

# A $\Delta clpB$ Mutant of *Francisella tularensis* Subspecies *holarctica* Strain, FSC200, Is a More Effective Live Vaccine than *F. tularensis* LVS in a Mouse Respiratory Challenge Model of Tularemia

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## Abstract

*Francisella tularensis* subsp. *tularensis* is a highly virulent pathogen for humans especially if inhaled. Consequently, it is considered to be a potential biothreat agent. An experimental vaccine, *F. tularensis* live vaccine strain, derived from the less virulent subsp. *holarctica*, was developed more than 50 years ago, but remains unlicensed. Previously, we developed a novel live vaccine strain, by deleting the chaperonin *clpB* gene from *F. tularensis* subsp. *tularensis* strain, SCHU S4. SCHU S4 $\Delta clpB$  was less virulent for mice than LVS and a more effective vaccine against respiratory challenge with wild type SCHU S4. In the current study, we were interested to determine whether a similar mutant on the less virulent subsp. *holarctica* background would also outperform LVS in terms of safety and efficacy. To this end, *clpB* was deleted from clinical *holarctica* strain, FSC200. FSC200 $\Delta clpB$  had a significantly higher intranasal LD<sub>50</sub> than LVS for BALB/c mice, but replicated to higher numbers at foci of infection after dermal inoculation. Moreover, FSC200 $\Delta clpB$  killed SCID mice more rapidly than LVS. However, dermal vaccination of BALB/c mice with the former versus the latter induced greater protection against respiratory challenge with SCHU S4. This increased efficacy was associated with enhanced production of pulmonary IL-17 after SCHU S4 challenge.

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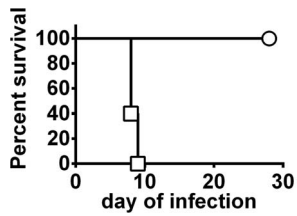
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## Introduction

*Francisella tularensis* is a facultative intracellular bacterial pathogen of mammals. Two subspecies, subsp. *holarctica* and subsp. *tularensis* are highly infectious for humans, and the latter can cause high mortality when inhaled [1]. Consequently, subsp. *tularensis* was developed as a biological weapon by several countries during the 20<sup>th</sup> century [2]. Tularemia caused by subsps. *holarctica* was endemic in the USSR during the early 20<sup>th</sup> century. To counter this threat, Soviet scientists developed an attenuated *holarctica* strain, strain 15, as a live vaccine. This vaccine was highly effective at preventing tularemia caused by subsp. *holarctica* [3]. However, its efficacy against subsp. *tularensis* was not assessed. Subsequently, the USA developed a phenotypically more defined live vaccine strain (LVS) from strain 15 [4]. LVS was tested extensively in human volunteers during the 1960s, and was shown to initially provide 80–90% protection that waned over time against substantial intradermal or inhaled inocula of subsp. *tularensis* strain, SCHU S4 [5,6]. For various regulatory reasons, LVS has never been licensed for public use [7]. Nevertheless, it is currently being reassessed for safety and immunogenicity, but not efficacy, in several clinical trials (<http://www.clinicaltrials.gov/ct2/show/NCT01150695?term=tularemia&rank=1>).

We and others have been investigating a variety of approaches to develop more defined, and more effective tularemia vaccines using murine models of respiratory infection with SCHU S4 as a preclinical screen (reviewed in [8]). Several of us, have focussed on devising defined attenuated mutant strains of SCHU S4 for use as live vaccines on the grounds that they contain antigens unique to subsp. *tularensis* that could be important targets for the protective immune response [9–11]. Several such mutants have been shown to be highly effective. In our hands, SCHU S4 $\Delta clpB$  has performed well in this respect [12–15].

A potential concern about SCHU S4 deletion mutants is their ability to revert to a virulent phenotype. In this regard, it has recently been shown that LVS is attenuated almost entirely because of partial deletion of a single gene [16]. Moreover, strain 15, the parent strain of LVS was given to millions of Russians with no documented evidence of its reversion [3]. Nevertheless, a defined vaccine strain on a subsp. *holarctica* background could help alleviate any lingering concerns, since it, like LVS, could at most revert to the less virulent subsp. *holarctica* phenotype. To determine whether such a strain remains an effective vaccine, we have deleted the *clpB* gene from a fully virulent subsp. *holarctica* strain, FSC200.



**Figure 1. Attenuation of FSC200 $\Delta$ clpB for mice.** BALB/c mice (n=5/group) were inoculated ID with  $10^7$  CFU of FSC200 $\Delta$ clpB (circle) or 100 CFU of the complemented strain (square) and monitored for survival.

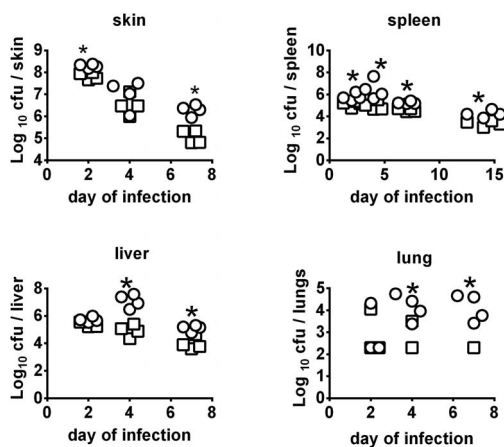
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Compared to LVS, FSC200 $\Delta$ clpB replicated to greater numbers in target organs, but was less lethal than the former via the respiratory route. As with SCHU S4 $\Delta$ clpB, ID immunization of BALB/c mice with FSC200 $\Delta$ clpB elicited greater protection than LVS against respiratory challenge with SCHU S4. Additionally, like SCHU S4 $\Delta$ clpB, enhanced protection by FSC200 $\Delta$ clpB versus LVS was associated with increased levels of IL-17 in the lungs after challenge with virulent bacteria. However, in a head-to-head comparison, FSC200 $\Delta$ clpB was less effective than SCHU S4 $\Delta$ clpB. Furthermore, FSC200 $\Delta$ clpB killed severely immunocompromised SCID mice more rapidly than either SCHU S4 $\Delta$ clpB or LVS.

## Materials and Methods

### Bacterial Strains

*F. tularensis* subsp. *tularensis* strain SCHU S4, and subsp. *holarctica* strain FSC200, were obtained from the Francisella Strain Collection, Umea University, Sweden. Both are clinical isolates, and both are highly virulent for mice ( $LD_{100} < 10$  CFU) [16,17]. The  $\Delta$ clpB deletion mutants of both strains were prepared by allelic replacement resulting in mutants with no foreign DNA as previously described [18]. Complementation was performed as we previously described [19]. LVS was a low passage stock prepared from NDBR lot #11 [20].



**Figure 2. In vivo growth kinetics of FSC200  $\Delta$ clpB vs LVS following ID vaccination.** BALB/c mice (n=4/group) were immunized ID with  $\sim 10^5$  CFU of FSC200  $\Delta$ clpB (circle) or LVS (square). Mice were killed on days 2,4,7, and 14 for bacteriology. \*, significantly higher burden vs LVS. Symbols not shown for organ burdens below detectable limits (100 CFU).

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### Mice & Ethics

Specific pathogen free BALB/c mice and BALB/c SCID mice were from Charles Rivers Laboratories, St. Constant, Que. Animal experiments described herein were approved by the National Research Council (NRC) Animal Care Committee (ACC) and conducted in a Canadian Council on Animal Care (CCAC) accredited facility. Mice were examined daily for signs of infection. Whenever feasible, mice were euthanized by CO<sub>2</sub> asphyxiation as soon as they displayed signs of irreversible morbidity. In our experience, such mice were at most 24 h from death, and the time to death of these animals was estimated on this premise. All work with SCHU S4, FSC200, and mutants thereof was performed in a small animal biocontainment level 3 facility certified by the Canadian Food Inspection Agency and the Public Health Agency of Canada.

### Immunization and Challenge

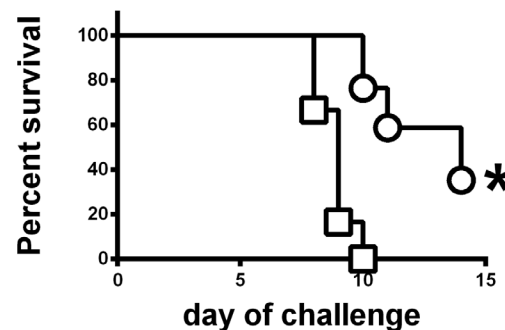
Mice were vaccinated with various doses of one or other of the vaccine strains administered intradermally (ID) in a volume of 50  $\mu$ l of saline, or intranasally (IN) in 20  $\mu$ l then chased with 20  $\mu$ l sterile saline. On the stated days post-vaccination, some mice were killed and skin, spleen, lung, and serum assessed for bacterial levels as described previously [14]. Additionally, some immunized mice were bled on day 28 post-vaccination and their sera used for immunoproteomics. Finally, representative mice from each group were challenged IN six weeks post-vaccination with wild-type SCHU S4 and either killed at intervals for pulmonary cytokine analysis or monitored for survival.

### Cytokines and Chemokines

Lung homogenates were clarified by centrifugation and 0.22  $\mu$ m filtration, and confirmed to be sterile by plating. Levels of pulmonary cytokines and chemokines in these samples were determined using Fluorokine Mouse 21-plex Cytokine Detection System (R & D systems, Minneapolis, MN) on a Luminex<sup>®</sup> 100 IS system (Luminex, Austin, TX). Cytokine/chemokine concentrations were calculated against the standards using Beadview<sup>®</sup> software version 1.03.

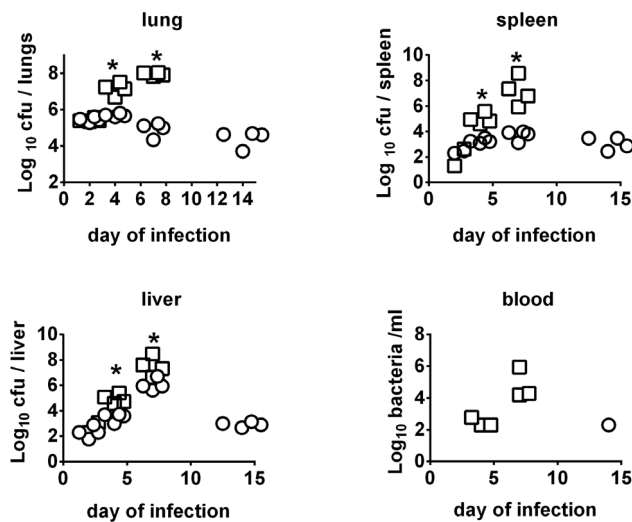
### Immunoproteomics

A total cell lysate of *F. tularensis* SCHU S4 was used as antigen for immunoproteomics studies as described in our recent work [14]. Proteins were separated in the pH range 4–7 using a linear



**Figure 3. Protective efficacy of ID vaccination with FSC200 $\Delta$ clpB versus LVS against respiratory challenge with SCHU S4.** BALB/c mice were immunized ID with  $10^5$  CFU of LVS (square; n=12) or FSC200 $\Delta$ clpB (circle; n=17) and challenged 6 weeks later IN with 86 CFU of SCHU S4. \*, Significantly longer survival than mice immunized with LVS.

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**Figure 4. Kinetics of SCHU S4 infection in mice immunized with FSC200  $\Delta$ clpB vs LVS.** BALB/c mice (n = 4/group) were immunized ID with  $\sim 10^5$  CFU of FSC200  $\Delta$ clpB (circle) or LVS (square). Six weeks later immunized and control mice were challenged IN with 86 CFU of SCHU S4. Mice were killed on days 2,4,7,14 after challenge and bacteriology performed on their organs. \*, significantly higher burden vs mice immunized with FSC200  $\Delta$ clpB. doi:10.1371/journal.pone.0078671.g004

gradient and then using a 12% SDS-PAGE. Proteins separated by 2D PAGE were electroblotted onto PDVF membranes and probed with murine sera at a dilution of 1:500, chosen on the basis of previous work. Reactive spots were visualized using the ECL Chemiluminescence kit (GE Life sciences USA). PDQuest software (Biorad, USA), was used to align protein stained gel images of with images of the developed blots. Immunoreactive spots were excised from matched protein stained 2D-PAGE gels and typically digested prior to analysis by nano-liquid chromatography nLC-MS/MS exactly as described in earlier work [14].

### Statistics

Statistical analyses were performed with GraphPad Prism version 6.0 software. Bacterial burdens and cytokine levels were compared by t-test on log transformed data. Survival curves were compared by Mantel-Cox test with Bonferroni correction as appropriate. In all cases, a corrected  $P < 0.05$  was considered statistically significant.

## Results

### Virulence of FSC200 $\Delta$ clpB Versus LVS for BALB/c Mice

Deleting the *clpB* gene from FSC200 created a highly attenuated strain and complementation restored virulence (figure 1 & figure S1 in File S1). LVS is also highly attenuated by the ID route, however it retains substantial virulence for mice via the IN route and was found to be significantly more lethal than FSC200 $\Delta$ clpB in this regard (figure S2 in File S1). The *in vivo* growth of an ID inoculum of  $10^5$  CFU FSC200 $\Delta$ clpB compared to LVS is shown by figure 2. By day 2, there had been a mean 630-fold versus 1600-fold increase in burden of LVS versus FSC200 $\Delta$ clpB at the site of inoculation ( $P < 0.05$ ). Bacterial burdens declined in the skin in both groups on days 4 and 7, but FSC200 $\Delta$ clpB persisted at a significantly higher level than LVS at the latter time-point. The skin was sterile in both groups by day 14 post inoculation. Both strains had disseminated to the spleen by day 2, and persisted

therein for at least 14 days. At all time-points, FSC200 $\Delta$ clpB was present at significantly higher levels than LVS in this organ. A similar situation was observed in the liver on days 4 and 7 after infection, but both strains were cleared from this organ by day 14. In the lungs, LVS and FSC200 $\Delta$ clpB were recovered from 3/4 mice on day 2, but the former was recovered only sporadically thereafter compared to the latter. Both strains had been cleared from the lungs by day 14. In the blood, 1 or 2 colonies (100–200 CFU/ml), were recovered from 1 or 2 mice on days 2 and 4 after vaccination. A similar result was obtained upon repeat (figure S3 in File S1).

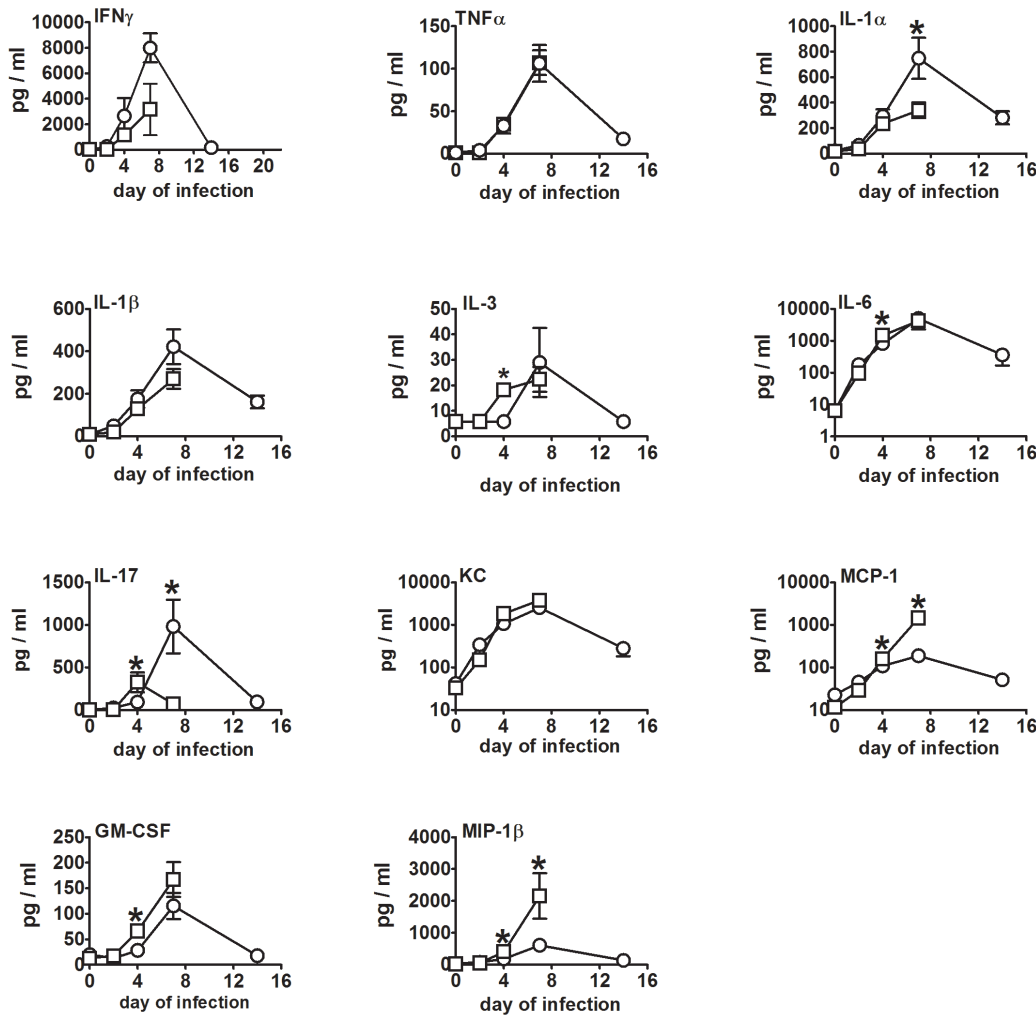
During the course of this experiment, most immunized mice remained completely asymptomatic except that one mouse immunized with LVS died on day 4, and a few others in both groups displayed mild pilo-erection between days 5 and 6. Additionally, using the skin reaction scale shown in figure S4 in File S1, only 3/25 mice immunized with FSC200 $\Delta$ clpB reached a score of 2 compared with 19/25 mice immunized with LVS.

### Efficacy of ID Vaccination with FSC200 $\Delta$ clpB Versus LVS Against Respiratory Challenge with SCHU S4

LVS is only approved for use in humans by the scarification method. To mimic this situation, and to allow more precise quantification of the inoculating dose, mice were immunized by the ID route. Six weeks after ID immunization with  $\sim 10^5$  CFU of FSC200 $\Delta$ clpB or LVS, mice were challenged IN with 86 CFU of SCHU S4. Some mice were killed on days 2, 4, 7, and 14 and organs and sera examined for bacteria, and the lungs for cytokines, and chemokines. All naive mice died on day 5 after challenge, no mice immunized with LVS survived past day 10, and some mice immunized with FSC200 $\Delta$ clpB died between days 10–14 (figure 3). By day 2 there had been obvious growth of SCHU S4 in the lungs of mice immunized with either LVS or FSC200 $\Delta$ clpB and it continued to grow in the lungs of the former mice until they succumbed to infection between days 8 and 10 after challenge (figure 4). In contrast, after day 2, surviving mice immunized with FSC200 $\Delta$ clpB were able to control SCHU S4 infection in the lungs, but not eliminate it during the ensuing twelve day observation period. A similar situation was seen in the spleen, whereas in the liver SCHU S4 grew up to day 7 in both groups of mice. Thereafter, infection was brought under control only by the mice immunized with FSC200 $\Delta$ clpB. Bacteria were recovered from the blood of most mice immunized with LVS on days 4 and 7 after challenge with SCHU S4, but only from the blood of a single mouse immunized with FSC200 $\Delta$ clpB.

### Cytokine and Chemokine Responses in Vaccinated Mice Following IN Challenge with SCHU S4

Previously, we showed that in BALB/c mice, vaccination with SCHU $\Delta$ clpB was more effective against respiratory challenge with wild-type bacteria than vaccination with LVS [13]. Additionally, we showed that SCHU $\Delta$ clpB protects BALB/c mice but not C57BL/6 mice against such challenge [14]. In both cases, the enhanced efficacy of SCHU $\Delta$ clpB was associated with significantly higher levels of IL-17 in the lungs seven days after challenge with SCHU S4. To determine if this was the case too for FSC200 $\Delta$ clpB versus LVS, we examined the lungs of vaccinated mice challenged IN with SCHU S4 for a panel of cytokines and chemokines (Figure 5). For the most part, cytokine and chemokine levels rose steadily over the course of the first 7 days of challenge in both groups, then declined between day 7 and 14 in surviving mice. The exceptions were IL-17 which peaked on day 4 in mice



**Figure 5. Kinetics of pulmonary cytokine production after challenge of vaccinated mice with SCHU S4.** BALB/c mice ( $n=4$ /group) were immunized ID with  $\sim 10^5$  CFU of FSC200  $\Delta$ clpB (circle) or LVS (square). Six weeks later immunized mice were challenged IN with 86 CFU of SCHU S4. Mice were killed on days 2,4,7,14 after challenge and Luminex performed on their lungs. \*, significantly higher level vs other vaccinated group. doi:10.1371/journal.pone.0078671.g005

immunized with LVS, and IL-5, IL-12p40, IL-12p70, IL-13, and RANTES which remained at or close to background throughout.

By day 2 after challenge with SCHU S4, pulmonary IL-6 and KC were significantly elevated over background levels in both groups of immunized mice, whilst IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , and IL-17 levels were significantly elevated only in mice immunized with FSC200 $\Delta$ clpB; MCP-1 was significantly elevated only in LVS-immunized mice. By day 4 after challenge with SCHU S4 pulmonary levels of IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-17, KC, MCP-1, MIP-1 $\beta$ , and TNF $\alpha$ , were significantly above background in both groups of immunized mice, whereas IL-3 and GM-CSF were significantly elevated only in mice vaccinated with LVS. The situation was the same on day 7 after challenge, except that GM-CSF and IL-3 were now significantly elevated in both groups. No mice immunized with LVS survived to day 14 after challenge with SCHU S4, at which point most cytokines and chemokines had dropped from day 7 levels in mice immunized with FSC200 $\Delta$ clpB. However, IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-17, KC, MCP-1, MIP-1 $\beta$ , and TNF $\alpha$  were still significantly above background levels at this time. We have observed similar results for LVS and SCHU S4  $\Delta$ clpB in previous studies [13,14].

A comparison of pulmonary cytokine and chemokine levels between the two vaccinated groups of mice showed that MCP-1, MIP-1 $\beta$ , GM-CSF, and IL-17 levels were significantly higher in the lungs of mice immunized with LVS versus FSC200 $\Delta$ clpB on day 4 after challenge with SCHU S4 as were the former two cytokines on day 7. However, IL-17 and IL-1 $\alpha$  levels were significantly higher in mice immunized with FSC200 $\Delta$ clpB on day 7 after challenge with SCHU S4. We have obtained similar results for IL-17 in BALB/c mice immunized with SCHU S4 $\Delta$ clpB [13].

### Immunoproteomics

Mice immunized ID with  $10^5$  CFU of FSC200 $\Delta$ clpB or LVS were bled four weeks later, and serum from each group was pooled separately and used to probe 2DE Western blots of a whole cell lysate of SCHU S4. The identified immunodominant proteins are listed in figure 6. No reactivity was observed with pre-immune sera. Some differences in the reactivity pattern were observed between the pools of sera from mice immunised with LVS vs FSC200 $\Delta$ clpB. Immunoreactive proteins included dihydrolipoamide succinyltransferase (FTT\_0077), 30S ribosomal protein S1 (FTT\_0183), chaperonin protein, GroEL (FTT\_1696) and hypothetical protein FTT\_1747. One of the serum pools from mice

immunised with FSC200 $\Delta$ clpB, recognised nine proteins that were not reactive at detectable levels with sera from LVS vaccinated animals. These proteins included three unknown proteins, and Elongation factor Ts (FTT\_0314), Succinyl CoA (FTT\_0504), phosphopyruvate hydratase (FTT\_0709), Fructose-1,6-bisphosphate aldolase (FTT\_1365), Malonyl CoA-ACP transacylase (FTT\_1374) and hypothetical protein FTT\_1778c). Finally, pooled sera from FSC200 $\Delta$ clpB immunised mice showed between 2–3 fold greater total intensity of immunoreactivity, compared with sera from LVS vaccinated animals.

### Efficacy and Safety of FSC200 clpB Versus LVS or SCHUS4 $\Delta$ clpB

To determine whether FSC200 $\Delta$ clpB elicits a similar level of protection as SCHU S4 $\Delta$ clpB, BALB/c mice were immunized ID with  $10^3$ ,  $10^5$ , or  $10^7$  CFU of one or other mutant, then challenged 6 weeks later IN with 105 CFU of SCHU S4 (figure 7). SCHU S4 $\Delta$ clpB was more protective than FSC200 $\Delta$ clpB at all test inocula, significantly so at doses of  $10^5$  and  $10^7$  CFU. Next, we assessed the residual virulence of SCHU S4 $\Delta$ clpB, FSC200 $\Delta$ clpB and LVS in SCID mice via ID and IN routes (figure 8) By the ID route, FSC200 $\Delta$ clpB was significantly more virulent than LVS which, in turn, was significantly more virulent than SCHUS4 $\Delta$ clpB. Relative virulence corresponded with bacterial burden (Table S1 in File

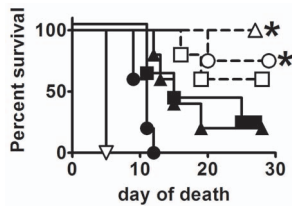
Locus Tag	Protein Name	FSC200	FSC200	LVS	LVS2
		$\Delta$ clpB	$\Delta$ clpB	Group 1	Group 2
FTT_0077	Dihydroliipoamide succinyltransferase	■	■	■	■
FTT_0137	Elongation factor Tu	■	■	■	■
FTT_0183c	30S ribosomal protein S1	■	■	■	■
FTT_0188	Cell division protein FtsZ	■	■	■	■
FTT_0314	Elongation factor Ts	■	■	■	■
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	■	■	■	■
FTT_0504c	Succinyl-CoA synthetase beta chain	■	■	■	■
FTT_0583/FTT_0350	OM associated protein/ DNA-directed RNA polymerase	■	■	■	■
FTT_0709	Phosphopyruvate hydratase	■	■	■	■
FTT_1269c	molecular chaperone DnaK	■	■	■	■
FTT_1365c	Fructose-1,6-bisphosphate aldolase	■	■	■	■
FTT_1374	Malonyl CoA-ACP transacylase	■	■	■	■
FTT_1539c	Hypothetical protein FTT_1539c	■	■	■	■
FTT_1696	Chaperone protein, groEL	■	■	■	■
FTT_1713c/FTT_1358c	intracellular growth locus, subunit B	■	■	■	■
FTT_1747	hypothetical protein FTT_1747	■	■	■	■
FTT_1778c	Hypothetical protein FTT_1778c	■	■	■	■
FTT_0721c	Peroxidase/catalase	■	■	■	■
FTT_0893	Phosphoribosylaminoimidazole synthetase	■	■	■	■
Unknown 5	no ID	■	■	■	■
Unknown 71	no ID	■	■	■	■

Relative intensity of immunoreactivity	
■	1-999
■	1000-1499
■	1500-4999
■	>5000

**Figure 6. Immunoreactivity of sera from BALB/c mice after vaccination with FSC200 $\Delta$ clpB or LVS.** Mice were bled 28 days after immunization with  $10^5$  CFU of FSC200 $\Delta$ clpB or LVS. Each pool contained sera from five mice. Western blots were developed and scanned, and immunoreactive areas recorded as relative immunoreactivity, measured by densitometry. Shown are the top ranked immunodominant proteins. The identity of each immunoreactive area was determined by alignment with an equivalent protein stained gel, and immunoreactive spots identified by nLC-MSMS of their tryptic digests.

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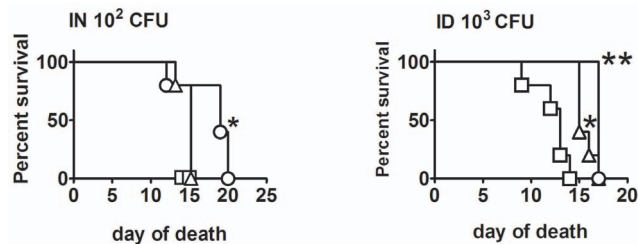
**Figure 7. Efficacy comparison of SCHU S4  $\Delta$ clpB versus FSC200  $\Delta$ clpB.** BALB/c mice ( $n=5$ /group) were immunized with  $10^3$  (squares),  $10^5$  (circles), or  $10^7$  (triangles) CFU of SCHU S4 $\Delta$ clpB (open symbols) or FSC200 $\Delta$ clpB (closed symbols). Immunized mice and naïve controls (inverted triangle) were challenged six weeks later IN with  $10^5$  CFU of SCHU S4. \*, significantly better survival versus mice immunized with FSC200 $\Delta$ clpB.

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S1). By the IN route FSC200 $\Delta$ clpB was significantly more virulent for SCID mice than SCHU S4 $\Delta$ clpB.

## Discussion

Cell-mediated immunity is required for protection against *F. tularensis* subsp. *tularensis* [21]. A single vaccination by scarification with *F. tularensis* LVS elicits essentially life-long cell-mediated immunity in humans, whereas humoral immunity disappears within ten years [22,23]. However, human volunteer studies conducted during the 1960s indicated that the protective immunity provided by LVS against aerosol challenge with subsp. *tularensis* strain SCHU S4, waned within 6–12 months [5,6,24]. The anti-LVS antibody response was the sole immunological parameter assessed by the latter studies, and did not correlate with protection [25]. Because of these findings and the uncertain pedigree of LVS, we and others have been searching for alternative vaccine candidates. The major indication for vaccination against tularemia is to prevent a mass-casualty outbreak caused by the deliberate release of an aerosol of subsp. *tularensis* either on the battlefield or on civilians. A vaccine that is rapidly effective after a single dose would be preferable to combat both of the aforementioned scenarios. To this end, we developed a deletion mutant of SCHU S4 missing the gene for the chaperonin protein, clpB. It is much more effective and safer than LVS in a murine model of respiratory challenge with SCHU S4 [12–15]. Attempts to delete a second virulence gene from SCHU S4 $\Delta$ clpB have resulted in almost complete loss of protective properties even in situations where the absence of the other virulence gene alone causes only minor loss of virulence (Table S2 in File S1). For instance, 10 CFU of SCHU S4  $\Delta$ relA kills mice more slowly than



**Figure 8. Virulence of vaccine strains for SCID mice.** SCID mice were challenged ID or IN with SCHU S4 $\Delta$ clpB (circle), FSC200 $\Delta$ clpB (square), or LVS (triangle) and monitored for survival. \*, significantly increased survival versus FSC200 $\Delta$ clpB; \*\*, significantly increased survival versus other strains.

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the same sized inoculum of the wild-type, but SCHU S4 $\Delta$ clpB $\Delta$ relA elicits no protection against respiratory challenge with SCHU S4. This suggests that SCHU S4 $\Delta$ clpB has reached the limits of its attenuation for efficacy purposes. Others have encountered a similar problem when combining otherwise highly protective single mutations in SCHU S4 [10].

In our hands, SCHU S4 $\Delta$ clpB does not regain any measurable virulence or lose protective immunogenicity following multiple *in vivo* passages in mice, or when co-cultured multiple times with SCHU S4  $\Delta$ iglC, another highly attenuated mutant that possesses an intact *clpB* gene (data not shown). This indicates that the *clpB* deletion mutation is essentially irreversible. Nevertheless, a similar mutation on a subsp. *holarctica* background could be as effective as and potentially safer than SCHU S4  $\Delta$ clpB. The current study shows that this is not the case on either count. Subsp. *holarctica* strain FSC 200 is highly virulent for mice, and deleting its *clpB* gene results in severe attenuation. Like SCHU S4 $\Delta$ clpB, FSC200 $\Delta$ clpB persists longer and at higher levels in mice than LVS after ID inoculation, suggesting that the former might be more virulent. However, by other measures, namely IN LD<sub>50</sub> and skin reactogenicity, both  $\Delta$ clpB mutants are less virulent than LVS. FSC200 $\Delta$ clpB elicits greater protection than LVS against respiratory challenge with SCHU S4, and this is associated with the production of significantly higher levels of IL-17 in the lungs one week following challenge with virulent SCHU S4. This represents the third distinct scenario involving  $\Delta$ clpB mutants of *F. tularensis* in which we have observed this association between protection and late onset production of pulmonary IL-17. Previously, we have seen this phenomenon when comparing the superior efficacy of SCHU S4 $\Delta$ clpB versus LVS in BALB/c mice, and when comparing the superior efficacy of the former in BALB/c versus C57BL/6 mice [13,14]. In contrast, no significant differences were observed in pulmonary IFN $\gamma$  or TNF $\alpha$  levels in any of these three situations, despite the fact that these two cytokines are known to be critical for vaccine-induced protection against pulmonary challenge with virulent *F. tularensis*. Therefore, it might be that IFN $\gamma$ , TNF $\alpha$ , and IL-17 are all required to be present in appropriate amounts and at the appropriate time to ensure adequate protection against respiratory challenge with virulent bacteria. A similar phenomenon has been observed for live vaccine induced protection against *Yersinia pestis* where pluripotent CD4+ T cells secreting TNF $\alpha$  and IL-17 or TNF $\alpha$ , IFN $\gamma$  and IL-17 appear to be critical for host defense [26]. These three cytokines alone or combined are also known to be involved in vaccine-induced immunity against other intracellular and extracellular pulmonary bacterial pathogens [27–30]. Given the plethora of actions of all three cytokines, additional experimentation rather than excessive speculation will be required to determine their coordinated roles in immune defense against pulmonary *F. tularensis*.

FSC200 $\Delta$ clpB provides significantly inferior protection compared to SCHU S4 $\Delta$ clpB. This supports our hypothesis that immune responses to antigens specific to subsp. *tularensis* contribute to protection [31]. Additionally, FSC200 $\Delta$ clpB is more virulent than SCHU S4 $\Delta$ clpB for SCID mice, whilst LVS displayed intermediate virulence. The reason for the enhanced virulence of FSC200 $\Delta$ clpB for SCID mice is unclear, but suggests that clpB is a more critical virulence factor for subsp. *tularensis* than for subsp. *holarctica*. Additionally, the  $\Delta$ clpB mutant of the LVS strain has previously been characterized and found to be essentially avirulent [32]. Presumably, this is due to the *a priori* marked attenuation of LVS. In our hands LVS $\Delta$ clpB elicits excellent protection against dermal challenge, but not respiratory challenge with SCHU S4 (figure S5 in File S1).

Mice are much more susceptible to *F. tularensis* than humans, and could, therefore, require a substantially more robust immune response for effective protection. In this case, either  $\Delta$ clpB mutant might safely provide equally good protection in humans, though whether they would outperform LVS clinically remains to be determined, possibly by comparative testing in a non-human primate model of respiratory tularemia. In this regard, LVS has previously been shown to be effective in macaques [33], and several additional monkey models of tularemia have been recently published, but not yet used for vaccination studies [34–36].

## Supporting Information

**File S1 Figure S1.** Attenuation of FSC200 $\Delta$ clpB. BALB/c mice (n = 5/group) were challenged ID with 10<sup>7</sup> CFU of FSC200 $\Delta$ clpB (circle) or 10 CFU of complemented FSC200 $\Delta$ clpB (squares), and were monitored for survival. **Figure S2.** Intranasal virulence of FSC200 $\Delta$ clpB versus LVS. BALB/c mice were inoculated IN with 10<sup>4</sup> (open circle) or 10<sup>5</sup> (closed circle) CFU of FSC200 $\Delta$ clpB or 10<sup>3</sup> (open square) or 10<sup>4</sup> (closed square) CFU of LVS and were monitored for survival. \*, significantly shorter survival than mice challenged with the same dose of FSC200 $\Delta$ clpB. **Figure S3.** *In vivo* growth kinetics of FSC200 $\Delta$ clpB vs LVS. BALB/c mice (n = 4/group) were immunized ID with ~ 10<sup>5</sup> CFU of FSC200 $\Delta$ clpB (circle) or LVS (square). Mice were killed on days 2, 4, 7, and 14 for bacteriology. \*, significantly higher burden vs LVS. Dashed line

shows limit of detection. **Figure S4.** Skin reactogenicity score chart for *F. tularensis* strains. **Figure S5.** Protection against ID or IN challenge with SCHU S4 following immunization with SCHU S4 $\Delta$ clpB versus LVS $\Delta$ clpB. BALB/c mice were immunized ID with ~10<sup>5</sup> CFU of SCHUS4 $\Delta$ clpB or LVS $\Delta$ clpB. Immunized and control mice were challenged six weeks later with 1000 CFU ID or 40 CFU IN of SCHU S4 and were monitored for survival. \*, significantly longer survival than control mice; \*\*, significantly longer survival than mice immunized with LVS $\Delta$ clpB. **Table S1.** Growth of SCHU S4 $\Delta$ clpB, FSC200 $\Delta$ clpB, and LVS in the tissues of SCID mice. **Table S2.** Attenuation and immunogenicity profiles of selected SCHU S4 deletion mutants administered ID or IN to BALB/c mice.

(PDF)

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## Author Contributions

Conceived and designed the experiments: IG SMT AS WC. Performed the experiments: IG HS AS SMT WC. Analyzed the data: WC SMT. Contributed reagents/materials/analysis tools: IG SMT HS AS WC. Wrote the paper: SMT AS WC.

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