While the atypical LPS endotoxin expressed on *Ft* is a poor activator of TLR4, one study demonstrated that TLR4-deficient mice were more vulnerable to intracutaneous infection by LVS [Macela *et al.* 1996]. Hence, increases in the TLR4 receptor may be significant if increased receptor density raises the potential for LPS ligation and macrophage activation.

The increase in the costimulatory molecules on RAW cells, following treatment with the fixed bacterial immunogen *iFt* plus CTB, suggests the potential for enhanced antigen presentation. To confirm that *Ft* antigens was more readily presented to T lymphocytes, an antigen presentation assay was performed using the *Ft*-specific T-cell hybridoma FT256D10 cocultured with either *iFt* or LVS as a source of *Ft* antigens. Antigen presentation was increased in cells exposed to the fixed antigen while *Ft* LVS had no effect. These observations may be the byproduct of a hallmark of *Ft* pathogenesis: intracellular survival and replication in the cytosol of a host macrophage.

During the initial infection, *Ft* is sequestered within the phagasome, resulting in increases in TNFα and nuclear factor κB (NFκB) driven pro-inflammatory cytokine production [Telepnev *et al.* 2005]. The bacterium evades initial destruction and subverts the primary host defense through altering acidification and maturation of the phagosome and then escaping to the cytosol to replicate [Fernandes-Alnemri *et al.* 2010]. In coordination with entrance into the cytosol, *Ft* inhibits NFκB, thus modulating the host inflammatory reaction to the intracellular bacterium. In response to cytosolic localization of *Ft*, the mouse macrophage produces type I IFN and absent in melanoma 2 (AIM2) protein, leading to activation of the inflammasome [Asare and Kwaik, 2010; Sjostedt, 2006]. As a result of inflammasome activation, the host macrophages undergo caspase-1 and death-fold containing adaptor protein mediated cell death in an attempt to quell bacterial replication and expansion [Mariathasan *et al.* 2005]. This cytopathogenic effect has been observed after 24–48 h in the *Ft* LVS infected murine macrophage cell line J774 [Lai *et al.* 2001]. These phenomena may have resulted in the limited antigen presentation of *LVS* we observed in RAW 264.7.

While the *Ft* LVS strain can present challenges in the context of antigen presentation, the inactivated bacteria administered with CTB significantly increased antigen presentation and thus T-cell activation. This demonstrates the stimulation of cellular immunity invoked by CTB when administered with *iFt*, which could provide sufficient protection against lethal *Ft* challenge. In fact, when CTB is administered as an adjuvant with *iFt in vivo*, increased protection against *Ft* biovar A and biovar B challenge was noted [Bitsaktsis *et al.* 2009]. To further correlate our observations of CTB *in vitro* with protective cellular immunity, future evaluation of CTB coadministered with *iFT* in a murine model is required. Evidence of adjuvant-enhanced macrophage activation could be established through quantification of proinflammatory cytokines such as TNFα, IL-6, IL-12, and IL-1 in bronchoalveolar lavage fluid and lung homogenates. In addition, *in vivo* macrophage activation can be assessed through the expression of costimulatory molecules on the surface of peritoneal cells and alveolar macrophages following immunization with CTB and the immunogen. Furthermore, memory T-cell responses can be demonstrated *ex vivo* by monitoring T-cell expansion and cytokine secretion in isolated splenocytes exposed to *iFT*. However, the lack of enhanced antigen presentation of LVS by murine macrophages suggests that the role they play in protection observed *in vivo* is primarily due to enhanced proinflammatory cytokine production rather than providing the bridge between innate and adaptive immunity.

While evidence suggests CTB is a potent immunogen, certain challenges exist which have slowed the potential for FDA approval of CTB as an adjuvant. Foremost among these concerns are reports of side effects ranging from headaches and nasal bleeding to some rare cases of encephalitis [Scerpella *et al.* 1995]. Many of the side effects observed however may be overcome by optimizing dosing regimens or creating CTB-coupled vaccination strategies to provide a strong immune response while limiting side effects [Guo *et al.* 2012].

In summary, the adjuvant CTB administered with *iFt* showed *in vitro* evidence of enhancing cellular immunity through increases in proinflammatory immune-stimulating cytokines. CTB also appears to activate an antigen-specific response by increasing costimulatory molecules and antigen presentation, therefore generating pathogen-specific T-cell responses. These observations demonstrate mechanisms through which CTB could stimulate protection in an *in vivo* infectious disease model. Hence, utilizing an adjuvant like CTB may provide the necessary enhancement of the innate and adaptive facets of the immune response required to protect against *Ft* infection.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

Asare, R. and Kwaik, Y. (2010) Exploitation of host cell biology and evasion of immunity by Francisella tularensis. *Front Microbiol* 1: 145.

Bitsaktsis, C., Rawool, D., Li, Y., Kurkure, N., Iglesias, B. and Gosselin, E. (2009) Differential

requirements for protection against mucosal challenge with Francisella tularensis in the presence versus absence of cholera toxin B and inactivated F. tularensis. *J Immunol* 182: 4899–4909.

Clemens, D., Lee, B. and Horwitz, M. (2004) Virulent and avirulent strains of Francisella tularensis prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. *Infect Immun* 72: 3204–3217.

Crawford, A., Macleod, M., Schumacher, T., Corlett, L. and Gray, D. (2006) Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol* 176: 3498–3506.

Elkins, K., Cowley, S. and Bosio, C. (2003) Innate and adaptive immune responses to an intracellular bacterium, Francisella tularensis live vaccine strain. *Microbes Infect* 5: 135–142.

Fernandes-Alnemri, T., Yu, J., Juliana, C., Solorzano, L., Kang, S., Wu, J. *et al.* (2010) The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nat Immunol* 11: 385–393.

Gagliardi, M., Sallusto, F., Marinaro, M., Vendetti, S., Riccomi, A. and De Magistris, M. (2002) Effects of the adjuvant cholera toxin on dendritic cells: stimulatory and inhibitory signals that result in the amplification of immune responses. *Int J Med Microbiol* 291: 571–575.

Guo, L., Liu, K., Xu, G., Li, X., Tu, J., Tang, F. *et al.* (2012) Prophylactic and therapeutic efficacy of the epitope vaccine CTB-UA against Helicobacter pylori infection in a BALB/c mice model. *Appl Microbiol Biotechnol* 95: 1437–1444.

Hajjar, A., Harvey, M., Shaffer, S., Goodlett, D., Sjostedt, A., Edebro, H. *et al.* (2006) Lack of in vitro and in vivo recognition of Francisella tularensis subspecies lipopolysaccharide by Toll-like receptors. *Infect Immun* 74: 6730–6738.

Heinrich, P., Behrmann, I., Haan, S., Hermanns, H., Muller-Newen, G. and Schaper, F. (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374: 1–20.

Holmgren, J., Czerkinsky, C., Eriksson, K. and Mharandi, A. (2003) Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine* 21(Suppl. 2): S89–S95.

Kim, P., Eckmann, L., Lee, W., Han, W. and Kagnoff, M. (1998) Cholera toxin and cholera toxin B subunit induce IgA switching through the action of TGF-beta 1. *J Immunol* 160: 1198–1203.

Lai, X., Golovliov, I. and Sjostedt, A. (2001) Francisella tularensis induces cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication. *Infect Immun* 69: 4691–4694.

Lavelle, E., Jarnicki, A., McNeela, E., Armstrong, M., Higgins, S., Leavy, O. *et al.* (2004) Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent. *J Leukoc Biol* 75: 756–763.

Macela, A., Stulik, J., Hernychova, L., Kroca, M., Krocova, Z. and Kovarova, H. (1996) The immune response against Francisella tularensis live vaccine strain in LPS(N) and LPS(D) mice. *FEMS Immunol Med Microbiol* 13: 235–258.

Maeyama, J., Isaka, M., Yasuda, Y., Matano, K., Kozuka, S., Taniguchi, T. *et al.* (2001) Cytokine responses to recombinant cholera toxin B subunit produced by Bacillus brevis as a mucosal adjuvant. *Microbiol Immunol* 45: 111–117.

Mariathasan, S., Weiss, D., Dixit, V. and Monack, D. (2005) Innate immunity against Francisella tularensis is dependent on the ASC/caspase-1 axis. *J Exp Med* 202: 1043–1049.

Oyston, P., Sjostedt, A. and Titball, R. (2004) Tularaemia: bioterrorism defence renews interest in Francisella tularensis. *Nat Rev Microbiol* 2: 967–978.

Pechous, R., McCarthy, T. and Zahrt, T. (2009) Working toward the future: insights into Francisella tularensis pathogenesis and vaccine development. *Microbiol Mol Biol Rev* 73: 684–711.

Scerpella, E., Sanchez, J., Mathewson, I., Torres-Cordero, J., Sadoff, J., Svennerholm, A. *et al.* (1995) Safety, immunogenicity, and protective efficacy of the whole-cell/recombinant B subunit (WC/rBS) oral cholera vaccine against travelers' diarrhea. *J Travel Med* 2: 22–27.

Sjostedt, A. (2006) Intracellular survival mechanisms of Francisella tularensis, a stealth pathogen. *Microbes Infect* 8: 561–567.

Steiner, D., Furuya, Y. and Metzger, D. (2014) Host-pathogen interactions and immune evasion strategies in Francisella tularensis pathogenicity. *Infect Drug Resist* 7: 239–251.

Telepnev, M., Golovliov, I., Grundstrom, T., Tarnvik, A. and Sjostedt, A. (2003) Francisella tularensis inhibits toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages. *Cell Microbiol* 5: 41–51.

Telepnev, M., Golovliov, I. and Sjostedt, A. (2005) Francisella tularensis LVS initially activates but subsequently down-regulates intracellular signaling and cytokine secretion in mouse monocytic and human peripheral blood mononuclear cells. *Microb Pathog* 38: 239–47.

Wu, T., Hutt, J., Garrison, K., Berliba, L., Zhou, Y. and Lyons, C. (2005) Intranasal vaccination induces protective immunity against intranasal infection with virulent Francisella tularensis biovar A. *Infect Immun* 73: 2644–2654.