AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

BACTERIAL INFECTIONS

Deletion of the Major Facilitator Superfamily Transporter *fptB* Alters Host Cell Interactions and Attenuates Virulence of Type A *Francisella tularensis*

Phillip M. Balzano,[a] Aimee L. Cunningham,[a] Christen Grassel,[a] Eileen M. Barry[a]

[a]Institute for Global Health, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland, USA

ABSTRACT *Francisella tularensis* is a Gram-negative, facultative, intracellular coccobacillus that can infect a wide variety of hosts. In humans, *F. tularensis* causes the zoonosis tularemia following insect bites, ingestion, inhalation, and the handling of infected animals. The fact that a very small inoculum delivered by the aerosol route can cause severe disease, coupled with the possibility of its use as an aerosolized bioweapon, has led to the classification of *Francisella tularensis* as a category A select agent and has renewed interest in the formulation of a vaccine. To this end, we engineered a type A strain SchuS4 derivative containing a targeted deletion of the major facilitator superfamily (MFS) transporter *fptB*. Based on the attenuating capacity of this deletion in the *F. tularensis* LVS background, we hypothesized that the deletion of this transporter would alter the intracellular replication and cytokine induction of the type A strain and attenuate virulence in the stringent C57BL/6J mouse model. Here we demonstrate that the deletion of *fptB* significantly alters the intracellular life cycle of *F. tularensis*, attenuating intracellular replication in both cell line-derived and primary macrophages and inducing a novel cytosolic escape delay. Additionally, we observed prominent differences in the *in vitro* cytokine profiles in human macrophage-like cells. The mutant was highly attenuated in the C57BL/6J mouse model and provided partial protection against virulent type A *F. tularensis* challenge. These results indicate a fundamental necessity for this nutrient transporter in the timely progression of *F. tularensis* through its replication cycle and in pathogenesis.

KEYWORDS FPT mutants, *Francisella tularensis*, major facilitator superfamily, amino acid transport, tularemia

Francisella tularensis is a Gram-negative, facultative, intracellular coccobacillus that is the causative agent of the zoonosis tularemia (1, 2). Two subspecies of *Francisella*, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B), account for virtually all instances of disease in humans, although a third, *F. tularensis* subsp. *novicida*, is not infectious in humans but is often used as a laboratory model. While there is no documented case of human-to-human transmission, *F. tularensis* can be spread to humans via the inhalation of infected aerosols, the bite of insect vectors, the ingestion of contaminated food or water, and the handling of infected animals (3). Cutaneous exposure, most often via an insect vector, accounts for much of the disease burden and can lead to a protracted although rarely fatal infection (4, 5). Conversely, the inhalation of as few as 10 to 15 bacteria of the virulent type A strain can cause serious illness in humans, with a mortality rate approaching 50% if left untreated (6, 7). Several countries had utilized *Francisella* as a biological weapon during the middle of the 20th century due to its high virulence, low inoculum, and ease of delivery by the aerosol route (8). For this reason, the CDC has designated *Francisella tularensis* subsp.

Received 16 November 2017 **Returned for modification** 6 December 2017 **Accepted** 22 December 2017

Accepted manuscript posted online 8 January 2018

Citation Balzano PM, Cunningham AL, Grassel C, Barry EM. 2018. Deletion of the major facilitator superfamily transporter *fptB* alters host cell interactions and attenuates virulence of type A *Francisella tularensis*. Infect Immun 86:e00832-17. https://doi.org/10.1128/IAI.00832-17.

Editor Shelley M. Payne, The University of Texas at Austin

Address correspondence to Eileen M. Barry, ebarry@medicine.umaryland.edu.

tularensis a tier 1 category A select agent and a high priority for countermeasure development.

Currently, there is no licensed vaccine against *F. tularensis*, but development efforts continue in light of the potential threat to public safety. Past development efforts included component and killed whole-cell, or "Foshay," vaccines, but these vaccines were met with limited success (9–12). To date, the live-attenuated vaccine strategy has shown the most promise. One candidate strain derived from *F. tularensis* subsp. *holarctica* by the Soviet Union through the 1940s and 1950s was transferred to the United States in 1956 and further passaged and became known as "live vaccine strain" (LVS) (7). *F. tularensis* LVS was tested extensively in clinical trials and demonstrated the feasibility of the live-vaccine approach in conferring at least partial protection against type A challenge. Subsequently, multiple attenuated live vaccine candidates have been engineered by utilizing the *F. tularensis* subsp. *novicida*, *F. tularensis* LVS, and *F. tularensis* type A backgrounds (6, 13–22). These new vaccine candidates have shown various degrees of efficacy in animal models, and some are being further developed for use in humans. An additional outcome of these studies has been the discovery of novel information about the pathogenic process of *F. tularensis*, including alterations to and impacts on the pathogenic life cycle incurred by the targeted deletion of various genes in the attenuation process.

A hallmark of *Francisella* infection is the ability to induce phagocytosis via several routes to gain entry into many cell types, especially macrophages, where it rapidly escapes the phagosome to replicate to high numbers in the cytosol (23–27). Because of the distinctive tetra-acylated structure of its lipopolysaccharide lipid A component, *Francisella* is largely able to evade Toll-like receptor 4 (TLR4)-mediated recognition (28). Rather, *Francisella* initiates signaling through TLR2, resulting in the production of proinflammatory cytokines (29, 30), and, in a manner dependent on guanylate binding proteins (GBPs), STING, and mitochondrial reactive oxygen species, activates the AIM2 inflammasome (31–34). The virulence of *Francisella* stems from its ability to multiply to high numbers in the cytosols of infected cells, especially macrophages (35). As a result, many attenuation attempts have targeted metabolic or nutrient acquisition genes (13, 15–17, 36–38). We demonstrated previously that deletions in three of eight *Francisella* phagosomal transporter (*fpt*) genes, encoding members of the major facilitator superfamily (MFS) of secondary transporters, in the *F. tularensis* LVS background resulted in significant *in vitro* and *in vivo* phenotypes: the intracellular replication of these strains in macrophages and hepatocytes was reduced, and the mutants were attenuated in BALB/c mice. Furthermore, immunization of mice with *F. tularensis* strain LVSΔ*fptB*, LVSΔ*fptE*, or LVSΔ*fptG* protected mice against homologous lethal challenge (13). We hypothesized that engineering deletion mutations in one of the most promising of these genes, *fptB*, an isoleucine transporter (38), in the *F. tularensis* type A background would result in a protective vaccine candidate strain. Here we report that an *F. tularensis* strain SchuS4 mutant lacking *fptB* demonstrates altered intracellular replication and cytokine release kinetic profiles in macrophages and is attenuated in mice, indicating its essential role in the intracellular life cycle and virulence of *F. tularensis*.

RESULTS

Generation of type A *Francisella tularensis* SchuS4 strains with an *fpt* deletion. An *F. tularensis* type A strain with an unmarked deletion of *fptB* was constructed by using allelic exchange technology as described previously (13). The deletion of the target gene and the generation of *F. tularensis* mutant strain SchuS4Δ*fptB* were confirmed by PCR using primers originating in both gene flanks and intragenically (see Fig. S1 in the supplemental material). The substrate for *fptB* has been reported to be isoleucine in both *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *novicida* (38). Given the 99% identity between the type A and type B gene sequences, we first confirmed that isoleucine was the substrate for *fptB* using Chamberlain's defined medium (CDM); the growth of *F. tularensis* SchuS4Δ*fptB* was reduced compared to that

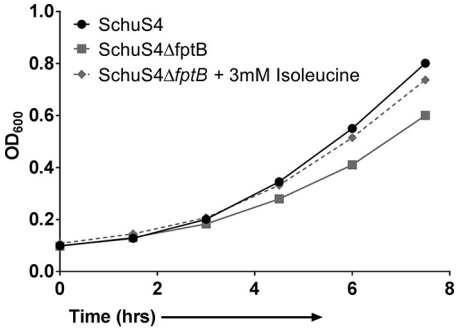

FIG 1 Growth in Chamberlain's defined medium. Growth kinetics of WT *F. tularensis* SchuS4 and SchuS4Δ*fptB* in Chamberlain's defined medium with or without supplementation with 3 mM isoleucine were examined. Data are from a single representative experiment ($n = 3$).

of the wild type (WT) in CDM but could be restored to WT levels by the addition of exogenous isoleucine (Fig. 1).

Mutant strain SchuS4Δ*fptB* is deficient in intracellular replication and cellular escape in macrophages. The virulence of *Francisella* is dependent, in part, on its ability to replicate to high numbers intracellularly, especially in macrophages (35). The replication kinetics of the *fpt* mutant strain was compared to that of WT *F. tularensis* SchuS4 initially in THP-1 human macrophage-like cells and HepG2 human hepatic carcinoma cells. In order to observe only the initial round of invasion and replication, a bactericidal concentration of gentamicin was maintained in the culture medium to prevent cell-to-cell spread. Under these conditions, there were significantly higher numbers of the mutant strain in THP-1 macrophages at 24 h postinfection (hpi) than of the WT strain ($P < 0.0001$) (Fig. 2A). WT *F. tularensis* doubled 3 to 4.5 times in the first 24 h, compared

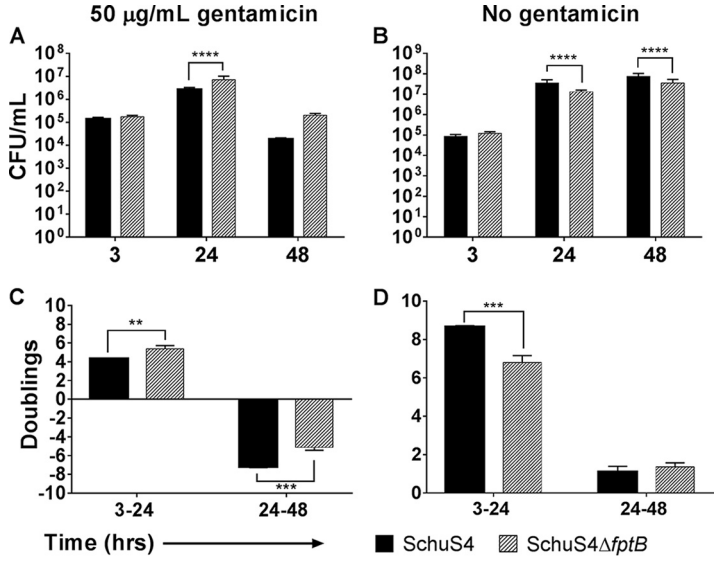

FIG 2 *F. tularensis* SchuS4Δ*fptB* exhibits altered intracellular replication kinetics in THP-1 cells. THP-1 cells were infected with the *F. tularensis* WT strain or SchuS4Δ*fptB* at an MOI of 100 for 2 h. Cells were then washed twice with PBS and incubated with 50 μg/ml gentamicin either for 1 h followed by washing and incubation without antibiotics (B and D) or for the duration of the experiment (A and C). Intracellular bacteria were enumerated at 3, 24, and 48 hpi, and the number of doublings was calculated between each interval. Data are presented as means ± standard errors of the means with three biological replicates for each experiment. Data are from a single representative experiment ($n = 5$). **, $P < 0.01$; ***, $P < 0.001$; ***, $P < 0.001$; ****, $P < 0.0001$ (average CFU were analyzed by two-way ANOVA, and calculated doublings were analyzed by a two-sided *t* test with Benjamini, Krieger, and Yekutieli *P* value adjustment).

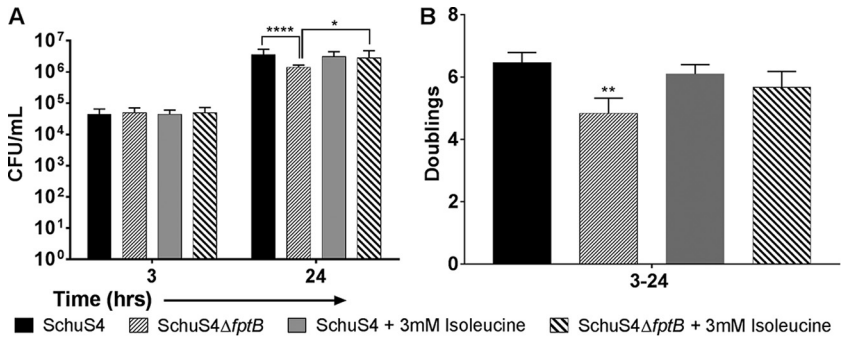

FIG 3 Excess isoleucine restores *F. tularensis* SchuS4Δ*fptB* replication kinetics to WT levels in THP-1 cells. THP-1 cells were infected at an MOI of 100 for 2 h. Cells were then washed twice with PBS and incubated with 50 μg/ml gentamicin for 1 h before being returned to medium lacking gentamicin. Intracellular bacteria were enumerated at 3, 24, and 48 h postinfection. Data are presented as means $\pm$ standard errors of the means with three biological replicates for each experiment. Data are from a single representative experiment ($n = 2$). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ (average CFU were analyzed by two-way ANOVA, and calculated doublings were analyzed by a two-sided *t* test with Benjamini, Krieger, and Yekutieli *P* value adjustment).

to 5 to 5.4 times for *F. tularensis* SchuS4Δ*fptB*, significantly more than the WT strain ($P < 0.01$) (Fig. 2C).

When cell-to-cell spread was allowed to proceed by the removal of gentamicin from the medium postinvasion, *F. tularensis* SchuS4Δ*fptB* exhibited a significant ($P < 0.0001$) replication defect as evidenced by reduced numbers of intracellular bacteria and significantly fewer doublings ($P < 0.001$) through 24 h (Fig. 2B and D). Without gentamicin, *F. tularensis* SchuS4Δ*fptB* doubled only 6.8 times, compared to 8.7 times for the WT (Fig. 2D). These data suggest that *F. tularensis* SchuS4Δ*fptB* has a reduced intracellular replication rate compared to that of the WT and is delayed in cellular escape. *trans* complementation of the *fptB* gene with an intact plasmid-borne gene copy restored WT replication kinetics for the mutant strain in THP-1 cells (see Fig. S2 in the supplemental material). Furthermore, the addition of 3 mM isoleucine, the reported substrate for FptB (38), to the culture medium of THP-1 cells infected with *F. tularensis* SchuS4Δ*fptB* was able to rescue the replication defect in a fashion similar to that in broth assays. The addition of exogenous isoleucine resulted in equivalent numbers of intracellular bacteria for *F. tularensis* SchuS4Δ*fptB* and the WT at 24 hpi (Fig. 3A). Similarly, the calculated doubling rate for the mutant in the presence of isoleucine was significantly higher than that of the mutant without isoleucine ($P < 0.05$) and statistically equivalent to that of the WT (Fig. 3B).

Finally, intracellular growth kinetics were tested in the human hepatic carcinoma cell line HepG2. Previous work demonstrated that the LVSΔ*fptB* mutant exhibited a replication defect compared to the parental LVS strain, with fewer mutant bacteria being present intracellularly at 24 and 48 hpi. In contrast, SchuS4Δ*fptB* did not exhibit a growth defect or delayed cell escape in this cell type (data not shown).

***F. tularensis* SchuS4Δ*fptB* induces delayed cell death in THP-1 cells.** To examine more closely the escape delay phenotype displayed by *F. tularensis* SchuS4Δ*fptB*, invasion assays with THP-1 macrophages were carried out under cell spread-limiting conditions with time points every 6 h during 33 h of infection. Positive doubling rates were interpreted as an indication that replication was outpacing escape, and negative doubling rates were interpreted as an indication that escape was outpacing replication. Supernatants were assayed for the release of lactate dehydrogenase (LDH), an intracellular enzyme whose release is indicative of cell death (39, 40). Supporting our previous findings with THP-1 cells, *F. tularensis* SchuS4Δ*fptB* exhibited a significant growth defect that was quantified as reduced intracellular bacterial numbers, evident by 15 hpi ($P < 0.05$) (Fig. 4A), and reduced doublings at between 3 and 9 hpi (Fig. 4B). *F. tularensis* SchuS4Δ*fptB* continued to double for 12 h beyond that of the WT, exhibiting increasing numbers of CFU (Fig. 4A) and positive doubling values (Fig. 4B)

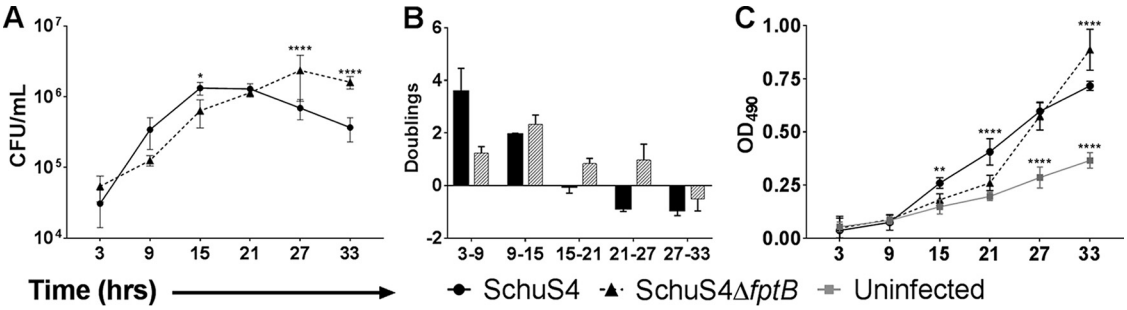

FIG 4 *F. tularensis* SchuS4Δ*fptB* induces a delay in cell death and LDH release. THP-1 cells were infected with the *F. tularensis* WT strain or SchuS4Δ*fptB* at an MOI of 100 for 2 h. Cells were then washed twice with PBS and incubated with 50 μg/ml gentamicin for the duration of the experiment. Intracellular bacteria were enumerated at 3, 9, 15, 21, 27, and 33 hpi; doublings were calculated between each time interval; and supernatants were analyzed for LDH release. Data are presented as means ± standard errors of the means with three biological replicates for each experiment. Data are from a single representative experiment ($n = 4$). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ (average CFU were analyzed by two-way ANOVA, and calculated doublings were analyzed by a two-sided *t* test with Benjamini, Krieger, and Yekutieli *P* value adjustment).

through 27 hpi, 12 h beyond that of the WT. In fact, *F. tularensis* SchuS4Δ*fptB* reached significantly higher peak intracellular numbers than did the WT ($P < 0.0001$). The release of LDH from *F. tularensis* SchuS4Δ*fptB*-infected cells was similarly and significantly ($P < 0.0001$) delayed compared to that from WT-infected cells (Fig. 4C). The release kinetics indicate a 12-h delay in the onset of cell death.

To further assess the differences in cellular escape between *F. tularensis* SchuS4Δ*fptB* and the WT, bacteria were sampled from the supernatants of infected THP-1 cells cultured without antibiotics as well as from the intracellular compartment. As described above, there were significantly fewer *F. tularensis* SchuS4Δ*fptB* than WT CFU by 15 hpi ($P < 0.0001$) (Fig. 5A). By 15 hpi, significantly more WT *F. tularensis* bacteria were found in the supernatants of infected cells ($P < 0.01$) than SchuS4Δ*fptB* bacteria (Fig. 5B). This increase in the number of extracellular WT *F. tularensis* bacteria correlates with the significant increase in LDH release seen previously at the 15-hpi time point (Fig. 4C). These results support a role for *fptB* in intracellular replication and timely escape from the cell.

***F. tularensis* SchuS4Δ*fptB* elicits a delayed early proinflammatory response in host cells.** The production of TLR2-dependent cytokines and the activation of STING and the AIM2 inflammasome are necessary for the initiation of the host cytokine response to *F. tularensis* infection (30, 41–43). The altered intracellular replication kinetics of *F. tularensis* SchuS4Δ*fptB* in macrophages suggested that infection with the

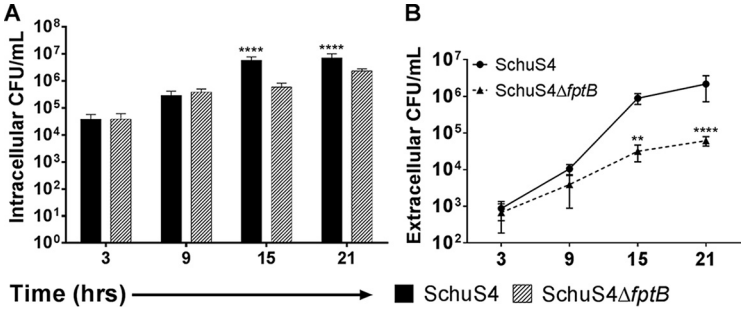

FIG 5 *F. tularensis* SchuS4Δ*fptB* is delayed in release to infected cell supernatants. THP-1 cells were infected with the *F. tularensis* WT strain or SchuS4Δ*fptB* at an MOI of 100 for 2 h. Cells were then washed twice with PBS and incubated with 50 μg/ml gentamicin for 1 h before being washed twice more with PBS and being returned to medium lacking gentamicin. Intracellular and extracellular bacteria were enumerated at 3, 9, 15, and 21 hpi. Data are presented as means ± standard errors of the means with three biological replicates for each experiment. Data are from a single representative experiment ($n = 2$). **, $P < 0.01$; ****, $P < 0.0001$ (average CFU were analyzed by two-way ANOVA, and calculated doublings were analyzed by a two-sided *t* test with Benjamini, Krieger, and Yekutieli *P* value adjustment).

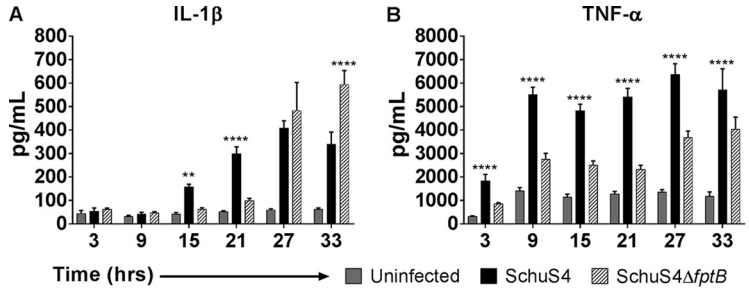

FIG 6 *F. tularensis* SchuS4Δ*fptB* elicits altered inflammatory responses. THP-1 cells were infected at an MOI of 100 for 2 h. Cells were then washed twice with PBS and incubated with 50 μg/ml gentamicin for the duration of the experiment. Cell-free supernatants were collected at 3, 9, 15, 21, 27, and 33 hpi, and cytokine contents were measured by using sandwich ELISAs. Data are presented as means ± standard errors of the means with three biological replicates for each experiment. Data are from a single representative experiment ($n = 5$). **, $P < 0.01$; ****, $P < 0.0001$ (by two-way ANOVA).

mutant strain would likely result in modified interactions with innate immune components. The levels of secretion of two important early proinflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), were measured in supernatants from the infected THP-1 samples from the above-described assay. The release of IL-1β was delayed in *F. tularensis* SchuS4Δ*fptB*-infected cells until 27 hpi, 12 h later than when WT-infected cells began secreting IL-1β at a rate above that of uninfected cells (Fig. 6A). At 27 hpi, IL-1β secretion from cells infected with *F. tularensis* SchuS4Δ*fptB* reached levels equivalent to that of the WT. The timing of IL-1β release for both the mutant and the WT coincides with that of LDH release, demonstrating a correlation between cell death and mature IL-1β release. Similarly, infection of THP-1 cells with WT *F. tularensis* induced a rapid and robust induction of TNF-α. In contrast, *F. tularensis* SchuS4Δ*fptB* induced the release of significantly less TNF-α than in WT-infected cells at all time points measured ($P < 0.0001$), despite increasing numbers of intracellular bacteria (Fig. 6B).

***F. tularensis* SchuS4Δ*fptB* is replication deficient in primary macrophages.** In order to bridge studies between the THP-1 cell line and *in vivo* experiments in mice, the replication kinetics of the *fpt* mutant strains was also examined in human monocyte-derived macrophages (hMDMs) and bone marrow-derived macrophages (BMDMs) from C57BL/6J mice. We hypothesized that growth attenuation in primary cells would be predictive of attenuation *in vivo*. Significantly fewer intracellular *F. tularensis* SchuS4 Δ*fptB* mutant bacteria ($P < 0.001$) than WT bacteria were counted at 24 hpi (Fig. 7A) in hMDMs. Correspondingly, the number of doublings for *F. tularensis* SchuS4Δ*fptB* was significantly lower than that for the WT at between 8 and 24 hpi (1.5 versus 4.2 doublings, respectively; $P < 0.05$) (Fig. 7B). Similar trends were observed in mouse BMDMs. *F. tularensis* SchuS4Δ*fptB* elicited significantly fewer intracellular CFU at 24 hpi (Fig. 7C). This corresponded to a significant reduction in the calculated doubling rate between *F. tularensis* SchuS4Δ*fptB* and the WT at between 8 and 24 hpi (1.2 versus 6.1 doublings, respectively; $P < 0.0001$) (Fig. 7D).

SchuS4Δ*fptB* is highly attenuated in the C57BL/6J mouse model. The altered intracellular replication kinetics and cytokine induction patterns suggested a role for *fptB* in *Francisella* virulence. The C57BL/6J mouse model was used to assess pathogenic defects of these mutant strains, as WT type A *F. tularensis* has an intranasal 50% lethal dose (LD_{50}) of <10 CFU in this model (44). Groups of 4 mice were inoculated intranasally with 5×10^1 CFU of the WT or 5×10^1 to $>7 \times 10^6$ CFU of the mutant strains. WT-inoculated mice steadily lost weight over the course of 5 days before succumbing to infection on day 5, exhibiting overt signs of sickness beginning late on day 4 or early on day 5. In contrast, no mice inoculated with *F. tularensis* SchuS4Δ*fptB* up to the highest dose succumbed to infection (Table 1). No weight loss or clinical signs of sickness were evident in any *F. tularensis* SchuS4Δ*fptB*-inoculated mice, demonstrating attenuation and an LD_{50} of $>7.6 \times 10^6$ CFU.

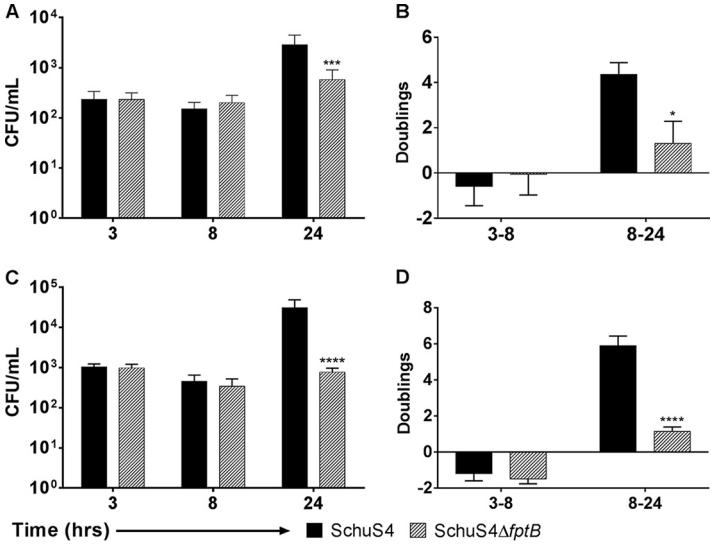

FIG 7 *F. tularensis* SchuS4Δ*fptB* is deficient in replication in primary cells. Human MDM cells (A and B) and mouse BMDM cells (C and D) were infected at an MOI of 10 for 2 h. Cells were then washed twice with PBS and incubated with 50 μg/ml gentamicin for 1 h before being returned to medium lacking gentamicin. Intracellular bacteria were enumerated at 3, 8, and 24 h postinfection. Data are presented as means ± standard errors of the means with three biological replicates for each experiment. Data are from a single representative experiment ($n = 4$). *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ (average CFU were analyzed by two-way ANOVA, and calculated doublings were analyzed by a two-sided *t* test with Benjamini, Krieger, and Yekutieli *P* value adjustment).

***F. tularensis* SchuS4Δ*fptB* vaccination protects mice against virulent *F. tularensis* SchuS4 challenge.** Given the high level of attenuation of SchuS4Δ*fptB*, we assessed the ability of this strain to protect mice against lethal challenge. Groups of 4 mice in two independent experiments were inoculated with a single dose of ~1×10^6 CFU of *F. tularensis* SchuS4Δ*fptB* before subsequent WT challenge 28 days later with doses ranging from 7 CFU to 360 CFU. Ten of 26 vaccinated mice from two separate experiments survived challenge, as did 1 unvaccinated control, resulting in an efficacy value of 28% (Table 2). An increased delay in the time to death was observed in a challenge-dose-dependent manner (Table 2). All protected mice showed no overt signs of illness, with little to no loss of weight following challenge. These data suggest that *F. tularensis* SchuS4Δ*fptB* has a limited protective capacity but demonstrates the first example of protection by an MFS transporter-targeted vaccine strain from type A challenge.

DISCUSSION

The formulation of a licensed, efficacious, live-attenuated vaccine against *Francisella tularensis* has remained an elusive goal despite increased efforts since the anthrax

TABLE 1 Attenuation of the *fptB* deletion strain in C57BL/6J mice

Strain	Intranasal dose (CFU)	No. of surviving mice/total no. of mice
SchuS4	1.10×10^1	0/2
SchuS4	1.90×10^1	0/4
SchuS4	4.30×10^1	0/4
SchuS4	5.30×10^1	0/4
SchuS4Δ*fptB*	5.40×10^1	3/3
SchuS4Δ*fptB*	3.10×10^2	4/4
SchuS4Δ*fptB*	5.60×10^2	3/3
SchuS4Δ*fptB*	1.01×10^3	4/4
SchuS4Δ*fptB*	8.70×10^3	4/4
SchuS4Δ*fptB*	9.80×10^4	4/4
SchuS4Δ*fptB*	1.02×10^5	2/2
SchuS4Δ*fptB*	1.06×10^6	4/4
SchuS4Δ*fptB*	7.60×10^6	3/3

TABLE 2 Protection against wild-type challenge after vaccination with SchuS4Δ*fptB*

Vaccine inoculum (CFU)	Challenge dose of SchuS4 (CFU)	No. of surviving mice/total no. of mice	Delays in time to death of individual mice (days)	Avg delay in time to death (days)
7.30×10^5	7	2/5	8, 9, 8, >14, >14	4
7.30×10^5	70	1/4	7, 6, 6, >14	2
7.30×10^5	162	2/5	6, 6, 6, >14, >14	1.67
PBS	350	0/3	4, 4, 5	
1.14×10^6	39	2/4	7, 10, >14, >14	3.5
1.14×10^6	78	1/4	6, 7, 9, >14	2.33
1.14×10^6	360	2/4	7, 6, >14, >14	1.5
PBS	39	1/4	5, 5, 5, >14	

bioterror attacks of 2001. Previous studies in humans with *F. tularensis* LVS in the 1960s demonstrated the proof of concept for the protective capacity of the live-attenuated vaccine strategy and pointed the way forward for the engineering of live-attenuated strains harboring targeted deletions (6, 13, 14, 19, 45). Here we investigated the potential of one MFS transporter, FptB, as an attenuating target in the virulent type A strain and the effects of its loss on the intracellular life cycle of type A *F. tularensis*. Our previous studies demonstrated the importance of the *fptB* gene in the *F. tularensis* LVS background for both *in vitro* replication kinetics and *in vivo* virulence (13). *F. tularensis* LVSΔ*fptB* exhibited a severe replication defect in multiple cell types, and the strain was both attenuated and protective in the BALB/c model (13). We hypothesized that given the high protein identity between the *F. tularensis* LVS and type A strains for this protein (99% for FptB), similar phenotypes would be observed in the clinically relevant type A background.

F. tularensis SchuS4Δ*fptB* displayed a significant growth defect in macrophages, both cell line derived and primary, similar to observations with the *F. tularensis* LVS-derived mutant, but, in contrast to our previous findings with LVS, did not harbor a replication defect in HepG2 cells (13, 38). The restriction of intracellular growth defects to macrophages and not hepatic cells may stem from the fact that the substrate of FptB, isoleucine, is regulated in macrophages. Isoleucine is one of nine amino acids that are depleted by activated macrophages (46). This, coupled with the fact that excess isoleucine in culture rescues the growth of *F. tularensis* SchuS4Δ*fptB*, implies that isoleucine is a rate-limiting nutrient for the intracellular replication of *Francisella tularensis* and that there is likely another, previously hypothesized (38), lower-affinity transporter that contributes to the transport of isoleucine in the absence of FptB. It may be that FptB functions as a high-affinity transporter to scavenge isoleucine intracellularly, even at depleted levels.

In addition to an intracellular replication defect, these data support an escape delay for SchuS4Δ*fptB*. Multiple studies have demonstrated that *F. tularensis* does not replicate until it escapes from the phagosome (47–49). The positive intracellular replication rate of SchuS4Δ*fptB*, measured as early as 3 to 9 hpi, demonstrates that the mutant strain has escaped from the phagosome, similarly to the WT, and is replicating in the cytosol albeit at a reduced rate. However, the mutant continues to replicate intracellularly for an additional 12 h beyond when WT *F. tularensis* is primarily escaping. Further evidence demonstrating a delay in the emergence of *F. tularensis* SchuS4Δ*fptB* in infected cell supernatants compared to the WT, and a corresponding delay in the onset of LDH release from mutant-infected cells compared to WT-infected cells, supports a delayed-cellular-escape phenotype. Specifically, at 21 hpi, *F. tularensis* SchuS4Δ*fptB* and the WT strain are present at similar intracellular numbers, but only the WT strain triggers the release of LDH above background levels, suggesting that the WT strain is killing cells while the mutant is accumulating intracellularly. The delay in cytosolic escape observed with the *fptB* mutant strain has not been reported previously for *Francisella*. Extensive work by multiple laboratories has revealed the process by which

Francisella escapes the phagosome early in the replication cycle, which host and bacterial genes are key to this process, and the consequences of delays at this stage (50–54). However, escape from the host cell at the final stage of the intracellular cycle remains an aspect of the *Francisella* life cycle that is poorly understood. Current models of escape from macrophages involve type A *Francisella* replicating to high numbers before triggering cell death mediated through caspase 3 (55), during which the bacteria are presumed to be released from the cell (35, 56, 57). While the escape process appears to be tied to the cell death machinery, the control of the timing of cell death and subsequent escape includes both host and bacterial factors. Exactly which molecular steps define the escape path for *F. tularensis* remain understudied. It has been demonstrated that *Francisella* triggers autophagy in mouse embryonic fibroblasts and then associates with the autophagic machinery to harvest this microenvironment for nutrients, especially amino acids (58–61). The involvement of autophagy in the *F. tularensis* life cycle and release in human cells remains largely unexplored but is an intriguing cell process to explore.

A fundamental key to the virulence of *Francisella* is its ability to invade and replicate intracellularly in many cell types, especially macrophages, where host responses have been extensively studied, revealing the induction of a potent yet atypical inflammatory response that can ultimately lead to sepsis and hypercytokinemia (62, 63). The avoidance of TLR4, yet the stimulation of TLR2/6, AIM2, and STING, produces an immune response that can be less than effective for host survival (28–30) and highlights the extreme virulence of this bacterium. Infection with the *fpt* mutant strain elicited altered inflammatory cytokine secretion: *F. tularensis* SchuS4Δ*fptB* infection resulted in a 12-h delay in IL-1β secretion and a significantly reduced TNF-α response across all time points measured. The level of attenuation of SchuS4Δ*fptB* in the C57BL/6J mouse model was $>$700,000 times higher than that of the WT. This high level of attenuation exhibited by *F. tularensis* SchuS4Δ*fptB* places it among the most attenuated type A-derived vaccine strains (64) and confirms the importance of intracellular replication and host cell interactions early in infection. Although we have not yet reached the lethal dose for this strain, challenge results suggest that *F. tularensis* SchuS4Δ*fptB* may be overattenuated. Historically, the prime difficulty of live-attenuated vaccine development has been achieving the optimal balance of safety/attenuation and immunogenicity/protection. The reduced inflammatory response elicited by *F. tularensis* SchuS4Δ*fptB* in the early stages of macrophage infection compared to the one elicited by the WT may indicate that an altered host cell interaction at the time of initial macrophage infection does not guarantee the onset of a long-term protective response.

These data represent the first utilization of an MFS transporter, an amino acid transporter specifically, as an attenuating target in the virulent *F. tularensis* type A background. Previous efforts were centered upon biosynthetic (16, 18, 65), capsule (19, 66, 67), and virulence (68) genes, but transporters represent a largely untapped source of potentially attenuating targets. Importantly, nutrient transporters, including those of the MFS, may be of increased importance to intracellular pathogens. It is appreciated that bacteria residing in the intracellular space undergo genome reductions, resulting in the shedding of genes involved in transport and metabolism at a rate that is much higher than that of genes involved in replication and defense (69). This implies that any remaining transporter and metabolite genes are necessary for the survival and, possibly, the virulence of the organism. The type A strain of *Francisella tularensis* is predicted to have 35 MFS transporters and 182 transporters in total (70), representing close to 12% of the SchuS4 protein-coding sequences. This represents a very large pool of potential novel targets for not just vaccines but also antibiotic therapies. Intriguingly, similar gene proportions exist in many clinically relevant pathogenic organisms.

F. tularensis SchuS4Δ*fptB* is the first MFS-based live-attenuated strain in the type A background to demonstrate protection against WT type A *Francisella* challenge. While the efficacy was relatively modest following vaccination with this strain, these data support the idea of targeting nutrient transporters as part of an attenuation strategy.

These strains represent insightful tools allowing more intimate study of the intracellular life cycle of *F. tularensis*, especially the understudied molecular steps leading to cellular escape.

MATERIALS AND METHODS

Bacteria and growth conditions. Bacterial strains utilized in this study are listed in Table S1 in the supplemental material. *Francisella tularensis* SchuS4 (BEI, Manassas, VA) was preserved at −80°C in Mueller-Hinton broth (MHB) (BD Microbiology Systems, Sparks, MD) with 15% glycerol added. Complete MHB includes 1% IsoVitaleX (BD, Cockeysville, MD), 0.1% glucose, and 0.25% ferric pyrophosphate and was used for all liquid cultures. Mueller-Hinton agar (MHA) (BD Microbiology Systems, Sparks, MD) was used for solid cultures and was augmented as defined above but also contained 10% defibrinated sheep blood (Lampire Biological Laboratories, Pipersville, PA). When selection for electroporants was being undertaken, kanamycin (Km) was added to MHA to a final concentration of 10 μg/ml. Suicide plasmids used in this study (Table S1) were preserved in *Escherichia coli* DH5α and grown in Luria-Bertani (LB) broth (BD Microbiology Systems, Sparks, MD) supplemented with 50 μg/ml kanamycin.

Deletion of *F. tularensis* genes. *F. tularensis* was transformed via electroporation as previously described (13). Briefly, *F. tularensis* was first grown on MHA at 37°C in 5% CO_2 for 2 days and then resuspended in 1 ml of 0.5 M sucrose. Bacteria were pelleted and washed three times in 0.5 M sucrose before being resuspended in a final volume of 300 μl. One hundred fifty microliters of a suicide plasmid (13) prepared from 500-ml LB broth cultures (utilizing a Qiagen [Germantown, MD] Midi-Prep kit) was added to this mixture. Electroporation was performed at 1.75 kV, 25 μF, and 600 Ω. After electroporation, cells were allowed to recover in MHB for 2 h at 37°C in 5% CO_2 and then plated onto kanamycin-containing MHA plates to select for cointegrants. Cointegration of the plasmid was confirmed by using PCR. Plasmid curing was accomplished by growth in MHB containing 10% sucrose. These cultures were plated onto MHA supplemented with 8% sucrose, and colonies arising here were screened by PCR to confirm the loss of the targeted gene and plasmid. Primers utilized for screening can be found in Table S2 in the supplemental material.

Growth curves. Growth curves comparing WT *F. tularensis* and *fpt* mutant strains were performed by using Chamberlain's defined medium (CDM) (Teknova, Hollister, CA) or CDM supplemented with 3 mM isoleucine. Bacteria were grown on MHA plates and resuspended to a starting optical density at 600 nm (OD_{600}) of 0.l in liquid medium. Cultures were incubated with shaking at 37°C in 5% CO_2 for 7.5 h, with OD_{600} readings being taken every 1.5 h.

Intracellular survival assays. The ability of *fpt* mutant strains to survive and replicate intracellularly was evaluated in the human THP-1 macrophage cell line (ATCC, Manassas, VA), the human HepG2 hepatic carcinoma cell line (ATCC, Manassas, VA), hMDMs, and BMDMs from C57BL/6J mice.

THP-1 cells were cultivated in RPMI 1640 (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 0.1% 1,000× 2-mercaptoethanol (Gibco, Gaithersburg, MD) and maintained at 37°C in 5% CO_2. Three days prior to the beginning of an assay, THP-1 cells were differentiated by using phorbol myristate acetate (PMA) (Sigma-Aldrich) at a concentration of 50 ng/ml for 24 h. Medium was then changed to supplemented RPMI 1640 as described above. For assays to assess bacterial replication, cells were infected with either *F. tularensis* SchuS4 or *fpt* mutants at a multiplicity of infection (MOI) of 100 for 2 h. Following the 2-h infection, cells were washed twice with phosphate-buffered saline (PBS) and incubated in RPMI 1640 containing 50 μg/ml gentamicin (Gibco, Gaithersburg, MD) for 1 h. At 3 hpi, cells were washed again twice with PBS and placed into fresh RPMI 1640 lacking gentamicin. Intracellular bacterial replication was assayed at 3, 24, and 48 h postinfection by lysing cells with a solution containing 0.02% SDS in PBS, followed by serial dilution and plating of the bacteria onto MHA. Culture supernatants were saved for downstream analyses. Bacterial doublings were calculated by utilizing the formula $[\log_{10} T_n - \log_{10} T_{(n-1)}] \times 3.32$, where T_n is the number of CFU at one time point and $T_{(n-1)}$ is the number of CFU at the prior time point. For assays to assess bacterial escape kinetics, the infection and washing steps were repeated as described above through 3 hpi. Gentamicin-containing medium was not washed off cells, and thus, cells were left in 50 μg/ml gentamicin for the duration of the assay. CFU were sampled as described above at the time courses indicated in the figure legends.

To assess the appearance of bacteria in the supernatants, THP-1 cells were infected and washed as described above. Gentamicin-containing medium was removed at 3 hpi, and cells were placed into antibiotic-free medium until the enumeration of CFU at 3, 9, 15, and 21 hpi. Supernatants were removed from the wells at the times specified above, serially diluted, and plated onto MHA. Intracellular bacteria were then enumerated as described above.

HepG2 cells were cultivated in minimal essential medium (MEM) supplemented with 10% FBS and maintained at 37°C in 5% CO_2. To assess the intracellular survival abilities of *fpt* mutant strains compared to that of WT *F. tularensis*, HepG2 cells were also seeded at a density of 1×10^6 cells per well in 12-well plates and infected with either *F. tularensis* SchuS4 or *fpt* mutant strains at an MOI of 300 for 2 h. After the 2-h infection period, cells were washed twice with PBS and then incubated in MEM containing 50 μg/ml gentamicin for 1 h. Next, cells were either left in gentamicin-containing medium for the duration of the experiment or washed twice with PBS and then incubated in antibiotic-free MEM for the duration of the experiment. Bacterial replication was assayed at 3, 24, and 48 h postinfection by lysing the cells with 0.02% SDS–PBS and plating the bacteria onto MHA.

Supplemented intracellular survival assays. The ability of isoleucine supplementation to rescue the intracellular replication of *F. tularensis* SchuS4Δ*fptB* was examined in THP-1 macrophage-like cells. THP-1 cells were prepared and infected as described above. Immediately before the start of the assay,

all cells were washed and placed into either standard RPMI 1640 or RPMI 1640 supplemented with 3 mM isoleucine. Such medium conditions were maintained throughout the entirety of the assay before CFU enumeration at 24 h postinfection.

Isolation and culture of mouse bone marrow-derived macrophages. Femurs were extracted from 6- to 8-week-old C57BL/6J mice (University of Maryland, Baltimore [UMB], Veterinary Resources Breeding Facility) and flushed with Dulbecco's minimal essential medium (DMEM) supplemented with 10% low-endotoxin FBS (Gemini Bioproducts, West Sacramento, CA), 30% L929 cell supernatants (as a source of colony stimulating factor 1 [CSF-1]), 1% nonessential amino acids (ThermoFisher Scientific, Waltham, MA), 1% HEPES (ThermoFisher Scientific, Waltham, MA), and 1% penicillin-streptomycin (ThermoFisher Scientific, Waltham, MA). Bone marrow cells were passed through a 70-μm nylon mesh (ThermoFisher Scientific, Waltham, MA) to remove debris and placed into a T175 flask (Costar, Corning, NY) for culture and differentiation. Fresh medium was added on the day after extraction and every other day subsequently for at least 1 week. The day before an assay, cells were scraped, spun down at 1,100 rpm ($125 \times g$) for 10 min, and resuspended at a concentration of 5×10^5 cells per ml in medium lacking penicillin-streptomycin. Cells were plated at a concentration of 5×10^5 cells per well in a 24-well plate (Costar, Corning, NY).

Isolation and culture of human monocyte-derived macrophages. Monocyte-derived macrophages (MDMs) were isolated from 100 ml whole human blood gathered in EDTA tubes (BD Microbiology Systems, Sparks, MD) by the University of Maryland, Baltimore, Center for Vaccine Development clinical staff. Blood was diluted 1:2 in PBS before being added to 15 ml of Ficoll (GE Healthcare, Laurel, MD) in SepMate tubes (Stem Cell Technologies, Cambridge, MA). SepMate tubes were spun at $1,200 \times g$ for 15 min at room temperature. Red blood cells were depleted by using ammonium chloride-potassium (ACK) lysis buffer (Gibco, Gaithersburg, MD). Isolated MDMs were cultured for 1 week before the start of an assay in T75 flasks (Costar, Corning, NY) with RPMI 1640 supplemented with 10% low-endotoxin FBS (Gemini Scientific, West Sacramento, CA), 1% nonessential amino acids (ThermoFisher Scientific, Waltham, MA), 1% HEPES (ThermoFisher Scientific, Waltham, MA), and 1% sodium bicarbonate (ThermoFisher Scientific, Waltham, MA). Medium was changed daily. Two days before the start of an assay, cells were scraped from the flask, enumerated, and plated at a density of 5×10^5 cells per well in a 24-well plate (Costar, Corning, NY). These studies were approved by the University of Maryland, Baltimore, Institutional Review Board.

Complementation of mutants. A plasmid containing *fptB* under the control of the *F. tularensis guaB* promoter, named p*FT906-fptB* (13), was electroporated into the *F. tularensis* SchuS4 mutant strain as described above. The complemented mutant was then tested via an invasion assay in THP-1 macrophage-like cells for reversion to WT growth mechanics.

Intracellular replication time course assay. The timing of host cellular escape of *fpt* mutant strains compared to that of WT *F. tularensis* SchuS4 was evaluated in THP-1 macrophage-like cells. Differentiated THP-1 cells were seeded at a density of 1×10^6 cells and then infected with either *F. tularensis* SchuS4 or the *fpt* mutant strain at an MOI of 100 for 2 h. Following the 2-h infection, cells were washed twice with PBS and then incubated in RPMI 1640 containing 50 μg/ml gentamicin for the remainder of the assay. Supernatants were collected for subsequent measurement of levels of LDH and cytokine release, and CFU were determined by lysing the cells with a 0.02% SDS solution in PBS, followed by serial dilution and plating of the bacteria onto MHA at 3, 9, 15, 21, 27, and 33 h postinfection.

Lactate dehydrogenase release assay. LDH release was measured by using a CytoTox 96 kit (Promega, Madison, WI) according to the manufacturer's protocol. Measurement of samples was done by using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA) at a 490-nm wavelength.

Cytokine ELISAs. To assay the secretion of IL-1β and TNF-α from THP-1 cells, enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) were used. Supernatants collected at 3, 9, 15, 21, 27, and 33 h postinfection were assayed according to the manufacturer's protocol and read by using a VersaMax plate reader at 450 nm, with correction set at 540 nm.

Mouse survival studies. Six- to eight-week-old male and female C57BL/6J mice were housed in the University of Maryland animal biohazard safety level 3 (ABSL-3) facility for the duration of the studies. All experiments were performed according to protocols approved by the UMB Institutional Animal Care and Use Committee (IACUC). To ascertain whether the *fpt* mutant strain was attenuated, a mouse infection model was used. Groups of 4 C57BL/6J mice per dosage concentration (2 males and 2 females) were anesthetized and inoculated intranasally with either WT *F. tularensis* SchuS4 or the *fpt* mutant suspended in 20 μl of PBS. Mice were monitored daily for survival and clinical signs of infection (weight loss, lethargy, and ruffling of fur) for 14 days postinfection.

Mouse health was scored based on the following criteria: condition 1 for normal activity, where mice run freely and energetically around the cage and resist when picked up; condition 2 for mice that are slower than usual and offer less resistance when picked up; condition 3 for mice that exhibit a hunched posture, move very slowly, and display ruffled, dull fur and squinted eyes; and condition 4 for mice that are almost entirely sedentary, hunched, and either unresponsive or barely responsive to prodding and that have fur that is very ruffled and dull. Mice reaching a clinical score of 4 or losing $>$20% of their body weight were euthanized as required by the UMB IACUC.

Mouse challenge studies. Six- to eight-week-old male and female C57BL/6J mice were immunized as described above by using a single vaccination regimen. Mice received $\sim 1 \times 10^6$ CFU of *F. tularensis* SchuS4Δ*fptB* and were challenged with WT *F. tularensis* SchuS4 intranasally 4 weeks later with the doses outlined in Table 2. After challenge, all mice were monitored daily to check for clinical signs and weight loss as described above. The delay in the time to death for vaccinated, challenged mice was calculated by using the average day of death postchallenge for the unvaccinated control group and the average day

of death for vaccinated mice that succumbed to challenge. The average day of death for each group was calculated, and the average day of death for the control group was subtracted, giving the delay in the time to death per group. Mice that survived challenge were excluded from these calculations.

Statistical analysis. By using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA), two-way analysis of variance (ANOVA) with Tukey's posttest was performed to assess statistical significance between log-transformed bacterial CFU counts, LDH release levels, and cytokine ELISA data ($P < 0.05$). A two-sided *t* test was utilized to determine significance between calculated bacterial doubling values ($P < 0.05$). Calculated *P* values for the *t* tests were adjusted by using the Benjamini, Krieger, and Yekutieli method with a false discovery rate of 1%. In all instances, WT *F. tularensis* SchuS4 served as the reference strain for all statistical tests.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00832-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This work was supported by NIH NIAID grants 1R01AI102966, R01AI123129, and 5T32AI095190-04.

We acknowledge Diana Lam and William Blackwelder for guidance on the biostatistical analyses contained in this work and Stefanie Vogel for critical review of the manuscript.

REFERENCES

1. Wherry WB, Lamb BH. 2004. Infection of man with bacterium tularense. J Infect Dis 189:1321–1331. https://doi.org/10.1093/infdis/189.7.1321.
2. Steiner DJ, Furuya Y, Metzger DW. 2014. Host-pathogen interactions and immune evasion strategies in Francisella tularensis pathogenicity. Infect Drug Resist 7:239–251. https://doi.org/10.2147/IDR.S53700.
3. Bosio CM. 2011. The subversion of the immune system by Francisella tularensis. Front Microbiol 2:9. https://doi.org/10.3389/fmicb.2011.00009.
4. Ellis J, Oyston PCF, Green M, Titball RW. 2002. Tularemia. Clin Microbiol Rev 15:631–646. https://doi.org/10.1128/CMR.15.4.631-646.2002.
5. Evans ME, Gregory DW, Schaffner W, McGee ZA. 1985. Tularemia: a 30-year experience with 88 cases. Medicine (Baltimore) 64:251–269. https://doi.org/10.1097/00005792-198507000-00006.
6. Hornick RB, Eigelsbach HT. 1966. Aerogenic immunization of man with live tularemia vaccine. Bacteriol Rev 30:532–538.
7. Sjöstedt A. 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci 1105:1–29. https://doi.org/10.1196/annals.1409.009.
8. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763–2773. https://doi.org/10.1001/jama.285.21.2763.
9. Foshay L. 1950. Tularemia. Annu Rev Microbiol 4:313–330. https://doi.org/10.1146/annurev.mi.04.100150.001525.
10. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. 1961. Tularemia vaccine study. II. Respiratory challenge. Arch Intern Med 107:702–714. https://doi.org/10.1001/archinte.1961.03620050068007.
11. Conlan JW, Shen H, Webb A, Perry MB. 2002. Mice vaccinated with the O-antigen of Francisella tularensis LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine 20:3465–3471. https://doi.org/10.1016/S0264-410X(02)00345-6.
12. Fulop M, Manchee R, Titball R. 1995. Role of lipopolysaccharide and a major outer membrane protein from Francisella tularensis in the induction of immunity against tularemia. Vaccine 13:1220–1225. https://doi.org/10.1016/0264-410X(95)00062-6.
13. Marohn ME, Santiago AE, Shirey KA, Lipsky M, Vogel SN, Barry EM. 2012. Members of the Francisella tularensis phagosomal transporter subfamily of major facilitator superfamily transporters are critical for pathogenesis. Infect Immun 80:2390–2401. https://doi.org/10.1128/IAI.00144-12.
14. Reed DS, Smith LKP, Cole KS, Santiago AE, Mann BJ, Barry EM. 2014. Live attenuated mutants of Francisella tularensis protect rabbits against aerosol challenge with a virulent type A strain. Infect Immun 82:2098–2105. https://doi.org/10.1128/IAI.01498-14.
15. Santiago AE, Cole LE, Franco A, Vogel SN, Levine MM, Barry EM. 2009. Characterization of rationally attenuated Francisella tularensis vaccine strains that harbor deletions in the guaA and guaB genes. Vaccine 27:2426–2436. https://doi.org/10.1016/j.vaccine.2009.02.073.
16. Santiago AE, Mann BJ, Qin A, Cunningham AL, Cole LE, Grassel C, Vogel SN, Levine MM, Barry EM. 2015. Characterization of Francisella tularensis Schu S4 defined mutants as live-attenuated vaccine candidates. Pathog Dis 73:ftv036. https://doi.org/10.1093/femspd/ftv036.
17. Pechous R, Celli J, Penoske R, Hayes SF, Frank DW, Zahrt TC. 2006. Construction and characterization of an attenuated purine auxotroph in a Francisella tularensis live vaccine strain. Infect Immun 74:4452–4461. https://doi.org/10.1128/IAI.00666-06.
18. Pechous RD, McCarthy TR, Mohapatra NP, Soni S, Penoske RM, Salzman NH, Frank DW, Gunn JS, Zahrt TC. 2008. A Francisella tularensis Schu S4 purine auxotroph is highly attenuated in mice but offers limited protection against homologous intranasal challenge. PLoS One 3:e2487. https://doi.org/10.1371/journal.pone.0002487.
19. Twine S, Shen H, Harris G, Chen W, Sjostedt A, Ryden P, Conlan W. 2012. BALB/c mice, but not C57BL/6 mice immunized with a ΔclpB mutant of Francisella tularensis subspecies tularensis are protected against respiratory challenge with wild-type bacteria: association of protection with post-vaccination and post-challenge immune responses. Vaccine 30:3634–3645. https://doi.org/10.1016/j.vaccine.2012.03.036.
20. Barry EM, Cole LE, Santiago AE. 2009. Vaccines against tularemia. Hum Vaccin 5:832–838. https://doi.org/10.4161/hv.10297.
21. Cong Y, Yu J-J, Guentzel MN, Berton MT, Seshu J, Klose KE, Arulanandam BP. 2009. Vaccination with a defined Francisella tularensis subsp. novicida pathogenicity island mutant (ΔiglB) induces protective immunity against homotypic and heterotypic challenge. Vaccine 27:5554–5561. https://doi.org/10.1016/j.vaccine.2009.07.034.
22. Cunningham AL, Dang KM, Yu J-J, Guentzel MN, Heidner H, Klose KE, Arulanandam BP. 2014. Enhancement of vaccine efficacy by expression of a TLR5 ligand in the defined live attenuated Francisella tularensis subsp. novicida strain U112ΔiglB::fljB. Vaccine 32:5234–5240. https://doi.org/10.1016/j.vaccine.2014.07.038.
23. Celli J, Zahrt TC. 2013. Mechanisms of Francisella tularensis intracellular pathogenesis. Cold Spring Harb Perspect Med 3:a010314. https://doi.org/10.1101/cshperspect.a010314.
24. Balagopal A, MacFarlane AS, Mohapatra N, Soni S, Gunn JS, Schlesinger LS. 2006. Characterization of the receptor-ligand pathways important for entry and survival of Francisella tularensis in human macrophages. Infect Immun 74:5114–5125. https://doi.org/10.1128/IAI.00795-06.
25. Clemens DL, Lee B-Y, Horwitz MA. 2005. Francisella tularensis enters

macrophages via a novel process involving pseudopod loops. Infect Immun 73:5892–5902. https://doi.org/10.1128/IAI.73.9.5892-5902.2005.
26. Schulert GS, Allen L-AH. 2006. Differential infection of mononuclear phagocytes by Francisella tularensis: role of the macrophage mannose receptor. J Leukoc Biol 80:563–571. https://doi.org/10.1189/jlb.0306219.
27. Pierini LM. 2006. Uptake of serum-opsonized Francisella tularensis by macrophages can be mediated by class A scavenger receptors. Cell Microbiol 8:1361–1370. https://doi.org/10.1111/j.1462-5822.2006.00719.x.
28. Hajjar AM, Harvey MD, Shaffer SA, Goodlett DR, Sjöstedt A, Edebro H, Forsman M, Byström M, Pelletier M, Wilson CB, Miller SI, Skerrett SJ, Ernst RK. 2006. Lack of in vitro and in vivo recognition of Francisella tularensis subspecies lipopolysaccharide by Toll-like receptors. Infect Immun 74:6730–6738. https://doi.org/10.1128/IAI.00934-06.
29. Cole LE, Shirey KA, Barry E, Santiago A, Rallabhandi P, Elkins KL, Puche AC, Michalek SM, Vogel SN. 2007. Toll-like receptor 2-mediated signaling requirements for Francisella tularensis live vaccine strain infection of murine macrophages. Infect Immun 75:4127–4137. https://doi.org/10.1128/IAI.01868-06.
30. Katz J, Zhang P, Martin M, Vogel SN, Michalek SM. 2006. Toll-like receptor 2 is required for inflammatory responses to Francisella tularensis LVS. Infect Immun 74:2809–2816. https://doi.org/10.1128/IAI.74.5.2809-2816.2006.
31. Crane DD, Bauler TJ, Wehrly TD, Bosio CM. 2014. Mitochondrial ROS potentiates indirect activation of the AIM2 inflammasome. Front Microbiol 5:438. https://doi.org/10.3389/fmicb.2014.00438.
32. Weiss DS, Henry T, Monack DM. 2007. Francisella tularensis: activation of the inflammasome. Ann N Y Acad Sci 1105:219–237. https://doi.org/10.1196/annals.1409.005.
33. Man SM, Karki R, Malireddi RKS, Neale G, Vogel P, Yamamoto M, Lamkanfi M, Kanneganti T-D. 2015. The transcription factor IRF1 and guanylate-binding proteins target AIM2 inflammasome activation by Francisella infection. Nat Immunol 16:467–475. https://doi.org/10.1038/ni.3118.
34. Meunier E, Wallet P, Dreier RF, Costanzo S, Anton L, Rühl S, Dussurgey S, Dick MS, Kistner A, Rigard M, Degrandi D, Pfeffer K, Yamamoto M, Henry T, Broz P. 2015. Guanylate-binding proteins promote AIM2 inflammasome activation during Francisella novicida infection by inducing cytosolic bacteriolysis and DNA release. Nat Immunol 16:476–484. https://doi.org/10.1038/ni.3119.
35. Chong A, Celli J. 2010. The Francisella intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation. Front Microbiol 1:138. https://doi.org/10.3389/fmicb.2010.00138.
36. Russo BC, Horzempa J, O'Dee DM, Schmitt DM, Brown MJ, Carlson PE, Xavier RJ, Nau GJ. 2011. A Francisella tularensis locus required for spermine responsiveness is necessary for virulence. Infect Immun 79:3665–3676. https://doi.org/10.1128/IAI.00135-11.
37. Gesbert G, Ramond E, Rigard M, Frapy E, Dupuis M, Dubail I, Barel M, Henry T, Meibom K, Charbit A. 2014. Asparagine assimilation is critical for intracellular replication and dissemination of Francisella. Cell Microbiol 16:434–449. https://doi.org/10.1111/cmi.12227.
38. Gesbert G, Ramond E, Tros F, Dairou J, Frapy E, Barel M, Charbit A. 2015. Importance of branched-chain amino acid utilization in Francisella intracellular adaptation. Infect Immun 83:173–183. https://doi.org/10.1128/IAI.02579-14.
39. Ozanic M, Marecic V, Lindgren M, Sjöstedt A, Santic M. 2016. Phenotypic characterization of the Francisella tularensis ΔpdpC and ΔiglG mutants. Microbes Infect 18:768–776. https://doi.org/10.1016/j.micinf.2016.07.006.
40. Lai X-H, Golovliov I, Sjöstedt A. 2001. Francisella tularensis induces cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication. Infect Immun 69:4691–4694. https://doi.org/10.1128/IAI.69.7.4691-4694.2001.
41. del Barrio L, Sahoo M, Lantier L, Reynolds JM, Ceballos-Olvera I, Re F. 2015. Production of anti-LPS IgM by B1a B cells depends on IL-1β and is protective against lung infection with Francisella tularensis LVS. PLoS Pathog 11:e1004706. https://doi.org/10.1371/journal.ppat.1004706.
42. Cole LE, Santiago A, Barry E, Kang TJ, Shirey KA, Roberts ZJ, Elkins KL, Cross AS, Vogel SN. 2008. Macrophage proinflammatory response to Francisella tularensis live vaccine strain requires coordination of multiple signaling pathways. J Immunol 180:6885–6891. https://doi.org/10.4049/jimmunol.180.10.6885.
43. Rathinam VAK, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA. 2010. The AIM2 inflammasome is essential for host-defense against cytosolic bacteria and DNA viruses. Nat Immunol 11:395–402. https://doi.org/10.1038/ni.1864.
44. Qin A, Scott DW, Mann BJ. 2008. Francisella tularensis subsp. tularensis Schu S4 disulfide bond formation protein B, but not an RND-type efflux pump, is required for virulence. Infect Immun 76:3086–3092. https://doi.org/10.1128/IAI.00363-08.
45. Kim T-H, Pinkham JT, Heninger SJ, Chalabaev S, Kasper DL. 2012. Genetic modification of the O-polysaccharide of Francisella tularensis results in an avirulent live attenuated vaccine. J Infect Dis 205:1056–1065. https://doi.org/10.1093/infdis/jir620.
46. Nishiyama A, Yokote Y, Sakagami H. 2010. Changes in amino acid metabolism during activation of mouse macrophage-like cell lines. In Vivo 24:857–860.
47. Santic M, Molmeret M, Klose KE, Jones S, Kwaik YA. 2005. The Francisella tularensis pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell Microbiol 7:969–979. https://doi.org/10.1111/j.1462-5822.2005.00526.x.
48. Lindgren H, Golovliov I, Baranov V, Ernst RK, Telepnev M, Sjöstedt A. 2004. Factors affecting the escape of Francisella tularensis from the phagolysosome. J Med Microbiol 53:953–958. https://doi.org/10.1099/jmm.0.45685-0.
49. Lindgren M, Tancred L, Golovliov I, Conlan W, Twine SM, Sjöstedt A. 2014. Identification of mechanisms for attenuation of the FSC043 mutant of Francisella tularensis SCHU S4. Infect Immun 82:3622–3635. https://doi.org/10.1128/IAI.01406-13.
50. Golovliov I, Baranov V, Krocova Z, Kovarova H, Sjöstedt A. 2003. An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells. Infect Immun 71:5940–5950. https://doi.org/10.1128/IAI.71.10.5940-5950.2003.
51. Clemens DL, Lee B-Y, Horwitz MA. 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. https://doi.org/10.1128/IAI.72.6.3204-3217.2004.
52. Santic M, Molmeret M, Abu Kwaik Y. 2005. Modulation of biogenesis of the Francisella tularensis subsp. novicida-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-γ. Cell Microbiol 7:957–967. https://doi.org/10.1111/j.1462-5822.2005.00529.x.
53. Clemens DL, Lee B-Y, Horwitz MA. 2009. Francisella tularensis phagosomal escape does not require acidification of the phagosome. Infect Immun 77:1757–1773. https://doi.org/10.1128/IAI.01485-08.
54. Chong A, Wehrly TD, Nair V, Fischer ER, Barker JR, Klose KE, Celli J. 2008. The early phagosomal stage of Francisella tularensis determines optimal phagosomal escape and Francisella pathogenicity island protein expression. Infect Immun 76:5488–5499. https://doi.org/10.1128/IAI.00682-08.
55. Wickstrum JR, Bokhari SM, Fischer JL, Pinson DM, Yeh H-W, Horvat RT, Parmely MJ. 2009. Francisella tularensis induces extensive caspase-3 activation and apoptotic cell death in the tissues of infected mice. Infect Immun 77:4827–4836. https://doi.org/10.1128/IAI.00246-09.
56. Belhocine K, Monack DM. 2012. Francisella infection triggers activation of the AIM2 inflammasome in murine dendritic cells. Cell Microbiol 14:71–80. https://doi.org/10.1111/j.1462-5822.2011.01700.x.
57. Abu Kwaik Y, Asare R. 2011. Exploitation of host cell biology and evasion of immunity by Francisella tularensis. Front Microbiol 1:145. https://doi.org/10.3389/fmicb.2010.00145.
58. Checroun C, Wehrly TD, Fischer ER, Hayes SF, Celli J. 2006. Autophagy-mediated reentry of Francisella tularensis into the endocytic compartment after cytoplasmic replication. Proc Natl Acad Sci U S A 103:14578–14583. https://doi.org/10.1073/pnas.0601838103.
59. Steele S, Brunton J, Ziehr B, Taft-Benz S, Moorman N, Kawula T. 2013. Francisella tularensis harvests nutrients derived via ATG5-independent autophagy to support intracellular growth. PLoS Pathog 9:e1003562. https://doi.org/10.1371/journal.ppat.1003562.
60. Alkhuder K, Meibom KL, Dubail I, Dupuis M, Charbit A. 2009. Glutathione provides a source of cysteine essential for intracellular multiplication of Francisella tularensis. PLoS Pathog 5:e1000284. https://doi.org/10.1371/journal.ppat.1000284.
61. Santic M, Abu Kwaik Y. 2013. Nutritional virulence of Francisella tularensis. Front Cell Infect Microbiol 3:112. https://doi.org/10.3389/fcimb.2013.00112.
62. Mares CA, Ojeda SS, Morris EG, Li Q, Teale JM. 2008. Initial delay in the immune response to Francisella tularensis is followed by hypercytoki-

nemia characteristic of severe sepsis and correlating with upregulation and release of damage-associated molecular patterns. Infect Immun 76:3001–3010. https://doi.org/10.1128/IAI.00215-08.
63. Sharma J, Mares CA, Li Q, Morris EG, Teale JM. 2011. Features of sepsis caused by pulmonary infection with Francisella tularensis type A strain. Microb Pathog 51:39–47. https://doi.org/10.1016/j.micpath.2011.03.007.
64. Marohn ME, Barry EM. 2013. Live attenuated tularemia vaccines: recent developments and future goals. Vaccine 31:3485–3491. https://doi.org/10.1016/j.vaccine.2013.05.096.
65. Ireland PM, LeButt H, Thomas RM, Oyston PCF. 2011. A Francisella tularensis SCHU S4 mutant deficient in γ-glutamyltransferase activity induces protective immunity: characterization of an attenuated vaccine candidate. Microbiology 157:3172–3179. https://doi.org/10.1099/mic.0.052902-0.
66. Lindgren H, Shen H, Zingmark C, Golovliov I, Conlan W, Sjöstedt A. 2007. Resistance of Francisella tularensis strains against reactive nitrogen and oxygen species with special reference to the role of KatG. Infect Immun 75:1303–1309. https://doi.org/10.1128/IAI.01717-06.
67. Rasmussen JA, Fletcher JR, Long ME, Allen L-AH, Jones BD. 2015. Characterization of Francisella tularensis Schu S4 mutants identified from a transposon library screened for O-antigen and capsule deficiencies. Front Microbiol 6:338. https://doi.org/10.3389/fmicb.2015.00338.
68. Qin A, Zhang Y, Clark ME, Rabideau MM, Millan Barea LR, Mann BJ. 2014. FipB, an essential virulence factor of Francisella tularensis subsp. tularensis, has dual roles in disulfide bond formation. J Bacteriol 196:3571–3581. https://doi.org/10.1128/JB.01359-13.
69. Merhej V, Royer-Carenzi M, Pontarotti P, Raoult D. 2009. Massive comparative genomic analysis reveals convergent evolution of specialized bacteria. Biol Direct 4:13. https://doi.org/10.1186/1745-6150-4-13.
70. Elbourne LDH, Tetu SG, Hassan KA, Paulsen IT. 2017. TransportDB 2.0: a database for exploring membrane transporters in sequenced genomes from all domains of life. Nucleic Acids Res 45:D320–D324. https://doi.org/10.1093/nar/gkw1068.