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Differential ability of novel attenuated targeted deletion mutants of *Francisella tularensis* subspecies *tularensis* strain SCHU S4 to protect mice against aerosol challenge with virulent bacteria: effects of host background and route of immunization

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Abstract

Francisella tularensis subspecies *tularensis* is a highly virulent facultative intracellular pathogen of humans and a potential biological weapon. A live vaccine strain, *F. tularensis* LVS, was developed more than 50 years ago by pragmatic attenuation of a strain of the less virulent *holarctica* subspecies. LVS was demonstrated to be highly effective in human volunteers who were exposed to intradermal challenge with fully virulent subsp. *tularensis*, but was less effective against aerosol exposure. LVS faces regulatory hurdles that to date have prevented its licensure for general use. Therefore, a better defined and more effective vaccine is being sought. To this end we have created gene deletion mutants in the virulent subsp. *tularensis* strain and tested them for their ability to elicit a protective immune response against systemic or aerosol challenge with the highly virulent wild-type subsp. tularensis strain, SCHU S4. Both oral and Intradermal (ID) primary vaccination routes were assessed in BALB/c and C3H/HeN mice as was oral boosting. One SCHU S4 mutant missing the heat shock gene, *clpB*, was significantly more attenuated than LVS whereas a double deletion mutant missing genes *FTT0918* and *capB* was as attenuated as LVS. In general mice immunized with SCHU S4*ΔclpB* were significantly better protected against aerosol challenge than mice immunized with LVS. A single ID immunization of BALB/c mice with SCHU S4*ΔclpB* was at least as effective as any other regimen examined. Mice immunized with SCHU S4*Δ0918ΔcapB* were generally protected to a similar degree as mice immunized with LVS. A preliminary examination of immune responses to vaccination with LVS, SCHU S4*ΔclpB*, or SCHU S4*Δ0918ΔcapB* provided no obvious correlate to their relative efficacies.

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1. Introduction

The *tularensis* and *holarctica* subspecies of the facultative intracellular bacterial pathogen *Francisella tularensis* cause tularemia, a severe infectious disease of humans and many other mammals. In particular, human typhoidal tularemia, thought to be caused by inhalation of the more virulent *tularensis* subspecies had an historical mortality rate of 30% or more when left untreated [1]. This led to the development of of subsp. *tularensis* as a biological weapon during the first half of the 20th century [2]. There was a concomitant search for effective vaccines against it. In human volunteers, whole killed bacteria and extracts thereof significantly alleviated infection initiated via the skin, but not via the lungs [3-6]. In contrast, a pragmatically-attenuated *holarctica* strain, *F. tularensis* LVS, fully protected volunteers against systemic challenge and partially protected them against aerosol challenge. LVS remains the only anti-tularemia vaccine to have been manufactured on a commercial scale in the USA, but currently is only available to at risk laboratory personnel via the Special Immunization Program of the US Department of Defense. In part, this is due to the fact that the basis for its attenuation and its mechanism of action remain poorly understood. In recent years, there has been increased concern about the potential abuse of *F. tularensis* by terrorists. This has led to renewed efforts to produce licensable vaccines.

The natural distribution of *F. tularensis* subsp. *tularensis* is confined to North America, and clinical cases of tularemia caused by its inhalation are extremely rare making it impossible to assess vaccine efficacy via clinical trials. The United States Food and Drug Administration devised the so called Animal Rule for such eventualities (http://www.fda.gov/cber/rules/humeffic.htm). The Animal Rule allows for the exclusive use of animal models to demonstrate vaccine efficacy for rare diseases provided that the mechanism of action of the vaccine in animals predicts its efficacy in humans. With the aforementioned considerations in mind, for the past several years we have been trying to produce novel defined attenuated live vaccines by targeted deletion of virulence genes from the prototypical subsp. *tularensis* strain, SCHU S4, using a murine model to screen for attenuation and efficacy. Mice have been the mainstay of *F. tularensis* infections and immunity research for the past 25 years and have been the sole model host used to evaluate efficacy of vaccines against *F. tularensis* subsp. *tularensis* during that time.

Previously, we and others have shown that systemic immunization of some mouse strains, e.g. BALB/c mice, with LVS provides excellent long-term protection against systemic, but not aerosol challenge with virulent subsp. *tularensis* strains of the pathogen [7-9]. This inability to replicate the long-lasting clinical efficacy of transdermally-administered LVS against pulmonary challenge with subsp. *tularensis* using a well-established small animal model of tularemia could seriously hamper the development of any novel anti-*Francisella* vaccines against inhalation-initiated infection under the Animal Rule. In particular, none of the other small animal models of tularemia developed during the past 50 years has shown any obvious advantage over mice [10]. Historically, certain non-human primate models of tularemia were reported to better mimic the protection against pulmonary challenge elicited in humans by LVS [4,11], and might ultimately need to be further developed to satisfy the Animal Rule. However, for ethical and economic reasons, such models are impractical for early stage vaccine discovery.

In the case of LVS, protection against pulmonary tularemia can be slightly improved by using C3H/HeN mice in place of BALB/c mice [8], or by pulmonary or oral vaccination of the latter with LVS [9,12,13]. Additionally, we have shown that a spontaneously-attenuated strain of SCHU S4 or a targeted deletion mutant missing the gene *FTT0918*, administered intradermally (ID) can provide partial protection to BALB/c mice against aerosol challenge with fully virulent subsp. *tularensis* [14]. However, these vaccine strains were either as undefined as LVS or

retained an unacceptable level of residual virulence to be considered clinically useful. To overcome these problems, we have continued to generate targeted deletion mutants of SCHU S4, and to screen them for attenuation via the ID route. For the current study, two deletion mutant strains, SCHU S4*ΔFTT0918ΔcapB* and SCHU S4*ΔclpB*, attenuated to a similar level as LVS by the ID route were generated. Herein, we examine whether they can be shown to elicit better protection than LVS in BALB/c or C3H/HeN mice against aerosol challenge with SCHU S4 using clinically-relevant routes of vaccination, to thereby allow for their further development in accordance with the Animal Rule. The targets were chosen because they were known to be important virulence determinants in *F. tularensis*. We have previously demonstrated that FTT0918 is a major virulence factor for *F. tularensis tularensis*, but SCHU S4 *Δ0918* was 100-fold less attenuated than LVS when administered to mice via the ID route. [14]. The ClpB chaperone fulfills important cellular functions by solubilization and refolding of aggregated proteins by virtue of its protease function and is a virulence determinant in other facultative intracellular bacteria such as *Salmonella enterica* serovar Typhimurium [15] and *Listeria monocytogenes* [16]. Several publications have also identified its important contribution in *F. tularensis* LVS [17,18] or *F. novicida* [19-21] intracellular growth and virulence. The *capB* gene has also been implicated in LVS or *F. novicida* virulence [17,21]. It shows a 38% amino acid identity with the capsule biosynthetic gene *capB* of *Bacillus anthracis* [22]. However, there is no direct evidence that it contributes to capsule formation in *F. tularensis*.

2. Materials and Methods

2.1 Mice

Female BALB/c and C3H/HeN mice were purchased from Charles Rivers Laboratories (St. Constant, Quebec) and entered experiments at 6-8-weeks of age. Mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

2.2 Bacterial strains

The ATCC 29684 isolate of LVS was used for comparison with SCHU S4-based vaccines. Spontaneously attenuated SCHU AV and deletion mutant SCHU S4*ΔiglC* have been described by us previously [14]. The former elicits significant protection against ID or aerosol challenge with *F. tularensis* subsp *tularensis*. In contrast, SCHU S4*ΔiglC* lacks the ability to disseminate from the skin inoculation site and fails to elicit protection [14]. They were included in the current study as positive and negative controls respectively. In-frame deletions of the *clpB* and *capB* genes were constructed by allelic exchange using a previously described method for *ΔiglC* [23] based on integration and excision of a suicide plasmid carrying upstream and downstream sequences of the target gene. The upstream and downstream regions of each of the genes were amplified by PCR. The PCR fragments for each gene contained complementary sequences in the 3′ end of the upstream fragment and the 5′ end of the downstream fragment which were annealed during a second round of PCR. After restriction enzyme digestion and purification, the PCR fragments were cloned into the suicide vector pDMK2, which was later transformed into *Escherichia coli* S17-1. Conjugation to *F. tularensis* SCHU S4 was carried out as described previously [23]. Conjugants were selected on media containing 10 μg/ml kanamycin and 50 μg/ml of polymyxin B and confirmed by PCR. To select for a second recombination event, conjugants were plated on medium containing 5% sucrose and the deletion of the genes identified by PCR and the exact location verified by sequencing. The *ΔcapB* mutation was introduced into the *ΔFTT0918* strain to generate a double mutant [14]. The strategy led to the deletion of 1073 out of the 1218 bp of *capB* and 2463 out of the 2580 bp for *clpB*.

For the present study, stock cultures of all strains were prepared by growing them as confluent lawns on cystine heart agar supplemented with 1% (w/v) hemoglobin (CHAH). Bacteria were harvested after 48 h incubation at 37°C into freezing medium consisting of modified Mueller Hinton broth containing 10% w/v sucrose. Stocks were aliquotted in volumes of 1 ml and stored at −80°C at a concentration of 10^{10}-10^{11} CFU / ml.

2.3 Immunization and challenge

We have previously shown that LVS has an oral LD_{50}>10^8 CFU for BALB/c mice [13], and at this dose it elicits protection against systemic and aerosol challenge with fully virulent *F. tularensis* subsp. *tularensis.* The ID LD_{50} is also >10^8 CFU, and 10^5 CFU of LVS administered ID completely protects against systemic challenge, but only prolongs survival by 1-2 days following aerosol challenge, with subsp. *tularensis* [7,8,12,14]. Prior studies from us also showed that LVS elicits a statistically insignificant improvement in protection in C3H/HeN *versus* BALB/c mice [12] and that SCHU AV administered ID to BALB/c mice provides an insignificant improvement in protection than LVS against aerosol challenge with subsp. *tularensis* [14]. Based on the aforementioned findings we chose 10^5 and 10^8 CFU as the ID and oral immunizing doses for all of the test vaccine strains and BALB/c and C3H/HeN mice as the model hosts for determining their efficacy. For oral immunization, mice were gavaged once with one or other vaccine strain suspended in 0.2 ml saline. ID inocula were injected into a fold of skin in the mid-belly in a volume of 0.05 ml saline. Aerosol challenges were performed using an InTox Products nose-only exposure chamber as previously described [7,8,12,14]. The protocol results in the delivery of ~20 CFU of SCHU S4 to the lower airways of mice. All animal work was performed in a federally-licensed and Select-Agent-approved small animal containment level 3 facility. In the present study, mice were examined daily for signs of infection and whenever feasible were euthanized by CO_2 asphyxiation as soon as they displayed signs of irreversible morbidity. For bacteriology, a 1 cm^2 piece of skin surrounding the inoculum site, and livers, spleens, and lungs were removed, minced with scissors, and homogenized using aerosol-proof homogenizers. Organ homogenates were diluted in sterile saline and plated on CHAH. To determine the extent of bacteremia, whole blood collected by cardiac puncture at the time of necropsy was diluted 1:10 in sterile water to lyse host blood cells. The lysate was further diluted in saline and plated as above.

2.4. Statistics

Survival curves were compared using the Mantel-Cox log rank test. Differences in bacterial burdens, serum cytokine levels or serum antibody titres were compared by One-way ANOVA with Tukey's post test, using GraphPad Prizm 5 software. In all cases, differences were considered significant at $P<0.05$.

3. Results

3.1 Safety of live vaccine strains

During initial screening, all BALB/c mice survived ID challenge with 10^5 CFU of any of the mutant strains employed in the present study whereas at an ID dose of 10^7 CFU 1/5 mice immunized with SCHU S4 *ΔFTT0918ΔcapB* died. Thus, all of the test mutants had an estimated ID LD_{50} of ≥10^7 CFU for BALB/c mice *versus* < 10 CFU for wild-type SCHU S4. SCHU S4*ΔiglC* was included as a negative control for potential non-specific protective effects of vaccination with live attenuated mutants of SCHU S4. In contrast, SCHU AV was included as a positive control. It is a highly attenuated spontaneous mutant of SCHU S4 that retains its ability to elicit robust protection against systemic challenge and partial protection against aerosol challenge with *F. tularensis* subsp. *tularensis* [14]. LVS was included as a reference strain. For the present study, mice received ID inocula of 10^5 CFU. Previously, we showed that LVS, but not SCHU AV or SCHU S4*ΔiglC* at this dose, elicited obvious necrosis at the

site of injection and visible signs of infection (ruffled fur) in BALB/c mice [14]. SCHU S4 *ΔFTT0918ΔcapB* was similar to LVS in this regard, whereas SCHU S4*ΔclpB* was similar to SCHU AV (not shown). LVS at an ID dose of 10^5 CFU killed a few BALB/c (2/15) mice, whereas all C3H/HeN mice survived this inoculum. The opposite result was observed with SCHU AV which killed 2/15 C3H/HeN mice, but none of the BALB/c mice. Both mouse strains survived ID immunization with SCHU S4*ΔclpB* and SCHU S4 *ΔiglC*. Surprisingly, SCHU S4 *ΔFTT0918ΔcapB* was very virulent for C3H/HeN mice compared to BALB/c mice (10^5 CFU ID killed 14/15 versus 3/15, respectively). In a follow-up experiment, 7/15 C3H/HeN mice died following ID immunization with 10^3 CFU SCHU S4 *ΔFTT0918ΔcapB* (not shown). Previously, we had shown that BALB/c mice survived oral immunization with 10^8 CFU of LVS and subsequently demonstrated some protection against aerosol challenge with *F. tularensis* subsp. *tularensis* [13]. Therefore, we chose this as the test dose for oral immunization with all of the mutants in the current study. By this vaccination route, SCHU AV and SCHU S4 *ΔiglC* were completely avirulent for both BALB/c and C3H/HeN mice. Likewise, LVS and SCHU S4 *ΔclpB* were completely attenuated for BALB/c mice, but each killed 5/15 C3H/HeN mice. Finally, SCHU S4*ΔFTT0918ΔcapB* demonstrated some degree of virulence for both BALB/c and C3H/HeN mice at an oral dose of 10^8 CFU, killing 3/15 and 6/15 mice, respectively.

3.2 Efficacy of ID vaccination against aerosol challenge

Previously, we showed that BALB/c mice immunized ID with LVS or SCHU AV, but not SCHU S4*ΔiglC*, survived a subsequent ID challenge with 1000 LD_{50} of fully-virulent *F. tularensis* subsp. *tularensis* [7,8,12,14]. In the present study, this was determined to be the case too for SCHU S4*ΔclpB* and SCHU S4*ΔFTT0918ΔcapB*; 100% of mice immunized ID with 10^3 or 10^5 CFU of either mutant survived a subsequent ID challenge with 1000 CFU SCHU S4. To determine the degree of protection against inhalation tularemia elicited by ID immunization with 10^5 CFU, mice were challenged 6 weeks post-vaccination with a low dose (~20 CFU) aerosol of SCHU S4. Note that for this experiment we used C3H/HeN mice that survived ID immunization with 10^3 CFU of the less attenuated SCHU S4 *ΔFTT0918ΔcapB* administered 5 weeks earlier to allow for contemporaneous challenge with other test groups. The results are shown in Table 1. All (n=6) naïve BALB/c and C3H/HeN mice died on day 5 of challenge. All vaccine candidates elicited significant protection ($P \leq 0.04$), against aerosol challenge in BALB/c mice, compared to naïve mice or mice immunized with the negative control strain SCHU S4*ΔiglC*. BALB/c mice immunized with SCHU S4*ΔclpB* showed the best median survival (>28 days) and this was significantly greater than the protection elicited by LVS ($P=0.038$), but not than that elicited by SCHU AV or SCHU S4 *ΔFTT0918ΔcapB*. In C3H/HeN mice, LVS, SCHU AV, and SCHU S4*ΔclpB* but not SCHU S4*Δ0918ΔcapB*, elicited a statistically significant increase in survival compared to naïve mice or mice immunized with SCHU S4*ΔiglC* ($p \leq 0.03$). As we, and others, have previously shown [8,12,24], LVS produced a statistically insignificant, improvement in median survival in C3H/HeN versus BALB/c mice challenged by aerosol with SCHU S4. In contrast, all of the SCHU S4-based vaccines elicited a statistically insignificant increase in median survival in BALB/c *versus* C3H/HeN mice. This result is consistent with our earlier findings with SCHU AV and SCHU S4*Δ0918 versus* LVS [14].

3.3 Efficacy of oral vaccination against aerosol challenge

Previously, we showed that most BALB/c mice immunized once orally with 10^8 CFU of LVS were fully protected against low dose aerosol challenge with *F. tularensis* subsp. *tularensis*, but this immunity waned substantially after 4 weeks [13]. Others have recently reported similar findings [25]. Therefore, to determine whether any of the other potential vaccine strains in the current study might be superior to LVS in this regard, aerosol challenges were performed 6 weeks post vaccination, when LVS-elicited protection would be expected to have markedly

diminished. The results are shown in Table 2. All control mice died on day 5 of challenge, as did all C3H/HeN mice immunized with LVS, SCHU AV, and SCHU S4*ΔFTT0918ΔcapB;* 2/5 of C3H/HeN mice immunized PO with SCHU S4*ΔiglC* survived to day 6, but this was not statistically significant. All BALB/c mice immunized with SCHU AV or SCHU S4*ΔiglC* died on day 5 whereas 2/5 BALB/c mice immunized with either LVS or SCHU S4*ΔFTT0918ΔcapB* survived to day 7, but this was not statistically significant. In contrast, C3H/HeN and BALB/c mice immunized PO with SCHU S4 *ΔclpB* survived significantly longer than all other groups of mice ($P \leq 0.025$). The different mean survival times between these two mouse strains (12 vs 16 days) was not statistically significant. In the case of LVS and SCHU AV, ID vaccination elicited a statistically significant increase in survival compared to PO vaccination ($P \leq 0.02$) in both BALB/c and C3H/HeN mice.

3.4 Effect of oral boosting on vaccine efficacy

Eight weeks after ID or PO vaccination, some mice were re-immunized PO with 10^8 CFU of the homologous mutant strain. In contrast to primary PO immunization, no mice died following oral boosting. Six weeks post-boosting, mice were exposed to an aerosol of SCHU S4 and their survival was monitored (Tables 3 and 4). BALB/c mice ID primed and orally boosted with LVS, SCHU S4*Δ0918ΔcapB*, or SCHU S4*ΔclpB* survived significantly longer than naïve mice or mice primed and boosted with SCHU AV or SCHU S4*ΔiglC* ($P \leq 0.04$). This was also the case for C3H/HeN mice immunized and boosted with SCHU S4*ΔclpB* ($P \leq 0.003$). Additionally, BALB/c and C3H/HeN mice primed and boosted with SCHU S4*ΔclpB* survived significantly longer than mice primed and boosted with LVS ($P = 0.03$ and 0.003 respectively). Compared to ID priming alone, BALB/c mice primed and boosted with SCHU AV survived SCHU S4 challenge longer ($P=0.023$) as did C3H/HeN mice primed and boosted with LVS ($P<0.002$) or SCHU S4*ΔclpB* ($P=0.049$). In all other cases there was no significant difference in survival times. Mice orally immunized and boosted with LVS or SCHU S4*ΔclpB* survived significantly longer than naïve mice or mice immunized with SCHU AV or SCHU S4*ΔiglC* ($P \leq 0.014$). Moreover, C3H/HeN mice orally immunized and boosted with SCHU S4*ΔclpB* survived significantly longer than mice so immunized with LVS ($P=0.003$) or SCHU S4*Δ091ΔcapB* ($P=0.008$). In BALB/c mice no significant differences in survival were observed between mice orally primed or orally primed and boosted for any of the test vaccines. In contrast oral priming and boosting of C3H/HeN mice with LVS ($P=0.014$) or SCHU S4*ΔclpB* ($P=0.047$) significantly increased survival compared to oral priming alone.

3.5 Course of infection in vaccinated mice

Based on the preceding results, it is clear that for SCHU S4-based vaccines, neither oral immunization nor oral boosting, nor the use of C3H/HeN mice conferred any survival advantage over a single ID immunization of BALB/c mice. Therefore, since single transdermal administration of LVS by scarification is currently the sole indication for clinical use, all further studies used only ID immunized BALB/c mice. Moreover, since SCHU S4*ΔiglC* elicited no protection, and SCHU AV was inferior to SCHU S4 *ΔFTT0918ΔcapB* or SCHU S4*ΔclpB*, these control groups were not pursued further. Next we confirmed on two additional occasions our initial observation that ID immunization of BALB/c mice with SCHU S4*ΔclpB* elicited significantly better protection than ID immunization with LVS against aerosol challenge with SCHU S4. In both additional cases, mice immunized with SCHU S4 *ΔFTT0918ΔcapB* showed a slight, but insignificant increase in median survival versus mice immunized with LVS (not shown). Understanding the basis for the superior performance of SCHU S4*ΔclpB versus* LVS in this model could provide insights into mechanisms or correlates of protection.

We have previously examined the infection kinetics of LVS in ID immunized mice [7]. Therefore, we were interested to determine the *in vivo* growth characteristics of SCHU S4 *ΔFTT0918ΔcapB* and SCHU S4*ΔclpB* (Figure 1). Overall, *in vivo* growth kinetics of SCHU

S4 *ΔFTT0918ΔcapB* and SCHU S4*ΔclpB* were similar to each other and to LVS [7]. Sera from mice immunized ID with 10^5 CFU of LVS, or SCHU S4 *ΔFTT0918ΔcapB*, or SCHU S4*ΔclpB* were collected on days 2,4,7,14 post vaccination and examined for the presence of interferon gamma (IFNγ) as previously described [26]. On day 7 post-vaccination only, circulating levels of IFNγ were significantly greater in mice immunized with SCHU S4 *ΔFTT0918ΔcapB versus* mice immunized with SCHU S4*ΔclpB* or LVS (figure 2). Sera were also collected 28 days post vaccination and IgG and IgM ELISA titres against killed SCHU S4 determined as previously described for rabbits [27]. IgG /IgM titres ±SD for mice immunized with SCHU S4 *ΔFTT0918ΔcapB* (936 ± 274 / 677± 483), SCHU S4*ΔclpB* (1175 ±283 / 428 ±80), or LVS (1814 ±764 / 411± 142) were compared by one way ANOVA. IgG titres were significantly greater for LVS- *versus* SCHU S4 *ΔFTT0918ΔcapB*- immunized mice (P=0.04).

Next we examined the course of infection initiated by inhalation of wild-type SCHU S4 in naive and immunized mice (Table 5). All three vaccines effectively conferred control of the intense bacteremia observed on day 4 of primary infection. By day 2 of infection, mice immunized with SCHU S4*ΔclpB* harbored significantly fewer bacteria in their lungs than naïve mice or mice immunized with LVS (P<0.05, One way ANOVA with Tukeys post-test). On day 4 of infection, lung burdens were similar in naïve and LVS immunized mice, and burdens in mice immunized with either of the other two attenuated strains were significantly lower than this (P<0.05). LVS-immunized mice harbored >100-fold fewer bacteria in their livers and spleens than naïve mice at day 4 (P<0.05), and mice immunized with either SCHU S4 *ΔFTT0918ΔcapB* or SCHU S4*ΔclpB* harbored significantly fewer bacteria than LVS-immunized mice (P<0.05). Naïve mice did not survive beyond day 5. On day 7 of infection, mice immunized with LVS or SCHU S4*ΔFTT0918ΔcapB* harbored similar numbers of bacteria in the lungs which were significantly higher than the burden in the lungs of mice immunized with SCHU S4*ΔclpB*. Mice immunized with SCHU S4*ΔclpB* were better controlling infection in the liver and spleen than mice immunized with SCHU S4*ΔFTT0918ΔcapB* at this time which, in turn, were performing significantly better than LVS-immunized mice (P<0.05). No LVS immunized and only one SCHU S4 *ΔFTT0918ΔcapB* immunized mouse survived to day 10 of infection. At this time, mice immunized with SCHU S4*ΔclpB* harbored similar numbers of bacteria in the lungs, liver and spleen as at day 7. Only between days 10-15 did these mice begin to reduce the bacterial burden in the lungs to the low levels seen in the liver and spleen throughout the course of infection. Similar results were obtained on two separate occasions.

4. Discussion

The current study examined the ability of novel defined live vaccine candidates to protect mice from ID or aerosol challenge with the highly virulent *F. tularensis* subsp. *tularensis* strain, SCHU S4, relative to the protection induced by LVS. LVS has been extensively tested in humans and experimental animals, predominantly mice, for efficacy against infections caused by subsp *tularensis* initiated via transdermal or pulmonary portals of entry [3-13]. Extensive immunological studies have also been conducted on human and murine hosts immunized with LVS, but to date, no common correlate of protection has been demonstrated. For humans, further vaccination and challenge studies might be required to break this impasse, but such studies will face considerable regulatory hurdles compared to animal studies. Most human studies have employed single dose vaccination by scarification as the method of LVS administration, and this remains the sole clinically indicated use. By this route LVS elicits complete protection against infections initiated via the skin, and partial protection against exposure to an aerosol of the pathogen. The former situation can be recapitulated in mice, the latter less so. In humans, LVS administered by inhalation or by ingestion also protects against systemically- or inhalation-initiated infection [reviewed in 13,32]. In mice, these routes of LVS administration appear to afford greater protection than transdermal routes against airborne

challenge with subsp. *tularensis* [13]. Currently, several live anti-viral or anti-bacterial vaccines are administered by inhalation or ingestion [28-31]. Moreover, both routes potentially offer a more tolerable means of vaccination than hypodermic injection. Additionally, for LVS at least, the oral route seems to be at least as safe as the ID route. In contrast, LVS is more virulent for humans and experimental animals when administered directly to the lungs [5,33, 34]. This appears to be the case too with SCHU S4-based vaccines; in our hands the IN LD_{50} for LVS and SCHU S4 *Δ0918ΔcapB* is ~ 10^3 CFU, and for SCHU S4*ΔclpB* ~10^6 CFU*)*. Another potential problem with oral or pulmonary administration of live attenuated strains of *F. tularensis* is that it exposes the vaccine to a variety of normal flora from which it might acquire homologs of the virulence genes that were deleted in order to attenuate it. Therefore, transdermal injection still appears to be the most rational route for administering live vaccines against *F. tularensis*, especially when coupled with the extensive clinical data for LVS given via this versus other routes.

LVS administered ID or orally to mice has been shown to elicit partial protection against aerosol challenge measured as enhanced survival for a day or so [7,8,12,14]. Therefore, it might be possible to predict efficacy of novel vaccines against inhalation tularemia in humans by comparing their efficacy relative to LVS in mice. As proof-of-concept for this possibility, in the current study we have generated defined experimental live vaccine strains by deleting specific virulence genes from fully virulent *F. tularensis* subsp. *tularensis* strain SCHU S4 and compared them against LVS for their ability to elicit protection against pulmonary challenge following ID or oral vaccination using two mouse strains for which such efficacy has been previously demonstrated by one or other route. In particular, two deletion mutants, SCHU S4 *Δ0918ΔcapB* and SCHU S4*ΔclpB*, showed levels of attenuation similar to LVS (>1-million-fold versus wild-type SCHU S4 by the ID route) and were as effective as LVS at combating ID challenge with >1000 LD_{50} of the fully virulent pathogen. Against an aerosol challenge, SCHU S4 *ΔclpB* significantly outperformed LVS when given ID or orally to BALB/c mice. In C3H/HeN mice both vaccines performed at a similar level when given ID, but SCHU S4*ΔclpB* was significantly better than LVS when given orally. Indeed, it was the only strain to elicit any measurable protection when given orally. ID primed and orally boosted mice of both strains were significantly better protected by SCHU S4*ΔclpB versus* LVS. Oral boosting of orally primed mice also resulted in better protection for mice immunized with SCHU S4*ΔclpB* versus LVS, but this only reached statistical significance in C3H/HeN mice. SCHU S4 *Δ0918ΔcapB* elicited similar protection as LVS in BALB/c mice regardless of vaccination regimen, but the results were more mixed in C3H/HeN mice. Furthermore, C3H/HeN mice did reveal SCHU S4 *Δ0918ΔcapB* to be less attenuated than SCHU S4*ΔclpB*, a phenomenon which was masked in BALB/c mice. No vaccination regimen offered any obvious advantage over single dose ID immunization of BALB/c mice, and results with this model reflected the majority finding of all of the others that were examined. Therefore, this model was adopted for all further studies. In this model, the superiority of SCHU S4 *ΔclpB versus* LVS correlated with an enhanced ability of mice immunized with it to control a subsequent aerosol challenge with SCHU S4. Surprisingly, by day 10 of infection, the lungs of mice immunized with SCHU S4*ΔclpB* still contained 1 million francisellae, a number that was reduced 1000-fold by day 14. Thus, even mice that were fully protected had difficulty killing *F. tularensis* in the lungs. A similar situation has been reported for inhaled *Mycobacterium tuberculosis* [35] and *Listeria monocytogenes* [36] in systemically immunized mice possibly suggesting a common defect in the ability to express anti-bacterial immunity in the lungs *versus* other organs.

A preliminary examination of the immune responses elicited by each vaccine provided no obvious reason for the superior ability of SCHU S4*ΔclpB* to elicit protective pulmonary immunity against inhaled *F. tularensis*. More detailed analyses are underway. Finally, to belay regulatory concerns about possible reversion to a wild-type virulent phenotype, it will probably be necessary to delete additional unlinked virulence genes from SCHU S4*ΔclpB*. However,

further attenuation of this mutant could lead to an unacceptable loss of immunogenicity. Indeed, such issues have plagued live vaccine development against other intracellular bacteria [37-39]. Nevertheless, live vaccines against *S.* Typhi containing multiple gene deletions have been developed for human use [40-41].

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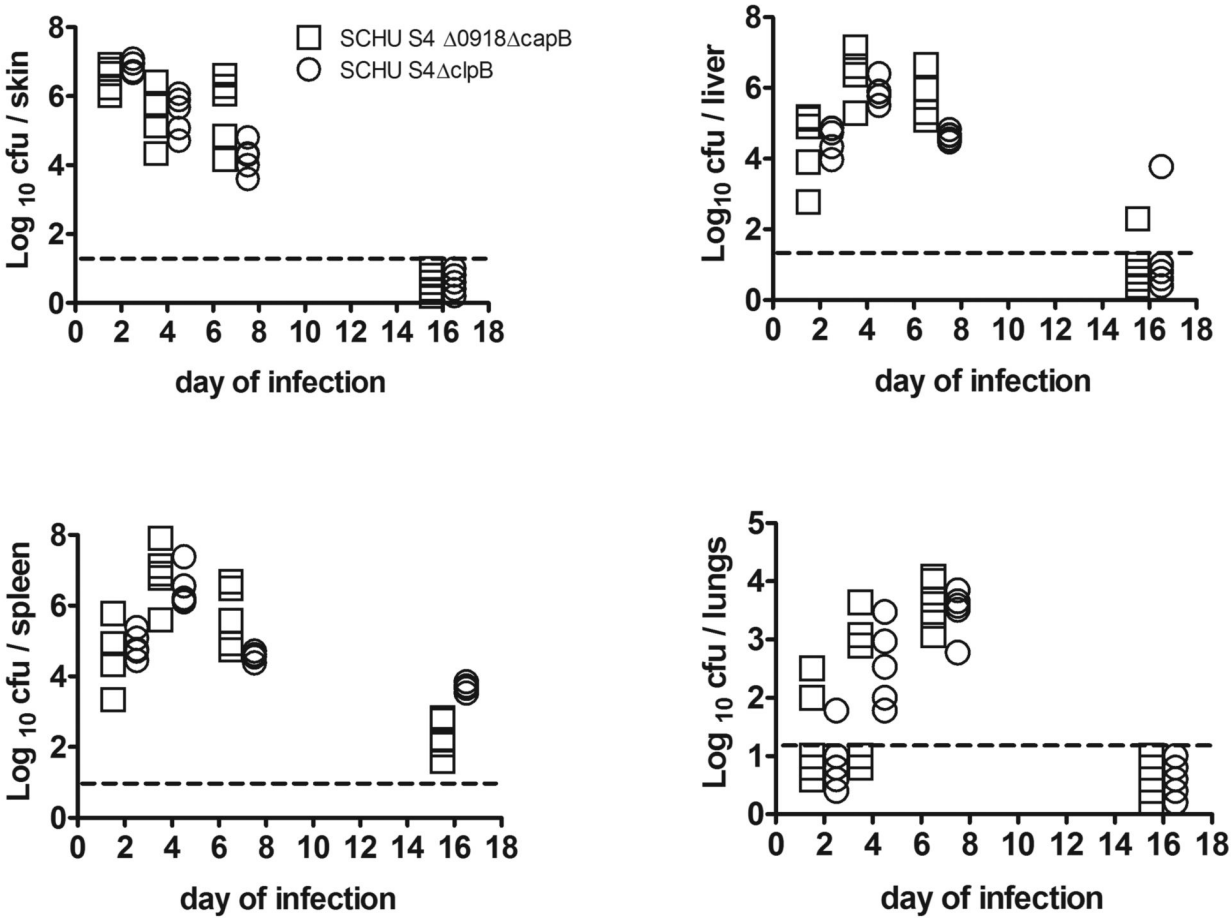

Figure 1.
Course of sublethal infection in mice immunized ID with 10^5 CFU of SCHU S4 *Δ0918ΔcapB* (squares) or SCHU S4 *ΔclpB* (circles). n=4 mice per group.

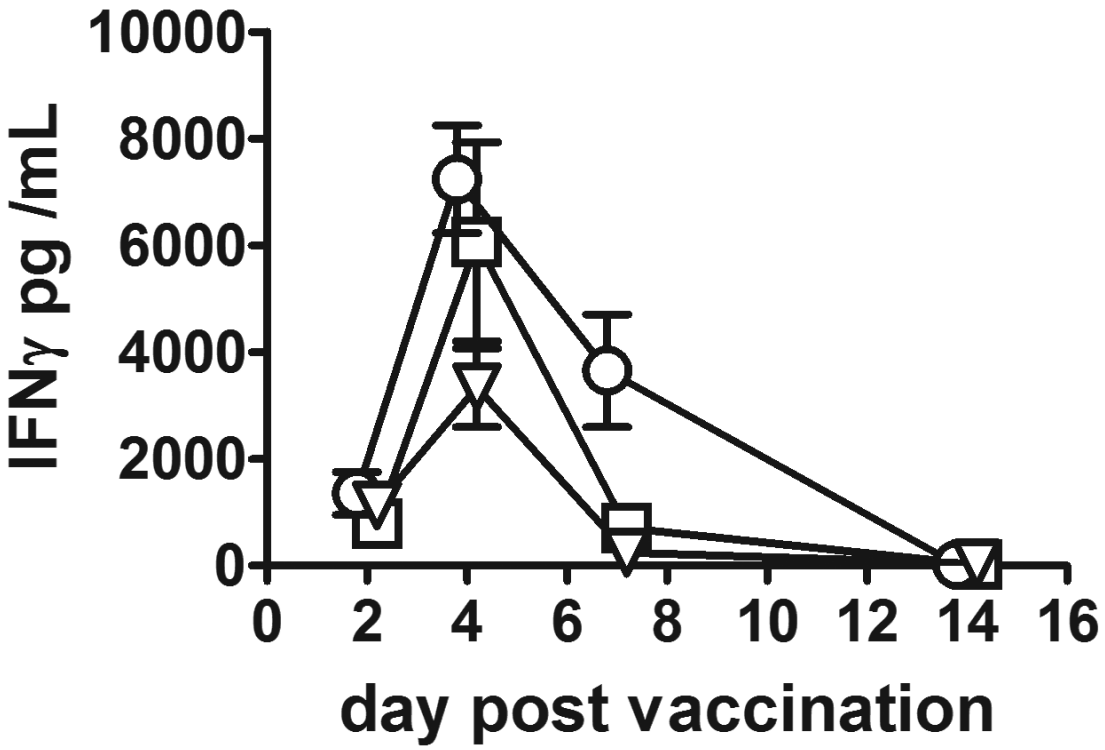

Figure 2.
Change in serum IFNγ levels following ID immunization with 10^5 CFU of SCHU S4 *ΔFTT0918ΔcapB* (squares), SCHU S4 *ΔclpB* (circles), or LVS (triangles). Mean +/− SEM, n=5/group.

Table1

Survival of ID immunized mice following aerosol challenge with SCHU S4.

Mouse strain	vaccine	Time to death of individual mice (days)	Median time to death (days)
BALB/c	none	5,5,5,5,5,5	5
BALB/c	LVS	7,7,8,9,12	8[1]
BALB/c	SCHU AV	6,9,10,10,12	10[1]
BALB/c	SCHU S4*Δ0918ΔcapB*	5,7,8,>28,>28	8[1]
BALB/c	SCHU S4*ΔclpB*	8,11,>28,>28,>28	>28[1,2]
BALB/c	SCHU S4*ΔiglC*	5,5,6,6,6	6
C3H/HeN	none	5,5,5,5,5,5	5
C3H/HeN	LVS	9,9,11,12,14	11[1]
C3H/HeN	SCHU AV	6,6,6,7,>28	6[1]
C3H/HeN	SCHU S4*Δ0918ΔcapB*[3]	5,5,5,9,9	5
C3H/HeN	SCHU S4*ΔclpB*	5,8,10,11,14	10[1]
C3H/HeN	SCHU S4*ΔiglC*	5,5,5,6,6	5

Mice were immunized ID with 10^5 CFU of one or other vaccine and challenged 6 weeks later by aerosol with 20 CFU of SCHU S4.

[1] significantly greater survival ($P \leq 0.03$) by Mantel-Cox log rank test) than for naive mice or mice immunized with SCHU *ΔiglC*.

[2] significantly greater survival than mice immunized with LVS ($P = 0.04$).

[3] These mice were immunized with 10^3 CFU.

Table 2

Survival of orally immunized mice following aerosol challenge with SCHU S4.

Mouse strain	vaccine	Time to death of individual mice (days)	Median time to death (days)
BALB/c	none	5,5,5,5,5,5	5
BALB/c	LVS	5,5,5,7,7	5
BALB/c	SCHU AV	5,5,5,5	5
BALB/c	SCHU S4 *Δ0918ΔcapB*	5,5,5,7,7	5
BALB/c	SCHU S4*ΔclpB*	9,9,16,>28,>28	16[1]
BALB/c	SCHU S4ΔiglC	5,5,5,5,5	5
C3H/HeN	none	5,5,5,5,5,5	5
C3H/HeN	LVS	4,5,5,5,5	5
C3H/HeN	SCHU AV	5,5,5,5,5	5
C3H/HeN	SCHU S4*Δ0918ΔcapB*	5,5,5,5	5
C3H/HeN	SCHU S4 *ΔclpB*	5,9,12,13,16	12[1]
C3H/HeN	SCHU S4*ΔiglC*	5,5,5,6,6	5

Mice were immunized *per os* with 10^8 CFU of one or other vaccine and challenged 6 weeks later by aerosol with 20 CFU of SCHU S4.

[1] significantly greater survival ($P \leq 0.03$) than for naive mice or mice of the same strain immunized with any of the other vaccine strains.

Table 3

Survival of ID immunized and orally boosted mice following aerosol challenge with SCHU S4.

Mouse strain	vaccine	Time to death of individual mice (days)	Median time to death (days)
BALB/c	none	5,5,5,5,5,5	5
BALB/c	LVS	6,7,8,8	7.5[1]
BALB/c	SCHU AV	6,6,6,6	6
BALB/c	SCHU S4*Δ0918ΔcapB*	6,8,8,9	8[1]
BALB/c	SCHU S4*ΔclpB*	7,9,11,11,>28	11[1,2]
BALB/c	SCHU S4*ΔiglC*	5,6,6,6	6
C3H/HeN	none	5,5,6,6,6,6,	6
C3H/HeN	LVS	5,6,7,7,7,8	7
C3H/HeN	SCHU AV	5,5,6,7	5.5
C3H/HeN	SCHU S4*Δ0918ΔcapB*	6,6,>28,>28	17
C3H/HeN	SCHU S4*ΔclpB*	8,13,16,16,19	16[1,2]
C3H/HeN	SCHU S4*ΔiglC*	5,6,6,6,7	6

Mice were immunized ID with 10^5 CFU of one or other vaccine, boosted eight weeks later orally with 10^8 CFU and challenged 6 weeks after this by aerosol with 20 CFU of SCHU S4.

[1] significantly greater survival ($P \leq 0.04$) than for the same strain of naive mice or mice immunized with SCHU AV or SCHU S4*ΔiglC*.

[2] significantly greater survival ($P \leq 0.03$) than for the same strain of mice immunized with LVS.

Table 4

Survival of orally immunized and orally boosted mice following aerosol challenge.

Mouse strain	vaccine	Time to death of individual mice (days)	Median time to death (days)
BALB/c	none	5,5,5,5,5,5	5
BALB/c	LVS	5,6,7,8,8	7[1]
BALB/c	SCHU AV	5,5,5,5,5	5
BALB/c	SCHU S4*Δ0918ΔcapB*	5,5,11,13	8
BALB/c	SCHU S4*ΔclpB*	5,6,11,19,>28	11[1]
BALB/c	SCHU S4*ΔiglC*	5,5,5,5,5	5
C3H/HeN	none	5,5,5,5,5,5	5
C3H/HeN	LVS	5,6,7,8,8	7[1]
C3H/HeN	SCHU AV	5,5,5,5,5	5
C3H/HeN	SCHU S4*Δ0918ΔcapB*	5,5,5	5
C3H/HeN	SCHU S4*ΔclpB*	11,15,>28,>28,>28	>28[2]
C3H/HeN	SCHU S4*ΔiglC*	5,5,5,5,5	5

Mice were immunized orally with 10^8 CFU of one or other vaccine, boosted eight weeks later by the same route with 10^8 CFU and challenged 6 weeks after this by aerosol with 20 CFU of SCHU S4.

[1] significantly greater survival ($P \leq 0.02$) than for the same strain of naive mice or mice immunized with SCHU AV or SCHU S4*ΔiglC*.

[2] significantly greater survival than for the same strain immunized with any other vaccine strain ($P \leq 0.008$).

Table 5

In vivo growth of *F. tularensis* SCHU S4 following aerosol challenge of vaccinated mice.

Vaccine group	Tissue	Log10 SD bacterial burden on day post-aerosol-challenge:				
		2	**4**	**7**	**10**	**14**
Naive	lungs	6.09± 0.11	7.71± 0.20			
LVS		6.0± 0.17	7.52± 0.07	8.31± 0.20		
Δ0918γΔcapB		5.65± 0.14[1,2]	6.37± 0.21[1,2]	8.24 ± 0.08	8.60 (1/1)	
ΔclpB		4.79 ± 0.93[1,2]	5.94 ± 0.46[1,2]	6.27± 0.96[2,3]	6.91 ± 0.96	3.97± 1.83
Naive	liver	4.04± 0.88	7.99 ± 0.25			
LVS		3.07 ± 0.58	5.77 ± 0.13[1]	7.94± 0.73		
Δ0918ΔcapB		2.68 (3/4)	4.25± 0.45[1,2]	6.23 ± 0.41[2]	7.66 (1/1)	
ΔclpB		1.87 (2/4)[1]	4.26 ± 0.41[1,2]	4.14± 0.64[2,3]	5.33± 0.70	3.70 (3/4)
Naive	spleen	3.85± 1.0	8.26 ± 0.98			
LVS		2.13± 0.76[1]	5.75 ± 0.17[1]	7.54 ± 0.92		
Δ0918ΔcapB		2.36 (3/4)	4.10 ± 0.18[1,2]	5.50 ± 0.37[2]	6.23 (1/1)	
ΔclpB		2.43 ± 0.46[1]	3.76 ± 0.38[1,2]	3.63 ± 0.58[2,3]	4.52± 0.82	3.41 ± 1.43
Naive	Blood /mL	2.82± 0.94	7.19 ± 0.03			
LVS		3.47 (1/4)[1]	3.22 ± 0.87[1]	4.22 (2/4)		
Δ0918ΔcapB		<2.0[1]	3.0 (1/4)[1]	3.91± 0.78	3.56 (1/1)	
ΔclpB		2.85 (1/4)[1]	2.0 (1/4)[1]	2.0 (1/4)	4.38 (2/4)	<2.0 (0/4)

Mice (n=4 / group were immunized ID with 10^5 CFU of one or other attenuated strain of *F. tularensis*, then challenged 6 weeks later with an aerosol of SCHU S4.

[1] burden significantly lower than in naïve mice

[2] burden significantly lower than in LVS-immunized mice

[3] burden significantly lower than in SCHU S4*Δ0918ΔcapB* immunized mice