A Francisella tularensis Live Vaccine Strain That Improves Stimulation of Antigen-Presenting Cells Does Not Enhance Vaccine Efficacy

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Abstract

Vaccination is a proven strategy to mitigate morbidity and mortality of infectious diseases. The methodology of identifying and testing new vaccine candidates could be improved with rational design and in vitro testing prior to animal experimentation. The tularemia vaccine, Francisella tularensis live vaccine strain (LVS), does not elicit complete protection against lethal challenge with a virulent type A Francisella strain. One factor that may contribute to this poor performance is limited stimulation of antigen-presenting cells. In this study, we examined whether the interaction of genetically modified LVS strains with human antigen-presenting cells correlated with effectiveness as tularemia vaccine candidates. Human dendritic cells infected with wild-type LVS secrete low levels of proinflammatory cytokines, fail to upregulate costimulatory molecules, and activate human T cells poorly in vitro. One LVS mutant, strain 13B47, stimulated higher levels of proinflammatory cytokines from dendritic cells and macrophages and increased costimulatory molecule expression on dendritic cells compared to wild type. Additionally, 13B47-infected dendritic cells activated T cells more efficiently than LVS-infected cells. A deletion allele of the same gene in LVS displayed similar in vitro characteristics, but vaccination with this strain did not improve survival after challenge with a virulent Francisella strain. In vivo, this mutant was attenuated for growth and did not stimulate T cell responses in the lung comparable to wild type. Therefore, stimulation of antigen-presenting cells in vitro was improved by genetic modification of LVS, but did not correlate with efficacy against challenge in vivo within this model system.

Introduction

The development of vaccines is essential to combat harmful infectious diseases [1]. Obtaining licensure after discovery of a vaccine, however, can take up to 20 years due to the stringent testing required to confirm the safety and efficacy of the vaccine [2]. To expedite this process, in vitro tests could be developed to define correlates of protection and identify more promising vaccine candidates. These assays would be particularly beneficial with vaccine candidates for highly pathogenic organisms, such as the bacterium Francisella tularensis, when challenge studies cannot be performed in humans because of contemporary regulations that govern clinical trials [3].

F. tularensis is the causative agent of tularemia [4]. This zoonotic disease is endemic in North America and parts of Europe and Asia, and outbreaks in these regions are frequently associated with the handling of infected animals or transmission by arthropod vectors [4,5]. F. tularensis is also classified by the Centers for Disease Control and Prevention as a Category A bioterrorism agent [6]. When inhaled, less than 10 organisms can cause an acute pneumonia that is lethal in up to 60% of infected individuals if left untreated [7]. The World Health Organization predicted that if virulent F. tularensis was aerosolized over a metropolitan area of five million people, more than 19,000 people would die and 250,000 individuals would be incapacitated [6]. An effective vaccine would be useful to reduce the number of naturally occurring tularemia cases and to protect against a possible intentional release.

To date, two different types of tularemia vaccines have been studied in humans. The Foshay vaccine consisted of chemically killed F. tularensis and was effective at reducing the incidence of laboratory-acquired tularemia cases from approximately 100% to 30% in the 1950s [8,9]. However, killed F. tularensis provided only minimal protection from aerosol type A Francisella challenge in a

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vaccine trial [10]. Researchers in the former Soviet Union took a different approach and developed a range of live attenuated 
Francisella strains to immunize people against tularemia [11]. One of these strains, a live attenuated strain of F. tularensis subsp. 
holocheilia, live vaccine strain (LVS), was superior to the Foshay-type vaccines at providing protection [10,12,13]. While two clinical studies involving small numbers of human vaccinees demonstrated effectiveness of LVS against aerosol challenge by virulent type A Francisella [10,12], a later study showed variable efficacy that diminished over time [14]. Vaccination of individuals by aerosol improved the efficacy of LVS but this required a high dose of 10^6 to 10^9 organisms which frequently resulted in severe adverse side effects [14].

Currently, LYS is not approved by the Food and Drug Administration (FDA) due to concerns about its undefined attenuation, mechanism of protection, and reversion frequency [15]. In order to obtain FDA approval, several groups are working to address these issues. Recent work by Salomonsson et al. identified two regions of difference, RD18 and RD19, which are deleted in LYS and account for its attenuation [16]. Additional studies improved the manufacturing process of LYS in compliance with good manufacturing practice guidelines [17]. This new lot of LYS was further characterized in human phase I clinical trials [18]. Researchers are also introducing mutations into LYS in order to improve its efficacy and bolster attenuation. One example is an LVS mutant deficient in iron superoxide dismutase (sodB). Compared to LYS, sodB increases median time to death and percent survival of C57BL/6 mice from pulmonary type A Francisella challenge [19]. As work toward the licensing of LYS continues, attempts have been made to replace LYS with a genetically defined, attenuated type A Francisella strain. For example, Schu S4 AFTT_1103 and Schu S4 ΔgelB, provide 75% and 60% protection, respectively, from virulent type A Francisella challenge in BALB/c mice [20,21]. Nevertheless, LYS remains the leading tularemia vaccine to date that has shown activity in humans [10,12].

A potential limitation of LYS as a vaccine is its relative stimulation of antigen-presenting cells (APCs). Published work has shown LYS stimulates murine and human DCs [22,23], though it is now known that culture conditions influence stimulation of innate immunity [24,25,26,27,28]. In contrast, other studies have shown that LYS suppresses the activation of murine macrophages [24,29,30,31,32] and dendritic cells (DCs) [32]. Murine macrophages and DCs cultured with LYS produce little to no proinflammatory cytokines in vitro compared to DCs cultured with other bacteria or TLR ligands [24,29,30,32]. Stimulation with TLR ligands such as Escherichia coli LPS fails to restore cytokine secretion by these cells suggesting that LYS is actively suppressing TLR signaling [24,25,29,32]. Another study noted that this suppression is due to downregulation of critical inflammatory signaling pathways involved in MAPK and NF-kB activation [30].

In this study, we tested whether in vitro screening of potential tularemia vaccine candidates for enhanced stimulation of APCs would improve a candidate’s immunogenicity, and ultimately protection after challenge. After initial testing of several LVS strains, we evaluated one genetic locus in detail with mutant strains that showed desirable vaccine characteristics in vitro, including attenuation in macrophages and enhanced DC stimulation. Despite these traits, they did not predict better protection against virulent type A Francisella challenge.

Materials and Methods

Ethics Statement

Human cells were purified from discarded buffy coats obtained from the Central Blood Bank (Pittsburgh, PA). The use of these samples was reviewed and approved by the Institutional Review Board of the University of Pittsburgh, which made a “no human subjects” determination and waived requirement for consent. All research involving animals was conducted in accordance with animal care and use guidelines, and animal protocols were approved by the University of Pittsburgh Animal Care and Use Committee (protocols 1003507 and 1002514).

Francisella strains and growth conditions

For cultivation of F. tularensis LVS strains and Schu S4, frozen stock cultures were streaked onto chocolate II agar plates and incubated at 37°C, 5% CO2 for 2–3 days. LYS strains were grown in Chamberlain’s chemically defined broth medium (CDM) [33] or MH broth [Mueller-Hinton broth (Difco) supplemented with 0.1% glucose, 0.025% ferric pyrophosphate (Sigma), and Iso-VitalEx (Becton Dickinson)] for in vitro infections. For mouse vaccinations, MH broth or TSb [trypticase soy broth (BD Biosciences) supplemented with 0.1% L-cysteine hydrochloride monohydrate (Fisher)] was used for culturing of LYS strains. Schu S4 was grown in MH broth for infections of vaccinated mice. Broth cultures were grown at 37°C with shaking for 14–18 hours. When required, antibiotics were added to the media at the following concentrations: kanamycin at 10 μg/ml, chloramphenicol at 5 μg/ml, and hygromycin at 200 μg/ml.

Generation of formalin-fixed Francisella tularensis Schu S4 (ffSchu S4)

Schu S4 was grown in MH broth as described above. Following overnight culture, bacteria were washed, resuspended in PBS (Gibco), and adjusted to an OD_600 of 0.3. Bacteria were then resuspended in 10% buffered formalin (Fisher) and incubated at 25°C for 10 min with shaking (200 rpm). Bacteria were washed five times and resuspended in PBS for an approximate concentration of 1–3×10^9 CFU/ml. Bacterial killing was confirmed by plating of formalin-fixed Schu S4 on chocolate II agar plates in which no colonies were observed following extensive incubation (data not shown). Prior to formalin fixation, an aliquot of the bacterial suspension was removed and tested for viable CFU by plating serial dilutions on chocolate II agar.

Construction of LVS mutants

Construction of LVS strain 1664d was described previously [34]. The F. tularensis LVS ΔcapC mutant was generated using homologous recombination with a suicide plasmid. This plasmid contained two segments homologous to regions flanking FTI-1415 and one third of the 5′ and 3′ ends of this ORF, surrounding a chloramphenicol acetyltransferase gene (cat) under the control of the F. tularensis groE promoter (Table 1). Linearized plasmid was electroporated into LYS [35] and double cross-over events were selected on cage heart agar with 5% defibrillated rabbit blood containing 2.5 μg/ml chloramphenicol. Recombination was confirmed by PCR (data not shown). To generate strain 13B17, plasmid pSD26 (a gift from Eric Rubin and Simon Dillon) was electroporated into LVS as previously described [36]. pSD26 is an E. coli plasmid delivery vector (oriE1, Ap’, that encodes a Himar1 transposase [37] and a transposon containing a kanamycin resistance cassette under the control of the F. tularensis groE promoter (Table 1). Following recovery in trypticase soy broth supplemented with 0.1% cysine, bacteria were plated on cysine heart broth with 5% defibrillated rabbit blood containing kanamycin (5 μg/ml). Colonies that emerged in the presence of kanamycin were isolated and screened for lack of response to extracellular spermine [36]. The selection
with 10% FBS, 25 mM HEPES, 1% non-essential amino acids, BMDCs were washed and resuspended in DMEM supplemented human serum, 25 mM HEPES, and 1% GlutaMAX. Murine washed and resuspended in DMEM supplemented with 1% flow cytometry. at 37°u resulting cells were greater than 90% CD11c+ (Miltenyi Biotec) per the manufacturer’s instructions. The DCs (BMDCs) were purified using CD11c magnetic beads with 500 U/ml GM-CSF was added every 2 days. Bone marrow

Infection of macrophages and DCs with F. tularensis LVS strains

Human monocytes were differentiated into macrophages and DCs by in vitro culture as described previously [34]. For generation of murine DCs, bone marrow was flushed from femurs and tibias of C57BL/6 mice with complete DC medium [DMEM supplemented with 10% heat-inactivated FBS, 25 mM HEPES, 1% non-essential amino acids, 1% sodium pyruvate, 1% GlutaMAX and 0.1% 2-mercaptoethanol (all from Gibco)]. Freshly isolated cells were washed and red blood cells were lysed with ACK Lysis Buffer (Gibco). After washing and counting, cells were resuspended in complete DC media supplemented with 500 U/ml GM-CSF (eBioscience) and seeded into T75 culture as described previously [34]. For macrophages and DCs were seeded into 24-well plates (Costar) at 5×10⁴ cells/well, respectively. Cells were incubated with 5% CO₂, allowing cultures to proceed 24, 48, and 72 hours) and DCs were prepared for flow cytometric analysis.

Inoculation of macrophages and DCs with F. tularensis LVS strains

For cytokine and flow cytometry experiments, human cells were washed and resuspended in DMEM supplemented with 1% human serum, 25 mM HEPES, and 1% GlutaMAX. Murine BMDCs were washed and resuspended in DMEM supplemented with 10% FBS, 25 mM HEPES, 1% non-essential amino acids, 1% sodium pyruvate, 1% GlutaMAX and 0.1% 2-mercaptoethanol (all from Gibco). DCs and macrophages were seeded into 24-well plates (Costar) at 5×10⁵ cells/well and infected with bacteria at an MOI of 10 for 24 hours prior to co-culture with T cells (see “Human DC-CD4 T cell co-culture”).
Flow cytometry and analysis of human monocyte-derived DCs

Surface markers on *F. tularensis*-infected human monocyte-derived DCs were evaluated by flow cytometric analysis. Following infection, DCs were removed from 24-well plates using a 2 mM EDTA solution. Cells were washed once and resuspended in FACS staining buffer [0.1% bovine serum albumin and 0.1% sodium azide in PBS]. Nonspecific antibody binding was blocked with human FeR Blocking Reagent (Miltenyi Biotec). Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (clone MT-101, AbD Serotec), phycoerythrin (PE)-conjugated anti-CD86 (clone IT2.2, Bioscience), PE-Cy7-conjugated anti-CD80 (clone 2D10.4, eBioscience), and PE-Cy7-conjugated anti-HLA-DR (clone LN3, eBioscience) at 4°C for 30 min. Isotype control antibodies were included in each experiment to confirm specificity of staining. After washing and fixing in 2% paraformaldehyde for 30 min at 4°C, cells were analyzed using a LSRII flow cytometer (BD Biosciences) and FlowJo Software (Tree Star). Statistically significant differences in CD80, CD86, and HLA-DR expression by infected DCs were determined by one-way ANOVA, followed by Bonferroni comparison of means.

Human DC-CD4+ T cell co-culture

DC-T cell co-cultures were performed similarly to previous studies [42,43,44]. CD4+ T cells were purified from human peripheral blood mononuclear cells that passed through the Optiprep gradient by positive selection using the Dynal CD4 Positive Isolation Kit (Invitrogen) per the manufacturer’s instructions. These cells were >95% CD3CD4+ T cells as assessed by flow cytometry. Purified CD4+ T cells were then stained with 2.5 µM CFSE for 10 min at 37°C, washed, and resuspended in complete T cell medium. CFSE-labeled T cells from a single donor were then added to DCs from a different donor that had been exposed to bacteria. DC-T cell co-cultures were performed in a 96-well round bottom plate at a ratio of 10:1 (2 × 105 T cells/2 × 10^6 DCs/well) for a period of 5 days at 37°C with 5% CO2. After harvesting supernatants, cells were washed once and resuspended in FACS staining buffer, treated with human FeR Blocking Reagent (Miltenyi Biotec), and stained with APC-conjugated anti-CD4 (clone OKT4, eBioscience) at 4°C for 30 min. After washing and fixing in 2% paraformaldehyde for 30 min at 4°C, fluorescence was measured using a FACS Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo Software (Tree Star). For T cell proliferation, CFSElow cells were measured in the CD4+ gate. Statistically significant differences in the percentage of proliferating T cells following co-culture with infected DCs were determined by one-way ANOVA, followed by Bonferroni comparison of means.

Mice

Six- to eight-week old female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in microisolator cages under specific pathogen-free conditions in a biosafety level-3 animal facility.

Immunization of mice

LVS and ΔFTL_0883 were cultured in MH broth or TSBc as described above. Mice were immunized subcutaneously (s.c.) or intratracheally (i.t.). Vaccinations were performed i.t. by oropharyngeal instillation as described previously [41]. A subset of mice was sacrificed at 2 hours post infection, and their lungs were homogenized and plated to confirm delivery of bacteria to the respiratory tract. Actual administered doses were determined by plating serial dilutions of the inoculum onto chocolate II agar plates.

Infection of mice with *F. tularensis* Schu S4

Schu S4 was grown in MH broth as described above. Mice were infected i.t. with 100 CFU of Schu S4 six weeks following LVS or ΔFTL_0883 vaccination. The actual dose was calculated by plating serial dilutions of the inoculum onto chocolate II agar plates. Following infection, mice were monitored daily for survival.

Measurements of bacterial burden in vivo

Bacterial burdens in the organs of mice vaccinated with LVS strains were measured as previously described [38,41]. Mice were sacrificed at the indicated time points and lungs, spleens, and livers were removed and homogenized in 1 ml (lungs, spleens) or 2 ml (livers) of TSBc. A portion of the organ homogenates were serially diluted and plated onto chocolate II agar plates. Plates were incubated at 37°C at 5% CO2 and individual colonies were enumerated.

**In vitro stimulation of lung cells from vaccinated mice**

Six weeks following vaccination with LVS or ΔFTL_0883, lungs were excised, minced, and incubated in RPMI (Gibco) supplemented with 12 mg type I collagenase (Gibco), 100 µg DNase I (USB), and 3 mM CaCl2 for 30 min at 37°C with shaking (170 rpm). The digested tissue was passed through a 40 µm cell strainer (BD Biosciences) to generate single cell suspensions. Erythrocytes were lysed with ACK Lysis Buffer (Gibco) and remaining cells were washed with RPMI. Viable cells were counted using trypan blue exclusion. Cells were resuspended in complete RPMI [RPMI supplemented with 10% heat-inactivated FBS, 25 mM HEPES, 1% non-essential amino acids, 1% sodium pyruvate, 1% GlhataMAX and 0.1% 2-mercaptoethanol (all from Gibco)] and seeded into 96-well round bottom plates at 1.5 × 10^4 cells/well. Lung cells from naive mice served as controls. BMDCs were generated as described above without CD11c magnetic bead purification. BMDCs were resuspended in complete RPMI and added at a 1:10 ratio (1.5 × 10^5 BMDCs/1.5 × 10^6 lung cells) to lung cells.

Preliminary experiments were performed with lung cells from LVS-vaccinated mice to determine the optimal antigen concentration and length of co-culture for this assay. Peak cytokine production was detected after 48 hours of co-culture with similar results observed at 72 hours (data not shown). Little to no cytokine production was detected from lung cells cultured with BMDCs and fSchu S4 below an MOI of 10 (data not shown). As a result, cells were incubated at 37°C with either media alone or fSchu S4 at dose of 10 CFU per cell. After 48 hour co-culture, supernatants were collected for analysis of cytokines and chemokines.

**Cytokine and Chemokine Assays**

DCs and macrophage supernatants were tested by ELISA using commercially available kits to measure TNF-α (R&D Systems), IL-12 p40 (human, R&D Systems; mouse, eBioscience), and IL-6 (human, R&D Systems) according to the manufacturer’s instructions. IFN-γ in supernatants from human DC-CD4+ T cell co-cultures was also measured by ELISA (human, R&D Systems). The limits of detection for the ELISAs were: human TNF-α – 15.6 pg/ml, human and mouse IL-12 p40 – 31.2 pg/ml, human IL-6 – 9.7 pg/ml, and human IFN-γ – 15.6 pg/ml. Cytokine and chemokine levels in lung supernatants from in vitro re-stimulation assays were determined by ELISA (mouse IFN-γ, R&D Systems; mouse IL-17A, Biolegend) or by using the Milliplex 32-plex Mouse
Cytokine/Chemokine Panel (Millipore) on a Bio-Plex 200 system (Bio-Rad Laboratories). Analyte concentrations were calculated against the standards using Milliplex Analyst software (version 3.5; Millipore). The limits of detection for the ELISAs were 31.2 pg/ml for mouse IFN-γ and 15.6 pg/ml for mouse IL-17A.

Statistically significant differences in cytokine production were identified by one- or two-way ANOVA followed by Bonferroni comparison of means.

Results

Limited inflammatory response of human DCs to LVS

We have shown previously that human macrophages have a limited capacity to produce proinflammatory cytokines following infection with LVS [25]. We hypothesized that human DCs would also be hyporesponsive to LVS stimulation. To test this, human macrophages and DCs were co-cultured with LVS, and then supernatants were harvested and analyzed for the proinflammatory cytokines TNF-α, IL-6, and IL-12p40. Similar to our findings with macrophages (Fig. 1A), LVS elicited little to no proinflammatory cytokines from human DCs (Fig. 1B). Pre-treating LVS with 100% human serum failed to enhance cytokine production (data not shown). As a positive control, human DCs were stimulated with E. coli [39]. E. coli-stimulated DCs produced significantly higher levels of all cytokines measured compared to untreated DCs or DCs cultured with LVS (Fig. 1B).

Identification of an immunostimulatory F. tularensis LVS strain

We hypothesized that a LVS mutant inducing a stronger proinflammatory response from APCs in vitro would be a more effective tularemia vaccine candidate. Surveying pre-existing LVS mutants generated in our laboratory, we tested several for their...
Intracellular bacteria. 13B47 was attenuated for growth at 13B47, and lysed at various times post infection to enumerate macrophages were infected with either wild-type LVS or strain 13B47. Similar results were observed when cytokine levels were measured 48 hours after infection (data not shown). Although the cytokine levels elicited by 13B47 were lower than those produced by cells stimulated with \textit{E. coli} (Fig. 1A–B), each was readily detected. Among the LVS strains tested, therefore, 13B47 stimulated the most proinflammatory cytokines from human APCs.

We next assessed whether the medium used to grow the bacteria would influence stimulation of DCs. LVS grown in media containing high levels of polyamines such as CDM stimulates low levels of proinflammatory cytokines from macrophages [25,27,38]. To address the effect culture conditions may have on the DC phenotypes observed here, LVS and 13B47 were cultured in CDM or MH broth prior to co-culture with human DCs. At various time points post infection, supernatants were harvested and analyzed for detection of IL-12p40. At 24 and 48 hours post infection, greater than 10-fold higher levels of IL-12p40 were produced by human DCs cultured with 13B47 compared to wild-type LVS (Fig. 1C). IL-12p40 production by DCs was higher regardless of whether 13B47 was cultured in CDM or MH broth (Fig. 1C). This result indicated that induction of cytokine production by 13B47 was not dependent on the growth medium used to culture this strain.

Maturation of DCs infected with \textit{F. tularensis} strain 13B47

In addition to the secretion of cytokines, DCs must undergo a process called maturation in order to efficiently prime T cells and initiate the adaptive immune response [45]. Among these alterations, the expression of MHC and costimulatory molecules increases. Since 13B47 stimulated cytokine production from human DCs, we next evaluated whether these cells also changed their surface phenotype in response to this mutant. The expression of CD80, CD86, and HLA-DR was measured on DCs following culture with either wild-type \textit{F. tularensis} LVS, 13B47, AcaPc, 1664d, or \textit{E. coli} as a positive control for maturation. LVS elicited little to no change in expression of maturation markers on the surface of human DCs (Fig. 2A–C). Similar results were observed with the LVS mutants AcaPc and 1664d (Fig. 2B, C). In contrast, the percentage of high-expressing cells and/or geometric mean fluorescence intensity increased after culture with 13B47 for CD80 and CD86 (Fig. 2). A similar trend of heightened expression of HLA-DR was also observed with 13B47-infected DCs (Fig. 2). Likewise, \textit{E. coli}-stimulated DCs increased expression of costimulatory molecules and MHC (Fig. 2). These data suggest that DCs undergo maturation after exposure to \textit{F. tularensis} strain 13B47 and, therefore, may be better suited to initiate an adaptive immune response.

Growth of 13B47 in human macrophages and DCs

Intracellular growth is a hallmark of pathogenic \textit{Francisella} strains. Although 13B47 stimulated APCs to secrete cytokines and upregulate costimulatory molecules, it was unclear if its intracellular growth was altered. To test this, human DCs and macrophages were infected with either wild-type LVS or strain 13B47, and lysed at various times post infection to enumerate intracellular bacteria. 13B47 was attenuated for growth at 24 hours post infection in human macrophages (Fig. 3). Surprisingly, 13B47 was still capable of replicating in human DCs (Fig. 3), albeit with a slightly slower rate compared to wild-type LVS (estimated generation time of 783 minutes versus 275 minutes for wild-type). These phenotypes could not be attributed to a general growth defect since 13B47 grew similar to wild-type LVS in bacterial growth medium (data not shown). These data suggest that, while the cytokine response to 13B47 is similar between macrophages and DCs, these cells differ in their ability to control growth of this mutant.

Enhanced activation of CD4\textsuperscript{+} T cells by \textit{F. tularensis} strain 13B47-infected DCs

Enhanced maturation of human DCs by 13B47 led us to hypothesize the resulting DCs would stimulate T cells more effectively. This was tested by measuring human CD4\textsuperscript{+} T cell proliferation and cytokine production following co-culture with allogeneic DCs pre-treated with LVS, 13B47, or \textit{E. coli}. T cell proliferation was measured by CFSE dilution after co-culture with infected DCs for 5 days as described previously [42,43,44]. An increase in the percentage of proliferating CD4\textsuperscript{+} T cells was observed following co-culture with 13B47-infected DCs compared to unstimulated CD4\textsuperscript{+} T cells (Fig. 4A and B). This increased percentage of proliferating CD4\textsuperscript{+} T cells was comparable to the level of proliferating T cells observed following co-culture with \textit{E. coli}-infected DCs (Fig. 4A and B). Similar rates of CD4\textsuperscript{+} T cell proliferation were observed after 7 days of culture with infected DCs (data not shown). In contrast, the percentage of proliferating CD4\textsuperscript{+} T cells following co-culture with LVS-treated DCs was not significantly different from the baseline level of proliferation observed with unstimulated DCs (Fig. 4B).

T cell activation following co-culture with infected DCs was also assessed by cytokine production. IFN-\gamma concentrations in the supernatants of the DC-T cell co-cultures described above showed a similar trend to the proliferation data. CD4\textsuperscript{+} T cells cultured with 13B47-infected DCs produced higher levels of IFN-\gamma compared to those stimulated with LVS-infected DCs (Fig. 4C). T cells stimulated with bacteria alone in the absence of DCs did not proliferate or produce measurable levels of IFN-\gamma (data not shown). The proliferation and cytokine production data together suggest DC maturation induced by 13B47 had measurable consequences on T cells in vitro.

Evaluation of the LVS FTL\_0883 deletion mutant as a tularemia vaccine

Protection from virulent type A \textit{Francisella} infection is largely dependent on the development of robust T cell-mediated immunity [46]. Based on the data obtained with human cells in vitro, we hypothesized that vaccination with 13B47 would prolong survival and improve T cell responses compared to LVS in mice challenged with virulent \textit{Francisella}. However, 13B47 is not optimal since it contains a transposon that could be unstable. To generate a more suitable vaccine candidate, an in-frame deletion mutant was created in LVS, \textit{ΔFTL\_0883}, that does not incorporate an antibiotic resistance marker [38]. Similar to human macrophages [38], more IL-12p40 and TNF-\alpha was produced by human DCs cultured with \textit{ΔFTL\_0883} than wild-type LVS (Fig. 5A–B). These cytokine levels were similar to, or greater than, that produced by DCs cultured with 13B47 (Fig. 5A–B). Moreover, IL-12p40 and TNF-\alpha levels continued to rise from 24–48 hours when DCs were cultured with \textit{ΔFTL\_0883} (Fig. 5B). To confirm the heightened stimulation of macrophages and DCs was due to deletion of FTL\_0883, an in cis-complementing construct (pJH1-FTL\_0883)
was generated and introduced into ΔFTL_0883 [38]. Complementation of ΔFTL_0883 with the wild-type copy of the gene significantly reduced IL-12p40 and TNF-α production by human macrophages [38] and DCs (Fig. 5B). Differential induction of IL-12p40 from human DCs by FTL_0883 mutants and wild-type LVS was also observed at a higher MOI of 500 (Fig. 5C).

Changes in CD80 and CD86 expression were also evaluated in human DCs cultured with either wild-type LVS or FTL_0883 mutants. As demonstrated previously in Fig. 2, expression of CD80 and CD86 was not altered on human DCs cultured with wild-type LVS at a low MOI (Fig. 5D). Culturing of human DCs with 13B47 or ΔFTL_0883, however, caused a statistically significant increase in expression of both costimulatory molecules on the surface (Fig. 5D). CD80 and CD86 expression were also higher on DCs cultured with 13B47 or ΔFTL_0883 than on LVS-infected DCs using a higher MOI (Fig. 5D). Lower levels of CD80 and CD86 were also measured on human DCs cultured with the ΔFTL_0883 strain complemented with a wild-type copy of the gene compared to DCs cultured with ΔFTL_0883 (Fig. 5D).

Deletion of FTL_0883 in LVS reduces its ability to replicate in human and murine macrophages [38]. Additionally, the LVS mutant containing a transposon insertion in the FTL_0883 gene, 13B47, was attenuated for growth in human macrophages and replicated slowly in human DCs (Fig. 3). To measure ΔFTL_0883 replication in human DCs, DCs were infected with either wild-type LVS, 13B47, ΔFTL_0883, or the complemented strain, and lysed at various times post infection to enumerate intracellular bacteria. ΔFTL_0883 replicated more slowly in human DCs than wild-type LVS, exhibiting at least 5-fold less growth 24 hours post infection (Fig. 5E). Similar results were observed 48 and 72 hours post infection with up to a 20-fold difference in growth between ΔFTL_0883 and LVS measured 72 hours post infection (Fig. 5E). The growth kinetics for ΔFTL_0883 and 13B47 in human DCs over the 72 hour period were indistinguishable (Fig. 5E). Complementation of ΔFTL_0883 with a wild-type copy of the gene restored growth of the mutant to near wild-type levels (Fig. 5E). In summary, 13B47 and ΔFTL_0883 were similar with 1) reduced growth in human DCs, 2) increased expression of CD80 and
challenge (Table 2). Although mice vaccinated s.c. with LVS and whether [47,48]. This experimental design allowed us to determine against a secondary challenge with a type A strain, we next assessed its ability to stimulate adaptive immune responses

CD86, and 3) stimulation of IL-12p40 and TNF-α production by human DCs.

To test whether the phenotypes observed in human DCs were species-specific, murine DCs were also tested with these LVS strains. IL-12p40 levels were higher in supernatants from murine DCs cultured with either 13B47 or ΔFTL_0883 compared to LVS (Fig. 5F). Growth of strains with mutations in FTL_0883 was also less than wild type in murine DCs, which was less than wild-type at 24, 48, and 72 hours post infection (Fig. 5G). Growth of the ΔFTL_0883 was nearly restored to wild-type levels by the complementing construct containing a wild-type copy of the gene (Fig. 5G). These results showed that human and murine DCs responded similarly to 13B47 and ΔFTL_0883.

Having established the in vitro phenotypes of the ΔFTL_0883 strain, we next assessed its ability to stimulate adaptive immune responses in vivo. C57BL/6J mice were vaccinated by either subcutaneous (s.c.) or respiratory (i.t.) routes with LVS or ΔFTL_0883. Mice were challenged six weeks later i.t. with the type A P. tularensis strain Schu S4. Vaccination of C57BL/6J mice with LVS prolongs survival but does not completely protect against a secondary challenge with a type A Francisella strain [47,46]. This experimental design allowed us to determine whether ΔFTL_0883 vaccination conferred better protection than LVS. All mice that received a sham vaccination with PBS succumbed to the Schu S4 infection within 5 days following challenge (Table 2). Although mice vaccinated s.c. with LVS and ΔFTL_0883 survived longer than sham-vaccinated controls, they still required euthanasia within 7 days of Schu S4 infection (Table 2). No survival differences were observed between animals vaccinated s.c. with LVS and ΔFTL_0883 (Table 2).

Vaccination by a respiratory route, however, showed statistically significant differences in protective efficacy. The median time to death of mice vaccinated i.t. with LVS was approximately 10–12 days following Schu S4 challenge (Table 2). This median time to death was double the median time to death for sham-vaccinated controls (5 days, Table 2) and was similar to previous work [47]. In contrast, mice vaccinated with ΔFTL_0883 survived for a median of 6 days (Table 2). Therefore, vaccination with ΔFTL_0883 by a respiratory route provided less protection than that elicited by wild-type LVS.

To investigate the differences in the protection elicited by the two strains, we evaluated bacterial burdens in the lung and peripheral organs following respiratory vaccination. LVS replicated exponentially in the lung for the first three days following i.t. immunization (Fig. 6). The lung bacterial burden remained steady until day 6 post immunization and then slowly began to decline up to day 10 (Fig. 6). Dissemination to the spleen and liver occurred at day 3 with LVS burden peaking at day 6 and being cleared by day 10 (Fig. 6). Despite comparable doses of bacteria used in the vaccinations, lower levels of ΔFTL_0883 were detected at all time points in the lung and beginning at day 3 in peripheral organs post immunization (Fig. 6). While clearance of LVS from the lung does not occur until 22 days post infection [49], ΔFTL_0883 was cleared more rapidly at approximately 10 days post infection (Fig. 6). Viable ΔFTL_0883 were measured in the spleens and livers of seven of eight mice by day 6, but none were detected in these organs at day 10 (Fig. 6). Therefore, LVS achieved higher numbers for a longer period of time in the lung and periphery following vaccination.

We next sought an immunological explanation for the performance of ΔFTL_0883 vaccination. We hypothesized wild-type LVS induced superior T cell responses than ΔFTL_0883, and measured cytokine and chemokine responses by lung cells after i.t. vaccination. Cells were harvested from the lungs of LVS- and ΔFTL_0883-vaccinated mice and were re-stimulated in vitro with fSchu S4. Cells from mice vaccinated with LVS produced higher amounts of IFN-γ with re-stimulation than cells from naive mice or those that received ΔFTL_0883 (Fig. 7). IFN-γ production by lung cells from mice vaccinated with ΔFTL_0883, however, was not statistically significantly different than naive controls (Fig. 7). Increasing the vaccination dose of ΔFTL_0883 by three-fold failed to improve IFN-γ responses by the lung cells (data not shown). Consistent with the IFN-γ results, the IFN-γ inducible chemokine MIG was also higher in cultures from mice vaccinated with LVS (data not shown). In contrast to IFN-γ, cells from both vaccination groups produced comparable amounts of IL-17 after re-stimulation. A 2–3 fold increase in IL-17 production was observed in lung cells from mice vaccinated with ΔFTL_0883 and LVS compared to naive controls (Fig. 7). No other statistically significant differences were consistently detected in the other cytokines and chemokines that were tested (data not shown). Therefore, the protection elicited by LVS against Schu S4 challenge correlated with IFN-γ production by lung cells after re-stimulation with antigen.

**Discussion**

Several studies, including this one, indicate LVS poorly stimulates innate immune cells [24,25,27,29,30,32]. This suggests insufficient activation of DCs could contribute to incomplete protection engendered by LVS. In this study, we sought to
improve vaccine efficacy with a LVS strain that stimulated APCs better than wild-type LVS. The LVS mutants used in this study (Table 1) were selected based on specific characteristics. All three of these genes (FTL_1415, FTL_1664, and FTL_0883) have been identified in negative selection screens in F. novicida and/or LVS to be necessary for growth and/or survival in mice [50,51]. The ΔcapC mutant was of interest because another LVS mutant in the capBCA operon, ΔcapB, afforded protection in BALB/c mice against challenge with the virulent Francisella strain Schu S4 [49]. Mutation of FTL_1664 in LVS resulted in diminished uptake by human DCs [34], which may impact DC activation. Recently, our laboratory has shown that LVS FTL_0883 mutants like 13B47 stimulate innate immune cells and are attenuated in vitro and in vivo, making this mutant a possible vaccine candidate [38]. Strains with mutations in the FTL_0883 locus of LVS showed promise based on in vitro results. The 13B47 and ΔFTL_0883 derivatives of LVS stimulated human DCs and macrophages (Fig. 1, 2, and 5), which was associated with better stimulation of T cells in vitro (Fig. 4). Contrary to our hypothesis, however, improving APC stimulation in vitro with the ΔFTL_0883 strain did not enhance protection in vivo. The median time to death doubled in mice vaccinated in the respiratory tract with LVS compared to naive animals. In contrast, the median time to death of mice vaccinated with ΔFTL_0883 was similar to naive animals (Table 2). Enhancing DC stimulation with ΔFTL_0883, therefore, failed to establish a protective immune response.

The poor performance of ΔFTL_0883 as a vaccine may be due directly to its attenuation. Mutation of the FTL_0883 locus in LVS attenuates growth in macrophages and DCs (Fig. 3, 5E, and 5G, and [38]). In addition, bacterial burdens in the lung and periphery of ΔFTL_0883-vaccinated mice are less than in animals receiving wild type (Fig. 6). Based on these findings, the attenuation and accelerated clearance of ΔFTL_0883 in vivo may prevent a sufficient adaptive immune response from being established. Consistent with this model, restimulation of lung cells isolated from ΔFTL_0883-vaccinated mice produced less IFN-γ than mice receiving wild type (Fig. 7). Since IFN-γ is a critical mediator of protective immunity against tularemia [48,52,53], the diminished IFN-γ response we observed following restimulation likely contributed to the lack of protection after vaccination with ΔFTL_0883.

Additional factors may also contribute to the vaccination results seen in this study. Though the molecular function of the protein encoded by FTL_0883 is unknown, it is possible that protective antigens may not be expressed since spermine responsiveness and transcription are altered after mutation of FTL_0883 [38]. Alternatively, different cytokine profiles stimulated in the host by the ΔFTL_0883 mutant may influence the vaccine performance. Inflammatory signals such as IL-12 can modulate T cell differentiation, promoting the generation of more short-lived effector cells compared to memory precursors [54]. The higher levels of IL-12 stimulated by ΔFTL_0883 (Fig. 5) may

**Figure 4. Enhanced proliferation and IFN-γ production by CD4+ T cells stimulated with LVS strain 13B47-infected DCs.** Purified CFSE-labeled CD4+ T cells from a single donor were co-cultured with either E. coli-infected, F. tularensis LVS-infected, or 13B47-infected DCs from a different donor at a ratio of 10:1 (2×10⁵ T cells/2×10⁴ DCs/well) for 5 days. (A) Representative dot plots showing loss of CFSE fluorescence versus CD4 staining on day 5 for each group from one experiment. (B) The mean percentages of proliferating CD4+ T cells were calculated (± SEM) from five individual experiments with different donors. (C) IFN-γ levels were measured in day 5 supernatants by ELISA. Data are presented as the mean ± SEM from four individual experiments with different donors that were represented in Figure 4B. BLD = below limits of detection of the ELISA. Statistically significant differences in mean percentages and GMFI for all groups were determined by one-way ANOVA, followed by Bonferroni comparison of means (*, p<0.05; **, p<0.01; ***, p<0.001).

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have shifted T cell development, impairing the development of memory cells. Each of these possibilities is consistent with the reduced IFN-γ observed during re-stimulation of lung cells with antigen in vitro. The mechanism(s) accounting for the poor recall responses observed with ΔFTL_0883 is currently being investigated.

Table 2. Survival of immunized mice following intratracheal Schu S4 challenge.a.

| Route            | Vaccine | Vaccination Dose | Time to Death of Individual Mice (days) | Median Time to Death (days) |
|------------------|---------|------------------|----------------------------------------|-----------------------------|
| Control          | PBS     | N/A              | 5, 5, 5, 5, 5                          | 5                           |
| Subcutaneous     | LVS     | 1×10^4           | 6, 7, 7, 7, 7                          | 7                           |
|                  | ΔFTL_0883 | 1×10^4         | 6, 7, 7, 7, 7                          | 7                           |
| Intratracheal    | Experiment 1 | LVS    | 1×10^3 | 10,12,12, >33, >33 | 12^b                           |
|                  |         | ΔFTL_0883 | 1×10^3 | 5, 6, 6, 7, 7 | 6                           |
|                  | Experiment 2 | LVS    | 2×10^3 | 9, 9, 10, 11, 12 | 10^b                           |
|                  |         | ΔFTL_0883 | 2×10^3 | 6, 6, 6, 7, 7 | 6                           |

aMice were immunized with either LVS or ΔFTL_0883 at the indicated dose and then challenged with 100 CFU of Schu S4 i.t.

bSignificant difference p<0.005 by log rank test.

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Figure 5. FTL_0883 deletion mutant, ΔFTL_0883, elicits maturation of DCs and is attenuated for growth similar to 13B47. Human (A–E) and murine DCs (F–G) were cultured with either LVS, 13B47, ΔFTL_0883, or ΔFTL_0883::pJH1-FTL_0883 at an MOI of 10 (A–B, D, and F) or 500 followed by gentamicin treatment (C–E, and F) or the indicated time points (B, and D–G). For cytokines, supernatants were harvested at 24 hours (A, C, and F) or the indicated time points (B), and IL-12p40 and TNF-α were measured by ELISA. For flow cytometry experiments (D), DCs were harvested 24 hours post infection and GMPFs for CD80 and CD86 were measured. For gentamicin protection assays (E, G), DCs were infected with LVS strains at an MOI of 500 and then lysed at indicated time points to enumerate intracellular bacteria. Data are presented as the mean ± SEM from at least two independent experiments. Statistically significant differences between groups were determined by one (A, C, D, and F) or two-way ANOVA (B, E, and G), followed by Bonferroni comparison of means (*, p<0.05; **, p<0.01; ***, p<0.001). BLD = below limits of detection of the ELISA.

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The results presented here with the FTL_0883 mutants share common outcomes with studies of other genetically altered Francisella. Mutation of \( \text{iglC} \) or \( \text{mglA} \), genes important for intracellular growth of Francisella, or deletion of the \( \text{purMC} \) purine biosynthesis operon resulted in highly attenuated strains that did not provide better protection than LVS against virulent Francisella challenge [55,56,57]. In contrast, vaccination with a \( \Delta \text{clpB} \) mutant in the Schu S4 background is superior to wild-type LVS [21]. A greater IFN-\( \gamma \) response was measured four days after challenge of mice vaccinated with the more successful \( \Delta \text{clpB} \) mutant than those vaccinated with LVS [58]. Coupled with our results, IFN-\( \gamma \) responses measured during restimulation could be a useful predictor of vaccine efficacy.

Several recent studies have shown that IL-17 is also required for control of \( F. \) tularensis growth and the generation of an effective Th1 response following pulmonary challenge [59,60,61]. Although the role of IL-17 in the immune response to acute \( F. \) tularensis infection has been characterized [59,60,61], its role in vaccination against tularemia remains to be elucidated. Paranavitana et al. demonstrated that PBMCs from LVS-vaccinated individuals produce high levels of IL-17 following \textit{in vitro} re-stimulation [62]. Similarly, we have shown that pulmonary vaccination of mice with LVS results in an increase in IL-17 compared to naïve controls (Fig. 7). Production of IL-17, however, did not correlate with vaccine efficacy since comparable levels of IL-17 were produced by cells from mice receiving wild-type or \( \Delta \text{FTL}_0883 \) vaccinations (Fig. 7). Additionally, neutralization of IL-17 in mice successfully protected by a Schu S4 \( \Delta \text{clpB} \) vaccine did not reduce survival after a pulmonary type A challenge despite increasing bacterial burden [58]. Therefore, IL-17 alone is not sufficient to predict vaccine efficacy.

Defining an optimal strategy for vaccine development remains a significant challenge for many pathogens. Improving APC stimulation using genetic modifications of LVS in this project failed to improve protection against a virulent \( F. \) tularensis strain. In addition, modeling vaccination and challenge \textit{in vitro} with human cells did not predict \textit{in vitro} responses in mice. Comparison of different vaccine strains and the protection conferred, however, confirmed IFN-\( \gamma \) production as a potential correlate of protection. A similar experimental approach by Shen et al. successfully characterized the immune response to Francisella strains that varied in vaccine efficacy [58]. Nevertheless, our current study and that of Shen et al. are limited by the conditions tested (the number of vaccine and mouse strains used), the limited number of output variables measured (relying primarily on multiplex cytokine measurements), and the timing of sampling (responses tested after challenge \textit{in vitro or in vivo}). This leaves open the possibility that more comprehensive investigations could yield additional insights. Recently, a systems-wide analysis of vaccine responses against yellow fever has met with significant success [63,64]. In this approach, genome-wide transcriptional studies using microarrays provided a broader assessment of in vivo host responses to vaccination [63]. A seminal application of these concepts to Francisella was also recently published by DePascale et al. [65]. Here, an \textit{in vitro} lymphocyte-macrophage co-culture was used to model the immune responses elicited by LVS vaccines of varying efficacies [63]. Analysis of 84 immunologically-relevant genes by real time PCR identified a list of immune mediators whose expression pattern correlated with protection from \( F. \) tularensis infection, including IFN-\( \gamma \) [65]. These higher order analyses, which integrate multi-parameter data sets of a variety of measurements, combined with traditional testing of specific
hypothetes will continue to yield insights into correlates of protection and biological response modifiers that may be exploited during acute infection and vaccination.

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Author Contributions

Conceived and designed the experiments: DMS GJN. Performed the experiments: DMS DMO JHC JMB MJB BCR DMO. Analyzed the data: DMS GJN. Wrote the paper: DMS GJN JHC JMB DMO.

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