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Vaccination with the Live Attenuated *Francisella novicida* Mutant *FTN0109* Protects against Pulmonary Tularemia

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Abstract

***Francisella tularensis* is considered a potential bioterrorism agent due to its low infectious dose, high mortality rate, and ability to be spread via the aerosol route. We characterized the *F. tularensis* subspecies *novicida* mutant strain *FTN0109* as a potential vaccine candidate against tularemia. This strain, which lacks an outer membrane lipoprotein, is attenuated *in vitro* and *in vivo*, as it exhibits reduced replication within murine J774 macrophages and has a pulmonary LD_{50} in BALB/c and C57BL/6 mice of $>10^5$ CFU (compared to WT parental strain U112, $LD_{50} < 10$ CFU). Intranasal immunization induced strong cellular responses (IFN-γ and IL-2 in splenocyte recall assays) as well as strong humoral responses. Vaccination with *FTN0109* also conferred complete protection in BALB/c mice against subsequent pulmonary challenge with 10 LD_{50} (60,000 CFU) of the murine virulent *Francisella* strain LVS. We also have demonstrated partial protection (50%) against the highly human virulent subspecies *tularensis* strain SCHU S4 (25 LD_{50}, 12,500 CFU) following intratracheal vaccination in the Fischer 344 rat, a second rodent model for tularemia. Overall, our results suggest that *FTN0109* serves as a potential putative vaccine candidate against pulmonary tularemia.**

Keywords

Tularemia, *F. tularensis*, Vaccines, Live Attenuated

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1. Introduction

There is a need for a vaccine against the potential bioterrorism agent *Francisella tularensis*. This Gram-negative coccobacillus is the etiological agent of the zoonotic disease tularemia, and is classified as a CDC Category A Select Agent due to its previous weaponization by the United States, Japan, and the Soviet Union [1]. Although tularemia manifests clinically in a variety of forms, pulmonary tularemia is the greatest cause for concern as it may be caused by inhalation of less than 10 CFU [2]. *F. tularensis* subsp. *tularensis* (Type A), is the most virulent subspecies in humans, with a 30% - 60% fatality rate if untreated [3]. In contrast, *F. tularensis* subsp. *novicida* (*F. novicida*) is essentially avirulent in healthy humans and is exempt from select agent status due to its inherent low virulence.

Live attenuated vaccines have been demonstrated to be effective against the pulmonary form of tularemia. The most successful example is the Live Vaccine Strain (LVS), developed in 1952 by the Soviet Union from subspecies holarctica by multiple passages *in vitro*. Questions of genetic stability and levels of protection have hindered approval for the use of LVS in humans [2] [4]-[7], and subsequently stimulated the search for alternative vaccine candidates against tularemia. Ideally, an efficacious live attenuated vaccine should induce potent cellular and humoral responses that protect humans against multiple forms of the disease, including pulmonary tularemia, while causing minimal adverse effects from vaccination.

We have previously shown that live attenuated strains of *F. novicida* can protect against pulmonary exposure to *F. tularensis* subsp. *tularensis* in the Fischer 344 rat model of tularemia [8]. Mice have been used extensively in the study of tularemia pathogenesis, but they are exquisitely sensitive to infection by all subspecies of *F. tularensis*, including subsp. *novicida* (pulmonary LD_{50} < 10 CFU for subsp. *tularensis*, *holarctica*, and *novicida*). In contrast, the Fischer 344 rat represents an attractive animal model for tularemia vaccine development; it exhibits sensitivities similar to humans for the various subspecies of *F. tularensis* [9]: the rat is sensitive to infection by the highly human virulent subsp. *tularensis* (pulmonary LD_{50} = 500 CFU) but resistant to infection by human avirulent subsp. *novicida* (pulmonary $LD_{50} \approx 5 \times 10^6$ CFU). Importantly, live strains of *F. novicida* administered via pulmonary or oral routes can protect against pulmonary exposure to *F. tularensis* subsp. *tularensis*in rats and non-human primates (cynomolgus macaques) [8]-[10], suggesting that *F. novicida* may represent an ideal candidate for a live attenuated tularemia vaccine.

F. novicida gene *FTN0109* encodes a glycosylated lipoprotein that has been shown to be important for virulence in several *Francisella* subsp. [11]. FTN0109 is orthologous to FTT1676 in subsp. *tularensis*, FTL0073 in LVS, and FTH0069 in subsp. *holarctica*. FTL0073 was identified in a signature-tag mutagenesis screen to be required for murine lung infections by LVS [12]. Transcriptional profiling identified FTT1676 as being upregulated during intracellular growth of subsp. *tularensis*, and inactivation of this gene prevented intramacrophage replication and virulence in mice [13]. Further studies demonstrated the efficacy of the Δ*FTT1676* strain to protect against low dose subsp. *tularensis* challenge in mice [14], showing 80% or 90% protection by the intradermal or intranasal routes, respectively. In this study, we demonstrate the protective efficacy of a *FTN0109 F. novicida* strain against subsequent heterologous LVS and subsp. *tularensis* pulmonary challenge in mice and rats, respectively. This protection is accompanied by potent cellular and humoral responses, leading to lowered bacterial burdens and pathology after subsequent pulmonary challenge.

2. Materials and Methods

2.1. Bacteria

F. novicida wild-type strain U112 was obtained from Dr. Francis Nano at the University of Victoria, Canada. The *FTN0109* mutant was obtained from an ordered transposon mutant library [15]. Subspecies *holarctica*-derived live vaccine strain (LVS, Lot # 703-0303-016) was obtained from Dr. Rick Lyons at Colorado State University. The highly human virulent subspecies *tularensis* strain SCHU S4 was obtained from the Centers for Disease Control and Prevention in Atlanta, GA. All strains were grown at 37˚C in tryptic soy broth or agar (BD Biosciences, San Jose, CA) supplemented with L-cysteine (1 g/L, Fisher Scientific, Fair Lawn, NJ). Dilution plating on this media was used to determine titers of all bacteria.

2.2. Animals

BALB/c mice (4 - 6 weeks) and Fischer 344 rats (6 - 8 weeks) were obtained from the National Cancer Institute

(Frederick, MD) and housed within the University of Texas at San Antonio AAALAC accredited vivarium. Animals were given food and water *ad libitum*, and all experiments were conducted with approval from the UTSA Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC).

2.3. Intramacrophage Replication

Murine macrophage J774 cells, maintained in D-10 medium (DMEM (Gibco, Grand Island, NY) + 10% FBS (Hyclone, Logan, UT)), were seeded into 96-well plates at a density of 2×10^5 cells/well and were incubated in a CO_2 incubator at 37˚C for 2 hrs to allow for adherence. Wild-type strain U112 and the live attenuated mutant *FTN0109* were added to cells at either 10 or 100 MOI, and incubated for 2 hrs to allow for uptake. Plates were washed and cells treated with gentamicin (20 µg/mL)-containing medium for 1 hr, then washed again and replaced with D-10 without gentamicin for the remainder of the assay. At defined time points (3 and 24 hrs post infection), cells were lysed with 0.2% deoxycholate solution and intracellular bacteria were determined by dilution plating.

2.4. LD_{50} Assessment

BALB/c mice (n = 6 per group) were anesthetized with 3% isoflurane in a rodent anesthesia chamber (Harvard Apparatus, Holliston, MA) and escalating doses (10^2 - 10^5 CFU suspended in a total of 25 µL PBS) of *FTN0109* were deposited on the nares of mice following previously described protocols [16] [17]. Mice were monitored daily for morbidity and mortality.

2.5. Intranasal Challenge of Mice

BALB/c mice were intranasally (i.n.) vaccinated with varying doses (10^2 - 10^5 CFU) *FTN0109* or mock vaccinated with PBS. Animals were rested for 30 days and then i.n. challenged with 60,000 CFU (approx. 10 LD_{50}) of LVS. Mice were monitored daily for morbidity and mortality.

2.6. Correlates of Immunity

BALB/c mice were i.n. vaccinated with 10^5 CFU of *FTN0109* or mock-vaccinated with PBS, then rested for 14 or 28 days for analysis of cellular and humoral responses, respectively. To analyze cellular responses, mice (n = 3 per group) were sacrificed to collect spleens. Single-cell suspensions of splenocytes were prepared and cultured for 72 hrs with the following antigens: Concanavalin A (a positive control mitogen, 1 µg/well), HEL (an unrelated antigen and negative control, 1 µg/well), and 2 doses (10^3 and 10^4 CFU) of UV-inactivated *FTN0109*. At the end of the incubation period, supernatants were collected and assayed for IFN-γ and IL-2 production using commercially available kits and their associated protocols (BD Biosciences). For analysis of humoral responses, mice were bled 28 days post-vaccination via the submandibular route to prepare sera. Serum ELISAs were conducted to obtain 50% binding titers to HEL (100 ng/well) or UV-inactivated *FTN0109* (10^6 CFU/well). Briefly, plates were coated with HEL or UV-*FTN0109* diluted in sodium bicarbonate buffer and incubated overnight at 4˚C. The next morning, plates were washed and blocked for 2 hrs with 5% fetal bovine serum and 0.1% Brij solution. Sera were serially diluted in blocking buffer across plates and incubated for 2 hrs. Plates were then washed and secondary antibody specific for either total antibody, IgG1, or IgG2a (all obtained from Southern Biotech, Birmingham, AL) was added for 1 hr. After another wash, TMB substrate (BD Biosciences) was added and plates were developed in the dark for 30 minutes, then read on an ELISA plate reader (Biotek Instruments) to assess 50% binding titer for each animal.

2.7. Bacterial Burden

Burden experiments were completed for both vaccination alone and vaccination with challenge. BALB/c mice were i.n. vaccinated with either 10^5 CFU *FTN*0109 or mock-vaccinated with PBS (for challenge burden only) and rested for 30 days prior to i.n. challenge with 60,000 CFU of LVS if necessary. At defined time points (days 3, 7, and 14 post-vaccination and day 1, 3, and 6 post-challenge), mice (n = 3/group/time point) were sacrificed and tissues collected. Whole organ homogenates were serially diluted and plated to enumerate organ bacterial burdens.

2.8. Histology

BALB/c mice (n = 3/group/time point) were i.n. vaccinated with 10^5 CFU *FTN0109* or mock-vaccinated with PBS and rested for 30 days prior to intranasal challenge with 60,000 CFU of LVS. Mice were sacrificed from each group at days 3 and 6 post-challenge and lungs were embedded in paraffin, then cut and stained for H&E histopathological analysis. Slides were imaged on a Zeiss Axioskop 2 plus microscope with representative images for each group shown.

2.9. Rat Vaccination and Challenge

Eight to nine week old Fisher 344 rats were anesthetized and intratracheally (i.t.) vaccinated with either 10^5 or 10^6 CFU *FTN0109*, 10^5 CFU U112, or mock-vaccinated with PBS, according to previously described protocols [9] [18]. Rats were rested for 30 days prior to i.t. challenge with 12,500 CFU (approx. 25 LD_{50}) of SCHU S4 in the UTSA ABSL-3 laboratory. Rats were monitored daily for morbidity and mortality.

2.10. Statistics

Statistical analysis was conducted using GraphPad Prism software. Kaplan-Meyer analysis was used to determine statistical significance of survival curves, and student's *t* test and two-way ANOVA were used to assess differences between groups for intramacrophage replication, organ burdens, and cellular and humoral responses. Data are represented as mean ± standard deviation for each group. All experiments were completed at least twice to assess for repeatability.

3. Results

3.1. *FTN0109* Is Defective for Intramacrophage Growth and Virulence in Mice

We determined the ability of the *FTN0109* mutant to replicate within macrophages, utilizing J774 murine macrophages. As shown in **Figure 1(a)**, the wild-type *F. novicida* strain U112 replicated robustly within macrophages at both 10 and 100 MOI, over a 24 hr time period ($p < 0.001$). In contrast, the *FTN0109* strain exhibited no net intracellular growth over the 24 hr time period, despite apparently enhanced entry of this strain into macrophages.

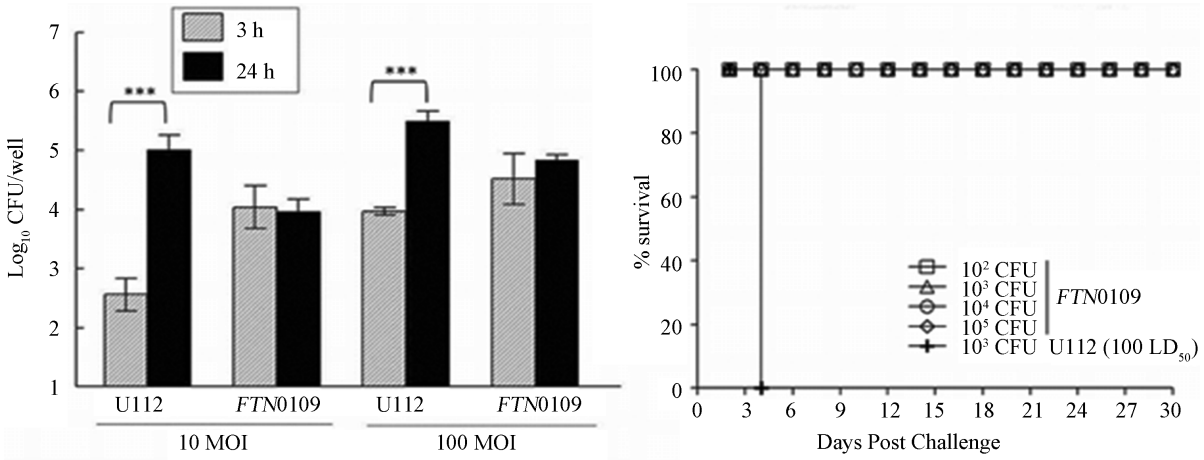


Figure 1. *FTN0109* is defective for intramacrophage growth and attenuated for virulence in mice. (a) J774 macropahges (2×10^5 cells/well) were seeded in 96-well plates and infected for 2 hrs with 10 or 100 MOI of either *FTN0109* or wild-type parent strain U112, washed, and treated for 1 hr with gentamicin-containing medium. Cells were washed and incubated with antibiotic-free medium. At defined time points (3 and 24 hrs post-infection) cells were lysed with 0.2% deoxycholate solution and dilution plating was used to enumerate intracellular bacteria. ***denotes $p < 0.001$; (b) BALB/c mice (n = 6 per group) were intranasally challenged with escalating doses of *FTN0109* (10^2 - 10^5 CFU) or with 10^3 of WT U112 and monitored daily for survival. Figures are representative of two independent experiments.

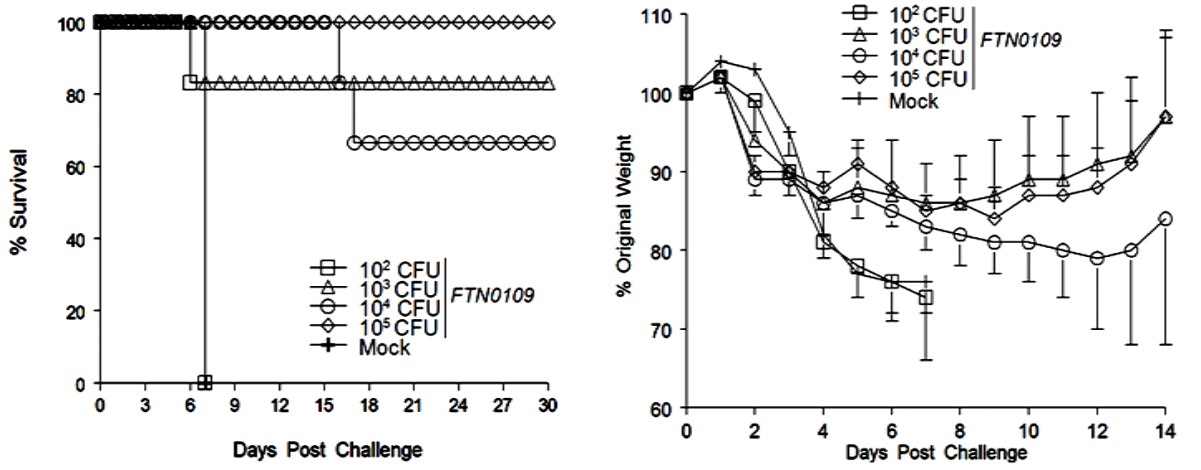
Given that the *FTN0109* strain was attenuated for intramacrophage growth, we assessed virulence *in vivo* by measuring the LD_{50} in BALB/c mice. Animals were i.n. inoculated with escalating doses from 10^2 - 10^5 CFU of *FTN0109* or with 10^3 CFU of the wild-type U112 strain (LD_{50} < 10 CFU in mice). As shown in **Figure 1(b)**, all mice infected with U112 succumbed by day 4. However, all animals infected with *FTN0109* survived, regardless of dose, demonstrating a 10,000-fold increase in LD_{50} (>10^5 CFU) compared to the wild-type strain. These data suggest that *FTN0109* is attenuated both *in vitro* and *in vivo*, and may be useful as a potential vaccine candidate.

3.2. *FTN0109* Protects against Subsp. *holarctica* Pulmonary Challenge in Mice

To determine whether inoculation of mice with the *F. novicida FTN0109* strain could provide protection against subsequent pulmonary challenge with a heterologous *F. tularensis* strain, we challenged vaccinated mice with a lethal dose of the subsp. *holarctica* LVS strain (LD_{50} = ~6000 CFU). Mice were vaccinated i.n. with *FTN0109* (10^2 - 10^5 CFU) or mock-vaccinated with PBS, and then rested for 30 days. Vaccinated mice were then challenged i.n. with 60,000 CFU (approx. 10 LD_{50}) LVS. As shown in **Figure 2(a)**, mice i.n. vaccinated with *FTN0109* exhibited increased protection to i.n. LVS challenge in a dose-dependent manner, with no protection conferred by 10^2 CFU vaccination, moderate protection from 10^3 - 10^4 CFU ($p < 0.01$ or $p < 0.001$ compared to mock for 10^3 and 10^4 CFU, respectively), and 100% protection with a 10^5 CFU immunization ($p < 0.001$ compared to mock). **Figure 2(b)** shows weight loss of vaccinated mice upon LVS challenge as a measure of morbidity, which correlated with survival. These results indicate that the *F. novicida* strain *FTN0109* can induce protective immunity in mice against challenge with a heterologous *Francisella* strain.

3.3. Correlates of Immunity in *FTN0109* Vaccinated Mice

We further characterized the correlates of immunity associated with i.n. vaccination of mice with a protective immunizing dose (10^5 CFU). We first assessed organ burdens of *FTN0109* after intranasal vaccination, and determined that the bacterium could not be recovered in organ homogenates by day 14 post vaccination (data not shown), indicating that *FTN0109* was cleared from the mouse by this time. Cellular immune responses were then assessed 14 days after vaccination, specifically assessing IFN-γ and IL-2 production, as these cytokines have been shown to be important for clearance of *F. tularensis* [19] [20]. As shown in **Figure 3**, splenocytes from *FTN0109*-vaccinated animals produced both IFN-γ and IL-2 in a dose-dependent fashion when co-cultured with UV-inactivated *FTN0109*, specifically producing 2490 ± 283 pg/mL of IFN-γ and 391 ± 72 pg/mL of IL-2 in response to 10^3 bacteria ($p < 0.001$ for IFN-γ in comparison to mock), and 5362 ± 139 pg/mL of IFN-γ and



(a) (b)

Figure 2. *FTN0109* vaccination can protect against pulmonary challenge with *F. tularensis* subsp. *holarctica* LVS in mice. BALB/c mice (n = 6 per group) were i.n. vaccinated with escalating doses of *FTN0109* (10^2 - 10^5 CFU) or mock-vaccinated with PBS and rested for 30 days followed by an i.n. challenge of 60,000 CFU LVS (approx. 10 LD_{50}). Mice were monitored daily for mortality (a) and morbidity (b).

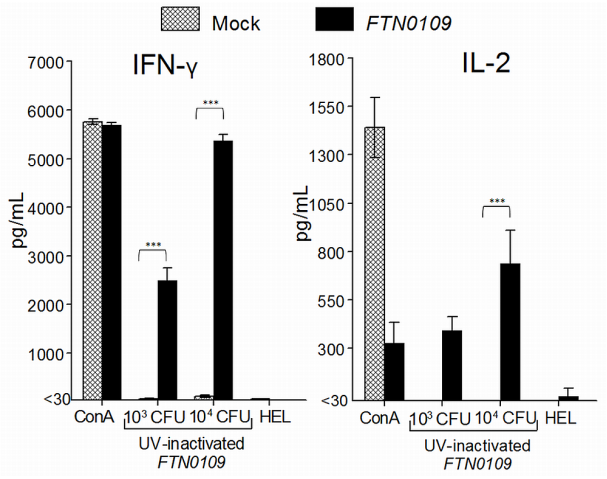


Figure 3. Intranasal vaccination with *FTN0109* induces antigen-specific cellular responses in mice. BALB/c mice (n = 3 per group) were vaccinated i.n. with 10^5 CFU *FTN0109* or mock-vaccinated with PBS and rested for 14 days. Mice were sacrificed and spleens collected to prepare single-cell suspensions. Splenocytes (10^6 cells/well) were cultured in triplicate for 72 hrs with either 1 µg of positive control mitogen Concanavalin A (ConA), 1 µg of unrelated antigen hen egg lysozyme (HEL), or two different doses (10^3 or 10^4 CFU) of UV-i nactivated *FTN0109*. Supernatants were collected and assayed by ELISA for IFN-γ and IL-2 production. *** denotes $p < 0.001$.

738 ± 170 pg/mL of IL-2 in response to 10^4 bacteria (both $p < 0.001$ in comparison to mock). Mock-vaccinated splenocytes had minimal responses to these conditions (64 ± 9 pg/mL and 107 ± 28 pg/mL of IFN-γ in response to 10^3 and 10^4 bacteria, respectively, with no detectable IL-2 responses at either dose). Both vaccinated and mock-vaccinated groups produced IFN-γ and IL-2 in response to culture with the mitogen ConA, and minimal responses were mounted to the unrelated antigen hen egg lysozyme (HEL). Thus, mice vaccinated i.n. with *FTN0109* mounted antigen-specific cellular responses following immunization.

Humoral responses of *FTN0109*-vaccinated mice were assessed at 28 days after vaccination. **Figure 4** shows that only vaccinated mice produced significant amounts of antibodies to *FTN0109*, with 50% binding titers of 1589 ± 664 for total antibodies ($p < 0.01$ compared to mock), 2842 ± 951 for IgG1 ($p < 0.001$ compared to mock), and 4251 ± 1197 for IgG2a ($p < 0.001$ compared to mock). Mice receiving PBS as a mock vaccine produced no detectable antibodies to *FTN0109*, and neither *FTN0109*- or mock-vaccinated animals produced detectable antibodies to the unrelated antigen HEL (data not shown).

3.4. Post-challenge Organ Burden and Pathology Are Lower in *FTN0109*-Vaccinated Mice

We examined organ bacterial burden and pathology following challenge with LVS in *FTN0109*-vaccinated mice. Animals were vaccinated i.n. with either 10^5 CFU *FTN0109*, or mock-vaccinated with PBS, and then rested for 30 days, followed by i.n. challenge with 60,000 CFU of LVS. At various time points, mice were sacrificed to determine organ bacterial burdens in lungs and spleen, and to assess pathology in the lung. As shown in **Figure 5**, mock-vaccinated, LVS-challenged mice had increasing bacterial burdens in the lungs and spleen over the time course before mice succumbed to infection about a week after challenge. In contrast, *FTN0109*-vaccinated animals had lower bacterial burdens in the lungs and spleen at days 3 and 6 compared to their mock-vaccinated counterparts, with significant differences compared to mock-vaccinated in the lung ($p < 0.05$ on days 1 and 3, $p < 0.01$ on day 6).

We also assessed lung pathology in *FTN0109*-vaccinated mice at various time points after LVS challenge. As shown in **Figure 6**, lungs of mock-vaccinated, LVS-challenged mice show perivascular cellular infiltration around the bronchioles at day 3, with tissue necrosis evident at day 6, shortly before mice succumbed to chal-

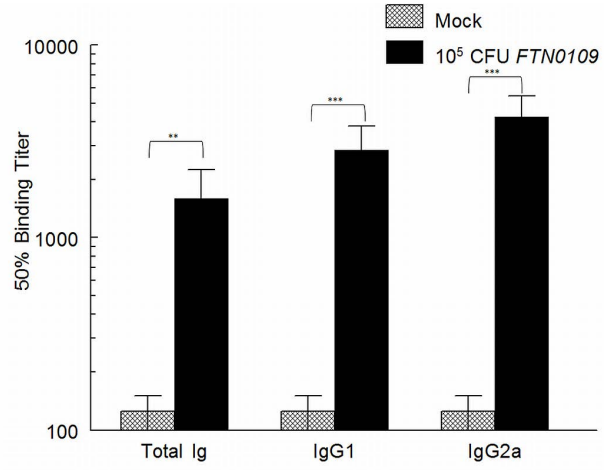


Figure 4. Intranasal vaccination with *FTN0109* induces systemic antibody production in mice. BALB/c mice (n= 6 per group) were intranasally vaccinated with 10^5 CFU *FTN0109* or mock-vaccinated with PBS and rested for 28 days. Mice were bled to obtain sera, and 50% binding titers for total antibody, IgG1, and IgG2a to HEL (100 ng/well) or UV-inactivated *FTN0109* (10^6 CFU/well) were determined by ELISA. ** denotes $p < 0.01$, *** denotes $p < 0.001$.

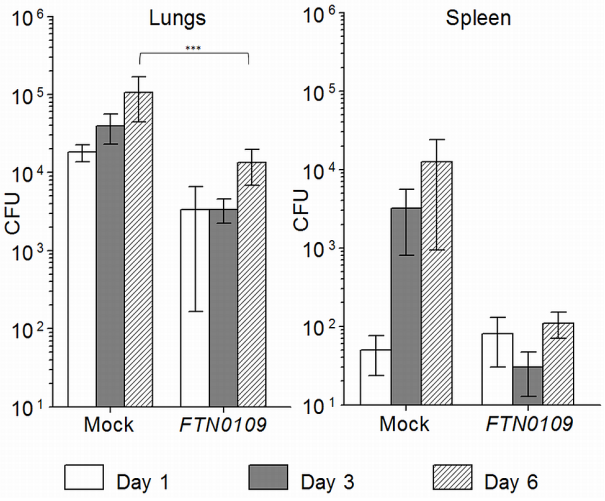


Figure 5. *FTN0109* vaccination reduces organ bacterial burden following LVS challenge in mice. BALB/c mice (n = 3 mice/group/time point) were i.n. vaccinated with 10^5 CFU *FTN0109* or mock-vaccinated with PBS and rested for 30 days prior to i.n. challenge with 60,000 CFU LVS. Animals were sacrificed at defined time points (days 1, 3, and 6 post-challenge) and lungs and spleens collected. Organs were homogenized and dilution plating was used to enumerate whole organ bacterial burdens. *** denotes $p < 0.001$.

lenge. In contrast, *FTN0109*-vaccinated mice exhibited lower levels of cellular infiltration into the lungs at day 3, and even lower levels of infiltration at day 6 post challenge. Additionally, there was no evidence of necrosis in the lungs of *FTN0109*-vaccinated mice at day 6 post-challenge. These data demonstrate that *FTN0109*-vaccinated mice have lower bacterial organ burdens and less lung pathology compared to mock-vaccinated mice upon LVS challenge.

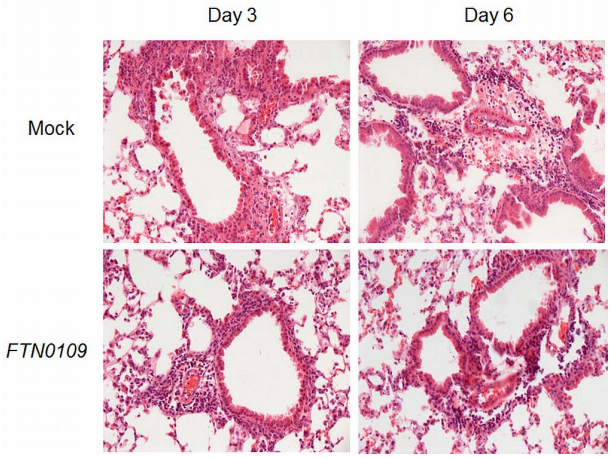


Figure 6. Vaccination with *FTN0109* reduces lung pathology following LVS challenge in mice. BALB/c mice (n = 3 mice/group/time point) were vaccinated i.n. with 10^5 CFU *FTN* 0109 or mock-vaccinated with PBS and rested for 30 days prior to i.n. challenge with 60,000 CFU LVS. Mice were sacrificed for lung collection on days 3 and 6 post-challenge. Paraffin-embedded sections were cut and H & E stained for histological analysis. Representative images from each group and time point are shown.

3.5. *FTN0109* Induces Protection against Pulmonary Challenge with *F. tularensis* Subsp. *tularensis* in Fischer 344 Rats

Due to the inherent sensitivity of mice to *F. tularensis*, murine protection studies using the highly human virulent subsp. *tularensis* strain SCHU S4 are generally limited to pulmonary challenges of around 100 - 200 CFU. In our hands, we did not see protection in C57BL/6 mice against SCHU S4 pulmonary challenge when animals received either a single priming dose or prime-boost vaccination regimen (10^5 CFU *FTN0109* i.n., once or twice 3 weeks apart, with challenge 6 weeks after initial immunizing dose, data not shown). To this end, Fischer 344 rats have been proposed as a useful animal model to evaluate vaccine candidates for tularemia [8] as this animal model allows for testing of a wider range of doses for pulmonary challenge (approx. 10^4 CFU). We utilized the Fischer 344 rat to determine if *FTN0109* vaccination could provide protection against the high human virulence SCHU S4 strain.

Fischer 344 rats were vaccinated i.t. with either *FTN0109* (10^5 or 10^6 CFU), the wild-type *F. novicida* strain U112 (10^5 CFU), or mock-vaccinated with PBS. Rats were rested for 30 days, and then challenged i.t. with 12,500 CFU (approx. 25 LD_{50}) of SCHU S4. Rats were monitored daily for morbidity and mortality. As shown in **Figure 7**, mock-vaccinated rats succumbed to SCHU S4 challenge in about a week, while rats vaccinated with 10^5 CFU wild-type *F. novicida* U112 were fully protected from SCHU S4 infection ($p < 0.001$ compared to mock). Importantly, i.t. vaccination with 10^5 CFU U112 (pulmonary $LD_{50} = 5 \times 10^6$ CFU) induces visible morbidity in rats from day 3 to day 8-9 post-vaccination (approx. 10% weight loss, porphyrin production around eyes, hunched posture, and ruffled fur), so this regimen would not be likely to serve as a successful putative vaccine candidate. To this end, rats vaccinated with 10^5 CFU *FTN0109* were not protected against i.t. SCHU S4 challenge. However, rats vaccinated with 10^6 CFU *FTN0109* exhibited 50% protection against pulmonary challenge with SCHU S4 ($p = 0.0526$ compared to mock), demonstrating dose-dependent protection by *FTN0109* against *F. tularensis* subsp. *tularensis* challenge.

4. Discussion

The *F. novicida FTN0109* strain induces protective immunity against heterologous challenge in both animal models for tularemia (against subsp. *holarctica* LVS in mice and subsp. *tularensis* SCHU S4 in rats). *FTN0109* demonstrates enhanced uptake into murine macrophages, possibly a result of enhanced binding to macrophage

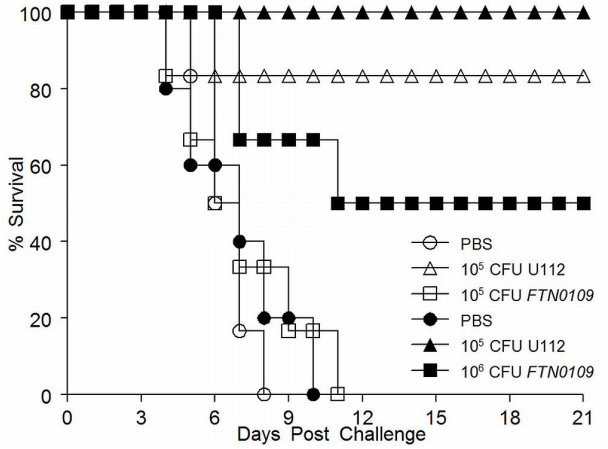


Figure 7. Intratracheal vaccination with *FTN0109* protects Fischer 344 rats against pulmonary *F. tularensis* subsp. *tularensis* SCHU S4 challenge. F344 rats (n = 6 per group) were intratracheally vaccinated with 10^5 CFU wild-type parent strain U112, 10^5 or 10^6 CFU *FTN0109* or mock-vaccinated with PBS and rested for 30 days prior to intratracheal challenge with 12,500 CFU (approx. 25 LD_{50}) of SCHU S4. Rats were monitored daily for morbidity and mortality. Both challenge results are shown (open vs. closed symbols).

mannose receptors or surface nucelolin [21]-[23]. However, the *FTN0109* strain was attenuated both *in vitro* and *in vivo*, and maintained a limited ability to disseminate post-immunization from murine lungs to the spleen and liver (data not shown); this mild dissemination to immune organs may aid in generating systemic antigen-specific immune responses like IFN-*γ* production and Th-1 type (IgG2a) responses, which are necessary to protect against a facultative intracellular pathogen such as *F. tularensis* [17] [24]-[26]. *FTN0109* vaccination induces protective cellular and humoral immune responses similar to those shown by *F. novicida* derived live attenuated vaccines Δ*iglB* and Δ*fopC* [8] [16] [27]. As seen with *F. novicida* mutant U112Δ*iglB* [8] [16], antibody responses to *FTN0109* differ between animal models, as rats mount a predominantly IgG2a response in sera with minimal IgG1 produced (data not shown) compared to a mixed IgG1/IgG2a response in mice. Importantly, both responses remain protective in their respective animal models and may simply allude to differences between these rodents in responding to *F. novicida*. Additionally, pilot studies conducted to elucidate the mechanism of protection of *FTN0109* in mice indicate that neither $CD4^+$ nor $CD8^+$ T cells alone contribute to the observed protection against lethal LVS challenge, and suggest that both $CD4^+$ and $CD8^+$ T cells may need to be inactivated to abrogate a protective immune response. No significant difference in survival are seen between *FTN0109*-vaccinated $CD4^{-/-}$ mice or $CD8^{-/-}$ mice compared to WT vaccinated controls, or between WT vaccinated mice which were depleted of $CD4^+$ or $CD8^+$ T cells at the time of challenge compared to WT vaccinated, mock-treated (non-depleted) controls (data not shown). To this end, the protective capacity of $CD4^+$ and $CD8^+$ T cells to compensate each other has been previously reported following LVS vaccination [18] [20] [28] [29].

The ability of *FTN0109* vaccination to protect against subsp. *tularensis* and *holarctica* pulmonary challenges lends credence to the use of *F. novicida*-based live attenuated vaccines against tularemia. Mounting evidence shows that *F. novicida* can be used as a successful platform for live attenuated vaccines, as our and Dr. Klose's laboratories also has demonstrated success with *novicida*-based *Francisella* Pathogenicity Island (FPI) mutant (U112Δ*iglB* and U112Δ*iglD*) as a live attenuated vaccine in mice [16], rats [8] [10] and non-human primates ,[10] and with another defined non-FPI mutant strain (U112Δ*fopC*) in mice [27]. Some of the major advantages of *F. novicida* as the platform for a live attenuated vaccine is that it has inherently low virulence in humans, requires only BSL-2 containment, and it is exempt from select agent status. *F. novicida* is easily manipulated genetically, and remains highly similar genetically to *F. tularensis* [30]. In contrast, live attenuated vaccine strains of subsp. *tularensis* and *holarctica* (except LVS strain) still require BSL-3 containment due to the uncertainty of reversion to a wild-type phenotype.

In addition, i.t. [9] and oral [31] administration of the WT *novicida* strain U112 has been shown to be effica-

cious as a vaccine in rats, although at the administered doses (10^5 CFU) it can cause moderate morbidity for 7 - 10 days post-vaccination and thus would require some attenuating mutation to enhance safety. Due to this morbidity associated with U112 vaccination in immunocompetent hosts, it is highly unlikely that WT U112 could be safely administered to an immune-compromised host. Vaccination with *FTN0109*, in contrast, induces minimal morbidity at either dose tested (10^5 or 10^6 CFU); animals appear healthy and gain weight following immunization. Considering that *FTN0109* is well-tolerated in F344 rats, future studies should test increased vaccine dosages (10^7 CFU) or a prime-boost regimen in order to generate greater protection against pulmonary SCHU S4 challenge. Additionally, it may be worthwhile to test *FTN0109* vaccine efficacy and safety in immunocompromised hosts.

The *F. novicida*-based mutant *FTN0109* may represent a useful vaccine candidate against tularemia. This vaccine represents the 4th successful putative tularemia vaccine derived from a *F. novicida* background, and as *FTN0109* has been proven efficacious in two independent animal models for tularemia, we argue that *F. novicida* may serve as an effective platform for live attenuated tularemia vaccines.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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